DETERMINATION OF LIFE CYCLE STAGES OF BODY CAVITY FLUKE CYCLOCOELIDS (CYCLOCOELIDAE) IN TERRESTRIAL SNAILS AND EXPERIMENTAL EXPOSURE OF DOMESTIC CHICKENS (GALLUS GALLUS)

A Thesis

by

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ABSTRACT

Zoos and other bird-holding facilities world-wide recently have reported serious health problems and frequently deaths of captive birds in exhibits associated with infections by introduced species of cyclocoel (Digenea) parasites. These adult flukes apparently have been introduced into these facilities by the importation of infected exotic birds. The larval stages of digenean species generally develop within either aquatic or terrestrial snails. A few of the life cycles of cyclocoelids that utilize aquatic snails as the molluscan host have been documented; however, very little is known about the life cycle of the species introduced into bird-holding facilities where the larval stages are produced in terrestrial snails. A more complete understanding of these terrestrial-based life cycles is essential for the effective prevention and treatment of infected birds. Ultimately, our objective is the development of practical control strategies for these parasites in zoos.

In this study, 16 of 53 terrestrial snails (30%) of the genus *Subulina* from Lincoln Park Zoo, Chicago, Illinois were found to be infected with cyclocoelid parasites of the genus *Szidatitrema*. Larval stages (redial generations, cercariae, metacercariae, and young adults) of the parasite taken from these snails were described and illustrated. Metacercariae were suspended in a saline solution administered to 15 one-day old chickens. Five control chickens were given saline solution. Fecal samples were taken weekly from experimental and control chickens to determine if eggs were present through sedimentation and floatation procedures. Blood samples were obtained from

chickens every other week starting in the second week post-exposure. A total of eight chickens were necropsied starting on Day 67.

Experimental infections were not successful; there were no differences between levels of eosinophils or fibrinogen in experimental chicks compared to control chicks, and helminth eggs were not found in any fecal floatation. No adult flukes were found during necropsy. Further studies should include attempts to experimentally infect additional bird hosts including monk parakeets from the Schubot breeding colony at Texas A&M University, testing of different exposure methods, and determination of any potential secondary intermediate hosts or paratenic hosts. Once experimental bird hosts have been successfully infected in a laboratory setting, vaccines, drug trials, and potential detection methods for this fluke should be considered.

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1. INTRODUCTION

Exotic bird displays often enhance the public's experience at a zoo. Many people enjoy watching and reading about birds that they would not otherwise see in their natural habitat. It is a common practice for zoos to introduce exotic bird species from their natural habitats or from other zoos into exhibits and other captive facilities. Zoos and other bird-holding facilities have established breeding programs for threatened species of birds. Non-native plant species may also be imported in order to give the birds a more natural setting within the aviary. Birds imported into these facilities are placed in a separate quarantine facility prior to their release into exhibits where they are isolated under the supervision of a veterinarian for a period of at least 30 days (Colbert, 2010). The quarantined birds undergo a physical exam, blood and fecal testing for helminths and other pathogens that may pose a risk to other birds, and may also receive appropriate vaccines and anthelmintic drugs. Generally, these quarantine procedures catch any potentially harmful organisms, and infected birds are treated before being released into an aviary. A family of digenean parasites called dicrocoelids has previously been reported to have been the cause of deaths in zoo birds. However, there is a new species of parasitic flukes of the family Cyclocoelidae that are reported to have caused deaths of exotic birds housed in zoos, despite normal quarantine procedures. It is not known how these parasites are being introduced into zoos. In captive birds, cyclocoelids can reach high densities in short periods of time, causing severe health problems and ultimately resulting in death. Apparently low level infections of these flukes are not always

detected during quarantine testing, but when detected, treatment with doses of anthelmintic drugs used for intestinal parasites is not successful. Because it is common to transfer birds between zoos and the ease by which this is done, it is important to detect cyclocoelid infections early.

Parasitic flatworms of the family Cyclocoelidae are large-bodied flukes that are in the subclass Digenea. Previous studies of digenean parasites have determined that these worms generally utilize a three-host life cycle, with a first intermediate host, a second intermediate host, and a definitive host. An intermediate molluscan host is used to develop non-sexual larval stages of the parasite, and a definitive avian host is used for sexual reproduction (Pearson, 1972). The snail host becomes infected by ingestion of eggs from adults shed in feces of infected definitive hosts or from an infective larval stage that hatches from these eggs. Sexually reproducing cyclocoelids produce eggs from which miracidia hatch, which are infective to snails. Once a snail comes into contact with miracidia, the life cycle within the snail begins. Miracidia develop into rediae or germinal masses, which produce round cercariae that possess a tail (Pearson, 1972). The tails on the cercariae allow this larval stage to escape from the snail into the outside environment. Some cercaria do not leave the redia, but develop into metacercaria, the stage that is infective to avian hosts. Redia can contain cercariae, metacercariae, and also young adult parasites. Cercariae and metacercariae are ingested by the avian host either by eating an infected snail with these larval stages present, or by ingesting the larval stages from the environment. Depending on the specific species of cyclocoelid, the snail may be terrestrial or aquatic. The definitive hosts of cyclocoelids

with an aquatic intermediate host are wading birds. Land birds are the definitive host of the cyclocoelid species that use a terrestrial intermediate host. Much of the research on cyclocoelids has focused on the species that utilize an aquatic-based life cycle. There is little information on the life cycles of cyclocoel species in terrestrial snails and birds.

Although it appears that some species of cyclocoelids utilize a terrestrial-based life cycle, our knowledge of cyclocoelids is based largely on the species utilizing an aquatic snail intermediate host. Cyclocoelids appear to have an atypical life cycle for digeneans in that there are only two hosts in the life cycle: the snail intermediate host and the definitive bird host. Cribb et al. (2003) outlined 17 digenean superfamilies that utilize a secondary intermediate host in order to complete the life cycle. All stages up to young adults are apparently present in the terrestrial snail host of cyclocoelids. It is likely that no second intermediate host is required in these life cycles and that birds need only eat infected snails to become exposed to these flukes.

There are five genera of cyclocoels that have been classified thus far: *Circumvitellatrema* Dronen, Greiner, Ialeggio and Nolan, 2009; *Morishitium* Witenberg 1928; *Psophiatrema* Dronen and Kinsella, 2009; *Selfcoelum* Dronen, Gardner and Jiménez, 2006; and *Szidatitrema* Yamaguti, 1971. Two species in particular have been identified as the cause of exotic bird deaths in zoos. Both of these species use terrestrial snails and land birds to complete their life cycle. *Szidatitrema yamagutii* was found in white-necked myna, *Streptocitta albicollis* Viellot, 1818 (Sturnidae), and the bearded barbet *Pogonornis dubius* Gmelin, 1788 (Lybiidae), at the Audubon Zoo in New Orleans, Louisiana, U.S.A. (Dronen et al., 2006). The bird species in which this parasite

was found are Old World species, the bearded barbet native to Africa and the white-necked myna native to Indonesia, that were imported in the United States for exhibition in zoo aviaries. Both species of birds suffered severe respiratory distress. The bearded barbet died of air sac parasite trauma, and the white-necked myna died of parasitic air sacculitis (Dronen et al., 2006).

