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Investigations into the Life Cycle of Drepanocephalus Auritus with Notes on the Discovery of a New Snail Host Biomphalaria Havanensis in Mississippi

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Investigations into the life cycle of *Drepanocephalus auritus* with notes on the discovery of a new snail host *Biomphalaria havanensis* in Mississippi

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Investigations into the life cycle of *Drepanocephalus auritus* with notes on the discovery of a new snail host *Biomphalaria havanensis* in Mississippi

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Drepanocephalus auritus is a digenetic trematode parasitizing the double-crested cormorant, a piscivorous waterbird often found feeding on catfish aquaculture ponds in Mississippi. The aquatic snail *Planorbella trivolvis* was the only known intermediate host in Mississippi until a new snail host, *Biomphalaria havanensis*, was discovered releasing cercariae belonging to North and South American *D. auritus* haplotypes. In addition, previous work has reported *D. auritus* metacercariae begin to resolve in channel catfish 7-21 days post-infection. As a result, a 2-year study was undertaken to elucidate the life cycle of *D. auritus* and identify if channel catfish can serve as a true intermediate host. In year 1, the role of the channel catfish as a true intermediate host was established, as gravid adults were recovered from double-crested cormorants fed parasitized fish. In year 2, each step of the life cycle was completed, and developmental timelines for each life stage were established.

DEDICATION

To my husband, Brad Alberson, who encouraged me to do my best and was always there for me on both the good and the bad days. I am so grateful for your love, support and encouragement. I could not have done this without you. And to my beautiful son Gatlin Gray Alberson, I love you to the moon and back. I hope you grow up to love science as much as I do.

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TABLE OF CONTENTS

APPENDIX

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Channel catfish aquaculture

The commercial production of channel catfish *Ictalurus punctatus* is an important industry in the United States, especially in the southeast. In 2016, sales exceeded $385 million nationally, with Mississippi, Alabama, Arkansas, and Texas making up 96 percent of total sales (USDA, 2017). Water surface acres, although down 4% from 2016, totaled just over 60 thousand acres nationally, with the previously mentioned states totaling over 42 thousand water surface acres (USDA, 2017). Catfish aquaculture in Mississippi alone accounted for over $213 million in sales in 2016 with over 34 thousand acres devoted to catfish aquaculture (USDA, 2017). The majority of commercial catfish operations in Mississippi are located in the Mississippi River Alluvial Valley, or Delta region, in the northwest portion of the state, and the Black Prairie region located in the east central region.

The first catfish ponds in Mississippi were constructed in 1957 but commercial production did not begin until 1965, expanding rapidly after 1970 (Jackson, 2004). This expansion brought with it an increase in piscivorous bird populations, with losses attributed to depredation and the introduction of digenetic trematodes harmful to channel catfish. Birds most commonly found foraging on catfish production ponds include the American white pelican *Pelecanus erythrorhynchos*, great blue heron *Ardea herodias*,

great egret *Ardea alba*, and double-crested cormorant *Phalacrocorax auritus* (Glahn and King, 2004). Aquatic snails also inhabit these ponds, frequently found in vegetation along the margins. Both the marsh rams-horn snail *Planorbella trivolvis* and the ghost rams-horn snail *Biomphalaria havanensis* have been identified as intermediate hosts to digenetic trematodes infective to channel catfish, including *Bolbophorus damnificus* and *Austrodiplostomum ostrowskiae* (Overstreet et al. 2002; Levy et al. 2002; Griffin et al. 2012; Rosser et al. 2016a; Rosser et al., 2016b).

1.2 *Drepanocephalus* species

Drepanocephalus spp. are a group of digenetic trematodes belonging to the family Echinostomatidae. Since the inception of the genus *Drepanocephalus* by Dietz in 1909, only four species have been reported from North and South America: *Drepanocephalus spathans, Drepanocephalus olivaceus, Drepanocephalus auritus,* and *Drepanocephalus* sp. Dietz (1909) described the type-species of the genus, *Drepanocephalus spathans,* from a single specimen recovered from a Neotropic cormorant *Phalacrocorax brasilianus* in Brazil. Specimens of *D. spathans* have also been reported from Neotropic cormorants in Venezuela (Caballero and Díaz-Ungría, 1958; Lutz, 1928; Monteiro et al., 2011), Argentina (Ostrowski de Núñez, 1968; Drago et al., 2011), Colombia (Rietschel and Werding, 1978), Paraguay (Kostadinova et al., 2002), and Texas, U.S.A. (Fedynich et al., 1997), and from the double-crested cormorant *Phalacrocorax auritus* in Florida, U.S.A. (Threlfall, 1982), Texas, U.S.A. (Fedynich et al., 1997), North Carolina, U.S.A. (Flowers, 2004), Mississippi, U.S.A (Doffitt, 2009; O'Hear et al., 2014; Sheehan et al., 2016b), Alabama, U.S.A., Vermont, U.S.A., Minnesota, U.S.A. (Sheehan et al., 2016b), and Canada (Robinson et al., 2008; Robinson et al., 2010; Wagner et al., 2012). The larval

metacercaria stage has been reported in the tropical cichlid *Cichlasoma urophthalmus* from the Yucatan Peninsula, Mexico (Salgado-Maldonado and Kennedy, 1997) while the larval cercaria stage was first reported from *P. trivolvis* snails in Mississippi (Griffin et al., 2012).

The second *Drepanocephalus* sp. to be described was *D. olivaceus,* reported by Nasir and Marvel (1968) infecting the Neotropic cormorant in Venezuela. Other specimens, also from Neotropic cormorants, have been reported in Mexico (Ramos, 1995; Violante-González et al., 2011), and Brazil (Montiero et al., 2011).

Drepanocephalus auritus was described by Kudlai et al. (2015), sending the taxonomic classification of *Drepanocephalus* spp. into flux. The species description was based on 12 specimens recovered from double-crested cormorants (DCCO) collected from North Dakota and Mississippi. Kudlai et al. (2015) suggested *D. spathans* was limited to South America and previous specimens collected from DCCO in North America were likely the newly described *D. auritus.* Since the erection of *D. auritus*, adult specimens have been recovered from DCCO in Mississippi, Alabama, Minnesota, and Vermont (Sheehan, et al., 2016a), while the larval cercaria stage has been observed from the aquatic snail *Biomphalaria straminea* in Brazil (Pinto et al., 2016). Molecular sequence data linked *D. auritus* to previous reports, suggesting isolates previously identified in North America as *D. spathans* were actually the newly described *D. auritus* (Griffin et al. 2012; 2014; Kudlai et al. 2015; Pinto et al. 2016).

There have been two reports of a *Drepanocephalus* sp. from Brazil. Pinto et al. (2014) recovered *Drepanocephalus* sp. metacercariae from wild Nile tilapia *Oreochromis*

niloticus, and *Drepanocephalus* sp. cercariae was reported being shed from *B. straminea* (Pinto et al., 2016).

1.2.1 *Drepanocephalus auritus* life cycle

Unfortunately, there have been limited studies on the life cycle of any *Drepanocephalus* spp., including *D. auritus*. It is speculated that Doffitt et al. (2009) incidentally infected a DCCO during a life cycle study to identify potential avian hosts for *Bolbophorus damnificus*, a digenetic trematode of great concern for catfish producers. Birds were captured and given a dose of praziquantel to clear any helminths. Following treatment with praziquantel, birds were fed channel catfish naturally infected with *B. damnificus* metacercariae from a commercial catfish farm. Birds were sacrificed 21 days post-infection and a single adult *D. auritus* (reported by Doffitt et al. (2009) as *D. spathans*) was recovered from the intestine of a DCCO. At the time, *D. spathans* had not been reported to infect channel catfish. Researchers speculated that either the praziquantel dose was less effective against *D. spathans*, or *D. spathans* was able to infect channel catfish, and birds obtained the infection from ingesting naturally infected fish used in the study.

Griffin et al. (2012) was the first to identify cercariae of *D. auritus* (reported as *D. spathans*) shed from *P. trivolvis* in Mississippi. During the same study, it was reported that cercariae could successfully penetrate and infect juvenile channel catfish, often causing mortalities within 7 days post-infection (dpi). Metacercariae were observed in the cranial region of surviving fish, with the highest concentrations being found within the branchial cavity. Metacercariae were also found in the oral cavity, esophagus, periocular tissues, and occasionally within bone. In 2014, Griffin et al. studied the longevity of *D.*

auritus metacercariae in juvenile channel catfish and found the infection was short-lived, resolving as early as 7-21 days post-infection, with metacercariae found mostly in the cranial region. While metacercariae were observed as late as 70 days post-challenge, these metacercariae were limited to within bone and other immune privileged sites, consistent with the previous infectivity study performed by Griffin et al. (2012).

During a malacological study in Brazil, Pinto et al. (2016) observed *B. straminea* shedding cercariae morphologically consistent to and molecularly confirmed as *D. auritus*. An experimental life cycle study was carried out using laboratory reared guppies *Poecilia reticulata* as the second intermediate host, which were then fed to laboratory maintained chicks *Gallus gallus domesticus* and mice *Mus musculus*. Metacercariae were observed in the oral cavity, on the caudal fins, and on the gills of guppies 7 days post-infection. However, when metacercariae were fed to chicks and mice, a patent infection was not achieved.

1.2.2 The double-crested cormorant host

The double-crested cormorant *Phalacrocorax auritus* is a piscivorous waterbird often found inhabiting coastal and inland waters of North America. It is a large bird, ranging between 70-90 cm in length, and weighing between 1.2-2.5 kg, with males being slightly larger than females (Dorr et al., 2014). There are five subspecies of double-crested cormorant (DCCO) and within these subspecies, there is sizeable variation in body size (Hatch and Weseloh, 1999). Adults are black or dark brown and plumage may or may not have a greenish or bronze gloss (Johnsgard, 1993; Hatch and Weseloh, 1999; Dorr et al., 2014). Ornamental feather tufts, making up the double-crest on the head in pre-breeding adults, are variable, and only seen for a short time (Johnsgard, 1993; Hatch

and Weseloh, 1999; Dorr et al., 2014). The facial skin and gular region is orange-yellow with a black or mottled hooked bill, while legs and webbed feet are black (Johnsgard et al., 1993).

There are six species of North American cormorants. Of these, DCCO are the most numerous and widely distributed (Hatch, 1995; Hatch and Weseloh, 1999; Dorr et al., 2014), with breeding ranges located in five regions comprising Alaska, the Pacific Coast (British Columbia to Mexico), interior regions of Canada and the United States (Great Lakes to the northern prairies), the Atlantic Coast (Newfoundland to New York), and southeastern portion of the United States, including the Gulf Coast (Texas to Florida) and the Atlantic Coast (Florida to the Carolinas), and the Caribbean (Bahamas and Cuba) (Dolbeer, 1991; Johnsgard, 1993; Hatch and Weseloh, 1999; Wires and Cuthbert, 2006; Dorr et al., 2014). Migratory patterns of DCCO are variable. Most birds breeding in the interior and Atlantic Coast are highly migratory, while those along the Pacific Coast are only slightly migratory. DCCO populations in Florida, Mexico, Cuba, and Bahamas are considered largely sedentary (Johnsgard, 1993; Hatch, 1995; Hatch and Weseloh, 1999; Dorr et al., 2014).

Migratory populations often over-winter in the southeastern United States and Mexico, and can also be found in Cuba, Puerto Rico, and the Bahamas (Hatch and Weseloh, 1999; Dorr et al., 2014). These populations often utilize the Atlantic Coast and the Mississippi Flyway as primary migration routes (Johnsgard, 1993; King et al., 2010).

While utilizing the Mississippi Flyway, a migration route following the Mississippi River, beginning in Canada and ending at the Gulf of Mexico, DCCO come into contact with catfish aquaculture ponds, serving as ideal wintering grounds for

migrants due to the regular availability of catfish. Over-wintering DCCO are often found foraging on catfish aquaculture ponds in the Delta and Black Prairie regions of Mississippi due to their proximity to the Mississippi Flyway, a bird migration route that generally follows the Mississippi River from the Gulf of Mexico to central Canada, bisecting the Midwestern United States (Stickley and Andrews, 1989; Johnsgard, 1993; King, 1996; Hatch and Weseloh, 1999; Dorr et al., 2004; Glahn and King, 2004; King et al., 2010). While foraging on catfish aquaculture ponds, DCCO can consume 227 g- 454 g of 10 cm-20 cm catfish daily (Stickley, 1990; Glahn et al., 1995), with an average catch rate of 5 catfish/hour (Stickley et al., 1992). Foraging by DCCO negatively impacts the commercial catfish aquaculture industry, and estimates of monetary losses are variable, ranging from $1.9 million to $3.3 million annually in the early 1990's to $4.8 million annually in the late 1990's (Stickley and Andrews, 1989; Glahn and Brugger, 1995; Glahn et al., 2000).

1.2.3 *Drepanocephalus auritus* North American mollusk hosts

Only two mollusk intermediate hosts are known for *D. auritus*. In North America, the planorbid snails *Planorbella trivolvis* have been reported shedding cercariae of *D. auritus* (Griffin et al., 2012) while *Biomphalaria straminea* serves as a host in South America (Pinto et al., 2016). *Planorbella trivolvis* and *Biomphalaria havanensis* have been reported from commercial catfish aquaculture ponds in the southeastern United States (Levy et al., 2002; Overstreet and Curran, 2004; Flowers et al., 2005; Wise et al., 2006; Yost et al., 2009; Khoo et al., 2011; Griffin et al., 2012; Rosser et al., 2016a; Rosser et al., 2016b) often found inhabiting vegetation along the pond banks.

Planorbella trivolvis are a first intermediate host for several trematode species infective to channel catfish, including *Bolbophorus damnificus, Clinostomum marginatum*, and recently *D. auritus* (reported as *D. spathans*) (Venable et al., 2000; Levy et al., 2002; Overstreet et al., 2002; Hawke and Khoo, 2004; Overstreet and Curran, 2004; Flowers et al., 2005; Mitchell et al., 2007; Griffin et al., 2012). Likewise, the exotic snails *B. havanensis*, first reported inhabiting catfish ponds in the Mississippi Delta in 2009 (Yost et al., 2009), were also found inhabiting ponds in the Black Prairie region of Mississippi (Rosser et al., 2016a; Rosser et al., 2016b). In Mississippi catfish aquaculture ponds, *B. havanensis* have been reported as first intermediate hosts in the life cycles of *B. damnificus, Austrodiplostomum ostrowskiae, Tylodelphys* sp. and *Austrodiplostomum* sp. (Yost et al., 2009; Rosser et al., 2016a; Rosser et al., 2016b).

The type locality for *Biomphalaria havanensis* is Havana, Cuba. However, these snails are relatively uncommon in Cuba and more commonly distributed in Mexico and the southern United States (Yong et al., 1997). Populations have been reported in southern Florida, Louisiana, central, southern, and southwestern Texas (Malek, 1969) and Idaho (Bowler and Frest, 1992). They have also been reported in Puerto Rico, the Dominican Republic, and Venezuela (Malek, 1969; Yong et al., 2001). Currently there is some uncertainty to the *B. havanensis* nomenclature, as it is morphologically identical to *Biomphalaria obstructa* from Mexico (Yong et al., 1997; Yong et al., 2001), and it has been suggested that *B. obstructa* and *B. havanensis* are synonymous (Yong et al. 2001) and their susceptibility to *S. mansoni* infection remains unclear.

Biomphalaria spp. are of great importance due to their role as intermediate hosts of *Schistosoma mansoni* (Cram et al., 1945; Michaelson, 1976). It has been reported that

B. obstructa is refractory to *S. mansoni* infection (Basch, 1976), while some studies suggest *B. havanensis* may be susceptible (Cram et al., 1945; Basch, 1976). Brooks (1953) found that *B. havanensis* was susceptible to *S. mansoni* infection, however, larval death and resorption was observed in the snail host, hypothesizing that *B. havanensis* might not be a suitable host. At present, the identity of the *Biomphalaria* spp. present in catfish aquaculture in the southeastern United States has been the subject of debate, although deference has been given to *B. havanensis* over *B. obstructa.*

1.3 Snail control strategies in catfish aquaculture ponds

1.3.1 Chemical control

The most effective way to interrupt trematode life cycles in catfish aquaculture ponds is to eradicate the snail host. Treatments used to control snail populations in aquaculture ponds are either chemical or biological, with some treatments more effective than others. A copper sulfate-citric acid shoreline treatment, containing 589 g of copper sulfate mixed with 58.9 g of citric acid and applied along 10 m of pond shoreline in a 2 m swath, was shown effective at killing *P. trivolvis* when applied at temperatures between 26.5-28 C (Mitchell, 2002). Mitchell and Hobbs (2003) found using 589 g copper sulfate, applied along 10 m of shoreline in a 2 m swath, without citric acid was more effective at temperatures exceeding 21 C.

Whole pond application of copper sulfate is also an effective treatment against *P. trivolvis* populations, especially those that may be located outside the littoral zone. Wise et al. (2006) applied copper sulfate to 0.25 and 10.0 acre catfish ponds at a rate of 2.5 and 5.0 ppm and observed greater than 95% snail mortalities post-treatment, while snail mortality in untreated ponds failed to exceed 8.5%. Dead fish were observed in a single

0.25 acre pond that was treated with the high dose of copper sulfate, but no fish mortality was observed in the 10 acre ponds. These studies took place in the fall with diminished algal blooms and water temperatures between 20-23 C. Conversely, applying copper sulfate in this manner during the summer months, while algal blooms are dense, can lead to catastrophic oxygen depletion and should be applied with caution, largely as a treatment of last resort (Wise et al., 2006).

Hydrated lime, used dry or in a slurry, can be an effective treatment for the eradication of snail populations in catfish ponds as well. If applying dry, hydrated lime should be applied at a rate of 0.75 to 1 kg/m of pond bank and the treated area should be limited to 1 to 1.5 m from the margin of the pond (Wise et al., 2004). Slurried hydrated lime treatments are most effective against *P. trivolvis* at rates of 45 kg/30 m in a 1 m swath and 79 kg/30 m in a 2 m swath, with researchers observing snail mortalities of 97%-98% (Mitchell et al., 2007).

Other possible treatments include the use of natural molluscicides vulgarone B, from the plant *Artemisia douglasiana*, and *Erigeron speciosus* steam distillate. Studies have shown both products are lethal to *P. trivolvis*, however, it is not known how these products will affect catfish or mammals, and additional research is needed (Meepagala, et al., 2002; Meepagala et al., 2004). Recently tobacco dust, a waste product of the tobacco industry, was found effective at eliminating *P. trivolvis*, while not harming juvenile channel catfish (Kuhn et al., 2014). While promising, the logistical practicality of this treatment has not been established.

Venable et al. (2000) evaluated the use of common salt (NaCl) to increase pond salinity in order to control snail populations. Field trials were carried out in ponds

containing both catfish and snails to determine the effect salinity had on snail density. Laboratory experiments were also performed to determine if salinity affected the survival, reproduction and growth of *P. trivolvis*. During the field trials, very few, if any, snails were observed in ponds with a salinity of 2.5 ppt. It was also noted that juvenile snails were absent, meaning that a salinity of 2.5 ppt inhibits reproduction. In laboratory experiments, a salinity of 2.5 ppt had a negative effect on snail growth, survival, and reproduction, with egg cases observed, but failing to develop into juvenile snails. However, these salinities are nearly 25-30 times greater than what is commonly found in commercial aquaculture ponds in the southeastern United States.

1.3.2 Biological control

Along with chemical treatments, biological control methods employing black carp *Mylopharyngodon piceus* (Venable et al., 2000), redear sunfish *Lepomis microlophus*, and blue catfish *Ictalurus furcatus* (Ledford and Kelly, 2006) have been investigated. Black carp are mollusk-eating cyprinids used in Russia and Israel for mollusk control. Ponds averaging 4.5 ha, and having abundant populations of *P. trivolvis*, were stocked with 62 black carp fingerlings/ha. Six months after black carp were placed into ponds, it was noted that snail populations declined substantially compared to ponds that did not have black carp (Venable et al., 2000). Ledford and Kelly (2006) performed laboratory experiments comparing snail consumption between black carp, redear sunfish, and blue catfish. Sunfish, catfish, and carp were placed into individual aquaria containing 15 *P. trivolvis* snails for 48 hr, and then removed, euthanized, and stomach contents were examined. Black carp consumed the most snails (86%), while the redear sunfish consumed more than the blue catfish (47% and 27% respectively).

1.3.3 Other control methods

Ultrasound has also been evaluated as a means of controlling *P. trivolvis* populations in catfish aquaculture ponds. In 2002, Goodwiller and Chambers performed a series of experiments utilizing ultrasound as a source of high amplitude acoustics to kill snails. In laboratory tests, sonification killed 35% of snails instantly, and within 4 days, an extra 42% of the population had died. Experiments were conducted on snails housed in aquarium tanks and not aquaculture ponds containing catfish. While acoustics were shown to kill snails, it is not known how these acoustics affect catfish.

