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INTESTINAL DIGENETIC TREMATODES FOUND IN DOUBLE-CRESTED CORMORANT POPULATIONS IN THE MISSISSIPPI DELTA AND THE POTENTIAL IMPACT OF THESE PARASITES ON COMMERCIAL AND WILD FISH SPECIES FOUND IN THIS REGION

By

Mary McPherson O'Hear

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Sciences
in the Department of Basic Sciences
in the College of Veterinary Medicine

Mississippi State, Mississippi

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Double-crested cormorants have steadily increased in the Mississippi Delta. This bird serves as a definitive host for digenetic trematodes, many of which infect fish. To identify these digeneans in cormorants and determine the impact these infections have on wild fish in the Mississippi Delta, two surveys were done. Cormorants were collected for two years in the Mississippi Delta. At necropsy trematodes were collected and identified morphologically and molecularly as: *Austrodiplostomum ostrowskiae, Hysteromorpha triloba, Drepanocephalus spathans, Ascocotyle longa* and *Pseudopsilostoma varium*. Additionally, 14 fish species were collected from a Mississippi Delta lake. Fish were examined for parasites and *Posthodiplostomum minimum* metacercariae were found in multiple organs in 6/14 fish species. The 18S gene sequences of these metacercariae were identical to published *P. minimum* sequences, whereas the cytochrome oxidase I (COI) sequences matched published COI sequences for *Posthodiplostomum* sp. 3, 5 and 8, suggesting subspecies of *Posthodiplostomum* in this fish population.

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المنارة للاستشارات
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ... ii

LIST OF TABLES ... v

LIST OF FIGURES ... vi

CHAPTER

I. INTRODUCTION ... 1

Channel catfish aquaculture ... 1
Digenetic trematodes of freshwater fish in the Mississippi Delta ... 2
History of the double-crested cormorant ... 6
Digenetic trematodes of the double-crested cormorant ... 10
Objectives ... 16
References ... 18

II. MORPHOLOGICAL AND MOLECULAR IDENTIFICATIONS OF INTESTINAL DIGENETIC TREMATODES IN DOUBLE-CRESTED CORMORANTS FROM THE MISSISSIPPI DELTA ... 26

Abstract ... 26
Introduction ... 27
Materials and Methods ... 29
Bird Collection ... 29
Necropsy ... 29
Parasite Identification ... 30
Results ... 35
Discussion ... 44
Conclusion ... 48
References ... 51

www.manaraa.com

III. MORPHOLOGICAL AND MOLECULAR IDENTIFICATIONS OF DIGENETIC TREMATODES IN WILD FISH SPECIES COLLECTED FROM AN OX-BOW LAKE IN THE MISSISSIPPI DELTA 55

Abstract 55
Introduction 56
Materials and Methods 59
Fish Collection 59
Necropsy 60
Parasite Identification 61
Results 65
Discussion 71
Conclusion 77
References 80

IV. Conclusion 84

للاستشارات المنارة
www.manaraa.com

LIST OF TABLES

1.1. Reported trematodes of the double-crested cormorant (*Phalacrocorax auritus*) 17

2.1. Montly double-crested cormorant (DCCO) collection 33

2.2. PCR primers for amplification of 18S rRNA and mitochondrial COI genes 34

2.3. Digenetic trematodes found in the 35 double-crested cormorants, *P. auritus*, surveyed in this study 39

3.1. PCR primers for amplification of 18S rRNA and mitochondrial COI genes 64

3.2. *Posthodiplostomum minimum* metacercariae collected in freshwater fish from Caile Lake 68

3.3. COI gene sequence homology of *Posthodiplostomum minimum* found in fish species collected in Caile Lake 69

المنارة للاستشارات

www.manaraa.com

LIST OF FIGURES

2.1. Prevalence of digenetic trematodes found in the 35 double-crested cormorants surveyed in this study..38

2.2. Anterior region of an adult *Drepanocephalus spathans* collected from the intestine of a double-crested cormorant in this study showing distinctive head collar spines (A) 40

2.3. The large, muscular acetabulum of an adult *Drepanocephalus spathans* collected from the intestine of a double-crested cormorant in this study...40

2.4. Mean infection intensities of the digenetic trematodes found in the 35 double-crested cormorants surveyed in this study 41

2.5. Mean abundance of the digenetic trematodes found in the 35 double-crested cormorants surveyed in this study 41

2.6. An adult *Hysteromorpha triloba* collected from the intestine of a double-crested cormorant collected during the study demonstrating the triangular shaped body and small oral sucker (A)..42

2.7. An adult *Pseudopsilostoma varium* collected from the stomach of a double-crested cormorant in the study demonstrating a well-developed oral (A) and ventral sucker (B).......................................42

2.8. An adult *Austrodiplostomum ostrowskiae* collected from the intestine of a double-crested cormorant in the study demonstrating the small oral sucker (A) and distinct hindbody region (B). 43

2.9. An adult *Ascocotyle longa* collected from the intestine of a double-crested cormorant in the study demonstrating a pyriform body and circumoral spines (A)..43

3.1. *Posthodiplostomum minimum* metacercariae from a bluegill (*Lepomis macrochirus*) 68

www.manaraa.com

3.2. Prevalence of *Posthodiplostomum minimum* metacercariae found in freshwater fish from Caile Lake 69

3.3. Mean infection intensity of *Posthodiplostomum minimum* metacercariae found in freshwater fish from Caile Lake 70

3.4. Mean abundance of *Posthodiplostomum minimum* metacercaraie in freshwater fish from Caile Lake 70

المنارة للاستشارات

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CHAPTER I

INTRODUCTION

Channel catfish aquaculture

Commercial catfish production is a major industry in the southeastern United States, especially in the state of Mississippi. In 2009, Channel catfish (*Ictalurus punctatus*) was the leading aquaculture industry in the United States, earning over $372 million in sales in 2009. Mississippi accounted for 53% of total sales with 60,000 acres devoted to catfish aquaculture (USDA, NASS, 2010). The majority of Mississippi catfish aquaculture operations are concentrated in the northwestern corner of the state, in the flood plains between the Mississippi and Yazoo Rivers, a region often referred to as the Mississippi Delta.

Catfish aquaculture in Mississippi began in the 1960s and experienced rapid growth in the 1970s and 1980s. The growth of the commercial catfish industry has been accompanied by increasing numbers of piscivorous bird populations to this region. The birds most commonly observed feeding on the catfish ponds include the double-crested cormorants, *Phalacrocorax auritus*, American white pelicans, *Pelecanus erythrorhynchos,* great egrets, *Ardea alba*, and great blue herons, *Ardea herodias* (Glahn et al., 1999, 2000; King and Werner, 2001; King, 2005). In a survey done by the USDA, 70% of catfish producers considered the double-crested cormorant to be the primary wildlife species affecting channel catfish stock (Wywialowski, 1999). Double-crested

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cormorants and great blue herons are the most commonly reported wildlife species feeding on commercial channel catfish (Glahn and King, 2004).

Digenetic trematodes of freshwater fish in the Mississippi Delta

In addition to predation damages, these piscivorous birds also serve as the definitive hosts for a variety of digenetic trematodes, many of which infect numerous fish species, including channel catfish (Hoffman, 1999). Digenetic trematodes known to infect cultured channel catfish include *Bolbophorus damnificus*, *Bursacetabulus pelecanus*, *Hysteromorpha triloba*, *Diplostomum spathaceum* and *Clinostomum marginatum* (Overstreet and Curran, 2004; Wise et al., 2004). *Clinostomum marginatum* and *Diplostomum spathaceum* are considered production nuisances, as opposed to economic burdens (Wise et al., 2004).

Clinostomum spp. are relatively harmless to the infected fish, except when heavily infected (Hoffman, 1999). This digenetic trematode usually encysts under the dermis of the catfish (Overstreet and Curran, 2004). The rams-horn snail, *Planorbella trivolvis,* sheds *Clinostomum* cercariae and a wide variety of freshwater fish are capable of serving as the second intermediate host. The great blue heron, *Ardea herodias*, serves as the definitive host (Hoffman, 1999; Overstreet and Curran, 2004). *Clinostomum* spp. infect such a wide range of fish species, it is thought that this trematode is capable of infecting any species of freshwater fish (Hoffman, 1999).

Diplostomum spathaceum is commonly found in the intestines of gulls (Whyte et al., 1987) and also infects a wide range of freshwater fish (Hoffman, 1999). This trematode forms metacercariae in the lens of the eye and can cause blindness (Holloway

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and Leno, 1982). *Diplostomum spathaceum* cercariae are shed by Lymnaea snails (Whyte et al., 1987).

Hysteromorpha triloba is a commonly reported trematode in double-crested cormorants (Flowers et al., 2004; Threlfall, 1982). *H. triloba* forms large metacercariae in the musculature of several fish species, including *Catostomus* spp., *Hyborhynchus notatus, Ictalurus* spp. and *Notemigonus crysoleucas* (Hoffman, 1999). *H. triloba* metacercariae has a diplostomulum metacercariae that is frequently encapsulated by host fibrotic material (Overstreet and Curran, 2004). The freshwater snail, *Gyraulus hirsustus*, serves as the first intermediate host (Hugghins, 1954).

Bursacetabulus pelecanus (Overstreet and Curran, 2004) and *Bolbophorus damnificus* (Levy et al., 2002; Overstreet and Curran, 2004; Yost, 2008) are both digenetic trematodes of the American white pelican. *Bursacetabulus pelecanus* produces a metacercariae that develops in the brain, spinal cord, optic nerve and eye of the catfish after being penetrated by a cercariae emerging from the snail, *Gyraulus parvus* (Overstreet and Curran, 2004). The digenetic trematode, *Bolbophorus damnificus* has been associated with high mortalities in channel catfish and can lead to severe economic losses for commercial catfish producers (Overstreet et al., 2002). *Bolbophorus damnificus* metacercariae encyst under the dermis of the caudal and dorsal regions (Overstreet et al., 2002) and severe infections can cause mortalities 5-6 days post-infection (Yost, 2008). The American white pelican is the only known definitive host for *B. damnificus* (Overstreet and Curran, 2004; Yost, 2008) while the snails, *Planorbella trivolvis* and *Biomphalaria havanensis* (syn. *B. obstructa*) can both serve as the first intermediate hosts (Yost, 2008).

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Although the digenetic trematodes infecting channel catfish in the Mississippi Delta are well described, little is known about digenetic trematodes affecting wild fish species in this region and no parasitic surveys have been done pertaining to these fish species. Common digenetic trematodes reported to infect North American wild freshwater fish species include *Uvulifer ambloplitis*, *Posthodiplostomum minimum*, *Clinostomum* spp. and *Diplostomum spathaceum*.

Uvulifer ambloplitis metacercariae have been reported in many fish species in the families Centrarchidae, Cyprinidae and Esocidae (Hoffman, 1999). Metacercariae of *U. ambloplitis* are frequently seen as pinhead-sized black spots in the skin, tail base, fins and musculature of the fish (Hunter and Hunter, 1938). Sunfish (*Lepomis* spp.), bass (*Micropterus* spp.) and perch (family Percidae) are the most commonly affected species (Lemly and Esch, 1985.) *Uvulifer ambloplitis* cercariae are shed by the snail, *P. trivolvis*, and can cause mechanical damage and hemorrhage upon penetration into the fish host (Hoffman and Putz, 1965). Fish infected with the "black grub" tend to lose weight and body condition (Hunter and Hunter, 1938) and infected fish have increased oxygen requirements (Lemly and Esch, 1984). The definitive host for *U. ambloplitis* is the belted kingfisher, *Megaceryle alcyon* (Hoffman and Putz, 1965).

Posthodiplostomum minimum infect a wide variety of North American freshwater fish (Grizzle and Goldsby, 1996), but are mostly commonly found in centrarchid fishes (Hoffman, 1958). It is thought that there are two subspecies of this parasite; *Posthodiplostomum minimum mimimum*, which only infects fish of the family Cyprinidae, while *Posthodiplostomum minimum centrarchi* is primarily a parasite of Centarchidae fishes. These two subspecies were identified after artificial infection trials

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demonstrated specificity to the two fish families within the species (Hoffman, 1958). Currently, there is no published molecular data confirming the differences of DNA sequences in these two subspecies. *Physa* spp. snails shed *Posthodiplostomum minimum* cercariae and the definitive host for this parasite is the great blue heron, *Ardea herodias* (Spall and Summerfelt, 1970).

Metacercariae of *P. minimum* primarily encyst in the liver, kidneys and heart and mesenteries of the infected fish host (Avault and Smitherman, 1965; Spall and Summerfelt, 1970). *Posthodiplostomum minimum* can cause significant disease in fish, particularly in cases in which the metacercariae are so numerous that they cause the organs to become compressed. Heavily infected fish can have displaced organs, ascites, ruptured abdomens and mortality (Mitchell et al., 1982). Death can also occur if the liver and other organs are destroyed by large numbers of encysted metacercariae (Hoffman, 1958). It has also been postulated that penetration of *P. minimum* cercariae impairs immune function and can leave fish susceptible to secondary infections (Grizzle and Goldsby, 1996).

Some of the digenetic trematodes known to infect channel catfish are also frequently reported parasites of wild freshwater fish species. *Clinostomum* spp. and *Diplostomum spathaceum* have both been reported in numerous fish species (Hoffman, 1999). Double-crested cormorants are potential reservoir hosts for several of the digenetic trematodes that could cause severe pathology in both the commercial channel catfish and the wild fish species that are found in the waterways in the Mississippi Delta. However, no parasite survey has been done to confirm the presence of these parasites and to ascertain the potential parasitic impact this piscivorous bird has on fish in this region.

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With the increasing numbers of double-crested cormorants in the Mississippi Delta these birds may play a significant role in trematode infections in both wild and cultured fish in this region.

History of the double-crested cormorant

The double-crested cormorant is a fairly large waterbird with dark plumage that is abundant and widespread in North America. These birds are excellent swimmers that dive underwater to consume subsurface fish prey, and to a lesser extent amphibians, mollusks and crustaceans (Johnsgard, 1993). Double-crested cormorants are a long-lived bird with fairly high reproductive rates, producing two to three young per year (Price and Weseloh, 1986; Weseloh and Ewins 1994; Weseloh et al., 1995). These piscivorous birds typically reach sexual maturity around age three, although some birds begin breeding two years after hatching (Palmer 1962; Weseloh and Ewins, 1994; Weseloh et al., 1995). Double-crested cormorants nest either on the ground or in trees, but always require a nearby water source for foraging (Wires et al., 2001).

Double-crested cormorant populations have undergone extreme fluctuation during the past century, primarily due to human intervention. The double-crested cormorant population that winters in the Mississippi Delta breeds in the Great Lakes and central Canada (Dolbeer, 1991). This population of double-crested cormorants is commonly referred to as the interior population and accounts for 61 percent of the total breeding population of cormorants in North America (Hatch, 1995; Tyson, et al. 1999). Between 1925 and 1935, Mandall estimated that the interior population contained over 7,500 breeding pairs of cormorants (1936). Mandall's report was not a systematic survey and it

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is possible that small breeding colonies were overlooked. Subsequent surveys show cormorant populations continued to increase in the Great Lakes Region, especially on lakes in Manitoba, Canada (Tyson et al., 1999; Weseloh et al., 1995).