The second cyclocoelid parasite, *Circumvitellatrema momota* Dronen, Greiner, Ialeggio and Nolan, 2009, was reported from the air sacs of a blue-crowned motmot, *Momotus coeruliceps* Gould, 1836 (Momotidae), that died at the Philadelphia Zoo, Philadelphia, Pennsylvania, U.S.A. (Dronen et al., 2009). The blue-crowned motmot is native to Central and South America. It is interesting to note that this bird was hatched at the Riverbanks Zoo in Columbia, South Carolina, U.S.A. and spent four years at the Audubon Zoo before being transferred to the Philadelphia Zoo. This bird was born in the United States and was apparently exposed to the parasite in holding facilities within this country. Fluke eggs were found during a fecal examination at Audubon Zoo, which were confirmed later at the Philadelphia Zoo to be a species of cyclocoelid. *Circumvitellatrema momota* has also been isolated at the Montpellier Zoo in Montpellier, France (Libert et al., 2012).

Cyclocoelid parasites have infected an invasive species of the terrestrial snail *Subulina striatella* in bird exhibits in both the United States and in Europe. This species of snail is normally found in Africa. However, it apparently has been imported to Europe and North Amercia on plants that were brought into zoos and other bird-holding facilities to develop more natural settings for birds in exhibits (Libert et al., 2012).

Infected snails were found in an aviary where a blue-crowned motmot from the Montpellier Zoo had been attacked by its father and later died of a suspected air sac infection. Necropsy showed the bird in good body condition, but had numerous cyclocoelid trematodes in the lungs, air sacs, and around the heart. In addition, there were fluke eggs in the parabronchi, and the cause of death was determined to be bronchial obstruction and suffocation. It is believed that this snail species was introduced to this aviary with the planting of exotic trees from a dealer based in the Netherlands while it was being constructed (Libert et al., 2012).

It is not known for certain how cyclocoelids are introduced into zoos. One possible way is through the plants that are often introduced with the new birds. Cyclocoel-infected snails could be on the plants themselves or in the soil surrounding the plants. This would introduce the parasite to the aviary where the plants are placed, and potentially expose birds in that aviary to the parasite. Another possible route of introduction is through infected birds. The birds may enter the facility already infected with a cyclocoelid infection, especially if taken directly from its natural habitat. Although fecal testing has not revealed presence of eggs in infected birds, it is possible that eggs can be shed in the feces into the environment. Once the eggs are in the environment, they may be picked up by a snail in the soil to complete the life cycle. Because of the common practice of exchanging animals between zoos and the ease by which this is done, it is important to detect cyclocoelid infections early.

The complete life cycles of these types of parasites are not well known, but they do seem to spread rapidly among birds in the same facility. In order to better understand

this parasite and prevent avian deaths, it is important to determine the life cycles of these invasive parasitic helminths. Once a full picture of the life cycle is obtained, steps can be proposed to prevent disease and the development of diagnostic tests and effective treatments can be evaluated.

In this two-part study, the aspects of interest were determining the life stages of the parasite within the intermediate snail host and successfully inoculating domestic chickens with the cyclocoelid parasite to complete the information about the life cycle. Although dicrocoelids are not the focus of this study, we did find this family of parasite within most of our snails, often infecting the snails along with the cyclocoel parasite. We obtained information on types of infections present within each snail (cyclocoelid, dicrocoelid, or both), the snail length, and the stages of the life cycle found within the snails. From that data, we were able to create drawings of each life stage. We also experimentally infected chickens with the parasite and examined the comparative histopathology, comparative blood cell counts, and fecal samples. However, testing and necropsy results did not prove that we successfully infected the chickens. This study serves as a starting point for further investigations involving prevention and control methods.

2. LITERATURE REVIEW

The subclass Digenea (Class Trematoda) is one of the largest groups of the Platyhelminthes. There are more than 2,500 genera (Caira and Littlewood, 2001) that have been classified, and more are identified every year. Digenea consists of parasitic flukes with complex life cycles, with anywhere from one to four hosts, several larval stages, and morphological differences between each species (Cribb et al., 2003). The larval stages include redia, miracidia, cercaria, metacercaria. Most digeneans have two suckers, an oral sucker and a ventral sucker, and position within the fluke may be different between species. Differences in morphology where only one sucker is present or placement of suckers differs are used to distinguish digenean species. In addition to differences in morphology, the reproductive tract and the digestive tract are often used to classify species.

Digeneans are hermaphroditic, except for the schistosomes which have separate male and female worms. The male system is composed of paired round or tubular testes, a vas deferens, and a seminal vesicle, a sperm duct with prostate cells that may be surrounded with a cirrus (Caira and Littlewood, 2001). The female system is generally composed of an ovary, oviduct, seminal receptacle, ootype surrounded by Mehlis' glands, a vitelline system, and a uterus that opens to a genital pore (Caira and Littlewood, 2001).

Adult worms of many species have been found in numerous tissues and organs of vertebrate hosts, usually birds or fish, and more rarely in invertebrate hosts. Most

digeneans reside in the intestines, but they have also been found in the body cavity, gall bladder, lungs, air sacs, liver, and eye of definitive hosts (Cribb et al., 2003). Sexual reproduction occurs in the definitive host, ultimately producing eggs that are usually passed into the external environment, where they hatch into short-lived miracidia (Cribb et al., 2003). These miracidia can penetrate the molluscan intermediate host. A simple sac called a sporocyst develops from the miracidia. The sporocyst produces a second generation of either daughter sporocysts or rediae, which contain a mouthpiece (Cribb et al., 2003). A generation called cercariae is then produced from the sporocysts or rediae. Cercaria are equipped with a tail that allows them to migrate out of the molluscan host and infect the vertebrate host directly, or they become metacercaria which are ingested with the molluscan host or from the environment (Cribb et al., 2003). This outline of the reproductive stages is not, however, identical for all digeneans. The complexity of the life cycles has brought debate among taxonomists on the classification of many digeneans. A study done by Olson et al. (2003) revised the phylogeny of the subclass to consist of 77 nominal families and reclassified many orders, superfamilies, and families.

The Cyclocoelidae are classified under the superfamily Echinostomatoidea and order Plagiorchiida (Olson et al., 2003). The cercaria encyst as metacercaria in the intermediate molluscan host (Cribb et al., 2003), and metacercaria encyst in the molluscan host within the sporocyst or redia, depending on species (Caira and Littlewood, 2001). Cyclocoeldiae are large-bodied flukes that are generally found as adults in the air sacs, lungs, and infraorbital and nasal sinuses of their avian host (Caira and Littlewood, 2001). Cyclocoelids are given their name from a distinct morphological

feature. The intestinal ceca are fused at the posterior to form a cyclocoel, a structure that surrounds the body cavity almost completely (Caira and Littlewood, 2001).