Although there are several control strategies for *P. trivolvis* in catfish aquaculture, it is unknown whether or not these treatments are effective against *B. havanensis*. Since *B. havanensis* populations have been found inhabiting catfish ponds in both the Mississippi Delta and Black Prairie regions, and can serve as intermediate hosts for *B. damnificus* and *A. ostrowskiae* (Yost et al., 2009; Rosser et al., 2016a; Rosser et al., 2016b), further studies investigating the effectiveness of treatments eliminating these snails in catfish ponds are warranted.

1.4 Objectives

Drepanocephalus auritus has been found to experimentally infect channel catfish, causing mortalities in juveniles within 7 days post-infection (Griffin et al., 2012; 2014), although the life cycle of this digenetic trematode has not been experimentally confirmed. The piscivorous double-crested cormorant serves as the definitive host, and is often observed feeding on catfish aquaculture ponds in the Delta and Black Prairie regions of Mississippi. *Planorbella trivolvis* has been identified as a first intermediate host in North America (Griffin et al., 2012), while a *Biomphalaria* sp. (*B. straminea*) has been reported

as a snail intermediate host in South America (Pinto et al., 2016). Other than channel catfish, second intermediate hosts in North America are unknown, while in South America *D. auritus* was shown to experimentally infect guppies (Pinto et al., 2016). Currently, there is little data concerning the timeline of the *D. auritus* life cycle. While it is unknown how natural infections affect channel catfish, given this trematode can cause mortalities experimentally, it is important to establish timelines associated with the *D. auritus* life cycle so adequate control measures can be implemented.

The goal of this research was to experimentally confirm the life cycle of *Drepanocephalus auritus* and investigate the biology of these life stages in different systems. The first objective was a malacological survey to identity other potential snail intermediate hosts of *D. auritus*. The second objective was to perform an initial experimental life cycle study examining whether or not double-crested cormorants can be infected by ingesting *D. auritus* parasitized channel catfish. Lastly, the third objective focused on completing the life cycle in each host, while establishing developmental timelines for all life stages.

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CHAPTER II

NORTH AND SOUTH AMERICAN HAPLOTYPES OF *DREPANOCEPHALUS AURITUS* (DIGENEA: ECHINOSTOMATIDAE) ARE RELEASED FROM *BIOMPHALARIA HAVANENSIS* (MOLLUSCA: PLANORBIDAE) INHABITING CATFISH AQUACULTURE PONDS IN MISSISSIPPI, U.S.A.

2.1 Abstract

In July 2014, snails morphologically consistent with *Biomphalaria havanensis* were collected from 2 different catfish production ponds in the catfish farming region of eastern Mississippi. Phylogenetic analyses based on ITS1 and ITS2 fragments placed the snails within the *B. havanensis*/*obstructa* complex. Few snails shed cercariae morphologically and molecularly consistent with *Drepanocephalus* (formerly reported as *Drepanocephalus spathans*), an echinostomatid parasite of the double-crested cormorant *Phalacrocorax auritus* Lesson, 1831 and channel catfish *Ictalurus punctatus*. Juvenile channel catfish were challenged individually with ~275 cercariae of *D. auritus* per fish, and the presence of metacercariae was confirmed histologically. Parasite associated pathology was consistent with previous studies. Subsequent molecular analysis of five different gene targets confirmed morphological identifications. Previous research has suggested the existence of discrete North American and South American haplotypes of *D. auritus*. Both haplotypes were released by snails in this study.

2.2 Introduction

Digenetic trematodes play a significant role in disease related losses in catfish aquaculture in the southeastern United States (Overstreet and Curran, 2004). The trematode *Bolbophorus damnificus* Overstreet, Curran, Pote, King, Blend & Grater, 2002 has been linked to considerable production losses since its discovery in the late 1990s (Levy et al., 2002; Overstreet et al., 2002; Terhune et al., 2002). Even at low levels of infection, the presence of digeneans on commercial catfish operations can significantly reduce profitability (Wise et al., 2008).

Due to the regular availability of a confined food source, these ponds make ideal feeding and loafing grounds for many species of piscivorous birds, including the great egret *Ardea alba* Linnaeus, 1758, the great blue heron *Ardea herodias* Linnaeus, 1758, the American white pelican *Pelecanus erythrorhynchos* Gmelin, 1789, and the double-crested cormorant *Phalacrocorax auritus* Lesson, 1831 (Glahn and King, 2004). The marsh rams-horn snail *Planorbella trivolvis* Say, 1817 is regularly found inhabiting vegetation along the banks of commercial ponds and can serve as an intermediate host for a variety of digenetic trematodes including *B. damnificus* and *Drepanocephalus auritus* Kudlai, Kostadinova, Pulis & Tkach, 2015 (Schmidt and Fried, 1997; Hoffman, 1999; Mitchell, 2002; Overstreet et al., 2002; Overstreet and Curran, 2004; Flowers et al., 2005; Klockars et al., 2007; Griffin et al., 2012).

In addition to *P. trivolvis,* both *Physella gyrina* Say, 1821 and *Biomphalaria havanensis* L. Pfeiffer, 1839 have been reported from commercial aquaculture ponds in the southeastern United States (Overstreet and Curran, 2004; Wise et al., 2006; Yost et al., 2009). Increasingly, *B. havanensis* has been found inhabiting commercial catfish

ponds in the Black Prairie region of east Mississippi. Previous research has indicated *B. havanensis* can host the digenetic trematode *B. damnificus* (Yost et al., 2009; Rosser, et al., 2016b). Similarly, *Biomphalaria straminea* Dunker, 1848 has been identified as a first intermediate host for *Drepanocephalus* Dietz, 1909 in Brazil (Pinto et al., 2016). Cercariae of both *B. damnificus* and *D. auritus* have been shown infective to channel catfish (Levy et al., 2002; Griffin et al., 2012).

There has been some debate into the classification of *Biomphalaria* Preston, 1910 inhabiting the Gulf States of the southeastern United States. Previous reports of *B. havanensis* from catfish aquaculture have relied on morphology alone. Herein, we supplement morphological data with molecular and phenotypic data to offer a more comprehensive record of these putative emergent pests.

Moreover, with the recent description of *D. auritus* (Kudlai et al., 2015), the taxonomy of *Drepanocephalus* spp. in North and South America has recently undergone reorganization. Based on molecular sequence data, parasites reported as *Drepanocephalus spathans* Dietz, 1909 during surveys of digenetic trematodes in double-crested cormorants (O'Hear et al., 2014) and transmission experiments in juvenile channel catfish (Griffin et al., 2012; 2014) were actually the newly described *D. auritus* (Kudlai et al., 2015; Pinto et al., 2016). As a result, the host and geographic range of *D. spathans* is now uncertain.

The risks associated with large populations of *P. trivolvis* on catfish production systems have been well documented (Wise et al., 2004; 2008; 2013), however until recently, *Biomphalaria* spp. have been pests of minimal concern. The purpose of this study was to provide a more comprehensive record of *B. havanensis* recovered from

commercial catfish ponds in east Mississippi and to document their role in the transmission of *D. auritus* to channel catfish.

2.3 Materials and methods

2.3.1 Snail collection and identification

In July 2014, *B. havanensis* (n=1,740) were collected from two commercial catfish ponds (Pond C and Pond W) on separate aquaculture operations in Noxubee County, Mississippi (33°9'39.78"N; 88°29'33.63"W and 33°8'41.78"N; 88°26'42.09"W, respectively). Snails were transported live to the Mississippi State University College of Veterinary Medicine for identification and observation. Subsamples of these snails (4 from Pond C) were shipped to the University of North Alabama and identified based on morphological characteristics according to Burch (1989).

A separate subsample of snails (37 from Pond C; 35 from Pond W) was shipped to the University of New Mexico for phenotypic and molecular characterization. Genomic DNA (gDNA) was extracted from 20 *B. havanensis* collected in Mississippi using the E.Z.N.A. Mollusc DNA Kit (Omega Biotek, Norcross, Georgia) following the manufacturer's suggested protocol. Five out of 20 *B. havanensis* were infected with *D. auritus*. The remaining snails were negative as determined by shedding and dissection.

A large fragment (~1500 bp) of nuclear DNA containing partial 18S-ITS1-5.8S-ITS2-28S regions was amplified using ITS1-S and BD2 primers (DeJong et al., 2001). Amplifications were performed in 25-µl reactions consisting of 2.5 µl of 10X High Fidelity PCR buffer, 1 µl of 50 mM $MgSO_4$, 0.5 µl of dNTP mix, 5 pmols of each primer, 1U of Platinum *Taq* DNA Polymerase High Fidelity (ThermoFisher Scientific, Waltham, Massachusetts), 100-250 ng of template DNA and nuclease-free water to volume. The

amplification conditions were as follows: denaturation at 94 C for 2 min; 30 cycles at 94 C for 30 sec, 48.5 C for 30 sec and 68 C for 30 sec; and a final extension of 72 C for 7 min. Amplicons were visualized on 1% agarose gels containing GelRed nucleic acid dye (Biotium, Hayward, California) and purified using ExoSAP-IT (Affymetrix, Santa Clara, California). ITS1 and ITS2 were sequenced separately from the purified amplicon using the primer ITS1-S in combination with 5.8S-AS to sequence 18S-ITS1-5.8S, and 3SN in combination with BD2 to sequence 5.8S-ITS2-28S. Sequencing was performed on an Applied Biosystems 3130xl Genetic Analyzer (BigDye Terminator v3.1 Cycle Sequencing Kit, ABI, Grand Island, New York). Individual sequencing reads were aligned in Sequencher 5.0 (Gene Codes, Ann Arbor, Michigan) to generate contiguous sequences for each isolate. All primer sequences used for amplification and sequencing are listed in Table 2.1.

Table 2.1 Primers used to amplify the ITS1-5.8S-ITS2 cluster of the rRNA gene for *Biomphalaria* spp.

Primer	Sequence (5' to 3')	Target	Reference
ITS1-S	CCATGAACGAGGAATTCCCAG	ITS1	DeJong et al., 2001
BD2	TATGCTTAAATTCAGCGGGT	ITS2	DeJong et al., 2001
5.8S-AS	TTAGCAAACCGACCCTCAGAC	ITS1	DeJong et al., 2001
3SN	GCGTCGATGAAGAGCGCAGC	ITS2	DeJong et al., 2001

Phylogenetic analysis of 18S-ITS1-5.8S-ITS2-28S sequences were performed in MEGA6 (Tamura et al., 2013). Representative sequences from *Biomphalaria* spp. were downloaded from the National Center for Biotechnology Information non-redundant nucleotide (NCBI nr/nt) database and aligned with CLUSTALW v.2 (Larkin et al., 2007). Uninformative regions of ITS1 and ITS2, identified in DeJong et al. (2001), were

excluded from phylogenetic analyses. A total of 24 different nucleotide substitution models were evaluated and the best fit model was selected based on the lowest Bayesian Information Criterion score. Phylogenetic analysis was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980), a Gamma distribution (+*G*) and 50.26% of sites were allowed to be evolutionarily invariable (+*I*).

Susceptibility of *B. havanensis* to *Schistosoma mansoni* Sambon, 1907 was determined by exposing juvenile snails to the neotropical PR1 laboratory strain of *S. mansoni.* Twenty-four snails from catfish pond C were individually exposed to 10 miracidia of *S. mansoni* for 6 hr in 12-well culture plates containing artificial spring water. Four snails (2 at 2 days post-challenge and 2 at 21 days post-challenge) were dissected for observation of intramolluscan stages of *S. mansoni* and tested for the presence of *S. mansoni* ND5 mitochondrial genes by PCR (Lu et al., 2016). Remaining snails were kept in aquaria and fed red leaf lettuce *ad libitum* until 28 days post-challenge when snails were exposed to a light source for 2 hr to induce shedding of cercariae and signify patent infection with *S. mansoni*.

2.3.2 Identification of *Drepanocephalus auritus* cercariae

Individual snails were rinsed with reverse osmosis water and placed into 20-ml plastic vials (Diluvials, Elkay Laboratory Products, Hampshire, United Kingdom) containing 10 ml of autoclaved spring water (Ozarka, Wilkes Barre, Pennsylvania). Snails were kept at ambient temperatures (~25-27 C) for 48 hr and examined daily for the presence of cercariae morphologically consistent with *D. auritus* (Griffin et al., 2012; Pinto et al., 2016) using a stereomicroscope (Olympus SZ60, Olympus Optical Co., Ltd., Tokyo, Japan). Pools of cercariae from each infected snail were placed separately into 2-

ml microcentrifuge tubes and archived in 70% molecular grade ethanol (Sigma Aldrich, St. Louis, Missouri) for molecular analysis at a later date.

For each snail, approximately 10 individual cercariae were isolated from each archived pool and washed 3 times with molecular grade, nuclease-free water to remove residual ethanol. Genomic DNA was extracted from cercariae using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, California). Photomicrographs of ethanol fixed cercariae (n=14) were obtained using an Olympus BX-50 microscope (Olympus Optical Co., Ld., Tokyo Japan) equipped with an Olympus DP72 camera and DP2-TWAIN–cellSens software package (Olympus Optical Co., Tokyo, Japan) and measurements were obtained from representative images (Table 2.2)

Table 2.2 Measurements of *Drepanocephalus auritus* cercariae

		Drepanocephalus auritus		*Drepanocephalus spathans* (now *D. auritus*)	*Drepanocephalus* sp.
		Present study	Pinto et al. 2016	Griffin et al. 2012	Pinto et al. 2016
Host		*Biomphalaria havanensis*	*Biomphalaria straminea*	*Planorbella trivolvis*	*B. straminea*
Location		U.S.A.	Brazil	U.S.A.	Brazil
Fixation		Ethanol	Hot formalin	Ethanol	Hot formalin
Body	*L*	332±11 (315-355)	339±15 (314-362)	246 (234-266)	317±10 (307-334)
	W	120±7 (106-128)	171±4 (164-177)	110 (95-123)	146±8 (137-164)
Tail	*L*	440±34 (368-474)	539±16 (512-573)	556 (490-600)	482±7 (471-498)
	W	37±2 (33-41)	53±3 (48-60)	60 (54-68)	49±2 (45-53)
Oral sucker	*L*	41±3 (34-45)	42±2 (40-45)	–	42±1 (40-45)
	W	46±2 (42-48)	39±2 (37-42)	12 (11-13)	39±2 (47-62)
Ventral sucker	*L*	64±5 (54-70)	53±3 (50-58)	–	52±2 (48-58)
	W	68±9 (53-83)	58±5 (47-67)	43 (35-50)	55±3 (50-58)

*Measurements are in micrometers.
†The mean is followed by standard deviation with ranges in parentheses.
‡*L* length, *W* width.

PCR amplification and sequencing of cercariae rRNA (18S-ITS1-5.8S-ITS2-28S), cytochrome *c* oxidase subunit 1 (CO1), and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) gene targets were performed using primers listed in Table 2.3. Amplification was carried out in 25-µl reactions consisting of 22 µl Invitrogen™ Platinum® PCR Supermix High Fidelity (Life Technologies, Carlsbad, California), 20 pmols of each primer, and 1 µl of template DNA. The cycling conditions for 1F/5R primers consisted of an initial denaturation of 3 min at 94 C followed by 45

cycles of 94 C for 30 sec, 45 C for 30 sec, and 68 C for 1 min 30 sec. Cycling conditions for the Barker 3/Barker 4 primer set also incorporated a 3 min denaturation step at 94 C followed by 45 cycles of 94 C for 30 sec, 50 C for 30 sec, and 68 C for 1 min. Drep1579F/Drep2626R, Drep2456F/Drep3470R, and DrepCOI275F/DrepCOI887R primer sets used the same protocol as Barker3/Barker4 except annealing temperature was increased to 55 C for 30 sec, while cox1_schist5'/acox650r, NDJ11/ NDJ2a, LSU5/1500R, BD1/BD2, and JB3/JB13 primer sets employed an annealing temperature of 45 C for 30 sec, and the cox11/cox820 primer set employed an annealing temperature of 48 C for 30 sec followed by 68 C for 2 min. Amplification products were visualized after electrophoretic migration through a 1.2% agarose gel containing ethidium bromide (0.5 µg/ml) and either purified directly (QIAquick PCR Purification Kit; QIAGEN) or by gel extraction (QIAquick Gel Extraction Kit; QIAGEN). These purified amplification products were sequenced directly with primers used in the initial amplification (Eurofins MWG Operon, Huntsville, Alabama). Contiguous sequences were assembled using the SeqMan™ utility of the Lasergene software package (DNAStar, Madison, Wisconsin) and ambiguous base calls were annotated manually. Sequences generated for each isolate were compared to closely related trematode sequences available in the NCBI nr/nt database using the BLASTn utility (Altschul et al., 1990). Gene sequences generated in this study were deposited in the NCBI nr/nt database (Accession numbers KY677939-KY677977).

Table 2.3 Primers used to amplify ribosomal and mitochondrial genes of *Drepanocephalus auritus* cercariae

Primer	Sequence (5' to 3')	Target	Reference
1F	TACCTGGTTGATCCTGCCAGTAG	18S	Carranza et al., 1997
5R	CTTGGCAAATGCTTTCGC	18S	Carranza et al., 1997
Barker 3	TTAGAGTGTTCAAAGCAG	18S	Barker et al., 1993
Barker 4	GATCCTTCTGCAGGTTCACCTAC	18S	Barker et al., 1993
LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	28S	Littlewood et al., 2000
1500R	GCTATCCTGAGGGAAACTTCG	28S	Littlewood et al., 2000
BD1	GTCGTAACAAGGTTTCCCGTA	ITS	Morgan and Blair, 1995
BD2	TATGCTTAAATTCAGCGGGT	ITS	Morgan and Blair, 1995
NDJ11	AGATTCGTAAGGGGCCTAATA	ND1	Kostadinova et al., 2003
NDJ2a	CTTCAGCCTCAGCATAAT	ND1	Kostadinova et al., 2003
Drep1579F	GTCGCACGAAATTGAGCA	18S-ITS gap closure	This paper
Drep2626R	ACCCAAGCCACGACTATTTG	18S-ITS gap closure	This paper
Drep2456F	CTCGTGTGTCGATGAAGA	ITS-28S gap closure	This paper
Drep3470R	CTCCACCCGTTTACCTCTGA	ITS-28S gap closure	This paper
DrepCOI275F	CCTGCYTCGTTAAGGTTAGGG	CO1	This paper
DrepCOI887R	TCACCATCGTCACCGAACTA	CO1	This paper
cox1_schist5'	TCTTTRGATCATAAGCG	CO1	Lockyer et al., 2003
acox650r	CCAAAAAACCAAAACATATGCTG	CO1	Kudlai et al., 2015
cox11	CACCTTAATACCCGTCGGAAT	CO1	Pinto et al., 2016
cox820	AATATTATTTCCCCGGRAGTYT	CO1	Pinto et al., 2016
JB3	TTTTTTGGGCATCCTGAGGTTTAT	CO1	Bowles et al., 1995
JB13	TCATGAAAACACCTTAATACC	CO1	Morgan and Blair, 1998a

Phylogenetic analysis of *D. auritus* was based on CO1 sequences. Respective CO1 sequences from each isolate were compared to similar echinostomatid trematode sequences deposited in the NCBI nr/nt database. The 8 most closely related sequences identified using a BLASTn search were obtained and aligned (CLUSTALW) in MEGA6 (Tamura et al., 2013). *Isthmiophora hortensis* Asada, 1926 was chosen as an outgroup. As above, the best fit nucleotide substitution model was selected based on the lowest Bayesian Information Criterion score. Phylogenetic relationships were inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with bootstrap values obtained by 1000 replicates. Pairwise distances were calculated using MEGA6 based on the alignment of *Drepanocephalus* spp. used in constructing the phylogenetic tree.

2.3.3 Experimental fish challenge

Cercariae shed by *B. havanensis* from Pond C, morphologically consistent with *D. auritus,* were collected and pooled in 75 ml of autoclaved spring water (Ozarka) and gently stirred with a magnetic stir bar. Ten 100-µl aliquots were collected and the number of cercariae in each aliquot was counted using a stereomicroscope to approximate the number of cercariae per ml of inoculum. Parasite-free channel catfish fingerlings (~3-5 cm), reared for disease research at Thad Cochran National Warmwater Aquaculture Center in Stoneville, Mississippi, were used in experimental infections. Ten catfish were placed individually into 1-l plastic containers containing 300 ml dechlorinated municipal water with constant aeration. Individual fish were exposed to inocula of ~275 cercariae for 4 hr. Ten control fish were treated similarly, but were not exposed to any cercariae. At the end of the challenge, water from each container was decanted and fish were washed

with dechlorinated municipal water 3 times before being placed into recirculating aquaria and housed at ambient temperatures (~25-27 C) with constant aeration. All fish were euthanized 7 days post-challenge with an overdose of tricaine methanesulfonate (Western Chemical, Ferndale, Washington). The coelomic cavity was opened by sharp incision, and fish were fixed in 10% neutral buffered formalin, and archived for later histological analysis.