In the early 1940s, an official program to control double-crested cormorant numbers on Lake Winnipegosis was introduced. Local fishermen were also thought to be destroying cormorant nests throughout the Great Lakes region. During this time of official control, the number of breeding pairs of double-crested cormorants decreased by over 50 percent. This control program was considered a success and was discontinued in 1951 (McLeod and Bondar, 1953).

From the 1940s-1970s, cormorant populations across the country underwent a period of dramatic decline, due to human persecution and the widespread use of organochlorine pesticides, such as dichlorodiphenyltrichloroethane (DDT) (Weseloh 1983; Weseloh et al.,1995). Double-crested cormorants are extremely sensitive to pollutants that are bioaccumulated in fish prey (Fox et al., 1991; Ludwig et al., 1996). Female cormorants with elevated levels of DDT have decreased function of the shell gland, which results in eggshell thinning (Postupalsky, 1978; Weseloh et al., 1983). Subsequently the reproductive output of the interior population of double-crested cormorants was extremely low due to poor hatching success (Weseloh et al., 1983). The interior population of cormorants decreased approximately 80% during this time (Postupalsky, 1978).

Double-crested cormorant populations began to recover in the mid 1970s. In 1972, this migratory bird was added to the protected bird list of the U.S. Migratory Bird Treaty Act and was placed on the Blue List by the National Audubon Society (Tate and

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Tate, 1982). The use of organochlorine pesticides became prohibited during this time and DDT levels in the birds began to decline (Ludwig ,1984; Noble and Elliot, 1986). Increasing food availability, such as alewife, *Alosa pseudoharengus*, in the Great Lakes and channel catfish, *I. punctatus*, in the Mississippi Delta also aided double-crested cormorant recovery (Hobson et al., 1989; Ludwig 1984; Price and Weseloh 1986; Weseloh et al., 1995; Vermeer and Rankin, 1984). The double-crested cormorant populations in the Great Lakes increased from 89 breeding pairs in 1970 to approximately 93,000 breeding pairs in 1997 (Tyson et al., 1999).

Prior to the introduction of channel catfish aquaculture in the Mississippi Delta, the interior populations of double-crested cormorants wintered on the Gulf Coast (Dolbeer, 1991). However, the Christmas bird counts completed during the 1980s demonstrated that increasing numbers of these birds were spending the majority of winter season in the Delta region of Mississippi (Alexander, 1977-1990). The increasing numbers of double-crested cormorants in this region correspond to increasing acres devoted to catfish aquaculture (Price and Nickum, 1995).

Channel catfish farming has greatly benefited the double-crested cormorant. The higher forage base provided by catfish aquaculture potentially increases the survivability of juvenile birds during the winter (Duffy, 1995; Vermeer and Rankin, 1984; Weseloh and Ewins, 1994). Pre-migratory cormorants that feed on catfish ponds have been shown to have a better body condition than cormorants that reside in non-aquaculture areas (Glahn et al., 1999). Increasing numbers of double-crested cormorants have been observed in the Mississippi Delta year-round and small breeding colonies have been documented (Reinhold et al., 1998).

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The foraging of double-crested cormorants has a significant impact on the channel catfish aquaculture industry. Besides directly feeding on the fish, cormorants are also responsible for injuring fish and disturbing fish feeding patterns. Double-crested cormorants account for approximately 4% of fish losses each year and up to 33% loss of profits (Wywialowski, 1999). Estimations on monetary losses directly caused by double-crested cormorant predation vary, ranging from $2 million to $3.3 million dollars in the early 1990s (Glahn and Brugger, 1995; Stickley and Andrews, 1989). Glahn et al. (2000) estimated that double-crested cormorants cost catfish producers up to $25 million annually by the late 1990s.

Catfish producers utilize a variety of techniques to decrease avian predation on their farms. Propane exploders, pyrotechnics, ammunition, distress calls and human effigies are commonly used by producers to discourage loafing of piscivorous birds (Mott and Brunson, 1997). However, these strategies become less effective over time as the birds acclimate to them. These devices are most effective when frequently changed and moved around (Stickley and King, 1995). Despite the double-crested cormorant's current protected status under the Migratory Bird Treaty Act, catfish producers can obtain a permit for lethal control from the U.S. Department of Interior's Fish and Wildlife Service.

As the double-crested cormorant population continues to grow, there have been increasing concerns about the bird's effect on sport fisheries. However, there is little data that justifies this concern. Besides foraging on catfish, double-crested cormorants consume mostly gizzard shad, *Dorosoma cepedianum*, (Kirsch, 1995). A survey of the stomach contents of double-crested cormorants loafing on Lake Beluah in Bolivar

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County, Mississippi, demonstrated a preference for sunfishes, particularly bluegill, *Lepomis macrochirus* (Glahn et al., 1998). An additional survey of the stomach contents of cormorants inhabiting an ox-bow lake in Arkansas showed a preference for shad and yellow bass, *Morone mississippiensis*, (Fenech et al., 2004). To date, double-crested cormorants seem to have little impact on sport fish populations (Trapp et al., 1999).

Digenetic trematodes of the double-crested cormorant

Surveys: Piscivorous birds serve as definitive hosts to a variety of digenetic trematodes, many of which are pathogenic in fish. The parasites of the double-crested cormorant are well described morphologically (Yamaguti 1958), but little quantitative survey data exists for the double-crested cormorants that commonly forage on wild and commercial fish species in the Mississippi Delta.

Several surveys of endoparasites of the double-crested cormorant have been done in the U.S. (Table 1.1). In 1982, a quantitative survey on endoparasites was done on double-crested cormorants in Florida. However, the majority of the birds collected in this survey were a subspecies of the double-crested cormorant, *Phalacrocorax auritus floridanus*, which are non-migratory and reside in Florida year round (Threlfall, 1982). Another survey quantified the parasite load of twelve double-crested cormorants in Texas, an area that is included in the wintering grounds of the interior population of double-crested cormorants (Fedynich et al., 1997). Digenetic trematodes that have been reported in double-crested cormorants include *Drepanocephalus spathans*, *Hysteromorpha triloba*, *Austrodiplostomum ostrowskiae* and *Ascocotyle longa*.

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(Fedynich et al., 1997; Flowers et al., 2004; Threlfall, 1982). *Pseudopsilostoma varium* has been previously reported in neotropic cormorants (Fedynich et al., 1997).

Drepanocephalus spathans is a digenetic trematode commonly found in double-crested cormorants. This parasite was first reported in the neotropic cormorant, *Phalacrocorax brasilianus* in Brazil (Dietz 1909). *Drepanocephalus spathans* has also been found in neotropic cormorants in Venezuela, Columbia and Paraguay (Kostandinova et al., 2002; Lutz, 1928; Rietschel and Werding, 1978). This trematode was reported for the first time in the double-crested cormorant in Florida (Threlfall, 1982). In this survey, *Drepanocephalus spathans* was found in the intestines of approximately 9.21% of the birds surveyed with a mean infection intensity of 263, with the number of *D. spathans* ranging from 1-956/bird (Threlfall, 1982).

Another survey completed in 1997, found that both double-crested cormorants and neotropic cormorants in Texas were hosts for *D. spathans.* The prevalence of *D. spathans* in these birds was fairly low, with only 8% of double-crested cormorants and 8% neotropic cormorants being infected with this particular trematode (Fedynich et al., 1997). A survey of 47 neotropic cormorants in Brazil had a prevalence of 42.55% for *D. spathans*, with a mean infection intensity of 11.9 and an infection rate of 1-42 parasites per bird (Monteiro et al., 2011). *Drepanocephalus spathans* was also been reported in a population of double-crested cormorants residing in the Lake Erie region. The highest abundance of *D. spathans* in this population was 200, with male cormorants having both a higher abundance and prevalence of infection (Robinson et al., 2010). Double-crested cormorants in North Carolina have also been reported to be hosts for *D. spathans* (Flowers et al., 2004).

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Drepanocephalus spathans has distinctive morphological characteristics. It has an elongate body with maximum width at head collar level. Its tegument has small spines, with four angle spines, two pairs on each ventral lappet. The oral sucker is subterminal and spherical. The ventral sucker is muscular, cup-shaped, with a deep cavity. Testes are tandem and deeply lobed. Ovaries are small and transversely oval. Vitellarium is in two lateral fields of small follicles, between posterior margin of the ventral sucker and the posterior extremity (Kostandinova et al., 2002). The 18S gene sequence of *D. spathans* is currently the only sequence for this trematode in GenBank (Flowers, 2003). The life cycle for *D. spathans* is currently unknown.

Hysteromorpha triloba is a frequently reported parasite of both double-crested cormorants and neotropic cormorants. A single double-crested cormorant in Wisconsin was found to have 1,602 *H. triloba* in its intestine (Chandler and Rausch, 1948). This parasite has also been reported in double-crested cormorants in Florida and North Carolina (Flowers et al., 2004; Threlfall, 1982). The only quantitative data for infection of this digenetic trematode in double-crested cormorants was for a population of 76 double-crested cormorants residing in Florida year-round. *Hysteromorpha triloba* was found in the intestines of seven birds in this study, with a mean of 4,169 and a range of 1-19,727 (Threlfall, 1982).

Hysteromorpha triloba has a 3-host life cycle. Miracidia are highly specific to the snail, *Gyraulus hirsustus*. Artificial infection trials performed by Hugghins (1954) elucidated the life cycle of this digenetic trematode. Cercariae emerge 14 to 15 days after infection of the snail during the summer months and then penetrate a fish host. Metacercariae encyst throughout the muscles of fish and usually reach maturity in

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approximately 12 weeks. Fish in the families Cyprinidae and Ameiuridae are the preferred second intermediate host. A cormorant ingests fish containing metacercariae and eggs are shed in the feces within 60 hours post-infection (Hugghins, 1954).

Hysteromorpha triloba has a triangular body with the greatest diameter level to the ventral sucker. The hindbody is conical and the anterior extremity of the body is trilobate. The pharynx is small and the ventral sucker is well developed. The holdfast organ is funnel-shaped when protruded. Testes are tandem and the ovary is oval and located at the junction of the fore- and hindbody. Vitellaria is found in both the fore- and hindbody (Lutz, 1931). The 18S and COI gene sequences for *H. triloba* were recently published in GenBank (Locke et al., 2010, 2011).

Pseudopsilostoma varium is a digenetic trematode that is found in the stomach and proventriculus of neotropic cormorants (Fedynich et al., 1997). This parasite has also been reported in a common loon, *Gavia immer*, and the ring-necked duck, *Aythya collaris* (Linton, 1928; Noseworthy and Threlfall, 1978). In a survey of neotropic cormorants in Texas, 35% of birds were infected with *P. varium*. The mean infection intensity was 27.7, with a range of 1-78 (Fedynich et al., 1997).

Pseudopsilostoma varium has a fusiform body with an acetabulum that is slightly larger than the oral sucker. The pharynx is adjacent to the oral sucker and esophagus is very short. Testes are tandem at the middle of the hindbody. The cirrus pouch is anterodorsal to the acetabulum. The uterus is winding in the intercecal field between the anterior testis and the acetabulum. Vitelline follicles extend into lateral fields from pharynx to posterior extremity (Yamaguti, 1958). The life cycle of this parasite is unknown and there is currently no sequencing data available.

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Austrodiplostomum ostrowskiae is a digenetic trematode found in the intestines of double-crested cormorants. This parasite was previously identified to be *Austrodiplostomum mordax*, however a recent study has demonstrated that the morphology of *Austrodiplostomum* found in double-crested cormorants differs from those found in the neotropic cormorant (Dronen, 2009). A 1997 survey in Texas reported *A. mordax* in both the double-crested cormorant and the neotropic cormorant (Fedynich et al., 1997). However, subsequent examination of the specimens collected from the double-crested cormorants has shown that they are actually *A. ostrowskiae* (Dronen, 2009).

A 2009 survey of ten double-crested cormorants in Texas had a 100% prevalence of *A. ostrowskiae*. The mean infection intensity for this population of cormorants was 135, with a range of 9-225 (Dronen, 2009). The prevalence and intensity of *A. ostrowskiae* in the 1997 survey was much lower. Only 33% of double-crested cormorants were infected with *A. ostrowskiae*, with a mean infection intensity of 4.2 (Fedynich et al., 1997).

Austrodiplostomum ostrowskiae is described as having a small and bipartite body. It is finely spinose on the periphery and ventral surface from the anterior end to the midbody. The forebody is spatulate and concave ventrally. The hindbody is short and distinct. *Austrodiplostomum ostrowskiae* has a small, subterminal oral sucker and no acetabulum is present. The testes are tandem and approximately equal in width. The ovary is smooth and oval and is contiguous with anterior testes. The vitellarium is variable (Dronen, 2009). The life cycle of *A. ostrowskiae* is not yet elucidated and no molecular data for this parasite has been published.

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Ascocotyle longa (formerly *Phagicola longa*) is a digenetic trematode commonly found in both double-crested and neotropic cormorants. This parasite was first reported in a double-crested cormorant in Florida in 1964 (Hutton, 1964). In a later survey, two populations of double-crested cormorants were surveyed in Florida. The population located on the east coast of the state had a prevalence of 56% for *A. longa*. The mean infection intensity was 106, with a range of 1-400, while the second population, located on the west coast, had a prevalence of 38% for this parasite, with a mean infection intensity of 54 and a range of 1-488. This survey also showed that nestlings tended to have a higher intensity of infection (Threlfall, 1982).

Life cycle studies for *Ascocotyle longa* were completed in Brazil in 2010. The first intermediate host is the freshwater snail, *Heleobia australis*. Amphistome-type cercariae emerge from the snail and penetrate a mugilid fish (from the family Mugilidae) (Simoes et al., 2010). Metacercariae encyst in the heart, musculature, intestinal wall, liver gonads and mesenteries of the fish hosts (Scholz et al., 2001). A variety of birds and mammals can serve as a definitive host for this parasite, including humans (Simoes et al., 2010).

This parasite has been reported to cause human heterophyiasis, a severe gastro-intestinal disorder resulting from the consumption of mullet containing metacercariae (Simoes et al., 2010). The adult *Ascocotyle longa* has a pyriform body with a spinose tegument. The anterior extremity bears a crown armed with 16 spines. The genital pore is located in the pre-acetabular area. The vitellarium is longitudinal and follicular and located between the ovary and the testes. The testes are symmetrical in the posterior third

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of the body (Ransom, 1920). The 18S gene sequence for this digenetic trematode is published in GenBank (Dzikowski et al., 2004).

Objectives

The increasing numbers of double-crested cormorants (*Phalacrocorax auritus*) in the Mississippi Delta, potentially infected with high numbers of digenetic trematodes, could have a profound impact on the fish populations in this region. In order to ascertain the potential parasite impact of these birds, a parasite survey of the double-crested cormorants residing in this region is a necessity. This was accomplished by conducting a two-year survey to determine the species composition of adult digenetic trematodes in the gastrointestinal tracts of these double-crested cormorants and the intensity of infection. All trematodes will be identified using a combination of molecular and morphometric techniques.