Many species of Cyclocoelidae infect aquatic snail intermediate and definitive avian hosts. *Cyclocoelum mutabile* Zeder 1800 is a cyclocoelid that infects air sacs of coots (*Fulica* spp.). American coots, *Fulica americana* Gmelin, 1789 (Rallidae), range from Canada to the northern parts of South America. *Cyclocoelum mutabile* can infect the Eurasian coot, *Fulica atra* Linnaeus, 1758 (Rallidae), but its life cycle is not known (McLaughlin, 1976). In a 1976 study, laboratory-reared American coots were experimentally infected with *C. mutabile* metacercaria, and snail stages were collected from these coots during necropsy. Different types of snails were infected with the cyclocoel. It was found that miracidia infected planorbid snails more readily than physids or lymnaeids (McLaughlin, 1976). All of the coots became infected, and liver lesions were seen in many of these birds, although less noticeable after day 21 post infection (McLaughlin, 1976). The description of the life cycle during this study was very similar to the description given by Wooton in 1964, as stated by McLaughlin (1976). Lesions on the liver from juvenile and adult worms embedded in the air sacs suggested that the parasite migrates through the host before sexual maturity.

The migratory path of *C. mutabile* was studied using specimens collected from naturally infected coots. Snail stock cultures were experimentally infected with eggs, and then metacercaria from these snails were fed to laboratory-reared coots (McLaughlin, 1977). The coots were necropsied at intervals, and the organs were removed for examination. In this study, heart, liver, lung, gallbladder, spleen, kidney, and pancreatic

tissues were examined for infection. Flukes were found only in the lungs, liver, spleen, air sacs, and body cavity. At 12 hours post-infection, two flukes were found in the liver of a coot, and by day one the livers had raised lesions (McLaughlin, 1977). By day 15, half of the flukes that were recovered were found in the liver, and liver lesions were evident. This study found that after day 18, *C. mutabile* migrates to the air sacs. Although the exact route of migration from the gut to the liver is not known, it is suggested that the flukes encyst on the intestinal wall and penetrate through to the body cavity and then to the liver (McLaughlin, 1977). In a follow-up study, flukes taken from the coots at different post-infection intervals were observed and measured against each other as well as with the flukes from the 1977 study. The length of the flukes at 4 and 6 hours post inoculation in the previous study were similar to the lengths of flukes at one day post infection (McLaughlin, 1983). Their maximum size was attained at day 28 (McLaughlin, 1983). Growth and development of *C. mutabile* was similar to that of *Fasciola hepatica* until day 18 when the cyclocoelid leaves the liver (McLaughlin, 1983). The presence of eggs in some fluke specimens by day 18 suggest that *C. mutabile* reaches sexual maturity soon after leaving the liver (McLaughlin, 1983).

Another study of this cyclocoelid determined the age at which coots became susceptible. Juveniles of two months and adult coots, one group previously exposed and one group that was naïve, were experimentally exposed to metacercaria of *C. mutabile*. All juvenile coots became infected, while only 3 of 10 previously exposed coots and 2 of 8 naïve coots became infected (McKindsey et al., 1994).

The American goldeneye duck, *Bucephala clangula americana* Bonaparte, 1838 (Anatidae), has recently been reported to be infected with a cyclocoelid species. Like the American coot, the American goldeneye is a New World species. These ducks are native to North America, with ranges in the northern part of the country and along coasts, rivers, and lakes during winter. Cyclocoelids were removed from the bodies of two American goldeneye ducks that had been killed by hunters near Galveston, Texas in 2002 (Dronen and Blend, 2007). These specimens were compared to a cyclocoel species previously found in the American goldeneye in Minnesota in 1931. From comparisons, a description of *Opthalmophagus bucephali* Dronen and Blend 2007 as a new species was given. This species was determined to be *Opthalmophagus* because it possesses characteristics of the genus, such as tandem testes and vitelline follicles that follow the ceca and unite posteriorly (Dronen and Blend, 2007). Measurements of the parasite itself as well as internal structures differed slightly from other members of *Opthalmophagus*, so this parasite was classified as a new species. Dronen and Blend (2007) note that it is often difficult to classify digeneans due to similar characteristics, wide ranges in measurements, and the fact that many cyclocoelids often occur in low numbers in natural infections.

A new species of cyclocoelid was also reported from Lesser yellowlegs, *Tringa flavipes* Gmelin, 1789 (Scolopacidae). Five species of Cyclocoelidae, *C. mutabile*, *C. phasidi*, *Haematotrephus nittanyense, Haematotrephus halli,* and *Selfcoelum brasilianum*, have previously been reported in this species (Dronen et al., 2008). This is another wading bird species that is native to the New World, with ranges from western

and central Canada to northern United States territories including Alaska. It has also been found in South America. The new cyclocoelid species was described from flukes recovered from the air sacs of Lesser yellowlegs that were collected from Roger Mills County, Oklahoma and Manitoba, Canada in 1963. These fluke specimens were measured and compared to a previously described *Haematotrephus* species. No oral sucker or acetabulum was found on these specimens, and a seminal receptacle was also absent (Dronen et al., 2008). The flukes were determined to be significantly different from other *Haematotrephus* species, and was named *Haematotrephus selfi* Dronen, Gardner, and Jimenez, 2008, after the late Dr. J. Teague Self, professor, University of Oklahoma.

3. RESEARCH METHODS

3.1 Intermediate snail host collection and processing

Terrestrial snails of *Subulina* sp (Figure 1) infected with the Cyclocoelidae parasite were collected from free-flight aviaries at the Lincoln Park Zoo in February 2014 and shipped overnight to the Pathobiology Department, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University. Immediately upon arrival, the snails were transferred to Texas A&M University Department of Wildlife and Fisheries Sciences Parasitology Laboratory and processed for use in this study. Any snails received from Lincoln Park Zoo that were not used in the experimental study were kept in a terrarium maintained on a diet of lettuce on damp soil in the laboratory. The progeny of these cultured snails were dissected at intervals to determine if cyclocoelid infections were being transferred from adults to their offspring.

A total of 63 snails were used during this study. Fifty three snails were examined and used for experimental infection and 10 were progeny that were examined later for evidence of infection from adult to offspring. The lengths of the snails were recorded, along with the type of digenean (cyclocoelid or dicrocoelid) infection present within each snail. Infection type was determined by the appearance of different life stages collected from the snails. Metacercaria and juvenile adults present in snails were observed under a microscope to determine if they were cyclocoelids or dicrocoelids by differences in physical appearance. Metacercaria of cyclocoelids are round, while those of dicrocoelids are oval. Also, cercaria of cyclocoelids are produced by redia, and these

rediae contain all larval stages. The life stages of cyclocoelids were measured, and major structures were documented.

