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LIFE HISTORY STUDIES OF TWO DIGENETIC TREMATODES, BOLBOPHORUS DAMNIFICUS AND AN UNKNOWN CLINOSTOMOID SPECIES, THAT INFECT CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

By

Cynthia Michelle Doffitt

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the Department of Basic Science

Mississippi State, Mississippi

December 2011

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Pages in Study: 125

Candidate for Degree of Doctor of Philosophy

The commercial production of channel catfish (*Ictalurus punctatus*) is major industry in Mississippi. Infections of channel catfish with the digenetic trematode *Bolbophorus damnificus* have often been associated with heavy economic losses in the industry. To efficiently control transmission of this trematode, the avian hosts need to be identified. In the first study, two American white pelicans, two double-crested cormorants, two great blue herons, and two great egrets were fed channel catfish infected with *B. damnificus* metacercariae. The presence of *Bolbophorus damnificus* ova in pelican feces at three days post infection (dpi) indicated the pelicans had patent infections. Mature *B. damnificus* were recovered from the intestines of both pelicans at 21 dpi. No *B. damnificus* infections were observed in the other bird species. In a second study, 33 American white pelicans, 34 double-crested cormorants, 35 great blue herons, and 32 great egrets were collected in the Mississippi Delta. The prevalence of *B. damnificus* in the American white pelican was 93.9%, with an average of 158 *B. damnificus* found per bird (range 0-681). *Bolbophorus damnificus* was not found in any

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of the other bird species. The results of these two studies confirm that the AWPE is the only proven natural host for *B. damnificus*. In a third study, two previously undescribed cercariae were found infecting rams-horn snails in commercial catfish ponds. In challenge studies, channel catfish were exposed to both cercariae types. Only one type of cercariae (type I) was infective to channel catfish. The first evidence of type I metacercariae was seen histologically at 14 dpi and grossly at 21 dpi. Development continued until 120 dpi, when both gross examination and histology suggested that the metacercariae were mature. The type I metacercariae appeared to cause little host damage. Molecular analysis of the 18S rRNA gene region indicated that the type I cercariae and metacercariae may be a species of *Clinostomum.* The data generated in these three studies provides additional information that can be used in the development of efficacious management schemes to control digenetic trematodes infecting commercial catfish.

Key words: *Ardea alba, Ardea herodias, Bolbophorus damnificus*, *Clinostomum*, cercariae, Digenea, *Ictalurus punctatus,* metacercariae, *Pelecanus erythrorhynchos*, *Phalacrocorax auritus*, piscivorous birds, trematode

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DEDICATION

To my wonderful husband, Chris Doffitt, who always encouraged and supported me, even, and especially, when I wanted to give up. This achievement would not have been possible without you. Thank you for giving me the encouragement and time that I needed. I love you. To my precious son, Kale Bryce Doffitt, who has been my inspiration. A large part of what has kept me going is imagining you at my graduation and hoping that you would be proud. I love you with all of my heart.

To my mother, Dr. Wanda Goleman, who paved the way and encouraged me throughout. I followed your example in spite of knowing exactly what I was getting into (twice)! To my father, Harvey Goleman, for keeping me smiling. To my grandfather, Floyd Collins Carney, who taught me what it means to work hard and strive for excellence and precision. I love you! To my grandmother, Lois Carney, who has always loved me. To my mother-in-law, Judy Wilkes, who has always treated me as family. Thank you for everything. To my father-in-law, Gary Malcolm Doffitt, who left us too soon and whose conversations I will always treasure. To my sisters-in-law, Cindy Doffitt and Laura Doffitt, for your support and love.

To my major professor, Dr. Linda M. Pote. Thank you for sticking with me. I am honored to have been your student.

To all of my friends (you know who you are) who have listened to me laugh and cry. Thank you for keeping me sane.

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ACKNOWLEDGEMENTS

Many people helped with this research, and without them none of this would have been possible. I am grateful to all.

First, I thank my major professor, Dr. Linda M. Pote. She has been the best mentor I could have asked for, teaching and leading me throughout. I also thank my committee, both former and current members, for their guidance throughout this process: Dr. Stephen Demarais, Dr. Lester Khoo, Mr. D. Tommy King, Dr. Michael Mauel, Dr. Doug Minchew, and Dr. Carla Panuska.

Next, I would like to thank everyone else who helped along the way. Fellow graduate students, Ms. Angela Brandon, Ms. Barbara George, Dr. Matthew Griffin, Ms. Mary O'Hear, and Dr. Marlena Yost. Research Associates, Ms. Michelle Banes, Ms. Lorelei Ford, and Ms. Ellen Thornton. Employees of the USDA-NWRC, Dr. Scott Barras, Mr. Paul Fioranelli, Ms. Katie Hanson, Mr. Scott Lemmons, Mr. Russell Singleton, and Mr. Scott Woodruff. Student workers, Dr. Jackie Burns, Ms. Megan Anderson Childers, Dr. Jesse Grady, Dr. Lynette Goodwin, Ms. Shelley Meadors Griffin, Mr. Matt Mott, Ms. Katie O'Bringer Rees, and many, many others.

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TABLE OF CONTENTS

Page

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

I. INTRODUCTION 1

Larval Digeneans in Channel Catfish 3
Adult Digeneans in Piscivorous Birds 4
Bolbophorus spp. 4
Bolbophorus damnificus 9
Clinostomum spp. 13
Clinostomum marginatum 15
Transmission and Control 18
Bolbophorus damnificus 18
Clinostomum marginatum 20
Study Objectives 21
Objective 1: Potential for piscivorous birds to serve as definitive hosts to B. damnificus 22
Objective 2: Survey of *Bolbophorus damnificus* infections in wild piscivorous birds in the Mississippi Delta 22
Objective 3: Life history of an unknown clinostomoid species infecting channel catfish 23
Literature Cited 27

II. EXPERIMENTAL *BOLBOPHORUS DAMNIFICUS* (DIGENEA: BOLBOPHORIDAE) INFECTIONS IN PISCIVOROUS BIRDS 33

Abstract 33
Introduction 33
Materials and Methods 35

www.manaraa.com

Bird Collection and Care 35
Identification of Trematodes..38
Results..39
Discussion..43
Literature Cited 47

III. SURVEY OF BOLBOPHORUS DAMNIFICUS INFECTIONS IN PISCIVOROUS BIRDS IN MISSISSIPPI 50

Abstract 50
Introduction..50
Materials and Methods..55
Bird Collection and Necropsy..55
Bolbophorus damnificus Collection...56
Morphological Identification of *Bolbophorus damnificus*.........................57
Molecular Identification of *Bolbophorus damnificus* 57
Descriptive statistics 59
Results..60
Discussion..65
Literature Cited 69

IV. PRELIMINARY FINDINGS ON A CLINOSTOMOID-TYPE DIGENETIC TREMATODE INFECTING CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)..72

Abstract 72
Introduction..73
Clinostomum marginatum..74
Materials and Methods..75
Preliminary Study 75
Snail Collection and Care 75
Enumeration and Preparation of Cercariae 76
Fish Challenges..76
Fish Necropsies..77
Challenge Studies...77
Study 1 78
Study 2 78
Study 3 79
Morphological Identification of Cercariae..82
Morphological Identification of Metacercariae 82
Molecular Analysis 83
Histological Analysis..85
Results..86
Cercariae Types 86
Fish Challenges...87
Preliminary study 87

المنارة للاستشارات
www.manaraa.com

Short-term Challenge (Studies 2-3) 88
Long-term Challenge (Study 1) 91
Description of Cercariae 95
Description of Metacercariae 98
Morphological Comparisons...110
Molecular Analysis 112
Discussion 113
Literature Cited 118

V. CONCLUSION...122

المنارة للاستشارات
www.manaraa.com

LIST OF TABLES

1.1 Summary of Trematodes Reported in American White Pelicans, Double-crested Cormorants, Great Blue Herons, and Great Egrets....23

2.1 Estimated Number of *Bolbophorus damnificus* Metacercariae Consumed Per Bird during the Challenge Period. 40

3.1 Summary of Individual Avian Species Collected from the Mississippi Delta, by Species and Year. 55

3.2 Results of *B. damnificus* Survey in American White Pelicans, by Collection Period and Location....61

3.3 Results of 28S Ribosomal RNA and Internal Transcribed Spacer (ITS) Analysis of *B. damnificus* Collected from American White Pelicans....63

4.1 Summary of the Experimental Design for the Preliminary Study and Studies 1-3....80

4.2 Summary of Fish Necropsies. 81

4.3 Primer Sequences Used to Amplify Mitochondrial and Genomic Gene Regions in Cercariae and Metacercariae....84

4.4 Average Number of Cercariae Shed by *Planorbella trivolvis* during a 24-hour Period (Pooled by Type)....86

4.5 Morphometrics of Unknown Type I and Type II Cercariae....96

4.6 Morphometrics of Clinostomoid-type I Metacercariae....99

www.manaraa.com

LIST OF FIGURES

2.1 Daily *Bolbophorus damnificus* Egg Shedding Rates for American White Pelicans (*Pelecanus erythrorhynchos*) 1 and 2. 42

2.2 Adult *Bolbophorus damnificus* Recovered from AWPE 1. 43

2.3 Adult *Drepanocephalus spathans* Recovered from DCCO 1 43

3.1 *Bolbophorus damnificus* Adult Collected from American White Pelican 2-1...59

3.2 *Bolbophorus damnificus* Adult Collected from American White Pelican 2-2...59

3.3 Mean Abundance of *Bolbophorus damnificus* Infection in American White Pelicans, by Collection Month and Year. 64

3.4 Intensity of *Bolbophorus damnificus* Infections in American White Pelicans, by Collection Year and Intensity Class...................................64

4.1 Channel Catfish Infected with Pooled Type I and Type II Cercariae (Preliminary Study) at 31 dpi, Showing Hundreds of Metacercariae. 88

4.2 Cross Section of Fish Tissue at 7 dpi (Challenge Study 1) Showing Possible Cercariae Migration Tract...89

4.3 Cross-Section of Fish Tissue at 14 dpi (Challenge Study 3), Showing Developing Type I Metacercaria...90

4.4 Cross Section of Fish Tissue at 21 dpi (Challenge Study 3), Showing Developing Metacercaria with Ventral Sucker. 91

4.5 Cross Section of Fish Tissue at 40 dpi (Challenge Study 1), Showing Prominent Ventral Sucker in Metacercaria. 92

4.6 Cross Section of Fish Tissue at 80 dpi (Challenge Study 1), Showing Metacercaria with Basophilic Structure. 93

www.manaraa.com

4.7 Cross Section of Fish Tissue at 120 dpi (Challenge Study 1) Showing a Well-Developed Metacercaria....94

4.8 Type I Cercaria. Detail of Body, Showing Penetration Glands (pg), Eye Spots, Ventral Sucker Analogue, and Dorso-median Finfold (dmf)....97

4.9 Type I Cercaria. Whole Body, Showing Body Region, Trunk, Furcae, and Caudal Excretory Canal....97

4.10 Strigeid-type II Cercaria. Whole body, Showing Body Region, Trunk (t), Furcae (f), Penetration Gland (pg), and Ventral Sucker (vs)....98

4.11 Type I Metacercaria, Showing Oral Sucker (os), Eye Spots (es), Ventral Sucker (vs), and Immature Gonad (g) at 21 dpi in Study 3. 102

4.12 Type I Metacercaria, Showing Oral Sucker (os), Eye Spots (es), and Ventral Sucker (vs) at 31 dpi in the Preliminary Study. 103

4.13 Type I Metacercaria, Showing Oral Sucker (os), Pharyngeal Bulb (pb), Ventral Sucker (vs), and Developing Gonads (g) at 40 dpi in Challenge Study 1. 104

4.14 Type I Metacercaria, Showing Oral Sucker (os), Ventral Sucker (vs), and Developing Gonads (g) at 42 dpi in the Preliminary Study. 105

4.15 Type I Metacercaria, Showing the Oral Sucker (os), Oral Collar (oc), Pharyngeal Bulb (pg), Ceca (c), Ventral Sucker (vs), Anterior Testis (at), Posterior Testis (ts) and Uterus (u) at 73 dpi in the Preliminary Study....106

4.16 Type I Metacercaria, Showing Oral Sucker (os), Oral Collar (oc), Pharyngeal Bulb (pb), Ventral Sucker (vs), Ceca (c), Anterior Testis (at), Posterior Testis (pt), and Uterus (u) at 80 dpi in Challenge Study 1. 107

4.17 Type I Metacercaria, Showing Oral Sucker (os), Oral Collar (oc), Pharyngeal Bulb (pb), Ventral Sucker (vs), Ceca (c), Anterior Testis (at), Posterior Testis (pt), Uterus (u), and Uterine Sac (us) at 110 dpi in Challenge Study 1. 108

4.18 Type I Metacercaria Showing Oral Sucker (os), Oral Collar (oc), and Pharyngeal Bulb (pb) at 110 dpi in Challenge Study 1....108

4.19 Type I Metacercaria , Showing Anterior Testes (at), Posterior Testis (pt) Uterus (ut), Uterine Sac (ut) and Cirrus Pouch (cp) at 110 dpi in the Preliminary Study 1....109

المنارة للاستشارات
www.manaraa.com

4.20 Type I Metacercaria , Showing Oral Sucker (os), Oral Collar (oc), Pharyngeal Bulb (pb), and Ventral Sucker (vs) at 120 dpi in Challenge Study 1. 110

4.21 Type I Metacercaria , Showing the Uterine Sac (us) and Ventral Sucker (vs) at 120 dpi in Challenge Study 1. 110

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CHAPTER I

INTRODUCTION

The commercial culture of channel catfish (*Ictalurus punctatus*) is a major industry in the southeastern United States, especially in Mississippi (Wellborn, 1988). The majority of commercial catfish production in Mississippi is concentrated in the northwestern region of the state in the Mississippi alluvial flood plain, or "Delta." Currently, Mississippi leads the United States in catfish production. In 2008, Mississippi had a total of 64,000 acres (55% of the US total) used for commercial channel catfish production (Hanson and Sites, 2010). In 2010, Mississippi had 427 independent operations (26% of US total) (Hanson and Sites, 2010). In 2009, Mississippi catfish producers earned nearly 200 million dollars in sales, which represented approximately 52% of the industry total (Hanson and Sites, 2010).

The first commercial catfish pond in Mississippi was established in 1965 (Wellborn, 1988) and this commercialization continued to expand with rapid growth of this industry occurring during the late 1970s and 1980s. Accompanying this expansion has been a marked increase of fish-eating birds in the region and the subsequent predation of commercial catfish (Mott and Brunson, 1997). Predatory bird species of concern to channel catfish producers have changed over the years. Aerial surveys of waterfowl in the Delta from 1972-1983 found that various duck species were the major users of catfish ponds (Christopher et al., 1988). Currently, the birds most often observed feeding on channel catfish ponds are American white pelicans (*Pelecanus erythrorhynchos*), double-

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crested cormorants (*Phalacrocorax auritus*), great blue herons (*Ardea herodias*), and great egrets (*Ardea alba*) (Glahn et al., 2000b; Glahn et al., 1999b; King, 1996, 1997; King and Werner, 2001).

In a 1988 survey of Mississippi catfish producers, 87 % of the respondents indicated that avian predation was a major problem that warranted active control measures to reduce catfish losses (Stickley and Andrews, 1989). The results of a later survey (1998) indicated that 84% of the responding Mississippi catfish producers reported losses due to wildlife, including avian and mammalian predators. At that time, the most commonly reported avian predators were double-crested cormorants and great blue herons (Brewster, 1998).

Estimates of the potential catfish consumption rates of avian predators have been calculated (Stickley, 1990). American white pelicans (AWPE) can consume 450-1500 grams of small to brood-fish sized catfish per day (Glahn and King, 2004; Stickley, 1990). Double-crested cormorants (DCCO) can consume 230-450 grams of five-inch catfish per day and great blue herons (GBHE) may eat 340 grams of fingerling catfish per day (Stickley, 1990). Great egrets (GREG) consume 140 grams of fingerling catfish per day (Stickley, 1990). Great blue herons and great egrets are generally attracted to sick or weak catfish, suggesting that they may not have as great a monetary impact on catfish production as the other bird species (Glahn and King, 2004; Stickley, 1990). In addition to predation damage, these avian predators serve as hosts to numerous helminths, some of which may be infective to channel catfish (Table 1.1).

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Larval Digeneans in Channel Catfish

Several species of larval digeneans have been reported in channel catfish, including Apophallus venustus, Austrodiplostomum compactum, Bolbophorus confusus, Bolbophorus damnificus, Bursacetabulus pelecanus, Clinostomum marginatum, Crassiphiala ambloplitis, Diplostomum spathaceum, Hysteromorpha triloba, Ornithodiplostomum ptychocheilus, and Posthodiplostomum minimum (Hoffman, 1999; Overstreet and Curran, 2004). The most common trematodes found in channel catfish are better described than those less commonly reported. Austrodiplostomum compactum forms a diplostomulum metacercariae in the vitreous humor of the eye. Experimentally, infections have been reported in the central nervous system. Bursacetabulus pelecanus forms a diplostomulum metacercariae in the eye and central nervous system, specifically the brain, spinal cord, and optic nerve (Overstreet and Curran, 2004). Clinostomum marginatum metacercariae are found in subdermal tissues where they are either free or encapsulated in a host-derived cyst. There is no cyst of parasite origin. Diplostomum sp. metacercariae infect the lens of the eye (Overstreet and Curran, 2004). Hysteromorpha triloba forms a diplostomum metacercariae in the deep musculature, particularly near the vertebral column (Overstreet and Curran, 2004).

