ABSTRACT

CUPO, KATHERINE LYNN. Immunological Investigation of Protozoan Parasitic Infections in Poultry. (Under the direction of Dr. Chongxiao Chen).

Two intestinal protozoan parasites, *Histomonas meleagridis* which causes histomoniasis and *Eimeria* coccidia spp., are responsible for significant economic loss to the commercial poultry industry each year. *H. meleagridis* is an extracellular parasite that may be transmitted between chickens and turkeys. In the chicken, *H. meleagridis* typically does not cause clinical disease, but in the turkey, *H. meleagridis* causes severe histomoniasis which manifests as necrosis of the ceca and liver usually resulting in death. Different *Eimeria* spp., on the other hand, specialize in infecting either the chicken or the turkey but cannot be transmitted between the two poultry hosts. *Eimeria* infections are usually subclinical and result in reductions in bird performance and welfare. Currently, there are no countermeasures available for managing histomoniasis, while control strategies for *Eimeria* require rotating between vaccination for specific *Eimeria* species and administration of anticoccidial drugs throughout the life of the flock. *Eimeria* vaccines consist of live parasites which themselves cause mild reduction in early bird performance. Novel and improved countermeasure strategies are needed for improved management of both parasites in commercial poultry flocks. Development of a histomoniasis vaccine will require a better understanding of the host-pathogen interactions between either the chicken or the turkey and *H. meleagridis*. To reduce the negative side effects of *Eimeria* vaccination on bird performance, vaccination may be combined with the administration feed additives to improve intestinal health. Therefore, the objectives of this research were to investigate the immune responses of chickens and turkeys to *H. meleagridis* and to evaluate the effects of feed-grade sodium bisulfate on early turkey performance following *Eimeria* vaccination.

Immunological Investigation of Protozoan Parasitic Infections in Poultry

by
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DEDICATION

I dedicate this work to my parents, Michael A. Cupo and Teresa L. Blalock. For your unwavering love and support, for inspiring me to pursue my doctorate, and for believing in me even in times I didn't have the courage to believe in myself, thank you. To my fiancé, Brendan C. T. Mazaleski, for sharing in my struggles and successes, for being my soundboard at times and my critic at others, and for sticking it out with me through my entire graduate school experience. To my advisory committee; Dr. Chongxiao (Sean) Chen, Dr. Robert Beckstead, Dr. Frank Edens, Dr. Jeffrey Yoder, and Dr. Hilary Pavlidis, for your guidance and mentorship through this entire process. To my fellow graduate students for your unwavering friendship, spending countless hours in the lab and at the farm with me, and reminding me there is life beyond the lab, thank you.

BIOGRAPHY

Katherine Lynn Cupo, the daughter of Michael Anthony Cupo and Teresa Lynn Blalock, was born in West Chester, PA on August 31, 1993. She, her twin brother, Anthony, and her older brother Michael grew up in a West Chester suburban home. Katherine spent much of her time outdoors trekking through the woods, digging up earthworms in the garden, and climbing trees with her brothers. During those early years, Katherine developed a love for nature. In the fall of 2012, she enrolled at NC State to pursue a Bachelor of Science in Zoology. During her Junior year, she began working in Dr. Jeffrey Yoder's immunology lab at NC State's vet school. Through her experience in the lab, she developed an interest in disease and immunology research and decided to pursue a Master of Science and intended to apply to vet school following the completion of her degree. In August 2016, she joined Dr. Robert Beckstead's lab in the Prestage Department of Poultry Science at NC State to study the transmission of nematode and protozoan parasites in turkeys and chickens. After completing her master's research, Katherine decided to continue in Dr. Beckstead's lab for her PhD to study the innate immune response of turkeys to parasite infection rather than pursue a DVM. In the spring of 2020, the onset of the COVID-19 pandemic caused a delay her research schedule. The spring of the following year, Katherine altered her dissertation research plans while she transitioned to working under Dr. Chongxiao Chen to expand on her master's research to identify potential vectors of the cecal nematode and explore the transcriptional and cellular response of the chicken and turkey ceca and liver to infection with a cecal protozoan parasite.

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CHAPTER 1: Literature review

Introduction

Protozoal infectious diseases pose a persistent and important challenge for commercial production of poultry. Infection may cause mild to severe disease, and due to the highly intensive nature of commercial poultry production, infection with these parasites is elevated (Yegani and Korver, 2008). Two of the most important poultry protozoal parasites are *Histomonas meleagridis* and *Eimeria* coccidia. *H. meleagridis* can be transmitted between chickens and turkeys and results in greater devastation of turkey flocks (McDougald, 2005). *Eimeria* species are more host specific; however, because there are several distinct species causing disease in chickens or turkeys, *Eimeria* infection poses the largest threat to the poultry producer (Blake and Tomley, 2014). No control measures are currently available for histomoniasis while anticoccidial drugs and live vaccines are available for limiting coccidiosis (Blake and Tomley, 2014; Clark and Kimminau, 2017). In both cases development of novel vaccines or other countermeasures are needed to gain better control of parasite spread and disease. The immune responses initiated against either parasite is poorly understood; therefore, development of effective countermeasures for either parasite will depend on research to elucidate mechanisms of host-parasite interactions and immunostimulation. Here we review the life cycles of *H. meleagridis* and *Eimeria* species and the literature on immune development against either protozoan.

Pathology of histomoniasis in turkeys and chickens

Histomoniasis is a parasitic disease caused by the cecal protozoan *Histomonas meleagridis* that predominantly affects the ceca and liver in natural infections (McDougald, 2013). In the early stages of histomoniasis, when the parasite is entering the cecal mucosa and

underlying tissues from the cecal lumen, the tissue becomes inflamed, begins to thicken, and eventually leaks plasma proteins into the lumen where they agglutinate in yellow to white caseous aggregates (Clarkson, 1966; McDougald, 1998). During later stages of infection small lesions begin to appear on the liver as the infiltrating parasites are transported to the organ via the hepatic portal veins (McGuire and Morehouse, 1958; Clarkson, 1961). Around this time or immediately before, afflicted birds may begin to exhibit signs of infection such as excretion of sulfur-colored droppings, lethargy, drooping wings, standing with head held low with closed eyes, and weight loss (McDougald, 2005, 2013). In later stages of infection, the ceca become impacted with a thick caseous core and the mesenteries surrounding the ceca become inflamed and thicken (McDougald, 1998). The liver may be covered in numerous bullseye lesions or several much larger lesions. At this stage other organs may also become infected. *H. meleagridis* or histomoniasis lesions have been discovered in the proventriculus, spleen, kidneys, heart, lungs, bursa of Fabricius, pancreas, and brain of experimental inoculations (Tyzzer and Fabyan, 1920; Levine, 1947; McGuire and Morehouse, 1958; Peardon and Ware, 1969; Singh et al., 2008). Birds that are unable to recover from the infection generally die within 2 weeks of infection due to liver failure associated with the enterohepatitis caused by histomoniasis (McDougald, 2013).

Histomoniasis disease is observed routinely in commercial chicken and turkey operations, although it is more common and more severe in turkeys (Tyzzer and Fabyan, 1920; Tyzzer, 1934; McDougald, 1998). Chicken flocks often act as reservoirs for the parasite (Lund and Chute, 1970). In the absence of secondary infections or environmental stressors, most *H. meleagridis*-positive chickens may recover from an initial infection and never exhibit signs of disease while still maintaining the protozoan in the lumen of their ceca (McDougald, 2013;

Chadwick et al., 2020). Turkeys are more susceptible to development of severe histomoniasis disease and typically do not survive the infection. The different consequences of *H. meleagridis* infection in chickens and turkeys are indicative of differential host-pathogen interactions occurring between the two host species and *H. meleagridis*. A better understanding of the immunological responses of the chicken and the turkey will be crucial for novel countermeasure development.

Insufficiency of vaccination against histomoniasis disease

Vaccination is an effective countermeasure for protozoal infections in poultry. However, early studies of acquired immunity to histomoniasis indicated that inoculation with the virulent parasite only produced limited protection against subsequent infections. Protection provided by vaccination or prior infection with *H. meleagridis* cells decreased significantly after 2 months (Tyzzer, 1934). The protective potential of vaccination with *H. meleagridis* cells appears to be sufficient only in cases of intra-cloacal inoculation of poultry rather than by consumption of eggs from the cecal nematode, *Heterakis gallinarum*, the only known vector for *H. meleagridis* (Lund, 1959; Lund et al., 1966). However, Kendall later demonstrated protection to infection with pathogenic *H. meleagridis* delivered through administration of *H. gallinarum* eggs (Kendall, 1957). Tyzzer hypothesized, although it has not been demonstrated, that any protection conferred from earlier infection with *H. meleagridis* is lost after the host has cleared the infection and is no longer a carrier of the parasite (Tyzzer, 1934). According to this hypothesis, successful control of histomoniasis would be dependent upon contributing factors to barrier functions of the cecal mucosa as well as the activities of the cells and components of the avian innate immune system. However, limited research has been conducted in this area.

Immune response of chickens and turkeys to *Histomonas meleagridis*

The innate immune response to H. meleagridis

The cells and molecules that comprise the immune system can loosely be divided into innate and adaptive components. Immune activity against an invading pathogen is initiated by the innate immune system via recognition of conserved molecular patterns within molecules expressed by pathogens, termed pathogen associated molecular patterns (PAMPs) by receptors called pattern recognition receptors (PRRs) expressed by host cells. Activation of different PRRs produce distinct patterns of gene expression which direct the type and magnitude of the innate and adaptive immune cascades (Akira and Takeda, 2004). However, very little research has been conducted to identify innate immune pathways activated in chickens or turkeys infected with *H. meleagridis*. One report published in 2021 demonstrated that the transcription of certain Toll-like receptors (TLRs), the best characterized family of PRRs in avian and mammalian species, is altered by *H. meleagridis* infection in chickens and turkeys (Mitra et al., 2021). In that study, chickens and turkeys inoculated with high-pathogenic cultures of *H. meleagridis* were shown to have opposite transcriptional changes in TLR4 in the ceca. The turkey expressed significantly elevated levels of TLR4 transcripts at 4- and 10-days-post-inoculation (dpi) compared to the chicken, which downregulated transcription of this gene until 21 dpi. Several other TLRs can exhibit significantly upregulated levels of transcription in the chicken or turkey ceca at various time points as well, including TLR1B, TLR2B, and TLR3. Nevertheless, the transcriptional changes observed in that report require verification, and changes in TLR protein expression will require further evaluation before a conclusion about the role of different TLRs in the chicken's or the turkey's immune response to *H. meleagridis* can be determined.

Subsequent to PRR activation by pathogen PAMPs, stimulated cells will activate transcription of immune signaling molecules, called cytokines. The cytokine expression profile of the early innate immune response can be used to predict whether a T_H1 or T_H2 adaptive immune response will be promoted (Romagnani, 1997). Cytokines can be grouped into several categories. Some are pro-inflammatory and induce the proliferation and activation of immune cells while others are regulatory and suppress immune cell functions (Wigley and Kaiser, 2003). Depending on the combinations of certain cytokines, their interactions may be indicative of specific adaptive immune activation as well. Changes in cytokine transcription have been measured in the cecal tonsil, cecal pouches, spleen, and liver of experimentally infected chickens and turkeys. The transcription of pro-inflammatory cytokines, such as IL-6, IL-1B, and CXCLi2, is elevated early on in the chicken ceca, but these same cytokines are not upregulated until 4 or 5 dpi in the turkey ceca (Powell et al., 2009; Mitra et al., 2021). Expression or IL-10 and TGF-B4, cytokines which are generally associated with immunoregulation, are also upregulated very early in the chicken cecal tonsil while it is not upregulated at all and is even downregulated in the first few days of infection in the turkey cecal tonsil (Powell et al., 2009). Comparison of the expression of proinflammatory and regulatory cytokines in the liver and spleen of *H. meleagridis*-infected chickens and turkeys demonstrates a greater immune response to *H. meleagridis* outside the ceca in mid and late-stages of infection in the turkey compared to the chicken (Powell et al., 2009; Mitra et al., 2021). Cytokines that are indicative of an adaptive immune response are also differentially regulated in the *H. meleagridis* infected chicken and turkey cecal tonsil. IFN-γ stimulates development of T-helper type 1 (T_H1) lymphocytes and is observed to be elevated in the chicken ceca compared to the turkey for the duration of infection (Powell et al., 2009; Kidane et al., 2018). IL-13 and IL-4 by contrast, stimulate activation of T_H2

lymphocytes (Degen et al., 2005). IL-13 is elevated in the cecal tonsil of chickens and turkeys, especially during mid to late stages of infection but not IL-4 (Powell et al., 2009; Kidane et al., 2018).

Expression of cytokine genes in the liver follows two different trends for the chicken and the turkey. Fewer cytokines are expressed at lower levels in the chicken liver compared to the turkey. Expression of IFN-γ, IL-10, CXCLi2, and TGF-B4 are significantly upregulated in the turkey liver during the mid- to late- stages of infection, whereas the expression of these same cytokines is not significantly altered in the chicken liver at any stage of infection (Powell et al., 2009). However, expression of cytokine genes in the spleen more closely mirror that of the ceca in either species (Powell et al., 2009; Mitra et al., 2021)

The T-cell response to H. meleagridis

The helper T-cell (T_H) response induced by vaccination is generally polarized between two T_H subsets, T_H1 and T_H2 (Degen et al., 2005). Available research examining the immune response to histomoniasis has focused on evaluation of immunomodulation by inoculation with cell culture-attenuated *H. meleagridis* strains to serve as a vaccine. Therefore, those investigations mainly evaluated the T_H1 vs T_H2 response in birds inoculated with virulent or low-virulent (vaccine) strains of the parasite. However, characterization of the activation of T_H1 and T_H2 cells in the immune response to *H. meleagridis* infection has proven difficult because there are no distinguishing markers identifying T_H1 from T_H2 cells other than the cytokines they express. The cytokine response has been evaluated using RT-qPCR and *in situ* hybridization to quantify and localize the T_H1 and T_H2 cellular responses based on the expression of cytokines indicative the proliferation of either of these cell types. IFN-γ stimulates the activation of and is expressed by T_H1 cells while IL-13 and IL-4 are classically associated with T_H2 activation

(Degen et al., 2005). The data from those studies were conflicting as the profile of cytokine expression from the ceca, liver, and/or spleen of infected chickens and turkeys was variable between the studies.

Powell and colleagues reported that following delayed activation of immune activity, the turkey exhibited elevated levels of several cytokines including T_H1 and T_H2-specific IFN-γ, IL-13, and IL-4 in the liver (Powell et al., 2009). It was also shown that $CD4^+$ and $CD8^+$ T-cells infiltrated the liver tissue of turkeys following *H. meleagridis* influx from the ceca (Powell et al., 2009). Similarly, Kidane et al. observed that IFN-γ and IL-13 positive cells infiltrated the ceca and liver of *H. meleagridis* infected chickens and turkeys although quantities of these cells were elevated significantly in the turkey only (Kidane et al., 2018). The results of those two papers were obtained using different molecular approaches, but both indicated that T-cells are involved in the immune response to *H. meleagridis*. Although there is evidence for increased expression of T_H1 and T_H2 specific cytokines, Powell suggested that the turkey elicits a T_H2 response although IFN-γ was significantly elevated in the turkey liver from 6 to 12 dpi and not IL-13. Lagler et all, demonstrated that turkeys possessed IFN-γ positive $CD4^+$ and $CD8^+$ T-cells in the liver and spleen at 2 and 5 weeks post-infection with low and/or high virulence strains of *H. meleagridis* whereas only IFN-γ positive $CD4^+$ T-cells were present in the spleen in the chicken (Lagler et al., 2021). Those observations showed that IFN-γ producing cells play an important role in the immune response for both chickens and turkeys, but it also supports observations from previous research that indicated the immune response in the chicken liver is subdued compared to the turkey.

Kidane and colleagues reported that turkeys, but not chickens, infected with a highly virulent strain of *H. meleagridis* exhibited significantly elevated quantities of cells expressing

IFN-γ and IL-13 in the ceca and liver at different time points following infection compared to non-infected birds (Kidane et al., 2018). However, it was noted that chickens not infected with either low or high virulence strains of *H. meleagridis* naturally possessed higher numbers of IFN-γ positive cells in the cecal tissue compared to non-infected turkeys (Kidane et al., 2018). Those researchers proposed that the naturally high presence of this cellular phenotype to be a primed feature of the chicken's immune response to *H. meleagridis* invasion. This divergence between chickens and turkeys could explain, at least in part, why *H. meleagridis* infection in turkey cecal and liver tissue is more severe. This could also explain why a large degree of immune activity is observed in the turkey liver but not the chicken liver in terms of cytokine and T-cell presence. Future research will need to characterize the population of resident immune cells in the non-infected chicken and turkey cecal tissue.

The humoral response to H. meleagridis

The earliest analysis of humoral immunity in chickens and turkeys was conducted in 1963 by M. J. Clarkson. In that study, Clarkson demonstrated that turkeys, treated after *H. meleagridis* infection, and chickens that recovered naturally from *H. meleagridis* infection developed resistance to subsequent infection and produced serum antibodies against *H. meleagridis* (Clarkson, 1963). However, it was also demonstrated that transfer of serum antibodies to susceptible birds via intraperitoneal injection does not confer protection from histomoniasis (Clarkson, 1963; Bleyen et al., 2009). Windisch and Hess demonstrated that both chickens and turkeys generate IgG serum antibodies in response to *H. meleagridis* infection (Windisch and Hess, 2009). Together, those studies demonstrated that both chickens and turkeys are capable of generating a humoral response to *H. meleagridis* infection, but the protective capabilities of these antibodies are questionable. It is possible passive intraperitoneal injection of

antibodies is not an appropriate route for immunization since the parasite infiltrates the mucosa of the ceca. This could mean that immunization via other mucosa such as intranasal, ocular, or oral administration of future vaccine/adjuvants may be more effective.

Since chickens appear to better limit *H. meleagridis* infection in the ceca before a large number of parasites travel to the liver, it is possible that mucosal antibodies play a more significant role in limiting infection than circulating antibodies do. Windisch and Hess addressed this question by measuring IgM, IgG, and IgA in the serum as well as duodenum, jejunum, and ceca of specific-pathogen-free chickens (Windisch and Hess, 2010). They observed changes in IgG and IgA antibody titers in all three parts of the intestine at different time points after infection with virulent *H. meleagridis*. However, the earliest increase in all three of these antibodies was observed in the cecal tonsil during the first week post-infection. IgG titers peaked in the ceca at 14 dpi and remained elevated until termination of the trial at 42 dpi. IgA titers continually increased from 7 to 42 dpi. Therefore, it is possible the mucosal IgG and IgA antibodies play a more significant role in providing protection against subsequent tissue invasions of *H. meleagridis*.