Metacercaria are infective to the avian host, so this life stage was used to experimentally challenge chickens in the second part of this study. In order to collect metacercaria, snails were placed individually on a petri plate under a dissecting microscope so that the shell could be removed. Miracidia attached to the flesh of the snail were removed and opened to release the metacercaria. Once the metacercaria were identified and removed from the snail, they were placed in separate tubes with saline solution in order to create a parasite suspension. The metacercaria were suspended in 0.5mL normal saline at different doses: a low dose of 10, a mid-range dose of 100, and a high dose of 300. These suspensions were used to inoculate chickens in the second part of this study.

Figure 1 Snails of interest.

3.2 Experimental exposure and housing of domestic chickens

Twenty (20) one-day old male chickens were obtained from Hy-Line International in Bryan, Texas. These cockerels were hatched one day before inoculation of the cyclocoel parasite for this study. The birds were transported from the hatchery to the Texas A&M University Schubot Exotic Bird Health Center laboratory, where they were immediately inoculated with cyclocoelid parasite suspension. The chickens were divided into four groups of five animals each, with three groups (A-C) serving as experimental groups and one group serving as control (D). Because the parasite suspensions had been made the day before inoculation, 5-10 fresh metacercaria were taken from new snails and added to each suspension. The suspensions were drawn into a 1cc syringe, and a stainless steel oral gavage needle was attached. Administration of the suspension was by gavage into the proventriculus of fifteen chicks, each from one of the experimental groups. Group A was administered the low dose, Group B the mid-range dose, and three chickens of Group C the high dose. Two of the chickens in Group C received a mid-dose of dicrocoelid parasite solution. Chickens in Group D served as control and were administered 1cc of saline.

After inoculation, the chickens were transported to the housing aviary provided by the Texas A&M Schubot Center. Housing climate in the rooms was initially set at 34^{o}C with relative humidity between 40% and 60% as recommended by Hy-Line. These conditions were adjusted throughout the study to allow for normal chicken growth and development. Hanging wire cages were used to keep each group separate (Figure 2). All experimental groups were housed in one room of the aviary, while the control group was

housed in another room. Padding was added to the bottom of each cage for the comfort of the chickens. Cages for each group were swapped out for larger ones in order to accommodate for the chickens' growth. Food and water sources were freely available within each hanging cage. Each room was hosed out with water once a week in order to remove fecal material from the floor.

A colored band was placed on the left leg of each chicken for identification and for reference to recorded findings. Within each group, a bird got a different color: blue, yellow, purple, pink, and red. The colors were assigned a number 1-5 (blue = 1, yellow = 2, purple = 3, pink = 4, and red = 5) The bird identification number was created by combining the group letter with the color number (A1, B2, etc).

Figure 2 Hanging cage housing chickens.

3.3 Post-exposure observations and tests

Physical and behavioral changes were observed and recorded every other day. Any inactivity, breathing difficulty, abnormal feeding habits, or abnormal physical condition was recorded. Starting on Day 7 post-exposure, chickens were weighed once per week. Fresh fecal samples were taken from within the cage to decrease the chance of false negative test results from eggs drying out. Normal fecal sedimentation and fecal floatation procedures were used to determine presence of eggs. Physiological saline (0.85%) was added to tubes containing fecal samples from each group for sedimentation. The samples were broken up and strained into a new tube and allowed to form sediment at the bottom of the tubes. The top layer was removed, saline was added, and sediment formed at the bottom. This process was repeated until the top layer became clear. A few drops of sediment were added to a microscope slide, a coverslip was affixed on top, and the slide was examined under a microscope for fluke eggs. For floatation, fecal samples were added to a tube and $ZnSO_4$ (sp. gr. 1.18) was poured into the tubes to break up the samples. Cheesecloth was used to strain out large pieces of feces to form a suspension. The suspension was poured into a tube until it reached the top of the tube. A coverslip was placed on top of the tube. The tube was added to a balanced centrifuge and spun at 1,800rpm for five minutes. The coverslip was placed on a slide, and the slide was observed under a microscope for fluke eggs.

Blood collection began on Day 24, with 0.25mL of blood taken from each chicken. Blood was drawn every other week thereafter, for a total of four collections. Blood was taken from the right jugular vein using a 3cc syringe and a 25 gauge needle.

The samples were stored in a blood collection tube containing lithium heparin to prevent clotting, and were sent to the Clinical Pathology laboratory at Texas A&M University for complete blood count (CBC), including levels of fibrinogen (mg/dL). Fibrinogen is an acute phase reactant that serves as a marker for inflammation or tissue damage.

3.4 Necropsy and histology procedures

Based on the CBC levels and physical observations, eight chickens were selected for necropsy. Elevated fibrinogen levels (mg/dL), elevated eosinophil levels (absolute counts), and abnormal physical observations like poor comb growth and low body weight were used as selection criteria. Necropsies were performed between Day 67 and Day 82. The chickens were humanely euthanized per the Animal Use Protocol previously approved by Texas A&M University Institutional Animal Care and Use Committee. The chicken was given a body condition score, and normal avian necropsy procedures were followed. The body cavity and all organs were examined for juvenile worms and any abnormalities. The lungs, liver, kidneys, and eyes were removed and examined for evidence of migration or presence of parasite. Tissue samples of lung, liver, eye, and kidney were fixed to a slide, sectioned, and examined for histopathology. The entire digestive tract, trachea, lungs, brain, and eyes were placed in 10% formalin solution and sent to the Pathology laboratory. Tissue samples were stained for histopathology using Standard Operating Procedure 022 (SOP 022) of the TAMU Department of Veterinary Pathobiology. Samples were examined under a microscope after being stained with hematoxylin and eosin using standard H&E staining techniques.

4. RESULTS

4.1 Type of infection and life stages in snail host

All larval stages (rediae, cercariae, metacercariae, and juvenile adult) of *Szidatitrema* sp. were found to be present in 16 of the 53 (30%) snail hosts examined, suggesting that a second intermediate host where metacercaria would normally be found in most digenean life cycles is not required for exposure of the vertebrate definitive avian hosts by this species. Of the 53 snails, one was infected with only cyclocoelids (1.9%) and 29 were infected with only dicrocoelids (54.7%). There were 15 snails with infections of both cyclocoelid and dicrocoelid (28.3%), while seven were negative (13.2%) and one was dead (1.9%) (Table 1). About 30% of all snails dissected during this study were infected with cyclocoelids, including the snail infected with only cyclocoelids and all snails with double infections. All snails that were dissected had no evidence of sexual reproduction, as there were no eggs present.

Redial generations with developing cercaria were documented. The pharynx and cecum of the redia were easily distinguished. The free-swimming cercaria were also extracted from the snails. The tail and oral sucker were identified, and the ventral sucker was also seen. Many metacercaria were released from the snails. Each was clearly round, and an outer envelope was identified. Young adults were also extracted from the snails. The cyclocoel structure, merging of two ceca at the posterior end, that is distinctive of this parasite and the oral sucker were identified in the young adults.

