ABSTRACT

LOWERY, JUSTIN HENRY. Evaluating the Effects of *Cochlosoma anatis* Infection on Turkey Poult Gut Health and Performance (Under the direction of Dr. Lin Walker)

Cochlosoma anatis is a flagellated protozoan parasite of increasing concern in the United States turkey industry, causing the infectious disease, cochlosomiasis. Little research has been conducted regarding the parasite, with most characterizing its morphology, accompanied by field reports describing disease in commercial turkey production flocks. Symptoms of cochlosomiasis can be broadly described as enteritis throughout the small intestine, with its most classic symptom being runting and flock non-uniformity with high flock morbidity. The objective of this thesis was to characterize effects of cochlosomiasis in turkey poults up to 28 days of age and elucidate potential treatment avenues for further research. The first study in this thesis hypothesized that infection with C. anatis would lead to alterations in blood chemistry, nutrient digestibility, intestinal permeability, and histological morphology when inoculated with C. anatis at 14 days of age and assessed at 28 days of age. It was found that infection with C. anatis led to decreased body weight gain ($P<0.0001$) and feed intake ($P=0.0003$), with an increased feed conversion ratio ($P=0.0351$). Changes in blood carbon dioxide ($P=0.0004$), bicarbonate ($P=0.0006$), and excess base ($P=0.0024$) were seen, indicating potential stress on the blood pH balance in infected turkey poults. The calculated digestibility of amino acids was also lower with infection, though no changes were seen in intestinal permeability. Histopathological analysis revealed alterations of the villi in the duodenum and jejunum, suggesting a potential reduction in absorptive ability in these parts of the small intestine. The second study for this thesis hypothesized that infection with C. anatis at different ages would more accurately resemble the effects of cochlosomiasis as reported in case reports, and earlier infection would lead to more

severe disease compared to infection later in life. To test this hypothesis, turkey poults were inoculated with C. anatis at 0, 7, and 14 days of age, then grown to 28 days of age for data collection. Turkey poults inoculated earliest exhibited the worst disease effects seen through greater failure to gain weight ($P<0.0001$), and severely increased mortality to 70%, compared to 8% in non-infected poults. To conclude from these studies, it is apparent that infection with C. anatis resulted in changes to turkey poult performance parameters and physiological aspects. Additionally, more severe disease resulted from earlier infection with C. anatis. Future research is needed to better understand the physiological aspects of cochlosomiasis, however it appears the ability to maintain homeostasis is stressed. Additionally, it seems that efforts to prevent the introduction of C. anatis to turkey flocks at a younger age may greatly hinder the negative effects of infection.

Evaluating the Effects of *Cochlosoma anatis* Infection on Turkey Poult Gut Health and Performance

by
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Dr. Robert Beckstead

DEDICATION

To my family that has continually supported me in my greatest adventure yet and the friends who have helped shape me along the way.

BIOGRAPHY

Justin Henry Lowery is the son of Steve and Sharon Lowery and was born November 2nd, 1998, in North Richland Hills, TX. He grew up in Saginaw, TX, a small town just north of Fort Worth, and made frequent trips to Lincoln and Hastings, Nebraska to see his grandparents. He attended Saginaw Elementary School, Prairie Vista Middle School, and Saginaw High School, absorbing valuable information from the amazing teachers and friends he met along the way for which he is forever grateful. From both his parents, his brother, and their dog, Cassie, he developed an undying resolve to achieve his dreams and learned the meaning of hard work, kindness, respect, and love.

Justin completed his Bachelor of Science degree in 2021 at the University of Nebraska-Lincoln (UNL) where he majored in Microbiology with a minor in Veterinary Science. While at UNL, Justin worked at the Nebraska Veterinary Diagnostic Center in a diagnostic research microbiology laboratory focusing on ELISA development and MALDI-TOF analysis for infectious bovine keratoconjunctivitis (bovine pinkeye) under the direction of Dr. Dustin Loy and Dr. Matt Hille. These two individuals exposed Justin to the world of research and solidified Justin's desire to enter infectious disease research.

Following Justin's graduation from UNL, he sought to return to Texas and enroll in one of the medical schools in the state to pursue a PhD focused on viral infectious disease research, however acceptance to these schools was elusive. On May 17th, 2021, Justin embarked on his greatest adventure with the first email he sent to Dr. Lin Walker at North Carolina State University to work in her laboratory at the University. While working at NC State, Justin met many great people who have changed his life and will forever be his friends. After graduation, Justin plans to pursue his PhD under the direction of Dr. Lin Walker.

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 2

Chapter 3

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

CHAPTER 1

LITERATURE REVIEW

Introduction

Cochlosoma anatis is a flagellated protozoan parasite of significant importance to the United States turkey (*Meleagris gallopavo*) industry. *C. anatis* targets the intestinal tracts of ducks, wild birds, geese, and turkeys. Though the pathogenicity of *C. anatis* is debated, infestation of the avian gut with the parasite is grossly characterized as protozoal enteritis, with the specific term of cochlosomiasis. Disease symptoms associated with cochlosomiasis are failure to gain weight, runting (stunted growth), non-uniform bird size throughout the flock, diarrhea, lethargy, inflamed intestines, and an increased prevalence of co-infections (Bollinger and Barker, 1996; Gharagozlou and Dezfoulian, 2009; Beckstead, 2019). The exact timing of infection is non-descript; however, it is estimated that the parasite reaches its maximum concentration in the gut within 5 to 7 days following the onset of infection (Bollinger and Barker, 1996; Lindsay et al., 1999).

Through surveys of industry professionals and veterinarians, Clark et al. has demonstrated the rise in prevalence and overall concern by professionals in the commercial turkey industry regarding the health threat presented by flagellated protozoan parasites. The protozoan parasites of primary concern for the turkey industry are *C. anatis*, *Histomonas meleagridis*, the causative agent of the severe disease known has histomoniasis (blackhead), and *Eimeria*, the causative agent of coccidiosis, a major problem throughout all of poultry. In the latest Turkey Industry Annual Report (Clark and Froebel, 2022), infection with *C. anatis* has become the 13th-ranked overall issue facing the United States turkey industry with histomoniasis ranked as the 14th overall issue and coccidiosis ranking in the top 10. This is in continuation of

previous publications of the Turkey Industry Annual Report which has seen cochlosomiasis and steadily climb the rankings toward the most concerning issues facing the turkey industry over the past decade.

Likeness of *C. anatis* to other flagellated protozoans

Morphologically, *C. anatis* has been compared to other protozoan parasites, especially *Giardia lamblia*, the causative agent of giardia, an infectious disease largely characterized by potentially severe and fatal diarrhea, while also resulting in asymptomatic infection (Meyer, 1996; Adam, 2001). *G. lamblia* can be found on various surfaces as well as in soil, food, and water contaminated with feces of infected humans and animals ("Giardia | Parasites | CDC," 2022). The characteristic most related between *C. anatis* and *G. lamblia* is the presence of an adhesive disc that allows for the suctioning on of the parasite to the intestinal epithelium to establish infection (Pecka et al., 1996; Elmendorf et al., 2003).

Genetic relation between *C. anatis* and *G. lamblia* has been disproved by multiple research efforts (Pecka et al., 1996; Hampl et al., 2006). In the studies that support a classification with Trichomonads over Diplomads (the order containing *G. lamblia*), morphological, ultrastructural, and phylogenetic analyses have been used. Pecka et al. used scanning and electron microscopy to assess the morphology and ultrastructure of *C. anatis* (Pecka et al., 1996). The resulting conclusion noted ultrastructural homology of *C. anatis* with trichomonads, whereas homology with diplomonads was not seen (Pecka et al., 1996). Hampl et al. furthered this notion with a genetic analysis in which the 16S rRNA gene sequence from *C. anatis* was compared to those of other protozoa. The resulting similarities were highest (>94% bootstrap) among trichomonads (Hampl et al., 2006). While this data was unable to accurately

place *C. anatis* within a specific clade in the order Trichomonadida, the evidence strongly supported the classification of *C. anatis* with trichomonads.

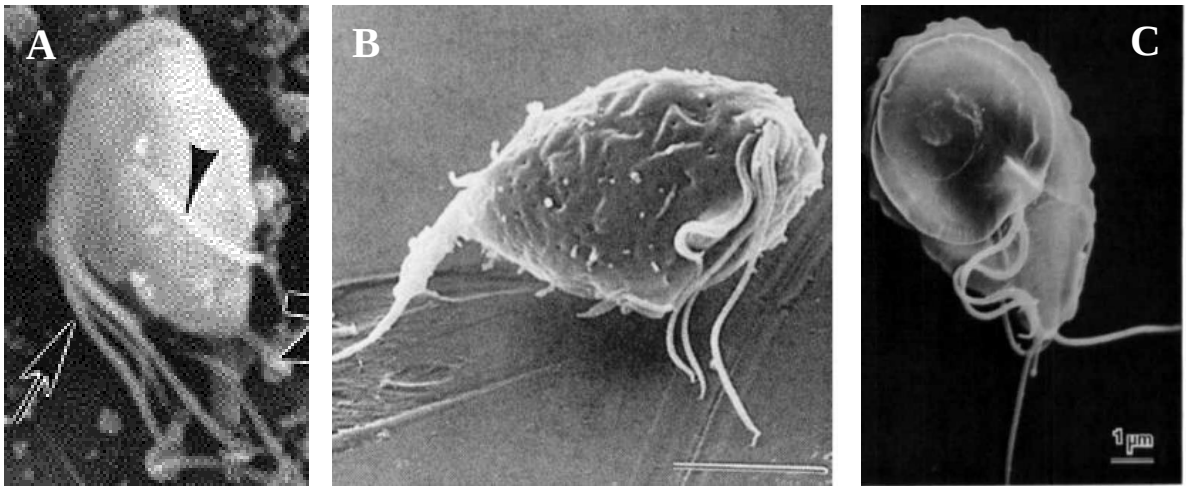

Figure 1. Scanning electron microscopy images of *Cochlosoma anatis* (A) (Lindsay et al., 1999), *Trichomonas vaginalis* (B) (Petrin et al., 1998), and *Giardia lamblia* (C) (Chavez et al., 1992).

Regardless of the classification of *C. anatis* and *G. lamblia*, the convergent evolution of similar adhesive discs may provide valuable insight to the pathogenic mechanisms of *C. anatis.* The adhesive disc of *G. lamblia* provides a robust attachment to the intestinal epithelium, allowing the parasite to colonize the intestinal tract without interference from normal peristaltic flow (Schwartz et al., 2012). This was tested through the characterization of the *G. lamblia* ventral disc protein composition and the use of electron microscopy to view attachment as it occurred through different timing of attachment and subsequent infection in hosts. It has also been shown that flagellar movement is not required to allow *G. lamblia* to remain attached to the host intestinal epithelium, highlighting the importance of the adhesive disc in infection by the parasite (House et al., 2011). Research following the methods of the studies presented here, with a focus on the protein composition of the adhesive disc and factors relating to attachment by *C. anatis* to the intestinal epithelium may be beneficial in the elucidation of *C. anatis* pathogenesis. If these pathogenic mechanisms are better understood, risk factors such as direct effects from the

parasite on the intestinal epithelium may provide insight to advanced risk for secondary infections during a primary *C. anatis* infection.