2.3.4 Histology

All fish were initially fixed in 10% neutral buffered formalin for at least 24 hr. Tissues were decalcified in Cal-Ex decalcifier (ThermoFisher Scientific, Fair Lawn, New Jersey) until the calcified portions were pliable upon digital manipulation. Decalcified tissues were rinsed in running water for a minimum of 3 hr before trimming. Fish were trimmed in ~5 mm cross sections starting rostrally and moving caudally, with small adjustments to thickness to ensure all major organs were sampled. Thinner sections were made for the head region to ensure multiple sections through the branchial chamber, identified in previous experiments as a predilection site for metacercariae of *D. auritus* (Griffin et al., 2012; 2014). Tissues were processed routinely, embedded in paraffin, sectioned at ~4 µm, stained with hematoxylin and eosin (H&E), and examined using light microscopy. Tissue sections were examined with a BX-50 Olympus microscope (Olympus Optical Co., Ltd), and representative images were captured with an Olympus DP72 camera and DP2-TWAIN–cellSens software (Olympus Optical Co., Ltd.).

2.4 Results

2.4.1 Snail identification and sequencing analysis

A total of 804 *Biomphalaria* sp. were collected from Pond C and 936 *Biomphalaria* sp. were collected from Pond W. Snails were morphologically identified as *B. havanensis,* which was supported by phylogenetic analysis of ITS sequences, placing the snails within the *B. havanensis/obstructa* complex.

Biomphalaria havanensis from Ponds C and W were sequenced to confirm morphological identification, establish evolutionary relationships and provide baseline genetic data for future studies. Of the 20 snails examined, 5 unique 18S-ITS1-5.8S-ITS2 sequence types were established. The Alberson isolate 1 sequence type was obtained from 2 *B. havanensis* from Pond W, both of which were infected with *D. auritus*. Six *B. havanensis* from Pond W possessed the isolate 2 sequence, none of which were infected with *D. auritus.* Isolate 3 and 4 sequences represent 2 and 5 non-infected *B. havanensis* from Pond C, respectively. Lastly, the isolate 5 sequence was obtained from 2 *B. havanensis* infected with *D. auritus,* and 3 *B. havanensis* that were not infected, all of which were from Pond C. All 5 18S-ITS1-5.8S-ITS2 sequence types fell within the *B. havanensis/B. obstructa* complex (Figure 2.1).

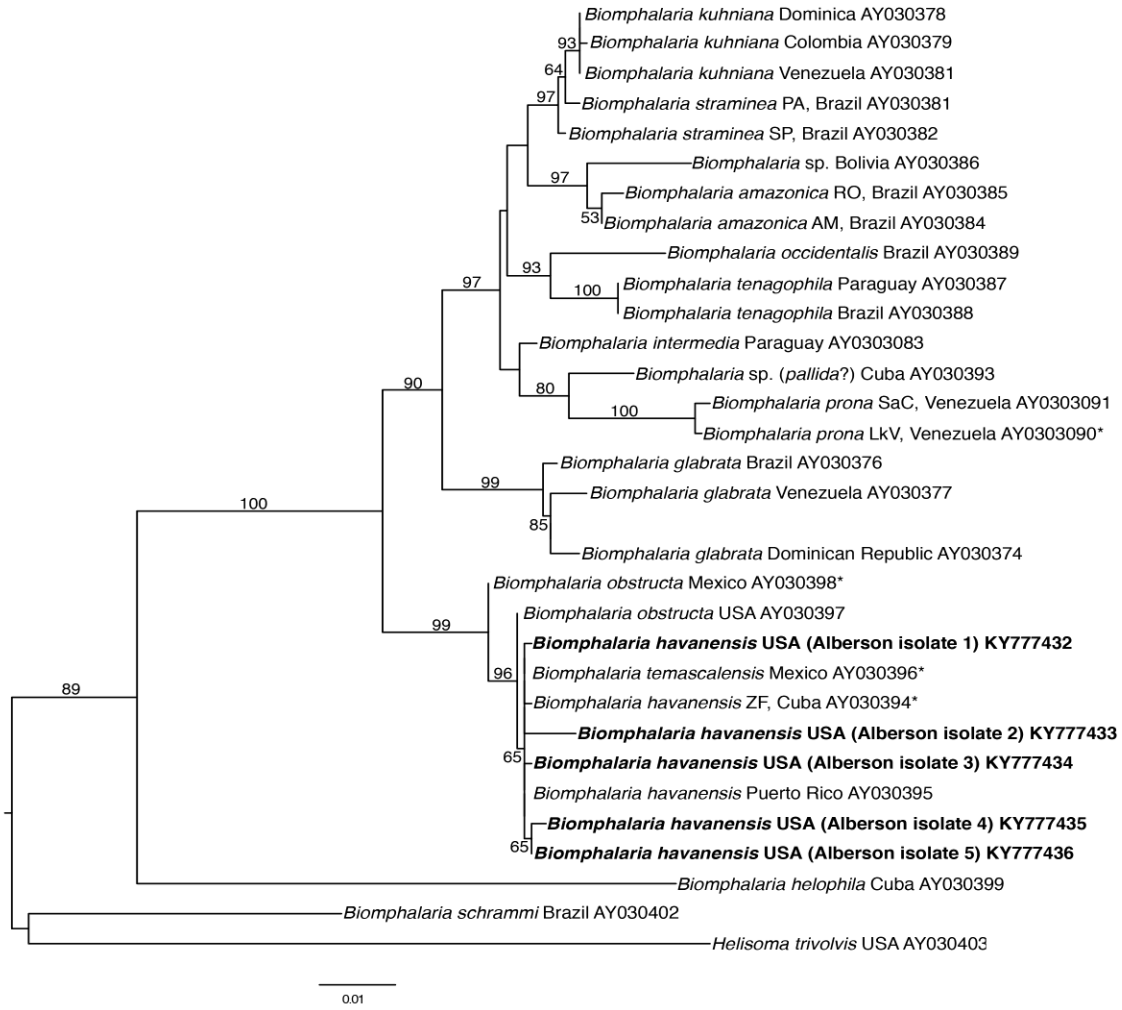

Figure 2.1 Maximum likelihood tree inferred from ITS1-5.8S rRNA, and ITS2 sequences.

Maximum likelihood tree inferred from ITS1-5.8S rRNA, and ITS2 sequences of isolates of *Biomphalaria havanensis* from Mississippi and published sequences from *Biomphalaria* spp. deposited into the NCBI nt/nr database. Isolates from this study are bolded. Specimen designations are in parentheses and GenBank accession numbers follow. Numbers above branches represent bootstrap values obtained by 1000 replicates. Taxa depicted by asterisks indicate specimens collected from that type locality.

Biomphalaria havanensis from catfish pond C were refractory to infection by *S. mansoni* in a laboratory setting. Successful penetration of *S. mansoni* was noted during exposures as miracidia disappeared from wells. At 2 days post-challenge to *S. mansoni*, 1

of 2 snails tested positive for *S. mansoni* DNA by PCR, while snails collected at 21 days post-challenge were conclusively negative. No snails were observed actively shedding cercariae of *S. mansoni*, suggesting the snails are able to eliminate the parasite after penetration thus preventing patent infection.

2.4.2 Cercariae identification and sequencing analysis

Eight (1.0%) *B. havanensis* from Pond C and 5 (0.5%) *B. havanensis* from Pond W were actively shedding cercariae morphologically consistent with *D. auritus* from previous studies (Griffin et al., 2012; 2014; Pinto et al., 2016). Digital measurements taken from 14 ethanol-fixed cercariae released from *B. havanensis* are listed in Table 2.2.

Sequencing of an 882 bp region of the CO1 gene matched gene sequences obtained from *D. auritus* specimens collected in North America and South America (Griffin et al., 2012; O'Hear et al., 2014; Kudlai et al., 2015; Van Steenkiste et al., 2015; Pinto et al., 2016). Eleven cercariae pools recovered from *B. havanensis* in Mississippi were consistent with a North American haplotype of *D. auritus* collected from *P. trivolvis* in Mississippi catfish aquaculture ponds (GenBank KR259644; Griffin et al., 2012; Pinto et al., 2016), sharing 99.5-100% nucleotide and 100% amino acid (a.a.) similarity with the North American haplotype. The remaining two cercariae pools shared 99.7% nucleotide (100% a.a.) similarity to a South American haplotype of *D. auritus* from *B. straminea* in Brazil (GenBank KP053255; Pinto et al., 2016). The intraspecific nucleotide variability of CO1 sequences for all isolates from *B. havanensis* recovered from catfish aquaculture ponds in Mississippi ranged from 0.0-3.1% (Table 2.4). Comparably, these isolates shared less than 90% (88.9-89.3%) nucleotide identity (99.3% a.a.) to an

unidentified *Drepanocephalus* sp. from *B. straminea* from Brazil (GenBank KP053256; Pinto et al., 2016) (Table 2.5).

Table 2.4 Pairwise similarity matrix of cytochrome *c* oxidase subunit 1 (CO1) nucleotide (bottom) and amino acid (top) sequences

Pairwise similarity matrix of cytochrome *c* oxidase gene													
Isolate	CD1	CD2	CD3	CD4	CD5	CD6	CD7	CD8	WD1	WD3	WD4	WD5	WD6
CD1	–	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
CD2	97.3%	–	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
CD3	96.9%	99.2%	–	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
CD4	97.8%	99.4%	99.1%	–	100%	100%	100%	100%	100%	100%	100%	100%	100%
CD5	97.5%	99.8%	99.4%	99.7%	–	100%	100%	100%	100%	100%	100%	100%	100%
CD6	97.4%	99.7%	99.4%	99.5%	99.9%	–	100%	100%	100%	100%	100%	100%	100%
CD7	97.4%	99.7%	99.5%	99.5%	99.9%	99.9%	–	100%	100%	100%	100%	100%	100%
CD8	97.4%	99.7%	99.5%	99.5%	99.9%	99.9%	100%	–	100%	100%	100%	100%	100%
WD1	97.4%	99.7%	99.5%	99.5%	99.9%	99.9%	100%	100%	–	100%	100%	100%	100%
WD3	97.4%	99.7%	99.5%	99.5%	99.9%	99.9%	100%	100%	100%	–	100%	100%	100%
WD4	100%	97.3%	96.9%	97.8%	97.5%	97.4%	97.4%	97.4%	97.3%	97.4%	–	100%	100%
WD5	97.4%	99.7%	99.5%	99.5%	99.9%	99.9%	100%	100%	100%	100%	97.4%	–	100%
WD6	97.3%	99.5%	99.4%	99.4%	99.8%	99.8%	99.9%	99.9%	99.9%	99.9%	97.3%	99.9%	–

Table 2.5 Sequence similarity of cytochrome *c* oxidase subunit 1 (CO1) gene

	GenBank Accession Number					
Isolate	KP053255*	KR259644†	KP683125‡	KM538090§	JX468067‖	KP053256¶
CD1	99.7%	97.4%	96.6%	97.2%	97.1%	89.3%
CD2	97.2%	99.7%	99.4%	99.5%	99.8%	89.1%
CD3	97%	99.5%	99.2%	99.3%	99.5%	88.9%
CD4	97.7%	99.5%	99.2%	99.5%	99.5%	89.3%
CD5	97.4%	99.9%	99.7%	99.8%	100%	89.2%
CD6	97.2%	99.9%	99.7%	99.8%	100%	89.1%
CD7	97.3%	100%	99.7%	99.8%	100%	89.1%
CD8	97.3%	100%	99.7%	99.8%	100%	89.1%
WD1	97.3%	100%	99.7%	99.8%	100%	89.1%
WD3	97.3%	100%	99.7%	99.8%	100%	89.1%
WD4	99.7%	97.4%	96.6%	97.2%	97.1%	89.3%
WD5	97.3%	100%	99.7%	99.8%	100%	89.1%
WD6	97.2%	99.9%	99.5%	99.7%	99.8%	89%

*KP053255 *Drepanocephalus auritus* HAP-H1; Pinto et al. 2016
†KR259644 *Drepanocephalus auritus* MJG-DA; Pinto et al. 2016
‡KP683125 *Drepanocephalus auritus* isolate 1; Kudlai et al. 2015
§KM538090 *Drepanocephalus auritus* voucher EC.IN.Pa.22.V27.19; Van Steenkiste, et al. 2015
‖JX468067 *Drepanocephalus spathans* CO1, now as *Drepanocephalus auritus*; O'Hear et al. 2014
¶KP053256 *Drepanocephalus* sp. HAP-H3; Pinto et al. 2016

Similarly, sequence analysis of a 439 bp region of the ND1 gene revealed the 11 isolates matching the North American haplotypes at CO1 also shared 100% identity at the nucleotide level to *D. auritus* released by *P. trivolvis* from the United States (GenBank KP053262; Pinto et al., 2016). Meanwhile, the two isolates sharing high sequence similarity to South American haplotypes at CO1 also shared 99.5% nucleotide (100% a.a.) sequence similarity at ND1 to a South American haplotype of *D. auritus* released by *B. straminea* in Brazil (GenBank KP053263; Pinto et al., 2016) (Tables 2.6, 2.7). Across all isolates intraspecific nucleotide variability ranged from 0.0-3.9%. When these

sequences were compared to *Drepanocephalus* sp. released by *B. straminea* in Brazil (GenBank KP053264; Pinto et al., 2016), nucleotide sequence similarity was 85.3-86% (98% a.a.).

Table 2.6 NCBI BLASTn and BLASTx comparisons (top hit) of nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) gene

	BLASTn				BLASTx			
	e-value	Coverage	Identity	GenBank	e-value	Coverage	Identity	GenBank
Haplotype 1*	0.0	100%	100% (439/439)	KP053262‡	9e-74	99%	100% (145/145)	AIY72193‖
Haplotype 2†	0.0	100%	99.5% (437/439)	KP053263§	9e-74	99%	100% (145/145)	AIY72193

*Isolates CD2, CD3, CD4, CD5, CD6, CD7, CD8; WD1, WD3, WD5, WD6
†Isolates CD1,WD4
‡KP053262 *Drepanocephalus auritus* MJG-DA; Pinto et al., 2016
§KP053263 *Drepanocephalus auritus* HAP-H1; Pinto et al., 2016
‖AIY72193 *Drepanocephalus auritus*; Pinto et al., 2016

Table 2.7 Sequence similarity of genetic groupings of nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) gene

	KP053262‡	KP053263§
Haplotype 1*	100%	95.7%
Haplotype 2†	96.1%	99.5%

*Isolates CD2, CD3, CD4, CD5, CD6, CD7, CD8; WD1, WD3, WD5, WD6
†Isolates CD1, WD4
‡KP053262 *Drepanocephalus auritus* MJG-DA; Pinto et al., 2016
§KP053263 *Drepanocephalus auritus* HAP-H1; Pinto et al., 2016

Moreover, sequences spanning 4,074 bp of the 18S, ITS1, 5.8S, ITS2, and 28S genes of cercariae released from *B. havanensis* in this study revealed a high degree of sequence similarity to sequences from *D. auritus* from *P. trivolvis* from the United States (GenBank AY245762; Flowers et al., 2004, (unpublished); GenBank JN993271; Griffin et al., 2012; GenBank KP053259; Pinto et al, 2016; GenBank KP683117; Kudlai et al., 2015; GenBank JN993268; Griffin et al., 2012) and *D. auritus* from *B. straminea* from Brazil (GenBank KP053260; Pinto et al., 2016). Minimal intraspecific variability was observed, as the *D. auritus* isolates in this study shared 99.8-100% sequence similarity across the entire set of ribosomal genes analyzed. In addition, there was limited nucleotide sequence variability (0.4%) when compared to *Drepanocephalus* sp. from *B. straminea* from Brazil (GenBank KP053261; Pinto et al. 2016) (Table 8).

Table 2.8 Sequence similarity of ribosomal RNA (rRNA) genes (18S-ITS1-5.8S-ITS2-28S)

Isolate	GenBank Accession Number						
	KP053259[*] (4267 bp)	KP683117[†] (2345 bp)	JN993268[‡] (1956 bp)	KP053260[§] (4267 bp)	KP053261[ll] (4267 bp)	AY245762[¶] (1967 bp)	JN993271[#] (1955 bp)
Current study (4074 bp)	100% (4068/4068 bp)	100% (2182/2182 bp)	100% (1898/1898 bp)	99.9% (4064/4068 bp)	99.6 % (4050/4068 bp)	99.8 % (1920/1923 bp)	99.9 % (1897/1898 bp)

[*]KP053259 *Drepanocephalus auritus* MJG-DA; Pinto et al., 2016
[†]KP683117 *Drepanocephalus auritus* isolate 1; Kudlai et al., 2015
[‡]JN993268 *Drepanocephalus spathans* HCC, now as *Drepanocephalus auritus*; Griffin et al., 2012
[§]KP053260 *Drepanocephalus auritus* HAP-H1; Pinto et al., 2016
[ll]KP053261 *Drepanocephalus* sp. HAP-H3; Pinto et al., 2016
[¶]AY245762 *Drepanocephalus spathans* 18S SSU rRNA, now as *Drepanocephalus auritus*; Flowers et al., 2004 (unpublished)
[#]JN993271 *Drepanocephalus spathans* YCA, now as *Drepanocephalus auritus*; Griffin et al., 2012

Phylogenetic inferences based on CO1 sequences clustered 11 of the 13 isolates of *D. auritus* recovered from *B. havanensis* with previous isolates of *D. auritus* from North America, while 2 of the isolates fell into a clade containing an isolate of *D. auritus* from Brazil. Meanwhile, the unidentified *Drepanocephalus* sp. from *B. straminea* in Brazil formed its own tribe, sister to *D. auritus* haplotypes from North and South America (Figure 2.2).

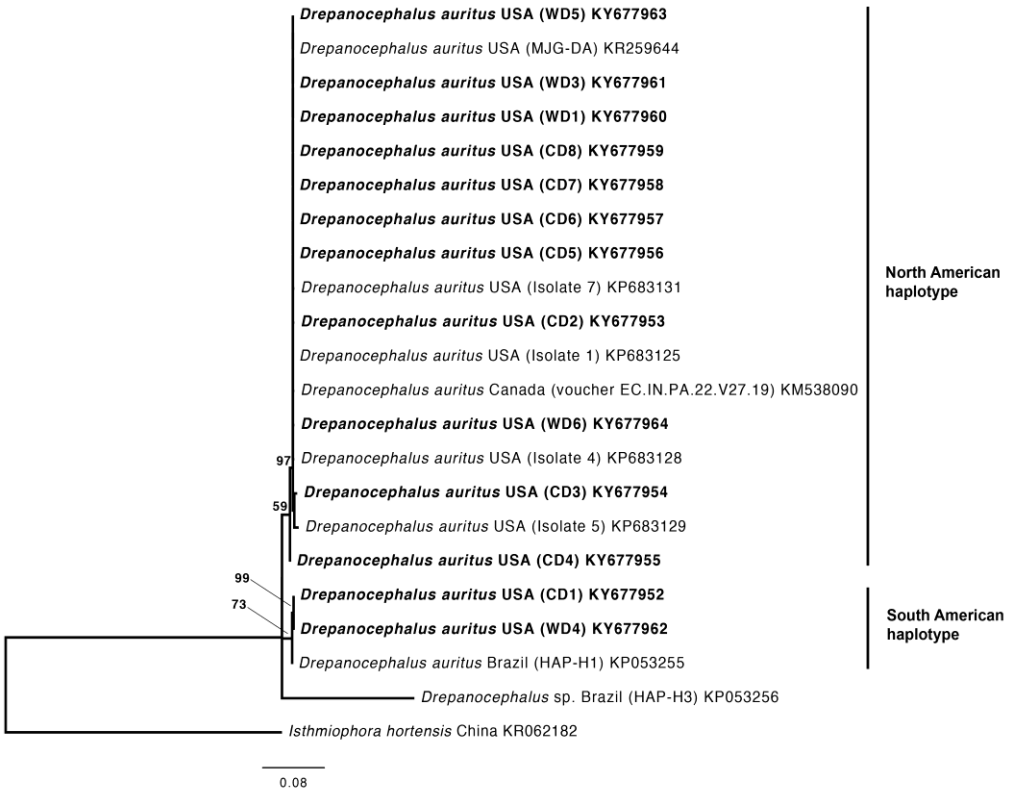

Figure 2.2 Maximum likelihood tree inferred from analysis of CO1 sequences

Maximum likelihood tree inferred from analysis of CO1 sequences of all isolates of *Drepanocephalus auritus* released by *Biomphalaria havanensis* in Mississippi and other *Drepanocephalus* spp. deposited into the NCBI nt/nr database. Isolates from current study are bolded. Specimen designations are in parentheses and GenBank accession numbers follow. Numbers above branches represent bootstrap values obtained by 1000 replicates.