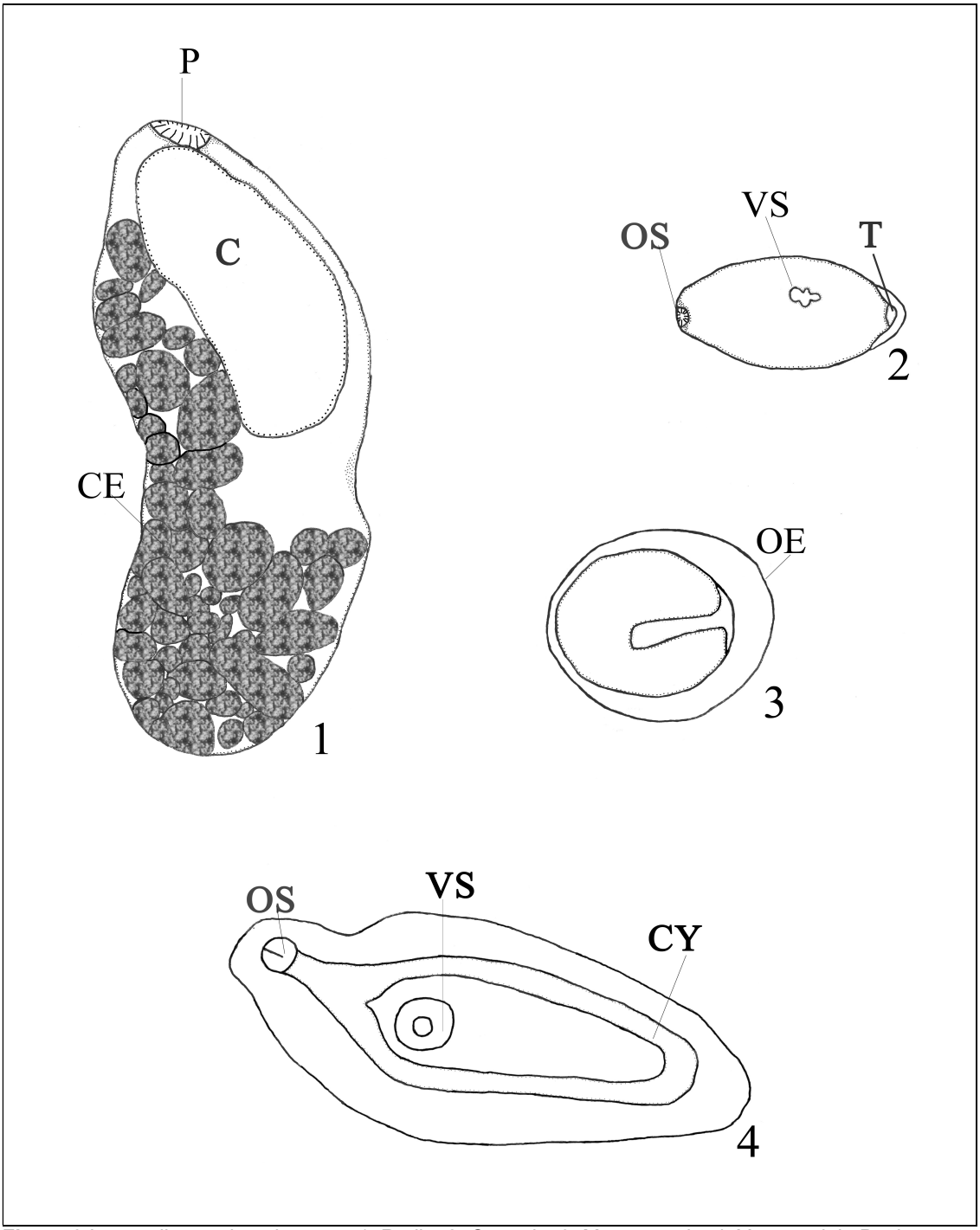

Figure 3 Intermolluscan larval stages. 1. Redia. 2. Cercaria. 3. Metacercaria. 4. Young adult. P: pharynx; C: cecum; CE: developing cercariae; OS: oral sucker; VS: ventral sucker; T: tail; OE: outer envelope; CY: cyclocoel.

Type of infection	Number of Snails
Cyclocoelid	1
Dicrocoelid	29
Both present	15
Negative	7
Dead	1

Table 1 Summary of infection types.

The average and range of the length of snails used for the study was 1.179cm and 0.9cm - 1.8cm, respectively. Table 2 lists the lengths of each snail along with the type of infection of that snail. Several snails not used in this study were kept in a terrarium with damp soil in order to determine if infection was passed from adult snails to offspring. Subsequent generations of these snails were dissected, and the flesh of the snails were extracted and examined for redial generations. The offspring of the snails that were dissected did not present evidence of cyclocoeil infection. All of these snails did have noted egg production, in contrast with the infected snails which were not producing eggs.

Snail length (cm)	Type of infection	Snail length (cm)	Type of infection
1.1	dicrocoelid	1.2	dicrocoelid
1.2	double	1.3	dicrocoelid
1.35	dicrocoelid	1.4	negative
1.5	dicrocoelid	1.2	dicrocoelid
1.25	double	1.3	dicrocoelid
1	dicrocoelid	1.4	dicrocoelid
1.35	dicrocoelid	1.3	dicrocoelid
1.2	dicrocoelid	1.3	double
1.2	dicrocoelid	1.2	double
1.05	dicrocoelid	1.2	double
1.05	negative	0.9	dicrocoelid
1.4	dicrocoelid	0.9	double
1.2	dicrocoelid	1.2	dicrocoelid
1.3	dicrocoelid	1	dicrocoelid
0.9	negative	0.9	dicrocoelid
1.2	double	1.1	double
1.05	dicrocoelid	1.2	dicrocoelid
1	cyclocoelid	1.4	dicrocoelid
0.9	double	1.3	double
1.05	dicrocoelid	1.4	double
0.08	negative	1.5	double
1.1	dicrocoelid	1.8	negative
1.4	negative	1.4	negative
1.3	double	1.4	dead
1.3	double	1.1	dicrocoelid
1.3	dicrocoelid	1.2	dicrocoelid
1.1	double		

Table 2 Snail lengths and infection type. Double denotes both cyclocoelid and dicrocoelid.

4.2 Experimental exposure of chickens

Fecal floatation and fecal sedimentation analyses did not indicate the presence of parasite eggs. Fibrinogen levels of all chickens, measured during the last two blood collections, did not indicate a difference between the control group and the experimental

group. Fibrinogen levels (mg/dL) for all groups are compared below in Figure 4. Levels marked as 0 indicate trace amounts of fibrinogen. There was insufficient data for eosinophil levels (absolute eosinophil count) due to sample clotting. Figures 5 through 8 indicate eosinophil levels for Groups A, B, C, and D, respectively. Levels marked as 0 indicate lack of result for that chicken due to clotting of the blood.

