ABSTRACT

FUDGE, CATHERINE. Understanding Histomoniasis Progression and Transmission in Turkeys (Under the direction of Dr. Chen and Dr. Beckstead).

Histomoniasis, caused by the protozoan parasite *Histomonas meleagridis*, is a disease in turkeys with high morbidity and mortality. With the removal of preventatives and therapeutic drugs for histomoniasis, there has been a resurgence of outbreaks reported across the United States. Stress is an inevitable part of poultry production and is understood to affect bird behavior, gut health, and disease susceptibility. However, there is a lack of understanding of stress factors in histomoniasis disease progression and transmission. The objectives of this dissertation were to study the role of stress factors on histomoniasis development and transmission and explore the potential new infection route of *H. meleagridis* in turkeys. To better understand the effects of stress on histomoniasis *in vivo*, turkey trials were performed with treatments designed to mimic many stress factors related to commercial production. In these studies, the effects of feed withdrawal, and reduced crude protein diets could increase infection rates and cecal lesions in turkeys. Furthermore, while feeding turkeys with reduced crude protein diets, the coccidiosis and feed withdrawal had an overall increase in mortality rate. This data emphasizes the importance of nutrition and gut health in histomoniasis disease progression.

Lateral transmission of *H. meleagridis* under these stress factors was not observed in this research setting. Failure to create a lateral transmission model led to reconsideration of the potential transmission pathway of *H. meleagridis* in turkeys during an outbreak. Coprophagic behavior in turkeys has led us to hypothesize that the fecal-oral route of infection could be a potential infection/transmission pathway during the commercial production. Oral infections of *H. meleagridis* were tested in culture media and cecal content to induce histomoniasis in turkeys. Frequent oral inoculations of turkeys (twice a day for 5 days in succession) with *H. meleagridis*

in cecal content had a 43% infection rate, higher than *H. meleagridis* in media (8%). Still oral infections routes showed a significant lower infection rate compared to the cloacal infection (100%). However, a follow up study using fresh cecal content carefully mixed with *H. meleagridis* and orally inoculated in turkeys revealed a 93% infection rate which is similar to the cloacal infection rate (90%). These infection rates indicate the oral infection could be possible. To understand the factors in infection rate via oral gavage, different isolates of *H. meleagridis* and infection frequency were explored. Results showed that Buford and BBAR2 had greater infection rates than the Michigan isolate. Increasing frequency of infection showed an upward trend in infection rates, with 4- and 5-days infection have infection rates close to 50% while one day oral infection had 20%. In summary, the current thesis found that stressors play a role in histomoniasis disease progression, and a potential new oral infection route was found which was demonstrated by oral gavage with *H. meleagridis* in cecal content. These findings will have an impact on the histomoniasis outbreak management for turkey industry.

Understanding Histomoniasis Progression and Transmission in Turkeys

by
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DEDICATION

I would like to dedicate this thesis to my family, who supported me through all my passions.

BIOGRAPHY

Catherine Grace Fudge, the daughter of Teresa Fudge and Steve Fudge, was born in Raleigh, NC, on December 29th, 1997. She grew up in Fuquay-Varina, NC, spending most of her days from the age of 7 working with horses. In high school she joined the local Future Farmers of America chapter, where she was introduced to poultry and poultry judging. In the fall of 2016, she enrolled at NC State and pursued a Bachelors in Poultry Science. She began working in a nutritional immunology lab in Scott Hall where she learned about turkey production and diseases that threaten this industry. The experiences in this lab inspired her to continue working as a graduate student in the same laboratory. She began her Masters in Poultry Science in 2020 under the direction of Dr. Robert Beckstead. Not long after beginning her Masters, Dr. Beckstead accepted a job in the industry. During this time of change, Dr. Chongxiao Chen stepped up and took over the research in the laboratory. She will be continuing to her PhD with Dr. Chongxiao Chen at the University of Georgia.

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I would like to thank Dr. Robert Beckstead for his unwavering support, his innovative thinking and encouraging out of the box ideas. I would also like to thank Dr. Chongxiao Chen. I want to say thank you for taking on the task of continuing the research in the laboratory and providing so much support and encouragement when work became difficult, or I felt like I was stuck. He took on three graduate students with no questions asked and made sure he made time for all of us even when his schedule was full. I will forever be thankful for his patience and kindness throughout my time here. I would also like to thank Christina Sigmon for always making sure I had what I needed to get my work done. To Dr. Katherine Cupo and Dr. Olivia Wedegaertner, thank you for providing advice, support, mentorship on how to succeed as a graduate student. I could have never done it without your help. To all my friends who were there for me when I was stressed and lending a listening ear, thank you. I would like to thank my parents for supporting me through all my schooling.

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LITERATURE REVIEW

Introduction

The protozoan, *Histomonas meleagridis* is the causative agent of histomoniasis, formerly known as enterohepatitis, typhlohepatitis, and blackhead disease. *H. meleagridis* was first described in the 1890s and was extensively studied at the beginning of the 20th century. In 1910, it was named *Amoeba meleagridis* (Smith, 1910). However, in 1922, further investigation by Tyzzer showed that this protozoan was not an amoeba, and was renamed *Histomonas meleagridis* (Ernest and Tyzzer, 1922). This protozoan infects gallinaceous birds throughout the world, and chickens and turkeys fall into this category (Dolka et al., 2015). Outbreaks of *H. meleagridis* in turkeys are often severe, with high morbidity and mortality, and could reach 100 percent within 3 to 4 weeks. Common pathology observed includes a caseous core within the ceca and raised, target-like rings on the surface of the liver (Figure 1)

Figure 1

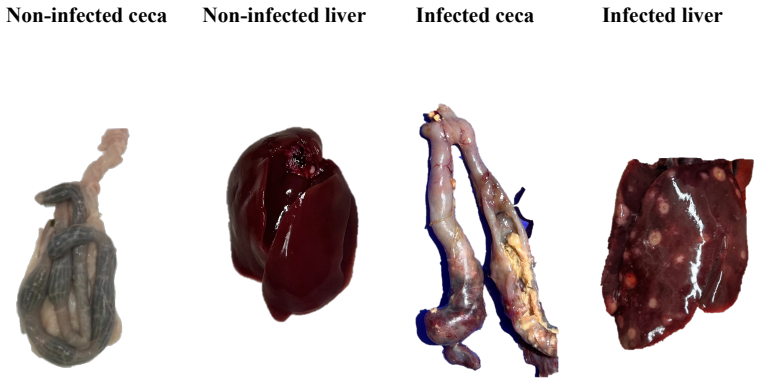

Figure 1. Comparison of non-infected systemic organs, ceca and liver, and classic lesions associated with *H. meleagridis* infection

After *H. meleagridis* was first described in 1893, histomoniasis became the number one cause of turkey mortality in the U.S. and Europe (Moore, 1896; Lund, 1977). Therapeutic drugs,

nitroimidazoles, and nitrofurans that successfully prevented and treated infections were discovered in the early 20th century. The application of these drugs during an outbreak successfully controlled the disease; histomoniasis was no longer a major concern. By the early 2000's, the European Union and the U.S. Food and Drug Administration banned the use of nitro-imidazoles and nitro-furans that treated histomoniasis in food animals. One preventative drug, Nitarsone, was officially removed from the market in 2016, and more than 600 outbreaks have been reported between 2016 and 2020. *H. meleagridis* has reemerged as a top pathogen of interest in turkey mortality. This resurgence has peaked researchers' interest in studying this parasite and raised many questions about *H. meleagridis*.

Early studies had logistical problems as turkeys were raised outside in pens, which allowed turkeys to be exposed to a vector, later identified as *Heterakis gallinarum*, which facilitated histomoniasis without challenge to the turkeys with verifiable infectious material (Graybill and Smith, 1920). When healthy turkeys, penned in areas previously housing *H. meleagridis*-infected turkeys, developed histomoniasis, researchers focused on a vector, *Heterakis papillosa* (now referred to as *Heterakis gallinarum*)(Graybill, 1920). Infections of turkeys with *H. gallinarum* were successful in producing histomoniasis. Research on histomoniasis then began to use oral inoculation of turkeys with embryonated *H. gallinarum* eggs to produce histomoniasis in turkeys (Graybill and Smith, 1920; Tyzzer and Collier, 1925). Culturing histomonads was first described in 1924 by Drbohlav (Hauck et al., 2010), allowing research trials infecting gallinaceous birds directly with cultured *H. meleagridis* (Hauck et al., 2010).

The high mortality during a histomoniasis outbreak is due to the transmission of *H. meleagridis* within a flock. Reducing the transmission is the key to reducing mortality. Although

the exact mode and cause of transmission within an outbreak of *H. meleagridis* has not been confirmed, several hypotheses on its transmission and environmental factors may induce an outbreak of histomoniasis (Welter, 1960; Denoncourt et al., 2014). Lateral transmission, which purportedly occurs in the absence of a vector, has been neither confirmed nor observed in field outbreaks. No evidence that direct cloaca to cloaca transmission might happen has been evidenced by recording turkey behavior in confinement rearing. However, a lateral transmission model was attempted in a laboratory setting (Hu et al., 2004; Hess et al., 2006; Armstrong and McDougald, 2011) with *H. meleagridis* transmitting from infected to non-infected birds without a vector. This review aims to summarize the research that has been done on histomoniasis lateral transmission and disease progression, which includes vector-borne transmission, co-morbidities, and conventional production stress factors affecting transmission.

Vector transmission

Heterakis gallinarum

Survival of *H. meleagridis* outside of the ceca is short. The longest survival times were reported in moist mediums, feces, and water, with livability averaging 9 hours at a maximum (Lotfi et al., 2012). With short survival periods outside the host, *H. meleagridis* relies on the common poultry cecal worm, *Heterakis gallinarum,* for long-term survival in the environment (Cupo and Beckstead, 2019). *H. gallinarum* was first reported in the late 1800's (Hasall, 1911) but was not connected to *H. meleagridis* as its vector until 1920 (Graybill and Smith, 1920). Though initial infection of turkeys does begin with *H. gallinarum*, the lateral transmission of *H. meleagridis* can occur without a vector in commercial production systems (McDougald and Fuller, 2018).

H. gallinarum is a nematode that cycles through the ceca of gallinaceous birds (Lund and Chute, 1974). After ingesting an embryonated egg, it hatches in the small intestine, where the larvae translocate to the ceca. Here they burrow into the ceca and develop sexually, with females producing ova around day 25 (Ernest and Fabyan, 1934). Eggs will then need to be shed into the environment and embryonated for two weeks before being ingested again and beginning the lifecycle (Saunders et al., 2000). Due to the nature of *H. gallinarum's* lifecycle, short-lived poultry, like broilers, are unlikely to be heavily infected with *H. gallinarum*. Once a chicken has consumed the *H. gallinarum* ova, the ova hatches and releases the *H. gallinarum* larvae and *H. meleagridis*. The larva and the protozoan migrate to the ceca, where they both can colonize the ceca, without causing severe disease (Lund, 1968). Heterakids become infected with *H. meleagridis* through unknown routes and become incorporated into the reproductive tract of females as well as the intestines of male heterakids (Cibbs, 1961). The prevalence of the cecal nematode in chicken populations has been found to range from 4 percent to 98 percent (Ogbaje et al., 2012; Shifaw et al., 2021). The prevalence of *H. meleagridis* in galliform populations has not been thoroughly analyzed, but some studies have found close to 30 percent of infected chickens harbor *H. meleagridis* (Hauck et al., 2010b; Badparva and Kheirandish, 2017). Hence, there is a higher chance that *H. gallinarum* eggs become infected with *H. meleagridis* during its life cycle in the ceca of chickens, facilitating the contamination of soils found under and around poultry houses.

During egg development, histomonads are enveloped within the outer chitin shell, where *H. meleagridis* remains viable if the egg remains intact (Lund, 1968). Female nematodes lay their eggs in the lumen of the ceca and are excreted when cecal content is dropped. Eggs remain viable within the environment for up to 3 years (Farr, 1960). For *H. gallinarum* eggs to spread *H.*

meleagridis, full embryonation must occur through contact with oxygen for two weeks (Graybill and Smith, 1920).