In addition to the bird survey, a parasite survey of a variety wild fish species will be performed on fish collected from an ox-bow lake in the Mississippi Delta that has a resident cormorant population. This survey will examine the potential link between the larval metacercariae stages in fish to the adult trematode stages found in the double-crested cormorant. The parasites collected in the fish survey will also be identified using molecular and morphometric techniques.

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Table 1.1 Reported trematodes of the double-crested cormorant (*Phalacrocorax auritus*)

Trematode	**Location in cormorant host**
Amphimerus sp.[1]	Liver
Ascocotyle longa[1,3]	Intestine
Austrobilharzia variglandis[2]	Blood
Austrodiplostomum compactum[6]	Intestine
Austrodiplostomum ostrowskiae[1,4]	Intestine
Clinostomum attenuatum[5]	Trachea
Clinostomum marginatum[3]	Trachea/lungs
Drepanocephalus spathans[1,3,6]	Intestines
Galacfosomum humbargi[9]	Intestine
Hysteromorpha triloba[1,3,6]	Intestine
Maritrema sp.[1]	Intestine
Mesoophorodiplostomum pricei[1]	Intestine
Mesostephanus appendiculatoides[3]	Intestine
Neogogatea kentuckiensis[7]	Intestine
Opisthorchis vitellotus[8]	Bile Ducts
Ornithobilharzia sp.[3]	Blood
Parascocotyle dimunita[5]	Intestine
Parorchis acanthus[3]	Cloaca
Phocitremoides butionis[1]	Intestine
Renicola sp.[3]	Kidney

[1]Fedynich et al.1997; [2]Barber and Caira, 1995: [3]Threlfall, 1982; [4]Dronen, 2009; [5]Hutton and Sogandares-Bernal, 1960; [6]Flowers et al., 2004; [7]Stunkard and Olson, 1972; [8]Chin, 1950; [9]Pearson, 1973.

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CHAPTER II

MORPHOLOGICAL AND MOLECULAR IDENTIFICATIONS OF INTESTINAL DIGENETIC TREMATODES IN DOUBLE-CRESTED CORMORANTS FROM THE MISSSISSIPPI DELTA

Abstract

Increasing numbers of double-crested cormorants in the Mississippi Delta have been observed over the past few decades. This piscivorous bird serves as a definitive host for numerous digenetic trematodes, some of which may induce pathology in a fish host. A two year survey of trematodes in 35 double-crested cormorants collected in the Mississippi Delta was completed. Trematodes were identified to the species level, using morphometric and molecular techniques, and enumerated. DNA sequencing of the 18S and COI genes was performed on collected trematodes. A total of 4,909 trematodes were collected, representing five distinct digenetic species. The five species of trematodes identified were: *Drepanocephalus spathans*, *Hysteromorpha triloba*, *Pseudopsilostoma varium*, *Austrodiplostomum ostrowskiae* and *Ascocotyle longa*. The most prevalent trematode of the double-crested cormorants was *D. spathans* (91.4%), followed by *H. triloba* (77.7%), *P. varium* (74.3%) *A. ostrowskiae* (57.1%). and *A. longa* (28.6%). Of these five digenean species, the life cycles are only known for *H.triloba* and *A. longa*.

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Novel DNA sequences of the COI gene were obtained for *D. spathans*, *A. ostrowskiae*, *P. varium* and *A. longa* adults. Using these DNA sequences, the identification and confirmation of the larval stages of these parasites in the fish and snail hosts will be possible.

Introduction

Channel catfish (*Ictalurus punctatus*) aquaculture is a major industry in the state of Mississippi. In 2009, sales of the Mississippi channel catfish industry was over $197 million, with approximately 60,000 water acres in production (USDA, NASS, 2010). Ponds used in channel catfish aquaculture are ideal feeding and loafing grounds for a variety of piscivorous birds. The birds most often observed feeding on these ponds include the double-crested cormorants, *Phalacrocorax auritus*, American white pelicans, *Pelecanus erythrorhynchus,* great egrets, *Ardea alba*, and great blue heron, *Ardea herodias* (Glahn et al., 1999, 2000; King and Werner, 2001; King, 2005) with the double-crested cormorants and great blue herons the most commonly reported species feeding on commercial catfish ponds (Glahn and King, 2004).

The steady increase in the double-crested cormorant populations since the 1970s has been partially attributed to the year-round readily available food source of commercial catfish these open ponds provide. Predation of these catfish has been linked to increases in survivability of juvenile birds (Duffy, 1995; Vermeer and Rankin, 1984; Weseloh and Ewins, 1994). Additionally, pre-migratory cormorants that feed on catfish ponds are in better body condition than cormorants that reside in non-aquaculture areas (Glahn et al., 1999). Not only have the numbers of double-crested cormorants increased

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but also some populations have altered their migratory patterns and are remaining in the Mississippi Delta year-round. Small breeding colonies have also been established in this region (Reinhold et al., 1998).

In addition to predation damages, double-crested cormorants also serve as definitive hosts to a variety of trematodes, many of which are pathogenic in fish. Currently there is little information on the helminth populations in the double-crested cormorants foraging on the ponds in the Mississippi Delta or the potential impact these parasites may have on fish populations in this region. Three surveys of parasites in double-crested cormorants have been previously reporteded in the U.S. A survey was completed on two double-crested cormorant populations in Florida, however, the majority of the birds collected in this survey were a subspecies of double-crested cormorant, *Phalacrocorax auritus floridanus*, which are non-migratory and reside in Florida year round (Threlfall, 1982). Another survey quantified the parasite load of twelve double-crested cormorants in Texas, an area that is included in the wintering grounds of the interior population of double-crested cormorants (Fedynich et al., 1997). The parasites were also collected in two double-crested cormorants around catfish ponds in North Carolina (Flowers et al., 2004).

The increasing numbers of double-crested cormorants in the Mississippi Delta, potentially infected with high numbers of several digenetic trematode species, could have a profound impact on the fish populations in this region. To address this potential impact, the digenetic trematode population and the level of parasite infections in this bird population needs to be determined. This was accomplished by conducting a two-year survey of the adult trematodes present in the gastrointestinal tracts of double-crested

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cormorants collected in the Mississippi Delta. All collected trematodes were identified using morphologic and molecular methods. DNA sequencing of the trematodes collected from the double-crested cormorant will aid in the identification of the larval stages of these parasites.

Materials and Methods

Bird Collection

Thirty-five adult double-crested cormorants, *Phalacrocorax auritus*, were collected when present (January-May 2003 and January-March 2004) in the Mississippi Delta region for two consecutive winters. Five birds were collected each month during these periods when possible (Table 2.1). Birds were killed and transported on ice to the parasitology laboratory at the College of Veterinary Medicine at Mississippi State University for necropsy.

Necropsy

The entire gastrointestinal tract from esophagus to cloaca was removed from each double-crested cormorant (DCCO). The stomach and intestines were then separated and processed individually. Stomach and intestines were opened longitudinally and contents were rinsed into a brass sieve with an aperture of 75 μm using reverse osmosis water. Additionally, extensive scraping of the mucosal surfaces of the stomachs and intestines was done to ensure all trematodes were dislodged and collected.

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All stomach contents were preserved in 10% formalin for later parasite collection and identification. At each collection (except on two collection dates Table 2.1) period the intestinal contents of three out of the five birds were immediately examined microscopically and all live parasites were collected. Representatives of each parasite species were placed in 70% molecular grade alcohol for further DNA analysis or for later staining for morphological analysis. Intestinal contents of the remaining two birds were preserved in 10% formalin for later parasite collection and identification (Table 2.1). Preserved intestinal and stomach samples were washed through a #200 stainless steel screen (aperture = 75 μm) to remove all formalin The wash was examined using a dissecting microscope (Olympus model SZ60, Olympus Imaging America, Inc., Center Valley, Pennsylvania) at 4X magnification. All parasites were removed and placed in 70% molecular grade ethanol for identification and enumeration.

Parasite Identification

Morphological identification: In order to obtain a morphological identification of the digenetic trematodes, collected specimens were stained with acetocarmine (Fisher Scientific, Pittsburgh, Pennsylvania). Specimens were placed in the stain for 8-10 hours and then destained with a 1% acid alcohol until organs became visible. The specimens were then progressed through a series of dehydrating ethanol washes (70%, 95% and 100% ethanol). Each dehydration step lasted approximately one hour. The trematodes were then cleared with Citri-solve (Omega Laboratories, Inc., Houston, Texas) and mounted on slides using Permount (ProSciTech, Thuringowa Central Queensland,

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Australia). Stained specimens were identified using taxonomic keys (Yamaguti, 1958; Schell,1985; and Gibson et al., 2002, 2005 and 2008).

Molecular identification: The molecular identification of the digenetic trematodes collected from the double-crested cormorants were based on DNA sequencing of both the nuclear ribosomal18S gene and the mitochondrial COI gene. Prior to extraction, specimens were rinsed in nuclease-free water. DNA was extracted from representative specimens previously preserved in 70% molecular grade ethanol using the Dneasy Blood and Tissue Kit (Qiagen, Valencia, California) according to the manufacturer's protocol. The purified genomic DNA was then suspended in 400 µl of AE elution buffer solution (10mM Tris-Cl, 0.5mM EDTA) (Qiagen, Valencia, California).

To ensure adequate coverage of the 18S gene, single PCR reactions were performed using three sets of primers: Worm A and Worm B, 18S-7 and 18S-A27 and 600F and 18S-11 (Table 2.2). The total PCR volume was 25 µl, which contained 10X buffer, 2.0 µl template, 0.625 U *Taq* polymerase (Hot Start Taq; Takara Bio, Inc., Shiga, Japan), 200 nM of each primer and 200 µM of each deoxynucleotide triphosphate. Polymerase chain reaction conditions were 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 54°C for 30 sec, 72° for 2 min, with a final cycle of 72°C for 5 min. PCR reactions were performed using a PTC-100 Peltier Thermal Cycler (MJ Research, Watertown, Massachusetts).

Amplification and sequencing of the COI gene was performed using the COI specific primers and PCR protocols of Moszczynska et al. (2009). All genomics were screened using two sets of primers; Plat-diploCOX1F and Plat-diploCOX1R and

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MplatCOX1dF and MplatCOX1dR (Table 2.2). The Plat-diploCOX1F and Plat-diploCOXR are specific for the family Diplostomatidae, while the MplatCOX1dF and MplatCOX1dR are generic platyhelminth primers. In most cases, each genomic sample amplified using only one of the two primers sets, depending on the species. The total PCR volume was 25 µl, which contained 1X buffer, 2.5mM $MgCl_2$, 2.0 µl template, 0.625 U of Platinum *Taq* Polymerase (Invitrogen, Carlsbad, California), 200 nM of each primer and 200 µM of each deoxynucleotide triphosphate. Polymerase chain reaction conditions were 94°C for 2 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, with a final extension of 72°C for 10 min.

All PCR products were visualized with Gelstar nucleic acid stain (Combrex BioScience Rockland, Inc., Rockland, Maine) on a 1.2% agarose gel. PCR products were purified using a Montage PCR Centrifugal Filter device (Millipore, Billerica, Maryland) prior to sequencing at Arizona State University DNA Laboratory (Tempe, Arizona). Sequences were assembled using the SeqMan of the Lasergene version 8.0 software package (DNASTAR, Madison, Wisconsin) and were edited manually. Results underwent a database search using BLAST (Altschul et al., 1990).

Prevalence, mean infection intensity and mean abundance were calculated as described by Margolis et al. (1982). Prevalence is the number of individuals infected with a particular parasite species divided by the number of hosts examined. Mean infection intensity is the total number of individuals of a particular parasite species divided by the number of infected hosts. Mean abundance is calculated by dividing the total number of a particular parasite species by the total number of hosts examined (Margolis et al., 1982).

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Table 2.1 Monthly double-crested cormorant (DCCO) collection

Month	Total DCCO collected	Location: County	No. processed immediately	No. preserved in formalin
Jan-03	5	Humphreys	3	2
Feb-03	5	Leflore	3	2
Mar-03	5	Leflore	3	2
Apr-03	2	Leflore	2	0
May-03	3	Leflore	3	0
Jan-04	5	Bolivar	3	2
Feb-04	5	Bolivar	3	2
Mar-04	5	Bolivar	3	2

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Table 2.2 PCR primers for amplification of 18S rRNA and mitochondrial COI genes

Primer Name	Primer Sequence 5'-3'	Region Amplified	References
Worm A	GCGAATGGCTCATTAAATCAG	18S	Littlewood and Olson 2001
Worm B	CTTGTTACGACTTTTACTTCC	18S	Littlewood and Olson 2001
18S-7	GCCCTATCAACTGTCGATGGTA	18S	Littlewood and Olson 2001
18S-A27	CCATACAAATGCCCCCGTCTG	18S	Littlewood and Olson 2001
600F	GCAGCCGCGGTAATTCCAGC	18S	Littlewood and Olson 2001
18S-11	CGGCCATGCACCACC	18S	Littlewood and Olson 2001
MplatCOX1dF	TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG	COI	Moszczynska et al 2009
MplatCOX1dR	CAGGAAACAGCTATGACTGAAAYAAYAIIGGATCICCACC	COI	Moszczynska et al 2009
Plat-diploCOX1F	CGTTTRAATTATACGGATCC	COI	Moszczynska et al 2009
Plat-diploCOX1R	AGCATAGTAATMGCAGCAGC	COI	Moszczynska et al 2009

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Results

The total number of trematodes collected from all birds was 4,909 which were distributed among five distinct digenetic species: *Ascocotyle longa* (Ransom, 1920), *Austrodiplostomum ostrowskiae* (Dronen, 2009), *Drepanocephalus spathans* (Dietz, 1909), *Hysteromorpha triloba* (Rudolphi, 1819) and *Pseudopsilostoma varium* (Linton, 1928). All birds were positive for trematodes and the number of trematode species per bird ranged from one to five. Two of the double-crested cormorants (5.7%) were hosts to a single trematode species. Five birds (14.3%) were infected with two species of trematodes; 11 birds (31.4%) were infected with three species of trematodes; 15 birds (42.9%) were infected with four species while only two birds (5.7%) were infected with all five species of trematodes.

The most prevalent of these species was *D. spathans,* which was collected from 32 of the 35 double-crested cormorants (91.4%). *Hysteromorpha triloba* was also highly prevalent with 27 of the 35 birds (77.7%) being hosts to this parasite. *Pseudopsilostoma varium* was collected from 26 out of the 35 birds (74.3%) and *A. ostrowskiae* was found in 20 of the 35 birds (57.1%). The least prevalent trematode species collected from this population of double-crested cormorants was *Ascocotyle longa* with only 10 of the 35 birds (28.6%) being infected with this species (Table 2.3 and Figure 2.1).

Drepanocephalus spathans was recovered from both the stomachs and intestines of the birds. The collected specimens (Figure 2.2 and 2.3) concurred with the morphological descriptions by Dietz (1909) and Kostandinova *et al*. (2002). The total count of this trematode species ranged from 1-346, with a mean infection intensity of

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81.5 ± 18.1 and a mean abundance for all 35 birds of 74.5 ± 17.0 (Table 2.3 ,Figures 2.4 and 2.5). DNA sequencing of this parasite supported the morphological identification. Resultant sequences of the 18S gene were a 99% match to the published *Drepanocephalus spathans* sequence in GenBank (Flowers, 2003b). The COI gene for *D. spathans* is unpublished, so no comparisons could be made with the COI gene sequence reported in this study.