Life Cycles of Flagellated Protozoan Parasites in Poultry

Protozoa are single-celled animals which infect all higher animals with one or more species (Yaeger, 1996) (Source). These cells are eukaryotic and have multiple morphologic variations that fulfill needs specific to the cell, including morphologies such as flagella, among others. The most common form of reproduction is asexual binary fission; however, some protozoa are capable of conducting sexual reproduction. The life stage of a protozoan is specific to the survival needs of the cell. When a protozoan is free-living or parasitizing a host, they are in the trophozoite stage. If a protozoan is pathogenic to another organism, it is most likely in the trophozoite stage of life as it is actively feeding from its host. Meanwhile, when the food source for a protozoan expires, some protozoa form a cyst. In this stage, the protozoan is still infectious, but has formed a protective coating that allows it to survive in unfavorable environments such as those outside the host or in conditions that would otherwise kill the cell. As the cyst matures and re-enters a favorable environment for growth, it releases multiple infectious cells that can restart the cycle, progressing from cyst-trophozoite intermediates to trophozoites, and back to the cyst stage (Yaeger, 1996). The cyst stage is important for understanding the epidemiology and transmissibility of specific protozoa as these parasites can remain in environments in spite of efforts to eradicate them.

The life cycle of *C. anatis* is not well known. This is due to the difficulty in culturing *C. anatis*, of which no methods are currently known. Therefore, to understand the life cycle of *C. anatis*, researchers must use microscopic techniques such as electron microscopy to observe various life stages as *C. anatis* grows in the host. Using scanning electron microscopy, Evans, et

al. identified morphological and life stages in *C. anatis* (Evans et al., 2006). It was previously determined that *C. anatis* replicates via longitudinal binary fission and that *C. anatis* in the trophozoite stage are overwhelmingly prevalent during infection (Kotlán, 1923). A cyst stage was also mentioned by Kimura, however additional supporting evidence for this claim was lacking (Kimura, 1934). The scanning electron microscopic images provided by Evans et al. assured the notion that *C. anatis* replicated via longitudinal binary fission. Furthermore, Evans et al. identified the trophozoite stage with subsequential rounding and pseudocyst stages, but did not describe a true cyst stage of the parasite. In the rounding stage, external structures begin to get internalized and compartmentalized by the cell. The pseudocyst stage was characterized as the complete internalization of the costa, adhesive disc, and anterior flagella which Evans et al. proposed as a method to reduce external surface area to prevent exposure to harmful environmental chemicals. It was also mentioned in this study that Granger et al. reached a similar conclusion for the pseudocyst stage of *Tritrichomonas fetus*, a related protozoan (Granger et al., 2000).

Transmission of *Cochlosoma anatis*

C. anatis survives poorly outside of the host. Trophozoites exposed to various conditions in tap water, distilled water, and Hanks Buffered Salt Solution (HBSS) at temperatures ranging from -20°C to 37°C resulted in a nonmotile, dead appearance when observed via light microscopy (Lindsay et al., 1999). Furthermore, trophozoites that were exposed to water for 5 minutes then inoculated into susceptible turkeys did not establish infection (Lindsay et al., 1999). This highlights the fragility of *C. anatis* to sudden changes in the environment. Lindsay et al. also conducted a study of litter transmission. In this study, 12 16 day-old naïve turkey poults were placed on litter 2 hours after the removal of turkeys with active *C. anatis* infections and

shedding live trophozoites in their feces. Seven of these turkey poults were euthanized and examined for infection at 8 days post-placement, 3 were examined at 11 days post-placement, and 2 were examined at 14 days post-placement. Furthermore, a subsequent study was conducted in which 8 day-old naïve turkey poults were placed in a box with a turkey infected with *C. anatis* and actively shedding live trophozoites. The naïve turkey poults were euthanized at 7 days post-placement and examined via necropsy (Lindsay et al., 1999). The findings from this study showed that only one of the three birds examined for litter transmission from vacated litter at day 11 post-placement had been infected by *C. anatis*. No other birds were infected from litter that previously housed *C. anatis*-infected turkeys. When turkeys were placed on litter with a turkey actively shedding *C. anatis* trophozoites, 7/8 naïve turkeys developed *C. anatis* infections by 7 days post-placement (Lindsay et al., 1999). Lindsay et al. also demonstrated efficacious infection of turkeys, chickens, and bobwhite quail using an oral inoculation technique (Lindsay et al., 1999). However, natural infections with *C. anatis* have not been documented in chickens or bobwhite quail. From this study, it appears that, due to the fragility of *C. anatis* outside of the host, within two hours of being excreted by the host on litter the trophozoites experience a significant decrease in infectivity or die from environmental exposure. The condition of litter used by Lindsay et al. was not specified. It is possible that the quality of litter (fresh vs caked, or wet vs dry) can influence the survivability of *C. anatis* through the retainment of moisture in caked litter as well as the availability of potential nutrients provided by excreta in caked litter. This can also be supported by the lack of survivability in water and HBSS as nutrient-poor conditions or incorrect pH. Conditions more similar to those found in the intestinal tract as those found in caked litter may facilitate the survival of *C. anatis* and allow for infection beyond the 2-hour limit specified by Lindsay et al.

Searching for Vectors of *C. anatis*

While the majority of early research in *Cochlosoma* has focused on species identification and morphologic classification, little research has prioritized the discovery of potential vectors for the spread of the parasite into susceptible turkey farms. Historically, wild birds such as ducks and various songbirds have been shown to harbor *C. anatis* in the environment, though their relation to the frequency of outbreaks of cochlosomiasis has not been determined.

McElroy, et al. developed a molecular PCR assay to detect *C. anatis* DNA in house flies and turkey specimens at commercial turkey production farms (McElroy et al., 2005). In this study, the researchers sequenced mitochondrial DNA from the 16S subunit ribosomal RNA of *C. anatis*. The *C. anatis* was obtained from infected turkeys. Testing was performed on house flies (*Musca domestica* L.), turkey intestines, and turkey fecal samples. The *C. anatis* used for the development of the PCR was identified via light microscopy. This study was validated through the use of turkey poults inoculated with *C. anatis*, which subsequently developed cochlosomiasis, and allowed for efficacious *C. anatis* DNA extraction from turkey poult intestinal scrapings and feces collections. House flies were collected from a farm experiencing a cochlosomiasis outbreak, and the total DNA extracted to attempt to acquire *C. anatis* DNA for PCR amplification. Of all poults tested (they were inoculated with *C. anatis* prior to testing), all were positive for *C. anatis* DNA in their intestines and feces. Furthermore, 8 of the 18 house flies tested showed amplification of *C. anatis* DNA. The amplicons were confirmed to be from the intended amplicon of the selected primer. While this study does not prove that house flies present in commercial turkey production are directly implicated in the transmission of *C. anatis* within or around turkey farms, it is possible, given that *C. anatis* DNA is present on the flies.

Further research is be needed to confirm the presence of live *C. anatis* trophozoites to solidify the notion that house flies might be vectors in *C. anatis* transmission.

Historical Observations and Discoveries in *Cochlosoma*

C. anatis was first identified in domestic ducks (Kotlán, 1923). In the report, the ducklings that contained *C. anatis* in their intestines also presented with coccidiosis, a disease of *Eimeria spp.*, a non-flagellated protozoan parasite of significant concern in the poultry industry. It is possible that infection with *C. anatis* may predispose its host to secondary infections such as coccidiosis, which can be detrimental to the host and complicate diagnostic and research efforts by preventing proper diagnosis of the primary pathogen. This idea draws support from Kimura (1934), who detailed a new species, *Cochlosoma rostratum*, which was also found in ducks and caused swelling and cattharal symptoms in areas with higher concentrations of the parasite among the intestinal epithelium (Kimura, 1934). In addition to considerations for co-infections with *C. anatis*, the pathology of the parasite in its host has been debated since its discovery. As in Kimura's description of *C. rostratum*, more significant signs of disease such as blood mixed with mucus around higher parasite concentrations were attributed to bacterial involvement in infection rather than effects from the parasite. Disease is also inconsistent between infected birds, leading researchers and veterinarians to question the virulence of *C. anatis*. However, previous research following reports of *C. anatis* prior to the 1940s using inoculation techniques with *C. anatis* and the research presented in this thesis support the notion that *C. anatis* acts as a primary pathogen with an elevated possibility for asymptomatic infection. One case, in 1945 Scotland, described a mass mortality event in a turkey farm housing turkey poults aged 2 to 10 weeks in which mortality approached 100% and the only pathogen-related organism recovered was *C. anatis* (Campbell, 1945).

In 1934, Kimura described the discovery and classification of a novel intestinal flagellate of the same genus as *C. anatis* and named it *Cochlosoma rostratum* (Kimura, 1934). *C. rostratum* was also found in the large intestine of the White Pekin and domesticated Muscovy ducks, like *C. anatis*. The ducks described by Kimura also harbored numerous trichomonads throughout the intestinal tract, indicating that the ducks used in this study contained a large amount of flagellated protozoan parasites within their intestines. Further mention of *C. anatis* was made in 1938 when Travis published work identifying two additional species: *Cochlosoma picae*, and *Cochlosoma turdi* (Travis, 1938). The discoveries by Travis were made in wild birds from the cloaca of an American magpie, and the cloaca of Eastern robins, respectively, both in the United States. All species identified through these research endeavors established the *Cochlosoma* genus and attributed a classic adhesive disc to members of the genus (Kimura, 1934; Travis, 1938).

In 1989, a significant discovery was made regarding the presence of *C. anatis* in the environment via the discovery of *Cochlosoma soricis* in shrews (Watkins et al., 1989). In this study, 183 small mammals were trapped in the Grand Teton National Park, sacrificed, and their intestines removed. Additionally, 13 Pekin ducks and 3 Rouen ducks were collected for the same observations as the small mammals. Of the 183 small mammals sampled, 6 of 7 tested *Sorex palustris* (American water shrew) and 3 of 7 *Sorex vagrans* (Vagrant shrew) showed the presence of a newly identified *Cochlosoma*-like organism, *C. soricis*. This *Cochlosoma* species was found in the duodenum and jejunum of the small intestine, and was absent from the last 5 centimeters of intestine. Of the ducks, only the 3 Rouen ducks contained *C. anatis* while the Pekin ducks showed no evidence of *C. anatis* infection or any other *Cochlosoma*-like organisms (Watkins et al., 1989).

In total, five species of *Cochlosoma* have been identified: *C. anatis, C. rostratum, C. picae, C. turdi,* and *C. soricis,* with the most significant species identified thus far being *C. anatis.* All species belonging to this genus target avian hosts except *C. soricis*, which resides in the shrew as far as the current published knowledge extends. None of these species have been demonstrated to transmit from an avian to a mammal or vice versa. Using the information from the various species of *Cochlosoma* identified from the environment presented thus far, it appears that the genus has a wide range of potential hosts. Therefore, it is possible that individual species within the genus *Cochlosoma* can also inhabit multiple organisms. It is essential that if the environmental presence of *C. anatis* is to be understood, multiple vectors and vector types ranging from birds to mammals must be evaluated.

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CHAPTER 2

Assessing *Cochlosoma anatis* Pathogenesis in Turkey Poults

ABSTRACT

Cochlosoma anatis, the causative agent of cochlosomiasis, is a flagellated protozoan parasite affecting the commercial turkey industry in the United States. Research concerning *C. anatis* is lacking, and no treatment for the disease is commercially available, requiring research focused on characterizing cochlosomiasis to identify potential targets for treatment. This study hypothesized that the harmful effects of cochlosomiasis would be attributable to changes in blood chemistry, nutrients digestibility, intestinal permeability, and histopathology of the poult gut during infection. To test this hypothesis turkey poults were placed at the day of hatch in isolator cages and inoculated with *C. anatis* at 14 days of age. Data collection occurred when the poults were 28 days old. Body weight gain and feed intake were reduced with infection ($P < 0.0001$; $P = 0.0003$, respectively), and the FCR was significantly increased ($P = 0.0351$). Uniformity was similar between infected and non-infected birds. Blood carbon dioxide ($P = 0.0004$), bicarbonate ($P = 0.0006$), and excess base in extracellular fluid ($P = 0.0024$) were increased with infection. Calculated digestibility values of amino acids were decreased with infection. No differences were observed in intestinal permeability. Differences in the histopathology of the duodenum and jejunum between infected and non-infected poults were observed. The results of this study showed that infection with *C. anatis* causes changes in blood chemistry related to pH homeostasis and alters the intestinal villi in the duodenum and jejunum.