In addition to measuring antibodies, a couple studies have reported on the presence of B-cells in infected chickens and turkeys. In chickens infected with virulent *H. meleagridis*, B-cells accumulate in the ceca but not the liver between 4 and 10 dpi (Kidane et al., 2018). Between virulent *H. meleagridis*-infected chickens and turkeys, B-cell numbers are elevated in the turkey ceca at 10 dpi, but numbers of this lymphocyte does not increase significantly in the chicken ceca at any time point after infection (Mitra et al., 2017). B-cells are significantly elevated in the turkey liver at 7 but not 10 dpi, while a trend of B-cell accumulation is evident in the chicken liver from 4 to 10 dpi, although these elevated numbers of B-cells are not significantly increased

above non-infected chickens (Mitra et al., 2017). The lack of consistency between experiments makes interpretation of the proliferation of B-cells in affected tissues difficult. It may be hypothesized, based on the insufficient increase in B-cell subsets in the ceca or liver of either the chicken or the turkey, that involvement of this cell type in either tissue may be minimal.

Life cycle and pathology of coccidiosis in turkeys

Coccidia are intracellular protozoan parasites that infect enterocytes of many animal species including chickens and turkeys, and cause damage to the intestinal lining (McDougald and Fitz-Coy, 2013). The species of coccidia that infect chickens and turkeys belong to the *Eimeria* genus of coccidia. Most of the species within this genus are highly species-specific and cannot be transmitted between chickens and turkeys. For instance, *E. adenoeides*, *E. innocua*, and *E. subrotunda* infect the turkey but not the chicken, quail, pheasant, or guinea fowl (Moore and Brown, 1951, 1952; Moore et al., 1954). *E. dispersa*, however, is the only *Eimeria* species that can infect several gallinaceous bird species (Tyzzer, 1929; Hawkins, 1952; Doran, 1978; Long and Millard, 1979). Most *Eimeria* species are also highly specialized with regards to what part of the intestine they infect. For example, *E. adenoeides* infects the ceca and, to a lesser extent, the ileum and colon while *E. meleagrimitis* mostly infects the jejunum, although some *E. meleagrimitis* gametocytes may infect epithelial cells in all parts of the intestine (Clarkson, 1958, 1959; McDougald, 2013).

Of the 7 *Eimeria* species known to infect turkeys (Chapman, 2008; McDougald and Fitz-Coy, 2013), *E. adenoeides*, *E. meleagrimitis*, *E. gallopavonis*, and *E. dispersa* are consistently viewed as the most economically important (Cook et al., 2010; McDougald and Fitz-Coy, 2013; Rathinam et al., 2015). Coccidiosis in young poultry may result in decreased feed intake and

weight gain, dehydration, increased mortality, and leaves the birds vulnerable to subsequent infectious diseases such as histomoniasis, salmonellosis, and necrotic enteritis (Long et al., 1977; Al-Sheikhly and Al-Saieg, 1980; Baba et al., 1982; McDougald and Hu, 2001). While young birds are highly susceptible to coccidiosis, older birds are more resistant to the disease most likely because they have developed immunity from earlier mild infections (McDougald and Fitz-Coy, 2013). In either case, young birds, 0 to 5 weeks of age, are most at risk for developing clinical coccidiosis. Therefore, severity of the infection in young birds is dependent upon the species of *Eimeria* and the number of oocysts consumed (McDougald, 1998; Williams, 2005; McDougald and Fitz-Coy, 2013).

Oocysts are resilient structures housing infective coccidia cells called sporozoites and are the only coccidia structures that survive outside the host body. The coccidia life cycle begins once sporulated oocysts are consumed by a susceptible host. The life cycle is the same among *Eimeria* species, although the number of asexual and sexual generations and length of each stage of the cycle varies, taking 4 – 6 days to complete (McDougald and Fitz-Coy, 2013). Following ingestion, the oocysts move through the upper digestive tract where several factors must be met for oocysts to release their sporozoites. The oocysts are exposed to the bird's body temperature (42°C) and elevated levels of CO_2, two physical criteria necessary to promote permeabilization of the oocyst wall and excystation (Ryley, 1972; Pyziel and Demiaszkiewicz, 2015). In addition, bile salts and trypsin activate the sporozoites contained within the oocyst and digests the sporocyst wall (Chapman, 1978; Müller and Hemphill, 2013). Each sporocyst releases two sporozoites (motile, infective cells) which initiate infection by infiltrating enterocytes (Bedrnik, 1969; Tierney and Mulcahy, 2003). Inside parasitophorus vacuoles within the host cells, the sporozoites develop into trophozoites (Bedrnik, 1969; Tierney and Mulcahy, 2003). These

trophozoites divide and mature into merozoites I which burst out of the host cells and reinfect new enterocytes lower in the intestine where they repeat the same process to produce a greater number of merozoites II (Levine, 1973). The number of times *Eimeria* repeat this asexual replication process varies depending on the species. *E. meleagrimitis*, for example, completes the asexual replication process for 5 generations before entering gametogony in which the merozoites develop into macrogametes and microgamonts which subsequently release microgametes (Madden and Vetterling, 1977). Many species complete only 2 asexual replication generations. The greatest pathology observed from coccidiosis is usually observed upon escape of merozoites II from host cells or during the production of oocysts in the subsequent sexual replication generation (Levine, 1973; McDougald and Fitz-Coy, 2013). The free microgametes enter surrounding epithelial cells and fertilize the macrogametes forming the zygote. These cells form the oocysts which rupture the epithelial cells and are shed into the environment with the feces (McDougald and Fitz-Coy, 2013). Once oocysts leave the host and are exposed to sufficient oxygen, moisture, and temperature, they begin to sporulate, a process in which the zygote divides and produces four sporocysts each containing two sporozoites (Waldenstedt et al., 2001).

Immune response of turkeys to coccidia

Because birds develop immunity following mild infection with *Eimeria* oocysts (Rose, 1979; Yun et al., 2000), it is evident that poultry are capable of generating an effective immune response to the parasite. Vaccination with low doses of cocci oocysts has been effective for limiting disease severity from natural infection; however, these vaccines rely on live, precocious parasites which have the potential to cause clinical pathology and mortality if too high of a dose

is given. Additionally, vaccination with one *Eimeria* species does not provide cross-protection for other *Eimeria* species, necessitating the vaccination with oocysts from multiple species (Fitz-Coy, 1992; McDougald and Fitz-Coy, 2013). This makes the production of live *Eimeria* vaccines laborious and costly. Development of vaccines which do not contain live parasite but stimulate a protective immune response will require an understanding of the host-pathogen interactions and the immune cascade generated in the chicken and turkey.

The cytokine response to Eimeria

The cytokine profiles of chickens infected with different *Eimeria* species indicate the importance of a proinflammatory response as well as coordination of T_H1 and T_H2 activation for protective immune development. Hong and colleagues observed large elevations in the expression of proinflammatory, T_H1, and T_H2 cytokines following primary infection with *E. acervulina* and/or *E. tenella* (Hong et al., 2006). Specifically, IL-1β has been shown to be an important proinflammatory cytokine upregulated following early, primary infection with *E. acervuline, E. tenella, and E. maxima* in chickens (Laurent et al., 2001; Hong et al., 2006).

In contrast to proinflammatory cytokine expression, production of regulatory cytokines such as IL-10, inhibit inflammation and immune activation (Rothwell et al., 2004; Couper et al., 2008). The timing and balance of proinflammatory and regulatory cytokine expression during a primary infection with *Eimeria* can determine the outcome of disease severity (Rothwell et al., 2004). Importantly, chicken lines that constitutively express elevated levels of the regulatory cytokine IL-10, exhibit greater coccidiosis and oocyst shedding during primary infection with *E. maxima* (Rothwell et al., 2004). This not only demonstrates importance of cytokine regulation throughout infection, but it also demonstrates the influence of genetic predisposition of different

chicken lines to generate an effective immune response or develop severe disease in response to pathogenic infection.

A T_H1-dominant adaptive immune response is activated in response to intracellular pathogens such as viruses and intracellular bacteria (Kaiser, 1996). *Eimeria* are intracellular protozoa; therefore, infection with these parasites is expected to elicit a strong T_H1 response. This is supported by the great upregulation of T_H1 cytokine gene expression observed in chickens upon primary infection with *Eimeria* oocysts. The expression of IFN-γ, a potent T_H1 cytokine, is greatly elevated in the intestines of chickens infected with *E. acervuline, E. tenella* and *E. maxima* (Laurent et al., 2001; Rothwell et al., 2004; Hong et al., 2006). The importance of T_H1 cytokines in the production of immunity to *Eimeria* infection is clearly demonstrated by the improved immunogenicity of vaccination with an *E. acervulina* subunit vaccine provided with T_H1 cytokine adjuvants (Ding et al., 2004). Balanced T_H1 and T_H2 activation is important for maintaining homeostasis, as an excessively T_H1-dominant immune response is associated with autoimmune disorders, and an excessively T_H2-dominant immune response is associated with allergic responses (Hwang et al., 2005). Therefore, it would be expected that some level of T_H2 activation is promoted over the course of *Eimeria* infection, although polarization of a T_H2 response is not protective against coccidiosis (Hong et al., 2006).

The T-cell response to Eimeria

T-cells play a crucial role in eliminating *Eimeria* parasites in primary infections and providing protective immunity to secondary infections. Specifically, $CD8^+$ cytotoxic T-cells are central to the elimination of invading *Eimeria* parasites and protective immunity against subsequent infections (Lillehoj and Bacon, 1991; Trout and Lillehoj, 1996). Chickens that are unable to produce $CD8^+$ T-cells suffer greater *Eimeria* invasion of intestinal epithelial cells and

shed greater numbers of oocysts compared to uncompromised chickens (Trout and Lillehoj, 1996). Numbers of circulating $CD8^+$ T-cells decrease in female turkey poults 4 days following inoculation with *Eimeria* oocysts while numbers of both $CD4^+$ T_H cells and $CD8^+$ T-cells are elevated in the blood 11 days-post inoculation (Gadde et al., 2009). The initial decrease in circulating $CD8^+$ T-cells is indicative of recruitment of these cells to the intestinal lining where *Eimeria* infection is occurring, although this would need to be confirmed by quantifying these cells in the intestinal tissue at this time point. The contribution of $CD4^+$ T-cells requires further study. While it has been demonstrated that both T_H1 and T_H2 $CD4^+$ T-cells are implemented to varying degrees in the production of protective immunity to different *Eimeria* infection in chickens (Hong et al., 2006), the mechanisms of both are not well understood.

The humoral response to Eimeria

It is well documented that chickens and turkeys generate a humoral immune response to coccidiosis, but the importance of antibodies in generation of protective immunity against subsequent infection is debated. The immunity of bursectomised chickens to secondary *Eimeria* infection is relatively uncompromised (Rose and Hesketh, 1979; Lillehoj, 1987), indicating an inconsequential role of the antibody response in development of host immunity to *Eimeria*. Due to the intracellular nature of these parasites, it is not surprising that antibodies do not play an important role in preventing future infections. Nevertheless, is has been reported that chickens produce serum and mucosal antibodies in response to primary infection with *Eimeria* oocysts (Lillehoj, 1987) Antibodies have also been shown to provide protection in the form of passive immunity via maternal antibodies (Smith et al., 1994a; b). Therefore, their contribution to improving immunity to coccidiosis warrants further study. IgA antibodies are secretory antibodies that are excreted into the mucosa interfacing the lumen (Corthésy, 2013). It has been

suggested that while antibodies do not clear parasite upon invasion of host tissues, IgA may help to limit the number of parasites that invade enterocytes (Girard et al., 1997).

Conclusions

Histomoniasis and coccidiosis in poultry are two prominent diseases that have high economic impacts on the poultry industry. Both parasites have distinct life cycles and etiologies. *H. meleagridis* infects the cecal tissue and travels to the liver causing damage in both organs while Eimeria species parasitize different parts of the intestinal tract resulting in different clinical and histological pathologies. Effective countermeasure development for either disease will require greater characterization of the immune responses induced by the parasites and an understanding of role of both cellular and humoral components of the immune system.

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CHAPTER 2: Transcriptome analysis of the cecal tonsil and liver of *Histomonas meleagridis*-infected chickens and turkeys

Abstract

Histomoniasis is an economically important parasitic infection in the poultry industry. The parasite, *Histomonas meleagridis*, can infect both chickens and turkeys; however, turkeys are more susceptible to developing severe disease from the infection than chickens. Development of novel countermeasures, such as vaccines, is a priority for limiting the impacts of the disease on the poultry industry. Before this can be achieved, a better understanding of the molecular interactions between the parasite and its hosts is needed. RNA sequencing and differential expression analysis was performed on the cecal tonsil and liver of *H. meleagridis*-infected broiler breeder chicks and turkey poults to compare effects of the infection on immune gene transcription between the two species. It was observed that *H. meleagridis* infection resulted in a greater alteration of global gene expression in the turkey cecal tonsil and liver than in those of the broiler breeder. Within the immune gene profile of differentially expressed genes, turkeys exhibited a greater increase in immune gene expression, especially cytokines, relative to the chicken. This data suggests the turkey exhibits a dysregulated immune response to *H. meleagridis* resulting from a cytokine storm.

Introduction

Histomoniasis (blackhead disease) is an economically important disease among poultry that causes necrosis of the ceca, then lesion formation in the liver and eventually death (Cushman, 1893). Histomoniasis is caused by invasion of the mucosal lining of the ceca by the protozoan parasite *Histomonas meleagridis*. Both chickens and turkeys may become infected

with the parasite. Chickens rarely display signs of disease, and most *H. meleagridis*-positive flocks may go undetected harboring the protozoan in their ceca asymptomatically (Kamil et al., 2006; Chadwick et al., 2020). *H. meleagridis*-positive chicken flocks serve as reservoirs for transmission to other flocks on the same or adjacent poultry farms. Conversely, turkeys rarely survive infection with *H. meleagridis*. Upon infection, they stop growing, excrete yellow fecal droppings, and eventually become listless and non-responsive before death, which occurs about two weeks later (Clarkson, 1962).

The mechanistic interactions between *H. meleagridis* and either poultry host are not understood, and it is not known why chickens are able to coexist with the parasite while turkeys cannot. The few studies that have been conducted to investigate the innate and adaptive immune responses in these species are conflicting and do not provide enough evidence to identify molecular pathways that could be involved in the chicken's successful clearance of the parasite from its tissues or the turkey's failure to do so (Powell et al., 2009; Kidane et al., 2016, 2018; Mitra et al., 2017). Identifying the immune pathways that are activated by *H. meleagridis* in the two hosts is necessary for development of targeted histomoniasis vaccines. Therefore, countermeasure development initiatives, such as generation of an effective histomoniasis vaccine, will not be feasible until an understanding of the molecular pathways involved in the immune response to *H. meleagridis* is gained.

Limited available data indicates that both chickens and turkeys produce *H. meleagridis*-specific antibodies, evidence of an adaptive immune response (Clarkson, 1963; Hess et al., 2008; Windisch and Hess, 2009; Powell et al., 2009; van der Heijden et al., 2010; Liebhart et al., 2013). However, it does not appear that those anti-*H. meleagridis* antibodies are able to clear the

parasite in either chickens or turkeys, suggesting that the innate response and/or cell-mediated adaptive response might be more crucial (Clarkson, 1963; Bleyen et al., 2009).

In mammals, infection with intracellular vs extracellular pathogens leads to polarization of T-helper cell types, T_H1 or T_H2 phenotypes, respectively (Mosmann et al., 2005). This division has also been reported in chickens (Degen et al., 2005). *H. meleagridis* is an extracellular parasite, suggesting that a T_H2 cellular response would be elicited over a T_H1 response. A T_H2 phenotype is generally associated with stronger humoral activation and elevated antibody titers. However, if it is in fact a stronger cell-mediated immune response that is most effective against *H. meleagridis* invasion, then a stronger T_H1 response should be observed in poultry that successfully clear the parasite. However, investigations into the T_H subsets activated by *H. meleagridis* infection of chickens and turkeys produced evidence that both T_H cell types may be stimulated (Kidane et al., 2016, 2018; Lagler et al., 2019). Further investigation of these cellular subset responses in chickens and turkeys is needed to determine which subset is significantly activated at different stages of infection. Available data suggests the turkey has a less effective or delayed innate immune response to *H. meleagridis* infection than the chicken (Powell et al., 2009). These studies provide pieces of information about immune signaling initiated in response to *H. meleagridis*, but they are insufficient to determine mechanism of immune activation in either species.

Previous studies have investigated specific components of immune signaling from different tissues, such as expression of specific cytokine transcripts and abundance of total T-cells and B-cells. However, a broader view of the immune response to *H. meleagridis* is needed. Therefore, RNA-sequence (RNA-seq) and differential expression analysis of the cecal tonsil and the liver from *H. meleagridis* infected chickens and turkeys, was performed to investigate

changes in the transcriptome of these two affected organs between the two species. The transcriptome of a tissue is the full range of mRNA expressed by several cell types at a given time. This method, as opposed to RT-qPCR, enables the entire transcriptome to be surveyed for genes that are significantly regulated in response to *H. meleagridis* infection rather than focusing on a few pre-selected genes. While this method does not enable transcription of important immune genes to be determined for specific immune cell subsets, it is a necessary first step in identifying mechanistic interactions between the host tissues and pathogen to focus future research efforts.

Materials and Methods

Animal inoculation and tissue sampling

All experimental procedures for poults were performed following the guidelines of the North Carolina State University Animal Care and Use Committee (19-631).

Broiler breeders

On the day of hatch, 120 female Ross 708SF broiler breeder chicks were divided into 2 groups and placed into litter floor pens with *ad libitum* access to unmedicated starter feed and water. Chicks had been vaccinated to control coccidiosis (*Eimeria acervulin*a, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria necatrix*, and *Eimeria tenella*) and Marek's disease at the hatchery. At 2 and 5 weeks of age, chicks received vaccinations for Newcastle disease and infectious bronchitis. At 25 days of age, the birds in the first group were inoculated intra-cloacally with 100,000 *H. meleagridis* cells/bird (inoculated treatment), and the birds in the second group were sham-inoculated intra-cloacally with water (control treatment). At 5- and 10-days-post-inoculation (dpi), 20 chicks from each group were euthanized, and at 20 dpi, 8 chicks from each

group were euthanized. The liver and both ceca from each euthanized bird were observed for signs of histomoniasis and assigned a histomoniasis score based on the 0 to 4 scale created by McDougald and Hu (McDougald and Hu, 2001). A score of 0 in the ceca or liver indicates no histomoniasis pathology. A score of 1 indicates low pathology with swelling of the cecal lining and a few small lesions in the liver, 2 indicates moderate pathology with inflammation of the cecal lining and numerous lesions in the liver, 3 indicates high pathology with sloughing of caseous exudate in the cecal lumen and many lesions in the liver, and 4 indicates severe necrosis of the ceca with a solid caseous core filling the lumen and lesion formation over the entire surface of the liver. Both cecal tonsils and an approximately 20 mg piece of the liver from each bird were placed in RNA-later (Invitrogen, Carlsbad, CA), held overnight at room temperature, then stored at -80°C for later RNA isolation.