Few studies have examined the pathology caused by larval digeneans in channel catfish. Of the few larval digeneans examined, *Bolbophorus damnificus* appears to be the most harmful (Overstreet and Curran, 2004). The experimental exposure of fingerling catfish to hundreds of *B. damnificus* cercariae, even for brief periods (5 minutes to 2 hours) can cause 100% mortality (Overstreet and Curran, 2004; Yost, 2008). In chronic infections in older channel catfish, the metacercarial cyst can become encapsulated by host connective tissue (Overstreet and Curran, 2004). Hundreds of *Bursacetabulus*

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pelecanus metacercariae have been observed migrating throughout the central nervous system (Overstreet and Curran, 2004). The clinical effects of these migrations are unknown. *Austrodiplostomum compactum* does not appear to cause direct harm to the catfish, but often the number of metacercariae that can develop in the eye are great enough to potentially impair vision (Overstreet and Curran, 2004).

Adult Digeneans in Piscivorous Birds

American white pelicans, double-crested cormorants, great blue herons, and great egrets individually and collectively harbor many species of adult trematodes (Table 1.1). Some of these trematodes are infective to channel catfish and are described below.

Austrodiplostomum compactum has been reported in double-crested cormorants (Overstreet and Curran, 2004). *Bolbophorus confusus* (Dronen et al., 2003) and *Bolbophorus damnificus* (Overstreet et al., 2002) have both been reported in American white pelicans. *Bursacetabulus pelicanus* has been observed in American white pelicans (Kinsella et al., 2004). Various species of *Clinostomum* have been reported in all study birds (Table 1.1). *Clinostomum marginatum* has been observed in double-crested cormorants (Threlfall, 1982) and great blue herons (Flowers et al., 2004). *Hysteromorpha triloba* have also been observed in double-crested cormorants (Overstreet and Curran, 2004). Similar to *Clinostomum* spp., Posthodiplostomum *minimum* has a wide range of bird hosts and has been reported in American white pelicans (Kinsella et al., 2004), great blue herons (Flowers et al., 2004), and great egrets (Sepulveda et al., 1999).

***Bolbophorus* spp.**

In 1994, channel catfish producers in Louisiana began reporting catfish losses caused by an unknown trematode. Although this parasite was probably present earlier, it

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was not until 1999 that the first catfish losses caused by trematodes in Mississippi were documented (Avery et al., 2001; Overstreet et al., 2002; Terhune et al., 2002). The causative agent of these losses was originally identified as *Bolbophorus confusus* based on the morphology of the metacercarial and cercarial stages found in infected channel catfish and the rams-horn snail (*Planorbella trivolvis*), respectively, and the presence of the American white pelican on ponds containing infected fish (Terhune et al., 2002).

Species of the genus *Bolbophorus* occur in piscivorous birds in both the eastern and western hemispheres. *Bolbophorus confusus* (Krause, 1914) Dubois, 1935 has been reported in American white pelicans (Fox, 1965; McNeil, 1948) and brown pelicans, *Pelecanus occidentalis*, (Dronen et al., 1999) in the United States. In Europe, *B. confusus* has been reported in the eastern great white pelican, *Pelecanus onocrotalus*, and potentially in the Dalmatian pelican, *P. crispus* (Overstreet et al., 2002). *Bolbophorus levantinus* has been reported in purple herons in Israel (Paperna and Lengy, 1963).

Bolbophorus confusus has been reported to naturally infect a variety of fish hosts representing several families, including salmonidae, esocidae, cyprinidae, catostomidae, percidae, and mugilidae (Fox, 1965; Olson, 1966). In laboratory settings, *B. confusus* was able to readily infect fish in the families poeciliidae and centrarchidae (Fox, 1965; Olson, 1966). Attempts to artificially infect *I. punctatus* (Ictaluridae), and *Cottus bairdi* (Cottidiae) resulted in poorly developed metacercariae, causing these hosts to be considered atypical for this trematode (Fox, 1965; Olson, 1966). In artificial infections, Fox (1965) described the life cycle of *B. confusus* based on the life stages he obtained from American white pelicans, rams-horn snails, *Helisoma trivolvis* (syn. *Planorbella trivolvis*), and infected rainbow trout (*Salmo gairdneri*). The life cycle description that follows was obtained primarily from those studies except where noted.

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Bolbophorus confusus ova obtained from infected pelican feces or excreted from live adult *Bolbophorus confusus* were generally ovoid and operculate, averaging 119 µm long x 72 µm wide. Fox reported that these measurements differed from those published by others but attributed the disparity to a difference in ova preparation prior to measurement. Development of miracidia and their release from the ova was found to be temperature dependent. Ova typically hatched, releasing miracidia, in 16-21 days at 21.1-23.9˚C; 14-18 days at 23.9-26.7˚C; 12–15 days at 26.7-29.4˚C; and in 12–13 days at 32.2˚C (Fox, 1965).

Miracidia were cylindrical, ciliated, and measured from 150-190 µm in length and 30-40 µm in width. Once released into the water, the miracidia were observed swimming in relatively straight paths for an average of 12 hours. Some were seen crawling on the rams-horn snails while others were observed entering the snails through shell apertures, but none were seen penetrating the snail body. Because the presence of snails seemed to have no effect on the swimming behavior of the miracidia, Fox concluded that the parasite locates its host by chance (Fox, 1965).

A single 4-5 µm long mother sporocyst was found in the mantle of each infected rams-horn snail. It was composed of 6-8 nodules containing germ masses and developing daughter sporocysts. The daughter sporocysts formed tangles, which blocked the digestive glands of the snails. Germ cells and developing cercariae were found within the bodies of the daughter sporocysts (Fox, 1965).

Cercariae used their tails to exit the daughter sporocyst via the birth pore with the anterior portion emerging first. Depending on the water temperature, cercariae emerged from the snails at 30-34 days post infection (dpi) at 21.1-23.9 ˚C or 35-51 dpi at 21.1-23.9˚C. Each snail released from zero to 1000 cercariae per day until its death. Death

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usually occurred within three days to three months, but averaged 15-30 days (Fox, 1965). The bodies of the cercariae averaged 257 µm long x 42 µm wide, while the tail stem averaged 280 µm long x 43 µm wide. A retractable ovoid penetration gland was observed at the anterior end of the cercariae. Emergence of the cercariae was not affected by light/dark cycles; consequently, it was concluded that the cercariae were not phototrophic. Resting cercariae oriented themselves with the tail facing upwards and the curved body facing downwards. Resting was interrupted by short periods of upward movement propelled by moving the tail in an oscillatory manner. When resting again, the cercariae were found at the bottom of the water column (Fox, 1965).

In artificial infections of rainbow trout using these cercariae, metacercariae were mainly found in the superficial musculature below the tegument but were also found in the brain, eyes, mouth, and on the fins. In these experiments, infected rainbow trout, metacercariae, and their cysts were fully developed 30-34 dpi at 21.1 ˚C. These metacercariae averaged 2120 µm long x 860 µm wide. Metacercariae of the genus *Bolbophorus* are unique in that they have a double-walled cyst. The inner wall of the cyst is of parasite origin and the outer of host origin (Fox, 1965). The term prodiplostomulum was coined to describe this double-walled metacercaria (Shoop, 1989).

To confirm the life cycle of *B. confusus*, Fox (1965) fed to an adult AWPE *Gambusia affinis* infected with *B. confusus* metacercariae. Patent adult trematodes recovered from the AWPE were identified as *B. confusus* and were found in the anterior small intestine three dpi. Based on the presence of ova in the feces, the infection lasted approximately two weeks. *Bolbophorus confusus* ova production appeared to reach a peak then decrease over time. Adult *B. confusus* measured 2.53 mm long, 0.61 mm wide at the forebody, and 0.39 mm at the hindbody. The forebody was trilobed with an oral

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sucker located on the median lobe. The ventral sucker was located on the midline of the forebody and was smaller than the oral sucker. A large oval holdfast organ was posterior to the ventral sucker (Fox, 1965).

Overstreet et al (2002) determined that the trematode infecting channel catfish differed significantly enough from other described *Bolbophorus* species to warrant the designation of a new species, *Bolbophorus damnificus*. The new species had a genital cone, genital bulb, and cyst wall of parasite origin, which placed it in the genus *Bolbophorus*. However, it differed from *B. confusus* (sensu stricto) by having larger ova (123–129 μm x 50–89 μm versus 90–102 μm x 55–72 μm) and vitellaria that does not reach the holdfast organ (Overstreet et al., 2002). In artificial life cycle studies, *Bolbophorus damnificus* ova measured 119.1 μm x 76.0 μm (Yost, 2008). Some of the previously reported *Bolbophorus* species in North American pelicans are probably *B. damnificus*, but that determination cannot be made due to the lack of preserved specimens (Overstreet et al., 2002). In addition to *B. damnificus*, another *Bolbophorus* species (designated *Bolbophorus* sp.) was found in American white pelicans and *P. trivolvis* in North America. A polymerase chain reaction (PCR) protocol was developed to differentiate between *B. damnificus* and *Bolbophorus* sp. (Levy et al., 2002). The process uses primers generated for a gene region that codes for the 18S rRNA (ribosomal RNA) subunit. *Bolbophorus damnificus* produces a 420 base pair (bp) amplicon while *Bolbophorus* sp. produces an 820 bp amplicon (Levy et al., 2002).

It is now uncertain which species of *Bolbophorus* was studied by Fox (1965). Based on ova measurements, it may have been *B. damnificus* or a mixed population of both *B. damnificus* and *Bolbophorus* sp. (Overstreet et al., 2002). Additionally, the trematodes examined by Fox (1965) did not infect channel catfish, but were infective to

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both the AWPE and rams-horn snail. The cryptic *Bolbophorus* sp. also infects AWPE and *P. trivolvis*. It has not been proven infective to channel catfish, but has caused mortality in green swordtail and guppies (Family Poeciliidae) and striped bass (Family Moronidae) (Levy et al., 2002). Mortality was seen in channel catfish that were exposed to mixed infections of *B. damnificus* and *Bolbophorus* sp. cercariae; however, it cannot be determined which of the two was the etiologic agent (Levy et al., 2002). In later studies (Yost, 2008), channel catfish were challenged with pure populations of *B. damnificus* that had been verified using molecular analyses. These challenges resulted in active infections and mortality in channel catfish. Although the identity of the species with which Fox worked is now questioned, his studies still provide valuable basic information about the general life cycle of the genus *Bolbophorus.*

Bolbophorus damnificus

The life cycle of *B. damnificus* has now been experimentally confirmed using both infection studies and molecular analysis. The definitive host is the AWPE, the first intermediate host is the rams-horn snail, *P. trivolvis*, and the second intermediate host is the channel catfish, *I. punctatus* (Overstreet et al., 2002). Studies have been performed to determine if these are the only species capable of serving as hosts for *B. damnificus*. In one study (Doffitt et al., 2009), AWPE, DCCO, GHBE, and GREG were challenged with metacercariae of *B. damnificus*. Only the AWPE was shown to develop a patent infection. The other bird species are thought to be refractory to *B. damnificus* infections. Additionally, the snail *Biomphalaria havanensis* has also been shown to be susceptible to experimental *B. damnificus* infections (Yost et al., 2009), but there have been no reports of *B. havanensis* naturally infected with *B. damnificus*.

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Similar to the life cycle study of *B. confusus* (Fox, 1965), a life cycle study of *B. damnificus* was done to confirm the life stages in *B. damnificus* using both molecular and morphometric analyses (Yost, 2008). Patent infections and ova shedding in the AWPE were observed 4-7 dpi. In this study, miracidia and cercariae development were shown to be temperature-sensitive. At 30˚C, miracidia emerged at 12 dpi but when temperatures were lowered to 22.0˚C and 23˚C miracidia hatched at 53 and 55 dpi, respectively. This same trend was seen with cercariae emergence from the rams-horn snail. Cercariae emerged at 23 and 27 dpi at 26.6˚C and 26.8˚C, respectively, and at 32 dpi when held at lower temperatures ranging from 23.9˚C to 26.7˚C. Viable, infective metacercariae were observed in channel catfish at 23 dpi (Yost, 2008).

Although *B. damnificus* infections were first reported to cause high mortalities in commercial catfish in Louisiana and Mississippi in the mid to late 1990s (Avery et al., 2001; Terhune et al., 2002), the pathology of this parasite is not clearly understood and the subsequent impact these parasite infections have on cultured channel catfish has not been well documented. Based on 2000-2009 yearly diagnostic case reports from the Mississippi State University College of Veterinary Medicine Fish & Diagnostic Laboratory at the Thad Cochran Warmwater Aquaculture Center; Stoneville, MS (Hanson and Sites, 2010), 2.4% (range 0.3-5.6%) of the catfish submitted have been infected with *Bolbophorus* sp. However, as channel catfish producers have become more familiar with the clinical signs of *Bolbophorus* sp. infections, fewer diagnostic cases were submitted. Therefore, the rates of infections based on case submissions may underestimate the number of commercial catfish infected and the economic impact of this parasite.

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Although not readily apparent, subclinical infections of *Bolbophorus* spp. may also be detrimental to the economic performance of catfish. Studies indicate that sublethal infections with *B. damnificus* make channel catfish more susceptible to other pathogens. Channel catfish fingerlings with concurrent *B. damnificus* and *Edwardsiella ictaluri*, the bacterial etiologic agent of enteric septicemia of catfish (ESC), infections had a higher mortality rate than those fish infected with only *E. ictaluri* (Labrie et al., 2004). As with *B. damnificus* infections, *E. ictaluri* can infect fish of all sizes, but tends to affect mainly fry and fingerling catfish. The seasonal timing of both infections overlap, with ESC more common in the spring and fall when *B. damnificus* cercariae emerge from their snail hosts (Labrie et al., 2004). The experimental exposure study demonstrated that the most efficient way to infect fish was when they were exposed to ESC at the same time as cercarial penetration (Labrie et al., 2004).

Metacercariae of *B. damnificus* can be seen grossly as small (~1 mm in diameter) papules beneath the skin. Metacercariae are usually seen in the musculature near the caudal fin, but can also be found in other regions of the body, especially in heavy infections (Wise et al., 2004). Reports of the pathology associated with *B. damnificus* infections in channel catfish have varied. In general, small fingerling catfish experience more morbidity and mortality than larger fish (over 0.25 kg (0.5 lb.)) (Tucker et al., 2004). In early studies using channel catfish that were naturally infected with *B. damnificus*, hemorrhaging was often associated with cercarial penetration and metacercarial cyst development. Exophthalmia and clear to yellow ascites may also be signs in heavy infections (Wise et al., 2004). In addition, damage to the posterior kidney, including renal tubule necrosis, inflammation, and hemorrhaging were seen (Overstreet et al., 2002; Terhune et al., 2002; Wise et al., 2004).

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In later challenge studies, using molecularly confirmed inoculums of *B. damnificus* cercariae, high mortality rates were observed in channel catfish fingerlings exposed to 100 or 200 *B. damnificus* cercariae with mortality rates of 100% observed in these groups at 5-6 dpi (Yost, 2008). Mortality rates in fish challenged with 25 to 50 cercariae were 0% and 7%, respectively, at six dpi (Yost, 2008). Histological analysis showed that inflammatory infiltrates were observed at the presumed cercarial entry points in the epidermis. Four to five dpi, mesenchymal cells were found around the developing metacercariae in the superficial muscular layers. Hemorrhage in the vessels surrounding the cercariae was also seen. In addition, progressive loss of hepatocyte vacoulation was observed in the liver, and the spleen showed signs of lymphoid cell loss. At six dpi, when death was observed in some of the catfish, the liver showed no signs of vacuolation and there were reduced numbers of lymphocytes detected in the spleen. Contrary to previous reports, no signs of renal tubular necrosis were seen in this study (Yost, 2008).

Catfish producers are generally more concerned with the high mortality rates associated with severe infections, but subclinical infections may have a more subtle negative economic impact on the industry. While older and larger catfish usually survive infections, feed intake is decreased, resulting in poor growth rates. Additionally, infected catfish may be unmarketable because meat quality is perceived to be compromised by the encystment of the metacercariae (Terhune et al., 2002). It has been shown that even light or moderate infections can cause considerable economic losses for producers (Wise et al., 2008). In light infections, defined as 1-33% prevalence, fish biomass production was reduced by 14% and net profits were reduced by 61%. In moderate and severe infections, the economic losses were such that producers did not earn enough money to cover the cost of production. Fish biomass was reduced in proportion with increasing disease

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severity with moderate infections, defined as 34-66% prevalence, causing a 35% reduction in fish biomass production. In severe infections, defined as 67-100% prevalence, fish biomass was reduced by 40% (Wise et al., 2008).

Clinostomum spp.

Members of the genus *Clinostomum* have wide host ranges. The hosts involved in the life cycle seem to vary based on the geographic location of the parasites, with the parasites using appropriate local hosts. Adults generally live in the oral cavity, pharynx, and esophagus of the definitive host, including piscivorous birds, reptiles, and mammals worldwide (Olsen, 1974). The first intermediate hosts are various species of aquatic snails, including *P. trivolvis* (Mitchell et al., 2007) and *Biomphalaria peregrina* (Dias et al., 2003). *Clinostomum* spp. metacercariae are common parasites of amphibians and freshwater fish worldwide (Cort, 1913; Hoffman, 1999), which serve as the second intermediate hosts. Many freshwater fish species, including channel catfish, have been reported to be infected with *Clinostomum* metacercariae (Dzikowski et al., 2004; Hoffman, 1999; Martinez, 1992). However, in a survey of channel catfish (*Ictalurus punctatus*) in Lake Erie, U.S.A., no species of *Clinostomum* were reported (Baker and Crites, 1976).

In the definitive hosts, adult members of the family Clinostomatidae have relatively large bodies with a distinctive collar-like fold surrounding the oral sucker. The pharynx is absent, but an esophagus is present. The ceca diverge pre-ventral sucker, extend down each side of the body, and may or may not have lateral branches. The testes are tandem with the ovary situated between them (Olsen, 1974). Operculate ova are unembroynated when released with the avian host feces (Osborn, 1912). Descriptions of

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the morphology of the miracidia are not available. The cercariae are clinostomoid-type with eyespots and a dorso-median finfold, but they lack a ventral sucker and pharynx. The trunk is at least as long as the furcae (Schell, 1985).