Hysteromorpha triloba was also present in the stomachs and intestines of the double-crested cormorants collected in this study. The collected specimens of *H. triloba* (Figure 2.6) matched the morphological descriptions of Rudolphi (1819) and Lutz (1931) with trematode counts ranging from 1-184; a mean infection intensity of 32.5 ± 9.4 and a mean abundance of 25.1 ± 7.7 (Table 2.3, Figures 2.4 and 2.5) The sequences obtained from the *H. triloba* identified in this study were a 100% match for both the 18S and COI genes of the *H. triloba* sequences published in GenBank (Locke et al., 2010; Locke et al., 2011).

Pseudopsilostoma varium was found in the stomachs of the double-crested cormorants. The collected specimens (Figure 2.7) matched the morphological descriptions of Linton (1928), with trematode counts ranging from 1-466; with a mean infection intensity of 40.5 ± 18.0 and a mean abundance of 30.1 ± 13.6 (Table 2.3; Figures 2.4 and 2.5). Currently, there is no sequencing data on this parasite published in the GenBank database. The 18S gene sequence *of P. varium* collected in this study was a 97% match to *Fascioloides magna*, *Petasiger phalacrocoracis*, *Fasciola gigantica*, *Euparyphium melis*, and *Isthmiophora hortensis*. The sequence of the COI

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gene for *P. varium* had no close matches to any digenean sequence currently published in GenBank.

Austrodiplostomum ostrowskiae was found in the intestines of the double-crested cormorants. The collected specimens of this parasite (Figure 2.8) matched the morphological description of Dronen (2009) with the trematode counts ranging from 1-44; with a mean infection intensity of 11.3 ± 3.0 and a mean abundance of 6.4 ± 1.9 (Table 2.3; Figures 2.4 and 2.5). The resultant sequence of the 18S gene of this parasite was a 100% match to *Diplostomum spathaceum* (Flowers, 2003a). However, the COI gene for *A. ostrowskiae* sequenced in this study was only an 88% match to *Diplostomum* sp. (Locke, 2010). The COI sequence for *A. ostrowskiae* is not published in GenBank, so no comparisons could be made with the COI gene sequences of *A. ostrowskiae* reported in this study.

Ascocotyle longa (syn. *Phagicola longa*) was recovered from the stomachs and intestines of the double-crested cormorants. Morphologic identifications (Figure 2.9) matched those described by Ransom (1920). The total count of this trematode ranged from 1-61, with a mean infection intensity of 14.8 ± 6.0 and mean abundance of 4.2 ± 2.0 (Table 2.3; Figures 2.4 and 2.5). The sequenced 18S gene of *A. longa* collected in this study was a 97% match to the published sequence of *Phagicola longa* (Dzikowski et al., 2004). The sequence of the COI gene for this parasite is not published on GenBank, therefore no comparisons could be made with the COI gene sequencing data reported for *A. longa* in this study.

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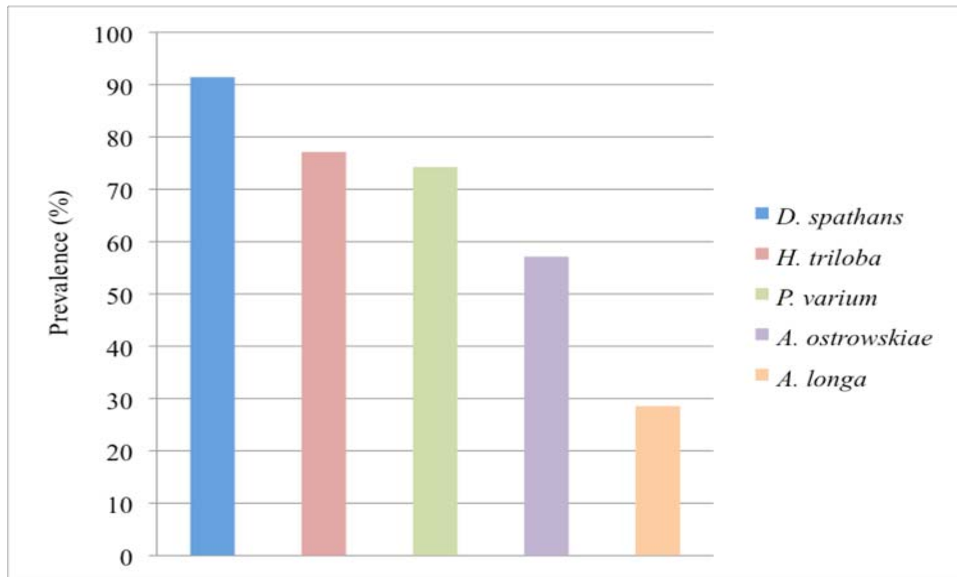

Figure 2.1 Prevalence of digenetic trematodes found in the 35 double-crested cormorants surveyed in this study.

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Table 2.3 Digenetic trematodes found in the 35 double-crested cormorants, *P. auritus,* surveyed in this study.

Trematode Species	[a]P(%)	Intensity, X ± SE	Abundance, X ± SE	Range	[b]Site of Infection	Percentage in Stomach
A. longa	28.6%	14.8 ± 6.0	4.2 ± 2.0	1-61	S, I	10.1%
A. ostrowskiae	57.1%	11.3 ± 3.0	6.4 ± 1.9	1-44	I	0%
D. spathans	91.4%	81.5 ± 18.1	74.5 ± 17.0	1-346	S, I	8.9%
H. triloba	77.7%	32.5 ± 9.4	25.1 ± 7.7	1-184	S, I	20.4%
P. varium	74.3%	40.5 ± 18.0	30.1 ± 13.6	1-466	S	100%

[a]P=prevalence
[b]S=stomach, I=intestine

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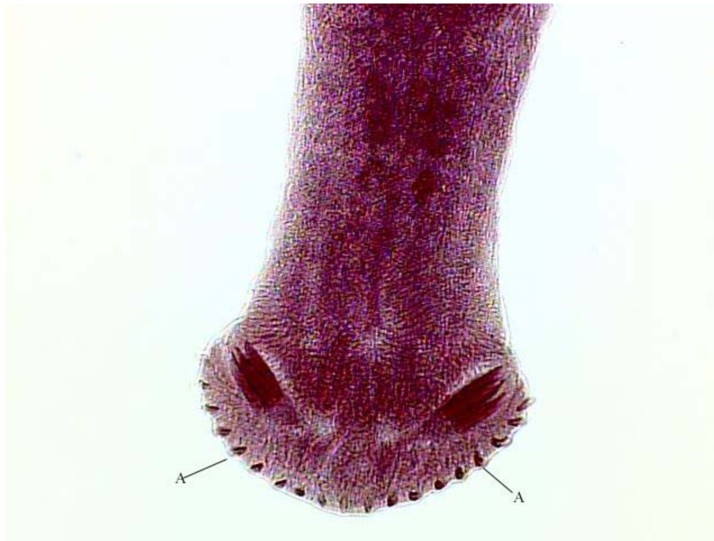

Figure 2.2 Anterior region of an adult *Drepanocephalus spathans* collected from the intestine of a double-crested cormorant in this study showing distinctive head collar spines (A).

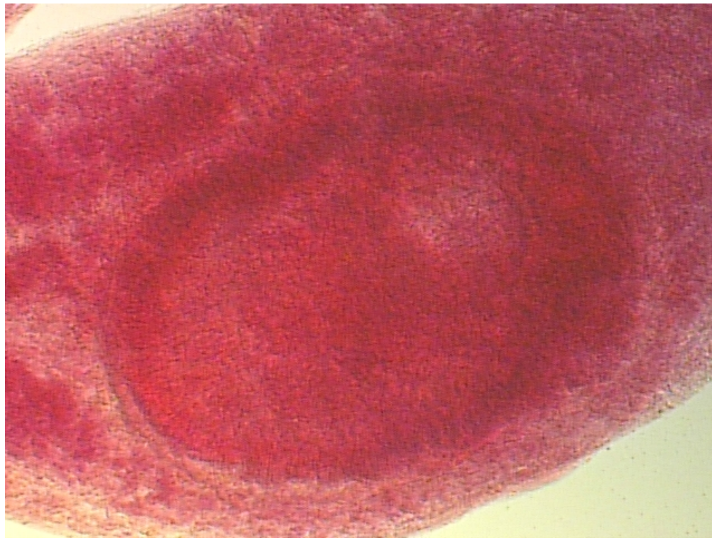

Figure 2.3 The large, muscular acetabulum of an adult *Drepanocephalus spathans* collected from the intestine of a double-crested cormorant in this study.

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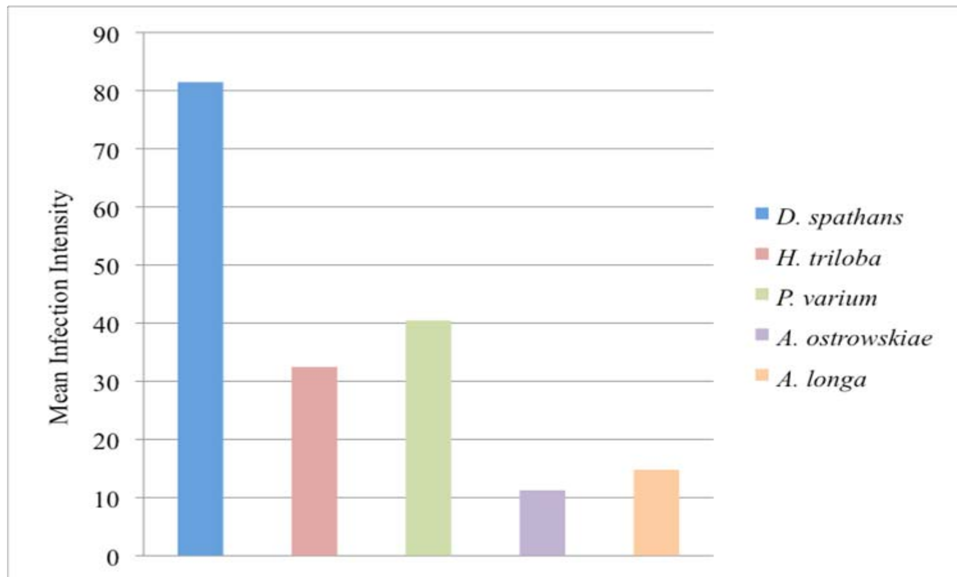

Figure 2.4 Mean infection intensities of the digenetic trematodes found in the 35 double-crested cormorants surveyed in this study.

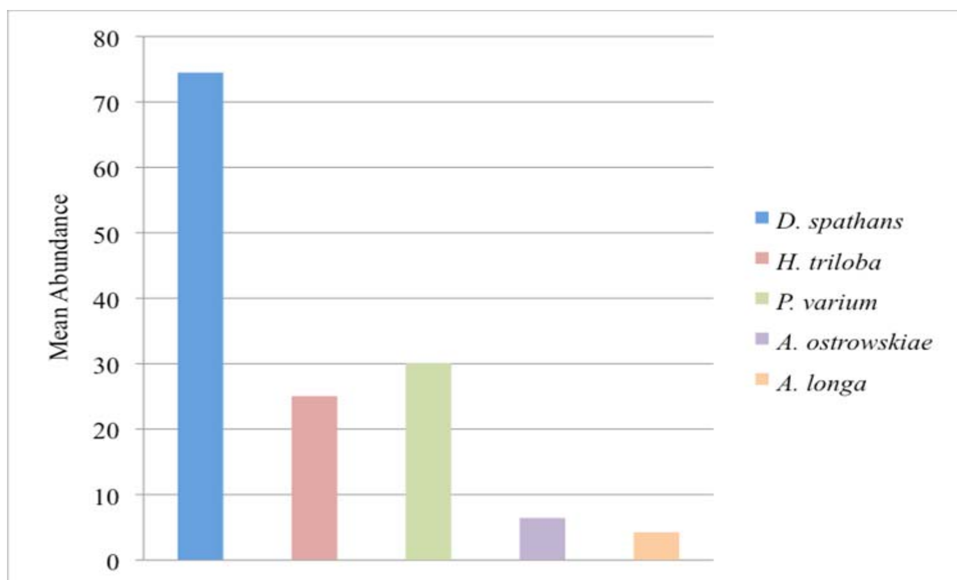

Figure 2.5 Mean abundance of the digenetic trematodes found in the 35 double-crested cormorants surveyed in this study.

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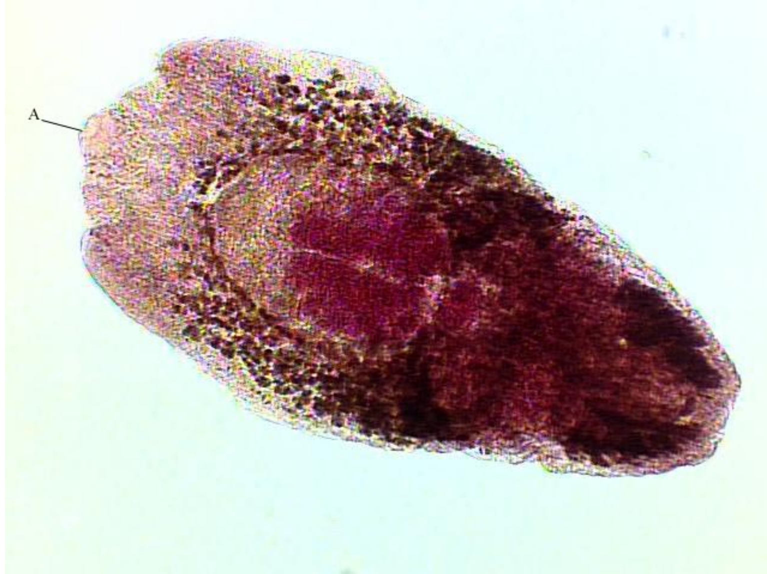

Figure 2.6 An adult *Hysteromorpha triloba* collected from the intestine of a double-crested cormorant collected during the study demonstrating the triangular shaped body and small oral sucker (A).

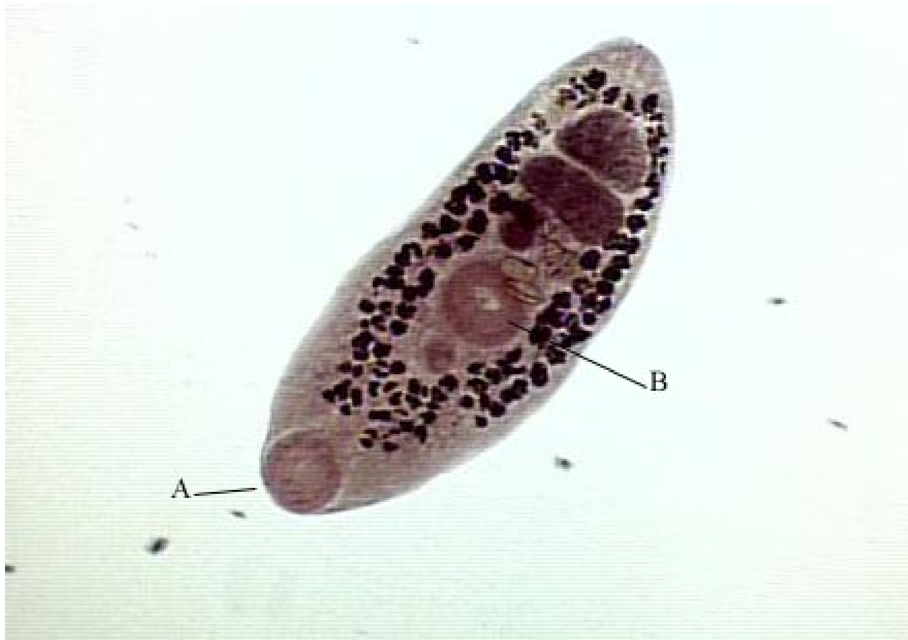

Figure 2.7 An adult *Pseudopsilostoma varium* collected from the stomach of a double-crested cormorant in the study demonstrating a well-developed oral (A) and ventral sucker (B).