Multiple attempts to infect turkeys with unembryonated *H. gallinarum* eggs failed to produce histomoniasis (Ernest and Fabyan, 1934). The mechanism by which *H. meleagridis* relies on embryonation of *H. gallinarum* for infection is not fully understood. Researchers hypothesize that enzymatic reactions occur externally within the gastrointestinal tract or intestinal stimulants signal for the egg to degrade from inside. Full cellular development within the *Heterakis* eggshell may be necessary for internal egg degradation to allow the larva to hatch (Cupo and Beckstead, 2019). If external enzymes were able to hatch the egg, then unembryonated eggs would be able to produce histomoniasis in turkeys, but unembryonated infections have failed to cause disease (Reid, 1968). Further research is needed to understand the molecular mechanism of egg hatching as well as the prevalence of *H. meleagridis i*n *H. gallinarum* eggs.

Both *H. gallinarum* embryonated ova and *H. gallinarum* nematodes, males, and females can infect turkeys with *H. meleagridis* when ingested. Embryonated eggs remain viable in the environment for long periods providing a readily available reservoir of vectoring capacity of *H. meleagridis* to brooded turkeys on the infested soils (Graybill and Smith, 1920; Tyzzer and Collier, 1925). Embryonated eggs are ingested by a host and travel through the gastrointestinal tract, where they hatch out in the small intestine, releasing a juvenile heterakid and *H. meleagridis* (Robert, 1937). Many galliformes, such as chickens and quail, could continue the life cycle of *H. gallinarum* after the consumption of embryonated eggs. However, turkeys succumb to *H. meleagridis* when consuming embryonated eggs and initiate an outbreak. *H.*

gallinarum does not complete its lifecycle in the turkey ceca due to the damage caused by *H. meleagridis* (Graybill and Smith, 1920; Press, 2017).

The vectors for Heterikas gallinarum

Both mechanical and biological vectors can carry heterakid eggs. Only one species, earthworms, are a confirmed paratenic host for *H. gallinarum* (Press, 1966). *Heterakis* ova in infested soil are consumed by earthworms and travel through the gastrointestinal tract where they hatch. Larva burrows into the 'earthworm's tissue and remain viable if the earthworm for the period that the earthworm is alive (Lund, 1966). Once the earthworm is consumed by a turkey, the larva can colonize the ceca, bringing *H. meleagridis* with it and producing histomoniasis.

The existence of other possible vectors has not been thoroughly researched. Other organisms, specifically insects and other invertebrates, have been suggested as potential facultative vectors of *Heterakis*. A common insect in poultry houses, the house fly, has been shown to be a mechanical vector for *Heterakis* ova (Frank, 1953). Flies naturally feed on fecal and cecal content, where *Heterakis* ova would be most concentrated (Piñero et al., 2009). Flies are covered in body hairs which may have the ability to pick up and carry the sticky parasites and protozoans for long distances (Khamesipour et al., 2018). Grasshoppers have also been shown to be able to carry *H. gallinarum* ova for up to 96 hours and remain infective (Frank, 1953).

One of the most common insects in poultry houses is the litter beetle, also known as the darkling beetle (Hess, 2008). Darkling beetles have been shown to carry numerous avian diseases, including *Eimeria* species, another protozoan pathogen (Goodwin, 1996). Darkling beetle larvae can serve as a mechanical vector for *H. meleagridis* for short periods without the cecal worm (Huber, 2007). PCR detection of cecal worm DNA in darkling beetles raised in contact with feces containing *Heterakis* eggs, showed the possibility of darkling beetles as a

potential vector for the cecal worm or its ova (Cupo and Beckstead, 2019). Further research looking for other *H. gallinarum* vectors would provide greater insight into the prevalence of *H. gallinarum* in the environment as well as methods of prevention.

These possible *H. gallinarum* vectors pose a risk for turkey houses near laying hen and broiler breeder operations. Recent epidemiological studies showed that a turkey house within 3 miles of a chicken house was 4.6% more likely to experience an outbreak of histomoniasis than a house outside of this diameter (Jones et al., 2020). Insects like darkling beetles, grasshoppers, flies, and earth worms can travel long distances in search of new food sources. Insects leaving chicken houses laden with *H. gallinarum* and possibly traveling to turkey houses, may bring the nematode eggs and *H. meleagridis* with them. Further research identifying which insects are the most common *H. gallinarum* vector would provide better information on which insects need to be a top priority for control.

H. gallinarum is currently the only known vector for *H. meleagridis* (Cupo and Beckstead, 2019). A closely related species, *Dientamoeba fragilis*, has an identified mammalian vector, mice (Clark et al., 2014). Mice directly fed *D. fragilis* cysts were colonized and shed the protozoan for up to 6 months in feces (Clark et al., 2014). Rodents, mice, and rats specifically are common around poultry houses and have a functioning cecum, which may be a possible reservoir for *H. meleagridis* outside of avian species (Escalante et al., 2016). Future research looking at the ability of mice or rats to be colonized by *H. meleagridis* and carry histomonads for long periods should be further researched.

Transmission in the absence of a vector

Cloacal route

Research of direct oral infection of *H. meleagridis* using infected liver tissues, cecal tissue, and feeding direct *H. meleagridis* cells lead to low or failed infections compared to infection with *H. gallinarum* (Tyzzer, 1925). Due to the high failure rates of direct oral infections of turkeys with *H. meleagridis* culture, prior to 2003, transmission of *H. meleagridis* was believed to only be possible through *H. gallinarum* (Swales, 1948). Lateral transmission of *H. meleagridis* from infected to non-infected birds in the absence of a vector was first observed in 2003 (Hu and McDougald, 2003). Following this observation, McDougald infected 2-week-old turkey poults with 100,000 H. meleagridis cells. To infect turkeys, a pipette tip was placed on the dorsal lip of the cloaca and the cell culture was pipetted onto the lip, and induced contractions pulled the culture into the colon. This newly developed laboratory model for infection produced 88% infection rates (Hu et al., 2004). The development of the cloacal drop method along with the failure of oral inoculations led to the conclusion that lateral transmission of *H. meleagridis* could occur between birds without an intermediate vector, though it was not determined whether the route was fecal-oral route or direct cloacal contact with *H. meleagridis*.

Although reverse peristalsis in avian species had been understood since the 60's (Akester et al., 1967) and further defined in the 90's (Duke, 1997), it had not been connected to any diseases as a possible way mechanism of transmission. McDougald demonstrated the turkey's ability to take up *H. meleagridis* into the body when material was placed on the lip of the cloaca and stimulated. This opened the door for the idea of cloacal transmission. The mechanism of reverse peristalsis is well understood as an evolutionary trait that allows birds to reduce water loss from feces (Sacranie et al., 2007). Through reverse peristalsis, fecal water content that has

moved into the colon and cloaca can be moved back into the ceca, where water is re-absorbed (Sacranie et al., 2007). Not only does reverse peristalsis allow for some but not all fecal material to be moved from the colon back to the ceca, but contents from the environment can also be taken up in the same manner (Sacranie et al., 2007). Though it was determined that turkeys could take up *H. meleagridis* cloacally, it is unclear whether bird-to-bird contact was necessary or if cloacal contact with droppings alone could manifest histomoniasis. The working hypothesis in this thesis is that bird-to-bird contact via the cloaca is not a viable mechanism for induction of histomoniasis.

Observations of lateral transmission were seen in both bird-to-bird contact research models as well as birds placed in contact with infectious fecal/cecal content (Hu and McDougald, 2003; Armstrong and McDougald, 2011). Bird-to-bird contact lateral transmission models of infected turkeys were kept in cages with non-infected birds for over two weeks, with infection rates close to 100% (McDougald, 2003). Birds placed in contaminated cages had lower infection rates. Turkeys placed in cages for 1 hour containing freshly dropped cecal content from infected turkeys had an infection rate of 12.5% (Armstrong and McDougald, 2011). Though short-term exposure led to lower infection rates, both bird contact and contact with droppings led to *H. meleagridis* infections.

Oral-fecal route

Coprophagic behavior is common in many avian species, and it is observed in many wild birds and domesticated fowl (Lamb, 2017; Kobayashi, 2019). Turkeys consume cecal droppings to utilize short-chain fatty acids and proteins produced by the microbiome in the ceca (Pan, 2013). However, cecal content can contain many pathogens, and these pathogens can be passed through the fecal-oral route when the cecal dropping content is consumed by a turkey. Some

species of *Eimeria* reproduce in the ceca, which are also shed in cecal content facilitating infections in turkeys (Chapman, 2008). *Tetratrichomonas gallinarum*, a closely related species to *H. melagridis*, is found in the ceca of turkeys and is also shed in cecal content. Oral experimental infections of chickens and turkeys with *T. gallinarum* produced infection rates of 90-100% (Friedhoff, 1991; Hess, 2011).

Though closely related species of protozoa are confirmed to transmit through the fecal-oral route, the oral transmission of *H. meleagridis* is highly debated, and not much research has been conducted to test this hypothesis. *H. meleagridis* requires a pH above 4 to remain viable and proliferate (Hauck et al., 2010). Because of this sensitivity to pH, it was hypothesized that oral transmission was not possible due to the acidic environment of the upper G.I. tract. The gizzard in most galliforms has a pH close to 3.5, below the survivability threshold for *H. meleagridis* (Mabelebele et al., 2014). Early oral inoculations of turkeys with *H. meleagridis* produced varying infection rates, with feces manifesting the greatest oral infection rates compared to infected tissue and egg albumin (Swales, 1948; Ernest and Fabyan, 2011). This indicated that turkeys must ingest large quantities of infected material to develop the disease. McDougald orally infected 2-week-old turkeys with 100,000 histomonads per bird and could not produce histomoniasis (Hu, 2004). On the other hand, Hess produced an 88% infection rate by infecting one-day-old turkey poults using 10,000 histomonads per bird from a monoculture. Oral infections of 2-week-old turkeys with 10,000 histomonads per bird were also observed, with a 50% infection rate after two weeks (Liebhart and Hess, 2009).

Because of varying oral infection rates, a cyst-like stage of *H. meleagridis* has been suggested as a possible way in which *H. meleagridis* can survive for more extended periods in excreted cecal material in unfavorable temperatures (Mehlhorn, 2008). Under slowly decreasing

temperatures, smaller, spherical stages of *H. meleagridis* were observed after approximately 6 hours (Munsch et al., 2009). Under light and transmission electron microscopy, condensed versions of *H. melagridis* were observed when the cells were placed under less favorable conditions (Zaragatzki et al., 2010). This may be the reason *H. meleagridis* can survive on surfaces for up to 9 hours on surfaces after being expelled from the ceca and may help it survive in the upper gastrointestinal tract. Additional research is needed to understand if the oral inoculation method seen in laboratory models can also occur in the field and if it is the main route of *H. meleagridis* transmission.

Pre-disposing factors affecting *Histomonas meleagridis* transmission and disease progression

Secondary infections

The interplay between protozoans and bacteria has been well established as an important part of many protozoan life and proliferation (Denoncourt et al., 2014). Research on co-infections with *H. meleagridis* and bacteria, viruses, or other protozoans is scarce. A common bacteria found in the small intestine and ceca of many poultry species, *Escherichia coli,* has the potential to cause disease through intestinal and hepatic damage (Arp, 1982). Overgrowth of E. coli also provides a preferred bacterial background for *H. meleagridis* growth (Ganas et al., 2012a). *H. meleagridis* relies on bacteria as its primary food source (Bilic and Hess, 2020a). Experiments with turkeys harboring only one bacterium, Bacillus cereus, observed 0% infection with *H. meleagridis*. On the other hand, turkeys harboring E. coli and Clostridium perfringens had 100% infection and even saw an increase in cecal lesion scores in chickens harboring the same bacteria and subsequently infected with *H. meleagridis* (Franker and Doll, 1964). It is suspected that specific bacterial populations may be necessary for the proliferation of *H.*

meleagridis in the ceca (Ganas et al., 2012b). *In* vitro cultures of *H. meleagridis* grown with varying bacteria affected overall cell counts. Increased histomonad numbers were observed when grown in culture with *Escherichia coli* followed by *Salmonella typhimirium* and *Pseudomonas aeruginosa* (Ganas et al., 2012).