Metacercariae, when mature, are nearly the size of adults with stout, linguiform bodies. They are convex on the dorsal side and concave ventrally. The oral sucker is surrounded by a collar-like fold. The pharynx is absent, but the esophagus is thicker at the posterior end (Hoffman, 1999), possibly giving the appearance of a pharynx (Osborn, 1912). The ventral sucker is large, relative to the body, and situated in the anterior third of the body. Two immature testes are present at the mid-hindbody, with the uterus positioned on the right side of the body (Hoffman, 1999).

The systematics of this genus is disputed at this time (Dzikowski et al., 2004) and has been reorganized numerous times since the first descriptions (Dowsett and Lubinsky, 1980; Gustinelli et al., 2010; McAllister et al., 2010). Currently, a major dispute is whether *Clinostomum complanatum* and *C. marginatum* are conspecific. These two species share many morphological characteristics, making sight identification difficult (Dzikowski et al., 2004) and have wide piscine host ranges that may overlap. Most reports of *C. complanatum* are in Old World fishes (Aohagi et al., 1992; Wajihullah et al., 1980). However, both species have been reported in the Americas (Dias et al., 2003; Salgado-Maldonado et al., 2005). The evolutionary relationship of these species in the Americas is further confused because some authors consider them to be synonyms (Dias et al., 2003; Dowsett and Lubinsky, 1980; McAllister et al., 2010), while others treat them as separate species (Bonett et al., 2011; Dzikowski et al., 2004). Recently, phylogenetic studies of these two species comparing the 18S rRNA and the cytochrome

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oxidase I (COI) gene regions seem to support the species distinction (Bonett et al., 2011; Dzikowski et al., 2004).

Clinostomum marginatum

The metacercariae infecting commercially produced channel catfish in Mississippi are reported to be *Clinostomum marginatum* and are commonly referred to as “yellow grub” (Lorio, 1989). They are diplostomum-type metacercariae with large bodies that form a 1-2 mm cyst that is yellowish in color. They can be found free or encapsulated in subdermal and muscle tissues, on the fins and gills, and in the visceral organs and oral cavity (Overstreet and Curran, 2004; Plumb and Rogers, 1990). When encapsulated, a host-derived cyst forms; there is no parasite-derived cyst (Larson et al., 1988). The cyst wall is permeable and enables the parasites to uptake glucose through either active or passive transport (Uglem et al., 1991).

The forebody of the metacercariae is cylindrical and the hindbody is broad and concave ventrally (Osborn, 1912). The ventral sucker is large and spherical. The tegument is made of non-cellular cuticula and may be spinous. The oral sucker is surrounded by a collar-like fold of tissue and, together, make up the oral field, which is used as an attachment organ (Osborn, 1912). As in the adult stage, the pharynx is absent and the esophagus is thin-walled anteriorly and thick-walled posteriorly. The ceca branch from the intestine and extend down each side of the body to the level of the excretory bladder. They have blind pouches that extend laterally on both sides and appear to be filled with a fine-grained yellowish substance that flows up and down the length of the ceca and is expelled following excystment (Osborn, 1912). The testes are tandem and

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pyramidal in shape and the ovary is oval and has an entire edge. The vitellaria is localized in the hindbody and is diffuse (Osborn, 1912).

Adult *C. marginatum* are morphologically similar to mature metacercariae. They are slightly larger and have mature reproductive organs. The ceca are usually filled with coagulated blood corpuscles, from feeding on the definitive host, giving them a darker color than in metacercariae (Osborn, 1912). Adults residing in the mouths of the definitive hosts have a smooth, thick tegument that is coated with bacteria that aid glucose uptake through facilitated diffusion (Uglem et al., 1991).

The ova have a distinct operculum and are unembryonated when released in the hosts' feces (Osborn, 1912). After miracidia hatch from the ova and penetrate the rams-horn snail, rediae develop. In an experimental infection, rediae of all maturity levels were observed at 60-70 days post infection (dpi). Some rediae contain other developing rediae or developing cercariae (Edney, 1950). The ability of the rediae to produce not just cercariae but also more rediae may explain the ability of infected snails to shed cercariae for up to 2 years. In this same infection trial, it was found that only young (less than two months old) snails were susceptible to *C. marginatum*. Snails aged 1-2 years were refractory to new infections, but often harbored older infections, presumably acquired when very young (Edney, 1950). *Clinostomum marginatum* reproduction was very successful in the snail host. One snail shed over 8000 cercariae in a six hour period and another had hundreds of rediae, in various stages of development (Edney, 1950).

Clinostomum marginatum infections generally have little to no impact on the profitability of commercial catfish production, especially when compared to the effects of *B. damnificus* infections. As a result, little information about the pathology caused by these trematodes is available. Low-level infections of *C. marginatum* in commercial

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channel catfish have been occasionally found in market-size catfish, but they do not cause any major pathological signs and mortality rates are low. In the rare case of heavy infections, stunted growth has been observed (Hawke and Khoo, 2004) and the fish may be unmarketable (Wise et al., 2004). Conversely, *C. complanatum* has been reported to cause significant morbidity and mortality in aquaculture systems in Asia. Affected fish species include sweet fishes (*Plecoglossus altivelis*) and cultured loaches (*Misgurnus anguillicaudatus*) (Kagei et al., 1984).

In the southeastern United States, the main definitive hosts for *C. marginatum* are great blue herons (Flowers et al., 2004; Uglem et al., 1991). Dias et al (2003) reported the great egret as a host of *C. complanatum*, which he considered to be conspecific with *C. marginatum,* in Brazil (Dias et al., 2003). The double-crested cormorant was reported to be a host of *C. marginatum* in a survey done in Florida (Threlfall, 1982). The only reported first intermediate host is the rams-horn snail (Mitchell et al., 2007). Many fish species spanning most piscine families have been reported as second intermediate hosts (Hoffman, 1999).

Clinostomum spp. have been reported in American white pelicans, double-crested cormorants, great blue herons, and great egrets. *Clinostomum attenuatum* and *C. complanatum* have been reported in American white pelicans (Kinsella et al., 2004) and great egrets (Sepulveda et al., 1999). *Clinostomum marginatum* has been reported in great blue herons (Flowers et al., 2004) and double-crested cormorants (Threlfall, 1982). There have only been a few documented cases of humans becoming infected after consuming raw infected freshwater fish (Chung et al., 1995; Park et al., 2009).

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Transmission and Control

The highly successful transmission of digenetic trematodes in commercial catfish ponds in the Mississippi Delta can be attributed to several factors. The relatively shallow water depth of commercial catfish ponds often allows vegetation to become established along the pond banks. This vegetation provides an ideal habitat for the rams-horn snail, which thrives in the vegetation in the spring and summer after emerging from dormancy in the pond sediment (George, 2008). It has been shown that the rams-horn snail remains in the pond environment year round, and that the emergence of the snails from the sediment coincides with the hatching of the miracidia from the trematode ova, thus enhancing the parasite's transmission (George, 2008).

Bolbophorus damnificus

Because the high catfish stocking density in most commercial catfish ponds provides a plentiful food source for piscivorous birds (Overstreet et al. 2002), the presence of infected migratory American white pelicans feeding on commercial catfish maintains a high level of *Bolbophorus* ova in the aquatic environment. Recently, attempts have been made to estimate the *B. damnificus* ova output of infected AWPE. Estimations have been difficult to make because most of these birds have been infected with more than one adult trematode. In an experimental infection study, an AWPE was infected with a single patent adult *B. damnificus*. The maximum ova shedding rate was 1680 eggs per gram of feces (epg) (Doffitt et al., 2009). American white pelicans have been shown to produce approximately 850 grams of fecal material per day (King, personal communication), indicating that the potential for the introduction of this parasite into catfish ponds is tremendous.

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Many methods to control or interrupt the life cycle of *B. damnificus* have been attempted. Due to the mobility of the pelicans and their protection under the Migratory Bird Treaty Act, attempts to control the avian host have been a challenge. Several non-lethal techniques have been attempted to reduce the numbers of piscivorous birds on catfish ponds, including verbal and noise-based harassment (King, 1997, 2005; Stickley and Andrews, 1989), draining fields used for loafing (King, 1997, 2005), and the placement of floating ropes across the ponds (Mott et al., 1995). These efforts have been largely ineffective for long-term determent of avian feeding and loafing because, since the early 1990s, pelicans have become more persistent foragers and increasingly acclimated to harassment efforts and will often move a short distance from the site of harassment and resume feeding or loafing behavior (King, 1997, 2005). Pelicans also feed on the ponds at night when producers are less vigilant (Wise et al., 2004). Although AWPE are protected under the Migratory Bird Treaty Act, permits may be obtained from the United States Fish and Wildlife Service to use lethal control measures on a limited number of birds (Wise et al., 2004).

The most effective trematode control measures have targeted the snail host, *Planorbella trivolvis*. Of the various control measures, the most efficacious have been chemical molluscicidal treatments. Copper sulfate ($CuSO_4$) applied to the pond shorelines at a rate of 2.5-5.0 parts per million (ppm) is effective at significantly reducing snail populations without harming the catfish (Wise et al., 2006). Slurried hydrated lime, when applied at a rate of 175 lb/100 feet in a 6 foot wide swath along the shoreline, reduced rams-horn snail populations by 98.3% (Mitchell et al., 2007). In both of these cases, care needs to be exercised during the warm months, as the toxicity of these chemicals to channel catfish increases with increasing water temperatures and higher salinities

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(Mitchell et al., 2007; Wise et al., 2006). Additionally, copper sulfate is also an algaecide that may kill the algal bloom, resulting in oxygen depletion (Wise et al., 2006). Increasing the salinity of the ponds to 2.5 ppt (parts per thousand) has also been shown to be an effective treatment, possibly by interfering with snail reproduction and/or development (Venable et al., 2000).

Vulgarone B, a compound distilled from the plant *Artemisia douglasiana*, has molluscicidal activity at 24 μM (LC_{50}) and 38 μM (LC_{90}), which is lower than the level that has been shown to be toxic to catfish fry (LC_{10} = 162.8 μM). Additional research is needed to determine the toxicity of vulgarone B to catfish, birds, and mammals and establish its half-life in pond water (Meepagala et al., 2004).

Biological control methods have also been proposed. The black carp, *Mylopharyngodon piceus*, when stocked at a rate of 12-50 fish per hectare, has been shown to be effective in reducing rams-horn snail populations in commercial catfish ponds. However, researchers concluded that the use of carp is inefficient because of their rapid growth rate, which can interfere with catfish harvesting. In addition, the status of the black carp as an exotic species requires that permits be issued for their use (Venable et al., 2000; Wise et al., 2004). The redear sunfish, *Lepomis microlophus*, may be useful in controlling snail populations, but the efficacy of this control measure has not been fully investigated (Wise et al., 2004).

Clinostomum marginatum

Treatment and control of *C. marginatum* is essentially the same as for *B. damnificus* and other trematodes with similar life cycles (Wise et al., 2004). However, attempts have been made to find pharmaceutical treatments for fish already infected with

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the metacercariae. Injections of 25 mg/kg praziquantel and 0.022 ml/kg ivermectin had no effect at 28 dpi, but at 5.5 months pi fewer metacercariae were noted in treated fish (Lorio, 1989). The results were similar when praziquantel was applied as a bath at a rate of 0.65 mg/L and exposure of 24 hours (Lorio, 1989). Another study tested the effects of praziquantel baths at a rate of 2 mg/L and exposure times of 2 and 4 hours. At 14 and 21 dpi, metacercariae numbers were reduced compared to control fish (Plumb and Rogers, 1990). In both studies, the authors concluded that, while the treatments reduced the numbers of metacercariae, they would not be cost effective in controlling *C. marginatum* infections. Furthermore, neither drug is approved for use in fish food.

Most channel catfish producers do not specifically focus control efforts on *C. marginatum* because it causes relatively little damage to the fish and infection rates throughout the industry are low. One possible reason for the low infection rates in the Mississippi Delta, despite having ideal conditions for the transmission of the parasite, may be that the main definitive host, the great blue heron, usually feeds on dead or weak channel catfish and *C. marginatum* typically does not cause mortality.

Study Objectives

Commercial catfish ponds have proven to be ideal ecosystems for the completion of many trematode life cycles because of the presence of numerous infected snails and piscivorous birds that are in constant contact with a concentrated catfish population. Although it is known that many of the digenetic trematode life cycles involve a fish, snail, and fish-eating bird, solving these life cycles has been a challenge because many of these hosts have multiple trematode infections and often the morphological characteristics of the snail stage (cercariae) and fish stage (metacercariae) are not enough

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to discern species. In order to control or eradicate these digenetic trematodes in commercial catfish, it is imperative that all of the hosts and life stages in these digenean life cycles be identified and the host-parasite relationships are understood. To fill in these gaps, this research will examine the life cycles of two digeneans found in commercial catfish, *Bolbophorus damnificus* and a clinostomoid species. The results of this research will confirm the avian host range and specificity of *B. damnificus* and further elucidate the life history and pathology of the clinostomoid species.

Objective 1: Potential for piscivorous birds to serve as definitive hosts to B. damnificus

In order to control the transmission of *B. damnificus*, it is necessary to identify all of the hosts, specifically the definitive hosts. This was achieved by attempting to experimentally infect two of each of the following bird species: American white pelicans (*Pelecanus erythrorhynchos*), double-crested cormorants (*Phalacrocorax auritus*), great blue herons (*Ardea herodias*), and great egrets (*Ardea alba*) with *B. damnificus* infected channel catfish.

Objective 2: Survey of *Bolbophorus damnificus* infections in wild piscivorous birds in the Mississippi Delta

Further confirmation of B. damnificus host specificity and host range in piscivorous birds was done to confirm that the AWPE is the only known host and determine if DCCO, GBHE, or GREG could serve as hosts under natural conditions. This was accomplished by conducting a two-year survey of gastrointestinal parasites of wild cormorants, herons, egrets, and pelicans collected in northwestern Mississippi.

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Objective 3: Life history of an unknown clinostomoid species infecting channel catfish

An unknown cercaria-type, isolated from Planorbella trivolvis collected from catfish ponds, was found to infect channel catfish, resulting in Clinostomum-like metacercariae. A series of challenge studies was done to confirm this infection and determine the pathology and life history of this unknown clinostomoid-type digenean.

Table 1.1 Summary of Trematodes Reported in American White Pelicans, Double-crested Cormorants, Great Blue Herons, and Great Egrets.

Parasite	AWPE[a]	DCCO[b]	GBHE[c]	GREG[d]
Amphimerus elongates		X[13, 14, 21]		
Amphimerus interruptus[*]				X[22]
Amphimerus sp.		X[5]		
Apharyngostrigea brasiliana				X[24]
Apharyngostrigea cornu[*]			X[6]	X[24]
Apharyngostrigea pipientis				X[24]
Apharyngostrigea simplex[*]			X[6]	
Apharyngostrigea sp.			X[9]	
Ascocotyle angrense				X[25]
Ascocotyle chandleri				X[24]
Ascocotyle diminuta[*]			X[NCP]	X[23, 24]
Ascocotyle gemina[*]	X[12]			X[24]
Ascocotyle leighi[*]	X[12]			
Ascocotyle longa[*]	X[12, 13, 18]	X[11, 27]	X[23]	X[11, 23]
Ascocotyle mcintoshi[*]				X[24]
Ascocotyle megalocephala[*]			X[23]	X[23]
Ascocotyle nana[*]	X[12]		X[7]	X[23]
Ascocotyle nunezae[*]			X[23]	X[23]
Ascocotyle pipientis				X[NCP]
Ascocotyle tenuicollis[*]			X[23]	X[23, 24]
Ascocotyle sp. [*]		X[5, 11]		X[11]
Austrodiplostomum compactum[*]		X[19]		
Austrodiplostomum mordax		X[5]		

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Table 1.1 (Continued)

Austrobilharzia variglandis		X[1]		
Bolbophorus confusus[*]	X[3, 10]			
Bolbophorus damnificus[*+]	X[19]			
Bolbophorus sp.	X[12, 19]			
Bursacetabulus pelecanus[*+]	X[3, 12]			
Bursatintinnabulus bassanus[*]	X[3, 12]			
Cladocystis trifolium[*]				X[8]
Clinostomum attenuatum	X[12]	X[11]		X[24]
Clinostomum complanatum	X[12]		X[NCP]	X[8, 24]
Clinostomum detruncatum				X[24]
Clinostomum heterostomum			X[NCP]	
Clinostomum marginatum[+]		X[27]	X[6, 9, 28]	
Clinostomum sp.				
Dendritobilharzia pulverulenta	X[12]			
Diasiella diasi				X[24, NCP]
Diplostomum ardeae[*]			X[4]	X[24]
Diplostomum compactum[*]		X[6]		
Diplostomum confusum	X[NCP]			
Diplostomum excavatum[*]				X[4]
Diplostomum heterostomum			X[NCP]	
Diplostomum longissimum			X[NCP]	
Diplostomum minimum[*]			X[NCP]	
Drepanocephalus spathans[*]		X[5, 6, 27]		
Drepanocephalus sp. [*]		X[NCP]		
Echinochasmus dietzevi[*]	X[12]			X[17]
Echinostoma				
Echinostomatidae				
Euamphimerus sp. [*]				X[24]
Galactosomum humbargari[*]		X[NCP]		
Galactosomum sp.[*]	X[12]			
Gigantobilharzia sp.	X[15]			
Hemistomum sp.				
Holostomum cornu			X[NCP]	
Holostomum sp.				
Hysteromorpha triloba[*+]		X[2, 5, 11, 27]		

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Table 1.1 (Continued)