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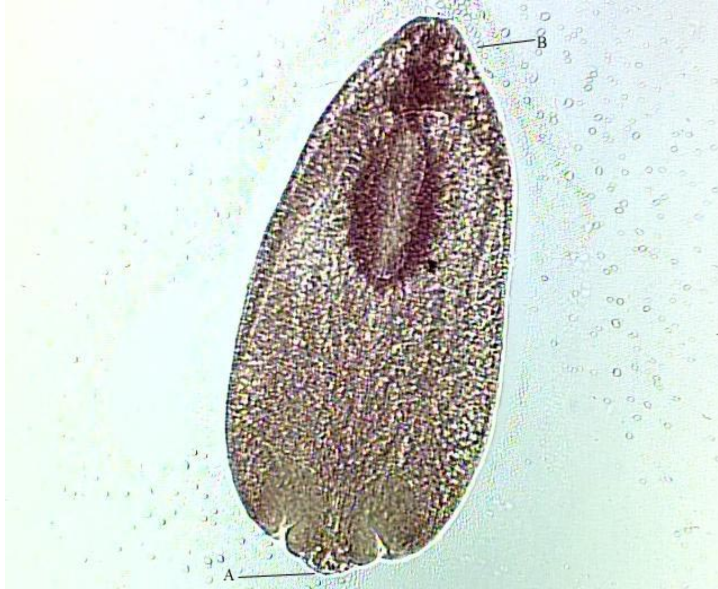

Figure 2.8 An adult *Austrodiplostomum ostrowskiae* collected from the intestine of a double-crested cormorant in the study demonstrating the small oral sucker (A) and distinct hindbody region (B).

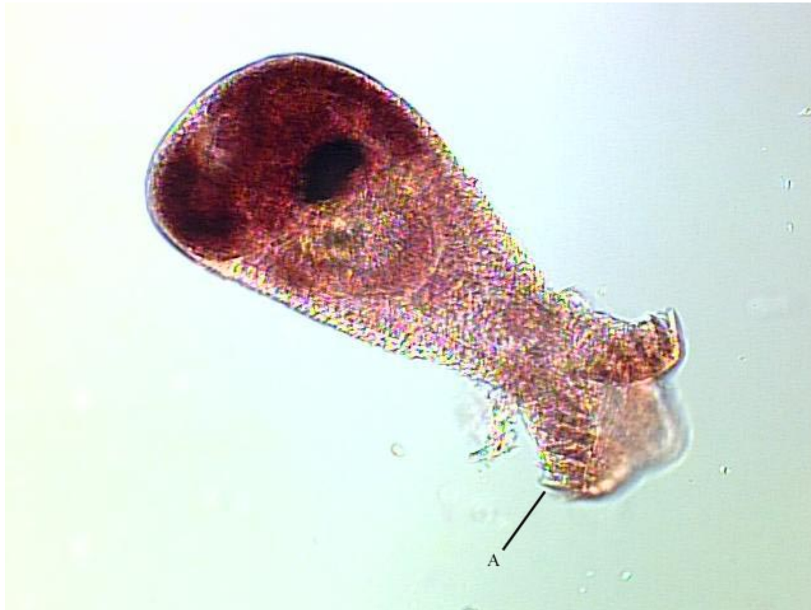

Figure 2.9 An adult *Ascocotyle longa* collected from the intestine of a double-crested cormorant in the study demonstrating a pyriform body and circumoral spines (A).

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Discussion

Although previous parasitic surveys of the double-crested cormorant have been completed in Texas, North Carolina and Florida (Fedynich et al., 1997; Flowers et al., 2004; Threlfall, 1982), this survey is the first report on the digenetic trematodes present in the double-crested cormorant population wintering in the Mississippi Delta. This survey found five different adult trematode species in this population of double-crested cormorants; *Ascocotyle longa*, *Austrodiplostomum ostrowskiae*, *Drepanocephalus spathans*, *Hysteromorpha triloba* and *Pseudopsilostoma varium.* The number of different trematode species seen in this survey is similar to those found in three previous surveys. Fedynich et al. (1997) described six species of intestinal trematodes in double-crested cormorants wintering in Texas, including *Ascocotyle* sp., *Austrodiplostomum ostrowskiae*, *Drepanocephalus spathans*, *Maritrema* sp., *Mesoophorodiplostomum pricei* and *Phocitremoides butionis*. Threfall (1982) described four species in double-crested cormorants that reside in Florida year-round, including *Drepanocephalus spathans*, *Ascocotyle longa*, *Mesostephanus appendiculatoides* and *Hysteromorpha triloba.* Only three digenetic trematodes were described from double-crested cormorants residing in North Carolina, however only two cormorants were sampled. The three digenetic trematodes recovered from these two double-crested cormorants were *Drepanocephalus spathans*, *Austrodiplostomum compactum* and *Hysteromorpha triloba* (Flowers et al., 2004).

The least prevalent trematode recovered from the Mississippi Delta double-crested cormorants was *Ascocotyle longa*, with only 28.6% of cormorants being infected

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with this digenean. The only survey data for this particular trematode was in a population of double-crested cormorants residing in Florida, which had prevalences ranging from 38-56% for *A. longa* (Threlfall, 1982). The mean infection intensity for the Florida double-crested cormorants ranged from 54-106 (Threlfall, 1982), compared to a mean infection intensity of 14.8 in the Mississippi Delta double-crested cormorant population.

Austrodiplostomum ostrowskiae was found in the intestines of 10 of the 25 double-crested cormorants (57.1%) in the Mississippi Delta. This infection rate was higher than a 1997 survey of double-crested cormorants in Texas, where 33% of the birds (4 out of 12) were infected with *A. ostrowskiae* (syn. *A. mordax*), but was lower than the 100% prevalence (10 birds) observed in a more recent Texas survey (Dronen, 2009). The mean infection intensity was also much higher in that 2009 survey, with 135 trematodes per infected bird (range 9-225) reported, compared to the reported mean infection intensity of 4.2 (range 1-8) in the 1997 survey (Dronen, 2009; Fedynich et al., 1997) and the mean infection intensity of 11.3, (range 1-44) reported in the Mississippi Delta double-crested cormorants sampled in this study.

The sequencing results of *A. ostrowskiae* demonstrated how sequencing the 18S gene alone could be misleading or result in an inaccurate identification. Although the 18S gene sequence for *A. ostrowskiae* was a 100% match to that of *Diplostomum spathaceum*, the differences in the both morphology of these trematodes and the COI gene sequences indicated that they are two different species. Adult *D. spathaceum* are found in the intestines of gulls (Whyte et al, 1987) and are morphologically very distinct from *A. ostrowskiae* (Niewiadomska, 1984). The COI gene sequence for *D. spathaceum* is not published on GenBank and as such cannot be compared to the COI gene sequence of *A.*

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ostroawskiae found in the Mississippi Delta population of double-crested cormorants. However, the COI gene sequence of *A. ostrowskiae* is only an 88% match to *Diplostomum* sp. 7, which provides further evidence that the 18S gene is too highly conserved to provide adequate data for molecular identification at the species level.

Drepanocephalus spathans was both highly prevalent and abundant in the double-crested cormorants in the Mississippi Delta with a prevalence of 91.4% and a mean infection intensity of 81.5. This differed greatly from the reported prevalence and intensities of *D. spathans* recovered from double-crested cormorants in Texas and Florida. Only one specimen of *D. spathans* was recovered from the intestine of a single double-crested cormorant out of 12 birds that were surveyed in Texas (Fedynich et al., 1997). The prevalence of *D. spathans* in the double-crested cormorants in Florida was slightly higher, with 9.2% of birds being infected. However, the mean infection intensity of 263 (range 1-956) in the Florida study (Threlfall, 1982) was much greater than that of the Mississippi Delta cormorants having an average infection intensity of 81.5 (range 1-346).

Hysteromorpha triloba was also found in significant numbers in the Mississippi Delta double-crested cormorants. *Hysteromorpha triloba* is a frequently reported parasite of cormorants (Chandler and Rausch, 1948; Flowers et al., 2004; Hugghins, 1956; Hutton, 1964), however the only quantitative data for this trematode was reported by Threfall (1982). Approximately 17% of the double-crested cormorants that were surveyed in that study in Florida were infected with *H. triloba*. The mean infection intensity was 4,169, with the as many as 19,727 found in a single bird (Threlfall, 1982). The prevalence in the Mississippi Delta double-crested cormorants was higher with

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77.7% of the birds infected with *H. triloba*, however the mean infection intensity of 32.5 was much lower than that of the Florida double-crested cormorants.

Pseudopsilostoma varium was found in the stomachs of the Mississippi Delta double-crested cormorants. None of the previous surveys reported this trematode in any of the double-crested cormorants sampled. Little is known about this digenetic trematode and the only quantitative data for it is from a population of neotropic cormorants, *Phalacrocorax brasilianus* residing in Texas (Fedynich et al., 1997). The prevalence in the Mississippi Delta double-crested cormorants was 74.3%, in contrast to 35% of the neotropic Texas cormorants infected. The mean infection intensity of *P. varium* in the neotropic cormorants was 27.7, while the mean infection intensity for the double-crested cormorants in this study was 40.5. The uppermost range of this trematode in the neotropic cormorant was 78 (Fedynich et al., 1997), while up to 466 *P. varium* were found in the stomach of one of the double-crested cormorants collected in this study.. Currently, little information exists on the life cycle of *P. varium*. The prevalence and abundance of this parasite in the Mississippi Delta population of double-crested cormorants strongly suggests that the second intermediate host is a fish species, however no studies have elucidated the life cycle of this parasite. The impact of this trematode on fish populations in the Mississippi Delta currently is unknown.

Previous surveys of double-crested cormorants reported that *D. spathans*, *H. triloba* and *A. longa* have only been found in the intestines of the birds. However, this survey has shown that a small number of these parasites may be present in the stomachs. Approximately 8.9% of *D. spathans*, 20.4% of *H. triloba* and 10.1% of *A. longa* collected were found in the stomachs. Although transported on ice, it was possible that the few

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hours that lapsed between euthanasia and necropsy (three hours) could have resulted in the parasites' migration to aberrant sites.

A few of the previously reported digenetic trematodes of the double-crested cormorants were not present in the surveyed Mississippi Delta population. However, differences in cormorant diets can potentially explain this variation. *Mesostephanus appendiculatoides* was reported in 38% of surveyed double-crested cormorants in Florida (Threlfall, 1982), but metacercariae have only been reported in brackish and saltwater fish species, not freshwater fish species (Hoffman, 1999). A single specimen of *Maritrema* sp. was collected from one double-crested cormorant from Texas (Fedynich et al., 1997). Several genera of crabs are reported to be the secondary intermediate host for this parasite (Ching, 1963; Etchegoin and Martorelli, 1999; Martorelli et al., 2004). *Mesoophorodiplostomum pricei* was also collected from a single double-crested cormorant in Texas (Fedynich et al., 1997) and the life cycle for this parasite is unknown. *Phocitremoides butionis* was found in a single double-crested cormorant in Texas (Fedynich et al., 1997) and the metacercariae of this trematode have only been found encysted in brackish water fish species (Shaw et al., 2005).

Conclusion

This survey is the first to document the prevalence and intensities of trematodes in the double-crested cormorants wintering in the Mississippi Delta. The predation impact of this piscivorous bird on the commercial catfish industry in this region has been well documented, however the parasites present in this bird population and the impact of these parasitisms is unknown. This two-year survey demonstrated that the double-crested

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cormorants in this region are hosts to high numbers of several species of trematodes, many of which are capable of infecting a wide variety of freshwater fish species, often causing pathology. The variation seen in helminth profiles of this population of cormorants compared to the double-crested cormorants surveyed in Texas and Florida may have been due to differences in habitat and diet. Most of the Mississippi Delta population of double-crested cormorants does not migrate further south than this region and subsequently brackish and saltwater fish and crustaceans are not usually components of their diet. Thus these birds would not have the opportunity to ingest the metacercariae of trematodes that utilize these species as intermediate hosts.

In this study novel sequences of the COI gene were obtained for several digenetic trematodes that have been reported in the double-crested cormorant. The COI sequences for *Drepanocephalus spathans, Austrodiplostomum ostrowskiae*, *Pseudopsilostoma varium* and *Ascocotyle longa* will be new additions to the GenBank database.

This research is also the first report of *P. varium* in the double-crested cormorant. The prevalence reported in this study of 74.3% would indicate this is not an incidental finding, but is a common parasite in this population of birds. Since the life cycle is unknown it is impossible at this point to determine the source of infection and the potential impact this parasite may have on fish populations.

This study confirmed the double-crested cormorants in the Mississippi Delta not only serve as definitive hosts for several trematodes, but often these birds are heavily parasitized with these adult trematode species. The heavy parasite burdens reported in this study did not appear to adversely affect the health of the birds, however, the impact these trematodes may have on commercial and feral (or wild) freshwater fish in the

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Mississippi Delta is still unclear. Many of the trematodes found in the Mississippi Delta double-crested cormorants in this study are reported to have a wide range of fish hosts and some of the snail hosts are also present in this region. *Ascocotyle longa* and *Hysteromorpha triloba* are known to induce pathology in fish, however the life cycles of *Austrodiplostomum ostrowskiae*, *Pseudopsilostoma varium* and *Drepanocephalus spathans* have not been elucidated. Further research is needed to ascertain the identity of the intermediate hosts in the life cycles of these parasites and the potential impact these parasites may have on the wild and cultured fish as they are introduced into the waterways in this region.

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CHAPTER III

MORPHOLOGICAL AND MOLECULAR IDENTIFICATIONS OF DIGENETIC TREMATODES IN WILD FISH SPECIES COLLECTED FROM AN OX-BOW LAKE IN THE MISSSISSIPPI DELTA

Abstract

The commercialization of the channel catfish industry has led to an increase number of piscivorous birds residing in the Mississippi Delta. These birds serve as definitive hosts to digenetic trematodes, many of which are capable of infecting a fish host. Prior to this survey, little information was available concerning the larval digeneans infecting wild freshwater fish in this region. In this survey fish were collected from a Mississippi Delta ox-bow lake, which had a large resident cormorant population. Fourteen fish species were collected and examined for parasites, which were identified morphologically and by DNA sequencing of the 18S and COI genes. *Posthodiplostomum minimum* metacercariae were found in multiple organs of six out of the 14 fish species collected which included: bluegills (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), orangespotted sunfish (*Lepomis humilis*), warmouths (*Lepomis gulosus*), longear sunfish (*Lepomis megalotis*) and threadfin shad (*Dorosoma petenense*). The 18S gene sequences of these metacercariae were 100% homologous to the published *P. minimum* sequence. The COI gene sequences of the metacercaraie matched published

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COI gene sequences for *Posthodiplostomum* sp. 3, 5 and 8, which may indicate multiple subspecies of *P. minimum* are present in this fish population.