Gnotobiotic poults, containing no bacteria, infected with embryonated heterakid eggs did not develop histomoniasis, and most of the poults showed no clinical signs of disease in the ceca or the liver. While turkey poults with fully functioning microbiomes had considerable disease signs, both cores and liver lesions (Society & Journal, 2014). These indicate the close relationship between bacteria and *H. meleagridis*.

Along with bacteria, other protozoal infections often occur alongside or before a histomoniasis infection. For instance, coccidiosis is one of the most prevalent diseases in commercial poultry production (Györke et al., 2013; Gharekhani et al., 2014). Experimental infection of chickens with *E. tenella* and *H. meleagridis* did not increase -related mortality but increased the number of birds with liver lesions (McDougald and Hu, 2001). However, for the transmission, turkeys previously infected with *E. adenoeides* and intermingled with *H. meleagridis* infected turkeys had decreased lateral transmission compared to turkeys without coccidiosis (McDougald and Fuller, 2005). It is well understood that *H. meleagridis* has a symbiotic relationship with many bacteria (Ganas et al., 2012a; Bilic and Hess, 2020b). *E. tenella* may have a more competitive nature with *H. meleagridis*, affecting *H. meleagridis* ability to proliferate in the ceca if *E. tenella* is already established in the ceca. Disease progression of birds directly inoculated with *E. tenella* and *H. meleagridis* was not examined but is an important factor to consider. Further research looking at bacterial and protozoal infections occurring prior to or alongside histomoniasis is needed to understand possible multi-species interplay. Coccidia

infection can disrupt the intestinal microbiome affecting certain populations of bacteria more than others- possibly the favored bacterial food of the histomonads (Lu et al., 2021).

Stress factors

External factors also play a role in lateral transmission between turkeys during an infection. After metronidazole was banned from the market as a histomoniasis treatment, reports of outbreaks show highly variable mortality levels within a flock between locations (Callait-Cardinal et al., 2007). A two-year study completed in France found that over 50 percent of histomoniasis outbreaks had less than 10 percent mortality, but other outbreaks had 100 percent mortality within a flock. This variability is not well understood, but many researchers believe that there are predisposing factors that affect the ability of *H. meleagridis* to transmit horizontally.

Stress has been suggested to trigger fatal histomoniasis in chickens and exacerbate histomoniasis in turkeys. One study focused on multiple stress factors and their effect on histomoniasis. Welter applied skip-a-day feeding (starving), *E. tenella,* variations in hot (38 °C) and cold (4 °C), anemia by blood withdrawals, vaccination with Fowl pox, cortisone injections, and splenectomy (chickens only) to chickens and turkeys (Welter, 1960). They found only the skip-a-day feeding in which turkeys were only fed every other day for 19 days, had greater histomoniasis-related lesions in the liver. The skip-a-day feeding may be leading to protein deficiency, exacerbating histomoniasis.

Although Welter found that feed withdrawal, when happening every other day influenced histomoniasis lesion scores, it was not studied how a single feed withdrawal event may affect a *H. meleagridis* infection. Feed withdrawal can alter many aspects of poultry health, including viscera and microbiota (Thompson and Applegate, 2006; Burkholder et al., 2008; Thompson et

al., 2008). After Welter's findings, short feed withdrawal periods have been studied relative to their effects on disease transmission. McDougald applied a 6-hour feed withdrawal prior to oral infections of turkeys, but the feed withdrawal did not lead to an increase in infection rates (Hu et al., 2004). No current research has thoroughly studied the effects of common stress factors on lateral transmission and disease progression of *H. meleagridis*. Further research is needed to understand the roles of these stressors on outbreak severity.

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CHAPTER 2

Role of stress factors on histomoniasis development and transmission in turkeys

ABSTRACT

The current study evaluated stress as a factor influencing histomoniasis development and lateral transmission of *H. meleagridis*. In the following experiments, half of turkeys were infected with 100,000 histomonad/bird in each pen to study the disease transmission and progression. Birds were either infected at 5-weeks-old and raised on floor pens (experiment 1&3), or at 2-weeks-old and raised in battery cages (experiment 2). No significant lateral transmission occurred during the three experiments, and the results were presented only for the directly infected birds. In experiment 1, multiple stress factors were evaluated as modulators of histomoniasis in turkeys. Stressors evaluated were high dietary electrolyte balance (DEB increased 100 mEq/kg) and decreased crude protein (5% CP reduction) in comparison to the positive control (PC) group and an18-hour feed withdrawal treatment, which all caused significantly greater infection rates and pathology in the ceca in comparison to PC. In experiment 2, the influence of a reduced crude protein diet in conjunction with stress factors was evaluated. All poults fed a poorly formulated diet had higher liver and ceca scores, which reflect the severity of the pathology associated with the disease, in comparison to the PC group. Feed withdrawal and *coccidial* challenge, in experiment 1 and 2, treatments increased mortality compared to PC. In experiment 3, 5 birds were fed diets containing 0 ppb, 6.26 ppb, or 19.82 ppb of naturally occurring aflatoxin. No significant differences among treatments were observed. In summary, the stress factors in these investigations did not induce lateral transmission of *H. meleagridis*. However, the results of these experiments demonstrated that reduced dietary crude

protein, feed withdrawal and *coccidial* infection facilitated a more robust progression of histomoniasis.

INTRODUCTION

Histomonas meleagridis, a vector-borne, anaerobic protozoan causes histomoniasis, also known as blackhead disease, in gallinaceous birds (McDougald, 2005). This disease leads to high mortality of domestic birds, causing significant economic losses for turkey producers. *H. meleagridis* colonizes the ceca, infiltrating the epithelial lining, leading to the development of a caseous cecal core. After accessing the lamina propria, the parasite can enter the capillaries and gain access to the hepatic portal vein, making it possible to infect the liver. In the liver, the accumulation of the histomonads in foci induces a massive influx of primarily polymorph nuclear cells represented by heterophils, basophils, possibly eosinophils, and mast cells, which then increase the inflammatory response of the liver, which is made manifest as bullseye-like lesions in the liver and results in liver failure (McDougald, 2005). Effective chemotherapeutics such as metronidazole and 3-Nitro-4-hydroxyphenylarsonic acid (roxarsone or 3-Nitro) were withdrawn from the market in the European Union (EU) and the United States of America (USA) with the last application of these therapeutic drugs in 2016 (Clark and Kimminau, 2017). Since then, the reports of histomoniasis outbreaks have increased with more than 500 known outbreaks since 2016 (Clark and Froebel, 2020). Outbreaks of histomoniasis vary in morbidity and mortality reported among the outbreaks, ranging from 10% to 100% flock loss in turkeys (Hauck and Hafez, 2013). Due to the lack of treatments and the increases in reported histomoniasis outbreaks, understanding the factors affecting the variability of infection rates in an outbreak as well as developing interventions for reducing outbreak-associated losses is of paramount importance.

H. meleagridis transmits between birds through infected cecal content or possibly via bird-to-bird contact (Hu and McDougald, 2003; Armstrong and McDougald, 2011). Even after 125 years of research on this disease, factors that play a role in histomoniasis transmission and progression are still not well-defined. In commercial poultry production systems, many stressors such as heat, handling, litter quality, cold, and aflatoxins are an inevitable part of the production system (Dietert et al., 1994). Managing stress is an important part of poultry welfare as stress has been shown to lead to greater disease susceptibility (Burkholder et al., 2008; Alhenaky et al., 2017). Each stress factor affects the bird differently depending on the duration of exposure and type of stressor experienced.

Previous studies, addressing some stressors, such as feed withdrawal, microbial infections, transportation, and aflatoxin-contaminated feed were shown to be high-risk factors for poultry health during rearing (Giambrone et al., 1985; Quist et al., 2000; Burkholder et al., 2008; Zhang et al., 2014). However, relatively few research investigations have been done to compare and understand stress factors associated with modern turkey production on histomoniasis progression and transmission. One such study suggested that coccidiosis exacerbated histomoniasis in chickens while decreasing lateral transmission and severity of disease in turkeys (McDougald and Fuller, 2005). Furthermore, feed restriction in turkeys may play a role in histomoniasis progression and infection (Welter, 1960). There is a paucity of research focusing on comparisons of different stressor effects on transmission and histomonasis progression in turkeys. A series of three studies were conducted to explore 1) the role of stressors during turkey production on the lateral transmission of *H. meleagridis* and 2) effects of stressors on histomoniasis progression.

MATERIALS AND METHODS

These experiments were completed at the Talley Turkey Education Unit at North Carolina State University. All research was approved by the North Carolina State University Institute of Animal Care and Use Committee (IACUC).

Inoculum preparation

Histomonas meleagridis cultures were previously collected from outbreaks in multiple locations in the Southeast U.S. and Mid-West US, and cryopreserved. Three isolates from Zeeland, Michigan (ZM.), Buford, Georgia (BF.), and Arkansas (BBAR2) were used for all experiments. Collected isolates were stored in 2 mL conical tubes with 5% of Dimethyl sulfoxide and frozen and stored in liquid nitrogen for one to two years. Isolates were removed from liquid nitrogen, thawed, and passed into 10 mL of modified Dwyer's media consisting of 0.8% (wt/vol) rice powder ('Bob's red mill, Milwaukie, OR, USA), 5% horse serum (Cytiva HyClone, Waltham, MA, USA), in Medium 199 with 'Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) (Hauck et al., 2010). Cultures were incubated at 42 °C for 48 hours. Each isolate was observed for growth and passed into a new flask containing modified 'Dwyer's medium. This process was repeated 3 times. Before the using the revived *H. meleagridis* to challenge turkeys transcloacally, *H. meleagridis* counts were determined using an improved Neubauer hemocytometer (Thermo-Fisher, Raleigh, NC). Each isolate was counted and diluted to yield 100,000 histomonads/mL. The three isolates were combined into one inoculum containing 1:1:1 ratio of the three *H. meleagridis* strains. The inoculum was stored in a portable incubator at 42 °C during the inoculation.

Inoculation procedure

For all three experiments, half of the turkeys in each pen were tagged individually for identification. Tagged birds were trans-cloacally inoculated with 1 mL of inoculum containing 100,000 mixed-strains of histomonads (pH 7). Inoculated birds were suspended by their legs and held inverted for 2 minutes after inoculation before being placed back in their pen. This procedure was to ensure that each bird successfully received the inoculum.

Experimental design

Experiment 1

A total of 480 one-day-old Nicholas turkey poults, obtained from an Aviagen hatchery in Lewisburg, WV, were allocated randomly to 8 experimental groups (3 replicates X 20 birds/ replicate). Experimental treatment descriptions are presented in Table 1. The experiment was conducted in floor pens containing fresh pine wood shavings. The formulation of the diets used in experiment 1 are shown in Table 4. Lighting and temperature were maintained based on Aviagen recommendation (Aviagen Management Guidelines for Growing Commercial Turkeys, 2016). For the first three days, lights were kept on 24 hours a day. From day 4 to termination, lights were kept on 16 hours of light, 8 hours of dark cycle. The temperature for the first week was 33 °C and lowered by 3 °C each week.

Table 1. Description of applied treatments in experiment 1.