INTRODUCTION

Cochlosoma anatis, a flagellated protozoan parasite primarily affecting the United States commercial turkey industry, annually results in significant losses for turkey producers. Infection with *C. anatis*, known as cochlosomiasis, is characterized by a non-uniform flock, runted individuals, drooping head and wings, diarrhea, and high flock morbidity (Bollinger and Barker, 1996; Gharagozlou and Dezfoulian, 2009; Beckstead, 2019). While increased mortality is uncommonly associated with cochlosomiasis, disruptions in the intestinal tract during infection is associated with a greater possibility of developing secondary coinfections that could be fatal for affected birds (Beckstead, 2022b). No commercial treatments are currently available to combat infection with *C. anatis*.

Blood chemistry analysis is an important tool used to assess physiological stress in animal and human medicine. The renal and pulmonary systems are involved in the maintenance of pH homeostasis (Hopkins et al., 2023). When abnormalities occur in the pulmonary system, such as hyperventilation (panting) or hypoventilation, gases in the blood are exchanged at a different rate (Patel et al., 2023). Panting results in an excess of CO_2 expelled from the body, removing an excess of acid, and disrupting and lowering the pH balance. The opposite is true for hypoventilation. Likewise, the renal system may stop functioning, resulting in a failure to remove acids from the blood, resulting in a lowering blood pH (Castro et al., 2023).

One of the classical symptoms of enteritis is the presence of diarrhetic symptoms. When this occurs, the body may be starved of electrolytes lost through the purge of fluids from the body. Electrolytes are essential for normal body functioning as they are involved in neural network activity, which functions in all body systems, including the regulation of blood pH in the renal and pulmonary systems (Shrimanker and Bhattarai, 2023).

It is suspected that disease factors such as a failure to gain weight in birds infected with *C. anatis* result from an inability to absorb or utilize adequate nutrients (Evans et al., 2006). Lesions in the duodenum and jejunum have been recorded, suggesting that epithelial damage from infection may be related to poorer nutrient acquisition (Cooper et al., 1995). *Giardia lamblia*, a morphologically similar parasite to *C. anatis*, has exhibited similar epithelial attachment activity to *C. anatis* and induces disease by blocking nutrient absorption channels. *G. lamblia* also increases intestinal permeability during infection in its host (Hardin et al., 1997; Scott et al., 2002). The increased permeability from *G. lamblia* allows for the passage of macromolecular structures through intestinal walls, causing significant disruptions in the host organism. It is possible that similar disease mechanisms are present during infection with *C. anatis*.

Histopathological analysis has been performed with *C. anatis* (Cooper et al., 1995). In the study by Cooper et al., histopathological lesions were observed. These lesions consisted of blunting and fusion of the villi, as well as infiltration of the lamina propria by various innate and adaptive immune cells, with increased mitotic cells in the crypt epithelium (Cooper et al., 1995). It was also noted that while most trophozoites were in the lumen, some were firmly attached to the intestinal epithelium. The lesions identified by Cooper et al. indicate potential mechanisms for disruptions of nutrient acquisition by inhibiting the function of villi in the intestinal epithelium.

MATERIALS AND METHODS

All research was approved by the North Carolina State University Institute of Animal Care and Use Committee (IACUC).

Collection and Storage of *C. anatis*

A mixture of *C. anatis* trophozoites collected from intestinal scrapings of turkeys from commercial turkey farms in North Carolina and Virgina actively infected with *C. anatis* were used in this study. The parasites were harvested from a 14 cm section of the ileum of infected turkeys by a mucosal scraping, then placed in 40°C phosphate buffered saline (PBS; VWR International, LLC, Radnor, PA, USA) . The mixture was preserved by mixing 1mL with 50 µl of dimethyl sulfoxide (DMSO; Fisher Scientific, Hampton, NH, USA) in a cryotube. The cryotube was then placed in a -80°C using a Mr. Frosty Freezing Container (Thermo Fisher Scientific, Waltham, MA, USA) for 24 hours before being moved to liquid nitrogen.

Experimental Design

One hundred ninety-two, one-day-old, off-sex Nicholas male poults were obtained from an Aviagen hatchery in Lewisburg, WV and placed into 16 isolator cages at 12 birds per cage with *ad libitum* feed and water. An additional 12 one-day-old poults were placed into an isolator cage to be used as seeder birds. The seeder birds were grown for 7 days and inoculated with *C. anatis*. At D14, *C. anatis* was harvested from the seeder birds and used to inoculate 8 of the 16 original cages, creating 8 cages infected with *C. anatis*, and 8 cages with non-infected birds. All 16 cages were also depopulated to 10 birds per cage to decrease the density of birds in each cage for adequate feed and water access. On day 21 of the study, the feed for all treatment cages was changed to a diet with 0.5% titanium dioxide (TiO_2), which was fed to all birds *ad libitum* for the remainder of the study. All birds were grown to 28 days of age.

Table 1. Feed formulations that were used for this study.

Ingredient	Control Diet (%)	Diet Supplemented with Titanium Dioxide (%)
Corn	51.00	50.74
Soybean Meal	17.90	17.81
Poultry by pro-Meal	20.35	20.25
Wheat Bran	5.00	4.97
Corn Micro Flush	0.55	0.55
Mono-Dicalcium Phosphate	1.49	1.49
Calcium Carbonate	1.30	1.30
Sodium Bicarbonate	0.15	0.15
Salt, Plain	0.09	0.09
DL-Methionine	0.21	0.21
Lysine	0.40	0.40
Threonine	0.06	0.06
Selenium	0.05	0.05
Choline Chloride	0.05	0.05
NCSU Vitamin Mix[1]	0.20	0.20
NCSU Mineral Mix[2]	0.20	0.20
Poultry Fat	1.00	0.99
Titanium Dioxide	--	0.5

[1]120 mg manganese, 120 mg zinc, 80 mg iron, 10 mg copper, 2.5 mg Iodine, and 2.5 mg cobalt per kg of diet.
[2]13,200 IU vitamin A, 4,000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid per kg of diet.

Inoculation

Frozen stocks of *C. anatis* were used for oral inoculation of D0-inoculated poults and seeder birds at placement. At D0, all inoculated poults received a frozen stock aliquot (1mL) of *C. anatis*. The seeder birds were then used to harvest *C. anatis* for inoculation of D7- and D14-

inoculated groups. The initial PBS-*C. anatis* mixture was diluted to a concentration of ~500,000 cells/mL. Each poult in the D7- and D14-inoculated groups received 1mL of prepared inoculum. All inoculums were kept at a temperature around 40°C and continuously mixed. The cells in the mixture are fully viable for roughly 30 minutes before the cells begin to gradually die. A new seeder bird was collected and its *C. anatis* harvested to create a new stock solution after 30 minutes if inoculation was not completed.

Data Collection

Individual body weights were recorded at D14 and D28 of the study. Total feed consumption from D14 to D28 was recorded. Feed conversion ratio and flock uniformity were calculated.

Table 2. Equations used to calculate feed conversion ratio (FCR) for feed efficiency and uniformity via the coefficient of variation.

Calculated Performance Parameter	Equation
Feed Conversion Ratio (FCR)	$\frac{Feed\ Intake}{Body\ Weight\ Gain}$
Flock Uniformity (Coefficient of Variation)	$\frac{Standard\ Deviation}{Average\ Flock\ Body\ Weight} * 100$

Blood Chemistry

Blood chemistry data was collected using an iSTAT machine (Abbott Laboratories, Abbott Park, Illinois, USA). Heparinized needles and syringes were used for blood collection (Beantown Chemical, Hudson, NH, USA). iSTAT CHEM8+ cartridges were used for blood analysis (Abbott Laboratories, Abbott Park, Illinois, USA). Parameters recorded included percent CO_2 (PCO2), percent O_2 (PO2), base excess extracellular fluid (BEecf), bicarbonate (HCO3), total carbon (TCO2) and specific blood oxygen (sO2%).

Digestibility using Titanium Dioxide

Titanium dioxide was incorporated into the normal diet at an inclusion rate of 0.5% starting at D21 and was supplied through D28. On D28, the ileal content from four birds per cage were pooled into 50 mL conical tubes and freeze-dried, then ground into a powder using a mortar and pestle. During the freeze-drying process, the machine malfunctioned. When this occurred, the conical tubes were removed and mistakenly placed in a 30°C incubator for roughly one hour before returning to the freeze-drying machine. The powdered digesta, as well as a powdered feed sample, were sent to the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories for amino acid profile, proximate, total sugars and carbohydrates, lipids, fats and oils, and elemental analyses. Apparent metabolizable energy (AMEn), apparent protein digestibility (APD), fat digestibility, fiber digestibility, and amino acid digestibility were calculated using the equations in **Table 3**.

Table 3. Equations used to calculate digestibility parameters from associated digesta analyses.

Calculated Digestibility Parameter	**Equation**
AMEn[1]	$(DE - \left(\left(EE \times \left(\frac{DT}{ET}\right)\right)\right) - 8.22 \times (DN - \left(EN \times \frac{DT}{ET}\right)) \times 10$
APD[2]	$(1 - \left(\frac{DT}{ET}\right) \times \left(\frac{EP}{DP}\right)) \times 100$
Fat	$(1 - \left(\frac{DT}{ET}\right) \times \left(\frac{EFa}{DFa}\right)) \times 100$
Fiber	$1 - \left(\frac{DT}{ET}\right) \times \left(\frac{EFi}{DFi}\right)) \times 100$
Amino Acid	$1 - \left(\frac{DT}{ET}\right) \times \left(\frac{EAA}{DAA}\right)) \times 100$

[1]AMEn = Apparent metabolizable energy corrected for nitrogen
[2]APD = Apparent protein digestibility
DE=Diet Energy, EE=Excreta Energy, DT=Diet Titanium, ET=Excreta Titanium, DN=Diet Nitrogen, EN=Excreta Nitrogen, EP=Excreta Protein, DP=Diet Protein, EFa=Excreta Fat, DFa=Diet Fat, EFi=Excreta Fiber, DFi=Diet Fiber, EAA=Excreta Amino Acid, DAA=Diet Amino Acid

FITC-d Intestinal Permeability Assay

An intestinal permeability assay using fluorescein isothiocyanate-dextran (FITC-d; Sigma-Aldrich, St. Louis, MO, USA) as an innate marker was performed on D28. The protocol used to perform this assay was adapted from those described by Liu et al (Liu et al., 2021) . Poults were orally inoculated with 1mL of FITC-d at a concentration of 4mg/kg, and 2-hours later their blood was drawn for analysis. Plasma was collected via centrifugation at 1500 RPM for 5 minutes and stored in black microcentrifuge tubes. Maximum excitation at 495nm and maximum emission at 519nm with a gain of 40 were used as measurement parameters. To determine the concentrations of FITC-d recovered from poult plasma, a standard curve was used.