Ignavia venusta				X[24]
Maritrema sp.		X[5]		
Mesorchis denticulatus*	X[12]			X[24]
Mesoophorodiplostomum pricei*		X[5]		
Mesostephanus appendiculatoides*		X[27]		X[11]
Mesostephanus microbursa*	X[3]			
Mesostephanus splendiculatoides*		X[NCP]		
Mesostephanus sp*.	X[12]	X[11]		
Microparyphium facetum	X[12]			X[24]
Neodiplostomum orchilongum*			X[NCP]	
Neogogatea kentuckiensis*		X[26]		
Opisthorchis speciosus			X[NCP]	
Ornithobilharzia sp.		X[27]		
Parascotyle dimunita*		X[11]		
Parorchis acanthus		X[27]		
Philophthalmus lacrymosus				X[24]
Phocitremoides butionis*		X[5]		
Pholeter anterouterus*	X[12]			X[24]
Posthodiplostomum boydae*				X[24]
Posthodiplostomum grande*				X[4]
Posthodiplostomum macrocotyle				X[NCP]
Posthodiplostomum minimum*+	X[12]		X[6, 9]	X[17, 24]
Posthodiplostomum nanum				X[24]
Posthodiplostomum opisthosicya*			X[4]	X[24]
Posthodiplostomum sp.				X[24]
Prosthogonimus sp.			X[NCP]	
Renicola thapari	X[12]	X[NCP]		
Renicola secundus	X[NCP]			
Renicola sp.	X[NCP]	X[27]		X[24]

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Table 1.1 (Continued)

Ribeiroia ondatrae	X[12, 18]			X[24]
Strigea pseudibis				X[24]

* = Intestinal trematodes in birds
[+] = Infective to channel catfish

[a] = American white pelican
[b] = Double-crested cormorant
[c] = Great blue heron
[d] = Great egret

[NCP] = U.S. National Parasite Collection

[1](Barber and Caira, 1995), [2](Chandler and Rausch, 1948), [3](Dronen et al., 2003), [4](Dubois, 1970), [5](Fedynich et al., 1997), [6](Flowers et al., 2004), [7](Font et al., 1984), [8](Garcia, 1993), [9](Georgi et al., 1986), [10](Hugghins, 1956), [11](Hutton, 1964), [12](Kinsella et al., 2004), [13](Kuiken and Danesik, 1999), [14](Kuiken et al., 1999), [15](Leigh, 1957), [16](Ponce de Leon, 1995), [17](Lumsden and Zischke, 1963), [18](McNeil, 1948), [19](Overstreet et al., 2002), [20](Overstreet and Curran, 2004), [21](Pense and Childs, 1972), [22](Ramos, 1995), [23](Scholz et al., 2001), [24](Sepulveda et al., 1999), [25](Sogandares-Bernal and Lumsden, 1963), [26](Stunkard and Olson, 1972), [27](Threlfall, 1982), [28](Uglem et al., 1991)

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CHAPTER II

EXPERIMENTAL *BOLBOPHORUS DAMNIFICUS* (DIGENEA: BOLBOPHORIDAE) INFECTIONS IN PISCIVOROUS BIRDS

Abstract

To determine potential definitive hosts of the digenetic trematode *Bolbophorus damnificus*, two American white pelicans (*Pelecanus erythrorhynchos*), two double-crested cormorants (*Phalacrocorax auritus*), two great blue herons (*Ardea herodias*), and two great egrets (*Ardea alba*) were captured, treated with praziquantel, and fed channel catfish (*Ictalurus punctatus*) infected with *Bolbophorus damnificus* metacercariae. Patent infections of *B. damnificus*, which developed in both American white pelicans at 3 days post infection (dpi), were confirmed by the presence of trematode ova in the feces. Mature *B. damnificus* trematodes were recovered from the intestines of both pelicans at 21 dpi, further confirming the establishment of infection. No evidence of *B. damnificus* infections was observed in the other bird species studied. This study provides further evidence that double-crested cormorants, great blue herons, and great egrets do not serve as definitive hosts for *B. damnificus.*

Introduction

Commercial aquaculture of channel catfish (*Ictalurus punctatus*) is an important industry in northwestern region of Mississippi, known as the "Delta" (Wellborn, 1988). After the first commercial catfish pond was established in 1965, the industry experienced rapid growth (Wellborn, 1988). As the number of commercial ponds increased, so did

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the presence of piscivorous birds (Glahn and King, 2004; Mott and Brunson, 1997; Overstreet and Curran, 2004). The birds of most concern are American white pelicans (*Pelecanus erythrorhynchos*), double-crested cormorants (*Phalacrocorax auritus*), great blue herons (*Ardea herodias*), and great egrets (*Ardea alba*) (Glahn et al., 2000a; Glahn et al., 1999b; King, 2005; King and Werner, 2001).

These birds may serve as definitive hosts to digenetic trematodes that are infective to channel catfish (Flowers et al., 2004; Kinsella et al., 2004; Overstreet et al., 2002; Overstreet and Curran, 2004; Sepulveda et al., 1999). When channel catfish producers in Louisiana and Mississippi first began reporting catfish losses, the losses were attributed to a trematode initially identified as *Bolbophorus* sp. (Avery et al., 2001; Terhune et al., 2002). Since that time, the results of a series of infection studies, which were confirmed using molecular analysis, have established the trematode *Bolbophorus damnificus* as the causative agent of these losses (Levy et al., 2002; Overstreet et al., 2002; Yost, 2008). These studies also confirmed that one of the definitive hosts for this parasite is the American white pelican, the first intermediate host is the rams-horn snail (*Planorbella trivolvis*), and the second intermediate host is the channel catfish (Overstreet et al., 2002; Overstreet and Curran, 2004).

In channel catfish, *B. damnificus* cercariae penetrate the skin and form prodiplostomulum metacercariae in the superficial layers of the musculature (Overstreet et al., 2002). Cercarial penetration and metacercarial development are commonly associated with hemorrhaging. In addition, kidney tubule necrosis and kidney inflammation may occur; however, the mechanism of this pathology is unknown (Overstreet et al., 2002; Terhune et al., 2002). High mortality rates were observed in severely infected fingerling catfish. Larger catfish exhibited less mortality, but decreases

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in feeding can result in poor growth rates. Additionally, the meat of infected catfish is often unmarketable due to damage caused by encysted metacercariae (Terhune et al., 2002).

Although the American white pelican has been confirmed as a definitive host for *B. damnificus*, other piscivorous birds that commonly feed on commercially produced catfish could potentially be hosts. In order to control the transmission of *B. damnificus*, it is necessary to identify the definitive hosts, which can introduce trematode ova into the aquatic environment. This research investigated the potential for double-crested cormorants, great blue herons, and great egrets to serve as definitive hosts for *B. damnificus.* These species were chosen because of their frequent association with commercial catfish production ponds and their potential for introducing digenetic trematodes into the fish population.

Materials and Methods

Bird Collection and Care

Two individuals of each bird species, American white pelican (AWPE), double-crested cormorant (DCCO), great blue heron (GBHE), and great egret (GREG), were live-captured from the Mississippi Delta (Table 2.1) using modified padded leg-hold traps and methods previously described (King et al., 1998). Birds were weighed, marked with uniquely numbered bands, and housed outdoors in 3.0 x 3.0 x 1.8 m pens lined with outdoor carpet and outfitted with misting systems. Pelicans and cormorants were provided with 1000 L recirculating filtered water tanks, whereas the herons and egrets were provided with 110 L tanks filled to 50 % capacity with fresh water every 2-3 days. The pens were specially designed for long-term studies on piscivorous birds and were

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located at Mississippi State University. Birds were fed a diet of specific pathogen free (SPF) channel catfish daily at approximately the following rates: AWPE, 1500 grams; DCCO, 600 grams; GBHE, 400 grams; GREG, 400 grams. The SPF channel catfish were obtained from enclosed hatcheries at Mississippi State University College of Veterinary Medicine in Starkville, Mississippi and the Thad Cochran National Warmwater Aquaculture Center in Stoneville, Mississippi. Each bird was observed daily for general health and body condition.

Following capture, birds were acclimated for 7 days prior to the initiation of the infection study (day 0). On day 0, each bird was given 26-30 mg/kg body weight of the anthelminthic praziquantel (Droncit® 34, Bayer Corporation, Shawnee Mission, Kansas 66201). During the acclimation period and continuing for the duration of the trial, fresh fecal samples were collected and examined daily for the presence of trematode ova using a modification of the fecal sedimentation method (Foreyt, 2001). One gram of fecal material collected daily from each bird was used. In order to remove excess fecal debris, a 0.5 gram homogenized sample of fecal material was washed with a 1% soap solution and allowed to sit undisturbed for 5 minutes before removing the supernatant. This process was repeated for 10 cycles. The fecal sample was then rinsed with distilled water and diluted to 10 ml. After the final water rinse, the sample was thoroughly mixed and a 1 ml aliquot of this preparation was quickly pipetted and viewed with a dissecting microscope (Olympus SZ60, Olympus America, Inc., Center Valley, Pennsylvania 18034-0610) to enumerate the trematode ova. The number of eggs per gram of feces (epg) was calculated according to the following formula: [(eggs in 1 ml)(10)]/weight (g) of feces.

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Channel catfish were collected from a commercial catfish pond in the Mississippi Delta that had been experiencing *B. damnificus* infections. A subsample of the channel catfish (n = 23) was examined prior to the challenge of the birds to confirm the presence and number of *B. damnificus* metacercariae. Metacercariae from this subsample of catfish were excised and enumerated and a single metacercaria from each of the sampled fish was randomly selected for molecular analysis to confirm that the catfish were infected with *B. damnificus*.

Seven days following praziquantel treatment (day 7), the birds were fed live catfish that were naturally infected with *B. damnificus* to simulate natural infections in these captive birds. The number of fish and metacercariae consumed by each bird varied based on their individual feeding rates (Table 2.1). The infected catfish were fed to the birds over a period of up to 7 days, with each bird being fed to satiation each day. Attempts to infect the birds ceased once they had eaten the preferred dose of 14 fish (approximately 182 metacercariae) or once the 7-day challenge period expired (day 14). After the challenge period expired, all birds were fed SPF fish for the duration of the trial.

Three weeks post-challenge (day 28), all birds were euthanized using carbon dioxide gas and then necropsied. The gastrointestinal tract from the esophagus to the cloaca of each bird was removed. The upper and lower gastrointestinal tracts were separated. The intestine was opened longitudinally, and the intestinal contents were gently rinsed through a #200 stainless steel screen (aperture = 75 μm) using dechlorinated water. All intestinal contents were immediately examined using a dissecting microscope, and all live parasites were collected and placed in 70% molecular grade ethanol in preparation for staining and/or molecular analysis.

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Identification of Trematodes

The molecular identification of the ova, metacercariae, and adult trematodes was based on polymerase chain reaction (PCR) using oligonucleotide primers specific to *B. damnificus* (Levy et al., 2002). Genomic DNA was isolated from individual parasites according to the Gentra Purgene kit manufacturer's instructions (Gentra Systems, Inc., Minneapolis, MN 55441). PCR amplifications were performed in 25 µL reaction volumes composed of 2.0 µL template DNA, 0.625 units Takara Hot Start Taq Polymerase (Takara Bio Inc., Japan), 2.5 µl Takara 10x PCR buffer (Takara Bio Inc., Japan), 200 µM dNTP mixture (Takara Bio Inc., Japan), 200 nM forward primer, 200 nM reverse primer, and nuclease-free water added *quantum satis* to 25 µl. Reactions were performed in a MJ Research PTC-100 Peltier thermal cycler (Bio-Rad Laboratories, Inc., Waltham, MA 02451) under the following conditions: 92˚C for 5 minutes followed by 34 cycles of 94˚C for 1 minute, 58˚C for 1 minute, 72˚C for 1 minute, with a final cycle of 72˚C for 5 minutes. The primers used were specific to *B. damnificus* (forward 5'-TCA GTT TCG AAC GAT GAT GA-3' and reverse 5'-CGG TCT ACG GTT CCA CC-3') (Levy et al., 2002). Both positive (known *B. damnificus* metacercariae) and negative (nuclease-free water) controls were used in each PCR reaction. PCR products were visualized on a 1.2% agarose gel, which was stained with Gelstar nucleic acid stain (Combrex BioScience Rockland, Inc.) and observed under ultraviolet light.

For morphological identification, adult trematodes were stained in acetocarmine for 12 hr, destained in acid alcohol, dehydrated in a graded alcohol series (70, 95, and 100% ethanol), cleared in Citri-solve (Omega Laboratories, Inc., Houston Texas 77080), and mounted on slides using Permount (ProSciTech, Thuringowa Central Qld. 4817 Australia). Identifications of stained *B. damnificus* specimens were based on published

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descriptions (Levy et al., 2002; Overstreet et al., 2002). One stained specimen collected from each American white pelican was deposited at the U. S. National Parasite Collection in Beltsville, Maryland, USA (USNPC 101433.00).

All procedures used in this study were approved by the US Department of Agriculture/Wildlife Services (USDA/WS) National Wildlife Research Center's (NWRC) Institutional Animal Care and Use Committee under NWRC QA-1138.

Results

The subsample (n=23) of channel catfish used in the challenge was found to be infected with an average of 13 (range 0-73) metacercariae per fish. A total of 18 of the 23 (78%) sampled catfish were confirmed to be infected with *B. damnificus*. No metacercariae were found in three of the catfish (13%). The two remaining sampled catfish (9%) were infected with metacercariae that were not *B. damnificus*, but are likely to be *Hysteromorpha triloba*, another larval digenetic trematode infecting channel catfish (Hoffman, 1999), based on the location of the trematode in the deeper musculature and the lack of a parasite derived cyst (Schell, 1985). Given that the metacercarial doses were based on a subsample of the channel catfish population used and that a few of the sampled channel catfish were not infected with metacercariae or with non-*Bolbophorus damnificus* metacercariae, all data pertaining to metacercarial doses are estimations. Although we identified three catfish as negative for metacercariae in our subsample, we may have underestimated the number of positive catfish in the population since the detection method relies on the microscopic gross examination of fish muscle tissue.

Three of the study birds received the full dose of 182 metacercariae (AWPE 1, GBHE 1, and GBHE 2). Three received nearly the full dose (AWPE 2, DCCO 1, and

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GREG 1 at 156, 143, and 156 metacercariae, respectively). Due to the individual feeding rates of the study birds, two of the study birds received lower doses (DCCO 2 and GREG 2) at 91 and 65 metacercariae, respectively (Table 2.1).

Table 2.1 Estimated Number of *Bolbophorus damnificus* Metacercariae Consumed Per Bird during the Challenge Period.

Bird	Challenge period (days)	Number of fish consumed	Estimated metacercariae dose*
AWPE 1[1]	7	14	182
AWPE 2	7	12	156
DCCO 1[2]	7	11	143
DCCO 2	7	7	91
GBHE 1[3]	5	14	182
GBHE 2	4	14	182
GREG 1[4]	7	12	156
GREG 2	7	5	65

* Estimated metacercariae dose = mean number of metacercariae/fish x number of fish eaten. [1]AWPE = American white pelican; [2]DCCO = double-crested cormorant; [3]GBHE = great blue heron; [4]GREG = great egret

Both American white pelicans (AWPE 1 and AWPE 2) shed *B. damnificus* ova beginning on day 10 (3 dpi); AWPE 1 ceased shedding by day 15 (8 dpi) (Figure 2.1), whereas AWPE 2 shed ova intermittently until day 24 (17 dpi) (Figure 2.1). The double-crested cormorants (DCCO 1 and DCCO 2), great egrets (GREG 1 and GREG 2), and one great blue heron (GBHE 2) did not shed trematode ova in the feces at any point during the study period. The other great blue heron (GBHE 1) shed low numbers of trematode ova intermittently (days 2, 4, and 6) following treatment with praziquantel; however, based on both morphology and molecular analysis, the ova were not those of *B. damnificus*.

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One adult *B. damnificus* was recovered from AWPE 1. It was stained and identified based on its morphology (Figure 2.2). Five adult *B. damnificus* were recovered from the intestine of AWPE 2. All adult trematodes were morphologically identical. Of these five trematodes, one was identified based on PCR, two were stained and identified morphologically, and the remaining three were archived in 70% molecular grade ethanol. No adult *B. damnificus* were found in the intestinal contents of either of the double-crested cormorants, great blue herons, or great egrets. However, a single gravid adult trematode (Figure 2.3) was recovered from DCCO 1. This trematode was stained for morphological comparison and identified as *Drepanocephalus spathans* (Jones et al., 2005; Kostadinova et al., 2002; Rietschel and Werding, 1978; Yamaguti, 1958).