For each sampling time point, only 1 cecal tonsil and 1 liver sample from 3 control and 3 inoculated birds selected based on the highest histomoniasis scores were used for RNA-sequencing. See **Table 1** for a list of the cecal and liver scores in birds used for tissue sampling at each time point (data previously published, Chadwick et al., 2020).

Turkey

An inoculation and sampling procedure similar to that described for the broiler breeders was performed with turkey poults in a different farm at a different time. On the day of hatch, 36 turkey poults were divided into 2 groups and placed into floor pens with *ad libitum* access to non-medicated feed and water. At 25 days of age, all the poults in the first group were inoculated intra-cloacally with 100,000 *H. meleagridis* cells, and the poults in the second group were sham-inoculated intra-coacally with water. At 5 and 10 dpi, 5 control poults and 6 inoculated poults were euthanized, and their livers and ceca were scored for histomoniasis lesions. At each

sampling time point, a small piece of liver and both cecal tonsils were collected into RNA-later as described above.

For each sampling time point, only 3 cecal tonsil and 3 liver samples from both treatments at each time point from chickens and turkeys were used for RNA-seq analysis. Samples from inoculated birds with the highest histomoniasis scores were selected to be used for RNA-seq analysis. See **Table 2** for a list of the histomoniasis scores in the ceca and liver of birds used for RNA-seq analysis at each time point.

RNA isolation, library preparation and RNA sequencing

Tissues were submitted to the North Carolina State Genomic Sciences Laboratory (Raleigh, NC, USA) for RNA extraction, Illumina RNA library construction and sequencing. Tissue samples were extracted using a Qiagen RNeasy Total RNA isolation kit with on column DNase treatment and eluted in nuclease-free water. Prior to library construction, RNA integrity, purity, and concentration were assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Chip (Agilent Technologies, USA). Purification of messenger RNA (mRNA) was performed using the oligo-dT beads provided in the NEBNExt Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, USA). Complementary DNA (cDNA) libraries for Illumina sequencing were constructed using the NEBNext Ultra Directional RNA Library Prep Kit (NEB) and NEBNext Mulitplex Oligos for Illumina (NEB) using the manufacturer-specified protocol. Briefly, the mRNA was chemically fragmented and primed with random oligos for first strand cDNA synthesis. Second strand cDNA synthesis was then carried out with deoxyuridine triphosphates (dUTPs) to preserve strand orientation information. The double-stranded cDNA was then purified, end repaired and tailed with adenine for adaptor ligation. Following ligation, the samples were selected for a final library size (adapters included) of 400-550 bp using

sequential AMPure XP bead isolation (Beckman Coulter, USA). Library enrichment was performed and specific indexes for each sample were added during the protocol-specified PCR amplification. The amplified library fragments were purified and checked for quality and final concentration using an Agilent 2100 Bioanalyzer with a High Sensitivity DNA chip (Agilent Technologies, USA). The final quantified libraries were pooled in equimolar amounts for clustering and sequencing on an Illumina HiSeq 2500 DNA sequencer, utilizing a 125 bp single end sequencing reagent kit (Illumina, USA). The software package Real Time Analysis (RTA) was used to generate raw base call files (BCLs), which were then de-multiplexed by sample into FASTQ files.

RNA-seq analysis and differential expression analysis

FASTQ files received from the NC State Genomic Sciences Laboratory were uploaded to CLC Genomic Workbench 12.0 (CLC) software (Qiagen, Hilden, Germany) in which all RNA-seq and differential expression (DE) analyses were conducted. For RNA-sequence analysis, the Illumina sequence reads for each tissue sample collected from chickens were aligned to the Ensembl GROg-6a annotated *Gallus gallus* genome. The Illumina sequence reads for each tissue sample collected from turkeys were aligned to the GCF_000146605.3 Turkey 5.1 annotated genome. The transcript abundance was estimated using an expectation-maximization (EM) algorithm and expression tracks were generated in the RNA-seq plugin. Principal component analysis (PCA) plots containing all the RNA-seq samples for each species were generated to evaluate the presence of a treatment effect (*H. meleagridis* vs sham-inoculation) on changes in RNA expression. Separate chicken and turkey DE analyses were conducted comparing inoculated to control samples for each tissue type at each time point in which a generalized linear model (GLM) is fit to the expression data and statistical analysis for the fitted GLM is performed

using a Wald test. To compare global changes in gene expression, Venn diagrams were generated for the cecal tonsil and liver tissue samples at each time point for each species using an FDR p-value ≤ 0.05.

Results and Discussion

Infection efficiency and pathology

Inoculated chickens and turkeys were confirmed to be infected with *H. meleagridis* based on the presence of histomoniasis lesions on the liver and/or ceca. In the broiler breeder trial, histomoniasis lesions were observed in 15 of the 20 chicks (75%) sampled at 5 dpi, 18 of the 20 chicks (90%) sampled at 10 dpi, and all 8 of the chicks (100%) sampled at 20 dpi. At 5 dpi, all infected chicks sampled had developed signs of histomoniasis in the ceca but not the liver. Only 1 out of the 18 infected chicks sampled at 10 dpi presented any lesions in the liver in addition to the ceca, and all 8 of infected chicks sampled at 20 dpi presented histomoniasis lesions in both organs. The severity of the pathology in the cecal tissue was already relatively high in the broiler breeder chicks by 5 dpi. 12 out of the 15 histomoniasis-positive chicks had histomoniasis scores of 2 or 3 in at least one of their ceca. At this early time point, histomoniasis lesion scores of 1 or 2 would be expected in the ceca of birds successfully infected using intra-cloacal injection of histomonads. When compared to the severity of turkey cecal lesions from the same sampling time point (described below), only 1 poult out of the 4 that presented histomoniasis lesions in the ceca had a score above 1. That is a difference of 80% to 25% positive cases that presented with moderate to high pathology in the ceca above a score of 1 in the chicken compared to the turkey. 15 out of the 18 infected chicks sampled 10 dpi had a score of 4 in at least 1 cecum. One chick had a score of 3 in both ceca. Two of the infected chicks had a score of 1 in only 1 cecum and 0

in the other. Among the 8 infected chicks sampled at 20 dpi, 6 had scores of 0 and 1 in each cecum and 1 in the liver. One chick had cecal scores of 1 and 4 and a liver score of 1. The last chick had a score of 0 and 4 in the ceca and 4 in the liver.

In the turkey trial, histomoniasis lesions were observed in 4 of the 6 poults (67%) sampled at 5 dpi and all 6 of the poults (100%) sampled at 10 dpi (**Table 2**). Poults that died during the trial were necropsied to determine the cause of death, and high or severe histomoniasis pathology was observed in each mortality. Like the infected chickens sampled at 5 dpi, all the infected poults sampled at this time point presented pathology in the ceca only and displayed no signs of disease in the liver. However, unlike the chicks, the histomoniasis scores in the turkey ceca were much lower on average, with 3 of the 4 infected poults having a score of 1 in both ceca and only 1 poult having a score of 3 in both ceca. The turkeys sampled at 10 dpi also presented severe histomoniasis pathology in the ceca similar to the chicks, but they also presented with lesions in the liver. Every poult sampled at 10 dpi, except for one, had a score of 4 in both ceca, of which two had liver scores of 3, while each of the others had hepatic pathology scores of 1, 2, and 4, respectively. The sixth poult had a score of 1 in both ceca and the liver. When compared to the progression and severity of histomoniasis pathology in the chicken ceca and liver, it appears that histomonads traveled to the liver of the turkey much sooner following infection. If this is the case, this is a key factor leading to the high mortality in *H. meleagridis*-infected turkeys that is not observed in chickens. It further represents the possibility of lack of evolutionary developmental resistance in the turkey as compared to the chicken, which has origins in geographic regions where histomonas has long been a parasite affecting birds.

Tissue sampling and RNA sequencing

1 cecal tonsil and 1 liver sample from selected birds (**Tables 1 and 2**) were submitted to the NC State Genomic Sciences Laboratory (Raleigh, NC, USA) for RNA extraction, Illumina RNA library construction and sequencing. Some of the RNA isolates were of low quality and had to be replaced, e.g., cecal tonsil RNA from chick 4 was replaced with cecal tonsil RNA from chick 6, liver RNA from #7 was replaced with liver RNA from 4. Several turkey RNA samples were replaced with RNA isolated from other poults. Cecal tonsil RNA samples from turkeys 1, 9, and 15 were replaced with cecal tonsil RNA samples from turkeys 4, 6, and 14, respectively. Liver RNA samples from turkeys 3, 7, 10, and 12 were replaced with liver RNA samples from turkeys 5, 6, 11, and 13, respectively. After submitting samples for RNA sequencing, the cecal tonsil and liver samples from turkey 17 were lost and RNA-seq reads were not obtained. Therefore, RNA-seq and DE analysis was performed with only 2 cecal tonsil and 2 liver samples from infected turkeys, at 10 dpi.

RNA-seq PCA plots

PCA plots were generated in CLC Genomic Workbench for all tissue samples collected over all time points for each species (**Figure 1**) to determine the effect of treatment and dpi on gene expression for the two host species. There is a clear division in the transcriptomes between liver and cecal tonsil tissues for both host species. Within the 2 tissues of the broiler breeder chicks, there was no clear separation among samples based on dpi or whether they were from inoculated or control birds. The lack of separation based upon treatment or day of sample collection may be attributed to genetic diversity that exists among broiler breeder chicks. To compensate for the degree of genetic diversity in the population, a much larger sample size would have been required, which was financially prohibitive. In the turkey ceca at 5 dpi and liver

at 10 dpi, there was a moderate partition between inoculated and control samples. The overlap between the transcriptomes of the two experimental groups also could be attributed to genetic diversity among the commercial turkey poults. The observation that some treatment effects exist among turkey samples, but not the broiler breeder samples, suggests that the turkey is exhibiting a more robust physiological response to this parasitic infection compared to the broiler breeder chicks. It appears that the turkey's physiological response is to redirect a large proportion of its resources to respond to *H. meleagridis* infection while the chicken is already significantly resistant and is more efficient than the turkey in its reactions to histomoniasis.

Differential gene expression (DEG) analysis

In the broiler breeder cecal tonsil at 5, 10, and 20 dpi, there were 6, 86, and 42 genes that were significantly up- or down- regulated, respectively, and in the chicken liver, at the same time points, 200, 542, and 4 genes were significantly up- or down-regulated. Whereas the expression of 1084 and 80 genes in the turkey cecal tonsil and 1817 and 2917 genes in the turkey liver were significantly modified at 5 and 10 dpi, respectively. A breakdown of the number of significantly up- and down-regulated genes by tissue and dpi for each host is provided in **Figure 2**. The number of DEGs in the turkey tissues was significantly greater than in the broiler breeder tissues at 5 and 10 dpi except in the cecal tonsil at 10 dpi, where the number of up-regulated DEGs in the turkey cecal tonsil dropped below that of the broiler breeder cecal tonsil. One possible explanation for this may be that the ceca of diseased turkeys may have reached a very severe stage of degeneration and, therefore, would have been unable to function properly.

Figure 3 presents the number of DEGs identified at each time point in the cecal tonsil and liver of the chicken and turkey. At 5, 10 and 20 dpi in the broiler breeder, 6, 86, and 42 DEGs were identified in the cecal tonsil and 200, 542 and 4 DEGs were identified in the liver,

respectively. At 5 and 10 dpi in the turkey, 1084 and 80 DEGs were identified in the cecal tonsil and 1817 and 2917 DEGs were identified in the liver, respectively. The far greater number of DEGs in the turkey cecal tonsil or liver compared to the respective tissues in the broiler breeder chickens is an indication of a physiological difference between the two species. Few of the DEGs identified in the broiler breeder cecal tonsil or liver were immune genes, further supporting the hypothesis that the chicken mounts an efficient innate immune response to *H. meleagridis* in the ceca. However, it is difficult to draw any substantiated conclusions about innate immune function from the broiler breeder cecal tonsil in this study, because samples were not collected until 5 dpi.

Few clues to the immune response of broiler breeder chickens are found in the data presented herein. Only two of the DEGs (MAL and CCL19-1) from the cecal tonsil at 5 dpi is directly associated with immune function. The MAL gene is associated with T-cell organelles and may suggest one or more T-cell subsets were activated at or before this time point (Alonso and Weissman, 1987). CCL19-1 is a chemokine involved in regulating migration of T- and B-cells (Rot and von Andrian, 2004; Schaerli and Moser, 2005). Only two of the DEGs (TGFβ2 and NOS2) identified in the cecal tonsil at 10 dpi may directly stimulate immunity. TGFβ2, has proinflammatory and regulatory properties depending on the concentration of its expression (Omer et al., 2000; Sanjabi et al., 2009, 2017). NOS2 is activated during a T_H1 but not T_H2 response to eliminate intracellular parasites (Chen et al., 2021). Similarly, only two of the DEGs at 20 dpi may be involved in an immune response, IGFBP4 (insulin like growth factor binding protein 4) and LECT2 (leukocyte cell derived chemotaxin 2). Therefore, this may suggest that an active immune response is no longer being propagated in the cecal tonsil of the broiler breeder chicks up to 10 dpi.

In the liver of the broiler breeder chicks, IL1R1, CCR2, STAT1, CCL19_1, IL6ST, and JAK2 are all immune-related genes which were identified at 5 dpi. JAKs and STATs are protein kinases involved in activation of gene transcription in many cell types including lymphocytes following stimulation from cytokine-receptor binding (Morris et al., 2018). IL1R1 is a receptor for IL1 cytokine which is involved in the activation of lymphocytes (Sims and Smith, 2010). It appears that the broiler breeder liver is responding to *H. meleagridis* infection by 5 dpi. However, the presence of the respective cytokine and receptor proteins would need to be quantified to support this observation. Several of the DEGs identified at 10 dpi may be associated with immune activation pathways depending on what intracellular signaling pathways to which they are contributing to in distinct cell types: IRF4, AvBD2, AvBD8, AvBD9, MADD, MAPKBP1, TNFAIP2, NLRC5, NFATC3, LECT2, and CARD11. Of note are the avian β-defensin genes. Powell and colleagues demonstrated that the early innate immune response in the chicken and turkey was not limited to cytokine expression based on the observation of significantly elevated levels of AvBD2 in the liver of both species during *H. meleagridis* infection (Powell et al., 2009). Here, we observed elevated expression of AvBD2 in the immune response of the broiler breeder chick, suggesting that β-defensins play an important role in immunity to *H. meleagridis* infection. No immune-related DEGs were noted in the broiler breeder liver at 20 dpi.

In contrast to the broiler breeder chicken, evidence of an active immune response was observed in the turkey cecal tonsil by 5 dpi. More immune-related DEGs were upregulated in this tissue at this time point compared to the chicken, including interferon regulatory factors (IRFs), chemokines, interleukins and interleukin receptors, tumor necrosis factor super family (TNFSF) members, immune gene transcription factors, CD4, CD44, LY96, TLR4 and NFκBIα.

However, 5 days later, only two immune-related genes were significantly, differentially regulated in response to *H. meleagridis*, C7 (a component of the complement system) and IL1RL1 (a cell surface receptor which may be involved in pro-inflammatory responses and helper T-cell effector functions) (Minutti et al., 2017; Lukassen et al., 2021). As mentioned previously, although several DEGs were observed at 5 dpi, it is not understood why there was such a great decrease in turkey cecal tonsil DEGs at 10 dpi. However, this decreased expression in immune-related DEGs at 10 dpi might be related to severe pathology in this tissue by this time point, which would suggest that the decrease might have been associated with degeneration of the tissue and not indicative of the progression of the immune response to *H. meleagridis*.

Similar to the broiler breeder chicken, it appears that the turkey initiated an immune reaction in the liver against *H. meleagridis* by 5 dpi. Several immune-related DEGs were identified in this tissue at 5 dpi in the turkey, which included TRAF3, TGFβR2, NFATC3, NFAT5, CD7, TRIM25, IL18, C6, TNFαIP8, TRIM2, IL6R, IL2Rβ, CD80, IL22Rα2, TNFαIP3, IGFβP1, STAT4, CSF1R, TRIM7, IL4I1, IRF6, and CCR7. The largest number of immune-related genes were identified in the turkey liver at 10 dpi listed in **Table 4**. Of note, C5, C8A and C8B are significantly down regulated while NFKB1, NFKB2, TLR4, IL10RA, IL10RB, JAK3, STAT1, NFATC1, CD4, IL4R were all upregulated in the turkey liver at this time point. The downregulated genes are components of the complement system. The upregulated genes include transcription factors, innate immune receptors, and cytokine receptors. It is clear that the turkey has the potential to activate immune responses in the liver at this stage.

Dysregulation of innate immune pathways

Because the data from this investigative report and previously published data on histomoniasis in poultry indicate that proper innate immune activation is essential for an

appropriate immune response against *H. meleagridis* (Powell et al., 2009), future research will need to focus on this component of the chicken's and turkey's immune response. The first cell type to respond to infection is the heterophil. Heterophils are the avian equivalent of mammalian neutrophils, which, upon phagocytosing any antigenic substance, signal to other innate and adaptive immune cells via cytokine secretion (Hachicha, 1998). Therefore, the response of these cells to *H. meleagridis* invasion may direct not only the impending innate response but also the type of adaptive immune response. Several studies have demonstrated that heterophils from salmonella-resistant chickens exhibit elevated levels of pro-inflammatory cytokine expression within the first 24 hours of infection compared to heterophils from susceptible chickens (Ferro et al., 2004; Swaggerty et al., 2005a; b). It is possible this early efficient activation of innate signaling by heterophils is critical to efficient activation of immune functions to prevent prolonged immunostimulation and severe pathology. The early functionality of heterophil responsiveness between chickens and turkeys requires investigation to determine if the heterophils in the turkey are less responsive to *H. meleagridis* than those in the chicken.

Regarding late-stage immune signaling, it is apparent from this limited analysis that the turkey elicits increased expression of cytokines which may lead to a large cellular immune response above that observed in the chicken. Only one cytokine is up regulated in the chicken liver at 5 dpi and another cytokine is upregulated in the chicken cecal tonsil at 10 dpi. In the turkey's cecal tonsil, IL16, IL18 and IL34, CCL19, and CCL20 were upregulated at 5 dpi, but no cytokines were significantly upregulated in this tissue at 10 dpi. In the turkey liver, IL18 was also upregulated at 5 dpi while IL16, IL8, IFN-γ, TGFβ2, TGFβ3, and CCL19 were up-regulated at 10 dpi. Therefore, the turkey exhibits greater elevation of cytokine synthesis. This suggests the immune response in the turkey is dysregulated. Thus, the role of cytokine proteins will need to

be quantified in the two tissues of both host species to determine if turkeys secrete a greater or lesser concentration of the various cytokines at these later stages of infection than the chicken.