Treatment	Description
NC	Negative control: non-infected control birds, no treatments applied and provided feed and water *ad libitum.*
PC	Positive control: Infected control birds, no treatments applied and provided feed and water *ad libitum.*
TS	Transportation stress: at 5 weeks of age turkeys in turkey load-out crates with approximately 8 birds/crate were placed in an open-air gooseneck trailer. Turkeys were driven over a local highway for 2 hours and then returned in their respective pens. This procedure was to simulate a transportation stressor often experienced during commercial production (movement from a brooder farm to a grow-out farm).
HE + RCP	High electrolytes and reduced crude protein: diets were formulated with increased dietary electrolyte balance (302 vs 200 mEq for control diet) and reduced crude protein (18% vs 23% for control diet) (Table 4). Feed was provided *ad libitum* to turkeys for the entirety of the trial.
FS	Feed withdrawal stress: one day post infection feed was withdrawn for 18 hours. Feed was provided *ad libitum* to turkeys for the rest of the trial.
BD	Butyric acid: butyric acid feed additive (3.75 g/Kg (Beautipearl, Kemin, Des Moines, IA, USA) was fed to turkeys for the entirety of the trial. Feed was provided *ad libitum* to turkeys for the entirety of the trial.
CS	Cold stress: turkeys were subjected to outdoor ambient temperatures ranging from 10-12 °C each evening. The heaters connected to this isolated area were shut off from 5 PM to 8 AM each day for a 12-day period following infection. Feed was provided *ad libitum* to turkeys for the entirety of the trial.
DS	Delay stress: on the day of placement, poults were withheld from pen placement and kept in chick boxes without access to feed or water. Weights were collected every 6 hours until 10% of their initial body weight was lost, which occurred at the 24-hour mark. Feed was provided *ad libitum* to turkeys for the rest of the trial.

At week 5, 10 poults/pen in infected treatment groups (21 pens) were tagged and infected trans-cloacally with 1 mL inoculum. Ten (10) sentinel birds were allowed to mingle with infected birds for the remainder of the trial. The mortality was recorded throughout

the trial. The trial was terminated at the end of week 10 when all remaining birds were euthanized, necropsied, and histomoniasis lesions for the liver and ceca were recorded.

Experiment 2

A total of 120 one-day-old Nicholas turkey poults were obtained from an Aviagen hatchery in Lewisburg, WV. The poults were allocated randomly to 4 experimental groups (3 replicates X 10 birds/replicate). Experimental treatment descriptions are presented in Table 2. The experiment was conducted in battery cages with each cage containing heavy paper over wire flooring. Pine wood shavings were spread over the paper to simulate floor-pen-like conditions. Lighting and temperature were maintained based on Aviagen recommendation (Aviagen Management Guidelines for Growing Commercial Turkeys, 2016). For the first three days lights were kept on for 24 hours a day. From day 4 to termination, lights were kept on 16 hours of light, 8 hours of dark cycle. The temperature for the first week was 33 °C and lowered by 3 °C each week.

Diet formulations for experiments 2 and 3 are shown in Table 5.

Table 2. Description of treatments applied in experiment 2.

Treatment	Description
NC	Negative control: non-infected control birds, no treatments applied and provided feed and water *ad libitum.*
PC	Positive control: infected control birds, no treatments applied and provided feed and water *ad libitum.*
RCP	Reduced crude protein: diet was formulated with reduced crude protein (Table 5). Feed was provided *ad libitum* to turkeys for the entirety of the trial.
RCP+C	Reduced crude protein + cocci: same feed as RCP treatment was provided *ad libitum* for the entirety of the trial. On day of *H. meleagridis* infection (2 weeks), turkeys were given a 5x dose of *Eimeria* (*E. adenoides, E. meleagrimitis)* vaccination (Immucox T, Ceva Animal Health, Lenexa, KS, USA).
RCP+W	Reduced crude protein+ feed withdrawal: same feed as RCP treatment was provided *ad libitum* for the entirety of the trial. One day post infection, feed was removed from the cage for 18 hours and then returned for the remainder of the trial.

On day 14, 5 poults/pen were subjected to trans-cloacal inoculation of 1mL of inoculum containing 100,000 histomonads. This procedure was done twice on day 14, at 9 AM and again at 1 PM to improve infection rate. The directly infected birds were tagged and to their respective cages to mingle with sentinel birds for the remainder of the trial. Mortality was recorded throughout the trial. At 5 weeks, all remaining birds were euthanized and scored for signs of histomoniasis.

Experiment 3

A total of 180, 5-week-old hens, obtained from a brooder facility in North Carolina, were randomly placed in 3 experimental groups (3 replicates X 20 birds/replicate). Experimental treatment descriptions are presented in Table 3. Aflatoxin levels were tested at the North Carolina Department of Agriculture and Consumer Services Food and Drug Protection

Division Laboratory, Raleigh, NC, USA. The diet formulation is shown in Table 3.3. The experiment was conducted in floor pens containing fresh pine wood shavings. Lighting and temperature were maintained based on Aviagen recommendations (Aviagen Management Guidelines for Growing Commercial Turkeys, 2016). For the first three days lights were kept on for 24 hours a day. From day 4 to termination, lights were kept on 16 hours of light, 8 hours of dark cycle. Temperature for the first week was 33 °C and lowered by 3 °C each week.

Table 3. Description of treatments applied in experiment 3.

Treatment	Description
APD	Aflatoxin reduced crude protein: the same RCP feed formulation from experiment 2 was produced for experiment 3 (Table 5). This diet was formulated with feed ingredients contaminated naturally with aflatoxin (19 ppb).
AFPD	Aflatoxin-free, reduced crude protein: RCP feed formulation produced with feed ingredients free of aflatoxin contamination (0 ppb).
AFPD + R	Aflatoxin-free, reduced crude protein, rancid fat: RCP feed formulation produced with feed ingredients free of aflatoxin contamination. Two weeks before the start of the experiment, feed was removed from storage bags and kept in feed box trucks. Box trucks were stored in an environment with an average room temperature of 35 °C and humidity ranging between 60-75%. Feed was stirred every day to increase oxygenation.

At 5 weeks of age, ten (10) hens/pen were subjected to trans-cloacal inoculation with 1 mL of inoculum containing 100,000 histomonads. Directly infected birds were wing tagged and returned to their respective pens to mingle with sentinel birds for the remainder of the trial. On day 16 post-challenge, the remaining tagged birds were re-infected to boost potentially the infection rates. All mortalities were recorded and necropsied for histomoniasis lesions in the ceca and liver. At 5 weeks post-challenge, all remaining birds were euthanized, and the livers and ceca were scored for signs of histomoniasis.

Diagnostics and data collection

All poults were examined daily for clinical signs of histomoniasis. Mortalities were recorded daily, and the dead were necropsied throughout the experiments and examined for signs of histomoniasis. At the end of each trial, all the birds were euthanized, and cecal and liver lesions were scored for histomoniasis and assigned a severity score between 0 and 4. For cecal lesions: 0 – no infection, 1 – slight thickening of the ceca but maintained functional fermentation, 2 – thickened cecal wall, small cecal core formation, still partially functioning, 3 – severely inflamed cecal wall, caseous core filling most of the ceca, 4 – a severe inflammation of the cecal wall, necrotic, friable, with the entire cecal lumen filled with a caseous core, complete loss of function. For liver lesions: 0 – no infection, 1 – less than 5 small foci, 2 – multiple foci throughout the liver lobes, 3 – larger and small foci, 4 – many large foci, necrotic lesions. Birds with a score greater than 1 in the ceca or liver, were considered positive for histomoniasis. The infection rate was calculated based on birds showing positive signs of histomoniasis in the ceca or the liver compared to the total number of birds inoculated with *H. meleagridis*.

Statistical analysis

Each replicate served as the experimental unit and data for all 3 trials were analyzed using the GLM procedure in SAS 9.4 (Cary, NC, USA). Mortality and infection rate data were transformed to arcsine before statistical analysis. Statistical differences between treatment means were separated using 'Duncan's multiple range test. For all tests, statistical differences were defined as $P < 0.05$.

RESULTS

In all three experiments, no significant lateral transmission was observed. The data only analyzed infected birds.

In experiment 1, FS (feed withdrawal) and HE + RCP (high electrolytes + reduced crude protein) had significantly higher infection rates in directly challenged turkeys (P=0.0168) compared to the PC (Table 6). FS had the greatest mortality (P =0.0144) compared to the rest of groups. The birds from the same group also have greater liver (P=0.0059) and ceca (P=0.0176) scores compared to the PC. No lateral transmission was observed in this experiment due to the application of different stressors.

In experiment 2, PC, RCP, RCP+C and RCP+W infection rates were not significantly different (P=0.1076) (Table 7). Mortality rates of PC, RCP, RCP+C, and RCP+W were not significantly different (P = 0.0894). The cecal scores of directly inoculated birds were higher in PF, RCP+C, and RCP+W compared to PC (P = 0.0315). Moreover, the liver score of directly inoculated birds in the RCP+C experimental group had significantly higher liver and ceca scores compared to P.C. (P = 0.0315). Interestingly, one case of lateral transmission was observed within the RCP experimental group. To capture the possible factors that could induce lateral transmission, we conducted experiment 3.

In experiment 3, again, no lateral transmission was found. There were no significant differences observed among treatments in infection rate, (P = 0.3975), mortality (P = 0.8220), liver scores (P = 0.5583) or cecal score, (P = 0.3003) (Table 8).

DISCUSSION

In this study, stressors often experienced by turkeys in commercial production were induced to better understand their effects on histomoniasis progression and transmission. Environmental and internal stressors have been previously defined as influential to poultry's gut health, sometimes causing unseen internal damage to the intestinal tract

(Burkholder et al., 2008). Limited data are available on the effects of commercial production stressors on histomoniasis development and transmission.

Lateral transmission

Lateral transmission of *H. meleagridis* in the absence of a vector has been replicated in a research model since the discovery of the cloacal drop method of infection (Hu et al., 2004). High lateral transmission rates have been previously observed in other laboratory models ranging from 75 to 85% (McDougald, 2005). However, lateral transmission has not been observed in the current research laboratory. We have not been able to explain this discrepancy, but we hypothesized that there were environmental or feed-related differences between these different laboratory settings. Differences in location, quality of feed ingredients, or poultry rearing environment may influence the lateral transmission of *H. meleagridis* in these two locations. Further research needs to be done to combine multiple factors or new stress factors on histomoniasis lateral transmission in turkeys.

Feed formulation and ingredient quality

Previously, our laboratory could produce 15 to 20% lateral transmission utilizing *H. meleagridis* isolates that have continuously been used and maintained in our laboratory infection model (Payne, 2017). To reproduce this model, a feed formulation like the one used by Payne (2017) in this laboratory that yielded high lateral transmission and combined the feeding of the modified diet in combination with different stressors. However, no lateral transmission was observed. High electrolytes in the feed lead to wet litter (Veldkamp et al., 2017) and potentially promote lateral transmission of *H. meleagridis*. No lateral transmission was observed in the birds fed with HE+RCP diet (high electrolytes and reduced crude protein). Nevertheless, the HE+RCP (high electrolytes and reduced crude protein) diet led to a 20%

greater infection rate compared to the PC group. The increased infection rate may be due to both high electrolytes, and 5% decrease in crude protein. Amino acids in the diet are important for gastrointestinal lining integrity, gut protein production, and immunomodulatory activity (Alagawany et al., 2020). Diets formulated with a reduced protein profile led to unbalanced supplies of amino acids and small peptides. A reduction in crude protein in the diet has been shown to increase intestinal permeability, leaving the bird more susceptible to bacterial translocation (Gilani et al., 2016; Chen et al., 2015). Possibly the reduced crude protein in the diets also created a condition for more abundant bacteria in the gut, which facilitated greater histomonas growth and infection (McDougald, 2005).

Because HE+RCP diet increased infection rate, it was important to understand whether the reduced crude protein in the diet played a vital role in histomoniasis infection. In experiment 2, the infection rates in all three treatments fed the 4% reduced crude protein diet (RCP, RCP+C, RCP+W) led to higher infection rate compared to the group fed with standard diets (PC). The reduction in crude protein may contribute to the loss of proper gut-barrier function, which may allow for an increased *Histomonas* growth and reactivity, which enabled them to more readily pass the epithelium into the hepatic portal system causing higher morbidity (Barekatain et al., 2019). Further studies are needed to understand the mechanism associated with dietary crude protein, bacteria and potentially increased *Histomonas* virulence. Due to the high infection rate caused by reduced dietary crude protein diet formulation, other efforts were made to further explore the effects of feed ingredient quality and feed storage on histomoniasis progression and lateral transmission. Unfortunately, there was no lateral transmission in experiment 3 when the birds were fed a 4% reduced crude protein diet. However, this diet did induce high infection rates in turkeys (60-67%). Feed analysis showed that APD and

AFPD + R feed had 19 ppb and 6 ppb aflatoxin, respectively, while AFPD feed had 0 ppb. For birds directly inoculated, aflatoxin did not increase the infection rate compared to birds given feed with no aflatoxin. Aflatoxins are known to cause low-level liver damage in turkeys, and compromise immunity at levels >400 ppb (Giambrone et al., 1985; Quist et al., 2000). For this study, low levels of aflatoxin may not lead to increased infection rates or have a significant impact on infections as the reduced crude protein.