Histology of the Turkey Poult Small Intestine During Infection

On D28, sections of the duodenum, jejunum, and ileum were used for histological analysis from one bird per cage. These sections were fixed in 10% formalin for 6 months and sent to the North Carolina State University College of Veterinary Medicine Histology Laboratory (Raleigh, NC, USA). Paraffin-embedded slices (5µm-thick) were stained using Hematoxylin and Eosin (H&E). Villi height and width, crypt depth, and muscularis depth were measured in 5 villi per section using ImageJ software (NIH, University of Wisconsin, Madison, WI, USA). Goblet cells and inter-epithelial leukocytes in the columnar epithelium layer of intestinal villi were counted per 100 cells in the ileum; lamina propria thickness was also measured (5 villi per section).

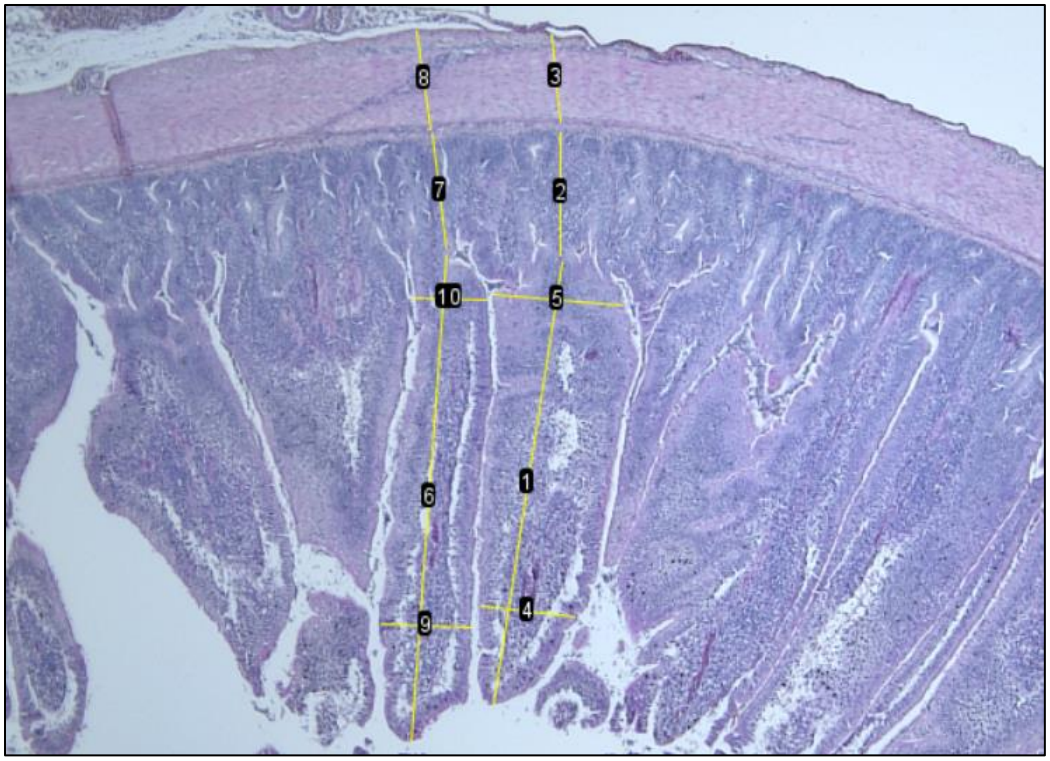

Figure 1. Pictured is an example of the measurement method used for obtaining measurements for histopathological calculations. Lines in the picture represent each measurement taken between two villi. 1&6 = villi height, 2&7 = crypt depth, 3&8 = muscularis depth, 4&9 = villi width at the top, and 5&10 = villi width at the bottom. Villi area was calculated as if it was a rectangle, requiring measurements for all 4 “sides.”

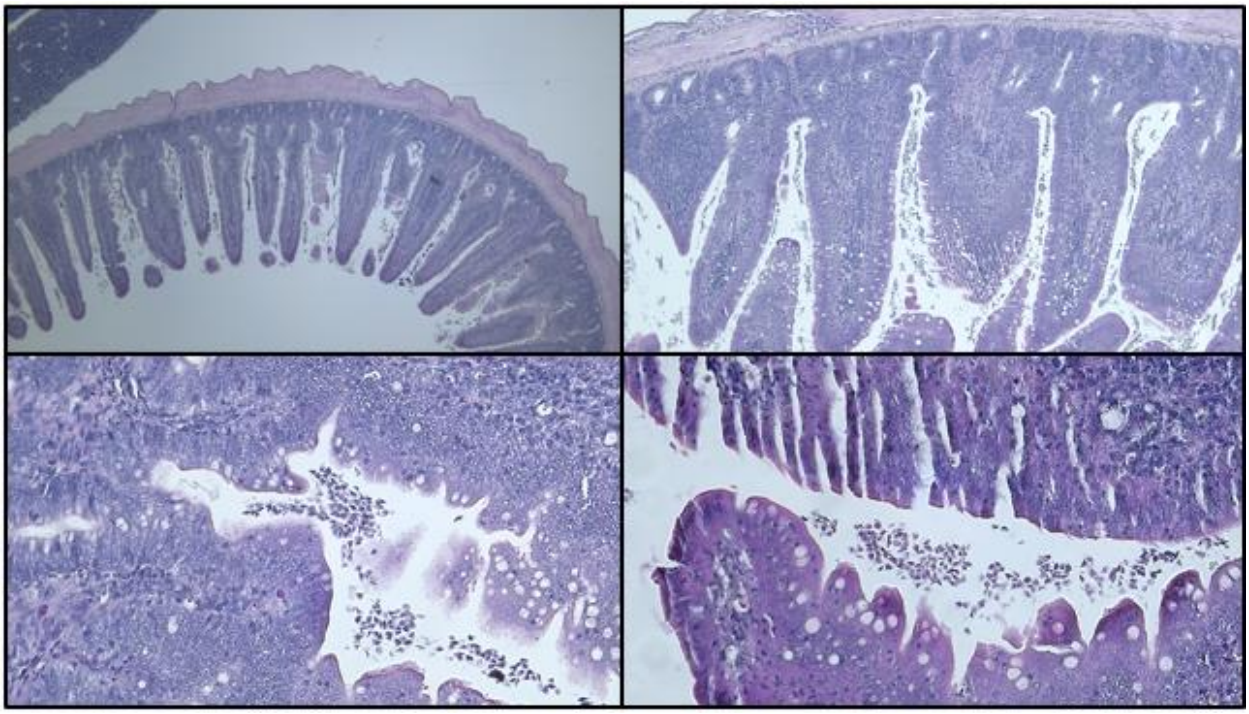

Figure 2. Intestinal villi of a bird infected with *C. anatis* showing the presence of the parasite in the intervillous spaces.

Table 4. Equations used to calculate parameters used to evaluate histopathology.

Histopathological Parameter	Equation
Villus Area	$\frac{Villus\ Width\ (Top) + \ Villus\ Width\ (Bottom)}{2} \times Villus\ Height$
Villus Height : Crypt Depth Ratio	$\frac{Villus\ Height}{Crypt\ Depth}$

Statistical Analysis

All statistical analyses were performed using JMP Pro 16 (Cary, NC, USA). Outliers were removed via a Grubb's Test, and the remaining data was screened for equal variance using a Levene Test for unequal variance. In cases where equal variance was assumed, a one-way analysis of variance with an associated pooled t-test was performed. In cases of unequal

variance, an independent t-test was performed. Statistically significant differences were determined when $P \leq 0.05$. Statistical trends were determined when the P-value was $0.05 \leq P \leq 0.1$.

RESULTS

Infected poults gained significantly lower weight compared to non-infected poults, with an average gain of 0.269 kg per bird and 0.411 kg per bird, respectively ($P<0.0001$). Feed intake was significantly lower in infected poults, consuming an average 0.715 kg of feed per bird compared to 0.927 kg per bird by the non-infected group ($P = 0.0003$). Together, using the calculation provided in **Table 2**, an average FCR for infected poults was determined to be 2.661, a significantly higher than non-infected poults, whose FCR was 2.161 ($P = 0.0351$). Lastly, in evaluation of uniformity using the coefficient of variation (CV) calculation from **Table 2**, uniformity was found to be similar between both treatment groups, with a CV of infected poults at 19.696 compared to a CV of non-infected poults at 20.178.

Ca, P, Na, fat, fiber, and AMEn were not significantly different between infected and non-infected poults tested. APD trended toward an increased digestibility in non-infected poults ($P = 0.0857$). Threonine ($P = 0.0009$), alanine ($P = 0.0216$), cysteine ($P = 0.0412$), valine ($P = 0.0190$), methionine ($P = 0.0096$), isoleucine ($P = 0.0077$), leucine ($P = 0.0163$), tyrosine ($P = 0.0124$), phenylalanine ($P = 0.0127$), and histidine ($P = 0.0070$) had higher digestibility values in the non-infected group. Lanthionine digestibility had a higher value in the infected group ($P = 0.0476$). Serine digestibility trended toward a higher value in the infected group ($P = 0.0567$).

The intestinal permeability assay revealed a similar concentration of FITC-d recovered from the blood plasma of tested birds ($P = 0.2038$). The average concentration of FITC-d

recovered from infected birds measured 95.14 ng/mL while the concentration from non-infected birds was 75.26 ng/mL.

In the duodenum, the average villus height was similar between non-infected and infected poults (P = 0.2252), as was the average muscularis depth (P = 0.6232), and average villus width (P = 0.1502). A statistical trend was seen between the average villus area of infected and non-infected poults where that of the non-infected poults was greater. Significant differences in the villi of the duodenum were seen with crypt depth, which was greater in the infected group (P = 0.0057), and the villus height-crypt depth ratio (V:C) was greater in the non-infected group (P = 0.0250). Average villus height (P = 0.5662), muscularis depth (P = 0.3493), average villus width (P = 0.3057), and average villus area (P = 0.9258) were all similar between both treatment groups in the jejunum. However, average crypt depth (P = 0.0110) was greater in the negative control group, and the V:C was greater in the infected group (P = 0.0037). All physical histological parameters measured in the ileum were similar between both treatment groups. Goblet cell and IEL presence in the ileum were similar, as was the lamina propria thickness.

Blood chemistry resulted in multiple significant differences between the treatment groups. Base excess in the extracellular fluid was higher in the infected group (P = 0.0024). Additionally, HCO_3 was significantly higher in the infected group (P = 0.0006). Total CO_2 was also increased in the infected group (P = 0.0004). The partial pressure of CO_2 trended toward an elevated value in the infected group (P = 0.0825). The pH, partial pressure of O_2, and specific O_2 in the blood were all similar between the treatment groups.

DISCUSSION

Infection with *C. anatis* is commonly associated with a failure to gain weight, as was shown in this study; however, the mechanism behind this association is unclear (Kimura, 1934;

Campbell, 1945; Cooper et al., 1995). A higher FCR in the current study is a direct reflection of a failure to effectively convert feed to body weight. Previous research has hypothesized that nutrients acquisition or absorption are at fault. The results of the research from the current study provide several potential explanations or routes for future research to explain the failure to gain weight.

Altered blood chemistry relating to pH is indicative of potential issues relating to the acid-base balance present in the bird. Increased CO_2 can arise from several physiological issues, such as increased metabolism without proper CO_2 removal or hypoventilation (Patel et al., 2023). HCO_3 in the blood is directly related to CO_2 as the product of CO_2 dissolution in blood is HCO_3. Therefore, in the analysis of blood chemistry relating to pH, the amount of CO_2 should be the focus. An increase in CO_2 in the blood likely resulted in the increase of excess base in the blood in the current study (Hopkins et al., 2023). Further research examining breathing rates, acid excretion, and renal functions in the turkey would be beneficial in isolating the cause if increased blood CO_2.