The evidence of excessive or persistent cytokine secretion in the turkey compared to the chicken is suggestive of a hyperinflammatory reaction. Hyperinflammation is the result of overstimulation of immune cells and results in excessive immune cell infiltration into infected tissues causing damage to the organs (Tavernier et al., 2019). In severe cases, systemic hyperinflammation may result in death. The overstimulation of the immune system by excessive cytokine secretion, hypercytokinemia, is referred to as a cytokine storm (Canna and Behrens, 2012). The largest number of cytokines are transcribed by the JAK/STAT second messenger pathway (Morris et al., 2018). JAK and STAT mRNA were significantly up-regulated in the turkey's cecal tonsil at 5 dpi but not the chicken's cecal tonsil at either time point (**Figure 4B and C**). However, JAK and STAT transcripts were significantly up-regulated in both the chicken's and the turkey's liver (**Figure 4**). JAK2 and STAT1 were up-regulated in the chicken liver at 5 dpi while STAT4 was up-regulated in the turkey liver at 5 dpi, and JAK3, STAT4, and STAT1 were all up-regulated in the turkey liver at 10 dpi. This is further evidence that innate immune signaling may play a larger role in determining the outcome of *H. meleagridis* infection. Overactivation of JAK/STAT signaling has been implicated in the cause of severe pathology and death in many diseases and suppressing JAK/STAT signaling has been demonstrated to reduce organ damage and mortality resulting from the over stimulation of the immune response. The acute respiratory distress syndrome (ARDS) observed in hospitalized COVID-19 patients is the result of a cytokine storm and subsequent systemic hyperinflammation (Kumar and Zhou, 2021). Since many of the cytokines involved in the COVID-19 cytokine storm are activated by JAK/STAT pathways, JAK/STAT inhibitors are currently being investigated in clinical trials for

their efficacy in reducing COVID-19 symptoms (Satarker et al., 2021; Kumar and Zhou, 2021). Therefore, it will be important to investigate the degree of JAK/STAT activation in the chicken and turkey regarding histomoniasis severity. If there is a significantly elevated level of JAK/STAT signaling occurring in the turkey relative to the chicken, JAK/STAT pathways may be targets for countermeasure development to reduce pathology from *H. meleagridis* infection in turkeys.

Conclusion

Histomoniasis is a prevalent and costly disease affecting chickens and turkeys but is especially common in turkeys causing severe pathology and high mortality. Understanding the immune responses in both hosts will be important for developing countermeasures to reduce or prevent morbidity and mortality from the disease. Using the broiler breeder chicken as a model of successful immune responsiveness to *H. meleagridis* infection, it has been demonstrated through RNA-sequence analysis that the turkey exhibits a prolonged, dysregulated immune response. The cause of this dysregulation may lie in the early innate activation of defenses against the pathogen. Future research will need to characterize the functionality of heterophils and early-acting polymorphonuclear leukocytes (PMNs) in these host species. Insufficient activation of these cells may lead to delayed immune activation and ultimately cause the dysregulated immune response in the turkey. Furthermore, evidence from this analysis suggest JAK/STAT activation may be involved in the dysregulated immune response in the turkey, possibly leading to hyperinflammation causing the severe pathology and organ damage observed in the liver and ceca of turkeys that succumb to the disease. Characterizing the cytokine response and propagation of cytokine secretion by JAK/STAT activation may provide targets for limiting

inflammation and pathology via inhibitors that limit JAK/STAT activation and reduce cytokine expression.

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Table 1: Histomoniasis scores for both ceca and the liver of each broiler breeder chick sampled at 5, 10, and 20 dpi for RNA-sequencing. Numbers in red indicate tissue samples used in the RNA-seq and differential expression analysis.

Broiler breeder

	5 dpi				10 dpi				20 dpi			
Treat.	**Bird**	**Ceca 1**	**Ceca 2**	**Liver**	**Bird**	**Ceca 1**	**Ceca 2**	**Liver**	**Bird**	**Ceca 1**	**Ceca 2**	**Liver**
Control	1	0	0	0	8	0	0	0	14	0	0	0
	2	0	0	0	9	0	0	0	15	0	0	0
	3	0	0	0	10	0	0	0	16	0	0	0
Infected	4	1	1	0 x2	11	4	4	0	17	1	0	1
	5	1	0	0	12	4	4	0	18	1	0	1
	6	1	2	0	13	4	4	0	19	1	0	1
	7	1	1	0								

Table 2: Histomoniasis scores for both ceca and the liver of each turkey poult sampled at 5 and 10 dpi for RNA-sequencing. Numbers in red indicate tissue samples used in the RNA-seq and differential expression analysis.

Turkey

	5 dpi				10 dpi			
Treatment	**Bird**	**Ceca 1**	**Ceca 2**	**Liver**	**Bird**	**Ceca 1**	**Ceca 2**	**Liver**
Control	1	0	0	0	10	0	0	0
	2	0	0	0	11	0	0	0 x2
	3	0	0	0	12	0	0	0
	4	0	0	0	13	0	0	0
	5	0	0	0				
Infected	6	3	3	0	14	3	3	1
	7	1	1	0	15	4	4	3
	8	1	1	0	16	4	4	4
	9	1	1	0	17	Both RNA samples lost		

Table 3: List of immune-related DEGs identified in the turkey liver at 10 dpi.

10 dpi turkey liver immune-related DEGs			
IRF5	IL8	NFKB2	TNFSF8
IFNG	NFKB1	TRIM8	TLR4
DRAM1	CD5	IL2RG	TRAF1
CD4	CD6	VCAM1	C5
IL10RB	IGF2	C8B	CD40
IL1R2	CD44	C8A	TNFRSF8
IL1RL1	TGFB3	TNFSF10	IFNLR1
TGFB2	TNFAIP2	IL16	IL10RA
IL20RA	IGF2BP3	IRF8	CD3E
ROS1	NOD1	IRF1	CXCR5
TNFRSF21	CCR5	JAK3	IGSF9
TGFBR1	CX3CR1	IGSF6	CD320
NFATC1	STAT1	IL4R	CCL19
CXXC5	ICOS	IL21R	TRIM14
CD83	CTLA4	TNFRSF13B	IL18BP
IRF4	CD28	TRIM41	IL17RC
LY96	CXCR4	TNFSF15	CD79A

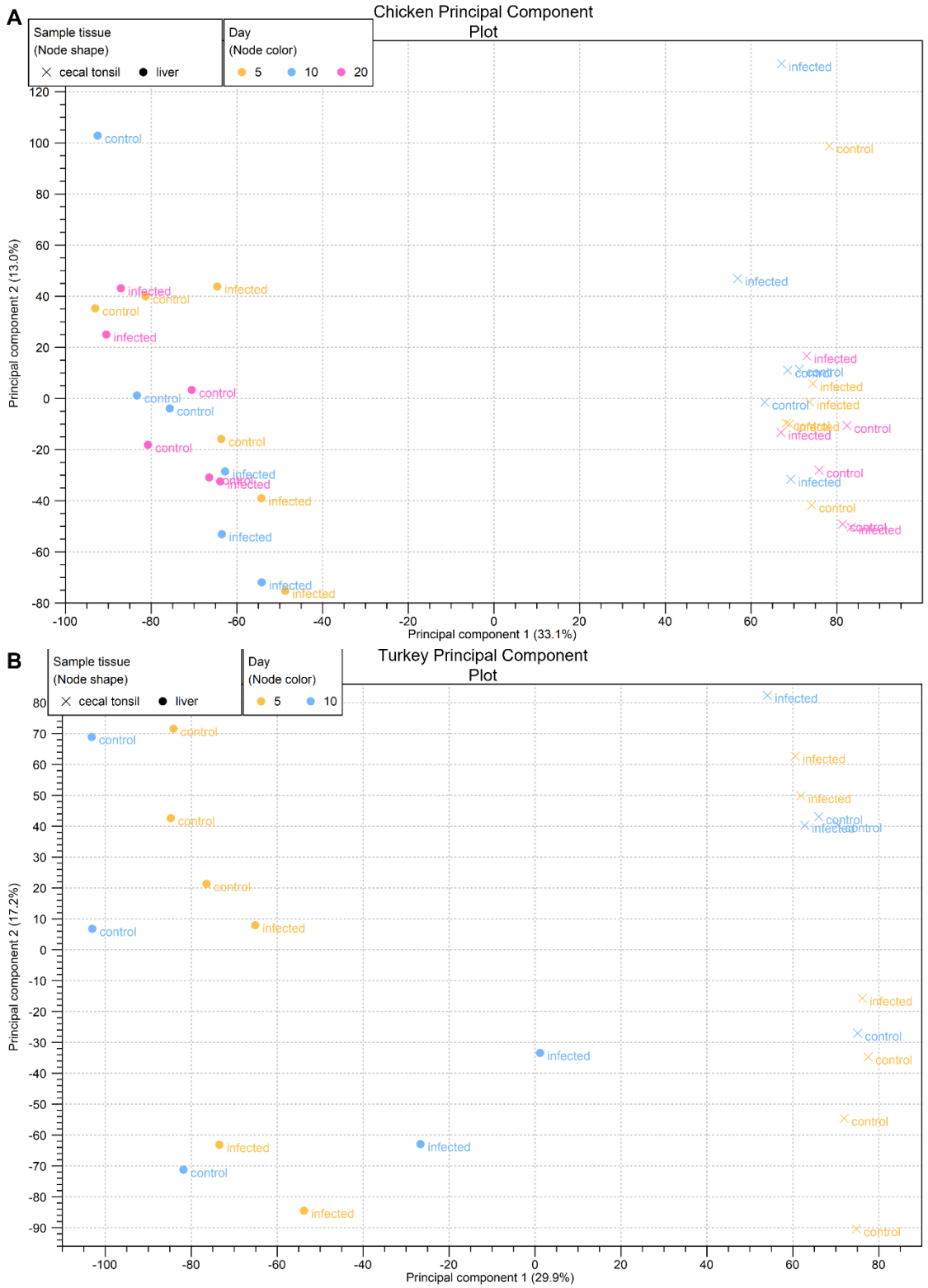

Figure 1: Principal component analysis (PCA) scatter plots for the cecal tonsil and liver tissue samples collected from infected and control chickens (**A**) and turkeys (**B**) at 5, 10, and 20 dpi*.

*Cecal tonsil and liver samples were unable to be collected from turkeys at 20 dpi.

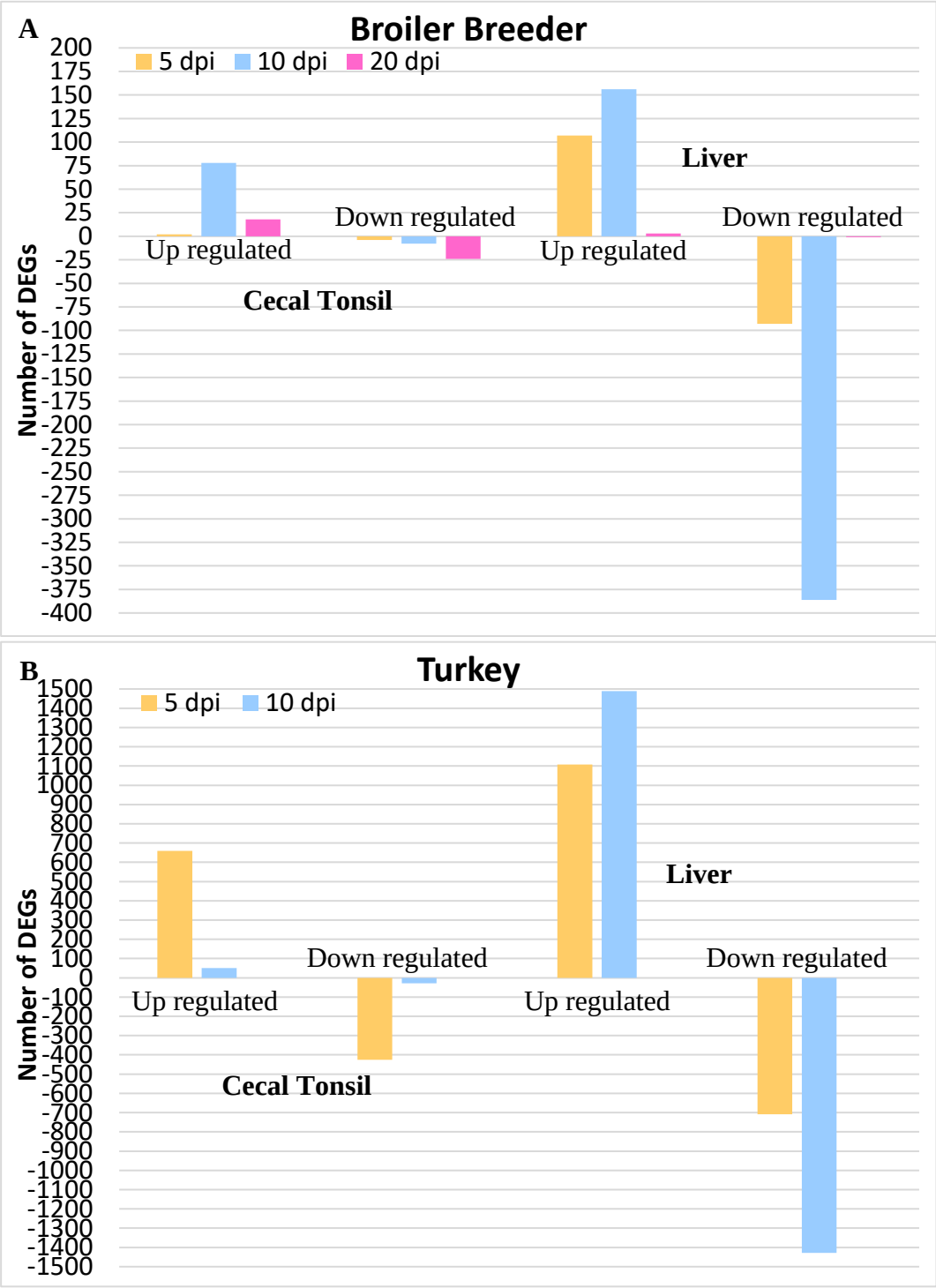

Figure 2: The number of up and down-regulated DEGs in the cecal tonsil and liver at each sampling time point for the chicken at 5 and 10 dpi (**A**) and turkey (**B**) based on an FDR q-value ≤ 0.05. Within either species, the largest degree of differential expression occurs in the liver at 5 and 10 dpi. Compared to the chicken, the turkey cecal tonsil and liver express a much larger number of DEGs. Note: Samples could not be collected from the turkey at 20 dpi.

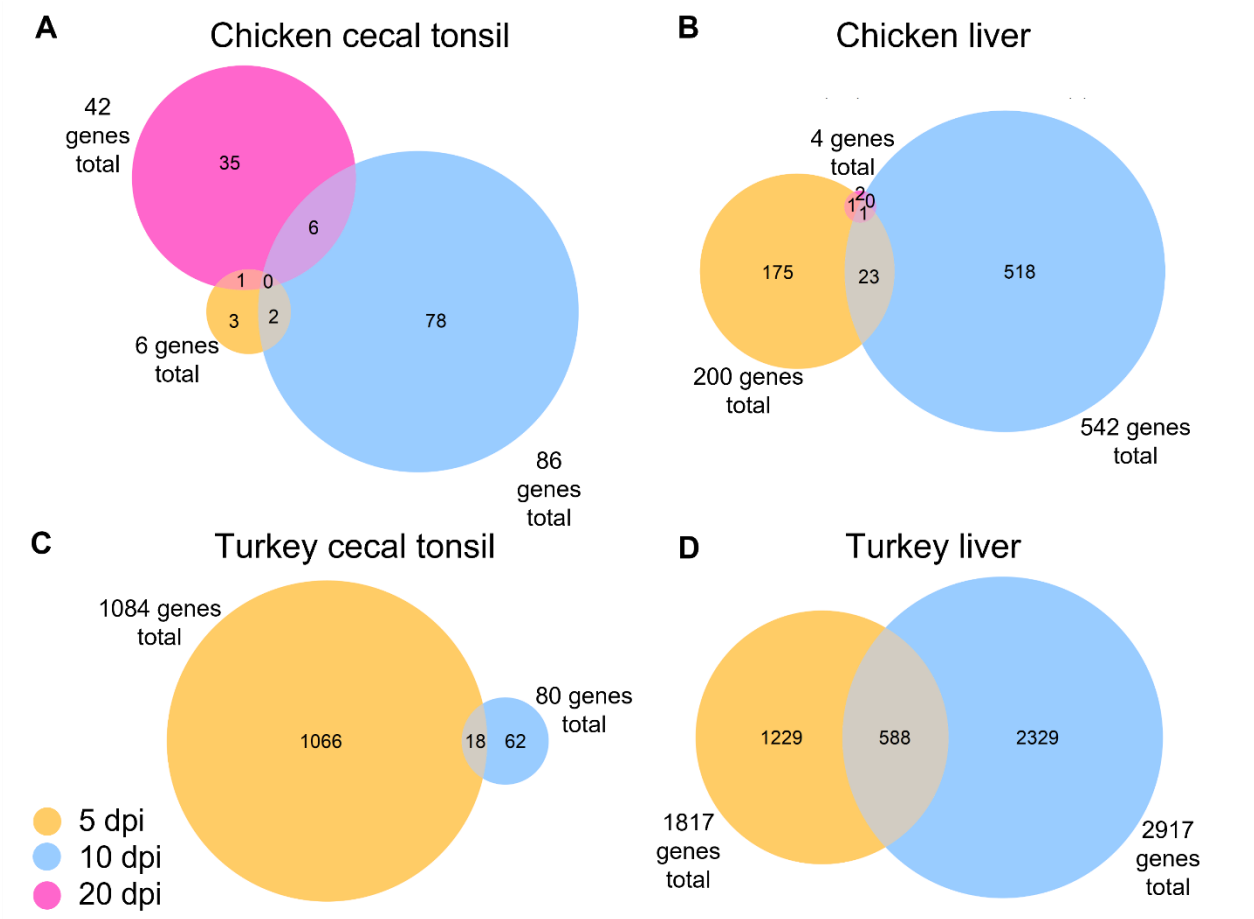

Figure 3: Venn diagrams displaying the number of unique and co-differentially expressed genes (DEGs) in the chicken and turkey cecal tonsil and liver at 5, 10, and *20 dpi. In the chicken cecal tonsil (**A**), the most shared DEGs occurs between 10 and 20 dpi, whereas the most shared DEGs in the chicken liver (**B**) occur between 5 and 10 dpi. In the turkey cecal tonsil (**C**), there are only 18 DEGs shared between both time points, whereas 588 DEGs are shared between 5 and 10 dpi in the turkey liver (**D**).
DEGs were considered significantly differentially expressed at an FDR q-value ≤ 0.05.
*Turkey cecal tonsil and liver samples were not collected at 20 dpi due to death of all turkeys by that time.