2.4.3 Experimental fish challenge

Of the 10 channel catfish individually exposed to cercariae from *B. havanensis*, 2 died within 48 hr of exposure. This is consistent with previous challenges, where mortality occurred within the first 7 days post-challenge (Griffin et al., 2012; 2014). However, post-mortem autolysis and tank mate cannibalism made them unsuitable for histological evaluation. The remainder of the fish (n=8) survived until the end of the trial. None of the control fish died. Surviving fish had no grossly visible signs of infectious disease.

2.4.4 Histology

Histological analysis showed multiple metacercariae in 6 of the 8 surviving experimentally exposed fish. The metacercariae and host inflammatory response were consistent with previous reports of experimental infection with *D. auritus* in channel catfish (Griffin et al., 2012; 2014). Based on histological examination, 2 fish were negative for *D. auritus*, as no metacercariae were observed in any tissue sections. Although multiple sections were taken of each fish, the lack of metacercariae could be an artifact of sectioning. As with previous studies, metacercariae were concentrated within the cranial region, with few, if any, located behind the head. These metacercariae were seen in the subdermal tissues just below the skin, within the branchial cavity, or in the periocular tissues (Figures 2.3 and 2.4). They were also observed within the lateral line system (Figure 2.5). Typically, these metacercariae incite an intense mononuclear inflammatory response. On occasion, metacercariae were observed encased within bone. In these instances, the inflammatory response immediately surrounding the metacercaria

was milder, although in some instances the inflammation was just as intense surrounding the bone.

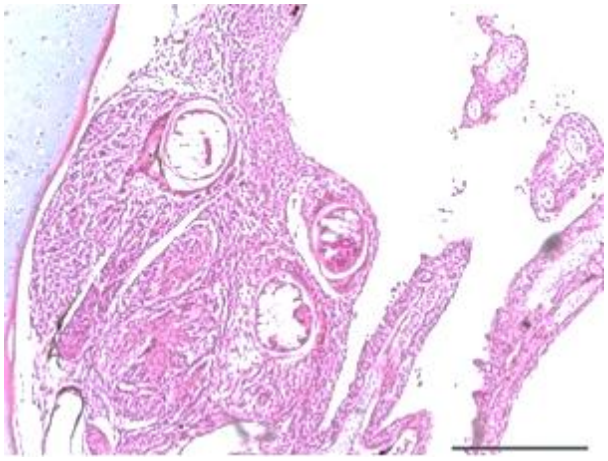

Figure 2.3 Three developing metacercariae of *Drepanocephalus auritus*

Developing metacercariae located in the branchial cavity together with an intense mononuclear inflammatory response (H&E; bar ~ 200µm).

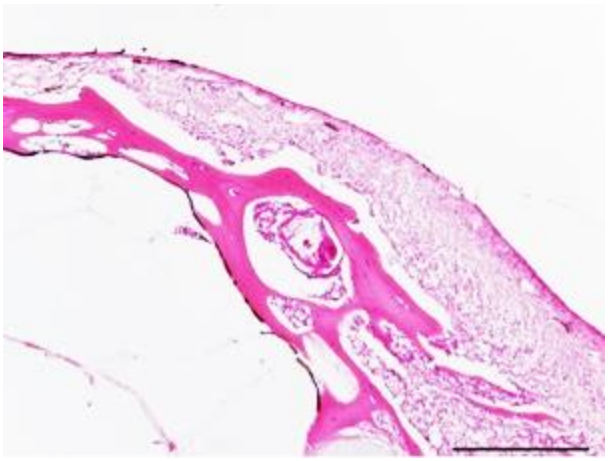

Figure 2.4 Metacercaria of *Drepanocephalus auritus* encased within bone

Note the inflammatory response immediately surrounding the metacercaria is relatively mild compared to the infiltrates surrounding the bone (H&E; bar ~ 200µm).

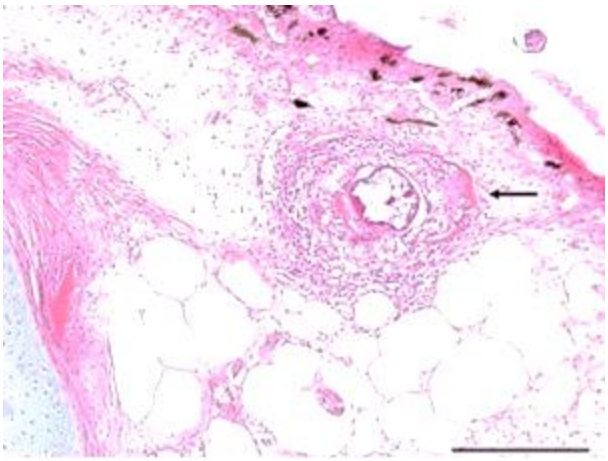

Figure 2.5 Subdermal location of a metacercaria of *Drepanocephalus auritus*

Metacercaria located in the cranial region of channel catfish. The lateral line system has been obliterated by the associated inflammatory response (arrow) (H&E; bar ~ 200µm).

2.5 Discussion

The taxonomic classification of *Drepanocephalus* spp. in North and South America is currently in flux. *Drepanocephalus auritus* has been reported as an intestinal parasite infecting the double-crested cormorant *Phalacrocorax auritus* (Threlfall, 1982; Flowers et al., 2004; Robinson et al., 2010; Wagner et al., 2012; O'Hear et al., 2014; Kudlai et al., 2015; Sheehan et al., 2016) and the marsh rams-horn snail *Planorbella trivolvis* (Griffin et al., 2012). A recent study by O'Hear et al. (2014) found 91% prevalence of *D. spathans* (=*D. auritus*) in double-crested cormorants in the Delta region of Mississippi. Experimental challenges exposing juvenile channel catfish to cercariae of *D. spathans* (=*D. auritus*) resulted in mortality in some fish (Griffin et al., 2012; 2014). In these studies, metacercariae were observed in the cranial region of surviving fish, with these metacercariae having a predilection for the branchial chamber, often located at the base of the branchial arches within, or adjacent to blood vessels, and the occlusion of which is thought to be the proximate cause of death (Griffin et al., 2012). A longevity

study using juvenile channel catfish demonstrated this infection to be short-lived, resolving as early as 7-21 days post-challenge, although metacercariae in some fish persisted up to 70 days post-challenge if located in immune privileged sites (Griffin et al., 2014). However, these studies took place prior to the description of *D. auritus* by Kudlai et al. (2015). Morphological and molecular analysis has determined the subject of these recent studies (Griffin et al., 2012; 2014; O'Hear et al., 2014) was not *D. spathans*, but rather the newly described *D. auritus* (Kudlai et al., 2015; Pinto et al., 2016).

Similar to the issues regarding the classification of *Drepanocephalus* spp., there is some uncertainty with respect to nomenclature regarding the *Biomphalaria* sp. found to be infected with *D. auritus*. The snails identified herein were morphologically consistent with descriptions of *B. havanensis* taken from the presumptive type locality for *B. havanensis*, at Zanja Ferrer, Havana, Cuba (Yong et al., 2001). These authors noted their descriptions matched those provided for *Biomphalaria obstructa* Morelet, 1849 by Paraense (1990) from the type locality for the latter species, Isla del Carmen, Mexico. Phylogenetically, DeJong et al. (2001) asserted there are two distinct species in Cuba, both of which have been historically referred to as *B. havanensis*, while Yong et al. (2001) considered *B. obstructa* to be a synonym of *B. havanensis*. The evolutionary histories of *Biomphalaria* spp. produced in this study (Figure 1) showed negligible differences among specimens from Zanja Ferrer, Havana, Cuba (GenBank AY030394), Temascal, Oaxaca, Mexico (GenBank AY030396), Texas (GenBank AY030397), and 5 isolates from Mississippi (GenBank KY777432-KY777436). As such, it seems prudent to call the specimens in this study *B. havanensis*. It should be noted our tree also indicates some variability among *B. obstructa* from the type locality Isla del Carmen, Mexico

(GenBank AY030398) and *B. obstructa/havanensis* from the southern United States, suggesting further definitive study is needed to ascertain if additional discrete taxa occur in the United States.

Most attempts to expose *Biomphalaria* spp. from the southern United States to *S. mansoni* have failed. This includes specimens from Texas (Sullivan and Hu, 1996), Louisiana (Malek, 1967), California (Basch et al., 1975) and Florida (Leigh, 1961). In most of these studies, the snails exposed have been referred to as *B. obstructa*. It is likely they should be referred to as *B. havanensis* using the reasoning described above. We also note that two studies have reported successful infections of *B. havanensis* to *S. mansoni*. In one of these cases, the snails originated from a now extinct colony from Baton Rouge, Louisiana (Cram et al., 1945), and another from Grenada (Richards, 1973). Sequence data for snails from either location is thus far not available, so it is not known if these snails represent schistosome-susceptible variants of *B. havanensis* or perhaps a different, misclassified yet closely related species.

Phenotypically, *B. obstructa* is considered refractory to infection with *S. mansoni*, while the susceptibility of *B. havanensis* is ambiguous (Basch, 1976). As such, the description of *B. havanensis* remains in dispute (Paraense and Deslandes, 1958; Yong et al., 1997; DeJong et al., 2001) and there exists multiple conflicting morphological descriptions (DeJong et al., 2001). Our results show *S. mansoni* is able to penetrate *B. havanensis* snails collected in this study but does not achieve a patent infection. In light of their morphological characters, resistance to *S. mansoni* infection and the lack of a definitive molecular classification between *B. havanensis* and *B. obstructa*, the snails in the current study are identified as *B. havanensis.*

Cercariae released by *B. havanensis* inhabiting catfish aquaculture ponds in the Black Prairie region of east Mississippi were deemed *D. auritus* based on shared sequence similarities ranging from 97.2-100% (Griffin et al., 2012; 2014; O'Hear et al., 2014; Kudlai et al., 2015; Van Steenkiste et al., 2015; Pinto et al., 2016) across five different gene targets (18S, ITS, 28S, CO1, and ND1). We identified two different genetic variants (haplotypes) based on CO1 and ND1 analysis. The first haplotype showed >99% nucleotide (100% a.a.) sequence similarity at the CO1 loci to *D. auritus* released by *P. trivolvis* from commercial catfish ponds in Mississippi (Griffin et al., 2012; Pinto et al., 2016). Nucleotide and amino acid sequence similarities between cercariae from this study and adult *D. auritus* recovered from a double-crested cormorant (Kudlai et al., 2015) and an archived museum specimen (Van Steenkiste et al., 2015) were 99.2-99.7% (100% a.a.) and 99.3-99.8% (100% a.a.), respectively.

The second haplotype showed >99% nucleotide (100% a.a.) identity to *D. auritus* released from *B. straminea* collected during a malacological survey in Brazil (Pinto et al., 2016). Nucleotide sequences of the CO1 gene of these two haplotypes differed by 1.8-2.7%, consistent with a previous study by Pinto et al. (2016) who suggested the existence of distinct North American and South American haplotypes of *D. auritus*. This current work suggests the Brazilian haplotype is not limited to South America, as both haplotypes are present in Mississippi. The level of variability found at the CO1 locus between both haplotypes from this study and *Drepanocephalus* sp. (GenBank KP053256) released by *B. straminea* in Brazil ranged from 10.7-11.1%, consistent with the range of interspecific variability reported by Pinto et al. (2016). Furthermore, phylogenetic analysis demonstrated the distinct clustering of these haplotypes as two separate clades.

Isolates from this study demonstrated congeneric nucleotide variability at the ND1 locus within ranges previously reported from *Echinostoma* Rudolphi, 1809 (Morgan and Blair, 1998a; 1998b; Detwiler et al., 2010). Moreover, all isolates were compared to published ND1 sequences of *D. auritus* from the United States and Brazil. The isolates from *B. havanensis* in Mississippi differed from North American and South American haplotypes of *D. auritus* by 0.0-3.9% and 0.5-4.3% respectively, but shared 100% similarity in amino acid sequence, suggesting they are conspecific. This intraspecific nucleotide variability is within the range reported for ND1 from both *D. auritus* (Pinto et al., 2016) and other echinostomes (Morgan and Blair, 1998a; 1998b; Detwiler et al., 2010). The level of interspecific nucleotide variability between isolates of *Drepanocephalus* sp. and *D. auritus* at the ND1 region previously reported by Pinto et al. (2016) ranged from 14.3-15.5%. This is consistent with the range of nucleotide variability (14.0-14.7%) reported in this current study, supporting the assertions by Pinto et al. (2016) that *D. auritus* and *Drepanocephalus* sp. isolate HAP-H3 are likely congeners but not conspecific.

Ribosomal RNA regions (18S, ITS1, 5.8S, ITS2, and 28S) from cercariae released from *B. havanensis* from this study were highly conserved. Additionally, isolates only differed from *Drepanocephalus* sp. released by *B. straminea* from Brazil by 0.4%, supporting the intimation that rRNA regions do not offer suitable resolution for identification to lower taxonomic levels. Intraspecific variation between isolates from this study and published rRNA sequences from *D. auritus* ranged from 0.0-0.2%, consistent with previous studies demonstrating limited intraspecific variability at the rRNA locus

for both the Echinostomatidae and Diplostomatidae (Morgan and Blair, 1995; Sorenson et al., 1998; Pinto et al., 2016; Rosser et al., 2016a).

The genetic variability of cercariae of *D. auritus* present in catfish aquaculture ponds is not surprising. The double-crested cormorant is widely distributed throughout North America, occurring in large numbers in interior and coastal portions of the United States and Canada (Dorr et al., 2014). Coastal populations, especially those on the Pacific Coast, are sedentary, whereas interior populations and those located on the Atlantic Coast are highly migratory (Johnsgard, 1993; Dorr et al., 2014). Breeding ranges are located in 5 regions including Alaska, the Pacific Coast, the Canadian and United States interior, the Atlantic Coast, and the southeastern United States and Caribbean, encompassing the Gulf Coast (Texas to Florida), Atlantic Coast (Florida, Georgia, and the Carolinas), the Caribbean, the Bahamas, and Cuba (Johnsgard, 1993; Wires and Cuthbert, 2006; Dorr et al., 2014). Breeding grounds of interior populations range from the Great Lakes to the northern prairies of the United States and Canada (Dolbeer, 1991).

Migrating interior populations often over-winter in the southeastern United States and northern Mexico, utilizing primary migration routes down the Atlantic Coast and the Mississippi Flyway, following the Mississippi River, beginning in Canada and ending at the Gulf Coast (Johnsgard, 1993; King et al., 2010). Inland lakes, rivers, and more recently, catfish aquaculture ponds located in the Mississippi Flyway serve as ideal wintering grounds for migrating double-crested cormorants (Dorr et al., 2004; King et al., 2010; Dorr et al., 2014). Oftentimes, wintering populations of double-crested cormorants overlap with populations of the neotropic cormorant *Phalacrocorax brasilianus* Gmelin, 1789 (Telfair II and Morrison, 2005), with populations found in South and Central

America, Mexico, and the West Indies. Year-round and breeding populations are also found in the United States in Arizona, New Mexico, Texas, Louisiana, Arkansas, and recently, Florida and Mississippi (Johnsgard, 1993; Coldren et al., 1998; Telfair II and Morrison, 2005; Hanson et al., 2010; Pranty et al., 2010). Although neotropic cormorants are mostly sedentary, some will move seasonally (Telfair II and Morrison, 2005). Neotropic and double-crested cormorant ranges overlap in the Gulf Coast region of Texas and Louisiana, and also in coastal regions of Mexico, Cuba, and the Bahamas. As such, it is likely piscivorous birds foraging on aquaculture ponds in Mississippi carry multiple genetic variants of intestinal parasites acquired while feeding in different geographic locales. The vast migratory range of double-crested cormorants likely drives the parasitic diversity seen in the snail populations in these ponds.

This current work demonstrates juvenile channel catfish can be infected by cercariae of *D. auritus* shed by naturally infected *B. havanensis* in catfish ponds. Metacercariae were visualized by histology 7 days post-challenge. Pathology was consistent with previous infection trials, with metacercariae located within the branchial arch and in the cranial region with minimal mortality (20%) occurring during the initial stages of infection (Griffin et al., 2012; 2014). Similar to previous studies, some metacercariae were also encysted within bone, which is a unique presentation. Portions of the lateral line system near the head are surrounded by bone (Grizzle and Rogers, 1976). It is speculated this association may facilitate the occasional intraosseous locations of these metacercariae, especially if the lateral line system is a portal of entry.

The biologic and economic implications of these infections to catfish aquaculture in the southeastern United States have yet to be determined. Likewise, the consequences

of this observed genetic variability are unclear. Fish were exposed to cercariae prior to molecular analysis. As such, catfish were unknowingly exposed to a pool of cercariae representing both genetic variants of *D. auritus*. At present, it is unknown if one haplotype is more successfully transmitted or more pathogenic to catfish than another and the associations between haplotype and virulence in catfish warrants further study.

Other digenetic trematodes infective to channel catfish, namely *B. damnificus* and *Austrodiplostomum ostrowskiae* Dronen, 2009, have also been reported from *B. havanensis* in commercial catfish ponds (Yost et al., 2009; Rosser, et al., 2016a; 2016b). Previous work demonstrated the presence of these digenetic trematodes in catfish production systems can have dire consequences for channel catfish health and commercial production (Wise et al., 2004; 2008; 2013). Until recently it was thought *P. trivolvis* was the only snail species of concern for catfish farmers. While Pinto et al. (2016) found *B. straminea* released cercariae of *D. auritus* and another unidentified *Drepanocephalus* sp. in Brazil, this is the first report of *B. havanensis* as a first intermediate host for *D. auritus* in North America. It is unknown if management practices aimed at eradication of *P. trivolvis* from catfish aquaculture ponds are effective against *B. havanensis*, and more work is needed to establish the true threat these snails pose to commercial catfish production. Regardless, the identification of a second snail host releasing both *B. damnificus* and *D. auritus* suggests aquaculturists must remain diligent in implementing adequate snail control measures on their operations.

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CHAPTER III

EXPERIMENTAL ELUCIDATION OF THE LIFE CYCLE OF *DREPANOCEPHALUS AURITUS* IN THE DOUBLE-CRESTED CORMORANT *PHALACROCORAX AURITUS*, THE CHANNEL CATFISH *ICTALURUS PUNCTATUS*, AND THE MARSH RAMS-HORN SNAIL *PLANORBELLA TRIVOLVIS*

3.1 Abstract

A 2-year study was undertaken to elucidate the life cycle of *Drepanocephalus auritus.* Snails collected from a commercial catfish operation were releasing *D. auritus* cercariae, and channel catfish were exposed individually. Double-crested cormorants were live captured, cleared of gastrointestinal helminth infections and fed *D. auritus* infected catfish. At the end of each trial, birds were sacrificed, and gravid trematodes recovered from experimental birds were identified as *D. auritus*. Miracidia hatched from ova collected from feces after 15 days at 25 C. Moreover, eggs incubated for two weeks at 4 C hatched in a similar time frame once they were brought to 25 C, suggesting dormancy at low temperatures. After exposure to ova, two snails shed *D. auritus* cercariae 89 and 97 dpi. Additionally, snails placed in ponds inhabited by cormorants shed *D. auritus* cercariae beginning 18 dpi. This work is the first experimental confirmation of the *D. auritus* life cycle.

3.2 Introduction

The digenetic trematode *Drepanocephalus auritus* (formerly reported as *Drepanocephalus spathans*) is a parasite of the double-crested cormorant *Phalacrocorax auritus* (Threlfall, 1982; Flowers et al., 2004; Robinson et al., 2010), the marsh rams-horn snail *Planorbella trivolvis*, and most recently the ghost rams-horn snail *Biomphalaria havanensis* (Threlfall, 1982; Flowers et al., 2004; Robinson et al., 2010; Griffin et al., 2012; Wagner et al., 2012; O'Hear et al., 2014; Kudlai, et al., 2015; Pinto et al., 2016; Sheehan et al., 2016; Alberson et al., 2017). The double-crested cormorant (DCCO) is a piscivorous migratory bird, widely distributed throughout North America (Dorr et al., 2014). Migrating populations over-winter in the southeastern United States and are often found foraging on commercial aquaculture ponds in the Delta and Black Prairie regions of Mississippi, U.S.A. due to the close proximity to the Mississippi Flyway, a bird migration route that generally follows the Mississippi River from the Gulf of Mexico to central Canada, bisecting the Midwestern United States (Stickley and Andrews, 1989; Johnsgard, 1993; King, 1996; Dorr et al., 2004; Glahn and King, 2004; King et al., 2010). While on these production ponds, DCCO can consume large amounts of catfish. Research suggests DCCO consume 227 g-454 g of catfish daily, with sizes ranging from 10 cm to 20 cm (Glahn et al., 1995; Stickley, 1990). The average catch rate for a DCCO on a production farm averages 5 catfish/hour, but can be as high as 28 (Stickley et al., 1992). Along with the consumption of large amounts of catfish, DCCO also injure and disrupt catfish feeding patterns reducing growth and production efficiency (Wywialowski, 1999).