Amino acids are essential to produce proteins in the body. Additionally, muscle formation is reliant on the ability of the turkey to effectively produce proteins (Castro and Kim, 2020). Chen et al. has stated that, during infection with *C. anatis*, the deficiency seen in body weight gain was reduced through the supplementation of added metabolizable energy in the diet (Schaeffer and Chen, 2022). The underlying mechanisms behind poor nutrients use during cochlosomiasis are unknown. However, the current study demonstrates that while energy utilization from the diet is unaffected with infection, amino acid usage is affected. Therefore, future research could address the deficit in amino acid digestibility by supplementing added dietary energy and measuring nutrient absorption.

On the other hand, an apparent decrease in amino acid digestibility may be a product of endogenous amino acid excretion during infection with *C. anatis*, or may be linked to blockage of absorption channels via parasite attachment to the epithelium. Currently, no methodology is available to test endogenous amino acid excretion directly (Ravindran, 2021). However testing with a nitrogen-free diet in which no amino acids or proteins are provided allows for the measurement of excreted endogenous amino acids and proteins (Ackerson et al., 1926). Future research could compare the endogenous amino acid loss during infection by direct comparison between healthy birds and those infected with *C. anatis*. Intestinal permeability was assessed in the current study to evaluate a more permeable gut as a potential explanation for failure to absorb nutrients or inadvertently release nutrients from the body. Because increased intestinal permeability was not observed in this study, it was not considered as a cause of lower body weight gain.

The intestinal epithelium fills multiple niches in the poult gut. Firstly, it provides a barrier between the gut lumen and the bloodstream, protecting the poult from sepsis, and selects specific nutrients to enter the bloodstream (Walton et al., 2018). This study revealed differences in the structures of the villi of the duodenum and jejunum associated with infection by *C. anatis*. Intestinal villi provide the framework of increased surface area in the intestinal lumen to enhance nutrient absorption. Disruption to these structures may directly affect the poult's ability to absorb the proper nutrients.

Previous histopathological findings related to cochlosomiasis remarked on the presence of immune cell invasion into the intestinal epithelium, however the concentrations and relative values of these cells in the epithelium were not reported (Cooper et al., 1995). In this study, no such observation was made. It is likely that some form of an immune response is occurring as

these poults presented with catarrhal enteritis and exhibited many of the classical symptoms of cochlosomiasis. Immune responses commonly result in one or a mixture of effects, such as reddening, increased temperature, pain, and swelling, some of which are seen with cochlosomiasis (Ciaccia, 2011). Further research, including more specific targeting to identify and enumerate additional types of immune cells and products active during infection, is suggested. This would allow for a better understanding of how the host's body recognizes and attacks *C. anatis* during infection and what specific target areas in the intestine and immune regulatory sites could lead to potential treatment options for cochlosomiasis.

CONCLUSION

The current study showed that infection with *C. anatis* leads to an increased FCR, apparent disruption of blood pH homeostasis, apparent decreased amino acid digestibility, and morphological changes in duodenal and jejunal villi. These results suggest that failure to gain weight during cochlosomiasis may be related to physiological disruptions in blood pH and altered intestinal villi morphologies. Future research should focus on the evaluation of the effects from cochlosomiasis on renal and pulmonary function and endogenous amino acid excretion during infection with *C. anatis*.

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Table 5. Performance parameters recorded from this study. BWG, FI, and FCR were recorded and calculated following inoculation with *C. anatis* (D14-D28). Uniformity was calculated at the end of the study (D28).

Treatment	BWG[1] (kg)	FI[2] (kg)	FCR[3]	CV[4]
Non-Infected	0.411 ± 0.017	0.927 ± 0.029	2.161 ± 0.149	20.178 ± 2.224
Infected	0.269 ± 0.017	0.715 ± 0.031	2.661 ± 0.149	19.696 ± 2.224
P-value	<0.0001	0.0003	0.0351	0.8804

[1]BWG = Gain in body weight following inoculation with *C. anatis.*
[2]FI = Total feed intake following inoculation with *C. anatis.*
[3]FCR = Feed conversion ratio
[4]CV = Coefficient of variation; used to assess flock uniformity

Table 6. Blood chemistry parameters were tested in this study. All values are averages from non-infected and infected treatment groups challenged with *C. anatis*. Values shown in this table are directly associated with the blood pH balance.

Treatment	BEecf[1]	HCO_3[2]	TCO_2[3]	pH
Non-Infected	-5.88 ± 1.22	20.05 ± 0.92	21.13 ±0.91	7.33 ± 0.03
Infected	0.86 ± 1.31	26.11 ± 0.99	27.43 ± 0.98	7.37 ± 0.03
P-value	0.0024	0.0006	0.0004	0.3215

[1]BEecf = Excess base present in the extracellular fluid.
[2]HCO_3 = Bicarbonate present in the blood.
[3]TCO_2 = Total carbon dioxide present in the blood.

Table 7. Blood chemistry parameters were tested in this study. All values are averages from non-infected and infected treatment groups challenged with *C. anatis*.

Treatment	PCO_2[1]	PO_2[2]	SO_2[3]
Non-Infected	38.2 ± 2.49	37.75 ± 3.25	64.13 ± 4.96
Infected	45.00 ± 2.66	36.86 ± 3.47	67.57 ± 5.30
P-value	0.0825	0.8540	0.6426

[1]PCO_2 = Partial pressure of carbon dioxide in the blood.
[2]PO_2 = Partial pressure of oxygen in the blood.
[3]SO_2 = Oxygen saturation of the blood.

Table 8. Calculated mineral digestibility of non-infected turkey poults and turkey poults infected with *C. anatis*.

Treatment	Ca	P	Na
Non-Infected	35.71 ± 3.26	53.98 ± 2.88	-0.23 ± 9.32
Infected	32.71 ± 3.57	47.19 ± 3.16	-21.58 ± 10.21
P-value	0.5504	0.1471	0.1570

Table 9. Calculated digestibility of fat and fiber, with apparent metabolizable energy[1] and apparent protein digestibility[2] of non-infected turkey poults and turkey poults infected with *C. anatis*.

Treatment	Fat	Fiber	AMEn[1] (%)	APD[2] (%)
Non-Infected	73.64 ± 4.55	-10.44 ± 7.14	2144.51 ± 84.70	73.14 ± 1.14
Infected	74.55 ± 4.26	-25.11 ± 6.68	2010.49 ± 79.23	70.13 ± 1.14
P-value	0.8870	0.1574	0.2687	0.0857

[1]AMEn = Apparent metabolizable energy, as calculated using the equation in **Table 5.**
[2]APD = Apparent protein digestibility, as calculated using the equation in **Table 5.**

Table 10. Amino acid digestibility calculated relative to Titanium present in the excreta compared between health turkey poults and turkey poults infected with *C. anatis*.

Amino Acid	Non-Infected		Infected		*P-value*	Difference
	Digestibility (%)	Std Error	Digestibility (%)	Std Error		
Taurine	50.6667	3.8538	52.0358	3.6049	0.7994	-1.3691
Hydroxyproline	60.3733	1.3992	63.4006	1.3088	0.1381	-3.0273
Aspartic Acid	74.9032	1.2209	72.4038	1.1421	0.1501	2.4994
Threonine**	71.8039	1.2420	66.3443	1.2420	0.0090	5.4596
Serine*	78.2458	2.0675	72.3239	1.9340	0.0567	5.9219
Glutamic Acid	78.7122	0.64886	78.3335	0.6488	0.6871	0.3787
Proline	73.1360	1.02550	71.0131	1.0255	0.1689	2.1229
Lanthionine**	63.4642	1.35130	67.6143	1.3513	0.0476	-4.1501
Glycine	71.6262	0.98538	70.0522	0.9853	0.2808	1.5740
Alanine**	76.3533	1.04750	72.4417	1.0475	0.0216	3.9116
Cysteine**	56.2239	2.15210	49.2653	2.1521	0.0412	6.9586
Valine**	73.5358	1.10960	69.2851	1.1096	0.0190	4.2507
Methionine**	85.8221	0.66490	82.9296	0.6649	0.0096	2.8925
Isoleucine**	75.5015	1.11310	70.4759	1.1131	0.0077	5.0256
Leucine**	78.2589	0.95617	74.4833	0.9562	0.0163	3.7756
Tyrosine**	79.4390	1.11520	75.0129	1.0431	0.0124	4.4261
Phenylalanine**	78.0983	0.96986	74.0874	0.9699	0.0127	4.0109
Ornithine	9.66440	17.3810	-0.56290	16.091	0.6742	10.2273
Lysine	82.0845	2.01770	77.4228	1.8873	0.1154	4.66170
Histidine**	79.7922	1.33170	73.9649	1.2457	0.0070	5.82730
Arginine	82.1113	0.86234	82.2091	0.8067	0.9352	-0.09780
Tryptophan	79.7008	0.75715	78.0465	0.7572	0.1483	1.65430

** = Statistically significant difference between the infected and non-infected groups.
* = Statistically trending difference between the infected and non-infected groups.

Table 11. Calculated values of average villus area and villus height/crypt depth ratio in each section of the small intestine collected at the end of the study.

Treatment	Duodenum		Jejunum		Ileum	
	VA[1]* (mm^2)	**V:C[2]**	**VA[1]* (mm^2)**	**V:C[2]**	**VA[1]* (mm^2)**	**V:C[2]**
Non-Infected	0.662 ± 0.061	9.81 ± 1.18	0.228 ± 0.022	4.04 ± 0.45	0.186 ± 0.016	4.35 ± 0.52
Infected	0.512 ± 0.057	5.63 ± 1.10	0.225 ± 0.022	6.34 ± 0.45	0.191 ± 0.016	4.96 ± 0.52
P-value	0.0922	0.0250	0.9258	0.0037	0.819	0.4254

[1]VA = Villus area, calculated as described in **Table 5.**
[2]V:C = Villus height/crypt depth ratio, calculated as described in **Table 5.**
* = A parameter for which the variances were unequal.

Table 12. Duodenal villi morphological measurements comparing turkey poults infected with *C. anatis* to non-infected turkey poults.

Treatment	Duodenum			
	VH (mm)	**CD (mm)**	**MD (mm)**	**VW (mm)**
Non-Infected	2.316 ± 0.139	0.258 ± 0.026	0.210 ± 0.013	0.287 ± 0.021
Infected	2.074 ± 0.129	0.379 ± 0.024	0.200 ± 0.012	0.244 ± 0.019
P-value	0.2252	0.0057	0.6232	0.1502

Table 13. Calculated duodenal villi measurements comparing turkey poults infected with *C. anatis* to non-infected turkey poults.

	Duodenum	
Treatment	**V:C**	**VA (mm^2)**
Non-Infected	9.813 ± 1.184	0.662 ± 0.061
Infected	5.630 ± 1.10	0.512 ± 0.057
P-value	0.0250	0.0922

Table 14. Jejunal villi morphological measurements comparing turkey poults infected with *C. anatis* to non-infected turkey poults.

	Jejunum			
Treatment	**VH (mm)**	**CD (mm)**	**MD (mm)**	**VW (mm)**
Non-Infected	1.116 ± 0.098	0.282 ± 0.015	0.163 ± 0.011	0.205 ± 0.012
Infected	1.198 ± 0.098	0.189 ± 0.015	0.148 ± 0.011	0.188 ± 0.012
P-value	0.5662	0.011	0.3493	0.3057

Table 15. Calculated jejunal villi measurements comparing turkey poults infected with *C. anatis* to non-infected turkey poults.

	Jejunum	
Treatment	**V:C**	**VA (mm^2)**
Non-Infected	4.038 ± 0.453	0.227 ± 0.022
Infected	6.344 ± 0.453	0.225 ± 0.022
P-value	0.0037	0.9258

Table 16. Ileal villi morphological measurements comparing turkey poults infected with *C. anatis* to non-infected turkey poults.