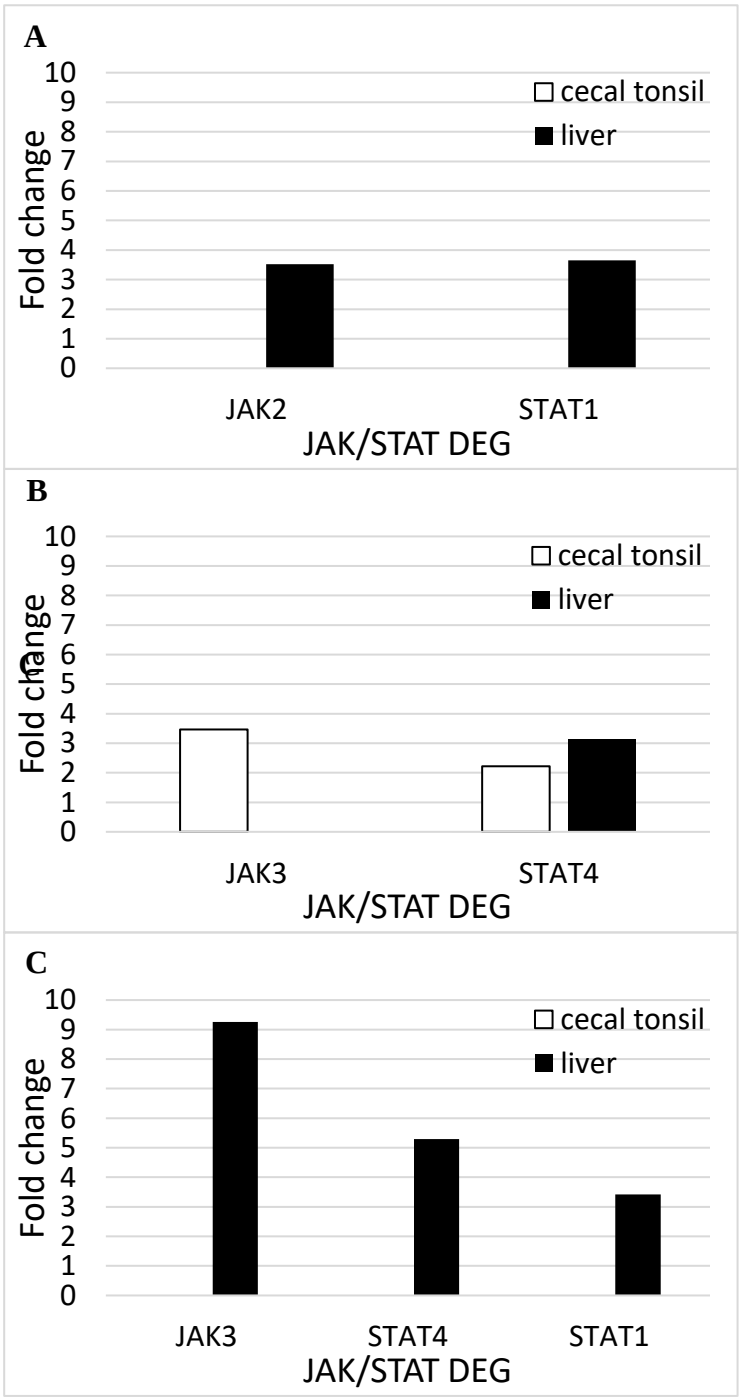

Figure 4: Fold change in JAK and STAT molecules in the cecal tonsil and liver of the chicken at 5 dpi (**A**) and turkey at 5 dpi (**B**) and 10 dpi (**C**).
DEGs were considered significantly differentially expressed at an FDR q-value ≤

CHAPTER 3: Immunocompetence and circulating leukocyte response in turkey poults 7 days after *Histomonas meleagridis* challenge

Abstract

Histomoniasis, caused by the protozoan parasite *Histomonas meleagridis*, has re-emerged as a costly communicable disease in turkeys following the removal of anti-histomonal chemotherapies from the market. Novel countermeasures are now being investigated for future control of this disease in commercial poultry. Vaccines have been effective for limiting disease caused by protozoal infections and serve as a proactive alternative to chemotherapeutic treatment of disease. Effective development of a histomoniasis vaccine has proven difficult due to a lack of knowledge for the interactions between the protozoan and host cells. Therefore, the goal of this study was to characterize the cellular immune response in the blood of turkey poults 7 days after inoculation with pathogenic cultures of *H. meleagridis*. First, the effects of *H. meleagridis* infection on the immunocompetence of turkey poults was measured using the phytohemagglutinin (PHA) skin swelling response test. The helper, cytotoxic, and regulatory T-cell response in the blood were then measured using flow cytometry analysis and granulocytes were enumerated on blood smears. The results from this study indicate that *H. meleagridis* infection suppresses the immunocompetence of turkeys 7 days after inoculation. Evaluation of circulating T-cells demonstrated that the percentage of cytotoxic T-cells was significantly altered in response to *H. meleagridis* infection at this stage of infection. Comparison of circulating lymphocytes to granulocytes suggests the turkey is responding to damage at 7-days-post-inoculation.

Introduction

Histomoniasis is an economically important infectious disease for chicken and especially turkey producers globally caused by the intestinal protozoan parasite *Histomonas meleagridis*. Following ingestion or cloacal uptake of *H. meleagridis* cells, the parasites travel to the cecal pouches where they multiply in the lumen and eventually penetrate the cecal mucosa and infect the epithelial tissue (McDougald, 1998, 2005; Hu et al., 2004). In these early stages of infection, the ceca become inflamed and leak proteins into the cecal lumen which accumulate as caseous exudate (Clarkson, 1966; McDougald, 1998). After 2 to 5 days, some invading *H. meleagridis* parasites enter the blood stream and travel via the hepatic portal vein to the liver (McGuire and Morehouse, 1958; Clarkson, 1961; McDougald, 1998). Liver accumulation of parasites and leukocytes (Singh et al., 2008), result in development of necrotic lesions. Eventually, in severe cases of disease usually observed in turkeys and in cases of additional stressors in chickens, accumulation of liver damage leads to failure of this organ and death of the bird (McDougald, 2013).

Previous research has demonstrated that both chickens and turkeys generate an immune response to *H. meleagridis* in the ceca and liver (Clarkson, 1963; Bleyen et al., 2009; Windisch and Hess, 2009, 2010; Powell et al., 2009; Kidane et al., 2018; Lagler et al., 2021). However, the turkey appears to generate a delayed immune response compared to the chicken (Powell et al., 2009). It has been hypothesized that this is the root cause of severe disease progression and death in the turkey which typically is avoided by the chicken. Mitra and colleagues have demonstrated that B-cell and T-cell distributions are significantly decreased in the ceca, liver, and blood of turkeys inoculated with virulent *H. meleagridis*, while only moderate and temporary changes in lymphocyte proportions were induced in the ceca of inoculated chickens (Mitra et al., 2017).

Powell and colleagues observed differential changes in the number of leukocytes infiltrating the liver of *H. meleagridis*-infected chickens and turkeys, but they also reported significant differences in cytokine mRNA expression leading to the conclusion that the turkey experienced greater pathology due to an inefficient innate immune response (Powell et al., 2009). While it is evident there are distinct cellular and molecular phenotypes between the chicken and turkey immune response to *H. meleagridis*, it is still not clear what is causing this divergence in immune activation.

Concerning the role of an adaptive immune response to *H. meleagridis*, the serum antibody response to histomoniasis is insufficient on its own to garner protective immunity (Clarkson, 1963; Bleyen et al., 2009). Therefore, it is presumed that the T-cell response is more critical in the adaptive immune response to histomoniasis. Regulatory T-cells which are characterized by the expression of CD4 and CD25 and lack of CD8 in poultry (Shanmugasundaram and Selvaraj, 2011) have not been evaluated in *H. meleagridis* infection of chickens or turkeys. These T-cells suppress rather than stimulate immune activity and are important in the regulation of immune responses (Shanmugasundaram and Selvaraj, 2011, 2012).

We hypothesized that the immunocompetence of the turkey is negatively affected by histomoniasis and that systemic cellular immune responses would be dominated by $CD4^+$ T-cells rather than $CD8^+$ T-cells and regulatory T-cells. Therefore, the objectives of this study were to evaluate the immunocompetence and systemic cellular immune response in turkey poults 7 dpi with pathogenic *H. meleagridis* in three separate experiments. First, as proof of concept, the immune responsiveness of poults infected with *H. meleagridis* was evaluated 7 dpi using the PHA-P (a mixture of 5 hemagglutinin isolectins which are composed of varying proportions of 2 PHA subunits, L and E; Yachnin et al., 1972) skin swelling test to measure immunocompetence

following *H. meleagridis* challenge. In the second experiment, $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ regulatory T-cell subsets were quantified in the blood of infected and non-inoculated poults 7 dpi via flow cytometry analysis. Based on an observation from this experiment, individual granulocytes and lymphocytes were quantified in blood smears taken from infected and non-infected poults at 7 dpi in a third experiment.

Materials and Methods

Birds and animal care

All experimental procedures for poults were performed following the guidelines of the North Carolina State University Animal Care and Use Committee (19-631). Three separate experiments were performed to (1) evaluate the immune responsiveness of poults infected; with *H. meleagridis* by measuring the skin swelling response to PHA-P injection (in two trials); (2) quantify T-cell subsets in peripheral blood; (3) estimate percentages of different leukocytes, including granulocytes, in peripheral blood. For all experiments, male white turkey poults were placed into isolation cages day of hatch and were given *ad libitum* access to non-medicated water and starter turkey feed for the duration of the experiments. Poults were allowed to acclimate to their environment until day 14, at which point any mortalities in the cages were replaced with poults from an extra isolation cage. In Experiment 1, 60 poults were placed in 6 cages with 10 poults per cage in two trials. For Experiment 2, 20 poults were placed in 2 cages with 10 poults per cage. For Experiment 3, 15 poults were placed in 2 cages with 10 in one and 5 in the other. Wing tags were placed through the patagium of the left wing for all poults in both trials of Experiment 1 and Experiment 3 to identify the individual birds.

H. meleagridis *inoculation*

Poults were intra-cloacally inoculated with 100,000 *H. meleagridis* cells in 1 ml of Dwyer's medium. In experiment 1, 3 cages (30 birds) were inoculated at 14 days of age, and the trial was repeated once with the same procedure and number of birds. In experiment 2, 1 cage (12 birds) was inoculated. In Experiment 3, the cage with 10 birds was inoculated at day 16. Infection of all inoculated poults were confirmed at the termination of each trial by necropsy and identification of necrotic lesions observed in the ceca and liver.

The PHA-P skin swelling test for immune responsiveness

For Experiment 1, the trial was repeated twice to measure the skin swelling response to intradermal PHA-P injection a week following *H. meleagridis* inoculation in three-week-old poults. In each trial, all 60 poults received intradermal PHA-P injections 6 days post inoculation (dpi) *H. meleagridis* inoculation, so that the differential skin swelling response to PHA-P injection following *H. meleagridis* infection could be determined. To prep the wing web for PHA-P injection, the feathers were removed from the upper patagium of the right wing for all 60 birds in each trial at 3 dpi. Three days following the removal of the patagium feathers (6 dpi), the pre-injection patagium thickness was measured using calipers. Two different calipers were used in either trial. In trial 1, a mechanical outside micrometer caliper was used. In trial 2, a microtome caliper that can accurately measure to within 0.01 mm was used. Then, approximately 100 µg of PHA-P (Sigma, St. Louis, MO) in 100 µl of 1x PBS was injected into the upper patagium of all poults within a 2-hour window. At approximately 27 to 29 hours following the first PHA-P injection (7 dpi), the post-injection patagium thickness was measured. Birds were raised to 4 weeks of age (14 dpi) and euthanized via cervical dislocation, then necropsied to

score the ceca and liver to confirm *H. meleagridis* infection. Index of stimulation (IS) was calculated for each bird using the following equation:

$$\left(\frac{postinjection - preinjection}{preinjection}\right) \times 100$$

Index of stimulation is a metric for comparing the inflammatory reaction to intradermal PHA injection based on the degree of swelling induced at the site of injection. IS values are positively correlated with inflammation.

In this experiment, isolation cage was the experimental unit. For statistical analysis in this experiment, the treatment was defined as the individual intra-cloacal inoculation with 100,000 *H. meleagridis* cells in 1 ml of Dwyer's medium or no inoculation at all on day 14. However, since birds were separated into three replicate cages per treatment per trial, the experimental unit was defined as the cage and the observational unit was defined as the individual bird. Data were analyzed using the MIXED procedure in SAS v9.4 (SAS Institute, Cary, NC, USA) with the main fixed effects of Trial, Treatment, and Trial x Treatment. To account for the correct degrees of freedom associated with the experimental unit, the cage) a random statement of Cage(Trial*Treatment) was applied to all the observational unit (the individual bird) data. LSMEANS were adjusted for Trial, Treatment, and Trial x Treatment. The PDIFF option was used for means separation and significance was defined at $P \leq 0.05$.

Flow cytometry for the enumeration of circulating T-cells

In Experiment 2, the percentages of circulating T-cell subsets one week following *H. meleagridis* inoculation were investigated. 3 ml of blood was collected via heart puncture into EDTA tubes from 8 inoculated (due to mortality prior to sample collection) and 10 non-inoculated poults at 7 dpi. Each poult was then euthanized via cervical dislocation and necropsied to score the ceca and liver for signs of histomoniasis. Only 4 inoculated poults

displayed signs of histomoniasis in the ceca and liver. Therefore, the blood samples from these 4 infected and 4 non-inoculated poults (selected at random) were used for flow cytometry analysis. The 3 ml blood samples were layered over 3 ml of room temperature Histopaque-1077 (Sigma Aldrich, St. Louis, MO) and centrifuged at 400 x g for 30 minutes at 21°C. The opaque plasma-Histopaque interface containing leukocytes was pipetted out using a Pasteur pipette and transferred to a clean 15 ml conical tube on ice. The cells were washed twice by adding 10 ml of cold 1x PBS followed by vortexing briefly, then centrifuging at 250 x g for 10 minutes at 4°C. The supernatant was decanted after each wash. The washed cell pellet was resuspended in 3 ml of cold 1x PBS, and a 1 ml 10x dilution was prepared from each sample to count the cells on a hemocytometer. The concentration of each 3 ml sample of cells was adjusted to $3x10^7$ cells/ml with cold 1x PBS.

For flow cytometry analysis of helper, cytotoxic, and regulatory T-cell subsets, a 1 ml aliquot ($3x10^7$ cells) of each sample was collected into microcentrifuge tubes and centrifuged at 250 x g for 10 minutes at 4°C. The supernatant was decanted, and the cells were resuspended in 1 ml of cold flow cytometry staining (FCS) buffer. 100 µl of each sample ($3x10^6$ cells) were transferred to clean microcentrifuge tubes. An additional seven 100 µl aliquots were pipetted into microcentrifuge tubes from one of the non-inoculated bird samples to serve as unstained, single fluorochrome, and isotype controls. The antibodies used for staining are provided in **Table 2**. The amount of each antibody added to the samples is provided in **Table 3**. CD4, CD8, and CD25 antibodies were added together to the experimental samples following resuspension of the cells in FCS buffer to total 300 µl. These three antibodies were also added to the respective single antibody controls at this time. The samples were vortexed briefly and incubated in the dark at 4°C for 30 minutes, then centrifuged at 500 x g for 30 minutes at 4°C. The cells were then fixed

and permeabilized using the cytofix/permeabilization kit (BD Biosciences, San Jose, California) as follows. The supernatant was decanted, and the cells were resuspended in 700 µl of BD cytofix/cytoperm buffer by vortexing, then incubated in the dark at 4°C for 20 minutes. The cells were centrifuged at 1000 x g for 5 minutes at 4°C and the supernatant was decanted. The cells were resuspended in 700 µl of BD perm/wash buffer and stored at 4°C overnight. The next day, the cells were centrifuged at 1000 x g for 5 minutes at 4°C to wash. All cell samples were resuspended in BD perm/wash buffer. CD3 or rat IgG1 antibodies were added to the experimental samples and CD3 control or isotype control, respectively, to total 300 µl. All other single antibody controls and the unstained control were resuspended in 700 µl of BD perm/wash buffer. The CD3/rat IgG1 stained cell samples were incubated in the dark at 4°C for 30 minutes, then centrifuged at 1000 x g for 5 minutes at 4°C. The supernatant was replaced with 700 µl BD perm/wash buffer. All stained and unstained samples were washed twice by centrifuging at 1000 x g for 5 minutes at 4°C then replacing the supernatant with another 700 µl of BD perm/wash buffer and repeating. After the final spin, the supernatant was replaced with 300 µl of BD perm/wash buffer and stored at 4°C for future flow cytometry analysis.

Stained cell samples were transferred to FACS tubes and analyzed on a Becton Dickinson LSRII analyzer (Becton Dickinson, Franklin Lakes, NJ). A representation of the gating hierarchy is presented in **Figure 1**. Cells were gated based on forward scatter (FSC) area and height then based on side scatter (SSC) area and height to remove doublets. Next, a lymphocyte gate and a total granulocytes gate was drawn based on FSC and SSC properties of the singlets. Total T-cells were gated from the lymphocytes based on staining of CD3ε. These T-cells were further divided into $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ populations. To quantify the regulatory-like T-cell population, $CD25^+$ cells were gated from the $CD4^+CD8^-$ T-cell population. Mean

percentages of each cell type from the parent cell population were calculated and are presented in **Table 4**.

For statistical analysis in Experiment 2, the treatment was defined as the individual intra-cloacal inoculation with 100,000 *H. meleagridis* cells in 1 ml of Dwyer's medium or no inoculation at all on day 14. Since the treatment was applied individually to each bird, the bird serves as both the experimental unit and the observational unit for this study. Data were analyzed using the GLIMMIX procedure in SAS v9.4 (SAS Institute, Cary, NC, USA) with the main fixed effect of Treatment. LSMEANS were adjusted for Treatment. The PDIFF option was used for means separation and significance was defined at $P \leq 0.05$.

Peripheral blood leukocyte response to H. meleagridis *infection*

Experiment 3 investigated the accumulation of leukocytes in the blood in the first week following *H. meleagridis* inoculation. For this trial, a drop of blood was drawn from each bird via wing veins to prepare a blood smear at 7 dpi. Each smear was stained with Camco Quick Stain II buffered differential Wright-Giemsa stain (Cambridge Diagnostic Products Inc., Fort Lauderdale, FL), according to the manufacturer's instructions. Birds were euthanized after smear preparation by cervical dislocation and necropsied to confirm *H. meleagridis* infection in the inoculated poults based on the presence of histomoniasis lesions in the ceca and liver. Only 5 inoculated poults were infected. The 5 blood smears from the infected poults and 5 smears from the non-inoculated poults were viewed under a bright field microscope with immersion oil at 1000x magnification to count heterophils, eosinophils, basophils, lymphocytes, and monocytes up to a total of 100 leukocytes per slide.