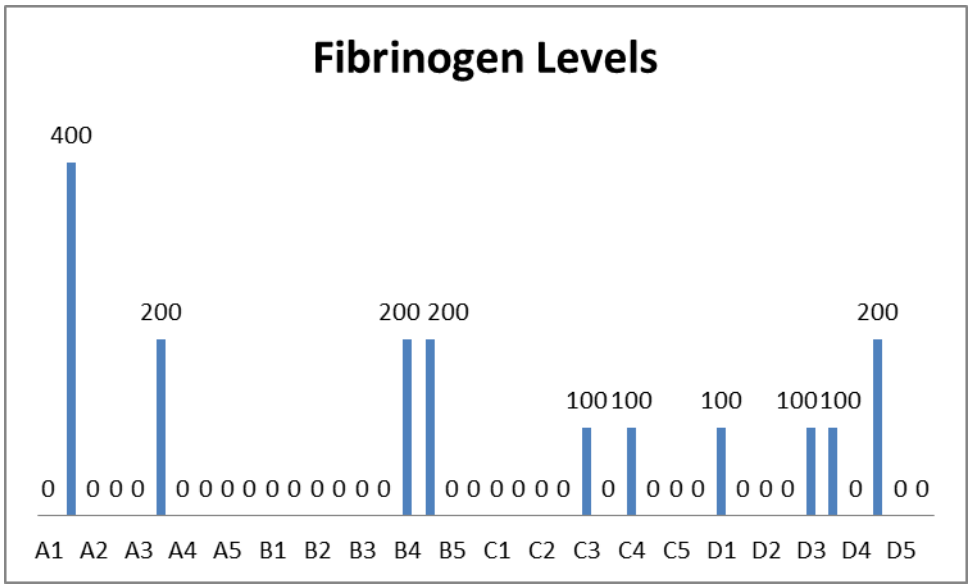

Figure 4 Fibrinogen levels. Two levels were measured for each chicken. '0' denotes trace amounts.

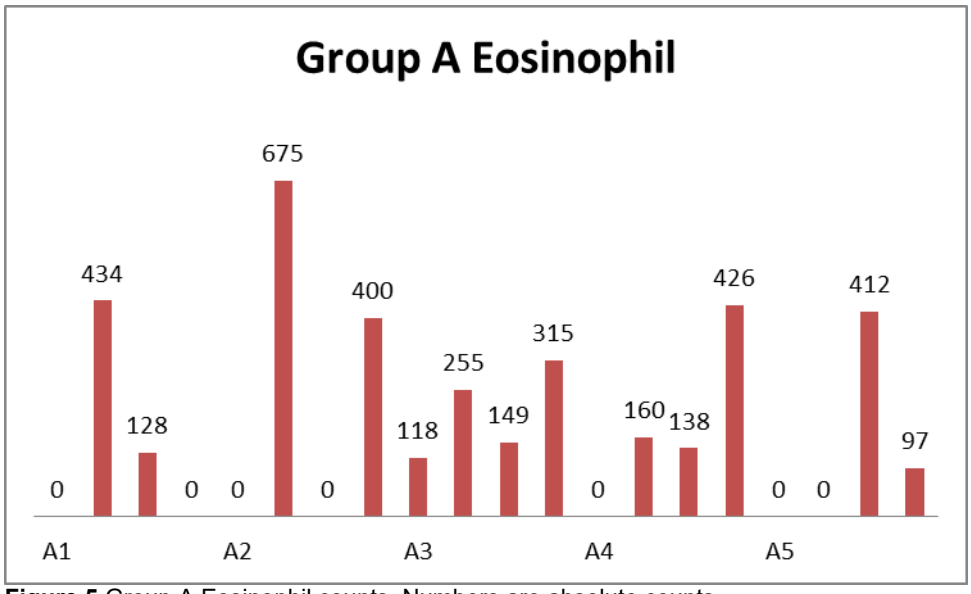

Figure 5 Group A Eosinophil counts. Numbers are absolute counts.

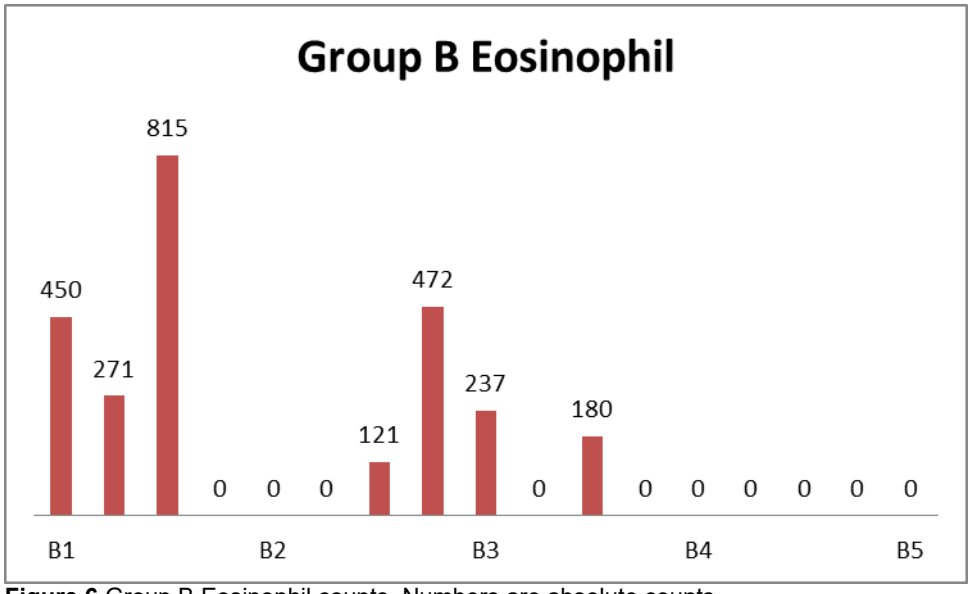

Figure 6 Group B Eosinophil counts. Numbers are absolute counts.

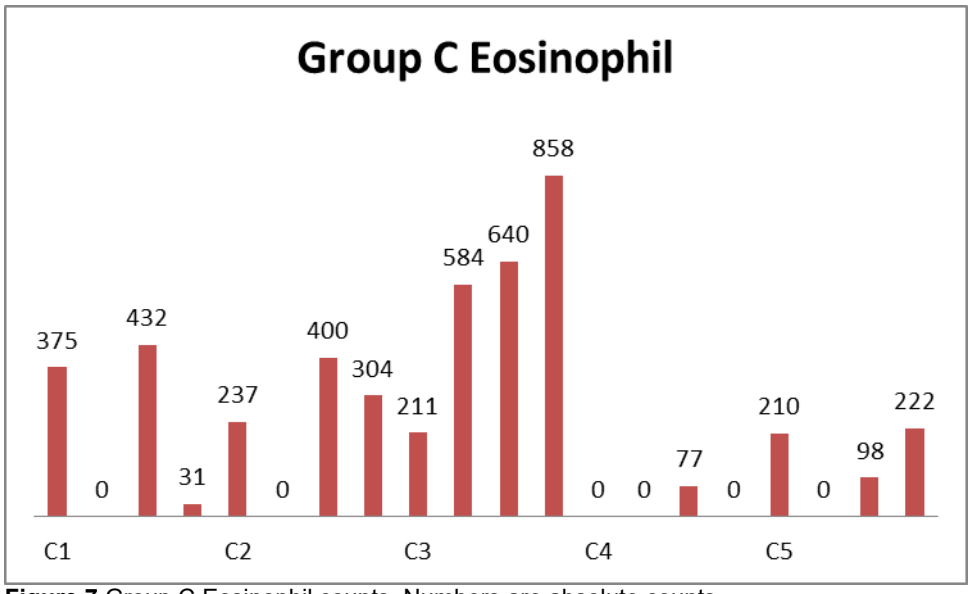

Figure 7 Group C Eosinophil counts. Numbers are absolute counts.