	Ileum			
Treatment	**VH (mm)**	**CD (mm)**	**MD (mm)**	**VW (mm)**
Non-Infected	0.941 ± 0.058	0.222 ± 0.017	0.168 ± 0.016	0.198 ± 0.011
Infected	0.936 ± 0.058	0.199 ± 0.017	0.156 ± 0.016	0.203 ± 0.011
P-value	0.9569	0.3382	0.6142	0.7543

Table 17. Calculated ileal villi measurements comparing turkey poults infected with *C. anatis* to non-infected turkey poults.

	Ileum	
Treatment	**V:C**	**VA (mm^2)**
Non-Infected	4.35 ± 0.52	0.191 ± 0.016
Infected	4.96 ± 0.52	0.191 ± 0.016
P-value	0.4254	0.8187

Table 18. Inflammation indicators present in the *C. anatis* infected ileum through the evaluation of innate immune cells (Goblet cells and inter-epithelial leukocytes; counted per 300 cells) and lamina propria thickness (counted from 5 villi per intestinal section).

Treatment	**Goblet Cell (%)**	**Inter-Epithelial Leukocytes (%)**	**Lamina Propria (mm)**
Non-Infected	29.40 ± 7.31	22.38 ± 4.75	0.117 ± 0.008
Infected	32.13 ± 7.31	29.38 ± 4.75	0.097 ± 0.008
P-value	0.7941	0.3155	0.1017

CHAPTER 3

Exploring the Effects of Infection with *Cochlosoma anatis* in Turkey Poults at Different Ages

ABSTRACT

Cochlosoma anatis, a flagellated protozoan parasite of rising importance in the United States commercial turkey production industry, is the causative agent of the enteric disease, cochlosomiasis. Previous research has been unsuccessful in elucidating underlying disease-causing mechanisms, though the disease pathogenesis suggests a failure to absorb or utilize nutrients in infected birds. The hypothesis of this study was that if turkey poults become infected with *C. anatis* at a younger age, the consequences of disease would be more severe compared to infection at an older age. To test this hypothesis, day-old poults were placed in isolation cages and inoculated with *C. anatis* at D0, D7, or D14, with a non-infected, negative control (NC) group. Poult performance was calculated, *C. anatis* was enumerated in the ileum and *Escherichia coli* and coliforms, *C. perfringens*, and *Salmonella* spp. were enumerated in the cecal tonsil. Body weight gain was lowest in D0-inoculated poults; weight gain for D7- and D14-inoculated poults was the next highest, and the NC group exhibited the greatest gain ($P<0.0001$). Feed intake was lower in D0-inoculated poults ($P=0.023$). Other performance parameters (FCR, and uniformity) were not significantly affected ($P=0.0644$; $P=0.0648$, respectively). *C. anatis*, *E. coli*, and *C. perfringens* were not statistically different between any groups, and *Salmonella* spp. was scarcely recovered. Mortality was increased the earlier turkey poults were inoculated with *C. anatis*, with a 70% mortality rate in D0-inoculated poults, 50% in D7-inoculated poults, 16% from D14-inoculated poults and 8% mortality in the NC group. This study showed that cochlosomiasis is more severe in turkey poults if they become infected at a younger age.

INTRODUCTION

Cochlosoma anatis is a flagellated protozoan parasite that causes cochlosomiasis, an enteric disease of turkeys. Cochlosomiasis is commonly characterized as an enteritis, complete with diarrhea, lethargy, inflamed intestines, and failure to gain weight resulting in runting and widespread flock non-uniformity accompanied by high morbidity (Bollinger and Barker, 1996; Gharagozlou and Dezfoulian, 2009; Beckstead, 2019). Cochlosomiasis is often identified with bacterial and protozoal coinfections such as various *Escherichia coli*, *Trichomonads*, and *Eimeria* spp. (Campbell, 1945; Duffy et al., 2005; Beckstead, 2019). However, *C. anatis* has also been identified as the only pathogen present in birds with classic symptoms of cochlosomiasis (Campbell, 1945; Gharagozlou and Dezfoulian, 2009).

Disease presentation *C. anatis*-infected turkeys varies drastically between individual birds, producing widespread uniformity in commercial production. This has driven a decades-long debate over the validity of *C. anatis* as a primary pathogen of turkeys. Campbell, et al. described a mass mortality event in a turkey flock in Scotland where *C. anatis* was the only pathogen-related organism recovered (Campbell, 1945). Other documentation and accounts of infection with *C. anatis* provide further evidence of the parasite as a primary pathogen with commonly referenced symptoms and disease signs including runting, diarrhea, and inflamed intestines (Campbell, 1945; Cooper et al., 1995; Bollinger and Barker, 1996; Gharagozlou and Dezfoulian, 2009). Altogether, the historical observations of *C. anatis* associated with enteritis in turkeys provide substantial evidence to regard *C. anatis* as a primary pathogen of turkeys.

Limited research evaluating *C. anatis* as a pathogen leaves a myriad of questions regarding its pathogenicity, especially when associated with coinfections. Thus far, experimental

infection of *C. anatis* with *Eimeria* and *Histomonas meleagridis* has been tested in the laboratory (Duffy et al., 2005; Schaeffer and Chen, 2022). However, field reports suggest other coinfections exists, such as infection with opportunistic enteric bacteria (Cooper et al., 1995). Therefore, it is essential to evaluate the presence of potentially pathogenic bacteria in the poult gut to understand bacterial population trends that may lead to later secondary infection.

In populations affected by outbreaks of infectious disease, individuals within the population rarely become infected at the same time. Previous studies conducted with *C. anatis* revealed no significant differences in the uniformity of *C. anatis*-infected and non-infected turkey poult groups when inoculation with *C. anatis* was performed at the same time (Lowery, 2022). This indicates that disparities in uniformity may not be directly attributable to disease effects alone. This study hypothesized that if turkey poults become infected earlier in life, they will be smaller and experience more severe disease than birds infected later in life.

MATERIALS AND METHODS

All research was approved by the North Carolina State University Institute of Animal Care and Use Committee (IACUC).

Collection and Storage of *C. anatis*

A mixture of *C. anatis* trophozoites collected from intestinal scrapings of turkeys from commercial turkey farms in North Carolina and Virgina actively infected with *C. anatis* were used in this study. The parasites were harvested from a 14 cm section of the ileum of infected turkeys by a mucosal scraping, then placed in 40°C phosphate buffered saline (PBS; VWR International, LLC, Radnor, PA, USA) . The mixture was preserved by mixing 1mL with 50 µl of dimethyl sulfoxide (DMSO; Fisher Scientific, Hampton, NH, USA) in a cryotube. The

cryotube was then placed in a -80°C freezer using a Mr. Frosty Freezing Container for 24 hours before being moved to liquid nitrogen.

Experimental Design

Two-hundred, one-day-old, off-sex Nicholas male poults, obtained from an Aviagen hatchery in Lewisburg, WV, were evenly placed into 20 isolator cages with *ad libitum* feed and water, and evenly divided into four treatments. Three treatments were inoculated with *C. anatis* at D0, D7, and D14 of the study. The other group was a non-infected negative control (NC). An additional 12, one-day-old poults were placed into an isolator cage to be used as seeder birds, since no methods are currently known for *in vitro* cultivation of *C. anatis*. All treatment cages were grown to 28 days of age, at which point all poults were humanely euthanized for data collection.

Table 1. Feed formulation used for this study.

Ingredient	Control Diet (%)
Corn	51.00
Soybean Meal	17.90
Poultry by pro-Meal	20.35
Wheat Bran	5.00
Corn Micro Flush	0.55
Mono-Dicalcium Phosphate	1.49
Calcium Carbonate	1.30
Sodium Bicarbonate	0.15
Salt, Plain	0.09
DL-Methionine	0.21
Lysine	0.40
Threonine	0.06
Selenium	0.05
Choline Chloride	0.05
NCSU Vitamin Mix[1]	0.20
NCSU Mineral Mix[2]	0.20
Poultry Fat	1.00

[1]120 mg manganese, 120 mg zinc, 80 mg iron, 10 mg copper, 2.5 mg Iodine, and 2.5 mg cobalt per kg of diet.
[2]13,200 IU vitamin A, 4,000 IU vitamin D3, 33 IU vitamin E, 0.02 mg vitamin B12, 0.13 mg biotin, 2 mg menadione (K3), 2mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B6, 55 mg niacin, and 1.1 mg folic acid per kg of diet.

Inoculation

Frozen stocks of *C. anatis* were used for oral inoculation of D0-inoculated poults and seeder birds at placement. At D0, all inoculated poults received a frozen stock aliquot (1mL) of *C. anatis*. The seeder birds were then used to harvest *C. anatis* for inoculation of D7- and D14-inoculated groups. The initial PBS-*C. anatis* mixture was diluted to a concentration of ~500,000

cells/mL. Each poult in the D7- and D14-inoculated groups received 1mL of prepared inoculum. All inoculums were kept at a temperature around 40°C and continuously mixed. The cells in the mixture are fully viable for roughly 30 minutes before the cells begin to gradually die. A new seeder bird was collected and its *C. anatis* harvested to create a new stock solution after 30 minutes if inoculation was not completed.

Data Collection

Performance

Individual body weights were recorded at D0, D14, D21, and D28 of this study. Extenuating circumstances prevented the recording of individual body weights at D7. Feed consumption was also recorded at the end of the study (D28). Performance was evaluated through calculations found in **Table 2**. Bird deaths were recorded as they occurred and removed from their cages.

Table 2. Performance parameter calculations for feed conversion ratio and flock uniformity. Flock uniformity was assessed by calculating the coefficient of variation between individual bird weights amongst all birds weighed per treatment.

Calculated Performance Parameter	Equation
Feed Conversion Ratio	$\frac{Feed\ Intake}{Body\ Weight\ Gain}$
Flock Uniformity (Coefficient of Variation)	$\frac{Standard\ Deviation}{Average\ Flock\ Body\ Weight} * 100$

Bacterial Collection and Analysis

Sterile necropsy was performed for bacterial collection from the cecal tonsil of two poults per cage. The cecal tonsil was excised and placed into a sterile 7oz Whirl-Pak bag with a filter (Nasco, Fort Atkinson, WI, USA). Each sample was immediately placed into a cooler on ice, then later macerated and mixed with PBS for a 4:1, PBS: sample weight-to-

weight ratio within 24 hours. To enumerate *C. perfringens*, serial dilutions were spread-plated on tryptose sulfite cycoloserine (TSC; Oxoid, Basingstoke, Hampshire, UK) agar supplemented with cycloserine (ThermoFisher Scientific, Waltham, MA) using the triple layer method (Valenzuela-Martinez et al., 2010) and incubated in an anaerobic environment at 37°C. Colonies matching the manufacturer's selection criteria were recorded and an estimation of the amount of *C. perfringens* colony forming units (CFUs) per mL calculated. *E. coli* and coliforms, and *Salmonella spp.* were enumerated using assays to estimate the most probable number of CFUs (MPN). For *E. coli* and coliforms, 0.9mL of Colilert broth (IDEXX Laboratories Inc., Westbrook, ME, USA) was placed into each well of a deep (2mL) 96-well plate (Greiner Bio-One North America, Monroe, NC, USA). In three adjacent wells, 0.1mL of cecal tonsil homogenate was transferred into the broth, and 10-fold serial dilutions were made in the next 6 wells. The plate was then incubated at 37°C for 24 hours and observed under a UV light. Wells that were yellow were considered positive for coliforms, and any wells that fluoresced were considered positive for *E. coli*. For *Salmonella* spp., 1mL of cecal tonsil homogenate was placed into three adjacent wells of a deep (2mL) 96-well plate. The other wells were then filled with 0.9mL of BD Difcp TT (Tetrationate) Broth Base (Fisher Scientific, Hampton, NH, USA) supplemented with iodine (Avantor, Radnor Township, PA, USA), and 10-fold serial dilutions were made from the initial homogenate to make 5 dilutions. The plate was then incubated at 42°C for 24 hours. Then, 1µL from each well was placed onto XLT-4 agar (Neogen, Lansing, MI) and incubated at 37°C for 24 hrs. All evaluations and calculations for enumeration were performed using the USDA Laboratory Guide Book, Most Probable Number Procedure and Tables (“Microbiology Laboratory Guidebook | Food Safety and Inspection Service,”).