Feed withdrawal

The effects of feed withdrawal in experiment 1 significantly increased infection rates, which were more than double the positive control rate (36.7% vs. 15.0%). Fasting has been shown to increase the likelihood of pathogen adherence to the intestinal wall, alter the microbiota within the lumen, and causes behavioral and physiological changes (Burkholder et al., 2008; Wang et al., 2021). This increase in infection rate could be due to dysbiosis caused by the feed withdrawal, leading to overgrowth of *Escherichia coli, Salmonella typhimurium* (Thompson et al., 2008). *H. meleagridis* growth depends on specific bacterial interactions, with *E. coli, C. perfringens, Bacillus subtilis (a fecal/ground based bacterium)* and *S. typhimurium* supporting *H. meleagridis* growth *in vitro* at higher rates (Ganas et al., 2012a). Co-infections of *H. meleagridis* and *E. coli* have been found to compromise the health of laying hens (Abdelhamid et al., 2020). In the current study, fasting induced bacterial overgrowth combined with simultaneous *H. meleagridis* infection ***could*** affect infections in birds, with increased lesions in organ systems.

Coccidiosis

In experiment 2**,** infection with LP diet led to a significant increase in mortality compared to the positive control group (87% vs. 47%, respectively). Infections with

cocci have previously been reported to cause dehydration, reduced nutrient absorption through the gastrointestinal tract, mortality, and reduction in growth (Chapman, 2008). Coccidia has been suggested to be one of the contributing factors to histomoniasis progression. Previous research in chickens showed an increase in histomoniasis related liver lesion severity when chickens were infected by *E. tenella* at low levels (McDougald and Hu, 2000).

Cecal coccidiosis in turkeys, and *coccidial* effects on histomoniasis development have not been well studied. There are many anecdotal field reports of *coccidial* infections occurring 2-3 weeks prior to histomoniasis outbreaks, but laboratory models have not explored this possibility. One study did find that cecal coccidiosis in turkeys led to a significant decrease in lateral transmission of *H. meleagridis.* Still, the investigators did not measure histomoniasis severity in directly inoculated turkeys infected with *H. meleagridis* and *E. adenoeides* (McDougald and Fuller, 2005). This may be due to cecal *Eimeria* outcompeting *H. meleagridis* for space or nutrients. A combination of *coccidial* infection and *H. meleagridis* challenge can pose a greater health challenge to turkeys than one infection alone. Further research is needed to understand the effects of *coccidial* infections on histomoniasis development in terms of minimum numbers of histomonads and or/*coccidial* organisms need to cause and exacerbate disease status and possibly be involved in bird-to-bird-related lateral transmission if it is really a true phenomenon in turkeys.

CONCLUSION

In the current study, the lack of lateral transmission among turkeys under various environmental and dietary regimes known to exacerbate histomoniasis development and progression does not abrogate the concept of lateral transmission of histomoniasis in turkeys. Thus, additional research is necessary to understand how lateral transmission might occur and

whether there are interactions among external factors that influence non-fecal/oral transmission efficiency.

Although lateral transmission was not observed in this research effort, the effects of feed withdrawal and reduced crude protein feed formulation increased the overall infection rate and cecal lesion scores of birds directly challenged via trans-cloacal infusion. Coccidiosis and feed withdrawal, along with reduced crude protein feed formulation had an overall increase in mortality rate. These findings clarify the effects of common stressors on turkey histomoniasis. Observations documented in this research may contribute to developing a strategy via stress reduction that would help alleviate the adverse effects of *H. meleagridis* infection in turkeys.

Table 4. Composition of diets for experiment 1.

	Treatment[1]	
	NC, PC, TS, DS, BD	HE + RCP
Ingredient	**%**	
Corn	38.15	45.4
Soybean meal	26.55	18.65
Soft wheat	20	20
Poultry meal	8.85	5
Mono-dicalcium phosphate	0	2.1
Hipho 2500 GT	0.06	0
Limestone	0.92	1.215
Mineral premix[2]	0.2335	0.2335
DL-Methionine	0.098	0.158
Choline chloride	0.2	0.2
Vitamin premix[3]	0.2	0.2
Selenium premix[4]	0.05	0.05
Potassium sulfate	0	1.14
Sodium bicarbonate	0	0.715
L-Threonine	0	0.064
L-Lysine	0	0.2565
Salt	0.4	0.0425
Poultry fat	4.3	4.6
Calculated nutrients		
ME kcal/kg	3102.35	3076.06
E.E. %	78.4	77.5
CP %	23.16	18.15
Lys %	1.15	0.99
Met %	0.46	0.45
Met + Cys %	0.79	0.62
Calcium %	0.70	1.13
Available Phosphorous %	0.24	0.16
Sodium %	0.22	0.27
Chloride %	0.35	0.12
Dietary electrolyte balance meq/kg	200.8	302

[1] NC = Negative control; PC = positive control; TS = transportation stress; HE+RCP = High electrolytes and reduced crude protein; FS = Feed withdrawal stress; BD = Butyric acid (0.375% Butipearl); CS = cold stress; DS = delay stress.
[2]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

Table 4 (Continued)

[3]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 IU.vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.

[4]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet.

Table 5. Feed formulations for experiments 2 and 3.

	Treatments[1]	
	Exp. 2: PC	Exp 2: RCP, RCP+C, RCP+W Exp. 3: APD, AFPD, AFPD+R
Ingredient	**%**	
Corn	47.04	54.3
Soybean meal	36.3	39.5
Poultry meal	10.85	0
Salt	0	0.4
Limestone	0	0.444
Mono-dicalcium phosphate	3.91	0
Sodium bicarbonate	0.405	0
Selenium premix[2]	0.05	0
Vitamin premix[3]	0.2	0.5
Mineral premix[4]	0.265	0.1
DL-Methionine	0.205	0.116
Choline Chloride	0.2	0
Soybean oil	0	2.424
Pet food grade poultry fat	0.575	0
Calculated macros		
ME Kcal/kg	2800	2908.9
E.E. %	5.16	4.86
CP %	26.94	22.68
Lys %	1.81	1.26
Met %	0.77	0.44
Met+Cys %	1.17	0.81
Calcium %	1.46	1.1
Available phosphorous %	1	0.81

[1]PC = positive control; RCP = reduced crude protein; RCP+C = reduced crude protein + cocci; RCP+W = reduced crude protein + feed withdrawal; APD = aflatoxin, reduced crude protein; AFPD = aflatoxin free, reduced crude protein; AFPD+R = aflatoxin free, reduced crude protein, rancid.
[2]Selenium premix=1 mg Selenium premix provides 0.2 mg Se (as Na2 SeO3) per kg of diet.
[3]Vitamin premix provides per kg of diet: 13,200 IU vitamin A, 4000 IU vitamin D3, 33 UI vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid.
[4]Mineral premix provides per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt.

Table 6. Experiment 1: Comparison of stress factors or feed formulation on histomoniasis progression: Effects on infection rates, mortality and pathological scores of ceca and liver at necropsy of birds.

			Lesion score	
Treatment[1, 2]	Infected (%)	Mortality (%)	Ceca	Liver
PC	15.0 ± 7.64^{b}	23.3 ± 8.82^{bc}	1.10 ± 0.57^{c}	1.10 ± 0.51^{b}
TS	16.7 ± 3.33^{b}	16.7 ± 5.77^{c}	1.30 ± 0.24^{bc}	0.97 ± 0.09^{b}
HE + RCP	35.0 ± 2.89^{a}	43.3 ± 8.82^{ab}	2.80 ± 0.23^{a}	2.30 ± 0.17^{a}
FS	36.7 ± 6.0^{a}	63.3 ± 12.02^{a}	2.60 ± 0.66^{ab}	2.50 ± 0.4^{a}
BD	15.0 ± 5.77^{b}	13.3 ± 8.82^{c}	1.30 ± 0.54^{bc}	0.91 ± 0.26^{b}
CS	13.3 ± 6.0^{b}	13.3 ± 8.82^{c}	1.03 ± 0.45^{c}	0.90 ± 0.40^{b}
DS	8.3 ± 1.67^{b}	13.3 ± 3.33^{c}	0.47 ± 0.03^{c}	0.73 ± 0.22^{b}
P-value	0.0168	0.0144	0.0176	0.0059

[1]PC = infected control group; TS = transportation (2 hours) stress; HE+RCP = high electrolyte and reduced crude protein diet; FS = feed withdrawal (18 hour) stress; BD = Butyric acid diet; CS = cold stress; DS = delayed placement stress.

[2]Infection rate, mortality, and lesion scores were calculated from the directed infected birds.

[a, b, c] Means within a column with different superscripts differ significantly $P<0.05$. The data was presented as means ± standard error. N=3

Table 7. Experiment 2: Comparison of feed formulation and stress factors on histomoniasis progression: Effects on infection rates, mortality and pathological scores of ceca and liver at necropsy of birds.

			Lesion score	
Treatment[1,2]	Infected (%)	Mortality (%)	Ceca	Liver
PC	26.7 ± 6.67	46.7 ± 6.67	0.93 ± 0.13[b]	0.67 ± 0.13[b]
RCP	73.3 ± 13.3	73.3 ± 13.3	2.85 ± 0.52[a]	1.62 ± 0.22[ab]
RCP+C	86.7 ± 6.67	86.7 ± 6.67	3.83 ± 0.17[a]	3.22 ± 0.42[a]
RCP+W	73.3 ± 17.6	93.3 ± 6.67	2.80 ± 0.83[a]	2.27 ± 0.84[ab]
P-value	0.1076	0.0894	0.0315	0.0315

[1]PC = infected control group; RCP = reduced crude protein feed formulation; RCP+C = RCP diet and 5x cocci vaccine dose on day of infection; RCP+W = RCP diet and 18-hour feed withdrawal 1-day post-infection.
[2]Infection rate, mortality, and lesion scores were calculated from the directed infected birds.
[a, b, c] Means within a column with different superscripts differ significantly $P<0.05$. The data was presented as means ± standard error. N=3

Table 8. Experiment 3: Comparison of feed formulation and feed storage methods on histomoniasis progression: Effects on infection rates, mortality and pathological scores of ceca and livers at necropsy of birds.

Treatment[1]	Infected (%)	Mortality (%)	Lesion score	
			Ceca	Liver
APD	60.0 ± 10.0	46.7 ± 3.33	3.8 ± 0.16	4.00 ± 0
AFPD	66.7 ± 3.33	60.0 ± 5.77	3.74 ± 0.18	3.87 ± 0.13
AFPD + R	53.3 ± 8.82	53.3 ± 8.82	3.51 ± 0.26	3.94 ± 0.06
P-value	0.3975	0.8220	0.3003	0.5583

Means within a column did not differ significantly P>0.05.
The data are presented as means ± standard error. N=3
[1]APD = feed formulated with a 4% CP reduction, produced using aflatoxin contaminated ingredients (19 ppb); AFPD = same feed formulation as APD, produced with aflatoxin-free ingredients (0 ppb); AFPD+R = APD feed formulation, produced using same feed ingredients at AFPD, stored for 2 weeks in high heat and humidity to encourage aflatoxin production (6ppb).
[2]Infection rate, mortality, and lesion scores were calculated from the directed infected birds.