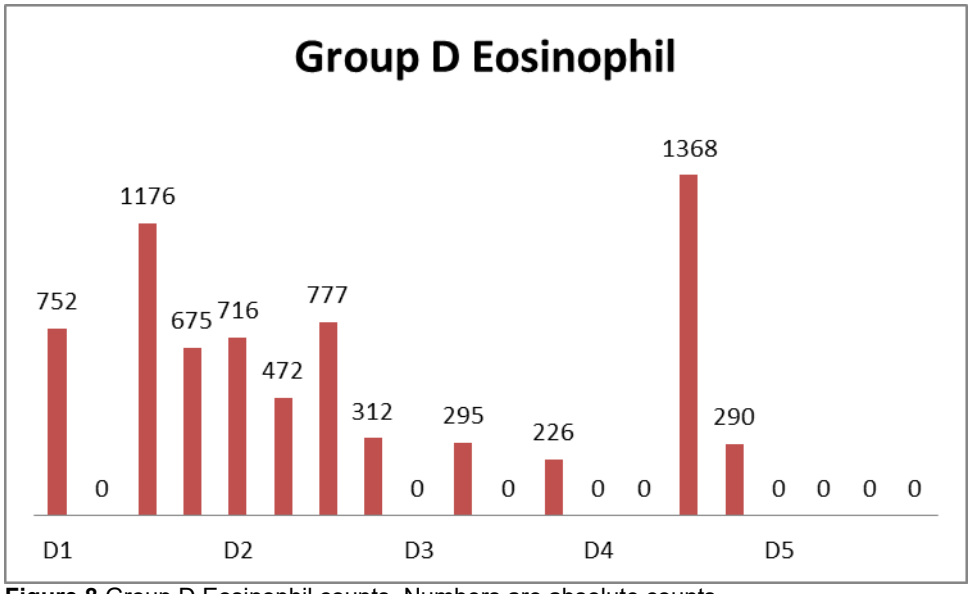

Figure 8 Group D Eosinophil counts. Numbers are absolute counts.

During the observation period, some physical differences were noted. On Day 7 post-inoculation, chickens in Group D (control) were somewhat more aggressive than those in Groups B and C (mid-range dose and high dose, respectively). Evidence of

molting was noted for all groups around Day 13. Aggressive behavior toward each other was noted for Group B on Day 17. On Day 33, Group D chickens were energetic and vocal, while chickens in all experimental groups were less active. Aggressive behavior among Group B was noted on Day 38. On Day 43, chicken B5 was noted to have an underdeveloped and pale comb, and chicken C5 had a somewhat underdeveloped comb. Physical underdevelopment of B5 was prominent throughout the study (Figure 9). All blood drawn from this chicken was noted to easily clot. On Days 46 and 48, Chicken A2 and Chicken C5 were also observed to have slower body and comb growth.

Figure 9 Chicken B5. Slow comb growth.

A list of chickens that were necropsied and results of those necropsies are summarized in Table 3. On initial examination during necropsy, no evidence of adult flukes was found in the body cavity of any chicken. Lungs and livers that were removed

from the body and examined appeared normal with regard to gross pathology. Figures 10 and 11 show necropsy findings of Chicken B5. Histopathological analyses did not indicate parasitic infection. No adult flukes or eggs were found by histopathology.

Chicken ID	Clinical History	Necropsy findings
A1	inoculated with low dose cyclocoeil solution	very fatty; ventriculus fatty; lungs pink; liver looks good; slightly enlarged heart
A4	inoculated with low dose cyclocoeil solution	small gonads; everything else looked normal
B4	inoculated with mid-dose cyclocoeil solution	large gonads; lungs pink
B5	inoculated with mid-dose cyclocoeil solution; underdeveloped, pale comb; blood tends to clot	dark, mottled lung; mottled liver; enlarged gall bladder
C1	inoculated with high dose cyclocoeil solution	everything looked normal
C3	inoculated with high dose cyclocoeil solution	lungs looked black, blood clots; liver looked good
C4	inoculated with dicrocoeil solution	everything looked normal
C5	inoculated with dicrocoeil solution; somewhat pale comb	none reported, looked ok

Table 3 Necropsy findings.

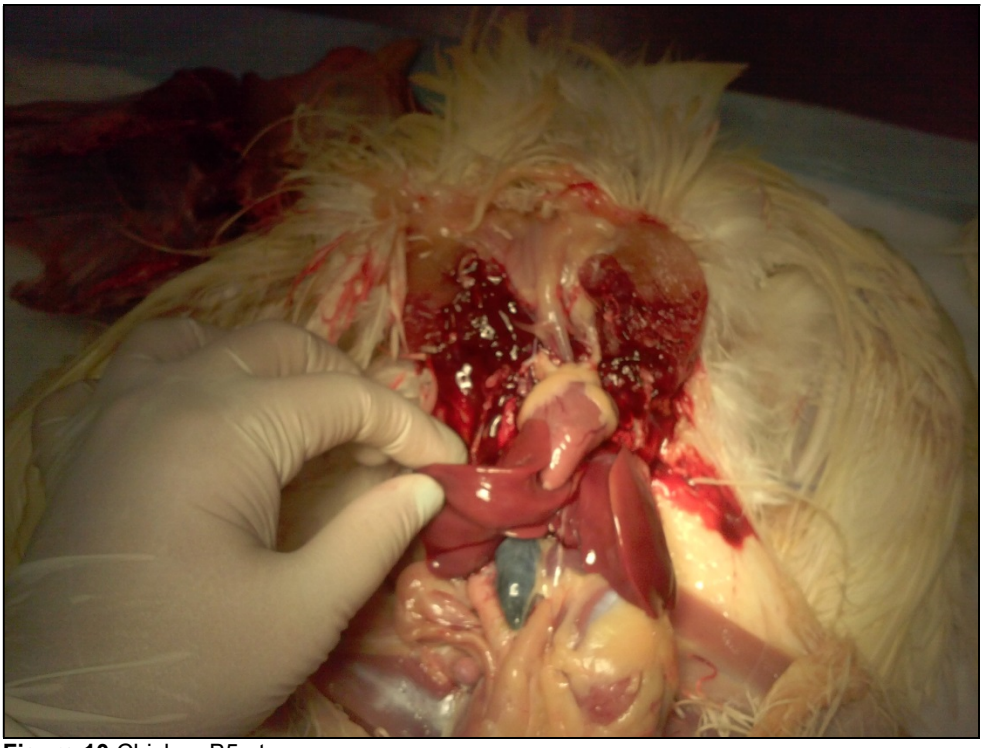

Figure 10 Chicken B5 at necropsy.