C. anatis Concentration

Poults used for cecal tonsil collection were also used for *C. anatis* enumeration. *C. anatis* cells were harvested according to the method described in **Collection and Storage of *C. anatis*.** The cells were then enumerated using a Neubauer hemacytometer according to manufacturer instructions ("Neubauer Haemocytometry,").

Statistical Analysis

Mortality rates were transformed using the Freeman-Tukey double arcsine transformation in JMP Pro 16 (Cary, NC, USA) to stabilize mortality variance and normalize the distribution. Statistical analysis was performed using a one-way analysis of variance and Duncan's multiple range test for mean separation in SAS 9.4 (Cary, NC, USA). Statistical significance was determined with a P-value of $P \leq 0.05$ and statistical trends were determined when $0.05 \leq P \leq 0.1$.

RESULTS

Birds inoculated with cochlosomiasis presented with depressed weight gain, lethargy, ruffled feathers, diarrhea, and catarrhal enteritis.

Individual body weights recorded throughout the trial revealed differences in average body weight. At D0 of the study, the average body weights were similar (P=0.0789; **Figure 1**). By D14, birds inoculated at D0 were significantly lower than all other treatment groups (P=0.0002; **Figure 1**). At D21, body weights were more differentiated, with the lowest belonging to D0-inoculated poults, and the next grouping consisting of D7- and D14-inoculated poults, with the NC group weighing the most. Additionally, at D21, D14-inoculated birds were not statistically different from the NC group, however the NC group weighed significantly greater than D0- and D7-inoculated birds (P<0.0001; **Figure 1**). At the end of the study (D28), the

lowest average body weight was seen in D0-inoculated birds, D7- and D14-inoculated birds belonged to the middle-weight tier, and NC birds weighed the most (P<0.0001; **Figure 1**).

Gains in body weight (**Figure 2**) showed a significant difference between birds inoculated with *C. anatis* and non-inoculated birds. From D0 to D14, body weight gain was dramatically lower in birds inoculated at D0, with all other groups showing similar weight gain (P=0.0002). From D14 to D21, weight gain in D0-inoculated birds was lowest, followed by a grouping of D7- and D14-inoculated birds, with the highest gain in NC birds (P=0.0008). Lastly, from D21 to D28, all inoculated birds exhibited similar body weight gains, with the gains in the NC group being greatest (P=0.0003).

Feed intake, FCR, and uniformity are presented in **Table 3**. Feed intake throughout this study was significantly lower in birds inoculated at D0, with no statistically significant differences observed between D7- and D14-inoculated birds and NC birds (P=0.0023). FCR trended toward an increased value (worse efficiency) in all infected birds, however no significant differences were seen (P=0.0644). Uniformity was also not significantly different between any of the treatment groups, but showed a trend in which the coefficient of variation was increased in the D0-inoculated group (P=0.0648).

Mortality (**Figure 3**) was significantly increased in D0- (70%) and D7-inoculated birds (50%) compared to D14-inoculated (16%) and NC birds (8%; P=0.0007). Mortalities per day visually spiked in the D0- and D7-inoculated groups within one week after inoculation (**Figure 4**).

No significant differences were observed in *E. coli* (P=0.1313), coliforms (P=0.6470), or *C. perfringens* (P=0.1233) populations between any of the treatment groups. However, less *E. coli* and *C. perfringens* were recovered in birds with earlier infection by *C. anatis* (**Table 4**).

Salmonella spp. was scarcely recovered from all treatment groups. No *C. anatis* was recovered from the NC group. No statistically significant differences in the concentration of *C. anatis* in the ilea were observed between the D0-, D7-, and D14-inoculated groups (**Table 4**).

DISCUSSION

Uniformity issues associated with cochlosomiasis in affected turkey flocks have been routinely recorded (Cooper et al., 1995; Evans et al., 2006; Gharagozlou and Dezfoulian, 2009). Previous reports of cochlosomiasis have remarked on separate incidences of runted flock individuals, as well as an associated mass mortality event (Campbell, 1945; Cooper et al., 1995; Evans et al., 2006; Gharagozlou and Dezfoulian, 2009; Beckstead, 2019). These incidences have been described in detail to show varying disease signs in birds infected with *C. anatis*, leading to the continued debate regarding the validity of *C. anatis* as a primary pathogen of turkeys.

Diseases such as coccidiosis have been shown to begin at a younger age (5 days after hatch) due to an immature immune system (Musa et al., 2010). Like *C. anatis*, coccidia are protozoan parasites of turkeys that target the intestine. Therefore, it is possible that protozoan parasites can cause infections within the first week of life in poultry. Different infection times were used in this study to better model the spread of infection. Maximum infectivity is regarded as the time during an infection where the pathogen reaches its maximum concentration in the host and symptoms appear (van Seventer and Hochberg, 2017). Since *C. anatis* reaches its maximum concentration in the host gut in 5-7 days following infection, 7-day periods between infection were used. The results of this study showed that cochlosomiasis can result in high mortality. Likewise, uniformity issues were re-created for the first time using varied infection times rather than simultaneous infection.

In the case report by Campbell et al., severe mortality was observed in turkey poults from two to ten weeks old (Campbell, 1945). Since the incubation period of *C. anatis* is 5-7 days, it can be assumed that infection with *C. anatis*, in at least some of the birds reported on, occurred within the first week of life since ages ran from 2 to 10 weeks old (Bollinger and Barker, 1996). Therefore, from this study, the reasoning behind the mass mortality from cochlosomiasis may be clearer – that the turkey poults were possibly infected with *C. anatis* at a younger age and thus experienced more severe disease. (Campbell, 1945).

Using this notion that turkey growth during infection with *C. anatis* is dependent upon the age when infection occurs, it may be possible to develop a growth standard that models turkey growth associated with *C. anatis* and the age at which the bird became infected with the parasite. If this is accomplished, a timeline may be established that is specific to an outbreak farm that estimates the time at which *C. anatis* was introduced to a farm. Together with environmental observations and data collection, specific environmental changes may be determined that precede outbreaks of cochlosomiasis, such as various weather patterns or wild animal movements. From this, a multitude of research avenues may be opened, such as narrowing the scope of potential vectors of *C. anatis* that allows for introduction of the parasite to naïve flocks, since no vectors of *C. anatis* are currently known.

One gap in cochlosomiasis research is the relationship between infection with *C. anatis* and depressed weight gain. Disease symptoms such as catarrhal enteritis are indicative of dysbiosis in the enteric microbiota (Li et al., 2020). Disruption of enteric microbiota has the potential to cause several issues in the intestine, either through the failure to break down nutrients through decreased bacterial metabolic activity or allow for the overgrowth of pathogenic bacteria in the gut, leading to secondary infections (Bailey, 2010). A secondary

infection could lead to intestinal epithelial damage, decreasing the absorptive capacity of nutrients by the intestinal epithelium. Past research has remarked on evidence of lesions during infection with *C. anatis* attributable to bacterial pathogenesis (Cooper et al., 1995). No evidence in this study, however, revealed differences in populations of *E. coli*, coliforms, *C. perfringens*, or *Salmonella* spp. with cochlosomiasis. To further explore this topic, evaluations of changes to the poult enteric microbiome would be beneficial to identify changes to the intestinal microbiota during infection with *C. anatis*.

CONCLUSION

This study showed that turkey poults infected with *C. anatis* at an earlier age may experience more deleterious effects from disease compared to infection at an older age. Additionally, it was shown that infection in turkey poults by *C. anatis* at different ages may lead to the common issues surrounding flock non-uniformity. These results suggest that the prevention of *C. anatis* entering turkey farms, especially during the first two weeks of placement, is crucial to optimal survival. Future research could focus on the determination of a standard growth timeline of turkeys infected with *C. anatis* at different ages. This would assist in estimating when *C. anatis* entered a turkey flock which could be used to evaluate environmental changes preceding infection to determine specific circumstances increasing the risk of cochlosomiasis outbreaks. Additionally, microbiome analysis may be performed to better understand changes to poult enteric microbiota to identify secondary issues arising from infection with *C. anatis*.

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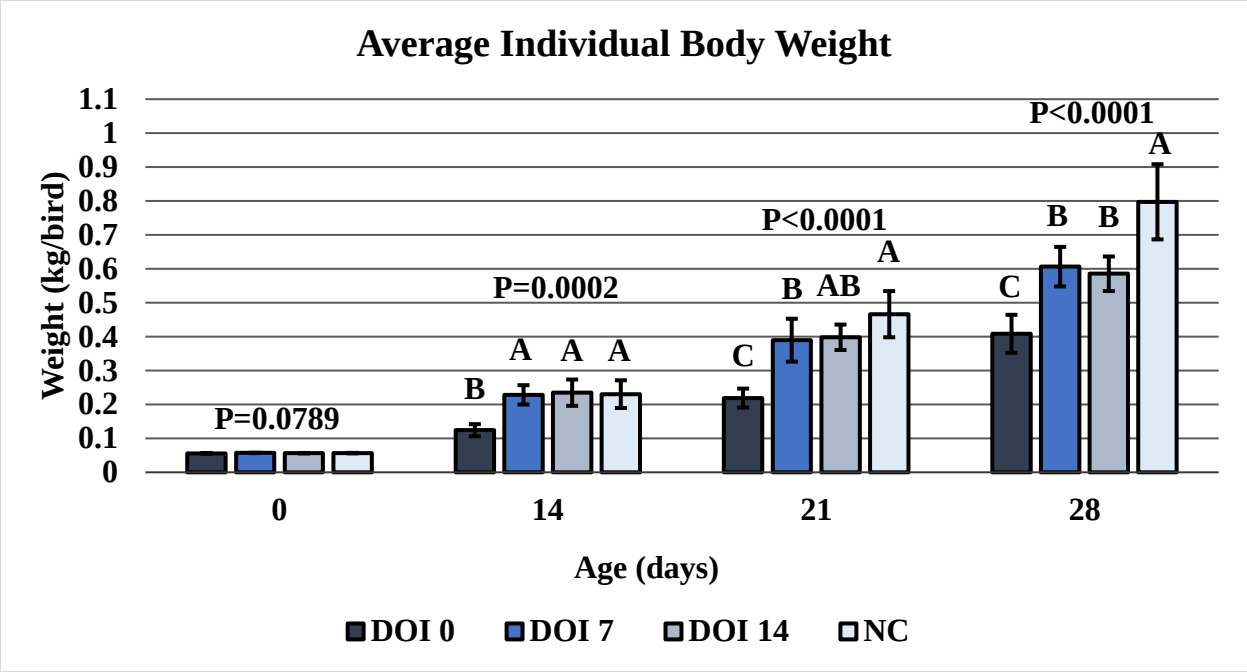

Figure 1. Turkey poults used in this trial were individually weighed at D0, 14, 21, and 28 of this study.