Along with the injury and consumption of catfish in production ponds, DCCO are also a definitive host for a variety of digenetic trematode species, many of which are pathogenic to fish. Several studies have reported multiple species of digenetic trematodes parasitizing DCCO (Threlfall, 1982; Fedynich et al., 1997; Flowers et al., 2004; Wagner et al., 2012; O'Hear et al., 2014; Sheehan et al., 2016a; Sheehan et al., 2016b). The echinostomatid *Drepanocephalus spathans* was commonly found during these studies, with prevalence rates ranging from 84%-91% across the Mississippi Delta, Saskatchewan Canada and the eastern United States (Wagner et al. 2012; O'Hear et al. 2014; Sheehan et al. 2016b). However, the taxonomic classification of *Drepanocephalus* spp. in North America is currently in flux due to the newly described *Drepanocephalus auritus* (Kudlai et al. 2015). Kudlai et al. (2015) suggests *D. spathans* is restricted to South America and the *Drepanocephalus* sp. reported in previous studies from North America represent *D. auritus* and not *D. spathans*. Recent work has supported these claims, and molecular surveys have confirmed that previous reports of *D. spathans* in North America were actually *D. auritus* (Griffin et al. 2012; 2014; O'Hear 2014; Kudlai et al. 2015; Pinto et al. 2016).

As with most digeneans, *D. auritus* utilizes an aquatic snail as a first intermediate host. Natural infections have been documented in *P. trivolvis* and *B. havanensis* from Mississippi, U.S.A. (Griffin et al., 2012; Alberson et al., 2017), and *Biomphalaria straminea* from Brazil (Pinto et al., 2016). Ubiquitous to commercial aquaculture ponds, *P. trivolvis* and recently *B. havanensis* are pests of major concern to catfish production, serving as intermediate hosts for *Bolbophorus damnificus* and *Austrodiplostomum ostrowskiae*, both infective to channel catfish *Ictalurus punctatus* (Yost et al., 2009;

Rosser et al. 2016a; Rosser et al., 2016b). The presence of *B. damnificus* in catfish production systems has been shown to be detrimental to catfish health, leading to significant production losses, even in mild infections (Wise et al., 2004; Wise et al., 2008; Wise et al., 2013).

Experimentally it has been shown that *D. auritus* infection can be detrimental to catfish as well, causing mortalities in juvenile channel catfish within 7 days post-exposure (dpe) (Griffin et al., 2012; 2014; Alberson et al., 2017). Metacercariae are mostly concentrated within the cranial region, located within the branchial cavity, periocular tissues, bone, and lateral line system (Griffin et al., 2012; 2014; Alberson et al., 2017). It is thought death is due to the occlusion of branchial vessels caused by metacercariae encysting within or adjacent to blood vessels located at the base of the branchial arches (Griffin et al., 2012). Infection with *D. auritus* is short-lived in juvenile channel catfish. In a recent longevity study, Griffin et al. (2014) found that infection began resolving as early as 7-21 dpe, but metacercariae in some fish persisted as long as 70 dpe if encysted within bone or other immune privileged sites.

The results of the longevity study brought about the question of whether or not channel catfish were a true intermediate host in the life cycle of *D. auritus*, which to date has not been experimentally confirmed. The purpose of this study was to investigate whether the channel catfish could truly serve as an intermediate host and establish developmental timelines for different *D. auritus* life cycle stages, establishing a baseline model for the study of trematode parasites in catfish aquaculture.

3.3 Materials and Methods

3.3.1 General procedures

3.3.1.1 Snail collection

In 2015, and again in 2016, *P. trivolvis* were collected from catfish production ponds in Lowndes County, Mississippi and brought back to Mississippi State University College of Veterinary Medicine. Snails were rinsed with reverse osmosis water and placed into 25-ml plastic vials (Diluvial, Elkay Laboratory Products, Hampshire, United Kingdom) containing 10 ml of autoclaved spring water (Ozarka, Wilkes Barre, Pennsylvania). Snails were kept at ambient temperatures (~25-27 C) for 48 hr and examined for the presence of cercariae with the use of a stereomicroscope (Olympus SZ60, Olympus Optical, Tokyo, Japan). Pools of cercariae morphologically consistent with *D. auritus* were collected from infected snails and archived in 50-ml tubes containing 70% molecular grade ethanol (Sigma Aldrich, St. Louis, Missouri) for molecular analysis.

3.3.1.2 Molecular identification

To expedite the initial identification process, a *Drepanocephalus* spp. specific PCR was designed, amplifying a 189 bp region of the cytochrome *c* oxidase subunit 1 (CO1) gene. Amplification was carried out using the forward primer 828F1 (5'-CCG CAC CAC CTA TCA TAC TTA AC-3') and the reverse primer 1017R (5'-GTG ACT TCT CAC GGG ATA ATT ATG A 3'). Approximately 10 cercariae were isolated from each archived pool and washed 3 times with molecular grade, nuclease free water, removing residual ethanol. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, California). The PCR consisted of 13 µl EconoTaq

PLUS GREEN 2X Master Mix (Lucigen Corporation, Middleton, Wisconsisn), 10 pmols of each primer, 5 µl of nuclease free water, and 5 µl of template DNA. Thermocycling profile consisted of an initial denaturation of 2 min at 94 C, followed by 39 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 45 sec, and a final extension for 5 min at 72 C. Amplification products were visualized under ultraviolet light on a 1.2% agarose gel containing ethidium bromide (0.5 µg/ml) and run concurrently with a 50 bp molecular weight ladder (HyperLadder™, Bioline, London, UK) to ensure the presence of the correct sized band.

Further molecular confirmation was performed by PCR amplification and sequencing of the CO1 gene using previously established primers and protocols (Table 3.1). Products were visualized as described above, directly purified using the QIAquick PCR Purification Kit (QIAGEN) and sequenced with the same forward and reverse primers used in the initial amplification (Eurofins MWG Operon, Huntsville, Alabama). Contiguous sequences were assembled using Geneious software version 9.1.5 (Biomatters Ltd., Auckland, New Zealand) with ambiguous base calls being manually annotated. Gene sequences generated from all specimens were compared to closely related trematode sequences by a Blastn search of the National Center for Biotechnology Information non-redundant nucleotide database (NCBI nr/nt) (Altschul et al., 1990).

Table 3.1 Primers used in sequencing of the cytochrome *c* oxidase subunit 1 (CO1) gene.

Primer	Sequence (5'-3')	Reference
cox11	CACCTTAATACCCGTCGGAAT	Pinto et al. 2016
cox820	AATATTATTTCCCCGGRAGTYT	Pinto et al. 2016
cox1_schist5'	TCTTTRGATCATAAGCG	Lockyer et al. 2003
acox650r	CCAAAAAACCAAAACATATGCTG	Kudlai et al. 2015

3.3.1.3 Channel catfish infection

For each challenge, cercariae were collected and pooled in autoclaved spring water (Ozarka), and gently stirred with a magnetic stir bar. Ten 100-µl aliquots were collected and the number of cercariae in each aliquot was counted using a stereomicroscope (Olympus SZ60, Olympus Optical) to approximate the number of cercariae per ml of inoculum. Specific pathogen free (SPF) channel catfish fingerlings (~3-5 cm) were placed individually into 1-l plastic containers containing 300 ml of dechlorinated municipal water with constant aeration. Fish were exposed individually to ~150 cercariae for 4 hr. At the end of each challenge, water from each container was decanted and the fish were placed into separate recirculating aquaria according to challenge day, and housed at ambient temperatures (~25-27 C) with constant aeration until fed to experimental DCCO.

3.3.1.4 Histology

Fish were euthanized with an overdose of tricaine methanesulfonate (Western Chemical, Ferndale, Washington), and the coelomic cavity was opened via sharp incision, and placed into 10% neutral buffered formalin. After fixation in 10% neutral buffered formalin for at least 24 hr, fish were decalcified using CalEx® (Fisher Scientific, Fair Lawn, New Jersey) until their calcified tissues were pliable upon digital manipulation. They were rinsed with running water for at least 3 hr prior to trimming in. Approximately 3-5 mm cross sections through the fish were made starting rostrally and moving caudally with small adjustments in thickness made to ensure sections bisected the major organs. Based on previous work, multiple thinner sections were made through the head region where the majority of *D. auritus* metacercariae are located. The sections

were processed routinely, embedded in paraffin and 4 µm sections were stained with Hematoxylin and Eosin (H&E) and examined using an Olympus BX-50-microscope (Olympus Optical Co., Ltd., Tokyo, Japan), and representative images were captured with an Olympus DP72 camera and DP2-TWAIN–cellSens software (Olympus Optical Co., Ltd., Tokyo, Japan).

3.3.1.5 Double-crested cormorant collection and care

Birds were transported to the NWRC's Avian Testing Facility located on the campus of Mississippi State University, and placed on experimental aviary ponds. After initial acclimation period, birds were weighed, equipped with individually numbered leg bands, and housed indoors in individual 3 X 6 X 2-m pens. Each pen was equipped with a 1000-l recirculating tank equipped with a ramp. Each bird was fed a daily ration of 600 g of channel catfish and observed daily for general health and body conditioning. Fecal samples were collected daily and examined for trematode ova using a modified fecal sedimentation method (Foreyt, 2001). Feces from each bird was weighed, homogenized and placed into individual 15-ml polypropylene conical bottom graduated centrifuge tubes (CELLTREAT Scientific Products, Pepperell, Massachusetts) and numbered according to each bird. A 1% soap solution, prepared using distilled water and liquid dish soap (Dawn® Dishwashing Liquid, Procter and Gamble, Cincinnati, Ohio) was added to each tube, mixed with the feces, and allowed to sit undisturbed for 5 min. Afterwards, the supernatant was removed. This process was repeated until the supernatant was clear. Once cleared, a final wash was performed using autoclaved spring water (Ozarka). After sitting for an additional 5 min, the supernatant was removed one final time and diluted with 10 ml of autoclaved spring water (Ozarka). The sample was stirred thoroughly, and

a 1 ml aliquot was removed and pipetted into a lined Petri dish for viewing using a stereomicroscope (Olympus SZ60, Olympus Optical) and trematode eggs were counted.

After a 10 d acclimation period, praziquantel (Droncit® 34, Bayer Corporation, Shawnee Mission, Kansas) was administered as a single dose to each bird at a rate of 34 mg/kg *per os* (PO) to clear any residual helminth infections. Daily fecal collections continued and sedimentation and egg counts were performed. Starting the day of praziquantel administration, birds were fed a diet of 600 g parasite-free catfish reared indoors for disease research at the Thad Cochran National Warmwater Aquaculture Center, Stoneville, Mississippi.

3.3.1.6 Double-crested cormorant necropsy

Birds were euthanized using carbon dioxide and the complete gastrointestinal tract was removed, from the esophagus to the cloaca. The stomach was separated from the intestines and both were processed individually. The stomach and intestines were opened longitudinally and the contents were rinsed into a 75-µm brass sieve using dechlorinated water. Additionally, the intestinal lining was manually scraped by hand 3 times to ensure trematodes were dislodged, and the contents were washed into the sieve. The contents were examined in a lined Petri dish under a stereomicroscope. All adult trematodes were collected and enumerated before being placed into 25-ml plastic vials (Diluvial) containing 0.09% saline.

3.3.1.7 *Drepanocephalus auritus* staining and identification

Recovered adult trematodes were relaxed in slightly boiling 0.09% saline. A sub-sample of trematodes recovered from each DCCO was fixed in 70% molecular grade

ethanol (Sigma Aldrich) for molecular analysis. Remaining trematodes were fixed in 10% formalin. A sub-sample of formalin fixed adults were stained with acetocarmine for at least 8 hr, destained in 1% acetic acid, and rinsed in increasing concentrations of ethanol (70%, 95%, and 100%) for at least 1 hr each. The specimens were cleared using Hemo-De (ThermoFisher Scientific) before being mounted on glass slides using Permount™ (Thermo Fisher Scientific). Photomicrographs of stained specimens were obtained using an Olympus BX41 compound microscope (Olympus Optical) equipped with a Nikon DS-Fi1 Color Camera with DS-L2 Camera Control Unit (Nikon Instruments Inc., Melville, New York) and measurements from gravid specimens were obtained from representative images according to Kudlai et al. (2015).

3.3.2 2015 Life Cycle Study

3.3.2.1 Birds and Fish

Five DCCO were live-captured by the USDA, APHIS, Wildlife Services, National Wildlife Research Center, Starkville, Mississippi, using methods previously described by King et al. (1994) from 3 different sampling sites in eastern Mississippi and western Alabama. DCCO were selected at random to serve as experimental (n=3) or control (n=2) birds. Fish challenges were performed over a 4-day period to produce *D. auritus* infected fish for DCCO challenge. A subsample of 20 fish from challenge days 1 and 2, and 10 fish from challenge days 3 and 4 were collected and euthanized with an overdose of tricaine methanosulfate (Western Chemical, Ferndale, Washington) at 5 days post-challenge for histological examination to enumerate metacercariae per fish.

Catfish fingerlings infected with *D. auritus* were pooled and fed to 3 experimental DCCO 8-12 days post-infection (dpi). Each experimental bird received 45 infected fish in

the a.m. and 46 infected fish in the p.m. resulting in an estimated inoculum of ~550 metacercariae/bird. Three DCCO were fed parasite-free catfish and served as controls. Experimental DCCO were observed eating the infected fish via a video monitoring system. Feces from each bird was collected daily and sedimentations were performed as above. To confirm successful infection, a single experimental bird was euthanized 8 dpi and necropsied, while others (2 experimental, 2 control) were euthanized 18 dpi.

3.3.2.2 Snails

Two separate infectivity trials were undertaken. In the first, 30 parasite-free *P. trivolvis* and 30 parasite-free *B. havanensis* from the snail colony at Mississippi State University College of Veterinary Medicine were housed in separate cricket tubes (Challenge Plastic Products, Incorporated, Edinburgh, Indiana) and placed into an outdoor experimental pond inhabited by DCCO at the NWRC Avian Testing Facility. Water temperatures ranged from 20-29 C (mean: 24.6 ± 2.7). Snails were taken out of the pond at ~14 d intervals and placed in 20-ml plastic vials (Diluvials) containing 10 ml of autoclaved spring water (Ozarka). Snails were kept at ambient temperatures (~25-27 C) for 48 hr and examined for the presence of cercariae with the use of a stereomicroscope (Olympus). After the 48 hr observation period was over, they were returned to the experimental pond. Snails remained in the pond for 53 d before being removed and taken back to the lab where they were housed in individual plastic vials (Diluvials) containing autoclaved spring water and observed daily for cercariae.

The second infectivity trial involved staggered exposures of small groups (n=10) of snails. Three groups of snails were housed in a cricket tube (Challenge Plastic Products, Incorporated), fed blanched romaine lettuce *ad libitum*, and placed into the

same experimental pond with water temperatures ranging from 28-34 C (mean: 31.3 ± 2.0). Snails from Group 1 were taken out of the pond and placed into 20-ml plastic vials (Diluvials) containing 10 ml of autoclaved spring water (Ozarka). Snails were kept at ambient temperatures (~25-27 C) for 48 hr and examined for the presence of cercariae with the use of a stereomicroscope (Olympus). After the 48 hr observation period was over, snails were returned to the experimental pond. A second group of 10 *P. trivolvis* (Group 2) were placed into the same experimental pond 7 d after Group 1, while Group 3 snails were placed into the pond 7 d after Group 2. Every 7 days, snails were removed from the pond, checked for the presence of cercariae for 48 h, then returned.

3.3.3 Life Cycle Study 2016

3.3.3.1 Birds and Fish

Four DCCO were live-captured at Cat Island, Green Bay, Wisconsin by the USDA, APHIS, Wildlife Services, National Wildlife Research Center, Starkville, Mississippi, using methods described by King et al. (1998), and placed onto an experimental pond already inhabited by a single DCCO previously live-captured for a separate study. Two separate fish challenges were performed 12 d apart due to a limited number of *D. auritus* infected snails. A subsample of 10 fish from each challenge was collected at 5 days post-challenge for pepsin digest (n=5) and histological examination (n=5) to enumerate metacercariae per fish. Fish for pepsin digest were euthanized with an overdose of tricaine methansulfonate (Western Chemical), heads were removed via sharp dissection and placed into a 125-ml Erlenmeyer flask containing 25 ml of a 0.5% pepsin solution. Bodies were cut into thirds via sharp dissection and placed into a 125-ml Erlenmeyer flask containing 50 ml of a 0.5% pepsin solution. Flasks were placed into a

40 C water bath and fish were allowed to digest overnight. Once digestion was complete the solution was poured into a lined petri dish and analyzed for metacercariae using a stereomicroscope (Olympus). Metacercariae were enumerated, collected, and archived in 70% molecular grade ethanol for molecular analysis at a later date.

Catfish fingerlings infected with *D. auritus* metacercariae were fed to 2 experimental DCCO on two separate occasions. The first experimental bird (XR30) received 40 infected fish and the second (XR44) received 38, equating to ~350 metacercariae/bird. Two DCCO were fed SPF catfish and served as controls. Again, experimental DCCO were observed eating the infected fish via a video monitoring system and feces from each bird was collected daily and processed as above. All DCCO (2 experimental, 2 control) were euthanized on study day 60 (XR30 = 43 dpi; XR44 = 31 dpi).

3.3.3.2 Snails

Once eggs were detected in experimental DCCO feces, experimental and control birds were moved to individual pens equipped with 1000-l tanks containing clean water. Experimental and control DCCO were allowed to defecate into individual tanks for 96 hr before being returned to their original pens. Once birds were removed, 80 SPF *P. trivolvis* snails from the snail colony at Mississippi State University College of Veterinary Medicine were placed into 4 separate cricket tubes (Challenge Plastic Products, Incorporated) (20 snails/tube). A single tube housing snails was placed into each 1000-l tank. Every 14 d the tubes were taken back to the lab and snails were placed in 20-ml plastic vials (Diluvials) containing 10 ml of autoclaved spring water (Ozarka). Snails were kept at ambient temperatures (~25-27 C) for 48 hr and examined for the

presence of cercariae with the use of a stereomicroscope (Olympus). After the 48 hr observation period was over, they were returned to their tubes and placed back into their respective tanks. Remaining snails were removed from tanks 44 d after placement and housed in individual 177-ml glass shakers containing ~60 ml autoclaved spring water (Ozarka) and kept at ambient temperatures (~25-27 C). Snails were fed a diet of blanched romaine lettuce *ad libitum* and checked daily for the presence of cercariae.

Two 19-l aquaria (1 experimental and 1 control) containing dechlorinated municipal water were set up in the laboratory and kept at ~23-25 C. Once trematode ova were observed in fecal samples collected from experimental birds via sedimentation, ova were collected from a subsample of feces using the Flukefinder® (Visual Difference, Moscow, Idaho). Eggs were enumerated under a stereomicroscope (Olympus) and 1,137 eggs were placed into the experimental aquarium. Feces from control birds was treated similarly and placed into the control aquarium. Fifteen SPF *P. trivolvis* snails were placed into each aquarium and were fed a diet of blanched romaine lettuce *ad libitum*. Snails were removed from tanks every 14 d and placed into individual 177-ml glass shakers as previously described for 48 hr and checked for the presence of cercariae.

Additionally, 10 SPF *P. trivolvis* and 10 SPF *B. havanensis* were housed separately in cricket tubes (Challenge Plastic Products, Incorporated), fed blanched romaine lettuce *ad libitum* and placed into an outdoor experimental pond inhabited by 5 DCCO at the NWRC Avian Testing Facility, with water temperatures ranging from 25-33 C (mean: 28.1 ± 1.5). Snails were removed from the pond weekly and placed into 177-ml glass shakers as previously described and checked for the presence of cercariae for 24 hr before being placed back into the tube and returned to the pond. A second group of 10 *P.*

trivolvis were housed in cricket tubes (Challenge Plastic Products, Incorporated) and placed into the pond 40 d following the first group and treated similarly.