Introduction

The commercial catfish industry in the Mississippi Delta region experienced rapid growth in the 1980s and 1990s (Mott and Brunson, 1997). This growth has been accompanied by a steady increase in piscivorous birds in this region. The bird species most commonly observed feeding on the catfish ponds include the double-crested cormorant, *Phalacrocorax auritus*, American white pelicans, *Pelecanus erythrorhynchus,* great egrets, *Ardea alba*, and great blue herons, *Ardea herodias* (Glahn et al., 1999, 2000; King and Werner, 2001; King, 2005). In a survey done by the USDA, 70% of catfish producers consider the double-crested cormorant to be the primary wildlife species affecting channel catfish (*Ictalurus punctatus*) stock (Wywialowski, 1999). Double-crested cormorants and great blue herons are the most commonly reported birds feeding on channel catfish in this region (Glahn and King, 2004).

While feeding and loafing on catfish ponds and other waterways in the Mississippi Delta, piscivorous birds are potentially introducing numerous digenetic trematodes into these waterways. Many of these digenetic trematodes are capable of infecting a variety of fish species, including channel catfish (Hoffman, 1999). Digenetic trematodes known to infect cultured channel catfish include *Bolbophorus damnificus*, *Bursacetabulus pelecanus*, *Hysteromorpha triloba*, *Diplostomum spathaceum* and *Clinostomum marginatum* (Overstreet and Curran, 2004; Wise et al., 2004). *Diplostomum*

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spathaceum and *Clinostomum marginatum* infections are usually mild and have not been noted to cause economic losses (Wise et al., 2004).

While the trematodes infecting channel catfish in the Mississippi Delta are well documented, little information is available concerning digenetic trematodes affecting wild fish species in this region. Currently no parasite surveys have been completed on wild fish species in the Mississippi Delta region. Most of the trematodes reported in freshwater fish species in the southeast region of the United States are based on diagnostic or case reports. Common trematodes reported in the wild fish in this region are *Uvulifer ambloplitis*, *Posthodiplostomum minimum*, *Clinostomum* spp. and *Diplostomum spathaceum*.

Uvulifer ambloplitis is a commonly reported parasite of fish in the families Centrarchidae, Cyprinidae and Esocidae (Hoffman, 1999). *Uvulifer ambloplitis* metacercariae are frequently seen as pinhead-sized black spots in the skin, tail base, fins and musculature of the fish (Hunter and Hunter, 1938). The freshwater snail, *P. trivolvis*, shed *Uvulifer ambloplitis* cercariae, which penetrates a fish host, causing mechanical damage and hemorrhage (Hoffman and Putz, 1965). The reported definitive host for *U. ambloplitis* is the belted kingfisher, *Megaceryle alcyon* (Hoffman and Putz, 1965).

Posthodiplostomum minimum metacercariae have been reported in a wide variety of North American freshwater fishes (Grizzle and Goldsby, 1996), especially in fish from the Centrarchidae family (Hoffman, 1958). *P. minimum* metacercariae encyst in the liver, kidneys and heart and mesenteries of the infected fish (Avault and Smitherman, 1965; Spall and Summerfelt, 1970). Severe infections of *P. minimum* can cause significant disease in fish, particularly in cases in which the metacercariae are so numerous that they

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cause the organs to become compressed. Heavily infected fish can have displaced organs, ascites, ruptured abdomens and mortality (Mitchell et al., 1982).

Numerous birds, great blue herons (*Ardea herodias*) (Spall and Summerfelt, 1970), great egrets (*Ardea alba*), snowy egrets (*Egretta thula*) common loons (*Gavia immer*), the black-crowned night herons (*Nycticorax nycticorax*) and the American white pelican (*Pelecanus erythorhynchus*) (Kinsella et al., 2004; Kinsella and Forrester, 1999; Ponce de Leon, 1995) have been reported as the definitive host for the adult of *P. minimum*. Several *Physa* species serve as the intermediate snail host for the *P. minimum* cercariae.

Generally *Clinostomum* spp. infections are subclinical and usually only associated with pathology in young heavily infected fish (Hoffman, 1999). *Clinostomum* spp. metacercariae usually encyst under the dermis of the catfish (Overstreet and Curran, 2004). The definitive host of *Clinostomum* spp. is the great blue heron, *Ardea herodias* (Hoffman, 1999; Overstreet and Curran, 2004). The intermediate snail host is the rams-horn snail, *P. trivolvis,* which shed *Clinostomum* cercariae, and are capable of infecting a wide variety of freshwater fish (Hoffman, 1999).

Diplostomum spathaceum infects a wide range of freshwater fish (Hoffman, 1999). Gulls serve as the definitive host for this trematode (Whyte et al., 1987). *D. spathaceum* forms metacercariae in the lens of the eye and can cause in blindness in the fish host (Holloway and Leno, 1982). Lymnaea snails are the first intermediate host for *D.spathaceum* (Whyte et al., 1987).

Double-crested cormorants and other piscivorous birds are potential reservoir hosts for several digenetic trematodes that could cause severe pathology in both

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commercial channel catfish and the wild fish species that reside in the natural waterways in the Mississippi Delta. The increasing numbers and persistence of piscivorous birds in the Mississippi Delta, particularly the double-crested cormorant, would strongly suggest that these birds might play a significant role in trematode infections in wild and cultured fish in this region. However, no survey has been done to confirm which trematodes are present in these wild fish populations and the level of parasitisms that may exist. In order to achieve this and establish a baseline, a study was done to determine the trematodes present in a wild fish population residing in a natural Mississippi Delta lake that had a resident population of double-crested cormorants feeding and roosting on the premises.

Materials and Methods

Fish Collection

Wild fish were collected from Caile Lake, an ox-bow lake located in Humphreys County, Mississippi (GPS coordinates: 33 degrees 18’ 17.29” N, 90 degrees 32’ 56.58” W). Caile Lake had been reported to have a history of a large resident population of double-crested cormorants (*Phalacrocorax auritus*). Fish were collected July 20, 2009 at five sites in the lake with five fish per species collected per site when possible. Using electrofishing techniques (Reynolds, 1983) fish were captured with nets, immediately euthanized with ethyl 3-aminobenzoate methanesulphonate salt (Sigma, St. Louis Missouri) and placed on ice. Collected fish were transported to the parasitology laboratory at the College of Veterinary Medicine at Mississippi State University for

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necropsy. On the day of fish collection, the following water quality data were recorded for the lake: temperature, dissolved oxygen, pH and chlorophyll-a and maximum pond depth.

Necropsy

The external surface and all organs (the liver, kidneys, heart, gills, muscle, eyes, gall bladder and mesentery) of each fish were examined grossly, using a magnifying lamp, for the presence of parasites and lesions. The fish were filleted and all muscle was sliced vertically in approximately 0.5 cm sections and examined microscopically for encysted parasite stages. All parasites or suspect lesions were removed for further microscopic examination. Any parasites found were placed in 70% molecular grade ethanol for morphological and molecular identification.

Using standard parasitological techniques, the gastrointestinal tract was then removed, opened longitudinally and rinsed into a brass sieve with an aperture of 75 μm using reverse osmosis water. The contents were either microscopically examined immediately and all parasites were removed or contents were preserved in 10% formalin for later examination. The serosa and mucosa of the entire intestinal tract was also examined microscopically for parasites and lesions. All parasites were collected and preserved in 70% molecular grade alcohol for further DNA analysis or for morphological analysis. All metacercariae were collected, enumerated and identified by morphometric and molecular methods.

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Parasite Identification

Morphological identification: In order to obtain a morphological identification of the metacecariae, collected specimens were stained with acetocarmine (Fisher Scientific, Pittsburgh, Pennsylvania). Specimens were placed in the stain for 8-10 hours and then destained with 1% acid alcohol and then progressed through a series of dehydrating ethanol washes (70%, 95% and 100% ethanol). The metacecariae were then cleared with Citri-solve (Omega Laboratories, Inc., Houston, Texas), mounted on slides using Permount (ProSciTech, Thuringowa Central Queensland, Australia) and examined microscopically.

Molecular identification: The molecular identification of the metacercariae collected from the fish was based on DNA sequencing of both the nuclear ribosomal18S gene and the mitochondrial COI gene. Prior to extraction, specimens were rinsed in nuclease free water. DNA was extracted from representative specimens previously preserved in 70% molecular grade ethanol. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California) according to manufacturer's protocol. The purified genomic DNA was then suspended in 400 µl of AE (10mM Tris-Cl, 0.5mM EDTA) buffer solution (Qiagen, Valencia, California).

To ensure adequate coverage of the 18S gene, single PCR reactions were performed using three sets of primers: Worm A and Worm B, 18S-7 and 18S-A27 and 600F and 18S-11 (Table 3.1). The total PCR volume was 25 µl, which contained 10X buffer, 2.0 µl template, 0.625 U *Taq* polymerase (Hot Start Taq; Takara Bio, Inc., Shiga, Japan), 200 nM of each primer and 200 µM of each deoxynucleotide triphosphate.

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Polymerase chain reaction conditions were 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 54°C for 30 sec, 72° for 2 min, with a final cycle of 72°C for 5 min. PCR reactions were performed using a PTC-100 Peltier Thermal Cycler (MJ Research, Watertown, Massachusetts).

Amplification and sequencing of the COI gene was performed using the COI specific primers and PCR protocols of Moszczynska et al. (2009). All genomics were screened using two sets of primers, Plat-diploCOX1F and Plat-diploCOX1R and MplatCOX1dF and MplatCOX1dR (Table 3.1). The Plat-diploCOX1F and Plat-diploCOXR are specific for the family Diplostomatidae, while the MplatCOX1dF and MplatCOX1dR are generic platyhelminth primers. In most cases, each genomic sample amplified using only one of the two primers sets, depending on species. The total PCR volume was 25 µl, which contained 1X buffer, 2.5mM $MgCl_2$, 2.0 µl template, 0.625 U of Platinum *Taq* Polymerase (Invitrogen, Carlsbad, California), 200 nM of each primer and 200 µM of each deoxynucleotide triphosphate. Polymerase chain reaction conditions were 94°C for 2 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, with a final extension of 72°C for 10 min.

All PCR products were visualized with Gelstar nucleic acid stain (Combrex BioScience Rockland, Inc., Rockland, Maine) on a 1.2% agarose gel. PCR products were purified using a Montage PCR Centrifugal Filter device (Millipore, Billerica, Maryland) prior to sequencing at Arizona State University DNA Laboratory (Tempe, Arizona). Sequences were assembled using the SeqMan of the Lasergene version 8.0 software

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package (DNASTAR, Madison, Wisconsin) and were edited manually. Results underwent a database search using BLAST (Altschul et al., 1990).

Prevalence, mean infection intensity and mean abundance were calculated as described by Margolis et al. (1982). Prevalence is the number of individuals infected with a particular parasite species divided by the number of hosts examined. Mean infection intensity is the total number of individuals of a particular parasite species divided by the number of infected hosts. Mean abundance is calculated by dividing the total number of a particular parasite species by the total number of hosts examined (Margolis et al., 1982).

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Table 3.1 PCR primers for amplification of 18S rRNA and mitochondrial COI genes

Primer Name	Primer Sequence 5'-3'	Region Amplified	References
Worm A	GCGAATGGCTCATTAAATCAG	18S	Littlewood and Olson 2001
Worm B	CTTGTTACGACTTTTACTTCC	18S	Littlewood and Olson 2001
18S-7	GCCCTATCAACTGTCGATGGTA	18S	Littlewood and Olson 2001
18S-A27	CCATACAAATGCCCCCGTCTG	18S	Littlewood and Olson 2001
600F	GCAGCCGCGGTAATTCCAGC	18S	Littlewood and Olson 2001
18S-11	CGGCCATGCACCACC	18S	Littlewood and Olson 2001
MplatCOX1dF	TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG	COI	Moszczynska et al 2009
MplatCOX1dR	CAGGAAACAGCTATGACTGAAAYAAYAIIGGATCICCACC	COI	Moszczynska et al 2009
Plat-diploCOX1F	CGTTTRAATTATACGGATCC	COI	Moszczynska et al 2009
Plat-diploCOX1R	AGCATAGTAATMGCAGCAGC	COI	Moszczynska et al 2009

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Results

A total of 131 fish representing 14 different species were collected from Caile Lake. Fish species included: threadfin shad (*Dorosoma petenense*), spotted gar (*Lepisosteus oculatus*), bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), common carp (*Cyprinus carpio*), longear sunfish (*Lepomis megalotis*), orangespotted sunfish (*Lepomis humilis*), shortnose gar (*Lepisosteus platostomus*), smallmouth buffalo (*Ictiobus bubalus*), bowfin (*Amia calva*), pugnose minnow (*Opsopoeodus emiliae*), warmouth (*Lepomis gulosus*), gizzard shad (*Dorosoma cepedianum*) and white crappie (*Pomoxis annularis*).

Of the 131 fish surveyed, 35 fish (26.7%) representing five different fish species were infected with a single species of metacercariae, which was identified as *Posthodiplostomum minimum.* Collected specimens of *P. minimum* (Figure 3.1) matched the morphological descriptions of Hughes (1928) and Palmieri (1976). Infected fish species included bluegill, largemouth bass, orangespotted sunfish, longear sunfish, warmouth and threadfin shad. The mean infection intensity for this population of fish was 57.6 ± 15.3; the mean abundance was 15.4 ± 4.6. The range for this metacercariae was 1-368 (Table 3.2).

Bluegills were most heavily infected with *P. minimum*, with 20 of the 25 (80.0%) of the fish sample infected. The mean infection intensity was 71.8 ± 23.1 metacercariae per fish with a range of 1-368. The mean abundance was 57.4 ± 19.3. *Posthodiplostomum minimum* metacercariae were found in the kidneys, liver, mesentery and muscle of the bluegills (Table 3.2, Figures 3.2-3.4). The resultant sequence of the

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18S gene was a 100% match to the published *P. minimum* sequence in GenBank (Flowers, 2003). DNA sequences of the COI gene showed that two species of *Posthodiplostomum* were present in this population of bluegills (Table 3.3). Seventeen of the twenty infected (85.0%) bluegills contained metacercariae that were a 100% match to *Posthodiplostomum* sp. 3. The remaining three infected bluegills were infected with *Posthodiplostomum* sp. 5 (Moszczynska et al., 2009). *Posthodiplostomum* sp. 3 metacercariae were found in the liver, muscle, mesentery and kidneys of the bluegills, while *Posthodiplostomum* sp. 5 was only found in the muscle of the fish.