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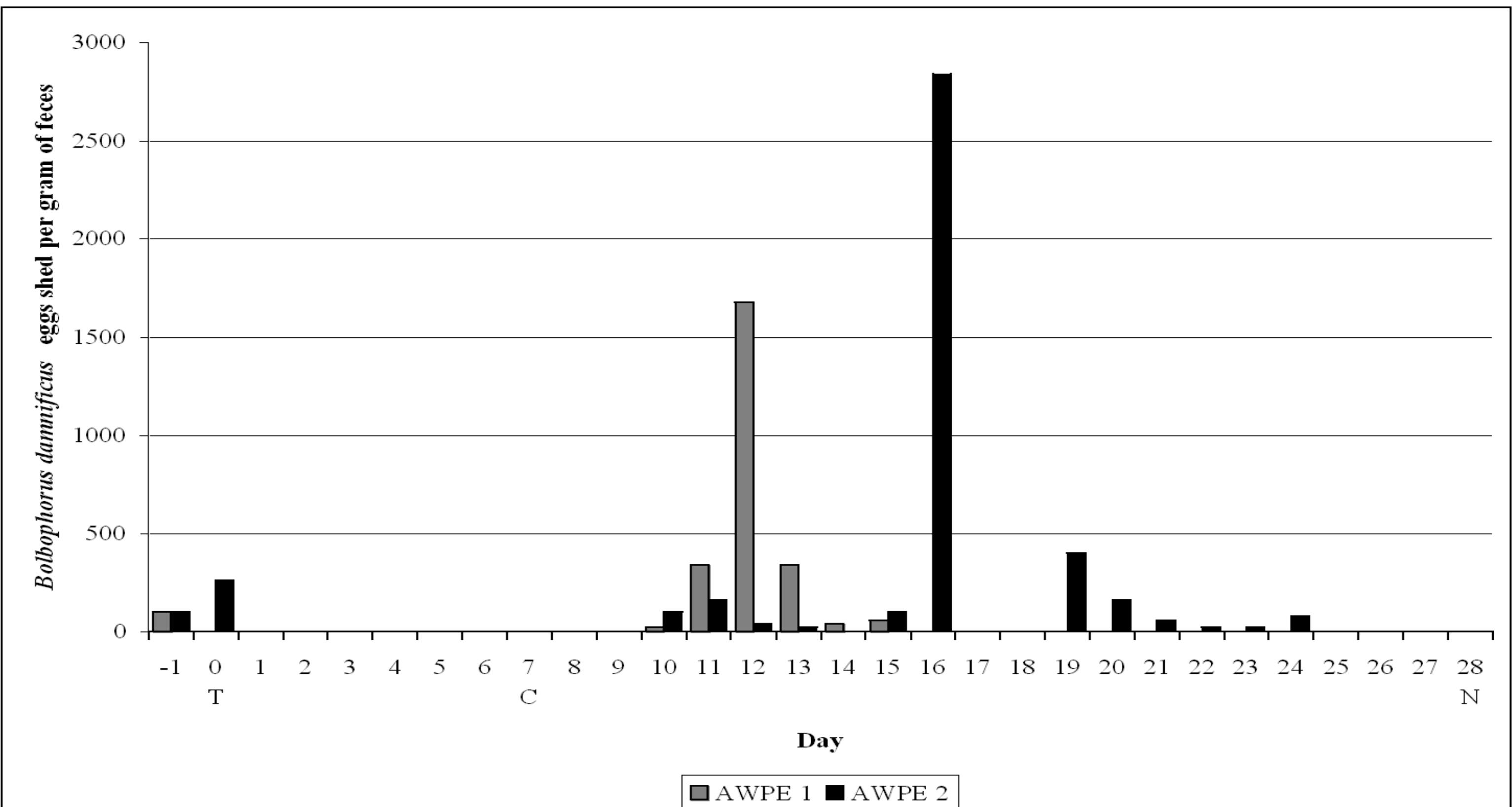

Figure 2.1 Daily *Bolbophorus damnificus* Egg Shedding Rates for American White Pelicans (*Pelecanus erythrorhynchos*) 1 and 2.

Reported as eggs shed per gram of feces (epg).
T = Praziquantel treatment
C = Challenge with live Bolbophorus damnificus infected channel catfish
N = Euthanasia and necropsy

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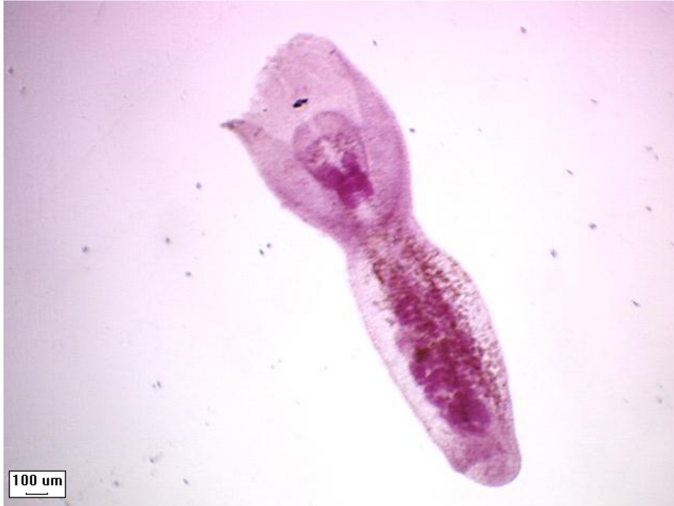

Figure 2.2 Adult *Bolbophorus damnificus* Recovered from AWPE 1.

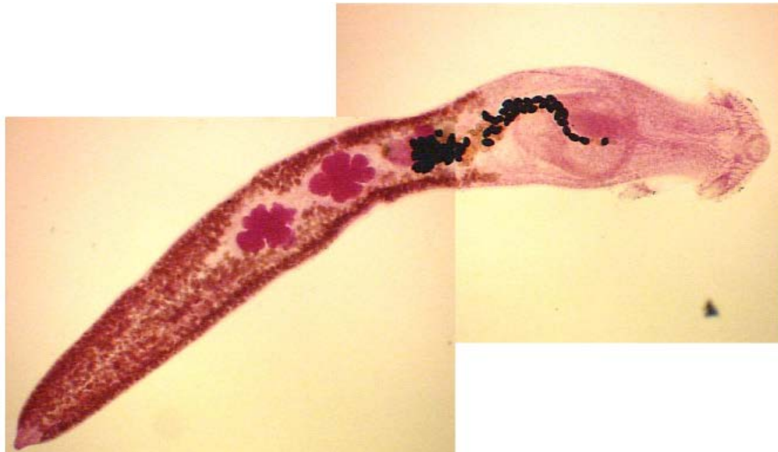

Figure 2.3 Adult *Drepanocephalus spathans* Recovered from DCCO 1

Discussion

The two American white pelicans were successfully infected with *B. damnificus*. Both exhibited patent trematode infections beginning on day 10 (3 dpi) and shed ova intermittently until day 24 (16 dpi). This timing of parasite maturation and ova production in the American white pelicans is similar to that of *B. damnificus* in previous studies (Overstreet et al., 2002; Yost, 2008). Following necropsy (day 28), the infections

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were verified by the presence of adult *B. damnificus* in the gastrointestinal tracts of both pelicans. At necropsy, a single adult *B. damnificus* was recovered from AWPE 1, which had a lower egg shedding rate (peak = 1680 epg) (Figure 2.1) for a shorter duration (3-8 dpi). In contrast, five adult *B. damnificus* were recovered from AWPE 2, which had a higher egg shedding rate (peak = 2840 epg) for a longer duration (3-16 dpi) (Figure 2.1).

The recovery of a single *B. damnificus* adult from AWPE 1 at necropsy may provide valuable insight into the life cycle of this parasite. Trematode ova counts obtained from fecal sedimentations of host fecal material, while demonstrating the presence of gravid adults within the avian host, are often confounded by the presence of several adult trematodes. However, the fecal trematode ova data generated from AWPE 1 may provide important information about the ova output of a single adult trematode. In this and previous studies (Yost, 2008), the fecal ova data from AWPE infected with multiple adult *B. damnificus* was cyclic with periods of intermittent shedding that continued for up to several weeks. However, the fecal ova data from AWPE 1 did not exhibit this cyclic pattern.

Although neither of the DCCO were positive for trematode ova in the feces, a single gravid adult trematode was recovered from the intestine of DCCO 1 at necropsy. This trematode was identified as *Drepanocephalus spathans*, which has been previously reported from this species (Fedynich et al., 1997; Flowers et al., 2004; O'Hear, 2011; Threlfall, 1982). Infections by trematodes in the genus *Drepanocephalus* have also been reported in other *Phalacrocorax* species (Kostadinova et al., 2002; Lamothe-Argymedo and Ponce de Leon, 1989; Nasir and Scorza, 1968). *Drepanocephalus spathans* has not been reported to infect channel catfish. The larval stage of this helminth has been reported in the cichlid fish, *Cichlasoma fenestratum* (Garcia, 1993; Salgado-Maldonado

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et al., 2005) and *Cichlasoma urophthalmus* (Salgado-Maldonado and Kennedy, 1997). However, the presence of this trematode was unexpected because all birds were treated with a dose of praziquantel previously shown to be efficacious against digenetic trematodes (Overstreet et al., 2002; Yost, 2008). The presence of *D. spathans* may indicate that praziquantel is less effective against this species. Another explanation is that *D. spathans* could be infective to channel catfish, despite the fact that it has not been previously reported in channel catfish. No trematodes were recovered from the gastrointestinal tract of DCCO 2.

No adult trematodes were recovered from either of the great blue herons at necropsy. However, GBHE 1 shed trematode ova intermittently following treatment with praziquantel on days 2, 4, and 6. These ova appeared non-viable and were confirmed to be non-*B. damnificus* ova using molecular analysis. The absence of ova in the feces of GBHE 1 pre- and post-challenge indicated a failure of *B. damnificus* to establish an infection in great blue herons. This was confirmed by the absence of adult trematodes in both of the GBHE at necropsy. No trematode ova were detected in the feces of either of the GREG during the study period and no adult trematodes were recovered from the intestines at necropsy.

Because it has been previously documented that AWPE serve as definitive hosts (Overstreet et al., 2002), their *B. damnificus* infections indicate that the metacercariae used for the challenge were infective and that the doses given were sufficient to induce an infection. American white pelicans (AWPE 1 and 2) were challenged with an estimated dose of 182 and 156 metacercariae, respectively (Table 2.1). These doses resulted in the maturation of one mature adult in AWPE 1 and five mature adults in AWPE 2. Although two of the study birds (DCCO 2 and GREG 2) received lower estimated doses of

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metacercariae, three birds (AWPE 1, GBHE 1, and GBHE 2) received the full estimated dose of 182 metacercariae. The remaining birds (AWPE 2, DCCO 1, and GREG 1) received comparable estimated doses ranging from 143 to 156 metacercariae (Table 2.1). Consequently, at least one individual of each bird species studied likely ingested a metacercariae dose sufficient to induce an infection had it been possible, as was evident by the successful infection of AWPE 2 with approximately 156 metacercariae. This demonstrates that the lack of *B. damnificus* patent infections in the double-crested cormorants, great blue herons, and great egrets was not due to an insufficient metacercariae dose.

We attempted to mimic the natural conditions necessary for transmission of *B. damnificus* to occur in commercial channel catfish production ponds. To that end, live channel catfish naturally infected with an estimated number of *B. damnificus* metacercariae were used to challenge the birds. The failure to infect DCCO, GBHE, or GREG with *B. damnificus* may have been related to the infection model used. Another issue that may have confounded the results is the relatively small avian sample size. Because facilities for housing the birds were limited and to minimize the number of protected birds used in the study, we chose to use two birds of each species.

This research provides further confirmation that AWPE serve as definitive hosts for *B. damnificus*. Additionally, we have demonstrated that DCCO, GBHE, and GREG, when subjected to the same experimental infection parameters as the AWPE, were refractory to *B. damnificus*, indicating that they are unlikely to serve as natural definitive hosts for this parasite. This information can be used by researchers and commercial channel catfish producers to focus control measures in an effort to reduce the impact of this parasite on the industry.

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CHAPTER III

SURVEY OF BOLBOPHORUS DAMNIFICUS INFECTIONS IN PISCIVOROUS BIRDS IN MISSISSIPPI

Abstract

Infections of channel catfish (*Ictalurus punctatus*) with the digenetic trematode, *Bolbophorus damnificus*, cause heavy economic losses in the aquaculture industry. To understand the transmission of this trematode, all possible avian definitive hosts need to be identified. A total of 33 American white pelicans (*Pelecanus erythrorhynchos*), 34 double-crested cormorants (*Phalacrocorax auritus*), 35 great blue herons (*Ardea herodias*), and 32 great egrets (*Ardea alba*) were collected monthly (January to March or April) for two years in areas of the Mississippi Delta (Humphreys, LeFlore, Sharkey, and Washington Counties) with concentrated commercial catfish operations. Following necropsy, the intestinal contents of all birds were examined for the presence of adult *B. damnificus*. The prevalence of this parasite in American white pelicans was 93.9% (31/33 birds infected), with an average of 158 *B. damnificus* found per bird (range: 0-681). *Bolbophorus damnificus* was not found in any of the other bird species sampled. This study provides further evidence that double-crested cormorants, great blue herons, and great egrets are refractory to *B. damnificus.*

Introduction

The commercial culture of channel catfish (*Ictalurus punctatus*) is a major agricultural industry in northwest Mississippi (Wellborn, 1988). Currently, Mississippi

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leads the United States in channel catfish production in terms of independent commercial catfish operations, land area, and sales (Hanson and Sites, 2010). Piscivorous birds are attracted to channel catfish production ponds because of the plentiful supply of prey. Currently, the birds most often observed feeding on channel catfish are double-crested cormorants (*Phalacrocorax auritus*), American white pelicans (*Pelecanus erythrorhynchos*), great blue herons (*Ardea herodias*), and great egrets (*Ardea alba*) (Glahn et al., 1999a; Glahn et al., 2000b; King, 1996, 1997; King and Werner, 2001).

Double-crested cormorants (DCCO) are the most common bird species at channel catfish production ponds in Mississippi (Barras, 2007) with populations peaking in February and March (Glahn and Brugger, 1995). Despite the enactment of the Cormorant Depredation Order by the United States Department of Agriculture (USDA) in 1993, cormorant populations more than doubled from 1995 to 1999 (Glahn et al., 2000b). Although DCCO consume, on average, less fish than AWPE (1 lb fish/day and 2.2 lb fish/day, respectively), the population as a whole is more destructive than AWPE, based on sheer numbers (Barras, 2007). Populations of AWPE are present in Mississippi from November through May with peak numbers present in February and March (King, 2005). Population sizes at both the wintering and breeding areas have increased 18-fold since 1985, which correlates with the expansion of the commercial catfish industry in this region (King and Grewe, 2001). Not only has this population increased but some of these birds are remaining year round with up to 2000 non-breeding individuals observed in the Mississippi Delta, indicating that some may not be completing the normal migratory pattern (King, 2005). Great blue herons (GBHE) are present in Mississippi year round, with population sizes peaking in mid-winter when non-resident populations migrate south (Glahn et al., 1999b). Great egrets (GREG) are present year round, however populations

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peak in the summer and fall (Glahn et al., 1999b). GBHE and GREG may have a lesser impact on channel catfish production, unless present in high numbers, than DCCO and AWPE because they consume less fish per day (0.77 lb fish/day and 0.66 lb fish/day, respectively) and usually feed on dead or diseased fingerling catfish (King, 2005).

As well as causing economic losses due to predation of channel catfish, these avian predators may introduce digenetic trematodes into the production system (Table 1.1). Of particular concern is *Bolbophorus damnificus*, which can reduce growth and feed consumption in channel catfish and cause high mortality rates in fish heavily infected in commercial production ponds (Overstreet et al., 2002; Terhune et al., 2002). The first losses associated with *B. damnificus* infections were documented in Mississippi in 1994 (Avery et al., 2001; Overstreet et al., 2002; Terhune et al., 2002).

The life cycle of *B. damnificus* has been experimentally confirmed using both infection studies and molecular analysis. In natural infections, the only confirmed definitive host is the American white pelican, *P. erythrorhynchos*, the first intermediate host is the rams-horn snail, *Planorbella trivolvis*, and the second intermediate host is the channel catfish, *Ictalurus punctatus* (Doffitt et al., 2009; Overstreet et al., 2002; Yost, 2008). The adult trematode is found in American white pelicans (AWPE). Trematode ova are shed in the pelicans' feces. The ova develop and hatch in an aquatic environment, releasing miracidia, which infect the rams-horn snail. Within the snail host, mother and daughter sporocysts develop and eventually release cercariae into the aquatic environment. In channel catfish, *B. damnificus* cercariae penetrate the skin and form prodiplostomulum metacercariae in the superficial musculature. Pelicans become infected and the life cycle is complete when they consume catfish infected with *B. damnificus* metacercariae (Doffitt et al., 2009; Overstreet et al., 2002; Yost, 2008).

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High mortality rates have been observed in naturally infected fingerling catfish and up to 100% mortality rates in experimentally infected fingerlings when exposed to as few as 100 cercariae/fish (Yost, 2008). Larger catfish are more likely to survive; however, they have poor growth rates due to reduced feeding behavior. Renal tubule necrosis and renal inflammation have been reported, although the mechanism is not understood (Overstreet et al., 2002; Terhune et al., 2002). Additionally, the presence of metacercariae in the muscle could render the catfish unmarketable for human consumption, resulting in increased economic losses (Terhune et al., 2002).

More subtle economic losses occur in catfish with subclinical infections of *B. damnificus*. In comparison to uninfected production ponds, those ponds experiencing infections resulted in a 31% overall reduction in fish production (Wise et al., 2008). Uninfected ponds yielded an average profit of $1,526. In this same study, losses of $1,123 and $781 were reported in severely and moderately infected ponds, respectively, indicating that even subclinical infections could contribute to economic losses.

The current production methods used by the commercial catfish industry contribute to the success of the transmission of *B. damnificus*. The relatively shallow water depth of commercial catfish ponds allows vegetation to grow along the pond banks, providing an ideal habitat for the first intermediate host, the rams-horn snail (George, 2008). The high catfish stocking density not only provides a readily available food source for piscivorous birds (Tucker et al., 2004), but when infection occurs in these ponds, there is also a concentrated population of fish with infective metacercariae.

Control of *B. damnificus* is primarily limited to molluscicides to eliminate the snail host, *P. trivolvis*, or targeting the potential avian definitive hosts. Chemical molluscicidal treatments include copper sulfate ($CuSO_4$) (Wise et al., 2006) and slurried

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hydrated lime (Mitchell et al., 2007). Although successful copper sulfate management schemes have been implemented, these treatments are not 100% efficacious, often require multiple treatments, and, depending on pond temperatures and salinities, can be toxic to the resident fish population (Mitchell et al., 2007; Wise et al., 2006). Recently, a *quantitative* polymerase chain reaction (qPCR) protocol specific for *B. damnificus* was developed for the detection of *B. damnificus* cercariae in the water and may be useful in detecting infected production ponds for targeted snail control efforts (Griffin et al., 2010).

Because the Migratory Bird Treaty Act limits lethal control measures, several techniques of varying success have been attempted to reduce the numbers of piscivorous birds on catfish ponds, including verbal and noise-based harassment (King, 1997, 2005; Stickley and Andrews, 1989), and draining fields used for loafing (King 1997, King 2005). These efforts have been largely ineffective for long-term determent of avian feeding and loafing on or near production ponds because the birds become habituated to harassment efforts and there is tendency for them to move to other loafing and feeding areas (King 1997, King 2005).