3.3.3.3 Trematode Ova

Drepanocephalus auritus ova (n=100) were collected and placed into a glass Petri dish containing autoclaved spring water (Ozarka), covered, and kept at ~23-25 C. The ova were checked daily for miracidium development and hatching using a stereomicroscope (Olympus Optical). Also, ova collected from fresh and cold stored (~4 C for 15 days) DCCO feces were placed individually into each well of a 24-well plate (ThermoFisher Scientific) containing autoclaved spring water (Ozarka) and kept at ~ 23-25 C. The glass Petri dish and 24-well plates were observed daily using a stereomicroscope (Olympus Optical) for miracidium development and hatching.

3.4 Results

3.4.1 Life Cycle Study 2015

3.4.1.1 Birds and fish

Developing metacercariae morphologically consistent with *D. auritus (*Griffin et al., 2012; 2014; Alberson et al., 2017) were present in 9 of the 10 fish. These were usually present within the lateral line system (Figure 3.1) as well as within the tissues of the branchial cavity. Infiltrates of moderate to large number of mononuclear cells immediately surrounding the metacercariae were associated at these sites and in adjacent tissues that were expanded or swollen, with loss or obliteration of the lateral line architecture at these sites. Besides the typical sites, rare metacercariae were also present in the base of the pelvic fin (Figure 3.2) and caudal to the abdominal cavity. The

metacercariae were enumerated from sections of the 10 fish, which were a subset of fish fed to the birds.

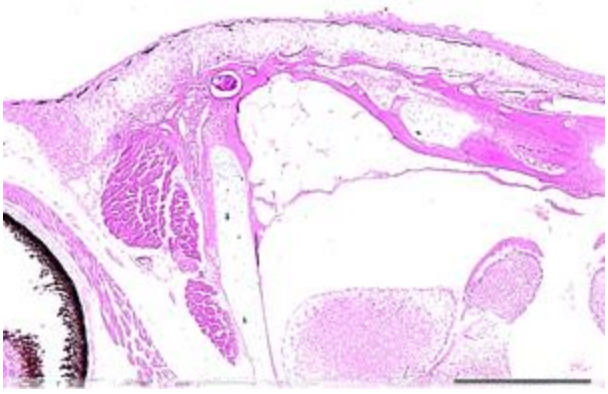

Figure 3.1 Developing *Drepanocephalus* auritus metacercaria

Developing *Drepanocephalus* auritus metacercariae located in the lateral line system is surrounded by bone in the cranium (H&E; bar ~500µm)

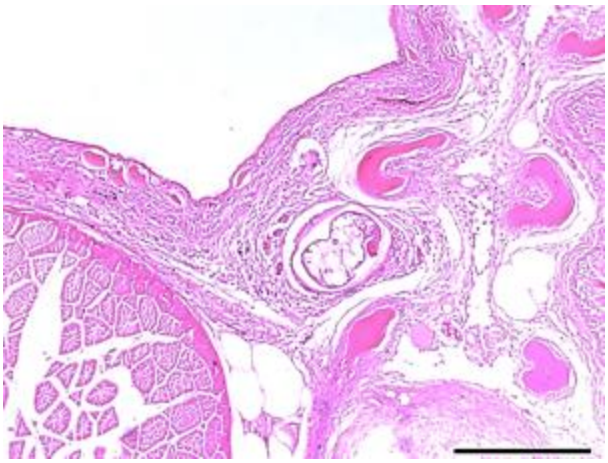

Figure 3.2 *Drepanocephalus auritus* metacercaria

Developing *Drepanocephalus auritus* metacercariae located at the base of the pelvic fin. This is not a typical site based on previous experimental exposures (H&E; bar ~200µm).

Numerous trematode ova were observed in the fecal samples prior to treatment with praziquantel (Figure 3.3). However, fecal samples were clear of ova 4 days after praziquantel treatment. After consuming *D. auritus* infected catfish, trematode ova were observed in the feces of 1 experimental bird on days 3, 4, 6, and 8 post-infection. At 7 dpi, 1 trematode ovum was observed in a different experimental bird. At 8 dpi dead adult *D. auritus* were observed in the feces of all experimental DCCO, resulting in the necropsy of one bird. Live, developing adult *D. auritus* (n=2,103) were recovered from this bird and the experiment was allowed to continue. Adult *D. auritus* (n=7) was observed again in the feces of a single remaining experimental DCCO 14 dpi, along with a single ovum 17 dpi. At 18 dpi DCCO were euthanized and at necropsy a total of 250 adult *D. auritus* were recovered from intestines of the two experimental birds (n=64 from 10R; n=186 from U66) (Figure 3.4). No adult trematodes were recovered from either control bird and no eggs were observed in the feces.

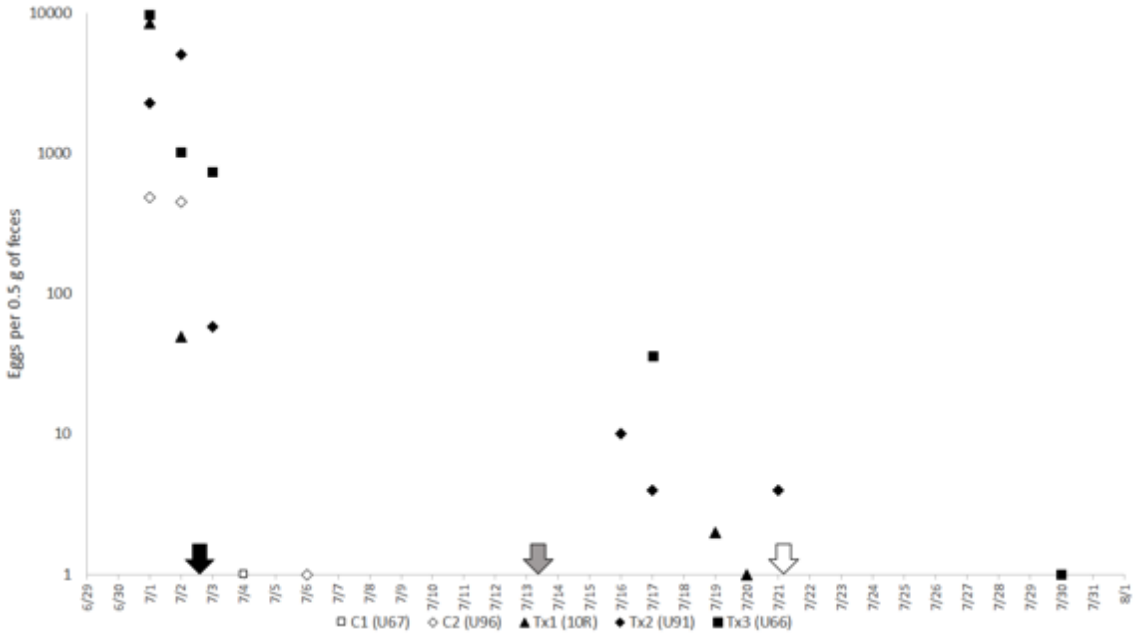

Figure 3.3 Fecal ova counts from 2015 life cycle study

Administration of praziquantel is indicated by the black arrow, feeding of parasitized fish by the gray arrow and necropsy of first experimental bird by the white arrow. Control birds (C) are designated by white markers, treatment (Tx) birds by black.

Figure 3.4 Adult *Drepanocephalus auritus*

Drepanocephalus auritus gravid adult recovered from the intestine of experimental double-crested cormorant at necropsy (bar ~500µm).

Sequence analysis of the CO1 gene from recovered adult specimens (n=5/bird) revealed 98.0%-100% similarity to published sequences of the North American *D. auritus* haplotype (Griffin et al., 2012; O'Hear et al., 2014; Kudlai et al., 2015; Van Steenkiste et al., 2015; Pinto et al., 2016; Alberson et al., 2017). A total of 53 adult *D. auritus* were stained (n=19 from U91; n=9 from 10R; n= 25 from U66). Although 5 stained specimens were gravid (n=1 from 10R; n=4 from U66), detailed measurements were obtained from only 4 gravid specimens since one specimen was damaged during the staining and mounting process. The following measurements were obtained from the stained gravid specimens according to Kudlai et al. (2015): body length (BL), body width at ventral sucker (BWVS), body width at anterior testis (BWAT), collar length (CL), collar width (CW), oral sucker length (OSL), oral sucker width (OSW), prepharynx length (PPL), prepharynx width (PPW), oesophagus lenth (OL), cirrus sac length (CSL), cirrus sac width (CSW), length of anterior portion of seminal vesicle (SV1L), width of

anterior portion of seminal vesicle (SV1W), length of posterior portion of seminal vesicle (SV2L), width of anterior portion of seminal vesicle (SV2W), ventral sucker length (VSL), ventral sucker width (VSW), ovary length (OVL), ovary width (OVW), Mehlis' gland length (MEL), Mehlis' gland width (MEW), anterior testis length (ATL), anterior testis width (ATW), posterior testis length (PTL), posterior testis width (PTW), egg length (EL), egg width (EW), forebody length (FORE), distance between ovary and posterior margin of ventral sucker (OVAR), and the distance between posterior margin of posterior testis and posterior extremity of body (TEND). Measurements of recovered adult trematodes were slightly less than those reported by Kudlai et al. (2015) (Table 3.2).

Table 3.2 Measurements of adult *Drepanocephalus auritus*

	Drepanocephalus auritus		
	Present study (2015)	Present study (2016)	Kudlai et al. (2015)‡
BL	4828±293 (4639-5334)	8101±444 (7497-8845)	8123 (6584-9129)
BWVS	665±22 (645-701)	972±58 (872-1056)	944 (779-1186)
BWAT	469±23 (431-490)	805±51 (737-898)	719 (531-974)
CL	474±26 (442-505)	625±32 (571-679)	638 (558-797)
CW	802±21 (769-827)	1249±79 (1132-1394)	1165 (991-1416)
OSL	212±7 (202-220)	312±11 (296-322)	274 (195-336)
OSW	181±8 (172-192)	289±16 (264-322)	264 (212-319)
PPL	62±18 (44-92)	68±13 (44-86)	73 (17-106)
PHL	204±5 (198-212)	313±11 (296-331)	287 (212-372)
PHW	134±7 (125-144)	199±11 (184-217)	171 (124-212)
OL	427±6 (421-436)	633±36 (556-680)	599 (53-761)
CSL	285±15 (264-307)	426±19 (398-463)	440 (320-578)

Table 3.2 (Continued)

CSW	199±6 (192-206)	312±26 (277-345)	257 (212-331)
SV1L	112±13 (97-128)	147±27 (100-191)	109 (88-159)
SV1W	96±22 (63-123)	92±7 (81-104)	104 (66-154)
SV2L	184±27 (158-227)	337±57 (234-444)	361 (278-498)
SV2W	167±13 (150-180)	207±33 (150-279)	193 (146-265)
VSL	620±16 (603-645)	856±84 (759-1034)	914 (797-1115)
VSW	543±9 (532-556)	822±45 (733-881)	692 (566-867)
OVL	129±21 (96-153)	264±17 (238-292)	225 (150-283)
OVW	98±9 (89-114)	207±13 (191-240)	186 (124-248)
MEL	162±17 (140-187)	259±27 (213-308)	182 (106-306)
MEW	116±14 (99-133)	251±30 (177-301)	252 (195-372)
ATL	345±25 (326-388)	545±39 (466-596)	560 (389-708)
ATW	246±17 (222-270)	409±32 (357-452)	421 (283-549)
PTL	364±39 (313-415)	620±28 (577-661)	579 (443-689)
PTW	244±14 (220-258)	427±37 (373-492)	410 (319-549)
EL	80±9 (65-90)	87±9 (68-99)	88 (74-99)
EW	53±1 (41-54)	54±6 (44-68)	52 (42-61)
FORE	1021±74 (921-1110)	1418±49 (1316-1482)	1459 (1239-1628)
OVAR	299±14 (280-319)	769±99 (679-953)	809 (531-1530)
TEND	1736±172 (1626-2033)	2870±275 (2501-3364)	2857 (2372-3264)

*Measurements in micrometers
†The mean is followed by standard deviation with ranges in parentheses.
‡Standard deviation not reported
BL – body length; BWVS – body width at ventral sucker; BWAT – body width at anterior testis; CL – collar length; CW – collar width; OSL – oral sucker length; OSW – oral sucker width; PL – prepharynx length; PPW – prepharynx width; OL – oesophagus length, CSL – cirrus sac length; CSW – cirrus sac width; SV1L – length of anterior portion of seminal vesicle; SV1W - width of anterior portion of seminal vesicle; SV2L - length of posterior portion of seminal vesicle; SV2W - width of anterior portion of seminal vesicle; VSL - ventral sucker length; VSW - ventral sucker width ; OVL - ovary length; OVW - ovary width; MEL - Mehlis' gland length; MEW - Mehlis' gland width; ATL - anterior testis length; ATW - anterior testis width; PTL - posterior testis length; PTW - posterior testis width; EL - egg length; EW - egg width ; FORE - forebody length; OVAR - distance between ovary and posterior margin of ventral sucker; TEND - distance between posterior margin of posterior testis and posterior extremity of body

3.4.1.2 Snails

In the initial infection study (2015), one snail from each species was observed shedding cercariae morphologically consistent with *D. auritus* 54 d after being placed into the experimental pond. In the staggered snail infectivity study (2015), 1 *P. trivolvis* each from Groups 1 and 2 began shedding cercariae morphologically consistent with *D. auritus* on days 26 and 18 post-exposure, respectively. No cercariae were observed from any snails from Group 3.

3.4.2 Life Cycle Study 2016

3.4.2.1 Birds and Fish

The number of metacercariae per fish was estimated from histological sections of a subset of parasitized fish. Often parasites were present in the lateral line system, particularly the head, with accompanying inflammation (Figure 3.5). Meanwhile, parasites present in the gills, often near the base of the gill filament (Figure 3.6), tended to lack inflammation, which when present was composed largely of macrophages and lymphocytes.

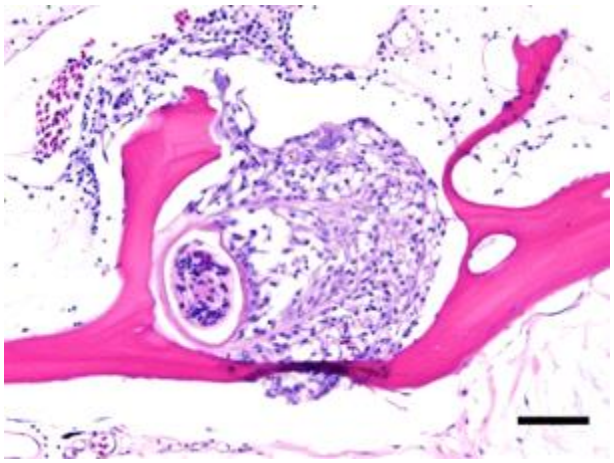

Figure 3.5 *Drepanocephalus auritus* metacercaria

Drepanocephalus auritus metacercaria present in the head with accompanying inflammation (H&E; bar ~50µm).

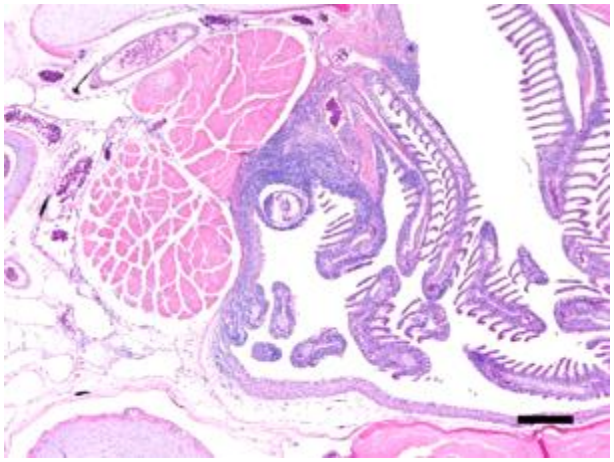

Figure 3.6 *Drepanocephalus auritus* metacercaria

Drepanocephalus auritus metacercaria present in the base of the gill (H&E; bar ~200 µm).

Metacercariae were also recovered by pepsin digest from 4 out of 5 fish from the first fish exposure and 5 of 5 fish from the second. All metacercariae were PCR positive

by *Drepanocephalus* spp. PCR and CO1 sequence from one metacercariae was a 99-100% match to *D. auritus* sequences in GenBank.

Numerous trematode ova were observed in feces collected from DCCO prior to praziquantel administration (Figure 3.7). After receiving praziquantel, DCCO fecal samples were cleared of trematode ova within 3 days. The first experimental DCCO (XR30) began shedding trematode ova 16 dpi (n=619 ova in 0.4g feces), while the second (XR44) began shedding ova 8 dpi (n=9 ova in 0.1g feces). The largest number of trematode ova was observed 13 dpi in bird XR44, with 2,383 ova shed in 0.3 g of feces. No ova were observed in feces collected from control birds after praziquantel administration.

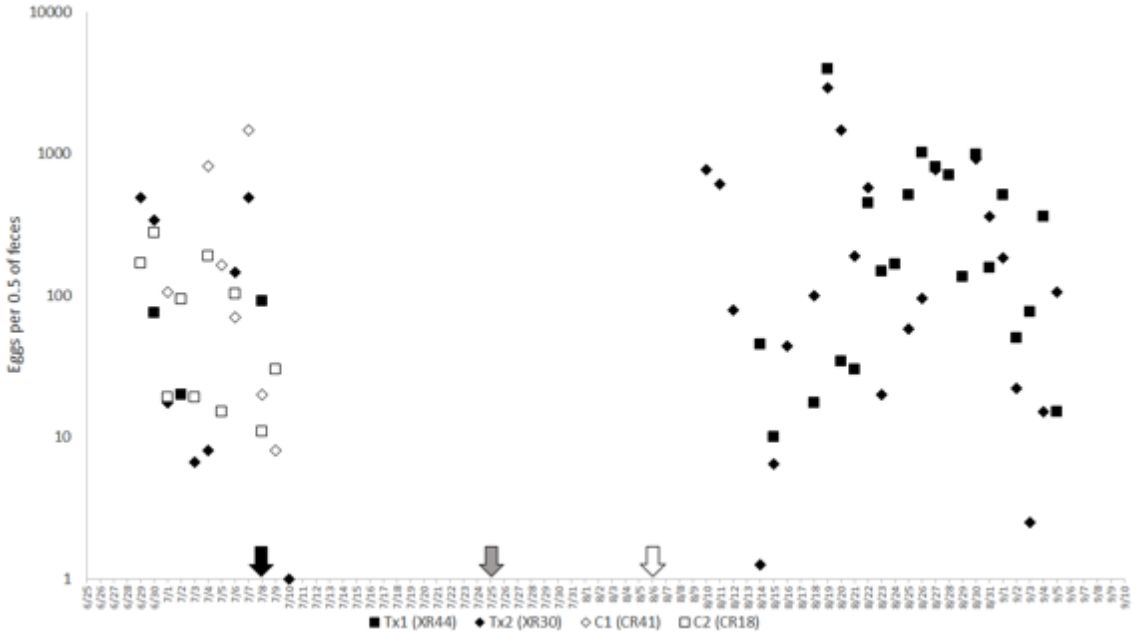

Figure 3.7 Fecal ova counts from 2016 life cycle study

Administration of praziquantel is indicated by the black arrow, feeding of parasitized fish to experimental bird XR30 by the gray arrow and feeding of parasitized fish to experimental bird XR44 by the white arrow

Upon necropsy, a total of 49 live adult *D. auritus* were recovered from intestines of both experimental DCCO. Twenty-nine adult *D. auritus* were recovered from XR30, while 20 were recovered from XR44. No trematodes were recovered from controls. PCR using primers encompassing ~189 bp region of the CO1 gene and specific for *Drepanocephalus* spp. confirmed specimens collected from experimental DCCO to be a *Drepanocephalus* sp. Sequencing of the CO1 gene further confirmed this finding, revealing the recovered adults to be a 99%-100% match to published *D. auritus* sequences in the NCBI nr/nt database.

All 10 stained *D. auritus* (n=5/bird) were gravid, but only 9 were photographed and measured on account of one specimen being damaged during the mounting process. As above, measurements obtained from the stained specimens were consistent with those reported by Kudlai et al. (2015) (Table 3.2) (Figure 3.8).