In Experiment 3, the treatment was defined as the individual intra-cloacal inoculation with either 100,000 *H. meleagridis* cells in 1 ml of Dwyer's medium or no inoculation at all on

day 14. Since the treatment was applied individually to each bird, the bird serves as both the experimental unit and the observational unit for this study. Leukocyte counts were analyzed using the GLIMMIX procedure in SAS v9.4 (SAS Institute, Cary, NC, USA) with the main fixed effect of Treatment. LSMEANS were adjusted for Treatment. The PDIFF option was used for means separation and significance was defined at $P \leq 0.05$. Heterophil to lymphocyte ratios were also analyzed between treatment groups using the MIXED procedure in SAS v9.4 with the main fixed effect of Treatment. LSMEANS were adjusted for Treatment. The PDIFF option was used for means separation and significance was defined at $P \leq 0.05$.

Results

The PHA skin swelling test

In Experiment 1, one poult from a control cage was culled after collecting the post-PHA measurement in trial 1. Four inoculated birds did not present histomoniasis lesions in the ceca or liver and were excluded from index of stimulation (IS) calculations. In the second trial, no poults died before post-PHA measurement, but 4 inoculated poults did not present histomoniasis lesions at 14 dpi and were excluded from IS calculations. There was no interaction between Treatment and Trial. However, there were main effects of each factor (**Table 1**). From the Treatment main effects, it can be determined that infected poults had a lower mean IS compared to non-inoculated poults. A significant difference in mean IS was also observed in the Trial main effects, where the mean IS in trial 2 was higher than in trial 1. The significant Trial effect is attributable to the use of two different types of calipers.

Flow cytometry analysis of circulating T-cells

In Experiment 2, blood was collected from 4 control poults and 4 *H. meleagridis*-infected poults 7 dpi to separate and quantify circulating leukocytes via flow cytometry analysis. The percentage of circulating granulocytes was significantly greater in infected poults while the percentage of circulating lymphocytes was significantly lower (**Table 4**). Within the $CD3^+$ T-cell population, there was a significantly lower percentage of $CD8^+CD4^-$ T-cells and a significantly higher percentage of $CD8^+CD4^+$ in the blood of infected poults (**Table 4**). Whereas there was no significant difference in the percentage of $CD4^+CD8^-$ and $CD4^-CD8^-$ T-cells between infected and non-inoculated poults at this time point. The percentage of $CD4^+CD8^-$ T-cells in the blood that were also $CD25^+$ was also not statistically different between infected and non-inoculated poults (**Table 4**).

Blood smear count of peripheral blood leukocytes

For experiment 3, a total of 100 leukocytes consisting of lymphocytes, heterophils, basophils, eosinophils, and monocytes were counted in a single blood smear for each of 5 infected and 5 non-inoculated poults. The mean percentage of each leukocyte subset was determined for both treatment groups. The percentage of lymphocytes was significantly lower in infected poults, as was observed in Experiment 2, while the percentage of heterophils was significantly greater in infected poults (**Table 5**). The percentage of all other circulating leukocyte cell types did not differ significantly between infected and non-inoculated poults. due to an increase in the concentration of heterophils in the blood (**Table 5**). The mean estimated ratio of heterophils to lymphocytes (H:L) in the blood of infected and non-inoculated poults, calculated as the number of heterophils divided by the number of lymphocytes, was 5.85 and 1.51, respectively. The ratio was significantly greater in the infected group (**Table 5**). Based on the increased density of heterophils observed in each field of view under 1,000x magnification

(**Figure 2**), it can be determined that the number of circulating heterophils increased in response to *H. meleagridis* infection.

Discussion

In all experiments, poults were allowed to grow and acclimate to their environment for at least 2 weeks prior to challenge to allow time for the immune system to mature. For example, heterophils, the most numerous of the avian granulocytes in the blood (Powell, 1987), require 14 to 21 days post-hatch to reach functional maturity (Lowry et al., 1997). Because the goal of this study was to evaluate changes in the percentages of circulating leukocytes in response to *H. meleagridis* infection, inoculating prior to 2 weeks could skew the analysis of the cellular immune response to the parasite. Clinical signs of histomoniasis and pathology develop in turkeys around 7 dpi, and peak mortality from histomoniasis occurs around 14 dpi (Mitra et al., 2017, 2021). Therefore, 7 dpi was selected as the singular time point for evaluating immune responsiveness and changes in quantities of circulating leukocyte in this artificial infection model.

The PHA-P skin swelling test

The phytohemagglutinin (PHA-P) test is a classical technique for comparing immune responsiveness or immunocompetence in sensitized animals and is especially suited to investigations of immune reactions in species where limited molecular tools for studying immunity are available. The profile of leukocytes infiltrating the tissue at the site of PHA-P injection changes dynamically over time and involves innate and adaptive immune cells (Martin et al., 2006). However, innate immune cells (such as heterophils, macrophages, and basophils) arrive within the first 6-12 hours following PHA-P injection then depopulate while lymphocyte

numbers remain elevated at 24 hours post-injection (McCorkle et al., 1980; Martin et al., 2006). PHA-P is also a known T-cell mitogen and does not activate B-cells *in vitro* (Geha et al., 1974). Therefore, swelling occurring 48 hours after intradermal injection of the mitogen is largely attributed to T-cell infiltration. Although the exact mechanism of inflammation induced by PHA injection is not well defined, Goto and colleagues demonstrated that swelling was significantly reduced in chicks that were thymectomized *in ovo* (Goto et al., 1978), suggesting that T-cells are responsible for a percentage of inflammation induced by the mitogen. The PHA test is used following host challenge with a pathogenic agent such as virus or bacteria to investigate the effect of the pathogen challenge on host immunocompetence. For example, in the case of frogs and salamanders inoculated with *Batrachochytridium dendrobatidis*, a fungus that causes chytridiomycosis, the skin swelling response to PHA-P injection is negatively correlated with the level of *B. dendrobatidis* infection (Fites et al., 2014; Venesky and Laskey, 2021).

In this study, a simplified protocol for the PHA skin swelling test (Smits et al., 1999) was used to determine if *H. meleagridis* infection reduces immunocompetence in turkey poults. The lower index of stimulation in *H. meleagridis* infected poults compared to non-infected poults indicates that *H. meleagridis* infection hinders the immune responsiveness of the birds. Because post-PHA measurements were taken 28-29 hours after injection, the reduction in inflammation in the infected birds may be attributable to a combination of innate and adaptive immune cells including heterophils and T-cells. To investigate the extent to which T-cells may be involved in the systemic immune response to *H. meleagridis* infection at this time point, flow cytometry analysis was conducted for Experiment 2 and percentages of circulating lymphocytes compared to monocytes, heterophils, basophils, and eosinophils were evaluated on blood smears in Experiment 3.

Flow cytometry analysis of circulating T-cells

Following identification of T-cells from total lymphocytes based on the expression of CD3, T-cell subsets were further resolved based on their expression of CD4 and CD8 to investigate changes in the concentration of $CD4^+CD8^-$ and $CD8^+CD4^-$ broadly functional T-cell types (Kaiser, 1996) in the blood of infected poults. Additionally, within $CD4^+CD8^-$ T-cells, regulatory T-cells (T_{reg} cells), which suppress immune activation, were also quantified. T_{reg} cells suppresses rather than stimulate immune cell proliferation and activation through direct cell-to-cell contact and through indirect signaling (Selvaraj, 2013). Therefore, changes in T_{reg} cell activity during an immune response are critical to control immune activities. Concentration of this T-cell type in the blood of infected chickens or turkeys has not been previously investigated.

Based on the predominantly extracellular nature of *H. meleagridis* (Lagler et al., 2021), it was hypothesized that $CD8^+CD4^-$ cytotoxic T-cells, which are an effective component of immune responses to intracellular pathogens (Kaiser, 1996; Tzelepis et al., 2006; Bangham, 2009), are not a highly effective component of the adaptive immune response to *H. meleagridis*. $CD4^+CD8^-$ T_H cells, on the other hand, are integral in the control and regulation of the adaptive immune response to intracellular and extracellular pathogens and are further divided into two or more functional subsets (Romagnani, 2000; Erf, 2004; Reiner, 2007). Therefore, the involvement of certain T_H subsets in the anti-*H. meleagridis* immune response was predicted to be reflected in the alteration of the absolute number of $CD4^+CD8^-$ T-cells in the blood of infected poults.

The decreased percentage of $CD8^+CD4^-$, elevated percentage of $CD8^+CD4^+$ and unaltered percentages of $CD4^+CD8^-$ in the blood of infected poults may indicate that the $CD8^+$ T-cell response is more greatly altered by *H. meleagridis* infection than that of $CD4^+$ T-cells at 7 dpi. This contrasts the idea that mechanisms against extracellular parasites rather than intracellular

parasites would be elicited in *H. meleagridis*-infected poultry. At 7dpi, *H. meleagridis* had already begun colonizing the liver as demonstrated by lesion accumulation in this organ of the infected poults (data not shown). Previous research in the turkey has demonstrated that, following *H. meleagridis* transport to the liver, macrophages, heterophils and T-cells accumulate in the liver surrounding *H. meleagridis* cells (Singh et al., 2008). Furthermore, in this same study, *H. meleagridis* were observed to be engulphed by immune cells in the ceca and liver. It is possible the percentage of $CD8^+CD4^-$ T-cells are decreased in the blood because these cells are leaving the circulation in greater numbers than $CD4^+$ T-cells to enter infected tissues. Therefore, the decreased percentage of circulating $CD8^+CD4^-$ T-cells may indicate the importance of the intracellular state of *H. meleagridis* on the modulation of the immune response at this time point. Additionally, this recruitment of $CD8^+$ cytotoxic T-cells may be at least partially responsible for necrosis caused to organs infected with *H. meleagridis* as has been proposed by Mitra and colleagues (Mitra et al., 2021). However, further research is needed to identify $CD8^+$ T-cells in *H. meleagridis* infected tissues and to characterize their interaction with the parasite.

Numbers of T_{reg} cells within the $CD4^+CD8^-$ population were not significantly altered by infection. This may indicate that the activity of T_{reg} cells is not significantly altered by *H. meleagridis* infection in the turkey poult at this time point. Previous research has demonstrated that turkeys elicit a delayed cytokine storm at the time *H. meleagridis* cells begin to colonize the liver whereas chickens exhibit a much earlier and less pronounced cytokine and cellular response (Powell et al., 2009; Mitra et al., 2017; Kidane et al., 2018). The relative lack of immune regulation elicited by unaltered percentage of T_{reg} cells in the blood of infected turkeys is further evidence that the turkey is actively propagating an immune response at this intermediate-late stage of disease development. However, the question remains whether the unaltered activity of

T_{reg} cells is a cause or a consequence of the progression to fatal histomoniasis in turkeys. Further research is needed to investigate the influence of immune regulation on the outcome of histomoniasis in the turkey.

Estimation of leukocytes in blood smears

From Experiment 2, it was noticed that the percentages of lymphocytes decreased while the percentage of granulocytes increased in *H. meleagridis*-infected poults. Experiment 3 was performed to further resolve the changes in circulating lymphocytes and granulocytes. Blood smears prepared with a coverslip and stained with Romanovsky (Wright's-Giemsa) stains are an accessible and reliable method for estimating percentages of circulating leukocytes in birds (Walberg, 2001). This technique was utilized to investigate changes in the proportions of different granulocytes with respect to lymphocytes by quantifying the number of each leukocyte type up to 100 total leukocytes in each smear. Results from this experiment demonstrated that the decreased percentage of lymphocytes and increased percentage of granulocytes observed from the flow cytometry analysis (Experiment 2) was the result of an increase in the concentration of heterophils in the blood (**Figure 5)**, leading to an increase in the ratio of heterophils to lymphocytes (H:L ratio). This result contrasts observations made in chickens infected with *H. meleagridis* which showed, via flow cytometry analysis of leukocytes, that circulating monocytes/macrophages increased and heterophils decreased significantly between 4 and 14 dpi (Mitra et al., 2017). It was suggested that the increased number of monocytes/macrophages in the blood of infected chickens was indicative of innate immune activation. Changes in circulating heterophils, however, may be indicative of stress, in this case resulting from damage to the ceca and liver. The H:L ratio is a commonly used indicator of stress in animals (Gross and Siegel, 1983; Campo et al., 2008). Heterophil concentration in the blood increases in response to

elevated stress leading to an elevated H:L ratio, while the opposite is true in the case of immune activation following infection (Gross and Siegel, 1983; Davis et al., 2008). The H:L ratio induced by stress or infection has been used to select for resistance to physical stress as well as salmonella infection in poultry (Al-Murrani et al., 2006, 2007; Thiam et al., 2021). Higher stress responses, and therefore higher H:L ratios, are associated with increased susceptibility to infectious disease in poultry raised for meat production (Huff et al., 2005). In the context of this study, the increase in the estimated H:L ratio observed in poults 7 dpi with *H. meleagridis* may be induced by damage caused to the ceca and/or liver and may indicate that the birds are responding primarily to damage rather than infection. Further investigation of the accumulation of heterophils in the blood, ceca and liver of *H. meleagridis*-infected poults is needed to determine if heterophils are leaving the blood to enter tissues damaged by *H. meleagridis* infiltration.

Conclusion

In summary, *H. meleagridis* infection in turkeys negatively affects immunocompetence as determined by PHA skin swelling analysis. Quantification of T-cell subsets via flow cytometry suggests that $CD8^+$ cytotoxic T-cells play a significant role in the immune response to histomoniasis 7 dpi. Furthermore, based on the quantification of leukocytes from blood smears, it was evident that poults exhibited a stress response to *H. meleagridis* infection at 7 dpi as demonstrated by an increased H:L ratio. These findings are suggestive of considerable immunopathology resulting in the ceca and liver of *H. meleagridis*-infected poults 7 dpi. It may be possible to limit the damage caused to these tissues by initiating an earlier immune response

or improving mucosal immunity at the cecal epithelium where infection is initiated to decrease the number of parasites that infiltrate the host.

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Table 1: Mean index of stimulation from trials 1 and 2 in Experiment 1.

Variable	**Index of Stimulation**	
Treatment	**Trial 1**	**Trial 2**
Non-Inoculated	108.13 ± 14.09	218.18 ± 14.09
Infected	58.14 ± 14.09	166.56 ± 14.09
Treatment Main Effect		
Non-Inoculated	163.15 ± 9.96[a]	
Infected	112.35 ± 9.96[b]	
Trial Main Effect		
Trial 1	83.14 ± 9.96[b]	
Trial 2	192.37 ± 9.96[a]	
Probability		
Trial Effect	<0.0001	
Treatment Effect	0.0080	
Trial*Treatment Effect	0.9819	

Means ± SEM

Means across rows within the same variable column with no common superscript differ significantly ($P < 0.05$).

Means within the same column with no common superscript differ significantly ($P < 0.05$).

Table 2: List of antibodies used in Experiment 2.

Antigen	**Clone**	**Species specificity**	**Isotype**	**Fluorochrome**	**Source of primary antibody**
CD3ε	CD3-12	Human	Rat IgG1	Pacific Blue	BioRad
CD4	CT-4	Chicken	Mouse (BALB/c) IgG1κ	Alexa Fluor 700	Southern Biotech
CD8	3-298	Chicken	Mouse IgG2bκ	R-phycoerythrin (PE)	Southern Biotech
CD25	AbD13504	Chicken	Human HuCAL Fab bivalent	fluorescein isothiocyanate (FITC)	BioRad
Horseradish peroxidase	N/A	N/A	Rat IgG1	Pacific Blue	BioRad

All antibodies were monoclonal, primary antibodies directly conjugated to a fluorochrome.
The Rat IgG1 antibody was used as an isotype control for the CD3ε antibody.

Table 3: Amount of each antibody added to experimental and control samples in Experiment 2.

Sample type	**Antibodies included per $3x10^7$ cell aliquot**
All experimental samples	1.5 µg CD3, 0.3 µg CD4, 3 µg CD8, 15 µg CD25
Unstained control	N/A
Isotype control	1.5 µg Rat IgG1 isotype
CD3 control	1.5 µg CD3 only
CD4 control	0.3 µg CD4 only
CD8 control	3 µg CD8 only
CD25 control	15 µg CD25 only

Table 4: Percentage of leukocytes quantified from whole blood via flow cytometry.

	Treatment		
Variable	**Non-Inoculated**	**Infected**	**P-value**
Granulocyte (%)	6.11 ± 0.06^{b}	41.16 ± 0.11^{a}	<0.0001
Lymphocyte (%)	53.73 ± 0.11^{a}	30.26 ± 0.11^{b}	<0.0001
CD8+CD4+ (%)	7.88 ± 0.44^{b}	10.91 ± 0.74^{a}	0.0102
CD8+CD4- (%)	16.01 ± 0.60^{a}	11.42 ± 0.75^{b}	0.0041
CD4+CD8- (%)	20.69 ± 0.66	23.11 ± 1.00	0.0864
CD8-CD4- (%)	55.42 ± 0.82	54.56 ± 1.18	0.5699
CD4+CD25+ (%)	2.34 ± 0.55	0.97 ± 0.48	0.1582

Means ± SEM

Means across rows within the same variable column with no common superscript differ significantly ($P < 0.05$).

Means within the same column with no common superscript differ significantly ($P < 0.05$).

Granulocytes and Lymphocytes are percentages of total singlet leukocytes.

CD8+CD4+, CD8+CD4-, CD4+CD8-, and CD8-CD4- cells are percentages of CD3+ lymphocytes (parent population not shown).

CD4+CD25+ T-cells are a percentage of CD4+CD8- T-cells.

Table 5: Percentage of leukocyte subsets from non-inoculated and infected poults based on survey of 100 total leukocytes counted from 1 blood smear per bird.

	Treatment		
Variable	**Non-Inoculated**	**Infected**	**P-value**
Lymphocytes (%)	34.6 ± 2.1^{a}	14.2 ± 1.6^{b}	<0.0001
Heterophils (%)	51.4 ± 2.2^{b}	72.6 ± 2.0^{a}	0.0001
Eosinophils (%)	0.0 ± 0.0	0.0 ± 0.0	1.0000
Basophils (%)	4.4 ± 0.9	2.0 ± 0.6	0.0686
Monocytes (%)	9.6 ± 1.3	11.2 ± 1.4	0.4317
Heterophil:Lymphocyte Ratio	1.51 ± 0.76^{b}	5.85 ± 0.76^{a}	0.0037

Means ± SEM

Within variable row, means with no common superscript differ significantly ($P < 0.05$).