The total damage caused to the channel catfish aquaculture industry by these avian predators includes direct predation on catfish, transmission of trematode infections, increased susceptibility to secondary infections, and the cost of control measures. In order to control this parasite in commercial catfish ponds, it is important to identify which of the four major species of predatory birds, *P. erythrorhynchos*, *P. auritus*, *A. herodias*, and *A. alba* are hosts for *B. damnificus* so that management schemes can be devised, targeting the correct avian hosts. To determine the role these four avian species may play in the transmission of this parasite, a two-year parasite survey of the four bird species, collected in close proximity to the commercial catfish production areas, was done.

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Materials and Methods

Bird Collection and Necropsy

Pelecanus erythrorhynchos (AWPE), *Phalacrocorax auritus* (DCCO), *Ardea herodias* (GBHE), and *Ardea alba* (GREG) specimens were collected from the northwest region of the Mississippi Delta (Bolivar, Humphreys, LeFlore, Sharkey, and Washington counties), using lethal methods, during the months of January to April of 2003 and January to March of 2004. During each monthly sampling period, a maximum of five individuals of a particular species were collected on alternating weeks, when possible. In total, 134 birds were collected (Table 3.1). Following collection, all birds were immediately placed on ice and transported to the Mississippi State University College of Veterinary Medicine (MSU-CVM) parasitology laboratory.

Table 3.1 Summary of Individual Avian Species Collected from the Mississippi Delta, by Species and Year.

Avian Species	2003	2004	Total
Pelecanus erythrorhynchos	20	13	33
Phalacrocorax auritus	19	15	34
Ardea herodias	20	15	35
Ardea alba	18	14	32
Total	**77**	**57**	**134**

Each bird was necropsied upon arrival at the MSU-CVM. The gastrointestinal tracts, from esophagus to cloaca, were removed. The gastrointestinal samples were divided in two: the upper digestive tract (esophagus and stomach) and the lower digestive tracts (intestine and ceca). The intestines were cut open longitudinally and intestinal contents were collected by gently scraping and rinsing the mucosal surface with

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dechlorinated water and rinsing the contents through a sieve (aperture = 75 μm) to capture the intestinal parasites.

Bolbophorus damnificus **Collection**

Following necropsy, all intestinal samples were either immediately preserved in 10% buffered formalin or prepared for live parasite collection. Of the approximately five bird samples of each bird species collected in a given weekly period, three were examined and live parasites were collected immediately. For the live collection, all of the intestinal contents were microscopically examined in approximately 20-40 ml subsamples. The subsamples were placed in petri dishes and examined using a dissecting microscope (Olympus model SZ60, Olympus Imaging America, Inc., Center Valley, PA 18034) and all parasites were collected, using either a glass pipette or metal probe. Subsamples of the collected helminths were either heat fixed and placed in 70% molecular grade ethanol in preparation for staining for morphological comparisons or placed directly in 70% molecular grade ethanol for later molecular analysis.

The intestinal contents of the remaining two birds at each collection period were placed in 10% buffered formalin to preserve the parasites until the samples could be microscopically examined later. At that time, the preserved intestinal samples were washed through a #200 stainless steel screen (aperture = 75 μm) to remove all formalin. The wash was examined using a dissecting microscope, and all parasites were removed and placed in 70% molecular grade ethanol for identification and enumeration. All helminths removed from each bird intestine were re-examined and all trematodes were sorted by parasite type using a dissecting microscope (Olympus model SZ60, Olympus Imaging America, Inc., Center Valley, PA 18034). A subsample (n=13 from pelican 2-1

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and n=24 from pelican 2-2) of the *Bolbophorus*-type trematodes were stained for morphological identification.

Morphological Identification of *Bolbophorus damnificus*

For morphological identification, *Bolbophorus*-type trematodes were stained in acetocarmine for 12 hr, destained in acid alcohol, dehydrated in a graded alcohol series (70, 95, and 100% ethanol), cleared in Citri-solve (Omega Laboratories, Inc., Houston, Texas 77080), and mounted on slides using Permount (ProSciTech, Thuringowa Central Qld. 4817 Australia). Identifications of stained *B. damnificus* specimens (Figures 3.1 and 3.2) were based on published descriptions (Levy et al., 2002; Overstreet et al., 2002). Further confirmation of species was done using molecular analysis.

Molecular Identification of *Bolbophorus damnificus*

To molecularly confirm the morphological identification of *B. damnificus*, molecular analysis was done on a subsample (n=20) of *B. damnificus* representing variations in morphology. Molecular identification of *B. damnificus* was based on polymerase chain reaction (PCR) using oligonucleotide primers specific to the 28S rRNA and internal transcribed region (ITS) gene regions (Mitchell et al., 2011). Parasites that were preserved in 70% molecular grade ethanol were washed in nuclease free water three times for 15-30 minutes each prior to DNA extraction, which was performed using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA 91355), according to the manufacturer's instructions. PCR amplifications were performed in 25 µL reaction volumes composed of 2.0 µL template DNA, 1.25 units of Amplitaq Gold *Taq* polymerase (Applied Biosystems, Carlsbad, CA 92008), 10x PCR buffer, 400nM of each primer, 50 µM of each deoxynucleotide triphosphate, 200 µM of $MgCl_2$, and nuclease-

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free water added *quantum satis* to 25 µl. Reactions were performed in a MJ Research PTC-100 Peltier thermal cycler (Bio-Rad Laboratories, Inc., Waltham, MA 02451) under the following conditions: 95˚C for 5 minutes followed by 35 cycles of 95˚C for 1 minute, 62˚C (for 28S rRNA primers) or 60.0˚C (for ITS primers) for 1 minute, 72˚C for 1.5 minutes, with a final cycle of 72˚C for 5 minutes. The 28S rRNA primers were as follows: 28SBolbo-FP1: 5'-ATC-GCT-GCG-GTC-CTC-CAC-CA-3' and 28SBolbo-RP1: 5'-CCT-GTG-GCC-GTT-TGG-CTG-CT-3'. The ITS primers were as follows: BolboITS-FP1: 5'-TCC-GTG-TTT-GGT-GGG-GTG-CC-3' and BolboITS-RP1: 5'-GCT-TGG-GTG-CGC-AAG-CAC-AC-3'. Both positive (known *B. damnificus* metacercariae) and negative (nuclease-free water) controls were used with each PCR reaction. PCR products were visualized on a 1.2% agarose gel stained with Gelstar nucleic acid stain (Combrex BioScience Rockland, Inc.) and observed under ultraviolet light.

PCR products were submitted to the Arizona State University DNA Lab for sequencing. Sequences were analyzed using Sequencher version 5 (Gene Codes Corporation, Ann Arbor, MI 48108). Edited consensus sequences were compared to other known sequences in BLAST (Basic Local Alignment Search Tool) (National Center of Biotechnology Information, Bethesda, MD 20894).

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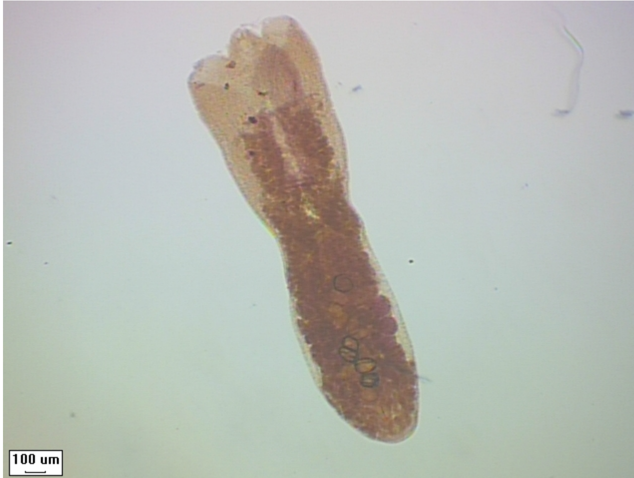

Figure 3.1 *Bolbophorus damnificus* Adult Collected from American White Pelican 2-1.

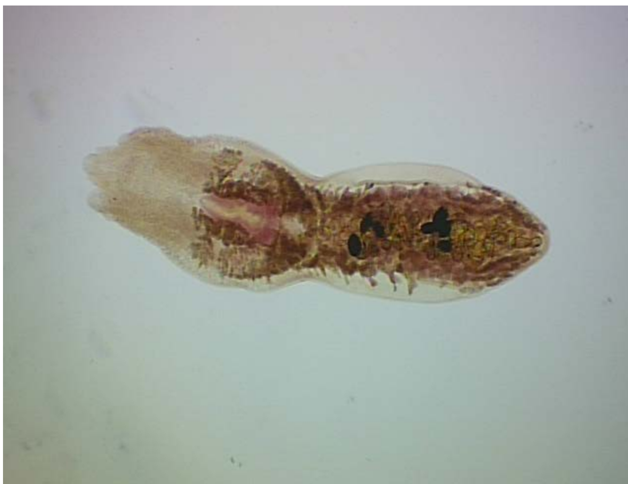

Figure 3.2 *Bolbophorus damnificus* Adult Collected from American White Pelican 2-2

Descriptive statistics

Parasite populations were described using descriptive statistics as previously defined (Bush et al., 1997). Prevalence is defined as the number of infected hosts divided by all sampled hosts. Intensity is the number of individual parasites in a single infected host. Mean intensity is the average intensity among all infected hosts. Abundance is defined as the number of individual parasites in all sampled hosts, both infected and

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uninfected. Mean abundance is the sum of parasites in all hosts divided by the number of sampled hosts, both infected and uninfected (Bush et al., 1997).

Results

Bolbophorus damnificus specimens were recovered from the upper small intestine of the American white pelican (*Pelecanus erythrorhynchos*), but not in any of the double-crested cormorants (*Phalacrocorax auritus*), great blue herons (*Ardea herodias*), or great egrets (*Ardea alba*). The prevalence of *B. damnificus* infections in the sampled American white pelicans (AWPE) was 93.9% (31 of 33). A total of 5,215 *B. damnificus* were collected from the 33 pelicans over the two-year period (Table 3.2). Over the course of the two-year study, the range was 0-681 adult *B. damnificus*, with a mean abundance of 158.0 ± 162.9 and a mean intensity of 168.2 ± 169.2 trematodes per bird. Only two AWPE out of the 33 (6.0%), collected in February of 2003 and 2004, were negative for *B. damnificus*.

In the first year (2003), 2595 adult *B. damnificus* were collected from 95.0% (19 of 20) pelicans sampled. The mean intensity of infection was 136.6 ± 112.6, and the mean abundance was 129.8 ± 113.7. The range was 0-409 *B. damnificus* adults per pelican. Mean abundance in 2003 peaked in March (mean abundance = 226.8; total *B. damnificus* = 1134) (Table 3.2, Figure 3.3). The highest intensity was seen in a pelican collected in April with a total of 409 *B. damnificus*.

In year two (2004), 2620 adult *B. damnificus* were collected from 92.3% (12 of 13) pelicans sampled. The mean intensity of infection was 218.3 ± 217.6, and the mean abundance was 201.5 ± 424.7. The range was 0-681 trematodes per pelican. Mean abundance in 2004 peaked in March (mean abundance = 273.8; total *B. damnificus* =

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1369) (Figure 3.3). The highest intensity was seen in a bird collected in January with a total of 681 *B. damnificus* (Table 3.2).

Overall, most pelicans were infected with less than 300 *B. damnificus*. In 2003, most pelicans were infected with 50-149 *B. damnificus*. Conversely, in 2004, most pelicans were infected with fewer than 50 *B. damnificus*. However, the highest intensities were seen in 2004 (Figure 3.4).

Of the twenty samples subjected to PCR analysis, nine were amplified by the primers specific to the 28S rRNA gene region and 12 were amplified by the primers specific to the ITS gene region (Table 3.3). All of the edited sequences for both gene regions were 100% matches to previously submitted *B. damnificus* sequences in GenBank, confirming the morphological identifications.

Table 3.2 Results of *B. damnificus* Survey in American White Pelicans, by Collection Period and Location.

Collection period	Collection location	Pelican sample #	Number of *B. damnificus* collected
January 2003	Washington Co	1-1	51
		1-2	135
		1-3	107
		1-4	2
		1-5	3
		Total	298
		Mean intensity	56.9 ± 60.3
		Mean abundance	56.9 ± 60.3
February 2003	Washington Co	1-6	94
		1-7	90
		1-8	0
		1-9	80
		1-10	38
		Total	302
		Mean intensity	75.5 ± 25.7
		Mean abundance	60.4 ± 40.4

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Table 3.2 (Continued)

March 2003	LeFlore Co	1-11	121
		1-12	317
		1-13	116
		1-14	294
		1-15	286
		Total	1134
		Mean intensity	226.8 ± 99.5
		Mean abundance	226.8 ± 99.5
April 2003	Sharkey Co	1-16	117
		1-17	142
		1-18	409
		1-19	30
		1-20	163
		Total	861
		Mean intensity	344.4 ± 365.6
		Mean abundance	344.4 ± 365.6
2003 total		2003 total	2595
		2003 mean intensity	136.6 ± 112.6
		2003 mean abundance	129.8 ± 113.7
January 2004	Sharkey Co	2-1	194
		2-2	681
		2-3	72
		Total	947
		Mean intensity	315.7 ± 322.2
		Mean abundance	315.7 ± 322.2
February 2004	Sharkey Co	2-4	59
		2-5	0
		2-6	173
		2-7	34
		2-8	38
		Total	304
		Mean intensity	60.8 ± 66.2
		Mean abundance	76.0 ± 65.6
March 2004	Humphreys Co	2-9	22
		2-10	270
		2-11	270
		2-12	208
		2-13	599
		Total	1369
		Mean intensity	273.8 ± 208.3
		Mean abundance	273.8 ± 208.3

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Table 3.2 (Continued)

2004 total	2004 total	2620
	2004 mean intensity	218.3 ± 217.6
	2004 mean abundance	201.5 ± 424.7
Grand total	Grand total	5215
	Total mean intensity	168.2 ± 169.2
	Total mean abundance	158.0 ± 162.9

Table 3.3 Results of 28S Ribosomal RNA and Internal Transcribed Spacer (ITS) Analysis of *B. damnificus* Collected from American White Pelicans.

Sample accession #	**Pelican sample #**	**28S**	**ITS**
834	2-1	-	-
835	2-1	X	X
836	2-1	X	X
837	2-1	-	-
838	2-2	-	-
839	2-2	-	-
840	2-2	-	-
841	2-2	-	-
842	2-9	-	-
843	2-9	-	X
844	2-9	-	X
845	2-9	-	-
846	2-10	X	X
847	2-10	X	X
848	2-10	X	X
849	2-10	X	X
850	2-11	X	X
851	2-11	-	X
852	2-11	X	X
853	2-11	X	X

\- = No match of sequence data with those in GenBank
X = 100% match of B. damnificus in this study with B. damnificus in GenBank

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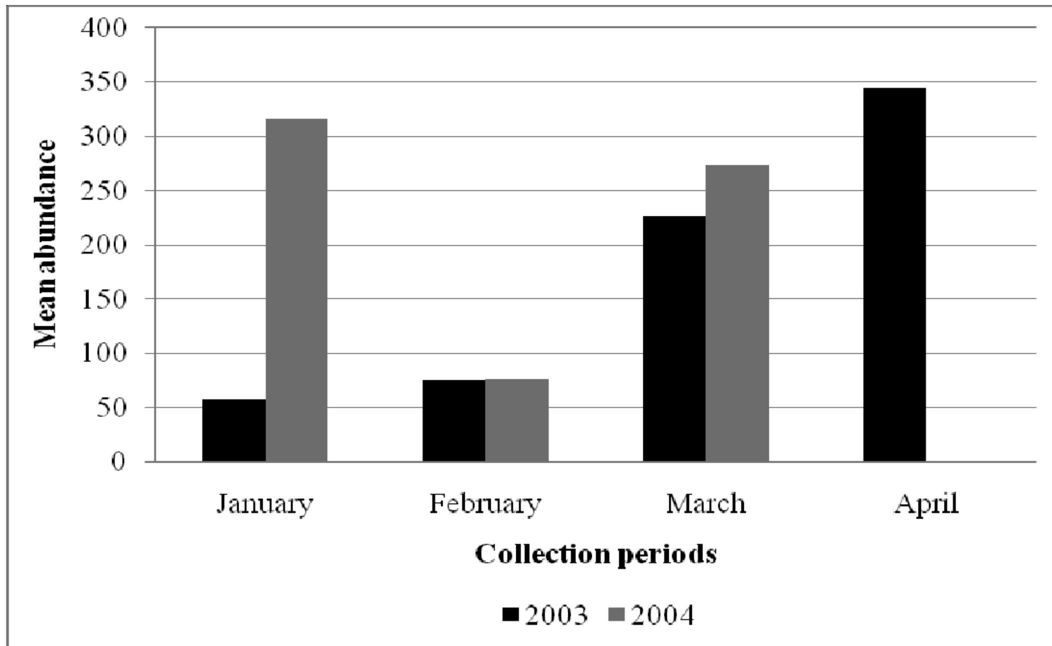

Figure 3.3 Mean Abundance of *Bolbophorus damnificus* Infection in American White Pelicans, by Collection Month and Year.