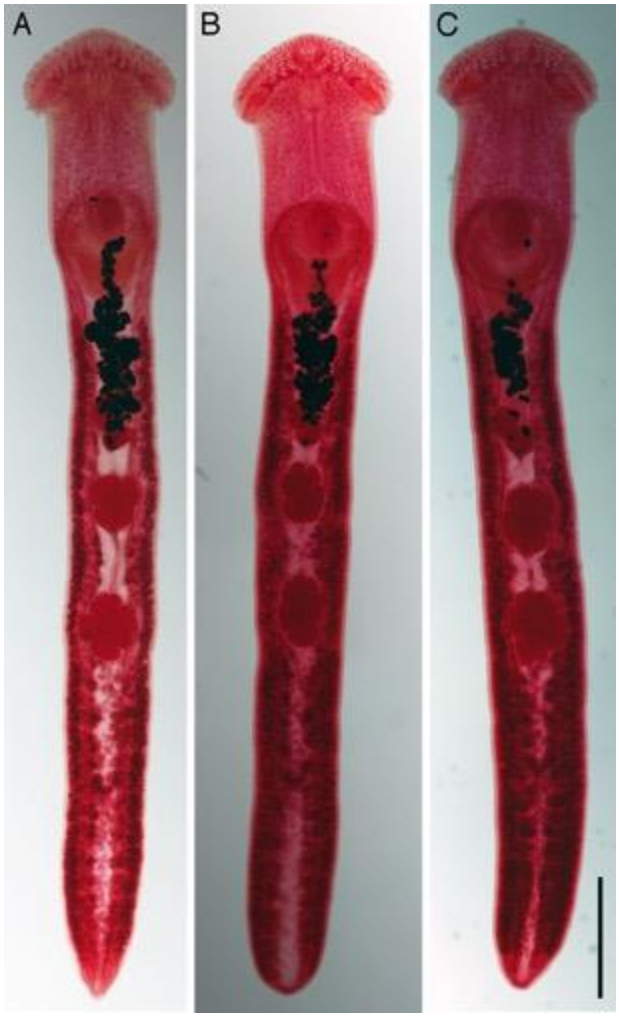

Figure 3.8 *Drepanocephalus auritus* adults

Three *Drepanocephalus auritus* adults recovered from intestines of experimental double-crested cormorants (bar ~1000µm)

3.4.2.2 Snails

The first group of *P. trivolvis* held in aviary tanks coincided with experimental bird XR30 and control bird CR18. They were held for 44 d before being removed. A second group of *P. trivolvis*, coinciding with experimental bird XR44 and control bird CR41, remained in the tanks for 39 d before removal. A single snail from the XR30/CR18 group began shedding cercariae consistent with *D. auritus* 97 d after

exposure to tank water containing DCCO feces. Sequencing of the CO1 gene revealed the cercariae were a 99%-100% match to published *D. auritus* sequences in the NCBI nr/nt database.

Similarly, a single snail exposed to trematode ova in the laboratory was observed shedding cercariae morphologically consistent with *D. auritus* 89 d after being exposed to *D. auritus* ova. Again, sequencing of the CO1 gene revealed the cercariae were a 99%-100% match to published *D. auritus* sequences in the NCBI nr/nt database.

Cercariae were not observed from any *B. havanensis* held in the aviary pond housing 5 DCCO. Comparatively, 5 *P. trivolvis* from the first exposure group shed cercariae consistent with *D. auritus* at 37 dpi (n=1), 44 dpi (n=3) and 51 dpi (n=1). Only one *P. trivolvis* from the second group was observed shedding cercariae consistent with *D. auritus* at 43 dpi.

3.4.2.3 Trematode ova

Two miracidia were observed 16 days post-inoculation from the 100 *D. auritus* ova held in sterile spring water in a glass petri dish at ambient temperatures (~23-25°C). Remaining ova were checked daily for another 18 d, but neither miracidia development nor hatching was observed, and ova began to deteriorate. For fresh feces, three miracidia hatched 15 d after being placed in a 24-well plate held at ambient temperatures (~23-25°C). A second hatched on day 17, while another hatched on day 21. Similarly, ova recovered from feces kept at ~4 C for 15 d released miracidia 15 d after being removed from cold storage (Figures 3.9 and 3.10).

Figure 3.9 *Drepanocephalus auritus* miracidium in ovum

Developing *Drepanocephalus auritus* miracidium present in ovum collected from experimental double-crested cormorant feces (40x magnification; bar ~100µm).

Figure 3.10 *Drepanocephalus auritus* miracidium

Miracidium hatched from ovum recovered from experimental double-crested cormorant feces (60x magnification; bar ~10µm).

3.5 Discussion

In experimental infections, it was found *D. auritus* cercariae penetrate and infect the juvenile catfish host, causing mortalities within the first 7 d post-exposure (Griffin et al., 2012; 2014; Alberson et al., 2017). In surviving fish, metacercariae are seen at 7 dpi, mostly in the cranial region (Griffin et al., 2012; 2014; Alberson et al., 2017). However, infection begins to resolve as early as 7-21 dpi (Griffin et al. 2014), raising the question of whether channel catfish can serve as an intermediate host in the *D. auritus* life cycle. At present, there have been no reported natural infections in channel catfish. During a similar infectivity study, Doffitt et al. (2009) reported a single adult trematode, morphologically identified as *Drepanocephalus spathans*, in a DCCO fed channel catfish naturally infected with *Bolbophorus damnificus*. They speculated that birds were either fed a *D. auritus* infected catfish, or gastrointestinal parasites were ineffectively cleared prior to inoculation. Moreover, Pinto et al. (2016) attempted to complete the life cycle, successfully infecting the guppy *Poecilia reticulata* with *D. auritus* cercariae and extracting metacercariae from the caudal fin and gills. Metacercariae were fed to chicks *Gallus gallus domesticus* and mice *Mus musculus*, but adult *D. auritus* were not recovered in hosts euthanized at 5 dpi. This current work confirms previous speculation and demonstrates channel catfish can serve as an intermediate host for *D. auritus*.

The work described herein also supports previous studies that suggest crowding can impact parasite size and maturation. The adults recovered in the 2015 study measured smaller than what was reported for *D. auritus* by Kudlai et al. (2015). It is suspected these adults were smaller in size due to crowding, a phenomenon first reported by Read (1951) in cestodes, wherein parasite size decreases as the number of parasites increase.

This crowding effect has also been reported in trematodes. Fried and Freeborne (1984) observed decreased body area of *Echinostoma revolutum* found in crowded sites versus non-crowded sites in intestines of chickens. Franco et al. (1988) also looked at the crowding effect of *E. revolutum* in golden hamster (*Mesocricetus auratus*). They found intraspecific crowding extends maturation time and reduces overall body size. Similarly, Valero et al. (2006) studied the effects of crowding on growth, pre-patent period, and the release of eggs of *Fasciola hepatica*. They reported the higher the number of parasites, the smaller the adults measured, and pre-patent period and egg production both decreased as the parasite numbers increased. Along those lines, Stillson and Platt (2007) looked at the effects of crowding on morphometric variability of *Echinostoma caproni* in mice and found size reductions in organs, body measurements, muscular structures, and eggs. During their study, Stillson and Platt (2007) recovered low numbers of adult *E. caproni* from a mouse infected with 300 metacercariae and suggested the possibility of the expulsion of juveniles due to an inflammatory response, and this response may play a role in crowding associated arrested development.

In the 2015 study, estimations of metacercariae numbers in fish suggest the possibility of DCCO ingesting up to ~ 550 metacercariae. Once *D. auritus* adults were observed in the feces of experimental DCCO at 8 dpi, one bird was sacrificed to evaluate infection. Over 2,000 *D. auritus* were recovered, suggesting histology is not an accurate method of metacercariae estimation. In the second life cycle study (2016), it was projected that each experimental bird could have received up to ~350 metacercariae, although these numbers are thought to be more accurate as they were largely derived from pepsin digests. At no time during this second study were adults seen in the feces and

at necropsy much fewer *D. auritus* were recovered. Furthermore, the adults recovered in the 2016 study were much larger than those recovered in the 2015 study, and were consistent with measurements reported by Kudlai et al. (2015), although it should be noted these necropsies occurred at 31 and 43 dpi, or 13 to 25 days later in 2016 than in 2015.

Similarly, ova measured from stained gravid adult *D. auritus* from both studies were within the range reported by Kudlai et al. (2015). In the 2015 study, ova were first observed in the feces of experimental DCCO 3 dpi. Ova continued to be observed on days 4, 6, 7, 8, and 17 dpi. During the second study (2016), ova were not observed until 16 dpi in the first DCCO infected, and 8 dpi in the second DCCO. It is speculated these early ova observed in fecal samples during the first study could be from residual feces leftover after the daily cleaning process and did not represent a patent infection, while later ova, observed on days 8 and 17, was consistent with the 2016 study and were more likely shed by gravid *D. auritus*. However, the absence of "residual" eggs in control pens and the fact these "residual eggs" were not observed until after birds were fed parasitized fish suggest these eggs represent patent infection the the presence of gravid adults, which was confirmed at necropsy.

Drepanocephalus auritus miracidia first emerged from ova collected from fresh DCCO feces 15 d after being placed in water held at ambient temperatures (~23-25 C), with the last miracidium emerging at 21 d. Miracidia also emerged from ova at day 15 following an initial 15 day incubation at 4 C prior to plating and subsequent incubation at ambient temperatures. Emergence of miracidia from ova kept at these lower temperatures is similar to miracidia emergence from freshly deposited ova. This likely represents a

dormant phase during cool temperatures. It is speculated that ova remain dormant, yet viable throughout the winter when water temperatures in catfish ponds are <10 °C, and miracidia emergence corresponds with rising water temperatures in the spring, which likely coordinates with the emergence of the snail host.

At warmer water temperatures (~27-30 C), cercariae were released from *P. trivolvis* as early as 18 d after being placed into experimental ponds. Comparatively, cercariae emergence took upwards of 51 d when water temperatures were <27 C or >30 C, suggesting an optimal range for intramolluscan development. In laboratory infections at ambient temperatures, it took 89 d from ova exposure to the release of cercariae (~23-25 C). Based on our observations here that miracidia hatch within 21 days at these temperatures, this would suggest an arrested development of 60-70 days compared to the 18 d incubation observed at ~27-30 C.

When water temperatures are optimal, it may only take the snail host ~2 weeks to become infected and begin releasing cercariae, but when water temperatures are low it could take as long as 97 d. Ova remain viable after being subjected to water temperatures below those that occur in catfish ponds during winter in Mississippi, and once water temperatures begin to rise, miracidium emergence takes a little over 2 weeks. However, the true length ova can remain viable at these lower temperatures requires further study.

This study also demonstrates the capacity for large numbers of *D. auritus* ova to be introduced into catfish production systems. O'Hear (2014) reported intensities in DCCO as high as 346 adult worms per bird. The shedding rates observed here for birds with much milder infections (20-29 adult worms per bird) still released >1,000 ova/g of feces (wet weight). According to studies by Brugger (1993) and Marion et al. (1994),

cormorants can defecate up to 30 g dry weight of feces daily. During a DCCO depredation study on catfish ponds in the Delta region of Mississippi, Stickley et al. (1992) reported as many as 85 cormorants on a pond in a single day. At low intensities, it is likely that up to 30,000 ova can be shed by a single DCCO daily, and a flock of 50 DCCO can shed up to 1.5 million eggs daily. Estimates of DCCO populations in the Delta region of Mississippi range from 30,000 to >80,000 birds over the course of a year (Glahn and Stickley, 1995; Glahn et al., 1996; Glahn et al., 2000; Dorr et al., 2008; Dorr et al., 2012). When one considers these estimates, the number of *D. auritus* ova introduced to catfish ponds can become numerous in a hurry.

Over the course of this study, each step of the *D. auritus* life cycle was completed, from natural infections in *P. trivolvis*, to successful recovery of gravid *D. auritus* in the double-crested cormorant definitive host and experimental infection of *P. trivolvis* in the laboratory from ova recovered from feces of experimentally infected DCCO. Observations from the ova laboratory study bring about the possibility that ova can remain dormant in catfish ponds during the winter months in Mississippi, with miracidia hatching somewhat synchronously once water temperatures warm up in the spring. This hatching also corresponds with snail emergence, making control of the snail hosts extremely important, since controlling the DCCO definitive host is logistically challenging. The information gathered from this research is useful to commercial catfish producers and fish health professionals, as data regarding cercariae release and miracidia emergence can be used to implement best management practices to reduce incidence and prevalence of digenetic trematodes in catfish aquaculture operations.

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CHAPTER IV

CONCLUSION

This research was the first to successfully link all life cycle stages of *Drepanocephalus auritus* in the marsh rams-horn snail *Planorbella trivolvis*, channel catfish *Ictalurus punctatus*, and double-crested cormorant *Phalacrocorax auritus*. Along with this information, a new intermediate host, *Biomphalaria havanensis*, was confirmed in channel catfish aquaculture ponds in east Mississippi. Moreover, this work demonstrated that *B. havanensis* recovered from commercial catfish operations in Mississppi is not a viable host for *Schistosoma mansoni.*

During a malacological survey carried out in two separate catfish ponds in east Mississippi, *B. havanensis* were found shedding cercariae morphologically consistent with and molecularly confirmed as *D. auritus*. Of the 1,704 *B. havanensis* collected and examined, 13 (1.5%) were releasing *D. auritus* cercariae, representing North and South American haplotypes. Channel catfish were challenged individually with ~275 cercariae/fish. Limited mortality occurred within the first 48 hr, consistent with previous studies. Fish were euthanized 7 days post-challenge and histology confirmed the presence of metacercariae in 6 of 8 fish. Metacercariae were observed in the branchial cavity, periocular tissues, and lateral line system, consistent with previous challenges using *D. auritus* cercariae released by *P. trivolvis*.

It is not surprising that *B. havanensis* was discovered releasing North and South American haplotypes of *D. auritus*. The double-crested cormorant is widely distributed across North America and can be founds as far south as Cuba. Oftentimes double-crested cormorants are found mixing with neotropic cormorant populations in the Gulf Coast region of Texas and Louisiana, as well as Mexico, Cuba, and the Bahamas. The double-crested cormorant's vast migratory range likely drives the parasitic diversity that is often seen in snail populations inhabiting catfish aquaculture ponds in Mississippi.

Drepanocephalus auritus cercariae have been shown to infect channel catfish experimentally, however, metacercaria development can begin to resolve as early as 7 days post-challenge. Based on these experimental observations, it was questionable whether channel catfish could serve as a true intermediate host in the *D. auritus* life cycle. The results of the initial *D. auritus* life cycle study (2015) revealed channel catfish are a suitable host for the propagation of this life cycle. Juvenile channel catfish were individually challenged with ~150 *D. auritus* cercariae/fish, and at 5 days post-challenge metacercariae were observed histologically. Infected fish were fed to 3 experimental double-crested cormorants. A single bird was euthanized at 8 days post-infection, while the remaining birds were euthanized at 18 days post-infection. Adult *D. auritus* were recovered from all experimental birds and their identity molecularly confirmed.

During the second life cycle study, all stages were observed. Juvenile channel catfish were challenged individually with ~100-150 *D. auritus* cercariae/fish, collected from naturally infected *P. trivolvis* snails. Metacercariae molecularly confirmed to be *D. auritus* were recovered by artificial digest and observed histologically at 5 days post-challenge. Infected fish were fed to experimental double-crested cormorants, and

trematode ova were observed in feces of experimental birds beginning at 8 and 16 days post-infection. Miracidia began hatching from ova within 15 days in water temperatures ranging from ~23-25 C. Two *P. trivolvis,* exposed to *D. auritus* ova, began releasing cercariae molecularly confirmed to be *D. auritus* 89 and 97 days after being exposed to ova, thus completing the life cycle. Upon necropsy, adult *D. auritus* were recovered from experimental double-crested cormorants and morphologically and molecularly confirmed.

Planorbella trivolvis and *B. havanensis* were observed releasing *D. auritus* cercariae 54 d after being placed into an experimental catfish pond inhabited by double-crested cormorants. During this initial infectivity trial, air temperatures ranged from 20 C to 29 C while water temperatures ranged from 13 C to 28 C. A second staggered infectivity trial, involving smaller groups of 10 *P. trivolvis*, was completed while water temperatures ranged from 28-34 C. During this trial, one snail from Groups 1 and 2 were observed releasing *D. auritus* cercariae on days 26 and 18, respectively. The following year, a second trial was completed and within 37 days of being placed into experimental catfish ponds inhabited by another batch of double-crested cormorants, a single *P. trivolvis* snail was observed releasing *D. auritus* cercariae. Three snails released cercariae at 44 dpi, and one released cercariae at 51 dpi. A single snail from Group 2, placed into the pond 40 days after Group 1, released cercariae at 43 dpi. Air temperatures during this trial ranged from 22 C to 40 C, and water temperatures ranged from 25 C to 33 C. According to this data, it would appear that during the late spring and early summer, while water temperatures are in the optimal range, it may take approximately 2-3 weeks for *P. trivolvis* to become infected with *D. auritus* and begin releasing cercariae.

The presence of digenetic trematodes in channel catfish aquaculture ponds has been proven consequential to catfish health. These ponds offer the perfect system for the propagation of trematode life cycles, including *D. auritus*. The most effective way to break these life cycles is through the eradication of the snail host. In order to effectively implement the best control strategy, it is important to gather as much information as possible on the hosts and timing of infection. This research provided data concerning the developmental timelines of *D. auritus* infection in the snail, fish, and bird host, and was the first experimental confirmation of this life cycle. Moreover, this work identified a new snail host in the *D. auritus* life cycle. This data is critical when devising management schemes for the control of trematode infections in channel catfish production.

APPENDIX A

DAILY OVA COUNTS FROM THE 2015 AND 2016

LIFE CYCLE STUDIES

Table A.1 Daily ova counts from 2015 life cycle study

Ova counts from double-crested cormorants

Date	Pen	Bird ID	Treatment/ control (Tx/C)	Feces weight (g)	Number of ova	Action
7/1/15	2	U67	C	–	N/S	
7/1/15	4	U96	C	0.5	486	
7/1/15	6	10R	Tx	0.5	8500	
7/1/15	10	U91	Tx	0.5	2272	
7/1/15	14	U66	Tx	0.5	9783	
7/2/15	2	U67	C	–	N/S	Received praziquantel
7/2/15	4	U96	C	0.5	449	Received praziquantel
7/2/15	6	10R	Tx	0.5	49	Received praziquantel
7/2/15	10	U91	Tx	0.5	5072	Received praziquantel
7/2/15	14	U66	Tx	0.5	1021	Received praziquantel
7/3/15	2	U67	C	0.5	NES	
7/3/15	4	U96	C	0.5	NES	
7/3/15	6	10R	Tx	0.5	NES	
7/3/15	10	U91	Tx	0.5	58	
7/3/15	14	U66	Tx	0.5	734	
7/4/15	2	U67	C	0.5	1	
7/4/15	4	U96	C	–	N/S	

Table A.1 (Continued)

7/4/15	6	10R	Tx	–	N/S
7/4/15	10	U91	Tx	–	N/S
7/4/15	14	U66	Tx	0.5	NES
7/5/15	2	U67	C	–	N/S
7/5/15	4	U96	C	–	N/S
7/5/15	6	10R	Tx	–	N/S
7/5/15	10	U91	Tx	0.5	NES
7/5/15	14	U66	Tx	0.5	NES
7/6/15	2	U67	C	0.5	NES
7/6/15	4	U96	C	0.5	1
7/6/15	6	10R	Tx	–	N/S
7/6/15	10	U91	Tx	0.5	NES
7/6/15	14	U66	Tx	0.5	NES
7/7/15	2	U67	C	0.5	NES
7/7/15	4	U96	C	–	N/S
7/7/15	6	10R	Tx	–	N/S
7/7/15	10	U91	Tx	0.5	NES
7/7/15	14	U66	Tx	0.5	NES
7/8/15	2	U67	C	0.5	NES

Table A.1 (Continued)

7/8/15	4	U96	C	–	N/S
7/8/15	6	10R	Tx	0.5	NES
7/8/15	10	U91	Tx	0.5	NES
7/8/15	14	U66	Tx	0.5	NES
7/9/15	2	U67	C	0.5	NES
7/9/15	4	U96	C	–	N/S
7/9/15	6	10R	Tx	–	N/S
7/9/15	10	U91	Tx	0.5	NES
7/9/15	14	U66	Tx	0.5	NES
7/10/15	2	U67	C	0.5	NES
7/10/15	4	U96	C	0.5	NES
7/10/15	6	10R	Tx	–	N/S
7/10/15	10	U91	Tx	0.5	NES
7/10/15	14	U66	Tx	–	N/S
7/11/15	2	U67	C	0.5	NES
7/11/15	4	U96	C	0.5	NES
7/11/15	6	10R	Tx	–	N/S
7/11/15	10	U91	Tx	0.5	NES
7/11/15	14	U66	Tx	0.5	NES

Table A.1 (Continued)