Largemouth bass were also heavily infected with *Posthodiplostomum minimum*, with 8 of the 15 collected largemouth bass infected (53.3%). The mean infection intensity in largemouth bass was 63.3 ± 31.2, while the mean abundance was 33.7 ± 18.2 (Table 3.2, Figures 3.2-3.4). The range of *P. minimum* metacercariae collected from largemouth bass was 2-268 and were found in the kidneys, liver and mesentery (Table 3.2). The resultant sequence of the 18S gene was a 100% match to the published sequence for *P. minimum* (Flowers, 2003). DNA sequencing of the COI gene for metacercariae collected from the largemouth bass were a 100% match (Table 3.3) to *Posthodiplostomum* sp. 8 (Moszczynska et al., 2009).

Only four orangespotted sunfish were collected during this survey, however three of them were infected with *P. minimum* metacercariae (75.0%). The mean infection intensity was 7.7 ± 3.5 and the mean abundance was 5.8 ± 3.2. The range of metacercariae collected from this species was 1-13 and were found in the heart and muscle (Table 3.2, Figures 3.2-3.4). The 18S gene sequence for the metacercariae collected from this fish species was a 100% match to the reported sequence of *P.*

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minimum in GenBank (Flowers, 2003). The DNA sequences of the COI genes (Table 3.3) were a 100% match to *Posthodiplostomum* sp. 3 (Moszczynska et al., 2009).

Five warmouths were collected during this survey, with two of the fish being infected with *P. minimum* metacercariae (40.0%). The mean infection intensity was 9.5 ± 1.5 and the mean abundance was 3.8 ± 2.4. The range of metacercariae collected in the warmouths was 8-11 and were found in the hearts of both infected fish (Table 3.2, Figures 3.2-3.4). The 18S gene sequence was a 100% match to the published *P. minimum* sequence in GenBank (Flowers, 2003). The COI gene sequence was a 100% match (Table 3.3) to *Posthodiplostomum* sp. 3 (Moszczynska et al., 2009).

Of the three longear sunfish collected, one was infected (33.3%) with *P. minimum* metacercariae. Thirty metacercariae were recovered from the muscle of this fish with a mean abundance of 10.0 ± 10.0 for *P. minimum* (Table 3.2, Figures 3.2-3.4). The 18S gene sequence of the metacercariae collected was a 100% match to the reported sequence of *P. minimum* (Flowers, 2003), while the COI gene sequence was a 100% match to *Posthodiplostomum* sp. 3 (Moszczynska et al., 2009)

Only one of the 25 threadfin shad collected was infected with two *P. minimum* metacercariae found in the mesentery of this fish. The mean abundance of *P. minimum* in this population of threadfin shad was therefore 0.08 ± 0.08 (Table 3.2, Figures 3.2-3.4). The 18S gene sequence was 100% homologous with published result for the 18S gene sequence and the COI gene sequence (Table 3.3) was 100% homologous with *Posthodiplostomum* sp. 3 (Moszczynska et al., 2009).

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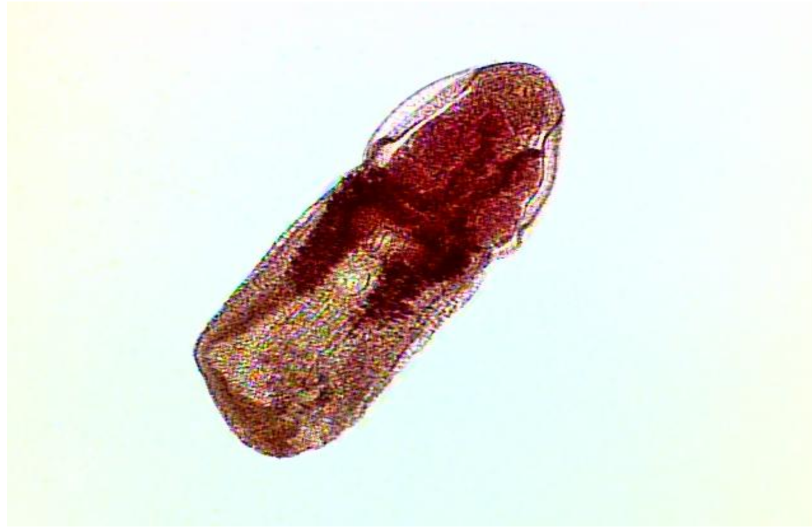

Figure 3.1 *Posthodiplostomum minimum* metacercariae collected from a bluegill (*Lepomis macrochirus*)

Table 3.2 *Posthodiplostomum minimum* metacercariae collected in freshwater fish from Caile Lake.

Fish Species (number collected)	P(%)	Intensity, X ± SE	Abundance, X ± SE	Range	Site of infection
Bluegill (25)	80.0%	71.8 ± 23.1	57.4±19.3	1-368	[a]K,[b]L, [c]Me, [d]Mu
Largemouth Bass (15)	53.3%	63.3 ± 31.2	33.7 ±18.2	2-268	K, L, Me
Orangespotted sunfish (4)	75.0%	7.7 ± 3.5	5.8 ±3.2	1-13	[e]H, Mu
Warmouth (5)	40.0%	9.5 ± 1.5	3.8 ±2.4	8-11	H
Longear sunfish (3)	33.3%	30	10.0 ±10.0	30	Mu
Threadfin shad (25)	4.0%	2	0.08 ±0.08	2	Me
All Fish Species	26.7%	57.6 ± 15.3	15.3 ± 4.6	1-368	H, K, L, Me, Mu

[a]Kidney, [b]Liver, [c]Mesentery, [d]Muscle, [e]Heart

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Table 3.3 COI gene sequnce homology of *Posthodiplostomum minimum* collected in fish species collected from Caile Lake.

Fish Species	[a]Prevalence of *Posthodiplostomum* spp.	COI gene	Site of infection
Bluegill	17/20	sp. 3	[b]K, [c]L, [d]Me, [e]Mu
Bluegill	3/20	sp. 5	Mu
Largemouth bass	8/8	sp. 8	K, L, Me
Orangespotted sunfish	3/3	sp. 3	[f]H, Mu
Warmouth	2/2	sp. 3	H
Longear sunfish	1/1	sp. 3	Mu
Threadfin shad	1/1	sp. 3	Me

[a]Number of fish with metacercariae that had 100% homology with COI gene sequences for *Posthodiplostomum* sp. 3, 5 or 8.
[b]Kidney, [c]Liver, [d]Mesentery, [e]Muscle, [f]Heart

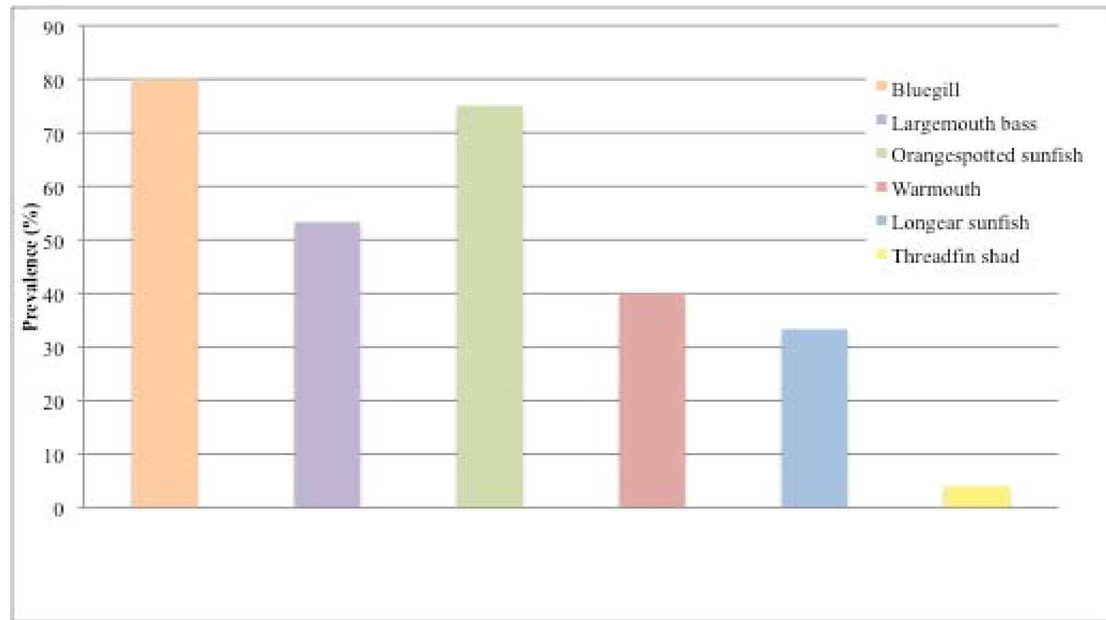

Figure 3.2 Prevalence of *Posthodiplostomum minimum* metacercariae found in freshwater fish collected from Caile Lake

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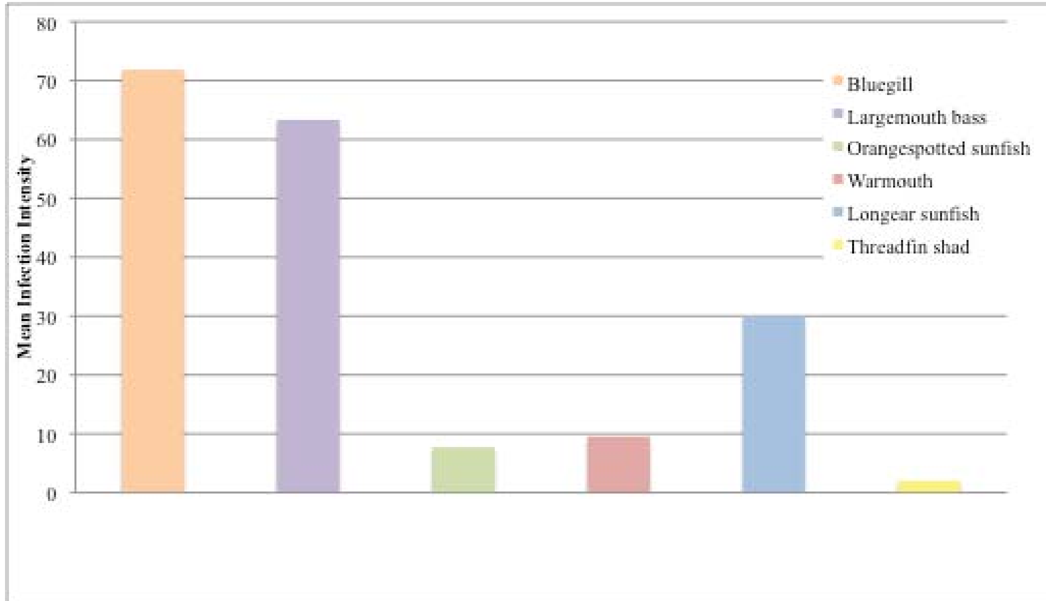

Figure 3.3 Mean infection intensity of *Posthodiplostomum minimum* metacercariae found in freshwater fish from Caile Lake.

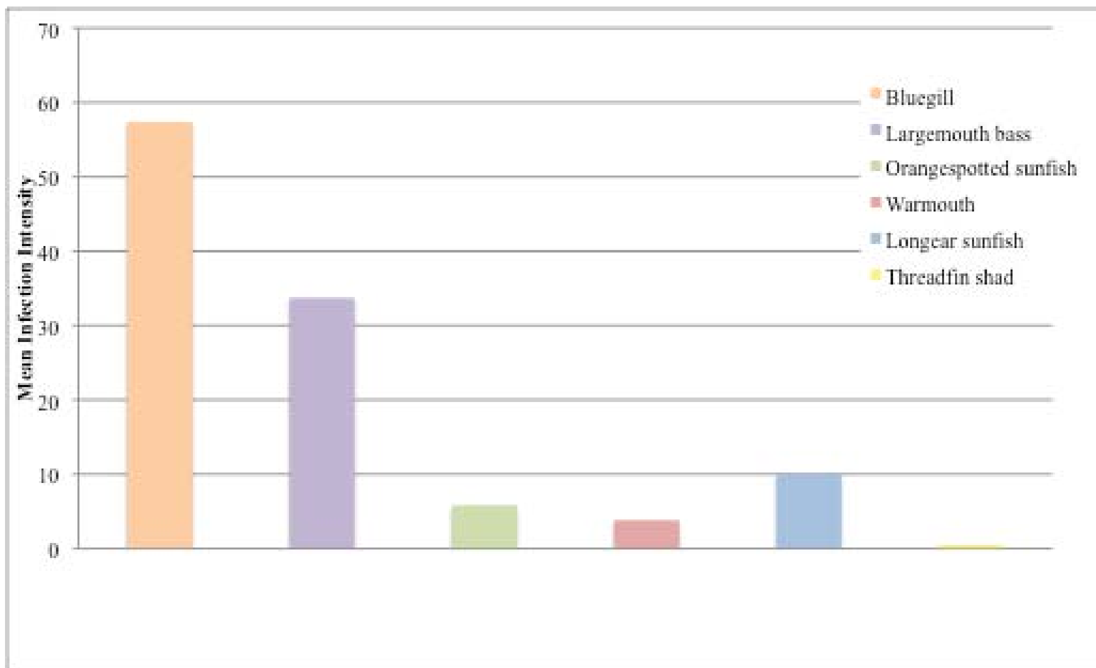

Figure 3.4 Mean abundance of *Posthodiplostomum minimum* metacercariae in freshwater fish from Caile Lake.

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On the day of sampling the maximum depth of the lake was 2.16 meters with an average water temperature of 25°C, a dissolved oxygen level of 5.91 mg/l, a pH of 3.95 and a chlorophyll level of 169.4 g/m^2.

Discussion

This survey is the first report on larval trematodes infecting wild fish species in an ox-bow lake in the Mississippi Delta. The results of this study indicated that *Posthodiplostomum minimum* may be both prevalent and abundant in freshwater fish in the Mississippi Delta region. This study not only confirms previous studies that have reported high prevalence and intensity of *P. minimum* in bluegills (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*) and warmouths (*Lepomis gulosus*), but also provides quantitative data for *P. minimum* infections in orangespotted sunfish (*Lepomis humilis*), longear sunfish (*Lepomis megalotis*) and threadfin shad (*Dorosoma petenense*).

The prevalence and intensities of *P. minimum* in other wild bluegill populations have been well documented. The results of this survey indicate that the prevalence and mean infection intensity of *P. minimum* in the ox-bow lake population of bluegills is comparable to what has been previously reported. Prevalence of *P. minimum* metacercariae in bluegills in the present study was 80.0%, compared to previous bluegill surveys done in California (Colley and Olson, 1963), Oklahoma (Spall and Summerfelt, 1970), Wisconsin (Amin, 1982), Iowa (Bailey, 1984) and Louisiana (Fischer and Kelso,

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1990), which had prevalences for *P. minimum* of 95.4%, 97.2%, 34.2%, 50.0%, 71.7%-94.2% and 24.1%, respectively.

The mean infection intensity of *P. minimum* in bluegills in the present study was 71.8 ± 23.1, which is comparable to data reported in previous surveys done in California (Colley and Olson, 1963), Oklahoma (Spall and Summerfelt, 1970), Arkansas (Cloutman, 1975), Iowa (Bailey, 1983) and Louisiana (Fischer and Kelso, 1990). The mean infection intensities of *P. minimum* reported in these surveys were 53.2, 79.7, 34.2, 24-839 and 3.0 ± 4.0, respectively.