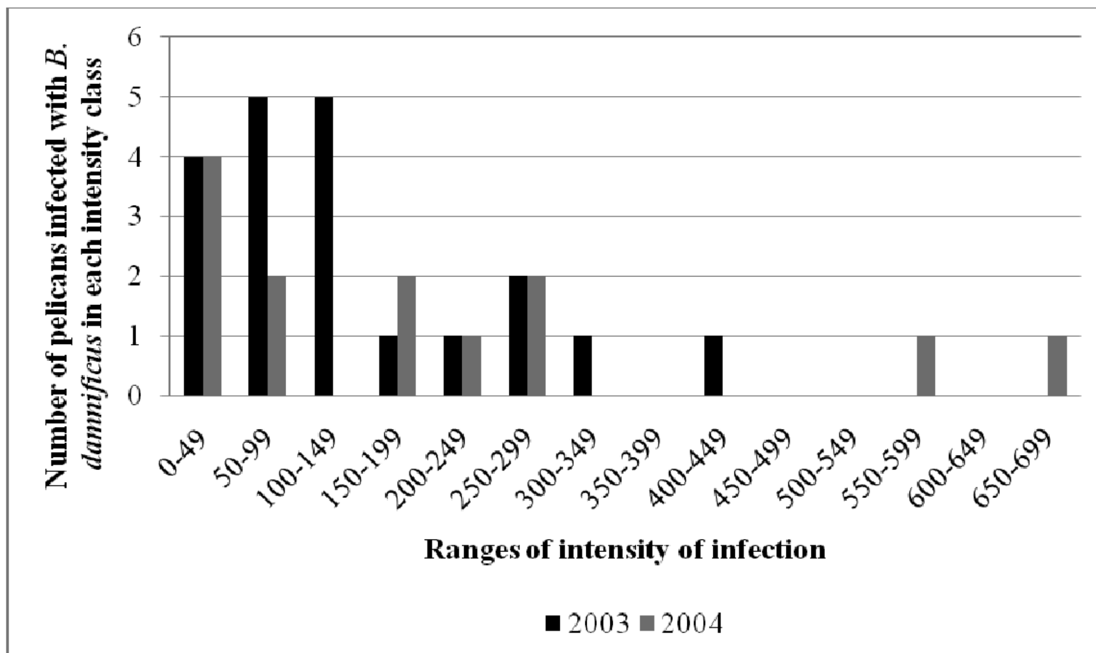

Figure 3.4 Intensity of *Bolbophorus damnificus* Infections in American White Pelicans, by Collection Year and Intensity Class.

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Discussion

Bolbophorus damnificus adults were found in the intestinal tracts of 94% of *Pelecanus erythrorhynchos* sampled. Conversely, zero *B. damnificus* were found in *Phalacrocorax auritus*, *Ardea herodias*, or *Ardea alba*. These findings are consistent with earlier work that attempted to experimentally infect these same four bird species with *B. damnificus* (Doffitt et al., 2009). In that study, two individuals of each species were fed channel catfish (*Ictalurus punctatus*) infected with *B. damnificus* metacercariae. At the end of the study, only the American white pelicans were found to have patent *B. damnificus* infections. The results of these two studies suggest that double-crested cormorants, great blue herons, and great egrets are refractory to *Bolbophorus damnificus*, while the American white pelican is the natural host.

Given the economic impacts on the aquaculture industry caused by these birds and the parasites that they harbor, this survey provided valuable information on the prevalence of *B. damnificus*. The prevalence (94%) and mean intensity (168.2 ± 169.2) (Table 3.2) reported in this study are greater than those previously reported (31%, 118 ± 233 (Kinsella et al., 2004) and 67%, 15 ± 5.7 (Dronen et al., 2003)). A possible explanation for the higher prevalence and intensity in this study could be that the population of AWPE from Mississippi has almost constant access to a large population of fish infected with *B. damnificus*, unlike the Florida or Texas populations.

While an accurate estimate is difficult to extrapolate, millions of *B. damnificus* ova may be introduced into channel catfish production ponds by AWPE. Past research demonstrated that a single adult *B. damnificus* could shed as many as1680 *B. damnificus* eggs per gram of pelican feces (epg) and in the same study another AWPE infected with five *B. damnificus* adults shed 2840 *B. damnificus* epg (Doffitt et al., 2009). Other

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research has shown that an AWPE can defecate up to 850 grams of feces per day (King, personal communication). Taking into consideration the potential ova output of this trematode, the amount of feces excreted per day by one pelican, and this research indicating an average of 158 adult trematodes per pelican in natural infections (Table 3.2), not only are these infected birds continually introducing these parasite ova into commercial catfish ponds but are doing so at an extremely efficient rate.

Commercial channel catfish ponds provide ideal conditions for the completion of the *B. damnificus* life cycle. These production ponds are concentrated along the Mississippi flyway and provide an ample and easily obtainable food source for the migrating AWPE. As indicated by this study, many of these birds are heavily infected and have the potential of introducing *B. damnificus* ova into the commercial ponds. These ponds have also been shown to be ideal habitats for the *B. damnificus* snail host, *P. trivolvis*, with vegetation-lined pond banks and mild winters that allow these snails to reproduce year-round (George, 2008). When infected, these snails can shed hundreds of cercariae per day for several months (George, 2008; Yost, 2008).

With the exception of pelican 2-2 in January 2004, which had the highest intensity (n=681) of all birds surveyed, there appeared to be a trend of increasing mean abundance from the winter to spring months (Table 3.2, Figure 3.3). An explanation for this trend could be that as the pelicans feed on infected catfish in the Mississippi Delta over several months, the intensity of infection increases for each individual bird, leading to an overall higher mean intensity. Earlier work has shown that pelicans, even after an effective anthelminthic treatment, can become reinfected with *B. damnificus*, which can survive in the bird host for at least three months (Yost, 2008), making this a plausible explanation.

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The present study is unique when contrasted with earlier helminthological surveys of AWPE. This study examined 33 freshly killed pelicans from Mississippi over a two-year period, making it the largest helminthological survey of AWPE, and the only one conducted in Mississippi. This is the first study that also used molecular methods to confirm morphological identifications of *B. damnificus*. Furthermore, the present study was the first to examine freshly killed pelicans, rather than sampling pelicans that were dead or dying, providing a more accurate account of the prevalence of *B. damnificus* in AWPE. A survey of pelicans in Florida was completed over the course of 19 years (Kinsella et al., 2004). A total of 29 dead or dying pelicans were collected. Sixteen were collected in 1998-1999 from Orange County where a massive die-off of 800-900 pelicans had occurred. The other 13 were collected from 10 counties from 1982-2002. *Bolbophorus* spp. (the authors did not identify these parasites to species) was reported in nine of 29 birds (prevalence of 31%), with a mean intensity of 118 ± 233 (range = 1-670). In Texas, six American white pelicans were collected from Galveston Bay (Dronen et al., 2003) over a 5 year period from 1994-1998. *Bolbophorus confusus* was reported with a prevalence of 67% (4 of 6) and a mean intensity of 15 ± 5.7 (range = 7-20). *Bolbophorus confusus* has been previously reported in American white pelicans (Fox, 1965; Hugghins, 1956; McNeil, 1948), but more recent reports suggest that *B. confusus* is only limited to Europe, and all past reports of *B. confusus* in North America may actually be *B. damnificus* (Overstreet et al., 2002). It is difficult to confirm this because most of these samples were not archived, except for those from Texas (Dronen et al., 2003), and none of these specimens were identified using molecular methods.

This study, in conjunction with a previous infection study (Doffitt et al., 2009), has shown that the AWPE is the only demonstrated host for *B. damnificus*, confirmed

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both molecularly and morphologically, and that DCCO, GBHE, and GREG are refractory to infection by this parasite. This information allows channel catfish producers to focus their avian control efforts on AWPE when attempting to reduce the damage caused by *B. damnificus*, rather than using resources to control the other bird species. In addition, the data suggests that the mean abundance in a population of pelicans may increase over a season as they feed on *B. damnificus* infected catfish, compounding the number of ova that may be released into the pond system. Furthermore, some AWPE may harbor prior infections of *B. damnificus* from the previous season when they migrated to Mississippi in the winter months, possibly serving as a reservoir host. This may have been the case with pelican 2-2, which was infected with 681 *B. damnificus* when it was collected in January of 2004 (Table 3.2). Overall, this study has provided valuable information about the abundance and intensity of *B. damnificus* in AWPE.

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CHAPTER IV

PRELIMINARY FINDINGS ON A CLINOSTOMOID-TYPE DIGENETIC TREMATODE INFECTING CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

Abstract

Clinostomum spp. metacercariae are common parasites of fish and amphibians worldwide. In Mississippi, *Clinostomum marginatum,* or "yellow grub," is occasionally seen in commercially produced channel catfish (*Ictalurus punctatus*). Recently, two cercariae types, a clinostomoid-type cercariae (type I) and a strigeid-type cercariae (type II), were found in snails collected from commercial catfish production ponds. To determine if these cercariae could infect catfish and cause pathology, a series of challenge studies were done. Specific pathogen free (SPF) channel catfish were exposed to both type I and type II clinostomoid cercariae for two hours and were necropsied at days 7, 14, 21, 31, 40, 43, 73, 80, 110 and 120 days post infection (dpi). At each necropsy, fish were examined to determine if metacercariae were present and determine pathology at each time point. During gross examinations, metacercariae were excised and preserved in 70% ethanol for molecular of the 18S rRNA, COI, and ITS gene regions and morphological analysis. The remaining fish were prepared for later histological examination. A subsample of the cercariae used for each challenge was collected for later morphological and molecular analysis. The first histological evidence of clinostomoid-type I metacercariae was at 14 days post infection (dpi). The first gross evidence was at 21 dpi.

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Development continued until 120 dpi, when both gross and histological analysis suggested that the metacercariae were fully or almost fully developed. No evidence of infection, neither grossly nor histologically, was observed in catfish challenged with the type II cercariae throughout the studies. Molecular analysis of the 18S rRNA gene region suggested that the two cercariae types are two different species. The type I cercariae and metacercariae were determined to possibly be a *Clinostomum* species, while the type II cercariae may be a species of *Apharyngostrigea*. Attempts to amplify the COI and ITS gene regions were unsuccessful. This research shows that these type I cercariae can infect channel catfish, while the type II cercariae are not infective. Type I metacercariae appeared to cause little host tissue damage. At 120 dpi, the last sampling point, 20 type I metacercariae were found in each of two fish, but no gross or histological pathology was observed, aside from the presence of the metacercariae. Although the type I cercariae and corresponding metacercariae were not identified to species, molecular and morphological data suggests that this parasite is a member of the genus *Clinostomum*. While this research confirms that there is another digenetic trematode infecting commercial catfish, there are still gaps in knowledge about its identification, pathology and life cycle. In order to determine the identity, pathology, and all of the hosts in the life cycle, further molecular work and long-term infection and field studies need to be done.

Introduction

Clinostomum spp. have a wide host range, and the hosts involved in the life cycle tend to vary based on the geographic location and the presence of appropriate local hosts. The first intermediate aquatic snail hosts include *Planorbella trivolvis* (Mitchell et al., 2007) and *Biomphalaria peregrina* (Dias et al., 2003). *Clinostomum* metacercariae are

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parasites of amphibians and freshwater fish (Cort, 1913; Hoffman, 1999), which serve as the second intermediate hosts. Many freshwater fish species, including channel catfish, *Ictalurus punctatus*, have been reported to be infected with *Clinostomum* spp. metacercariae (Dzikowski et al., 2004; Hoffman, 1999). Adult *Clinostomum* spp. generally live in the oral cavity, pharynx, and esophagus of piscivorous birds, reptiles, and mammals (Olsen, 1974). Rarely, human infections have been reported in people who have consumed raw infected freshwater fish (Chung et al., 1995; Park et al., 2009).

Clinostomum marginatum

The metacercariae that infect commercial channel catfish in Mississippi have been reported to be *C. marginatum,* or "yellow grub." They are diplostomum-type metacercariae with a distinct oral collar and large bodies that form a 1-2 mm cyst that is usually yellow in color. The cysts can be found free or encapsulated in subdermal and muscle tissues, on the fins and gills, and in the visceral organs and oral cavity (Overstreet and Curran, 2004; Plumb and Rogers, 1990). When encapsulated, a host-derived cyst forms, but they lack a parasite-derived cyst (Larson et al., 1988).

Clinostomum marginatum infections generally cause few negative consequences in commercial catfish production, especially when compared to the effects of *B. damnificus* infections. Because of this, little is known concerning the pathology caused by this parasite. When low-level infections of *C. marginatum* in commercial channel catfish are present, they do not cause any major pathology and have low mortality rates. However, in heavy infections, stunted growth may occur (Hawke and Khoo, 2004) and the fish may be unmarketable due to the presence of the metacercariae (Wise et al., 2004). Conversely, *Clinostomum complanatum* has been reported to cause significant

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morbidity and mortality in cultured loach aquaculture systems in Asia (Kagei et al., 1984). In the southeastern United States, the main definitive hosts of *C. marginatum* are great blue herons (*Ardea herodias*) (Flowers et al., 2004; Uglem et al., 1991). The first intermediate host is the rams-horn snail (*P. trivolvis*), and the second intermediate hosts include many fish species, representing several families (Hoffman, 1999; Mitchell et al., 2011).

Recently, cercariae were isolated from *P. trivolvis* snails collected from commercial catfish ponds in the Mississippi Delta (Doffitt et al., 2010). Further examination of these cercariae revealed that there were potentially two populations of these cercariae. This research was done to determine if these cercariae (type I and II) were infective to channel catfish and to determine the pathology, if any, associated with infection.

Materials and Methods

Preliminary Study

Snail Collection and Care

Rams-horn snails (*Planorbella trivolvis*) were collected from commercial channel catfish (*Ictalurus punctatus*) production ponds in Belzoni, MS (Humphreys County). The snails were transported to Mississippi State University College of Veterinary Medicine, where they were housed in individual dilu-vials (Krackler Scientific, Albany, NY 12201-1849) containing 10 ml of sterilized spring water. The water that housed the snails and potentially the cercariae were examined using a dissecting microscope (Olympus model SZ60, Olympus Imaging America, Inc., Center Valley, PA 18034) every 24 hours for the presence of cercariae. If a snail was positive, it was designated a number and all cercariae

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shed by that snail were collected daily for the duration of the shedding period. Each day, cercariae from each positive snail were examined microscopically to confirm shedding and the cercariae-type. Subsamples of these cercariae were collected and prepared for further molecular and morphological analysis to confirm cercariae-type. The remaining cercariae were used for subsequent challenge studies. Snails were provided with fresh sterilized spring water daily and fed blanched romaine lettuce every 2-3 days.

Enumeration and Preparation of Cercariae

To prepare and enumerate cercariae for use as the inoculum, viable cercariae of each cercariae-type were combined into pooled samples. The pooled samples were gently stirred and 10 - 0.1 ml aliquots were removed and placed on a concave glass slide. Using a dissecting microscope (Olympus model SZ60, Olympus Imaging America, Inc., Center Valley, PA 18034), cercariae in each 0.1 ml aliquot were enumerated to calculate the average number of cercariae/ml of sample volume. A subsample of the cercarial challenge inoculum was collected for molecular and morphological confirmation.

Fish Challenges

Specific pathogen free (SPF) channel catfish fingerlings (approximately 4 inches long) were obtained from the Mississippi State University College of Veterinary Medicine Fish Hatchery in Starkville, Mississippi. Fish (n=20) were challenged with approximately 6,000 cercariae (mixed type I and type II) cercariae/fish (Table 4.1). Fish were individually placed in plastic cups containing 200 ml of dechlorinated water with constant aeration. The challenge inoculum was added to the water and the fish were exposed to the cercariae for two hours. Following the two-hour exposure, all water was decanted quickly and the fish were rinsed three times with dechlorinated water. The fish

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were pooled by designated treatment groups and housed in indoor recirculating tanks until the termination of the trial. Fish were checked at least once/day and fed standard catfish ration *ad libitum*. If any of the catfish died before the termination of the trial, the fish were removed from the tank and examined following necropsy procedures described below.

Fish Necropsies

At necropsy, fish were sacrificed using a lethal dose of MS-222 (Finquel, Argent Chemical Laboratories, Redmond, VA 98052). At 31, 42, and 110 dpi, gross examination of catfish tissue, both external and internal, was performed on one of the challenged fish. Observations were made of gross external pathology of the skin, eyes, mouth, and gills. Internal examinations included gross and low-magnification microscopic observations of the muscle tissue, eyes, gills, and the internal organs, specifically the anterior and posterior kidneys, gastrointestinal tract, and pancreas. Further examination to confirm the presence of metacercariae in the muscle was done by filleting fish, which consisted of cutting 0.5 cm cross-sections on each side of the body, and examining these cross sections microscopically. Following necropsy, the fish were preserved in 10% buffered formalin. Any metacercariae found in the fish tissue were placed in 70% molecular grade ethanol, for later morphological or molecular analysis. No fish or metacercariae were prepared for histological analysis in the preliminary study.

Challenge Studies

In addition, a series of three challenge studies were done to confirm that the type I and II cercariae identified in the preliminary study were infective to channel catfish (*Ictalurus punctatus*). The protocols for collection and enumeration of cercariae and the

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basic fish challenge and necropsy model used in each challenge study (Studies 1-3) were identical to the preliminary study, except where noted (Tables 4.1 and 4.2). Additionally negative control fish were included in the all of the challenge studies and were treated identically to the challenged fish except that they were only exposed to dechlorinated water during the two-hour challenges.

Study 1

The fish were assigned to one of two groups of 15 fish/group. In the treatment group, fish were challenged with approximately 100 cercariae/fish (mixed type I and type II). The negative control fish (n=15) were exposed to dechlorinated water only (Table 4.1). Fish were necropsied at 40, 73, 80, and 120 days post-infection (dpi). At each necropsy, fish (n=5) from the treatment group and from the control group (n=2) were sacrificed (Table 4.2). At necropsy, two challenged and one control fish were examined internally and externally, as described in the preliminary study. Following necropsy, the examined fish were preserved in 10% buffered formalin. Any metacercariae found in the fish tissue were placed in 70% molecular grade ethanol for later morphological or molecular analysis. One of the challenged fish was preserved in McDowell's solution. The remaining challenged (n=2) and control fish (n=1) were slit open along the ventral aspect of the abdomen and preserved in 10% buffered formalin for later histological analysis.

Study 2

Study 2 consisted of two treatment groups of 10 fish/group. Treatment group 1 was challenged with approximately 200 clinostomoid-type I cercariae/fish, and treatment group 2 was challenged with approximately 200 strigeid-type II cercariae/fish. Negative

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controls (n=10) were exposed to dechlorinated water only (Table 4.1). At 7 dpi, the trial was terminated and fish were euthanized using MS-222. Five fish per group underwent a gross examination using procedures outlined in the preliminary study, while the remaining five were preserved in 10% buffered formalin for later histological analysis (Table 4.2). Any metacercariae found in the fish tissue were placed in 70% molecular grade ethanol, for later morphological or molecular analysis.