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CHAPTER 3: Oral inoculation of turkeys with *Histomonas meleagridis* induces histomoniasis

ABSTRACT

H. meleagridis could be excreted into the environment in cecal content, but consumption of cecal content containing histomonads has not been confirmed as a possible route of infection. To test the fecal-oral route of infection for *H. meleagridis,* four experiments were conducted. In Experiment 1, poults were placed in 3 experimental groups (10 birds x 3 replicates/treatment) consisting of *H. meleagridis*: intracloacal injection (CMI), oral inoculation (OIM), or oral inoculation with cecal content (OICC). Poults were inoculated with 50,000 histomonads at 8 AM and 5 PM for 5 days, which was initiated from 14-day of age. As expected, cloacal infections (CO) produced 100% infection rates. Infections using a cecal content mixture produced 43% infection rates, while the oral infection with *H. melegridis* in media led to 8% infection (P = 0.0006). The success of cecal content as the medium for oral inoculation led to Experiments 2 and 3. In Experiment 2, poults were assigned to 3 experimental groups (3 rep/trt). Treatments were *H. meleagridis* isolates in cecal content administered orally: Zeeland, Michigan (ZM), Buford, Georgia (BF), and Arkansas (BBAR2) - . Oral infection rates of each isolate were different significantly with BFC and ARC isolates producing higher infection rates compared to the ZM isolate (P=0.0136). Experiment 3 consisted of poults assigned to 5 treatments (1 rep/trt). Treatments were *H. meleagridis* and cecal content oral inoculation for: 1 day (1D), 2 days (2D), 3 days (3D), 4 days (4D) or 5 days (5D). Experiment 3 provided preliminary data showing that as the frequency of oral inoculations increased, the infection rates trended upward (1D 20%, 5D 40%). Further research is needed to understand whether oral transmission of *H. meleagridis*

occurs naturally in the field or whether there is genetic variation within isolates allowing for oral transmission.

INTRODUCTION

Histomonas meleagridis is a protozoal pathogen that affects gallinaceous birds, causing histomoniasis, commonly known as blackhead disease (Tyzzer, 1919). Outbreaks of histomoniasis in turkeys can be devastating, causing up to 100% mortality in a flock (Mcdougald, 1998). *H. meleagridis* is maintained in the environment by the cecal nematode, *Heterakis gallinarum*, with *H. meleagridis* surviving for up to 3 years in the nematode's egg (Farr, 1960). Once a turkey consumes *H. meleagridis* contaminated eggs, they hatch in the small intestine and release *H. meleagridis* (Cupo and Beckstead, 2019). Clinical signs of histomoniasis and peak mortality are seen within two weeks after initial infection (Hess et al., 2015). The high mortality in a turkey barn results primarily from the transmission of *H. meleagridis*. Initiation of a histomoniasis outbreak requires the cecal nematode, but lateral transmission might happen without the *Heterakis* vector. Past research has demonstrated, purportedly, that turkeys utilize cloacal drinking to imbibe cecal content excreted from infected turkeys (Hu et al., 2004; Armstrong and McDougald, 2011). *H. meleagridis* contaminated cecal content is moved into the colon and then into the ceca, through a mechanism called reverse peristalsis, to initiate histomoniasis (Sacranie et al., 2007). The current research model to initiate histomoniasis was developed in 2004 by McDougald. By placing inoculum on the dorsal lip of the cloaca, stimulating reverse peristalsis and relying on smooth muscle contractions to take *H. meleagridis* up into the turkeys body (Hu et al., 2004). Infection rates by this method have proved to be steady, with research using this method reporting infection rates of 80-100% (Hu and McDougald, 2003; Hu et al., 2006; Hauck and Hafez, 2013).

Though cloacal inoculation is used in research models, it has not been confirmed in the field. For this reason, the fecal-oral transmission route of *H. meleagridis* is also debated as a possible route of transmission. *H. meleagridis* is highly sensitive to pH environments below 4, with a drop in *H. meleagridis* proliferation (Hauck, 2010). When *H. meleagridis* is ingested, it will encounter an acidic environment in the proventriculus and gizzard of turkeys, with the gizzard pH being approximately 3.5 (Mabelebele, 2014). This pH falls below the minimum pH in which *H. meleagridis* can survive for a short period of time, and *H. meleagridis* would not survive the upper GI tract. However, in the field, *H. meleagridis* is not unprotected, it is secreted along with cecal content onto the turkey house litter-covered floor. Cecal content may provide a natural protective covering for *H. meleagridis* to survive the decreased pH of the gizzard and translocate to the ceca.

Swales summarized many of the original experiments with *H. meleagridis* in which feces, vectors, and culture were given to turkeys orally (Swales 1950). These experiments varied in infection rates from 75% (contaminated feces mixed into turkey feed, (Moore, 1896) to 0% (emulsified histomoniasis liver lesions fed back to turkeys). In 2004, Hu et. al, attempted to inoculate orally 2-week-old turkeys with 100,000 *H. meleagridis* in culture medium but could not recreate the oral route of infection, producing infection rates near 0%, even after a 6-hour feed withdrawal (Hu et. al., 2004). Coprophagic behavior is common in many avian species, which is observed in many wild birds and domesticated fowl (Lamb, 2017; Kobayashi, 2019). Turkeys consume cecal droppings to utilize short chain fatty acids and proteins produced by the microbiome in the ceca (Pan, 2013). Since cecal content is a natural carrier for *H. meleagridis* during an outbreak, and turkeys naturally consume cecal content, it is important to ascertain whether cecal content could be an optimal carrier for *H. meleagridis* and lead to an oral route of

infection. However, no experiments have been done using cecal droppings as the medium for oral inoculation of turkeys with *H. meleagridis*. It was hypothesized that there may be an alternative transmission pathway through the fecal-oral route, similar to routes of infection used by many other protozoa. The aim of this study was to determine whether oral inoculations of turkeys with *H. meleagridis* would induce histomoniasis and its involvement of factors involved in rates of infection via this fecal-oral route.

MATERIALS AND METHODS

All experiments were approved by the North Carolina State University Institute of Animal Care and Use Committee. For all three experiments, Nicholas turkeys were obtained from an Aviagen hatchery located in Lewisburg, WV. Experiment 1 was conducted in battery cages located at the North Carolina Agricultural Research Service Talley Turkey Education Unit at North Carolina State University. Experiments 2 and 3 were conducted in isolator cages located in the bird wing of Scott Hall in the Prestage Department of Poultry Science at North Carolina State University.

Inoculum preparation

Histomonas meleagridis cultures were collected previously from outbreaks in multiple locations in the Southeast US and Mid-West US, and cryopreserved. Three isolates from Zeeland, Michigan (ZM), Buford, Georgia (BF), and Arkansas (BBAR2) were used for Experiments 1, 3, and 4. Experiment 2 consisted of only one isolate per inoculum prepared. Isolates were removed from liquid nitrogen, thawed, and passed into 10 mL of modified Dwyer's media consisting of 0.8% (wt/vol) rice powder (Bob's red mill, Milwaukie, OR, USA), 5% horse serum (Cytiva HyClone, Waltham, MA, USA), in Medium 199 with Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) (Hauck et al., 2010). Cultures were incubated at

42 °C for 48 hours. Each isolate was observed for growth and passed into a new flask. This was repeated three times. Before each infection, *H. meleagridis* numbers were determined using a hemocytometer (Hausser Scientific, Horsham, PA, USA). Each isolate was counted and diluted to 50,000 histomonads/mL. The isolates were blended into one inoculum with approximately 1:1:1 ratio. The inoculum was stored in a portable incubator at 42 °C during the inoculation. Cecal content used for Experiments 1, 2, and 3 was frozen for 1 year prior to being thawed and used as medium. Cecal content for Experiment 4 was collected directly from turkeys confirmed to be negative for *H. meleagridis*, stored at 4 °C to discourage fermentation and used within a week of collection.

Inoculation methods

Intracloacal injection (CIM). Poults were inoculated intracloacally with 1 mL of inoculum containing 50,000 histomonads. Inoculated birds were suspended by their legs and held for 2 minutes after inoculation before being returned to respective their pen. This procedure was to ensure each bird had successful uptake of the inoculum.

Oral inoculation with culture media (OIM). For each poult, 1 mL of inoculum was pipetted into the esophagus. Poults were held until the inoculum was swallowed before being placed back in their respective cage.

Oral inoculation (OICC). A 1:1 ratio of cecal content and culture were gently mixed. 1 mL of the blended mixture was administered to each poult using a syringe and delivering the inoculum into the esophagus. Poults were held until inoculum was swallowed before their return to their respective cage.

Experimental design

Experiment 1

A total of 90 one-day-old poults were placed randomly in 3 experimental groups (3 replicates X 10 birds) in battery cages. Treatments were *H. meleagridis* isolate mixture (BF, BBAR2, and ZM) administered through intracloacal injection (CIM), oral inoculation with modified Dwyer's media (OIM) or oral inoculation with cecal content (OICC). Inoculations were administered at 8 AM and 5 PM from day 14 to 18. The experiment was terminated on day 32.

Experiment 2

A total of 90 one-day-old poults were placed randomly in 3 experimental groups (3 replicates X 10 birds) in isolator cages. Treatments consisted of BF *H. meleagridis* isolate in cecal content (BFC), BBAR2 *H. meleagridis* isolate in cecal content (ARC), or ZM *H. meleagridis* isolate in cecal content (ZMC). Oral inoculation was done at 8 AM and 5 PM from day 14 to 18. The experiment was terminated on day 32.

Experiment 3

A total of 50 poults were randomly placed in 5 experimental groups (1 cage X 10 birds) in isolator cages. Treatments consisted of *H. meleagridis* isolate mixture (BF, BBAR2, ZM) in cecal content and orally gavaged to turkey poults for: 1 day (1D), 2 days (2D), 3 days (3D), 4 days (4D), or 5 days (5D). One day of infection indicates *H. meleagridis* infection at 8 AM and 5 PM. Each treatment is infected for the number of days specified by the treatment: 1 day, 2 days, 3 days, 4 days, or 5 days. Day 1 (1D) infection began on day 14 and day 5 (5D) infection ended at day 18. The experiment was terminated on day 32.

Diagnostics

All poults were examined daily for clinical signs of histomoniais. Mortalities were necropsied throughout the experiments and examined for signs of histomoniais. Cecal and liver lesions were scored and recorded. All trials were terminated at day 28. Ceca and liver lesions were scored for histomoniasis. Cecal and liver lesions were assigned a numerical score of 0 to 4. For cecal lesions: 0 – no infection, 1 – slight thickening of the ceca but still functioning, 2 – thickened cecal wall, small cecal core formation, still partially functioning, 3 – Severely inflamed cecal wall, caseous core filling most of the ceca, 4 – severe inflammation of the cecal wall, necrotic, friable, with the entire cecal lumen filled with a caseous core, complete loss of function. For liver lesions: 0 – no infection, 1 – less than 5 small foci, 2 – multiple foci throughout the liver lobes, 3 – larger and small foci, 4 – many large foci, necrotic lesions. Birds with a score of greater than 1 in the ceca or liver were considered positive for histomoniasis. The infection rate was calculated based on birds showing positive signs of histomoniais in the ceca or the liver compared to the total number of birds inoculated with histomonads. Cecal and liver lesions scores were calculated based on birds showing positive signs in the ceca.

Statistical analysis

Each replicate served as the experimental unit and data for Experiments 1 and 2 were analyzed using the GLM procedure in SAS 9.4 (Cary, NC, USA). Arcsine transformation was conducted on infection rate and mortality percentages prior to analysis. Statistical differences between treatment means were separated using Duncan multiple range test. For all tests, statistical differences were defined as $P < 0.05$.

RESULTS

A series of studies were conducted to explore the possibility of infecting *H. meleagridis* via oral inoculation. In experiment 1, three infection routes with different carriers were tested

and compared. The results showed intracloacal inoculation with *H. meleagridis* (CIM) had the highest infection rate, at 100% (Figure 3.1A). The oral inoculation with *H. meleagridis* in media (OIM) yielded the lowest infection rate, at 8.7%. Meanwhile, oral inoculation of turkeys with *H. meleagridis* in cecal content (OICC) produced greater infection rates, at 43%, compared to the OI experimental group (P=0.0003). CIM mortality was 88%, statistically higher than the OIM and OICC groups, at 7% and 15% respectively (Figure 3.1 B) (P=0.0025). The liver and ceca scores followed the same trend with the CIM and OICC experimental groups both having a greater infection rate compared to the OIM group (figure 3.1 C and D) (P = 0.0018 and 0.0003, respectively). This experiment showed the possibility of the fecal-oral route of infection to induce histomoniasis with cecal content.