P. minimum infections in largemouth bass have also been widely documented. The prevalence and intensities of *P. minimum* in other wild largemouth bass populations are comparable to what was observed in the present study. The prevalence of *P. minimum* metacercariae in largemouth bass in the present study was 53.3% while previous surveys of *P. minimum* in largemouth bass in Texas (Ingham and Dronen, 1980), Oklahoma (Spall and Summerfelt, 1970), Louisiana (Fischer and Kelso, 1990) and Florida (Grizzle and Goldsby, 1996) report prevalences of 29-78%, 64.5%, 52.2% and 93.3-94.1% respectively.

The mean infection intensity of *P. minimum* in largemouth bass in the present study was 63.3 ± 31.2, which is similar to prior surveys of largemouth bass in Arkansas (Cloutman, 1975), Oklahoma (Spall and Summerfelt, 1970), Texas (Ingham and Dronen, 1980) and Louisiana (Fischer and Kelso, 1990) which reported mean infection intensities of 58.1, 21.5, 16.9-79.8 and 17.3 ± 27.3, respectively.

While *P. minimum* infections in bluegills and largemouth bass have been well documented, less is known about infections occurring in the longear sunfish and

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warmouths. In the present study, the prevalence of *P. minimum* in longear sunfish was 33.3%, with a mean infection intensity of 30, which was similar to an Oklahoma survey, which reported a prevalence of 42.3% of *P. minimum* metacercariae and an average intensity of 14.0 (Spall and Summerfelt, 1970). The only survey data for *P. minimum* infections in warmouths was conducted in a lake in northwest Arkansas. No prevalence data was reported in that survey, but the mean infection intensity of *P. minimum* in that population was 11.33, with a range of 1-44 (Cloutman, 1975) which was comparable to the mean infection intensity in this study of 9.5±1.5 and a range of 8-11.

Although *P. minimum* has been reported in 97 fish species from 18 families of freshwater fish (Hoffman, 1958), currently there is no data on *P. minimum* infections in orangespotted sunfish (*Lepomis humilis*) and threadfin shad (*Dorosoma petenense*). The prevalence of 75% and mean infection intensities of 7.7 ± 3.5 in the orangespotted sunfish would suggest that this parasite is ubiquitous in this fish population, however only four fish of this species were sampled in this study which may have been too small a sample size to determine prevalence. The low prevalence of 4% and a mean infection intensity of two in the 25 threadfin shad sampled may have indicated that this fish species was less susceptible to this parasite than other fish species such as the blue gill.

Of the eight fish species that were negative for *P. minimum* in this study (spotted gar, common carp, shortnose gar, smallmouth buffalo, bowfin, pugnose minnow, gizzard shad and white crappie), only one of these species has been previously reported to be a host for *P. minimum*. A California survey of 17 white crappies (*Pomoxis annularis*) reported that 94.1% of these fish were infected with *P. minimum*, with an average intensity of 3.2 (Colley and Olson, 1963). Four white crappies were collected in the

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present study, however all were negative for *P. minimum*. The white crappie was the only centrarchid fish species not infected with *P. minimum* in the present study, however the small number of white crappies surveyed may have been insufficient to detect the presence of *P. minimum* in this population of white crappies.

Two of the uninfected fish species in the current study, the shortnose gar and the common carp, have been previously reported to be negative for *P. minimum*. A survey of six shortnose gars and 18 common carp in an Iowa pond were all negative for the presence of *P. minimum* (Palmieri, 1976). In the present study, the two common carp and the 13 shortnose gar were examined and found to be negative for *P. minimum* infections. There are no previous reports on spotted gar, smallmouth buffalo, bowfin and gizzard shad being sampled for *P. minimum*. This is the first study to report the absence of *P. minimum* infections in these fish species.

Although *P. minimum* has a broad range of fish hosts and is often reported in large numbers in many freshwater fish species there is very little information about the pathology of these *P. minimum* infections. Significant disease due to *P. minimum* infections has been observed when organs become compressed due to the presence of large numbers of metacercariae (Mitchell et al., 1982), however the impact of mild to moderate infections has not been determined. As in previous reports, this survey also indicated that there appears to be little preference as to site specificity of *P. minimum* in its numerous fish hosts. In this survey *P. minimum* metacercariae were found in the kidneys, liver, mesentery, muscle and heart (Table 3.2), which confirms previous reports of *P. minimum* metacercariae encysting in the liver, kidney, heart, spleen, muscle and

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mesentery of numerous fish species (Avault and Smitherman, 1965; Spall and Summerfelt, 1970).

There is also very little information on the effects these heavy infections may have on its various fish hosts in the wild. Spall and Summerfelt (1970) found no correlation between the density of *P. minimum* metacercariae and the condition of the fish. The authors noted that the rate of *P. minimum* infections increases slightly with increasing age of the host. However, the pathologic effects of *P. minimum* on very young fish has not been determined. Pathologic effects associated with *P. minimum* are thought to result primarily from damage incurred during migration of larval stages and the growth of metacercariae (Spall and Summerfelt, 1970).

Artificial infections in which fish are exposed to large numbers of cercariae can induce mortality. Hoffman (1958) exposed approximately 6,000 *P. minimum* cercariae to two fathead minnows (*Pimephales promelas*) and the fish died at 12 and 22 days post-infection. A total of 5,917 metacercariae were recovered from one of the artificially infected fish. Additional artificial challenges in which two fathead minnows were exposed to 13,940 *P. minimum* cercariae per fish resulted in fish mortalities at 16.5 and 39 hours post-infection , respectively (Hoffman, 1958). Hoffman's artificial infections demonstrated that *P. minimum* infections could induce mortality in fish, however the exact cause of mortality in these fish was unclear. Infections of this magnitude are unlikely to occur in nature, and as such the impacts of *P. minimum* on the health of wild freshwater fish are difficult to ascertain (Hoffman, 1958).

The sequencing results of the metacercariae recovered from the fish in this study support the morphologic identification of *P. minimum.* The 18S gene sequences data of

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all of the *P. minimum* metacercariae recovered in this study were 100% homologous to the published sequence in GenBank (Flowers, 2003). The COI gene sequences for the *P. minimum* metacercariae isolated in this study indicated that three species of *Posthodiplostomum (Posthodiplostomum* sp. 3, *Posthodiplostomum* sp. 5 and *Posthodiplostomum* sp. 8) infected the fish in this study, when comparing these sequences to the eight published *Posthodiplostomum* COI sequences (*Posthodiplostomum* sp. 1-8), of Moszczynksa *et al.* (2009) in GenBank. These published COI gene sequences for *Posthodiplostomum* spp. (1-8) were obtained from specimens collected in the Saint Lawrence River Basin (Quebec, Canada). Besides a genus-level morphological identification, no additional information was provided on the morphology, the fish host, and site of infection for any of these eight specimens. Therefore no comparisons, other than molecular ones, could be made between the parasites sequenced in this study and those reported by Moszyczynska *et al*. (2009). While the COI gene sequences in the current study indicated that there may have been three species of *Posthodiplostomum* infecting the fish collected in this study, the morphological characteristics of the metacercariae were indistinguishable and identical to previous descriptions of *P. minimum*.

Previous life cycle studies have demonstrated that two subspecies of *P. minimum* may exist. One subspecies infects mostly centrarchid fish, while the other subspecies infects fish from the Cyprinidae family (Hoffman, 1958) but no molecular data currently exists that confirms that these species are different molecularly. In this current survey, three species of *Posthodiplostomum* (*Posthodiplostomum* sp. 3, *Posthodiplostomum* sp. 5 and *Posthodiplostomum* sp.8) were collected from five centrarchid fish species, including

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bluegills, largemouth bass, orangespotted sunfish, longear sunfish and warmouth. While, *Posthodiplostomum* sp. 1-8 are currently listed as distinct species, it is possible that they are actually subspecies of *P. minimum*. This supports Hoffman's hypothesis that there may be multiple strains of this parasite that cannot be distinguished morphologically (Hoffman, 1958). The results of the present study suggest that there may be further specificity among infected centrarchid fish and that mixed infections are possible.

Comparisons between the COI gene sequences of the *P. minimum* metacercariae collected from centrarchid fish to those of *P. minimum* metacercariae infecting fish of the Cyprinidae family need to be made. In the present study, two fish species of the Cyprinidae family were surveyed, the pugnose minnow (*Opsopoeodus emiliae*) and the common carp (*Cyprinus carpio*). Both of these fish species are not reported hosts of *P. minimum*, however intense infections have been documented in the closely related fathead minnows *(Pimephales* promelas) (Mitchell et al., 1982). In the Caile Lake survey, four pugnose minnows and two common carps were collected but none were infected with *P. minimum* metacercariae. Although the number of cyprinidae fish collected in this survey was small, the overall prevalence of *P. minimum* in this lake suggests that the strains of *P. minimum* observed in the centrarchid fish in this lake may not be infectious to the cyprinidae fish species.

Conclusion

This survey is the first report on larval trematodes infecting freshwater fish in an ox-bow lake in the Mississippi Delta. Prior to this survey, the species composition of larval trematodes infecting wild fish species in this region was unknown. The results of

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this survey indicate that *Posthodiplostomum minimum* is highly prevalent in wild freshwater fish in this region, especially fish from the family Centrarchidae.

The pathologic effects of *P. minimum* on fish in this region are unclear. High numbers of metacercariae were recovered from several fish species, however the fish appeared healthy despite their large parasite burdens. Future research is needed to ascertain the long term effects of these *P minimum* infections on the fish populations in this region.

The DNA sequencing results of the metacercariae collected from the ox-bow lake fish population are perplexing. Sequences of the 18S gene indicated the presence of a single species, *P. minimum* with 100% homology to this parasite. Further sequencing of the COI genes of the same metacercariae suggested that three different species of *Posthodiplostomum* (species 3, 5 and 8) may be present in this fish population. However the morphology of all the metacercariae collected from this fish population were identical and matched the morphological descriptions published on *P. minimum*. The different *Posthodiplostomum* COI gene sequences may indicate that multiple subspecies of *P. minimum* could exist. Unfortunately, very little information was provided about the fish host species, the location of the metacercariae in those fish hosts and morphology of the metacercariae accompanying the published COI sequences for *Posthodiplostomum* sp.3, *Posthodiplostomum* sp. 5 and *Posthodiplostomum* sp. 8 in Genbank. Therefore only the sequencing data could be compared between the metacerariae in this study and those sequenced by Moszyczynska *et al*. (2009). In order to elucidate the phylogeny of the *Posthodiplostomum* genus, not only is additional sequencing data needed on *Posthodiplostomum* metacercariae and adults isolated from other hosts, but it is

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imperative that these sequences be accompanied with morphological descriptions and complete and thorough host histories.

This initial survey provided baseline data on one prevalent digenean, *P. minimum,* of freshwater fish in the Mississippi Delta. However in order to get a complete picture of the biology of this digenean in all of its hosts in the Mississippi Delta, extensive surveys, that include data on the birds, fish and snail hosts, should be conducted in the waterways in this region.

Although this study only identified *P. minimum* in the wild fish collected, this research indicated that there are many unanswered questions about the significance of these heavy infections, with these parasites found in numerous organs in a wide range of fish species. Further research needs to be done to confirm the level of infection of *P. minimum* that causes pathology and determine if subspecies do exist, which can only be accomplished in artificial challenges, accompanied by molecular data.

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CHAPTER IV

CONCLUSION

This research was the first to attempt to link the life stages of digeneans found in naturally infected bird and fish hosts collected from the Mississippi Delta. To accomplish this, two parasite surveys were completed; a two-year survey of intestinal digeneans found in double-crested cormorants residing in the Mississippi Delta and a survey of digeneans found in fresh water fish in a Mississippi Delta ox-bow lake.

The cormorant survey demonstrated that this piscivorous bird is host to at least five different species of intestinal digenetic trematodes; *Ascocotyle longa*, *Austrodiplostomum ostrowskiae, Drepanocephalus spathans*, *Hysteromorpha triloba* and *Pseudopsilostoma varium*. All of these birds were infected with at least one of these species, with 42.9% infected with at least 4 species. *Drepancephalus spathans* was the most frequently found digenean in 91.4% of the birds, with a mean infection of 81.5. Not only were these birds frequently infected with several of these digeneans, but often the levels of infection were high, with parasite ranges of 1-466 (*P. varium*) and 1-346 (*D. spathans*), yet these high levels of infection did not seem to adversely affect the health of these birds.

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Of the five digenetic species found in the double-crested cormorants in this survey, the life cycles are only known for *H. triloba* and *A. longa*. The 18S gene sequences of these species reported in this study were 100% and 97% homologous to published sequences. The only previously published COI gene sequence for the five species found in this study was for *H. triloba*, which was 100% homologous with the *H. triloba* sequence reported in this study. This current study provides new COI sequencing data for adult *A. ostrowskiae*, *A. longa*, and *D. spathans* which can be used to molecularly identify life stages in the fish and snail host, thus potentially completing these life cycles.

The surveyed ox-bow lake is a known loafing site of double-crested cormorants, however these birds seemed to have little parasitic impact on the fish population. The only larval digenean recovered from this fish population was *Posthodiplostomum minimum*, which was found in multiple organs in 6 out of the 14 fish species sampled in this lake. Although the adult stage of this parasite has been frequently reported in the great blue heron (*Ardea herodias*), it has never been reported in double-crested cormorants. These results were unexpected when considering the number of double-crested cormorants reported roosting on this lake annually. This would suggest that the species composition of freshwater snails present in these natural waterways may play a key role in determining which digeneans will ultimately infect these wild fish hosts. Although only finding one digenean, *Posthodiplostomum minimum,* in the freshwater fish surveyed in this study was unexpected, many questions arose about this parasite pertaining to speciation, transmission, pathology, and the impact these parasites are having when found in high numbers in multiple organs in these wild fish populations.

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DNA sequencing results from the trematodes collected from the double-crested cormorants and the ox-bow lake freshwater fish demonstrate the difficulties in obtaining a genetic identification for these parasites. DNA sequences of the highly conserved 18S gene region can be unreliable in differentiating between species, however this gene is a useful starting point when attempting to identify an unknown trematode. In contrast, DNA sequences of the COI gene are highly variable and subsequently are more useful in differentiating species of digeneans. Sequencing the COI gene can also be problematic and the high variability observed in this gene makes primer design difficult. It is also unclear if differences observed in the sequences of the COI gene are truly indicative of distinct species. The variability of the COI gene may be sensitive enough to differentiate to the subspecies level. The resultant DNA sequences of the COI gene for the collected *Posthodiplostomum* metacercariae suggested that this may be the case.

Now that the major intestinal digenetic trematodes have been identified in the double-crested cormorants residing in the Mississippi Delta, additional work needs to be done to complete these life cycles and elucidate the impact these trematodes have on the fish in this region. In order to identify factors contributing to these parasitisms, more comprehensive parasite surveys of the bird, fish and snail hosts residing in these waterways need to be done. The sequencing data provided by this research can then be used as a tool to identify and confirm the hosts and life stages of these parasites.

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