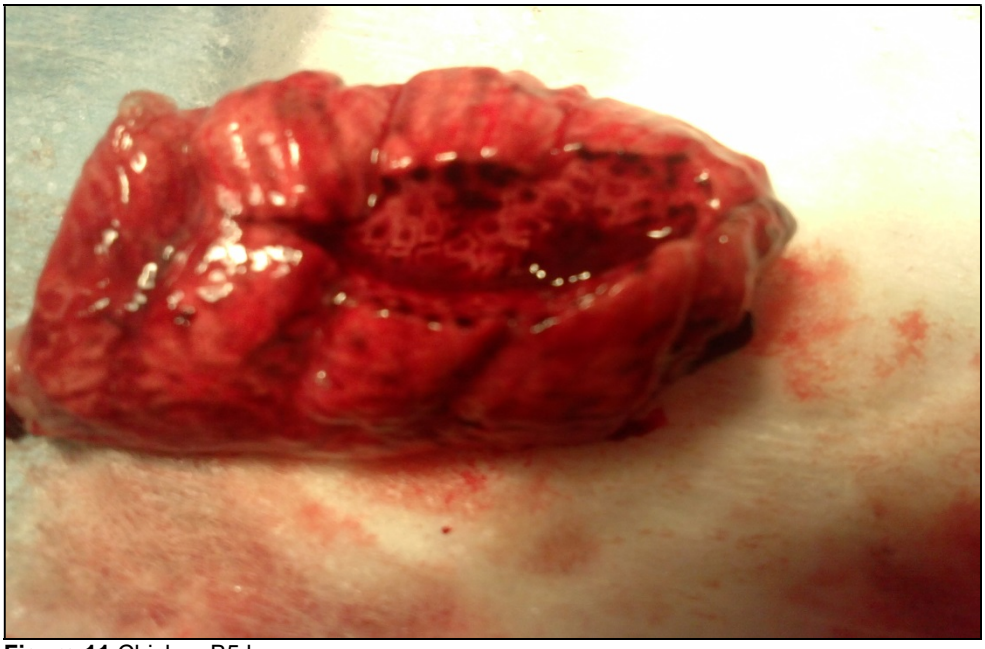

Figure 11 Chicken B5 lung.

5. SUMMARY

5.1 Conclusion

This study began with the examination of snails infected with cyclocoelid and dicrocoelid parasites. The two families of Digenea were distinguished from each other, and all larval stages of cyclocoelids found in the snails were identified. Each of the larval stages was seen in the snails, and the distinctive features of each were recorded. A majority of the snails used in the study were doubly infected, with only one being infected with only cyclocoel parasitic stages. About 30% of all snails dissected during this study were infected with cyclocoelids, including the snail infected with only cyclocoelids and all snails with double infections. The average snail length was measured as 1.179 cm, with a range from 0.9 cm to 1.8 cm. Subsequent generations of the snails kept in a terrarium did not present evidence of cyclocoeil infection. It appears that vertical transmission between infected snails and their progeny is not likely. It is probable that a snail must come into direct contact with the parasite in order for infection to occur.

In the second part of the study, we experimentally challenged domestic chickens with cyclocoelids collected from the snails in order to characterize the life cycle in an avian host. However, we did not obtain the information we anticipated. Fecal sampling did not indicate the presence of parasite eggs. Fibrinogen and eosinophil levels taken from blood collections throughout the study did not give indication of parasitic infection. In addition, adult flukes were not observed in the chicken carcasses. There were some

physical observations during the study and during necropsy that were questionable. However, we could not conclude that abnormal comb growth or abnormal livers were caused by the parasite. From these findings taken collectively, we concluded that domestic chickens are not a suitable host for this cyclocoelid parasite.

5.2 Discussion

Little is known about this species of parasite, as it is a newly recognized species. Zoos and bird-holding facilities have reported deaths of some birds infected with cyclocoelids even though the birds were quarantined before being placed in the facility. If the parasite cannot be contained and controlled, we may see a decline in many bird species that are kept at zoos and other bird-holding facilities. It is especially concerning if cyclocoelids infect threatened avian species that are part of breeding programs. Thus, it is important that the complete life cycle of this parasite is known in order to preserve the species at highest risk for extinction. In this experiment, we gathered information on the size of snails that are infected, what type of digenean infection each snail was carrying, and which life stages of the cyclocoel parasite we collected from the snails.

These results add to the knowledge of the life cycle of cyclocoel parasites. Most of the research that has been done on the life cycles of cyclocoelids has used the species that infect aquatic snails and bird hosts. In our study, we focused on gathering life stages of the parasite from terrestrial snails. We were successful in obtaining information on the larval stages of cyclocoels that mature within the snail intermediate host. We also challenged domestic chickens with the parasite, although this experiment was less

successful. From the second study, we concluded that chickens are not a suitable host for performing experimental exposures of cyclocoels for information on the parasite life cycle.

We were limited in the number of infected snails we received from Lincoln Park Zoo. Several factors can affect numbers of snails that can be recovered, including weather and disturbance of the soil in which they live. The snails that were recovered by the zoo were not guaranteed to be infected with the parasite in which we were interested. The only way to determine the type of infection of each snail is by dissection of the snail itself. We found some snails that were not infected or were not viable. This decreased the number of snails we had available to use for experimental exposure of chickens in the second part of the study. Also, we sought to establish a fluke infection comparable to those seen in naturally infected birds. However, we do not know the exact infectivity of this fluke and could not accurately determine the dose of metacercaria that is required to induce infection.

We were limited in the species of bird we could use in our study due to cost and accessibility. Our choice in model could have impacted the results of the study in several ways. We obtained domestic chickens from a local hatchery for use in this study because the species is easily obtained. Many domestic chicken species have been genetically altered for various reasons. Some chickens are altered to mature quickly in order to bring them to finishing phase within a few months of age. Free-range and backyard chickens that are not given antibiotics or anthelmintics are exposed to more parasites than those that are treated for parasitic infections. Since chickens have a higher incidence of being

exposed to pathogens from feeding off the ground, resistance to some diseases has become a survival mechanism. It is possible that chickens are not susceptible to this parasite because their immune system attacks the parasite before it can reproduce or do damage to the host. Additionally, it is possible that chickens may pass on pathogen resistance to their offspring. Different genetic factors can play a role in severity of infection, including species, gender, and age of the bird. All of these factors must be considered when designing an experiment.

Further research on this topic should focus on repeating the experimental infection study with monk parakeets. Testing different routes of exposure would be one adjustment to this study. Feeding whole snails instead of using gavage methods to expose the birds to metacercaria may provide a more natural route of infection. In addition, a secondary intermediate host other than a mollusk may be required to complete the life cycle of cyclocoel parasites. Exploring the possibility of another intermediate hosts, such as an ant species, may be considered. Once a successful infection has been achieved in a laboratory setting, vaccine and drug trials as well as improved detection methods may be of interest.

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