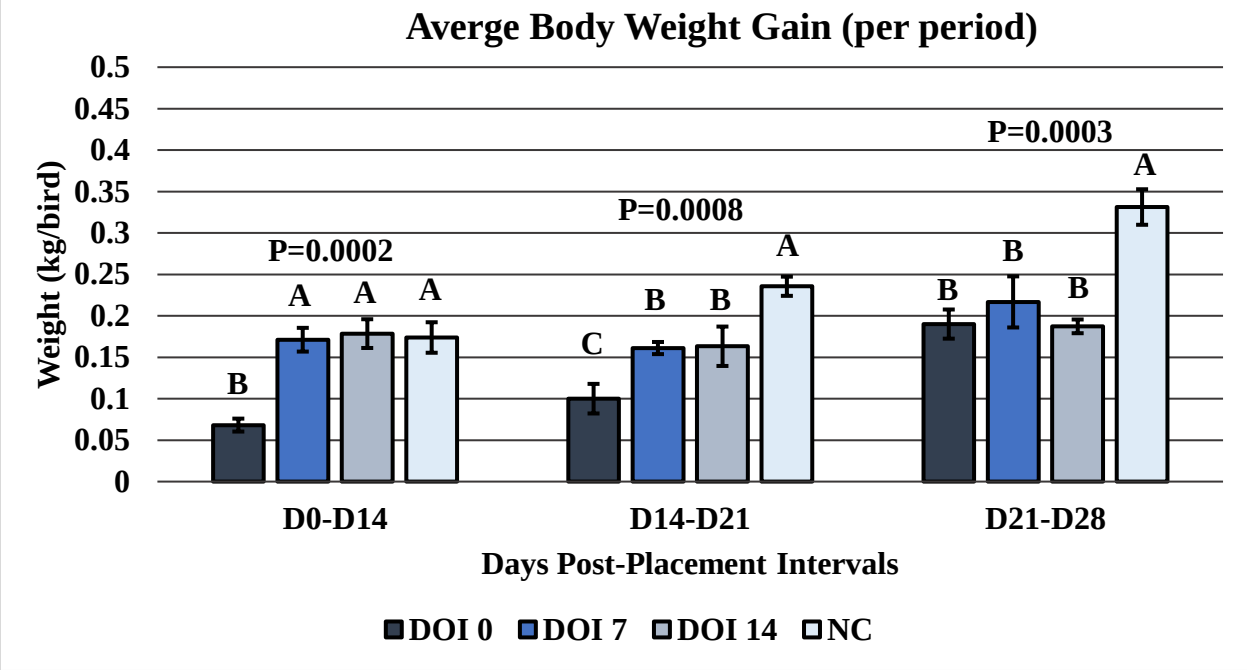

Figure 2. Average gain in body weight per bird in the time intervals between each weighing time throughout this study.

Table 3. Performance parameters measured and calculated for all treatments used in this study. BWG, FI, and FCR are relative to the entirety of the study (D0-D28). Uniformity was assessed only for values collected on D28.

Treatment	BWG[1] (kg/bird)	FI[2] (kg/bird)	FCR[3]	Uniformity
DOI 0	0.332 ± 0.030^{C}	0.847 ± 0.117^{B}	0.035 ± 0.005^{ab}	31.870 ± 7.760^{a}
DOI 7	0.549 ± 0.029^{B}	1.459 ± 0.117^{A}	0.038 ± 0.002^{a}	19.390 ± 2.756^{ab}
DOI 14	0.529 ± 0.023^{B}	1.422 ± 0.159^{A}	0.038 ± 0.002^{a}	18.049 ± 2.767^{b}
NC	0.741 ± 0.050^{A}	1.458 ± 0.158^{A}	0.028 ± 0.001^{b}	15.156 ± 1.623^{b}
P-value	<0.0001	0.0023	0.0644	0.0648

[1]BWG = Average body weight gain
[2]FI = Feed intake
[3]FCR = Feed Conversion Ratio

[A ,B, C] Indicates a statistically significant difference ($P \leq 0.05$)
[a, b ,c] Indicates a statistical trend ($0.05 \leq P \leq 0.1$)

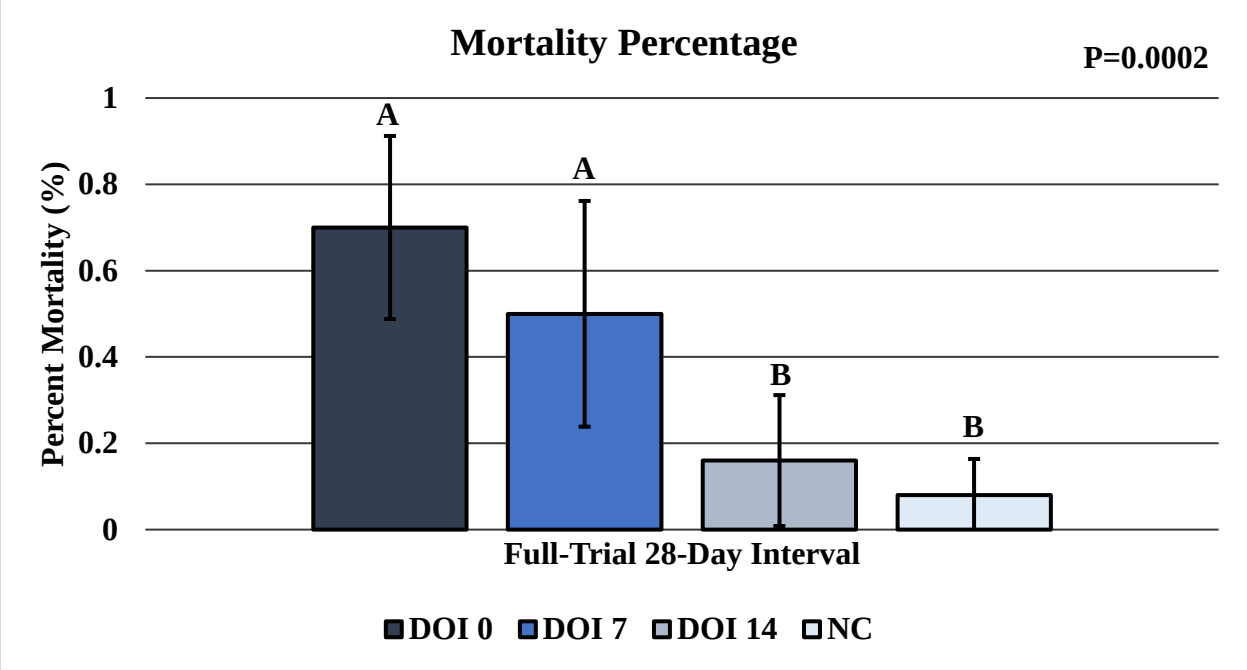

Figure 3. Mortality percentage for all treatments used in this study throughout the study.

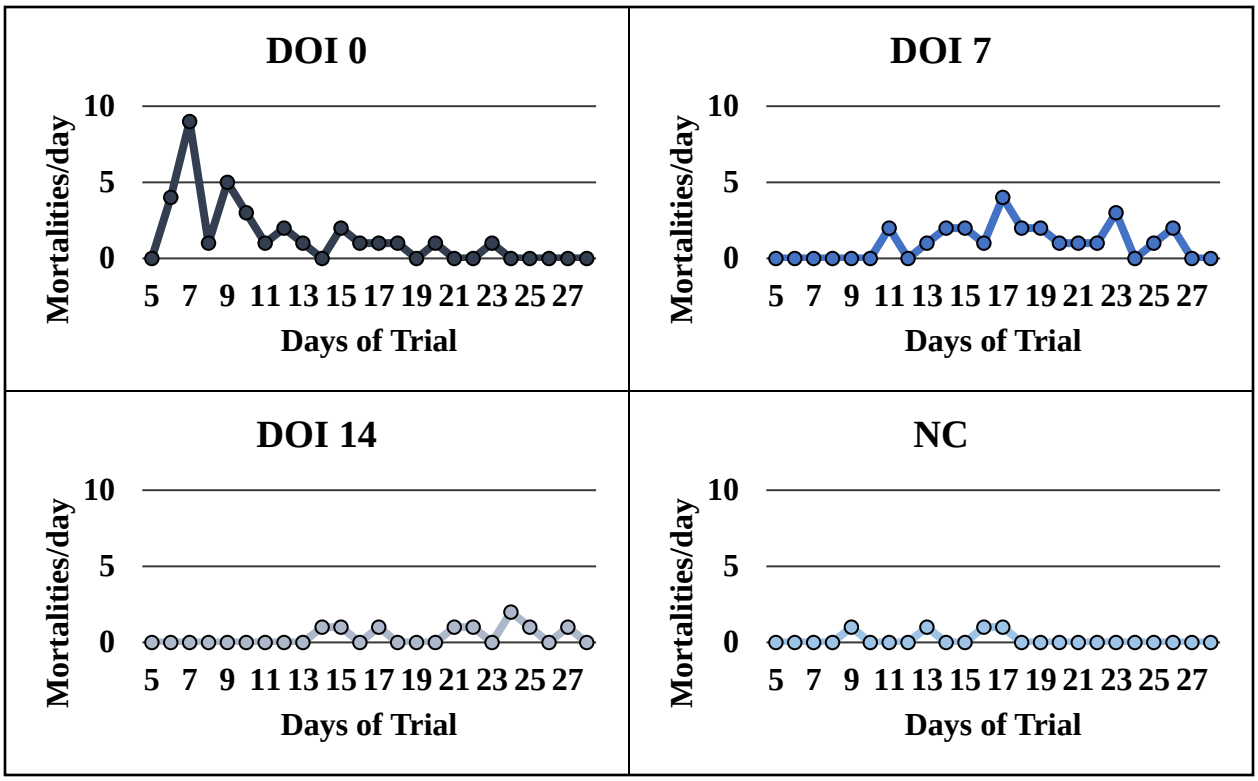

Figure 4. Deaths per day for all treatments used in this study.

Table 4. Bacterial enumeration in the cecal tonsil from each treatment group in this study.

Treatment	*C. anatis* (log Cells/mL)	*E. coli* (log MPN/g)	Coliform (log MPN/g)	*C. perfringens* (log CFU/g)
DOI 0	6.722 ± 0.096	2.461 ± 0.573	3.457 ± 1.387	2.160 ± 1.065
DOI 7	6.731 ± 0.091	3.000 ± 0.924	3.191 ± 0.830	2.741 ± 0.365
DOI 14	6.701 ± 0.139	3.126 ± 0.923	3.726 ± 1.265	3.013 ± 0.461
NC	---	4.085 ± 0.785	4.085 ± 0.785	3.735 ± 1.223
P-value	0.9083	0.1313	0.6470	0.1233

CONCLUSION

Infection with *C. anatis* regularly results in issues with turkey growth performance, especially in poults, as exhibited in this thesis. Overall, this thesis showed that infection with *C. anatis* negatively impacts growth performance and can cause disruptions to blood physiology and gut anatomy. Additionally, disease appears to become more severe when turkey poults are infected with the parasite at a younger age. Future research should focus on the supplementation of amino acids to reduce their potential loss due to disease, and blood chemistry should be further evaluated to better understand what is leading to disruptions of blood pH. Additionally, the gained knowledge regarding increased disease severity with earlier infection indicates that research could focus on creating a standardized expected weight progression of turkeys infected with *C. anatis* at different ages to determine when *C. anatis* enters a turkey flock. This would open research avenues for environmental studies. Lastly, microbiome analyses may be performed during infection with *C. anatis* to identify potential changes to the gut microbiome and target potential pre- and post-biotics for use in prevention and treatment of cochlosomiasis.