In Experiment 2, a study was designed to ascertain whether different isolates have various infection rates via the oral infection route. The results indicated *H. meleagridis* isolates BF (BFC) and BBAR2 (ARC) had statistically higher infection rates than the ZM isolate (Figure 3.2 A) (P = 0.0150). No difference in mortality was observed, and the various isolates did not vary in disease progression (figure 3.2 B). Cecal score averages for BFC, ARC, and ZMC were 2.49, 2.17, and 0.5, respectively (Figure 3.2 C). BFC and ARC cecal scores were statistically higher than ZMC (P = 0.0107). BFC, ARC, and ZMC liver scores were 2.6, 2.3, and 0.8, respectively (Figure 3.2 D) (P = 0.0650). In summary, the different tested isolates in these experimental settings showed various infection rates and lesion scores but did not have diverse impacts on mortality.

Experiment 3 was to explore the role of infectious challenge frequency on infection rate and disease progression in turkeys. Due to the limited space and high labor intensity, there were no replicates within treatments. The results were presented with average value. In Figure 3.3, an

upward trend from one day of oral inoculation (1D) to five days of oral inoculation (5D) was observed. It also showed, even only with two-time oral gavage, the infection rate could reach to 20%.

DISCUSSION

Many different mediums have been studied for *H. meleagridis* oral inoculations (Swales, 1948) with only one experiment utilizing the natural medium in which *H. meleagridis* is shed. Unspecified amounts of excreta were collected from infected turkeys and mixed into the feed of healthy turkeys. This was done for 2 weeks, with a 75% infection rate 3 weeks post infection (Turkeys, 1920). Previous research has shown the inability of *H. meleagridis* to survive in an environment with a pH less than 4 (Hauck et al., 2010a). The reported pH of the proventriculus and gizzard in turkeys is approximately 3.5 (Mabelebele et al., 2014). Anything consumed by the turkey, when consistently on feed, will remain in the proventriculus and gizzard for 2 hours before being neutralized in the duodenum. It is not likely that *H. meleagridis* will survive during this process. Cecal content pH ranges between 6.3 and 6.9 (Asare et al., 2021). The pH of the cecal content used in this research measured between 6.8-7.0. In addition to the physical protection, it is also possibly providing some buffering of HCl in the gizzard or barrier of protection to histomonads while being passed through the upper GI tract.

In this study, utilization of cecal content as a medium of infection was studied. The increase in infection rate observed in the turkeys OICC (orally inoculated with *H. meleagridis* in cecal content) compared to the OIM (unprotected *H. meleagridis*) may be due to cecal content providing a form of physical protection for *H. meleagridis* to survive the acidic environment of the upper GI tract. Inoculation of turkeys with *H. meleagridis* in cecal content produced increased infection rates compared to unprotected *H. meleagridis*. The cecal and liver lesions of

turkeys orally inoculated with cecal content were approximately one point higher than the group inoculated with unprotected *H. meleagridis* (Figures 3.1 D and E).

The cecal content used in the current experiments were stored at -20 °C for approximately one year. Another study with the same procedure as Experiment 1 but using fresh cecal content led to 90% infection rate. (Appendix A Figure 3.4 A) (P=0.6433). Oral inoculation with fresh cecal content increased infections by 50% compared to the oral inoculation with frozen cecal content (43%). Mortality of the CI group was 93% and FO was 40% (Appendix A figure 3.4 B) (P=0.1268). Average cecal scores of CI and FO were 3.7 and 3.9, respectively (Appendix A Figure 3.4 C) (P=0.8416). Average liver scores of CI and FO were 3.9 and 3.7.(Appendix A Figure 3.4 D) (P=0.4750). The difference between two trials might be due to the consistency of the fresh cecal content possibly provide better physical protection, because it was more viscous and stickier than the previously frozen cecal content. Secondly, research has shown interactions between *H. meleagridis* and certain opportunistic bacteria (Bilic and Hess, 2020). The presence of this bacteria in cecal content may play a role in *H. meleagridis* survival in the upper G.I. tract. Bacterial infections including C. perfringens can infect the upper G.I. tract of poultry, which may affect the microbiome or pH (Fossum et al., 1988). The capacity of fresh cecal content to protect *H. meleagridis* has not been previously studied nor has it been compared to frozen cecal content as a medium for oral inoculation. The mechanism that facilitated oral transmission is not completely understood at this time. Nevertheless, the current study provided evidence that cecal contents affords different levels of protection for *H. meleagridis* allowing it to survive passage through the acid upper gastrointestinal tract of the turkey.

Three field isolates were compared following infectious challenge through the oral route. Variation in infection rates and cecal and liver lesions were observed. With Buford (BFC) and

BBAR2 (ARC) isolates producing much higher infection rates, compared to Michigan (ZMC) (Figure 3.2 A). Previous research has found genetic variation among isolates depending on geographic region of origin (Wei et al., 2020). This suggests that there may be genetic differences among isolates that are reflected in terms of survivability of lower pH or ability to survive the entirety of the GI tract. It is yet to be ascertained whether there is a difference among isolates, genetic variations, virulence, and morphological variations that allow *Histomonas* isolate passage through the gastrointestinal tract of orally challenged birds. This research leaves no doubt that oral transmission is a distinct and viable means of transmission of histomonads to cause histomoniasis.

Previous study showed a singular challenge to turkeys with cecal material and cecal tissues collected from *H. meleagridis* infected turkeys. Lund (1956) orally challenged turkeys from 6 to 9 weeks of age with a singular dose of 10,000 histomonads in a slurry of cecal content and cecal tissues, producing a 24% infection rate. Meanwhile in our study we found increasing inoculation rates of *H. meleagridis* in cecal content produced an upward trend in infections rates.

The coprophagic behavior is common in the birds. In grouse, a bird species within the same family as turkeys, coprophagic behavior was observed in chicks 3 days after hatching and continued until 20 days of age (Kobayashi et al., 2019). Birds are naturally coprophagic, seeking out cecal content as a microbiome inoculum, as well as a source of short chain fatty acids (SCFAs) and B Vitamins (Pauwels et al., 2015; Sharma et al., 2019). Turkeys infected with *H. meleagridis* begin shedding cells in cecal content within one day after infection, with observed numbers reaching 20,000 histomonads per gram of cecal content (Landman et al., 2015). The frequent coprophagic behavior may lead to repeated consumption of litter materials, which

includes cecal contents containing *H. meleagridis*. This behavior may result in a significant amount of *H. meleagridis* consumed through this route.

Even though the frequency data (Experiment 3) were not statistically analyzed, it showed the possible relationship of increasing infection rate with increased oral inoculation frequency. During the commercial production, repeated consumption of litter materials is to likely happen during an outbreak. This preliminary data may further support, the possibility that healthy turkeys get infected by being exposed to the sick birds via repeated consumption of *H. meleagridis* contaminated cecal content. This research opens alternative routes of infectious challenge by oral administration of *H. meleagridis* in cecal material.

CONCLUSION

Cecal content produced greater infection rates than feeding unprotected *H. meleagridis* directly to turkeys. Comparisons of the three isolates used showed differences in infectivity when administered orally. Preliminary data also showed that with increasing infection frequency, infection rates also trended upwards. These three experiments provide evidence for the likelihood of oral transmission and provides evidence for the importance of litter management. Further research studying the coprophagic behavior of turkeys as well as oral transmission in field outbreaks is necessary to understand how to control lateral transmission.

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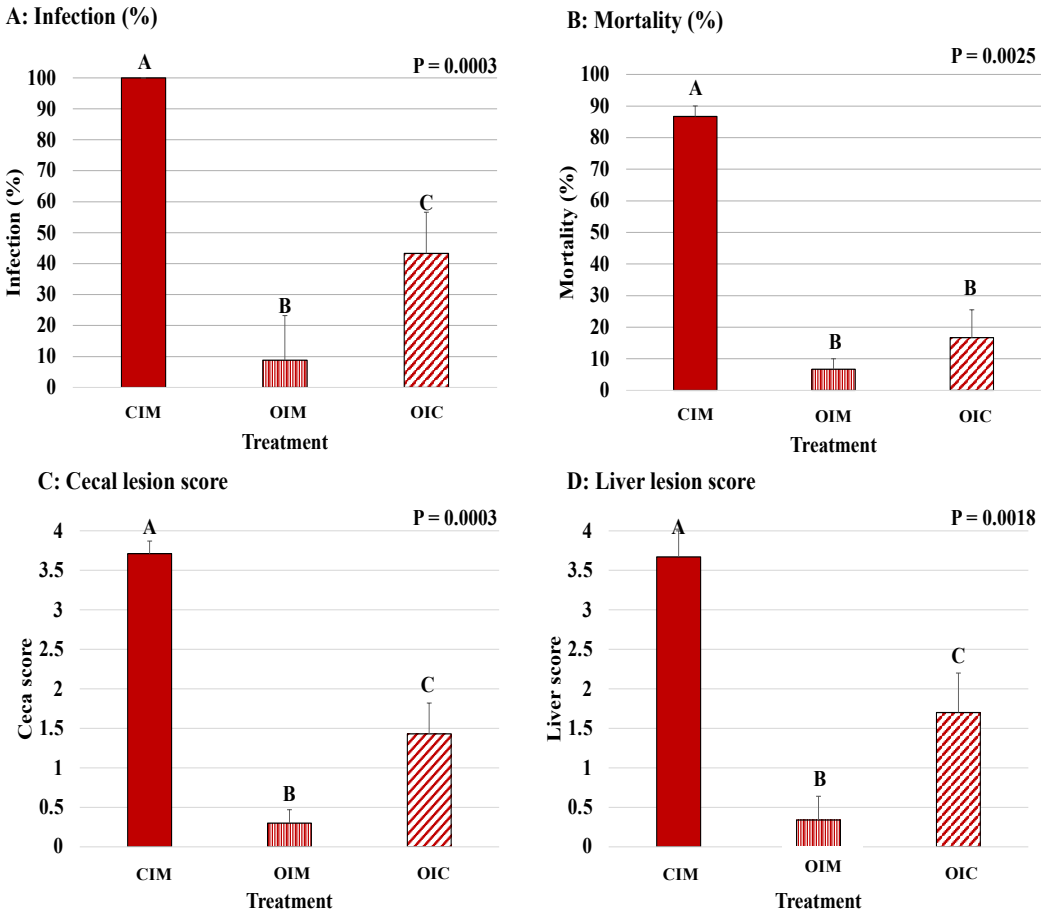
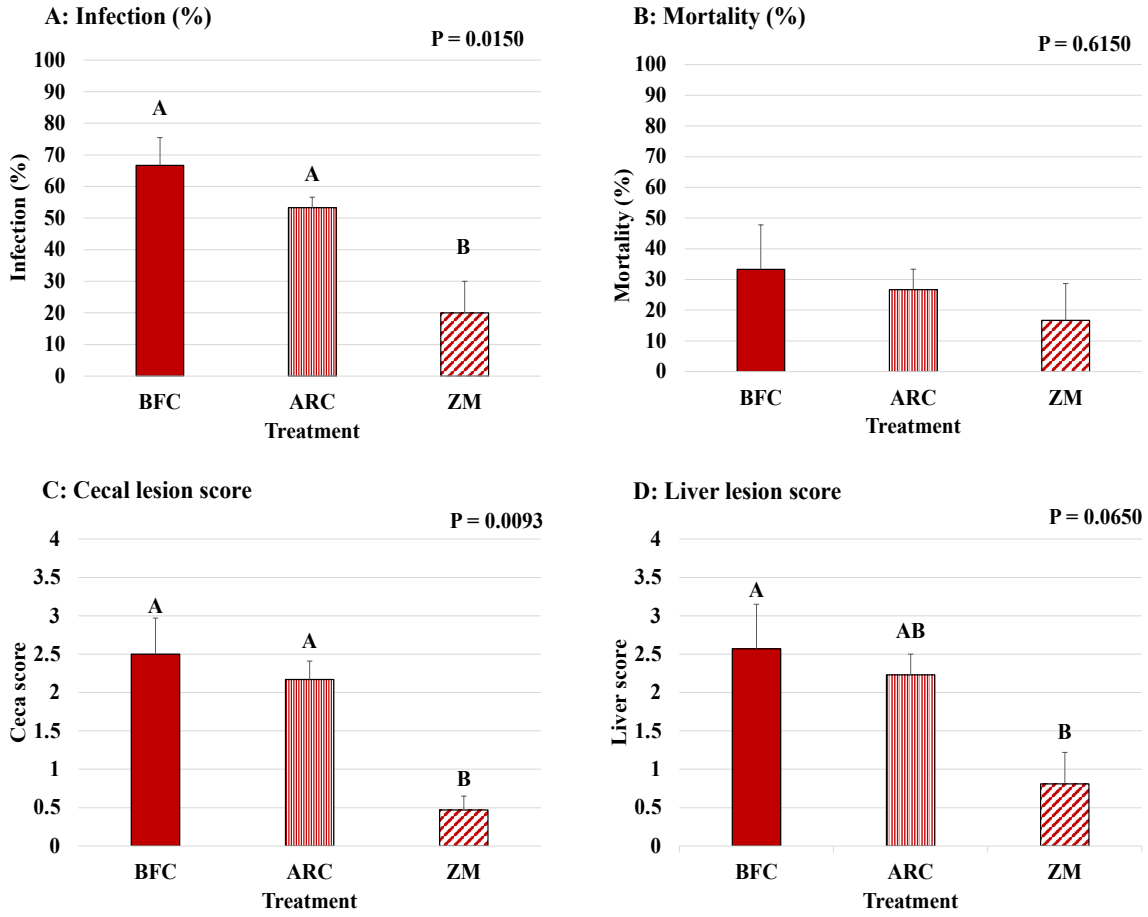
Figure 1: Experiment 1: Comparison of the route of infection on histomoniasis disease manifestation: Infection rate, mortality, cecal and liver scores.