7/12/15	2	U67	C	0.5	NES	
7/12/15	4	U96	C	0.5	NES	
7/12/15	6	10R	Tx	–	N/S	
7/12/15	10	U91	Tx	0.5	NES	
7/12/15	14	U66	Tx	0.5	NES	
7/13/15	2	U67	C	0.5	NES	Fed SPF channel catfish
7/13/15	4	U96	C	–	N/S	Fed SPF channel catfish
7/13/15	6	10R	Tx	0.5	NES	Fed 91 *D. auritus* infected catfish
7/13/15	10	U91	Tx	0.5	NES	Fed 91 *D. auritus* infected catfish
7/13/15	14	U66	Tx	0.5	NES	Fed 91 *D. auritus* infected catfish
7/14/15	2	U67	C	0.5	NES	
7/14/15	4	U96	C	0.5	NES	
7/14/15	6	10R	Tx	0.5	NES	
7/14/15	10	U91	Tx	0.5	NES	
7/14/15	14	U66	Tx	0.5	NES	
7/15/15	2	U67	C	0.5	NES	
7/15/15	4	U96	C	0.5	NES	
7/15/15	6	10R	Tx	0.5	NES	
7/15/15	10	U91	Tx	0.5	NES	

Table A.1 (Continued)

7/15/15	14	U66	Tx	0.5	NES
7/16/15	2	U67	C	0.5	NES
7/16/15	4	U96	C	0.5	NES
7/16/15	6	10R	Tx	0.5	NES
7/16/15	10	U91	Tx	0.5	10
7/16/15	14	U66	Tx	0.5	NES
7/17/15	2	U67	C	0.5	NES
7/17/15	4	U96	C	0.5	NES
7/17/15	6	10R	Tx	0.5	NES
7/17/15	10	U91	Tx	0.5	4
7/17/15	14	U66	Tx	0.5	36
7/18/15	2	U67	C	0.5	NES
7/18/15	4	U96	C	0.5	NES
7/18/15	6	10R	Tx	0.5	NES
7/18/15	10	U91	Tx	0.5	NES
7/18/15	14	U66	Tx	0.5	NES
7/19/15	2	U67	C	0.5	NES
7/19/15	4	U96	C	0.5	NES
7/19/15	6	10R	Tx	0.5	2

Table A.1 (Continued)

7/19/15	10	U91	Tx	0.5	NES	
7/19/15	14	U66	Tx	0.5	NES	
7/20/15	2	U67	C	0.5	NES	
7/20/15	4	U96	C	0.5	NES	
7/20/15	6	10R	Tx	0.5	1	
7/20/15	10	U91	Tx	0.5	NES	
7/20/15	14	U66	Tx	0.5	NES	
7/21/15	2	U67	C	0.5	NES	
7/21/15	4	U96	C	0.5	NES	
7/21/15	6	10R	Tx	0.5	NES	Dead adults passed in feces
7/21/15	10	U91	Tx	0.5	4	Dead adults passed in feces; euthanized and necropsied
7/21/15	14	U66	Tx	0.5	NES	Dead adults found in feces
7/22/15	2	U67	C	0.5	NES	
7/22/15	4	U96	C	0.5	NES	
7/22/15	6	10R	Tx	0.5	NES	
7/22/15	14	U66	Tx	0.5	NES	
7/23/15	2	U67	C	0.5	NES	
7/23/15	4	U96	C	–	N/S	
7/23/15	6	10R	Tx	0.5	NES	

Table A.1 (Continued)

7/23/15	14	U66	Tx	0.5	NES	
7/24/15	2	U67	C	–	N/S	
7/24/15	4	U96	C	0.5	NES	
7/24/15	6	10R	Tx	–	N/S	
7/24/15	14	U66	Tx	0.5	NES	
7/25/15	2	U67	C	0.5	NES	
7/25/15	4	U96	C	0.5	NES	
7/25/15	6	10R	Tx	0.5	NES	
7/25/15	14	U66	Tx	0.5	NES	
7/26/15	2	U67	C	0.5	NES	
7/26/15	4	U96	C	0.5	NES	
7/26/15	6	10R	Tx	0.5	NES	
7/26/15	14	U66	Tx	0.5	NES	
7/27/15	2	U67	C	0.5	NES	
7/27/15	4	U96	C	0.5	NES	
7/27/15	6	10R	Tx	0.5	NES	
7/27/15	14	U66	Tx	0.5	NES	7 adults passed in feces
7/28/15	2	U67	C	0.5	NES	
7/28/15	4	U96	C	–	N/S	

Table A.1 (Continued)

7/28/15	6	10R	Tx	0.5	NES	
7/28/15	14	U66	Tx	0.5	NES	
7/29/15	2	U67	C	–	N/S	
7/29/15	4	U96	C	0.5	NES	
7/29/15	6	10R	Tx	–	N/S	
7/29/15	14	U66	Tx	0.5	NES	
7/30/15	2	U67	C	–	N/S	Euthanized and necropsied; no trematodes found
7/30/15	4	U96	C	0.5	NES	Euthanized and necropsied; no trematodes found
7/30/15	6	10R	Tx	–	N/S	Euthanized and necropsied; 64 adult *D. auritus* recovered
7/30/15	14	U66	Tx	0.5	1	Euthanized and necropsied; 186 adult *D. auritus* recovered

(NES = no eggs seen; N/S = no sample)

Table A.2 Daily ova counts from 2016 life cycle study

Ova counts from double-crested cormorants						
Date	Pen	Bird ID	Treatment/ control (Tx/C)	Feces weight (g)	Number of ova	Action
6/29/16	11	XR44	Tx	0.1	NES	
6/29/16	13	XR30	Tx	0.5	486	
6/29/16	23	CR41	C	–	N/S	
6/29/16	25	CR18	C	0.6	202	
6/30/16	11	XR44	Tx	0.5	75	
6/30/16	13	XR30	Tx	0.5	341	
6/30/16	23	CR41	C	0.5	NES	
6/30/16	25	CR18	C	0.5	277	
7/1/16	11	XR44	Tx	–	N/S	
7/1/16	13	XR30	Tx	0.6	21	
7/1/16	23	CR41	C	0.1	21	
7/1/16	25	CR18	C	0.5	19	
7/2/16	11	XR44	Tx	0.5	20	
7/2/16	13	XR30	Tx	0.5	NES	
7/2/16	23	CR41	C	–	N/S	
7/2/16	25	CR18	C	0.5	94	
7/3/16	11	XR44	Tx	–	N/S	

Table A.2 (Continued)

7/3/16	13	XR30	Tx	0.3	4
7/3/16	23	CR41	C	–	N/S
7/3/16	25	CR18	C	0.4	19
7/4/16	11	XR44	Tx	–	N/S
7/4/16	13	XR30	Tx	0.5	8
7/4/16	23	CR41	C	0.1	163
7/4/16	25	CR18	C	0.5	190
7/5/16	11	XR44	Tx	–	N/S
7/5/16	13	XR30	Tx	–	N/S
7/5/16	23	CR41	C	0.5	163
7/5/16	25	CR18	C	0.5	15
7/6/16	11	XR44	Tx	–	N/S
7/6/16	13	XR30	Tx	0.5	146
7/6/16	23	CR41	C	0.1	14
7/6/16	25	CR18	C	0.5	61
7/7/16	11	XR44	Tx	–	N/S
7/7/16	13	XR30	Tx	0.3	295
7/7/16	23	CR41	C	0.5	1456
7/7/16	25	CR18	C	0.3	NES

Table A.2 (Continued)

7/8/16	11	XR44	Tx	0.5	91	Received praziquantel
7/8/16	13	XR30	Tx	0.1	NES	Received praziquantel
7/8/16	23	CR41	C	0.5	20	Received praziquantel
7/8/16	25	CR18	C	0.5	11	Received praziquantel
7/9/16	11	XR44	Tx	0.1	NES	
7/9/16	13	XR30	Tx	–	N/S	
7/9/16	23	CR41	C	0.5	8	
7/9/16	25	CR18	C	0.5	6	
7/10/16	11	XR44	Tx	–	N/S	
7/10/16	13	XR30	Tx	0.5	1	
7/10/16	23	CR41	C	–	N/S	
7/10/16	25	CR18	C	0.1	NES	
7/11/16	11	XR44	Tx	–	N/S	
7/11/16	13	XR30	Tx	0.5	NES	
7/11/16	23	CR41	C	0.5	NES	
7/11/16	25	CR18	C	0.3	NES	
7/12/16	11	XR44	Tx	0.1	N/S	
7/12/16	13	XR30	Tx	0.1	NES	
7/12/16	23	CR41	C	0.5	NES	

Table A.2 (Continued)

7/12/16	25	CR18	C	0.2	NES
7/13/16	11	XR44	Tx	0.1	NES
7/13/16	13	XR30	Tx	0.3	NES
7/13/16	23	CR41	C	0.3	NES
7/13/16	25	CR18	C	0.5	NES
7/14/16	11	XR44	Tx	0.1	NES
7/14/16	13	XR30	Tx	–	N/S
7/14/16	23	CR41	C	0.1	NES
7/14/16	25	CR18	C	0.5	NES
7/15/16	11	XR44	Tx	–	N/S
7/15/16	13	XR30	Tx	0.4	NES
7/15/16	23	CR41	C	–	N/S
7/15/16	25	CR18	C	0.5	NES
7/16/16	11	XR44	Tx	0.1	NES
7/16/16	13	XR30	Tx	–	N/S
7/16/16	23	CR41	C	0.5	NES
7/16/16	25	CR18	C	0.5	NES
7/17/16	11	XR44	Tx	0.1	NES
7/17/16	13	XR30	Tx	0.5	NES

Table A.2 (Continued)

7/17/16	23	CR41	C	0.5	NES
7/17/16	25	CR18	C	0.1	NES
7/18/16	11	XR44	Tx	0.1	NES
7/18/16	13	XR30	Tx	–	N/S
7/18/16	23	CR41	C	0.5	NES
7/18/16	25	CR18	C	0.1	NES
7/19/16	11	XR44	Tx	–	N/S
7/19/16	13	XR30	Tx	0.1	NES
7/19/16	23	CR41	C	0.5	NES
7/19/16	25	CR18	C	0.1	NES
7/20/16	11	XR44	Tx	–	N/S
7/20/16	13	XR30	Tx	0.1	NES
7/20/16	23	CR41	C	0.3	NES
7/20/16	25	CR18	C	0.1	NES
7/21/16	11	XR44	Tx	–	N/S
7/21/16	13	XR30	Tx	–	N/S
7/21/16	23	CR41	C	–	N/S
7/21/16	25	CR18	C	0.5	NES
7/22/16	11	XR44	Tx	–	N/S

Table A.2 (Continued)

7/22/16	13	XR30	Tx	0.1	NES	
7/22/16	23	CR41	C	0.2	NES	
7/22/16	25	CR18	C	0.4	NES	
7/23/16	11	XR44	Tx	0.1	NES	
7/23/16	13	XR30	Tx	–	N/S	
7/23/16	23	CR41	C	0.5	NES	
7/23/16	25	CR18	C	0.5	NES	
7/24/16	11	XR44	Tx	0.1	NES	
7/24/16	13	XR30	Tx	–	N/S	
7/24/16	23	CR41	C	0.4	NES	
7/24/16	25	CR18	C	0.2	NES	
7/25/16	11	XR44	Tx	–	N/S	
7/25/16	13	XR30	Tx	–	N/S	Fed 40 *D. auritus* infected catfish
7/25/16	23	CR41	C	–	N/S	
7/25/16	25	CR18	C	0.3	NES	
7/26/16	11	XR44	Tx	0.3	NES	
7/26/16	13	XR30	Tx	0.3	NES	
7/26/16	23	CR41	C	0.4	NES	
7/26/16	25	CR18	C	0.1	NES	

Table A.2 (Continued)

7/27/16	11	XR44	Tx	0.1	NES
7/27/16	13	XR30	Tx	–	N/S
7/27/16	23	CR41	C	0.4	NES
7/27/16	25	CR18	C	0.1	NES
7/28/16	11	XR44	Tx	–	N/S
7/28/16	13	XR30	Tx	0.1	NES
7/28/16	23	CR41	C	0.2	NES
7/28/16	25	CR18	C	0.3	NES
7/29/16	11	XR44	Tx	0.1	NES
7/29/16	13	XR30	Tx	–	N/S
7/29/16	23	CR41	C	–	N/S
7/29/16	25	CR18	C	0.4	NES
7/30/16	11	XR44	Tx	0.1	NES
7/30/16	13	XR30	Tx	0.2	NES
7/30/16	23	CR41	C	0.5	NES
7/30/16	25	CR18	C	0.5	NES
7/31/16	11	XR44	Tx	–	N/S
7/31/16	13	XR30	Tx	0.4	NES
7/31/16	23	CR41	C	0.5	NES

Table A.2 (Continued)

7/31/16	25	CR18	C	0.5	NES
8/1/16	11	XR44	Tx	0.3	NES
8/1/16	13	XR30	Tx	–	N/S
8/1/16	23	CR41	C	0.5	NES
8/1/16	25	CR18	C	0.1	NES
8/2/16	11	XR44	Tx	0.1	NES
8/2/16	13	XR30	Tx	–	N/S
8/2/16	23	CR41	C	0.4	NES
8/2/16	25	CR18	C	0.3	NES
8/3/16	11	XR44	Tx	0.4	NES
8/3/16	13	XR30	Tx	0.1	NES
8/3/16	23	CR41	C	0.5	NES
8/3/16	25	CR18	C	0.3	NES
8/4/16	11	XR44	Tx	0.4	NES
8/4/16	13	XR30	Tx	–	N/S
8/4/16	23	CR41	C	0.5	NES
8/4/16	25	CR18	C	0.1	NES
8/5/16	11	XR44	Tx	–	N/S
8/5/16	13	XR30	Tx	–	N/S

Table A.2 (Continued)

8/5/16	23	CR41	C	0.5	NES	
8/5/16	25	CR18	C	0.1	NES	
8/6/16	11	XR44	Tx	0.1	NES	Fed 38 *D. auritus* infected catfish
8/6/16	13	XR30	Tx	0.1	NES	
8/6/16	23	CR41	C	0.5	NES	
8/6/16	25	CR18	C	0.3	NES	
8/7/16	11	XR44	Tx	–	N/S	
8/7/16	13	XR30	Tx	0.1	NES	
8/7/16	23	CR41	C	1.0	NES	
8/7/16	25	CR18	C	0.2	NES	
8/8/16	11	XR44	Tx	–	N/S	
8/8/16	13	XR30	Tx	0.1	NES	
8/8/16	23	CR41	C	0.8	NES	
8/8/16	25	CR18	C	0.3	NES	
8/9/16	11	XR44	Tx	0.1	NES	
8/9/16	13	XR30	Tx	0.6	NES	
8/9/16	23	CR41	C	0.6	NES	
8/9/16	25	CR18	C	0.6	NES	
8/10/16	11	XR44	Tx	–	N/S	

Table A.2 (Continued)

8/10/16	13	XR30	Tx	0.4	619	
8/10/16	23	CR41	C	0.3	NES	
8/10/16	25	CR18	C	0.5	NES	
8/11/16	11	XR44	Tx	0.1	NES	
8/11/16	13	XR30	Tx	0.4	484	Moved to snail tank
8/11/16	23	CR41	C	0.6	NES	
8/11/16	25	CR18	C	0.6	NES	Moved to snail tank
8/12/16	11	XR44	Tx	0.1	NES	
8/12/16	13	XR30	Tx	0.4	63	
8/12/16	23	CR41	C	0.6	NES	
8/12/16	25	CR18	C	0.5	NES	
8/13/16	11	XR44	Tx	0.1	NES	
8/13/16	13	XR30	Tx	0.8	1	
8/13/16	23	CR41	C	0.5	NES	
8/13/16	25	CR18	C	0.5	NES	
8/14/16	11	XR44	Tx	0.1	9	
8/14/16	13	XR30	Tx	2.8	7	
8/14/16	23	CR41	C	0.5	NES	
8/14/16	25	CR18	C	0.3	NES	

Table A.2 (Continued)

8/15/16	11	XR44	Tx	0.1	2	
8/15/16	13	XR30	Tx	1.4	18	
8/15/16	23	CR41	C	0.5	NES	
8/15/16	25	CR18	C	0.3	NES	
8/16/16	11	XR44	Tx	0.3	NES	Moved to snail tank
8/16/16	13	XR30	Tx	1.4	122	Returned to pen
8/16/16	23	CR41	C	0.5	NES	Moved to snail tank
8/16/16	25	CR18	C	0.3	NES	Returned to pen
8/17/16	11	XR44	Tx	0.5	NES	
8/17/16	13	XR30	Tx	–	N/S	
8/17/16	23	CR41	C	0.9	NES	
8/17/16	25	CR18	C	0.1	NES	
8/18/16	11	XR44	Tx	0.2	7	
8/18/16	13	XR30	Tx	0.2	40	
8/18/16	23	CR41	C	0.8	NES	
8/18/16	25	CR18	C	1.0	NES	
8/19/16	11	XR44	Tx	0.3	2,383	
8/19/16	13	XR30	Tx	0.1	579	
8/19/16	23	CR41	C	0.5	NES	

Table A.2 (Continued)

8/19/16	25	CR18	C	0.5	NES	
8/20/16	11	XR44	Tx	0.5	34	
8/20/16	13	XR30	Tx	0.5	1,472	
8/20/16	23	CR41	C	0.5	NES	
8/20/16	25	CR18	C	0.5	NES	
8/21/16	11	XR44	Tx	0.1	6	Returned to pen
8/21/16	13	XR30	Tx	0.3	113	
8/21/16	23	CR41	C	0.5	NES	Returned to pen
8/21/16	25	CR18	C	0.1	NES	
8/22/16	11	XR44	Tx	0.3	268	
8/22/16	13	XR30	Tx	0.3	345	
8/22/16	23	CR41	C	0.5	NES	
8/22/16	25	CR18	C	0.5	NES	
8/23/16	11	XR44	Tx	0.3	89	
8/23/16	13	XR30	Tx	0.2	8	
8/23/16	23	CR41	C	0.5	NES	
8/23/16	25	CR18	C	0.5	NES	
8/24/16	11	XR44	Tx	0.5	165	
8/24/16	13	XR30	Tx	0.1	NES	

Table A.2 (Continued)

8/24/16	23	CR41	C	0.5	NES
8/24/16	25	CR18	C	0.1	NES
8/25/16	11	XR44	Tx	0.3	305
8/25/16	13	XR30	Tx	0.2	23
8/25/16	23	CR41	C	0.5	NES
8/25/16	25	CR18	C	0.2	NES
8/26/16	11	XR44	Tx	0.5	1,009
8/26/16	13	XR30	Tx	0.1	19
8/26/16	23	CR41	C	0.5	NES
8/26/16	25	CR18	C	0.5	NES
8/27/16	11	XR44	Tx	0.2	321
8/27/16	13	XR30	Tx	0.1	154
8/27/16	23	CR41	C	0.5	NES
8/27/16	25	CR18	C	0.5	NES
8/28/16	11	XR44	Tx	0.3	423
8/28/16	13	XR30	Tx	0.1	NES
8/28/16	23	CR41	C	0.5	NES
8/28/16	25	CR18	C	0.1	NES
8/29/16	11	XR44	Tx	0.1	27

Table A.2 (Continued)

8/29/16	13	XR30	Tx	0.6	NES
8/29/16	23	CR41	C	0.5	NES
8/29/16	25	CR18	C	0.4	NES
8/30/16	11	XR44	Tx	0.4	785
8/30/16	13	XR30	Tx	0.2	365
8/30/16	23	CR41	C	0.5	NES
8/30/16	25	CR18	C	0.5	NES
8/31/16	11	XR44	Tx	0.4	125
8/31/16	13	XR30	Tx	0.5	359
8/31/16	23	CR41	C	0.5	NES
8/31/16	25	CR18	C	0.5	NES
9/1/16	11	XR44	Tx	0.4	406
9/1/16	13	XR30	Tx	0.3	110
9/1/16	23	CR41	C	0.5	NES
9/1/16	25	CR18	C	0.1	NES
9/2/16	11	XR44	Tx	0.1	10
9/2/16	13	XR30	Tx	0.5	22
9/2/16	23	CR41	C	0.5	NES
9/2/16	25	CR18	C	0.3	NES

Table A.2 (Continued)

9/3/16	11	XR44	Tx	0.3	46	
9/3/16	13	XR30	Tx	0.2	1	
9/3/16	23	CR41	C	0.5	NES	
9/3/16	25	CR18	C	0.1	NES	
9/4/16	11	XR44	Tx	0.2	144	
9/4/16	13	XR30	Tx	0.1	3	
9/4/16	23	CR41	C	0.5	NES	
9/4/16	25	CR18	C	0.2	NES	
9/5/16	11	XR44	Tx	0.1	3	
9/5/16	13	XR30	Tx	0.3	63	
9/5/16	23	CR41	C	0.5	NES	
9/5/16	25	CR18	C	0.1	NES	
9/2/16	11	XR44	Tx			Euthanized and necropsied; recovered 20 *D. auritus* adults
9/2/16	13	XR30	Tx			Euthanized and necropsied; recovered 29 *D. auritus* adults
9/2/16	23	CR41	C			Euthanized and necropsied; no trematodes recovered
9/2/16	25	CR18	C			Euthanized and necropsied; no trematodes recovered