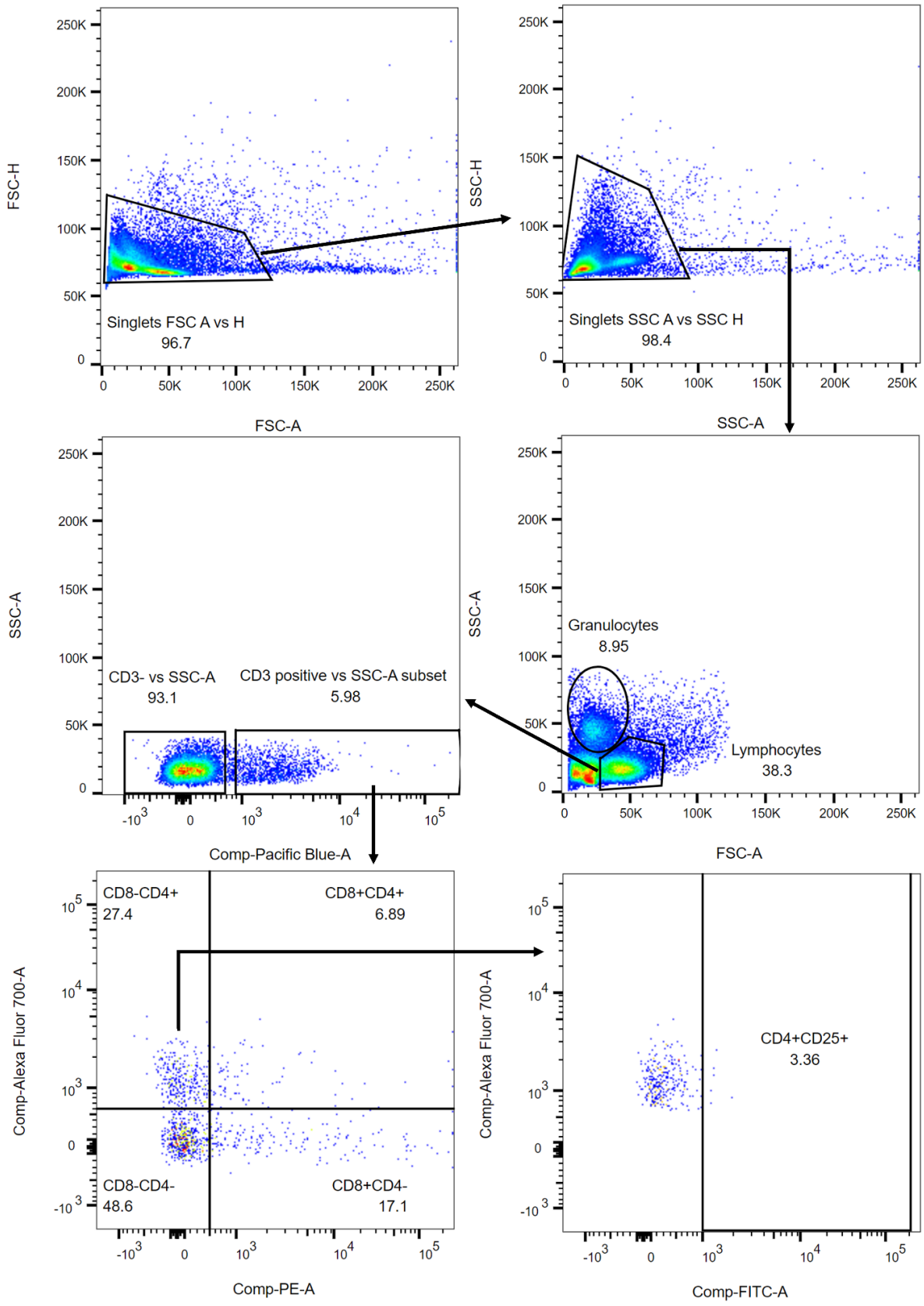

Figure 1: Representation of gating hierarchy for all leukocyte samples.

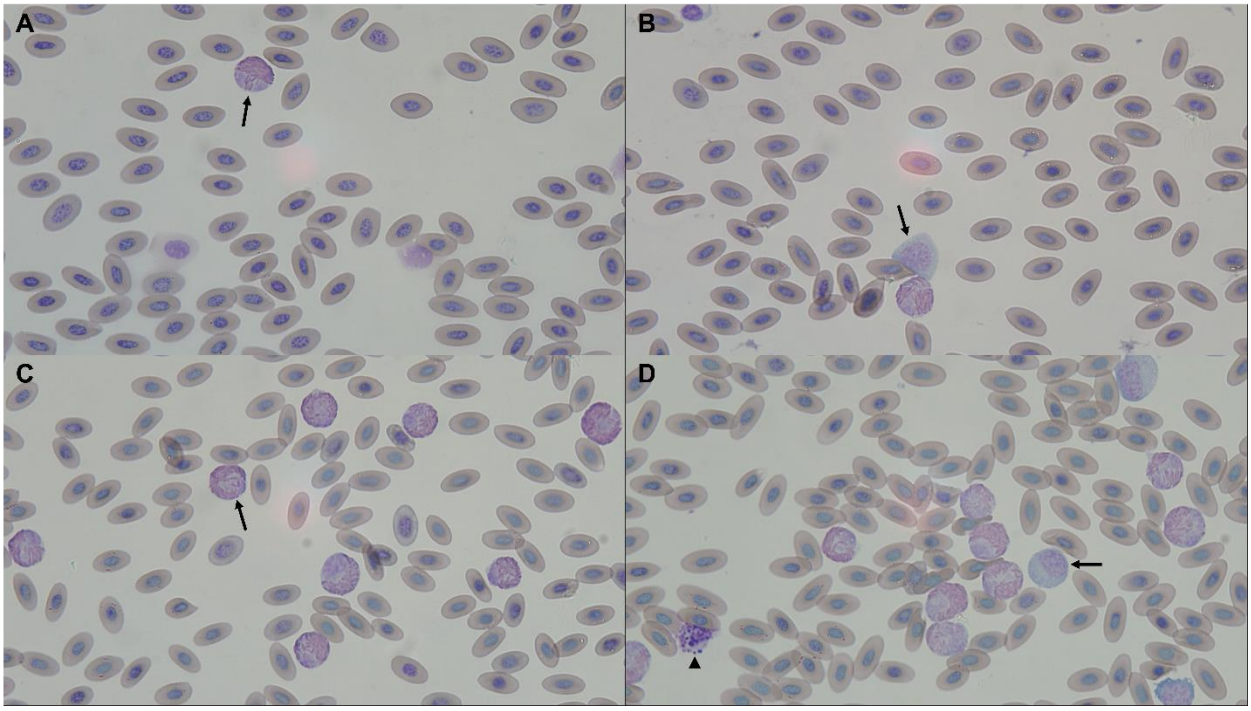

Figure 2: Representative fields of view in non-infected (A and B) and infected (C and D) blood smears viewed with immersion oil at 1,000x magnification. The arrow in A and C identify heterophils. The arrows in B and D identify monocytes. The arrowhead in D identifies a basophil.

CHAPTER 4: Evaluation of sodium bisulfate on the performance of turkeys with coccidiosis vaccination

Abstract

This study was performed to investigate the effects of dietary supplementation of sodium bisulfate on the performance of turkeys given a 2x dose of a coccidiosis vaccine. 960 day-of-hatch male Nicholas Select poults were weighed and randomly divided into 6 experimental groups with 8 replicates in a 2 × 3 factorial arrangement with non-vaccinated or vaccinated treatment (oral inoculation of 2x IMMUCOX®T at day 0) and dietary supplementation of 0% (control), 0.2%, or 0.4% feed-grade sodium bisulfate. Oocyst counting was performed on day 7 and 14 to confirm parasite cycling and vaccine viability. Body weight, feed intake, and feed conversion ratio (FCR) were evaluated at 0, 35 and 56 days. Body weight uniformity was also evaluated at 56 days, and livability was evaluated from 0 – 56 days. Data were analyzed by MIXED procedure in SAS v9.4. The results showed that 2x coccidiosis vaccination caused a failure to gain weight at 35 days of age, which was recovered at 56 days of age, and a lower FCR at 35 days of age. Vaccinated birds given 0.4% sodium bisulfate feed achieved higher body weights that were more similar to those of non-vaccinated groups at 35 days. Birds given 0.2% or 0.4% sodium bisulfate feed had lower feed conversion ratios compared to birds given the control diet from 0 – 56 days. Livability in vaccinated birds given 0.2% or 0.4% sodium bisulfate feed only decreased 2.14% and 1.43%, respectively. Body weight uniformity was not significantly altered between treatment groups or vaccinated groups at 56 days.

Introduction

Coccidiosis is a highly transmissible protozoan parasitic infection affecting the lining of the intestines. Coccidiosis is caused by ingestion of coccidia oocysts, each of which release four

infective parasite cells called sporozoites (McDougald and Fitz-Coy, 2013). The sporozoites invade epithelial cells lining the villi of the intestines where they undergo asexual replication, a process in which they produce new infective cells called merozoites that burst out of the host epithelial cells and infect other epithelial cells (Levine, 1973; McDougald and Fitz-Coy, 2013; Deplazes et al., 2016). Depending on the species and strain of coccidia, these parasites typically undergo two or more rounds of asexual replication before producing macrogametocytes and microgametocytes in the final stage of the life cycle (McDougald and Jeffers, 1976; El-Sherry et al., 2014, 2019). Because there is a greater number of merozoites infecting the epithelial cells after each replication cycle, the greatest amount of damage caused by the reproduction of these parasites inside host cells is caused during the last asexual replication cycle and gametogony (Levine, 1973).

E. meleagrimitis and *E. adenoeides* are two of the most prevalent and pathogenic coccidia species in turkeys (Edgar, 1986; McDougald and Fitz-Coy, 2013; Rathinam et al., 2015). *E. adenoeides* is the most pathogenic species of *Eimeria* infecting turkeys and primarily causes pathology in the lower intestinal tract (McDougald and Fitz-Coy, 2013). *E. meleagrimitis* is the most pathogenic *Eimeria* species infecting the upper digestive tract of turkeys; although, infections with this species alone are less severe than infection with *E. adenoeides* (McDougald and Fitz-Coy, 2013). Because coccidiosis results in reduced feed intake and weight gain as well as causes mortality in commercial flocks, the poultry industry invests a large sum to prevent infection and mitigate the negative effects of coccidiosis on bird growth performance.

Poultry producers alternate between vaccinating their flocks with a cocktail of live *Eimeria* species oocysts to boost flock immunity to natural infection and administering a coccidiostat in the feed to prevent infection. The weakened strains of *Eimeria* in the vaccine are

still able to replicate and cause damage to the intestines which, although more mildly, reduces early bird performance. Combination of feed additives that improve gut health and bird performance with the coccidia vaccine may be a practical approach to limit the negative effects of the vaccine while still boosting the young birds' immunity to subsequent infection with coccidia oocysts (Alfaro et al., 2007; Reisinger et al., 2011; Mathis et al., 2014; Ritzi et al., 2016; Roto et al., 2017; Tonda et al., 2018; Yang et al., 2020; Bafundo et al., 2021).

Sodium bisulfate ($NaHSO_4$), a dry granular acid salt, is typically administered as a litter amendment to reduce ammonia and has been investigated as a litter treatment for reducing *Campylobacter* and *Salmonella* colonization of broilers (Line, 2002; Kassem et al., 2012; Williams et al., 2012; Johnson et al., 2021). However, *Salmonella* populations in cecal contents have been demonstrated to be reduced by the addition of sodium bisulfate to the culture *in vitro* (Rubinelli et al., 2017). Administration of sodium bisulfate in poultry feed has also been demonstrated to reduce coccidiosis lesions and negative effects on bird performance in chickens challenged with *Eimeria* coccidia (Talghari et al., 2020; Chadwick et al., 2020; Suarez et al., 2021). Furthermore, administration of sodium bisulfate in the presence of a coccidia challenge has been shown to improve intestinal epithelium morphology and was not observed to affect coccidia cycling (Chadwick et al., 2020). However, the role of sodium bisulfate in coccidia challenges in turkey has not been researched. It is reasonable to assume a similar effect may be observed in turkeys challenged with *Eimeria*. Combination of sodium bisulfate administration in turkey feed and *Eimeria* vaccination may be a practical and effective means of generating flock immunity to natural *Eimeria* infection while mitigating the negative effects of vaccination on early poult performance.

To investigate the effects of combining sodium bisulfate administration and *Eimeria* vaccination on poult performance and the generation of *Eimeria* immunity, turkey poults were divided into a cocci-challenge and a non-challenge group. Poults placed in the cocci-challenge group were orally inoculated with a 2x dose of IMMUCOX®T vaccine, which administered a combined total of approximately 600 live oocysts of *E. meleagrimitis* and *E. adenoeides*, day of hatch. The vaccinated and non-vaccinated groups were both subdivided into 3 experimental groups with a basal starter diet not supplemented with sodium bisulfate (control diet) or supplemented with 0.2% or 0.4% sodium bisulfate. To prevent infection with naturally occurring oocysts in the environment, the basal diet provided to all non-vaccinated birds was medicated with 0.05% Amprolium. Oocyst shedding was measured in the first 2 weeks. Bird performance parameters were measured from 0 – 35, 35 – 56, and 0 – 56 days.

Materials and Methods

Animals, vaccination, and experimental diets

All experimental procedures for poults were performed following the guidelines of the North Carolina State University Animal Care and Use Committee (18-138-A). A total of 960 day-of-hatch male Nicholas Select poults (WV, US) were weighed and randomly divided into 6 experimental groups with 8 replicates (20 birds/replicate). The experimental design was a completely randomized block design with 2 × 3 factorial arrangement of non-vaccinated (with 0.0125% Amprolium) or vaccinated (oral inoculate with 2x IMMUCOX®T (Lenexa, KS, USA) at day 0) vaccination status groups and feed additive treatments of 0% (control), 0.2%, and 0.4% feed-grade sodium bisulfate (Jones-Hamilton Co., Walbridge, OH). Experimental diets and water were provided *ad libitum* from day 0 – 56. The diets were formulated to meet the nutrient requirements according to Commercial Nutrition Recommendations from Aviagen (2010). The

ingredients of stater diets are shown in **Tables 1 and 2** as percentages, and the chemical composition of the diets are shown in **Table 3**. Amprolium, a coccidiostat, was added to the feed of non-vaccinated birds to ensure they did not become infected with wildtype oocysts from the environment. Poults were observed twice a day to monitor general health, clinical signs of disease, adverse vaccine reactions, and mortality until trial completion. The lighting and temperature program was set according to the Management Guidelines of Aviagen (2021).

Oocyst counts

Fresh fecal samples were collected from 3 randomly selected birds/pen for all groups on days 7 and 14. Selected birds were placed on a cold metal surface to induce fecal expulsion. The feces were collected in individual sample bags and stored at 4°C until further processing. The oocyst counting was performed using McMaster chambers according to the protocol by Conway and McKenzie (Conway and McKenzie, 2007). In brief, 2 g of each fecal sample were soaked in 20 ml of tap water for 24 hours at 4°C, strained through muslin into conical tubes and centrifuged at 200 rcf in 15 ml of tap water to precipitate the solute. The solute was resuspended in 3 ml of 10% (w/v) NaCl solution and transferred to a McMaster chamber using a micropipette. Oocysts were enumerated at 40x magnification using a brightfield upright microscope. The data are presented as oocysts per gram (OPG) of feces.

Growth performance

Body weights pooled by pen and feed weights were recorded at 0, 35 and 56 days, and the average body weight, feed intake, and mortality-corrected feed conversion ratio (FCR) were calculated. On day 56, all birds were individually weighed to determine body weight uniformity for each pen. The individual body weight data was used to calculate a coefficient of variation for

each pen that was used in the subsequent analysis for body weight uniformity. Livability was determined for 0 – 56 days.

Statistical analysis

The oocysts shedding on days 7 and 14 were analyzed using one-way ANOVA using SAS v9.4 (SAS Institute, Cary, NC, USA). For this study, the experimental unit was defined as the pen. For all performance parameters under analysis, data were analyzed using the MIXED procedure in SAS v9.4 with the main fixed effects of Feed Additive, Vaccination Status, Feed Additive x Vaccination Status and Block. A significant Feed Additive x Vaccination Status interaction was detected for Day 0 body weight; therefore, a covariate of Day 0 body weight was applied to all other traits under analysis. The final model for all performance parameters, with the exception of body weight at day 0, contained the fixed effects of Feed Additive, Vaccination Status, Feed Additive x Vaccination Status and Block as well as the covariate for day 0 body weight. LSMEANS were adjusted for Feed Additive, Vaccination Status and Feed Additive x Vaccination Status. The PDIFF option was used for means separation and significance was defined at $P \leq 0.05$.

Results

Oocyst Counts

To validate the infection status of each treatment, fresh fecal samples were collected for oocyst shedding evaluation. The results showed the non-vaccinated control group had 0 OPG (data not shown). OPG for all other treatment groups was not significantly influenced by different levels of sodium bisulfate supplementation at 7 days and 14 days (**Table 4**).

Growth performance

The mean body weight of poults distributed into pens given 0.2% sodium bisulfate feed was significantly higher than the other treatments at placement (**Table 5**). Although not statistically significant, the mean body weight of poults distributed among non-vaccinated pens was higher than that of vaccinated pens at placement (**Table 5**). This resulted in an interaction effect between feed additive and vaccination at 0 days. For this reason, a covariate for day 0 bodyweight was included in the statistical model for all other performance parameters and for body weight at 35 and 56 days.

At 35 days, there was an interaction between feed additive and vaccination where vaccinated poults given 0.4% sodium bisulfate feed had higher body weights than either of the other vaccinated groups (**Table 5**). Between vaccinated and non-vaccinated groups, the mean body weight of vaccinated poults on control diet or 0.2% sodium bisulfate feed were significantly lower than those of the respective non-vaccinated treatment groups. The higher body weight of the vaccinated 0.4% sodium bisulfate group was more similar to the mean body weights of the non-vaccinated sodium bisulfate treatment groups. The main effects of vaccination at this age indicate that the 2x vaccination negatively affected body weight at this early life stage. No significant effects of vaccination or feed additive were observed on body weight at 56 days.

An interaction between feed additive and vaccination was observed for feed intake from 0 to 35 days (**Table 6**). Non-vaccinated poults given 0.4% sodium bisulfate feed had significantly lower mean feed intake per bird compared to the other two non-vaccinated treatment groups. No significant difference in feed intake was observed between vaccinated treatment groups. Between vaccinated and non-vaccinated groups, the mean feed intake per poult

in the vaccinated control group was significantly lower than that of the non-vaccinated control group over the first 35 days. No significant effects of vaccination or feed additive were observed from 35 – 56 days or 0 – 56 days (**Table 6**).

No interactions were observed at any age interval for FCR, but the main effects of feed additive and vaccination were significant at 0 – 35 days and 35 – 56 days (**Table 7**). In the earliest age interval, 0 – 35 days, poults given 0.4% sodium bisulfate feed had significantly reduced FCR compared to the control or 0.2% sodium bisulfate feed groups. Vaccination at this age interval resulted in higher FCR. By contrast, poults give the 0.2% sodium bisulfate feed had a significantly lower FCR compared to poults fed the control diet from 35 – 56 days. Vaccination at this later age interval reduced FCR. The main effects of sodium bisulfate treatment on overall poult performance, 0 – 56 weeks, indicate that inclusion of sodium bisulfate at either 0.2% or 0.4% reduced FCR over the control diet.

There was no significant effect of feed additive or vaccination on pen body weight uniformity at 56 days (data not shown). Lastly, the livability determined from 0 – 56 days for vaccinated groups given 0.2% or 0.4% sodium bisulfate feed were 97.86% and 98.57%, respectively. 0% mortality was observed in all other vaccinated and non-vaccinated treatment groups. The main effects of vaccination indicate that the greatest reduction in livability was caused by vaccination status opposed to inclusion of sodium bisulfate in the diet (data not shown).