CIM = Intracloacal inoculation, OIM = oral inoculation with *H. melagridis* in modified Dwyer's media, OICC = oral inoculation with cecal content

[a, b, c] Means within a column with different superscripts differ significantly $P<0.05$. The data was presented as means + standard error. N = 3.

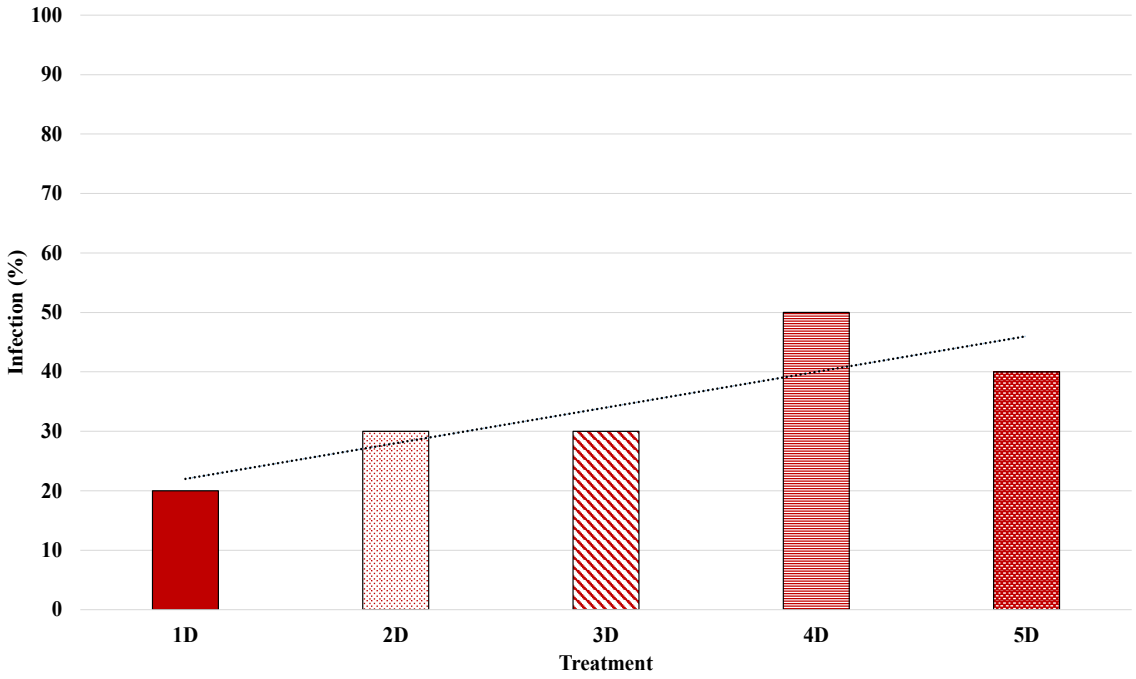
Figure 2: **Experiment 2**: Comparison of *H. meleagridis* isolate survivability of the oral route of inoculation: Infection rate, mortality, average day of mortality, cecal and liver scores.



BFC = Buford, Georgia *H. meleagridis* isolate, ARC = Arkansas *H. meleagridis* isolate, ZM = Zeeland, Michigan *H. meleagridis* isolate

[a, b, c] Means within a column with different superscripts differ significantly $P<0.05$. The data was presented as means + standard error. N = 3.

Figure 3: Experiment 2: frequency of oral inoculation: infection rates.



1D = Oral inoculation 8 AM and 5 PM for one day; 2D = Oral inoculation 8 AM and 5 PM for two days; 3D = Oral inoculation 8 AM and 5 PM for three days; 4D = Oral inoculation 8 AM and 5 PM for four days; 5D = Oral inoculation 8 AM and 5 PM for five days.

CONCLUSION

Histomoniasis outbreaks can potentially lead to major economic losses for turkey producers. Reports in variations of histomoniasis related mortality during an outbreak led to researchers to investigate factors affecting histomoniasis disease progression and its lateral transmission. The current study revealed that feed withdrawal, low protein with higher DEB diet, and low protein diet alone, increased *H. meleagridis* infection rates.

We also examined the fecal-oral route of infection using cecal content as the medium. Cecal content, when provided orally with *H. meleagridis* produced infection rates greater than *H. meleagridis* provided orally. Single isolate oral inoculation of turkeys led to variation in infectivity. Buford and BBAR2 isolates produced higher infection rates with higher cecal and liver lesion scores than the Michigan isolate. Furthermore, we investigated the effects of frequency of oral infections on infection rates. As infection rate frequency increased from one day to five days, the infection rate trended upwards as well.

The data presented through this dissertation emphasizes the need for further research in understanding the role of stress factors in histomoniasis progression and lateral transmission of *H. meleagridis.* Furthermore, cecal content has been shown to play an important role in the survival of H. meleagridis in the upper G.I. tract. Further identification of the exact mechanisms that provide this protection would provide greater insight into the specific route of transmission. This research is important for turkey producers, growers, nutritionists, and veterinarians because these findings reveal factors that may play a role in histomoniasis progression and the route of inoculation. While further research continues to investigate stressors that play a role in lateral transmission and disease progression, a focus on the stressors that had an impact on disease progression presented in this thesis may help control histomoniasis. Additionally, because *H.*

meleagridis enveloped in cecal content may allow for oral infection, litter quality and better litter management could be critical to control lateral transmission. Future research is still needed to better understand the relationship between stress and lateral transmission as well as the occurrence of fecal-oral transmission in histomoniasis outbreaks in the field.

APPENDIX

Appendix A

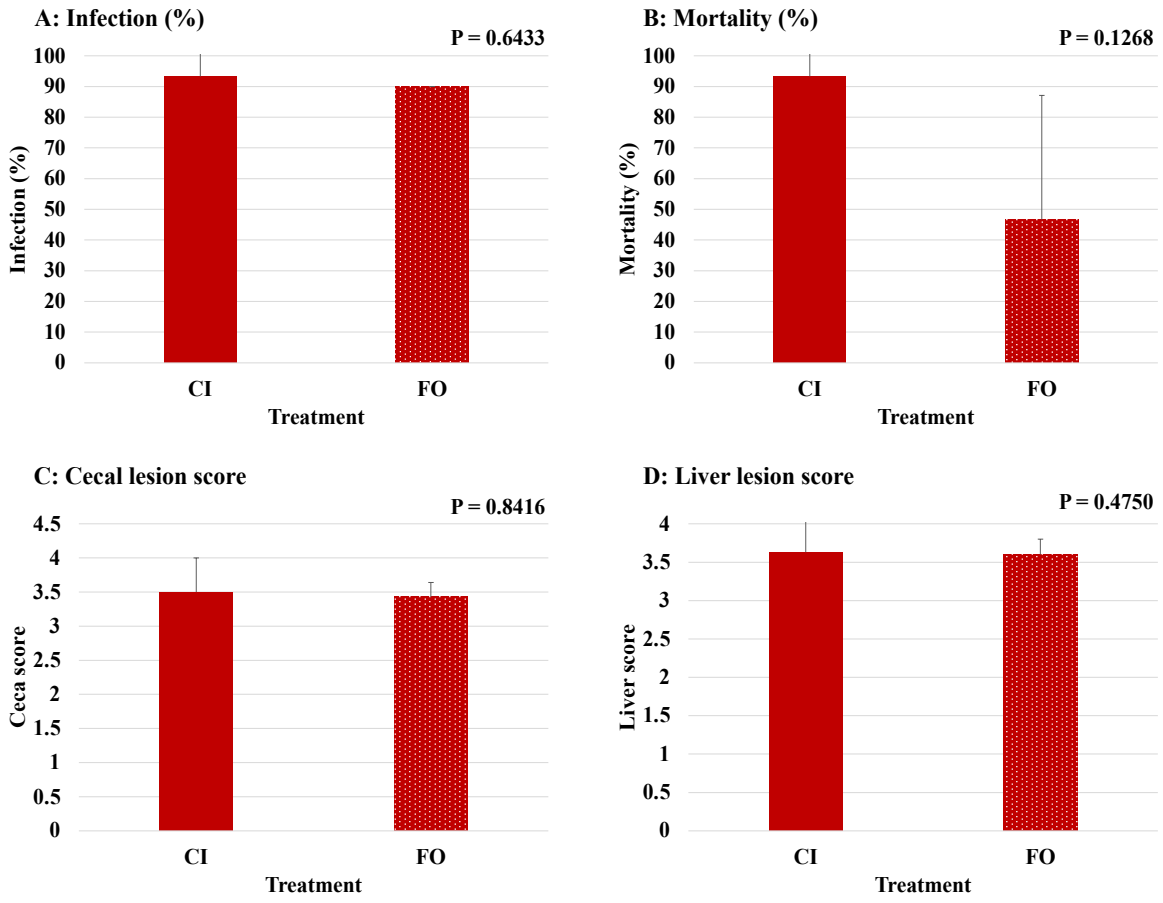
Experiment 4:

A total of 60 poults were randomly placed in 2 experiment groups (3 replicates * 10 birds). Treatments consisted of orally inoculated *H. meleagridis* isolate mixture (BF, BBAR2, ZM) in freshly collected cecal content (FO), or cloacal inoculation of the same isolate mixture (CI). Inoculation was done at 8 AM and 5 PM from day 14 to 18. The experiment was terminated on day 32.

Statistical analysis

Each replicate served as the experimental unit and data were analyzed using a student t-test in SAS 9.4 (Cary, NC, USA). For all tests, statistical differences were defined as $P < 0.05$.

Figure 1: Experiment 4: Comparison of cloacal inoculation and fresh cecal content: Infection rate, mortality, cecal and liver scores.



CI = Intracloacal inoculation; FO = fresh cecal content oral inoculation

[a, b, c] Means within a column with different superscripts differ significantly $P<0.05$. The data was presented as means + standard error. N = 3.