Discussion

The excretion of *Eimeria* oocysts in all vaccinated groups but not non-vaccinated groups confirms that non-vaccinated poults remained non-infected and that the vaccinated poults were

successfully infected and cycled oocysts in the litter. The decrease in early bird performance resulting from *Eimeria* infection observed in this study is likely due to the costs of immune activation against the parasites. Activation costs of immunity may be accrued due to reallocation of energy or nutrients away from other functions such as growth or reproduction and/or due to impaired nutrient acquisition (DiAngelo et al., 2009; Bashir-Tanoli and Tinsley, 2014). In fact, infection-induced anorexia, the behavior adopted by infected animals whereby they reduce their feed intake, may be the most significant cost of immune activation rather than resource reallocation to immune cells (Exton, 1997). This phenomenon was observed in the current study: FI of vaccinated control birds was significantly reduced compared to non-vaccinated control birds (**Table 6**). In addition, in the case of coccidiosis where the enterocytes are damaged by the replication of parasites (McDougald and Fitz-Coy, 2013), it is possible this damage exacerbates the reduced nutrient acquisition costs by decreasing nutrient absorption.

However, dietary supplementation of vaccinated birds with 0.4% sodium bisulfate significantly increased body weight compared to the vaccinated control group at 35 days (**Table 5**). This supports the hypothesis that the costs of immune activation can be controlled by improving intestinal morphology to maintain efficient nutrient absorption with sodium bisulfate. Feeding 0.4% sodium bisulfate to non-vaccinated birds reduced the FI compared to the non-vaccinated control from 0 – 35 days. Administration of sodium bisulfate in the feed has been shown to improve the intestinal morphology and BW of broilers infected with *Eimeria* (Chadwick et al., 2020). Furthermore, sodium bisulfate may also foster a healthier intestinal microbiota. In *in vitro* cultures of cecal content and microbial populations challenged with *Salmonella typhimurium*, treatment with sodium bisulfate inhibited the growth of *S. typhimurium* while altering the proportions of other microbial genera (Rubinelli et al., 2017). However, the

effects of sodium bisulfate on microbial populations *in vivo* are unclear (Kassem et al., 2012; Park et al., 2015). The positive effect of feed-grade sodium bisulfate on BW is likely a direct result of improved intestinal health which acts to mitigate the negative effects of coccidiosis challenge on nutrient absorption thereby offsetting the costs of immune activation.

While there was a significant effect of vaccination on body weight at 35 days, there was no significant effect of vaccination on body weight at 56 days. The purpose of coccidiosis vaccination is to enable young poults to build immunity to *Eimeria* early to allow the birds time to compensate for the reduction in body weight and body weight gain caused by *Eimeria* infection (Chapman et al., 2005). Chickens given a coccidiosis vaccination day of hatch experience the greatest decrease in performance at 3 weeks of age but recover to similar measures of body weight and body weight gain after 4 weeks (Lehman et al., 2009; da Silva et al., 2009; Gautier et al., 2020). Birds that are not vaccinated or given a coccidiostat have reduced body weight and body weight gain later in life than birds that are vaccinated or given a coccidiostat (Gautier et al., 2020). This decreased performance that occurs later in life for non-vaccinated/treated birds is what accounts for the greatest cost to poultry producers. Therefore, it is expected that body weight of vaccinated birds catches back up with the non-vaccinated (but coccidiostat-treated) birds in this study.

Supplementation of 0.4% or 0.2% sodium bisulfate in the feed was shown to improve the 0 – 35 day or 35 – 56 day FCR, respectively. Previous research has shown similar results: 0.25% to 0.75% sodium bisulfate supplemented in the feed of chickens in the absence of vaccination or pathogen challenge decreased FCR (Ruiz-Feria et al., 2011). This is in agreement with our current findings for vaccinated birds.

Conclusion

From this study, it can be determined that 2x IMMUCOX®T vaccination significantly impairs the growth performance of poults 35 days after vaccination, but this loss in performance is recovered by 56 days as expected for effective coccidiosis vaccination. Vaccinated poults given 0.4% sodium bisulfate feed did not fail to gain as much weight as the vaccinated control and vaccinated poults given 0.2% sodium bisulfate feed while maintaining low FCR and mortality up to 56 days of age. Therefore, dietary supplementation of 0.4% sodium bisulfate can mitigate negative effects of coccidiosis vaccination on early poult performance. This study demonstrates that the combination of dietary supplementation of feed-grade sodium bisulfate and coccidiosis vaccination could be an effective measure to alleviate the early performance losses caused by coccidiosis vaccination in turkeys. The long-term impact of this strategy on the performance of market weight turkeys with coccidiosis challenge needs further investigation.

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Table 1: Ingredient composition of the diets for Starter 1 feed (0 to 35 days)[1].

Ingredients (%)	T1	T2	T3	T4	T5	T6
Corn	19.50	19.50	19.50	19.50	19.50	19.50
Wheat	19.68	19.68	19.68	19.68	19.68	19.68
Soybean meal	37.98	37.98	37.98	37.98	37.98	37.98
Poultry meal	10	10	10	10	10	10
Fat	6.51	6.51	6.51	6.51	6.51	6.51
Limestone	1.23	1.23	1.23	1.23	1.23	1.23
Dicalcium Phosphate	2.59	2.59	2.59	2.59	2.59	2.59
Mineral Mix[2]	0.26	0.26	0.26	0.26	0.26	0.26
Vitamin Mix[3]	0.20	0.20	0.20	0.20	0.20	0.20
Se Mix	0.05	0.05	0.05	0.05	0.05	0.05
Choline CL	0.20	0.20	0.20	0.20	0.20	0.20
Lysine	0.46	0.46	0.46	0.46	0.46	0.46
Methionine	0.42	0.42	0.42	0.42	0.42	0.42
Threonine	0.13	0.13	0.13	0.13	0.13	0.13
CuSO4 Premix	0.07	0.07	0.07	0.07	0.07	0.07
Salt	0.40	0.40	0.30	0.30	0.20	0.20
Sand	0.26	0.31	0.16	0.21	0.06	0.11
Amprolium (25%)	0.05	0	0.05	0	0.05	0
Sodium bisulfate	0	0	0.20	0.20	0.40	0.40

[1] The experimental treatments were as follows: T1, Negative control (Amprolium 0.0125%); T2, Vaccinated control (2x IMMUCOX®T); T3, Amprolium + sodium bisulfate 0.2% (4 lb/ton); T4, vaccination + sodium bisulfate 0.2% (4 lb/ton); T5, Amprolium + sodium bisulfate 0.4% (8 lb/ton); T6, vaccination + sodium bisulfate 0.4% (8 lb/ton)

[2,3] Supplied the following per kilogram of diet: 9,000 IU of retinyl acetate, 2,000 IU of cholecalciferol, 12.5 IU of dl-α-tocopheryl acetate, 1.76 mg of menadione sodium bisulfite, 0.12 mg of biotin, 1.2 mg of thiamine, 3.2 mg of riboflavin, 6.4 mg of calcium d-pantothenate, 1.97 mg of pyridoxine, 28 mg of nicotinic acid, 0.01 mg of cyanocobalamine, 320 mg of choline chloride, 0.38 mg of folic acid, 60 mg of $MnSO_4 \cdot H_2O$, 80 mg of $FeSO_4 \cdot 7H_2O$, 51.74 mg of ZnO, 8 mg of $CuSO_4 \cdot 5H_2O$, 0.8 mg of iodized NaCl, 0.2 mg of Na_2SeO_3.

Table 2: Ingredient composition of the diets for Starter 2 feed (35 to 56 days)[1].

Ingredient	T1	T2	T3	T4	T5	T6
Corn	26.02	26.02	26.02	26.02	26.02	26.02
Wheat	19.68	19.68	19.68	19.68	19.68	19.68
Soybean meal	31.65	31.65	31.65	31.65	31.65	31.65
Poultry meal	10	10	10	10	10	10
Fat	6.9	6.9	6.9	6.9	6.9	6.9
Limestone	1.17	1.17	1.17	1.17	1.17	1.17
Dicalcium Phosphate	2.26	2.26	2.26	2.26	2.26	2.26
Mineral Mix[2]	0.26	0.26	0.26	0.26	0.26	0.26
Vitamin Mix[3]	0.20	0.20	0.20	0.20	0.20	0.20
Se Mix	0.05	0.05	0.05	0.05	0.05	0.05
Choline CL	0.20	0.20	0.20	0.20	0.20	0.20
Lysine	0.37	0.37	0.37	0.37	0.37	0.37
Methionine	0.34	0.34	0.34	0.34	0.34	0.34
Threonine	0.11	0.11	0.11	0.11	0.11	0.11
CuSO4 Premix	0.08	0.08	0.08	0.08	0.08	0.08
Salt	0.40	0.40	0.30	0.30	0.20	0.20
Sand	0.26	0.31	0.16	0.21	0.06	0.11
Amprolium (25%)	0.05	0	0.05	0	0.05	0
Sodium bisulfate	0	0	0.20	0.20	0.40	0.40

[1] The experimental treatments were as follows: T1, Negative control (Amprolium 0.0125%); T2, Vaccinated control (2x IMMUCOX®T); T3, Amprolium + sodium bisulfate 0.2% (4 lb/ton); T4, vaccination + sodium bisulfate 0.2% (4 lb/ton); T5, Amprolium + sodium bisulfate 0.4% (8 lb/ton); T6, vaccination + sodium bisulfate 0.4% (8 lb/ton)

[2,3] Supplied the following per kilogram of diet: 9,000 IU of retinyl acetate, 2,000 IU of cholecalciferol, 12.5 IU of dl-α-tocopheryl acetate, 1.76 mg of menadione sodium bisulfite, 0.12 mg of biotin, 1.2 mg of thiamine, 3.2 mg of riboflavin, 6.4 mg of calcium d-pantothenate, 1.97 mg of pyridoxine, 28 mg of nicotinic acid, 0.01 mg of cyanocobalamine, 320 mg of choline chloride, 0.38 mg of folic acid, 60 mg of $MnSO_4 \cdot H_2O$, 80 mg of $FeSO_4 \cdot 7H_2O$, 51.74 mg of ZnO, 8 mg of $CuSO_4 \cdot 5H_2O$, 0.8 mg of iodized NaCl, 0.2 mg of Na_2SeO_3.

Table 3: Calculated nutrient values of the diets for Starter 1 and 2 (0 to 56 days).

Calculated nutrient values	Starter 1 (0 to 5 weeks)	Stater 2 (6 to 9 weeks)
Crude Protein	29	26.37
ME kcal/kg	3050	3149.13
Crude Fat	9.11	9.6
Digestible Lysine	1.75	1.54
Digestible Methionine	0.8	0.71
Digestible M+C	1.13	1.01
Digestible Tryptophan	0.32	0.28
Digestible Isoleucine	1.07	0.96
Digestible Threonine	1.01	0.9
Digestible Arginine	1.89	1.57
Digestible Valine	1.31	1.05
Calcium	1.49	1.38
Av P	0.76	0.69
Sodium	0.19	0.19
Chloride	0.27	0.26

Table 4: The effects of sodium bisulfate on cocci shedding from turkeys given a 2x dose of IMMUCOX®T at 7 and 14 days.

Vaccination + Treatment	Week 1 (mean OPG)	Week 2 (mean OPG)
Vaccinated control	80783	60335
Vaccinated + sodium bisulfate	84413	12987
Vaccinated + sodium bisulfate	96021	69651
SEM[1]	20052	27294
P-value	0.8615	0.4448

[1] SEM: standard errors of the mean

Table 5: Body weight of turkey poults at 0, 35, and 56 days of age.

Variable	Body Weight (g): Day 0		Body Weight (g): Day 35		Body Weight (g): Day 56	
Treatment	**Non-Vaccinated**	**Vaccinated**	**Non-Vaccinated**	**Vaccinated**	**Non-Vaccinated**	**Vaccinated**
Control	52.81 ± 0.34^{b}	53.67 ± 0.37^{a}	1804 ± 28^{x}	$1572 \pm 29^{b,y}$	4438 ± 75	4278 ± 78
0.2% Sodium Bisulfate	$55.00 \pm 0.34^{a,x}$	$53.44 \pm 0.34^{ab,y}$	1762 ± 34^{x}	$1653 \pm 27^{b,y}$	4445 ± 90	4518 ± 71
0.4% Sodium Bisulfate	53.44 ± 0.34^{b}	52.60 ± 0.37^{b}	1758 ± 27	1738 ± 32^{a}	4430 ± 71	4391 ± 84
Feed Additive Main Effect						
Control	53.24 ± 0.25^{b}		1688 ± 20		4358 ± 53	
0.2% Sodium Bisulfate	54.22 ± 0.24^{a}		1708 ± 22		4481 ± 57	
0.4% Sodium Bisulfate	53.02 ± 0.25^{b}		1748 ± 21		4410 ± 55	
Vaccination Main Effect						
Non-Vaccinated	53.75 ± 0.20		1775 ± 16^{a}		4438 ± 42	
Vaccinated	53.24 ± 0.21		1654 ± 17^{b}		4396 ± 45	
Probability						
Feed Additive Main Effect	0.0032		0.1152		0.3249	
Vaccination Main Effect	0.0817		<0.0001		0.5109	
Vaccination x Feed Additive Interaction	0.0044		0.0055		0.4001	

[a-c] Means across treatment rows within the same vaccination column with no common superscript differ significantly ($P < 0.05$)

[x-z] Means within the same treatment row across variable columns with no common superscript differ significantly ($P < 0.05$)

Means ± SEM

Table 6: Feed intake per turkey poult for 0-35, 35-56, and 0-56 days of age.

Variable	FI/Bird (g): Day 0-35		FI/Bird (g): Day 35-56		FI/Bird (g): Day 0-56	
Treatment	Non-Vaccinated	Vaccinated	Non-Vaccinated	Vaccinated	Non-Vaccinated	Vaccinated
Control	2759 ± 45[a,x]	2605 ± 47[y]	4948 ± 73	4853 ± 75	7728 ± 115	7497 ± 117
0.2% Sodium Bisulfate	2717 ± 54[ab]	2636 ± 43	4821 ± 87	4833 ± 74	7512 ± 137	7499 ± 117
0.4% Sodium Bisulfate	2632 ± 43[b]	2733 ± 50	4750 ± 69	4794 ± 81	7456 ± 108	7663 ± 128
Feed Additive Main Effect						
Control	2682 ± 32		4901 ± 52		7613 ± 81	
0.2% Sodium Bisulfate	2677 ± 34		4827 ± 58		7506 ± 91	
0.4% Sodium Bisulfate	2682 ± 33		4772 ± 54		7559 ± 84	
Vaccination Main Effect						
Non-Vaccinated	2703 ± 25		4840 ± 41		7565 ± 64	
Vaccinated	2658 ± 27		4826 ± 44		7553 ± 69	
Probability						
Feed Additive Main Effect	0.9918		0.2105		0.6948	
Vaccination Main Effect	0.2503		0.8281		0.9003	
Vaccination x Feed Additive Interaction	0.0264		0.6628		0.2069	

[a-c] Means across treatment rows within the same vaccination column with no common superscript differ significantly ($P < 0.05$)

[x-z] Means within the same treatment row across variable columns with no common superscript differ significantly ($P < 0.05$)
Means ± SEM
FI: Feed Intake

Table 7: Feed Conversion Ratio of turkey poults between 0-35, 35-56, and 0-56 days of age.

Variable	Adj. FCR (points): Day 0-35		Adj. FCR (points): Day 35-56		Adj. FCR (points): Day 0-56	
Treatment	**Non-Vaccinated**	**Vaccinated**	**Non-Vaccinated**	**Vaccinated**	**Non-Vaccinated**	**Vaccinated**
Control	1.573 ± 0.024	1.716 ± 0.025	1.882 ± 0.034	1.792 ± 0.034	1.758 ± 0.023	1.762 ± 0.023
0.2% Sodium Bisulfate	1.597 ± 0.029	1.642 ± 0.023	1.804 ± 0.040	1.668 ± 0.034	1.719 ± 0.027	1.655 ± 0.023
0.4% Sodium Bisulfate	1.547 ± 0.023	1.619 ± 0.027	1.780 ± 0.032	1.795 ± 0.037	1.687 ± 0.021	1.724 ± 0.025
Feed Additive Main Effect						
Control	1.645 ± 0.017^{a}		1.837 ± 0.024^{a}		1.760 ± 0.016^{a}	
0.2% Sodium Bisulfate	1.619 ± 0.018^{ab}		1.736 ± 0.027^{b}		1.687 ± 0.018^{b}	
0.4% Sodium Bisulfate	1.583 ± 0.018^{b}		1.787 ± 0.025^{ab}		1.706 ± 0.017^{b}	
Vaccination Main Effect						
Non-Vaccinated	1.572 ± 0.014^{b}		1.822 ± 0.019^{a}		1.721 ± 0.013	
Vaccinated	1.659 ± 0.014^{a}		1.752 ± 0.020^{b}		1.714 ± 0.014	
Probability						
Feed Additive Main Effect	0.0460		0.0328		0.0100	
Vaccination Main Effect	0.0001		0.0175		0.7081	
Vaccination x Feed Additive Interaction	0.2038		0.0746		0.0927	

[a-c] Means across treatment rows within the same vaccination column with no common superscript differ significantly ($P < 0.05$)

[x-z] Means within the same treatment row across variable columns with no common superscript differ significantly ($P < 0.05$)

Means ± SEM

FCR: Feed Conversion Ratio

CONCLUSION

Histomoniasis and coccidiosis are two prevalent and impactful diseases in the poultry industry that require further investigation to understand the interactions between the poultry hosts and the protozoan parasites that cause these diseases. Through the research presented in this dissertation, it has been demonstrated that *Histomonas meleagridis* infection in turkeys induces a cytokine storm syndrome during the mid-to-late stages of disease that does not occur in the more histomoniasis-resistant chicken. This cytokine storm may be at least partly responsible for the severity of pathology and high mortality in the turkey. It is also evident that within the turkey, the percentage of circulating $CD4^+$ T-cell subsets, including regulatory T-cells, does not change in response to histomoniasis while the percentage of $CD8^+$ T-cell subsets are altered 7 days following inoculation with the parasite. The change in the percentage of $CD8^+$ T-cells in the blood of infected turkeys may indicate an important role of these T-cell types in the immune response to *H. meleagridis*. Further research is needed to determine how $CD8^+$ T-cells interact with the protozoan parasite. Notably, the increased ratio of heterophils to lymphocytes in *H. meleagridis*-infected turkeys suggests that the turkey's immune system is responding to damage caused by the parasite in the affected organs, rather than to the infection at 7 dpi. Lastly, the research presented in the final chapter of this dissertation shows that administration of feed-grade sodium bisulfate to coccidiosis-vaccinated turkeys helps mitigate the negative effects of vaccination on early poult performance and overall feed conversion ratio from 0 to 56 days of age.