Study 3

Identical procedures outlined in the preliminary study for the collection and isolation of cercariae, the fish challenges, and necropsies were used in study 3. Study 3 consisted of two treatment groups and one negative control group. Treatment group 1 (n=25) was challenged with approximately 200 clinostomoid-type I cercariae/fish; Treatment group 2 (n=20) was challenged with approximately 200 strigeid-type II cercariae/fish and the control group (n=15) was exposed to dechlorinated water only (Table 4.1). After the two-hour exposure period, fish were rinsed, combined by groups, and transported to recirculating tanks until trial termination.

At 14 dpi, ten fish from each treatment group and seven negative control catfish were sacrificed. Gross examination of five fish per treatment group and two negative control fish was performed following procedures outlined in the preliminary study. The remaining five fish from each treatment group and the two fish from the control group were preserved in 10% buffered formalin for later histology (Table 4.2). Any metacercariae found in the fish tissue were placed in 70% molecular grade ethanol for later morphological or molecular analysis.

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At 21 dpi, ten fish from treatment group 1 and four negative control fish were euthanized; At 24 dpi, ten fish from treatment group 2 and four negative control fish were euthanized. Five fish per treatment group and two fish per negative control group underwent gross examinations. The remaining five fish per treatment group and two negative controls were preserved in 10% buffered formalin for later histology (Table 4.2).

Table 4.1 Summary of the Experimental Design for the Preliminary Study and Studies 1-3.

Study #	Treatment groups	Approximate cercariae dose	# of fish challenged
Preliminary	Pooled cercariae (types I and II)	6000	20
1	Pooled cercariae (types I and II)	100	15
	Control	0	6
2	Type I	200	10
	Type II	200	10
	Control	0	10
3	Type I	200	20
	Type II	200	20
	Control	0	15

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Table 4.2 Summary of Fish Necropsies.

Study #	Termination (dpi)	Treatment groups	# of fish necropsied
Preliminary	31[1]	Pooled cercariae (types I and II)	1
	42	Pooled cercariae (types I and II)	1
	110[1]	Pooled cercariae (types I and II)	1
1	40	Pooled cercariae (types I and II)	5
		Control	2
	73[1]	Pooled cercariae (types I and II)	1
	80	Pooled cercariae (types I and II)	5
		Control	2
	120	Pooled cercariae (types I and II)	4
		Control	2
2	7	Type I	10
		Type II	10
		Control	10
3	14	Type I	10
		Type II	10
		Control	7
	21	Type I	10
		Type II	10
		Control	8

[1]Died prior to scheduled termination date.

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Morphological Identification of Cercariae

Prior to each challenge study, type I and II cercariae were differentiated from one another based on behavior (swimming pattern) and morphology. Unstained cercariae were observed using a light microscope at 200x. Cercariae that swam with periods of rest, lacked a ventral sucker, and possessed eyespots were designated type I. Cercariae that swam with no periods of rest, had a ventral sucker, and lacked eyespots were designated type II.

In preparation for detailed morphological studies, both types of cercariae were stained with acetocarmine for 10-15 minutes, then photographed and measured using an Olympus light microscope model BX60 and Olympus camera model number U-CMAD-2 (Olympus Imaging America, Inc., Center Valley, PA 18034).

Morphological Identification of Metacercariae

Morphological identification of metacercariae collected in the preliminary study and challenge studies 1-3 was based on stained specimens. Parasites were stained in acetocarmine stain for 6-8 hours, destained with 1% acid alcohol, and then dehydrated using a series of ethanol washes (70%, 95%, and 100%). Parasites were then cleared using Citru-solve (JBS Industries, Pawleys Island, SC 29585) and mounted on slides using Permount (Fisher Scientific, Pittsburgh, PA 15275). Photographs and measurements of these metacercariae were made at 21, 31,40, 42, 73, 80, 110, and 120 days post infection using an Olympus light microscope model BX60 and Olympus camera model number U-CMAD-2 (Olympus Imaging America, Inc., Center Valley, PA 18034). Morphological comparisons were made using taxonomic keys (Bray et al., 2008; Gibson et al., 2002; Jones et al., 2005; Yamaguti, 1958).

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Molecular Analysis

A subsample of the cercariae and metacercariae used in the preliminary study and each challenge study (1-3) was collected for molecular analysis. Individual cercariae or metacercariae were placed in 1.5 ml microfuge tubes containing 600 µl cell lysis solution (Qiagen PureGene). DNA extraction of metacercariae and cercariae was performed using the Gentra Puregene kit (Gentra Systems, Minneapolis, MN 55441) according to the manufacturer's instructions.

PCR (polymerase chain reaction) for each sample was performed in 25 µl reaction volumes composed of 0.625 units Takara Hot Start Taq polymerase (Takara Bio, Inc., Shiga, Japan), 2.5 µl Takara 10x PCR buffer, 200 µM of each deoxynucleotide triphosphate, 200 nM of each primer, 2-5 µl genomic DNA, and nuclease free water added *quantum satis* to 25 µl. Oligonucleotide primers specific to the 18S rRNA gene were used in PCR in preparation for DNA sequencing (Littlewood et al., 1999) (Table 4.3). PCR was performed using a MJ Research PTC-100 Peltier thermal cycler (MJ Research, Minnesota) under the following conditions: 94˚C for 5 minutes, 94˚C for 30 seconds, 54˚C for 30 seconds, and 72˚C for 2 minutes, with steps 2-4 being repeated 40 times, followed by 72˚C for 5 minutes. The PCR products were visualized on a 1.2% agarose gel and stained using GelStar (Fisher Scientific, Pittsburgh, PA 15275). PCR was performed on subsamples of clinostomoid-type I cercariae (n=5), strigeid-type II cercariae (n=5) and resultant type I metacercariae (n=5).

Additionally, the same genomic samples were PCR-amplified using two sets of cytochrome oxidase subunit I (COI) barcode primers and two sets of internal transcribed spacer (ITS) primers (ITS1 + 5.8S + ITS2 rRNA) using PCR as previously described (Moszczynska et al., 2009). PCR for each sample was performed in 25 µl reaction

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volumes composed of 0.6 units of Platinum Taq polymerase (Invitrogen Corporation, Carlsbad, CA 92008), 1x PCR buffer, 2.5 mM $MgCl_2$, 50 µM of each deoxynucleotide triphosphate, 1.25 pmol of each primer, 2-5 µl genomic DNA, and nuclease free water added *quantum satis* to 25 µl. Oligonucleotide primers specific to the COI barcode gene region and the ITS1 + 5.8S + ITS2 rRNA gene region were used in PCR in preparation for DNA sequencing (Moszczynska et al., 2009) (Table 4.3). PCR was performed using a MJ Research PTC-100 Peltier thermal cycler (MJ Research, Minnesota) under the following conditions: 94˚C for 2 minutes, 94˚C for 30 seconds, 50˚C for 30 seconds, and 72˚C for 1 minute, with steps 2-4 being repeated 35 times, followed by 72˚C for 1 minute. The PCR products were visualized on a 1.2% agarose gel and stained using GelStar (Fisher Scientific, Pittsburgh, PA 15275).

Table 4.3 Primer Sequences Used to Amplify Mitochondrial and Genomic Gene Regions in Cercariae and Metacercariae.

Gene Region	Primer name	Primer sequence
18S rRNA[1]	Worm A	5'-GCG ATT GGC TCA TTA AAT CAG-3'
	Worm B	5'-CTT GTT ACG ACT TTT ACT TCC-3'
COI barcode[2]	MplatCOXidF	5'-TGT-AAA-ACG-ACG-GCC-AGT-TTW-CIT-TRG-ATC-ATA-AG-3'
	MplatCOXidR	5'-CAG-GAA-ACA-GCT-ATG-ACT-GAA-AYA-AYA-IIG-GAT-CIC-CAC-C-3'
	Plat-diploCOXiF	5'-CGT-TTR-AAT-TAT-ACG-GAT-CC-3'
	Plat-diploCOXiR	5'-AGC-ATA-GTA-ATM-GCA-GCA-GC-3'
ITS1 + 5.8S + ITS2 rRNA[2]	BR (F)	5'-GTA-GGT-GAA-CCT-GCA-GG-3'
	Dig11 (R)	5' -GTA-ATA-TGC-TTA-AGT-TCA-GC-3'
	D1 (F)	5'-AGG-AAT-TCC-TGG-TAA-GTG-CAA-G-3'
	D2 (R)	5'-CGT-TAC-TGA-GGG-AAT-CCT-GG-3'

[1](Littlewood et al., 1999)
[2](Moszczynska et al., 2009)

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PCR products were submitted to the Arizona State University DNA Laboratory (Arizona State University School of Life Sciences, Tempe, AZ 85287-4501) for genomic sequencing using an Applied Biosystems 3730 capillary sequencer (Applied Biosystems, Carlsbad, CA 92008). Sequences were edited using Sequencher (Gene Codes Corporation, Ann Arbor, MI 48108) and aligned using Se-Al (version v2.0) and Clustalw. Aligned consensus sequences were compared to other known sequences in BLAST (Basic Local Alignment Search Tool) (National Center of Biotechnology Information, Bethesda, MD 20894).

Histological Analysis

At necropsy, all fish were examined grossly and all findings were recorded. The coelomic cavity was opened ventrally and the fish were placed in 10 % neutral buffered formalin for a minimum of 24 hours. The fish were trimmed and four cross sections of each fish were taken. The areas where these cross sections (at the level of the eye, at the level of the dorsal commissure of the operculum, at the level just caudal to the pectoral girdle and anterior to the dorsal fin, and between the pelvic fin and the anal fin) were chosen to ensure that the major organs could be examined histologically and also typically reflected where the heaviest concentration of parasitic cysts were located. The tissues were placed in cassettes and processed routinely by dehydration in a graded series of ethanol solutions of increasing strength, followed by clearing in a series of xylenes, embedding in paraffin and sectioning at six μm. Prepared slides were then stained with hematoxylin and eosin (H & E) (Luna, 1968).

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Results

Cercariae Types

In the preliminary study, 105 of the snails collected from Belzoni, MS were infected with some type of digenetic trematode cercariae. Three of the four cercarial types had been commonly observed in previous routine snail surveys conducted in our laboratory. Thirty-five snails were infected with *Bolbophorus* spp., 18 were infected with an amphistome-type cercariae, and seven were infected with *Clinostomum marginatum*. Forty-five snails were infected with the fourth type, a previously unobserved morphologically distinct cercariae type. After 24 hours, cercariae from all snails infected with of each species were enumerated (Table 4.4). The unknown cercariae type produced an average of 472 cercariae per ml (average of 262 cercariae per snail).

Table 4.4 Average Number of Cercariae Shed by *Planorbella trivolvis* during a 24-hour Period (Pooled by Type).

Cercariae type	Number of infected snails	Average cercariae/ snail	Average cercariae/ml
Pooled Unknown-types I and II	45	262	472
Bolbophorus spp.	35	5760	504
Amphistome-type	18	1150	92
Clinostomum marginatum	7	28.5	2

After further observation of the unknown cercariae, a behavioral difference was noted. One population of cercariae swam intermittently with periods of rest and was designated type I, and the other swam continually without resting and was designated type II. Visual examination of unstained cercariae using a compound light microscope revealed morphological differences, as well. Type I cercariae lacked a ventral sucker, but

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possessed two eyespots. Conversely, type II cercariae possessed a ventral sucker, but lacked eyespots.

Fish Challenges

<u>Preliminary study</u>

At 31 dpi, one fish (Figure 4.1) challenged with a pooled inoculum of clinostomoid-types I and strigeid-type II died prematurely and was necropsied. Grossly, ascites, excess mucus, and slight hemorrhaging at the fin rays were observed. Hundreds of metacercariae were present in the superficial tissue layers, mainly near the caudal fin and vent and on the abdomen, gills, operculum, oral cavity, eye orbit, and between fin rays. No metacercariae were observed in the internal organs. Some of the metacercariae were encysted, while others were not. Since it was not feasible to remove all metacercariae, approximately 195 metacercariae were excised. Fourteen of those metacercariae were stained with acetocarmine and the remaining metacercariae were placed in 70% molecular grade ethanol; the fish was preserved in 10% buffered formalin.

At 42 dpi, one fish (n=1) was terminated. It had exophthalmia and live metacercariae could be seen moving in the subdermal tissues. Metacercariae were collected from the operculum and gill area, ocular cavity, dorsal fin, abdominal area, and tail area. Many, but not all metacercariae were excised. Of the excised metacercariae, 14 were stained with acetocarmine, 31 were preserved in McDowell's solution, and 10 were prepared for molecular analysis. All other excised metacercariae were preserved in 70% molecular grade ethanol. The fish was preserved in 10% buffered formalin.

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At 110 dpi, one fish from the preliminary study died prematurely. Many, but not all metacercariae were excised from the fish tissue. Of those that were excised, 29 were stained. The fish was preserved in 10% buffered formalin.

Figure 4.1 Channel Catfish Infected with Pooled Type I and Type II Cercariae (Preliminary Study) at 31 dpi, Showing Hundreds of Metacercariae.

Short-term Challenge (Studies 2-3)

On day 7 dpi (challenge study 2), fish challenged with type I (n=5/10) or type II (n=5/10) cercariae and the control fish (n=10) were necropsied. In fish examined grossly (n=5/challenge group), no visible lesions were found externally on the body surface or internally in any organs and no metacercariae were observed on or within the fish. Furthermore, histological findings of the remaining five fish per challenge group and control group showed no evidence of developing metacercariae. There was, however, minimal to mild mononuclear inflammatory infiltrate around some of the myocytes, perhaps an indication of the migration tract of type I cercariae (Figure 4.2).

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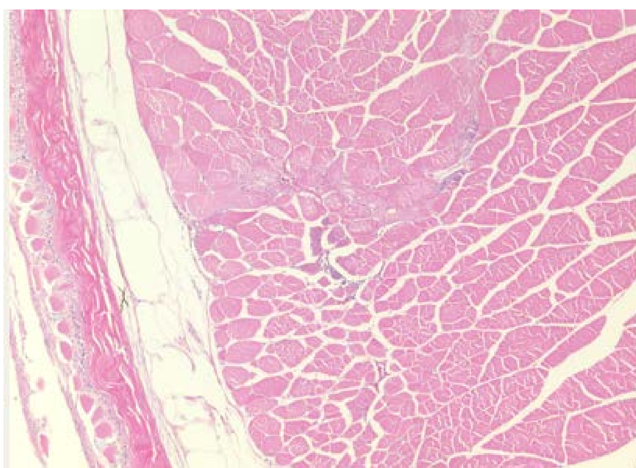

Figure 4.2 Cross Section of Fish Tissue at 7 dpi (Challenge Study 1) Showing Possible Cercariae Migration Tract

On day 14 dpi (challenge study 3), fish challenged with type I (n=5/10) or type II (n=5/10) cercariae and the control fish (n=7) were necropsied. Fish examined grossly (n=5/challenge group and n=7 control group) appeared normal with no visible lesions found externally on the body surface or internally in any organs and no metacercariae were observed on or within the fish. Histological findings of the remaining fish (n=5/challenge group and n=3 control fish) showed the growth of type I metacercariae appeared to be relatively slow and histopathological lesions were limited to the metacercarial site within the superficial musculature. The developing metacercariae were evident but there was often no inflammation seen at the metacercarial site. A clear space was present between the parasite and the surrounding mildly compressed myocytes. Separating the myocytes from the metacercariae was a single cell thick pale basophilic membrane. The metacercariae (55-60 x 65-75 μm) had a prominent eosinophilic tegument (~ 1.5 μm thick) with no spines evident. An epithelial lined cavity, that in some sections appeared to have two chambers (presumptively paired cecum), contained eosinophilic amorphous to fibrillar material that was sometimes seen on sections through

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the metacercariae (Figure 4.3). No evidence of infection was observed for the strigeid-type II challenged fish.

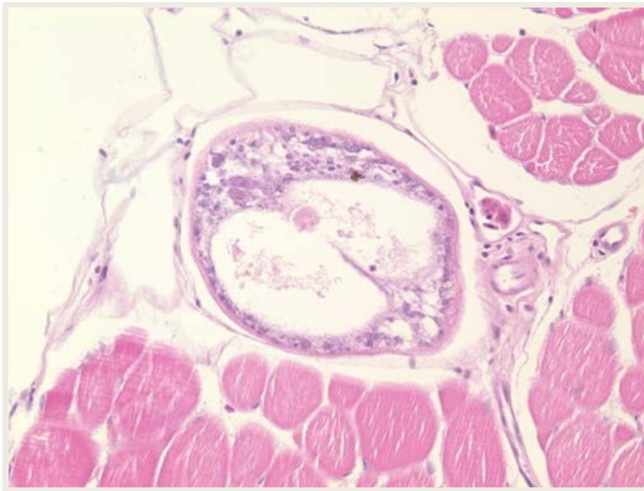

Figure 4.3 Cross-Section of Fish Tissue at 14 dpi (Challenge Study 3), Showing Developing Type I Metacercaria.

At 21 dpi (study 3), none of the channel catfish challenged with type II cercariae showed any signs of infection. However, pathology was noted in the catfish challenged with type I. In fish examined grossly (n=5/challenge group and n=5 control group), one of the challenged fish had one metacercaria on the gill operculum. No other signs were noted. No metacercariae or signs of infection were detected in the remaining four challenged fish. The remaining fish examined histologically (n=5/challenge group and n=3 control group), revealed that there was usually no inflammatory infiltrate around the developing type I metacercariae (Figure 4.4). In some instances, however, there was mild mononuclear inflammatory infiltrate but this was dependent on the metacercarial developmental site. Sites closer to the skin often, but not always, had an inflammatory component. A developing sucker, characterized by a circular structure with a stoma surrounded by eosinophilic cells (myocytes) arranged in a radial pattern around the stoma

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with overlapping, large, oval, vesicular nuclei in a pseudostratified pattern in the periphery, was sometimes seen in the sections (Figure 4.4).

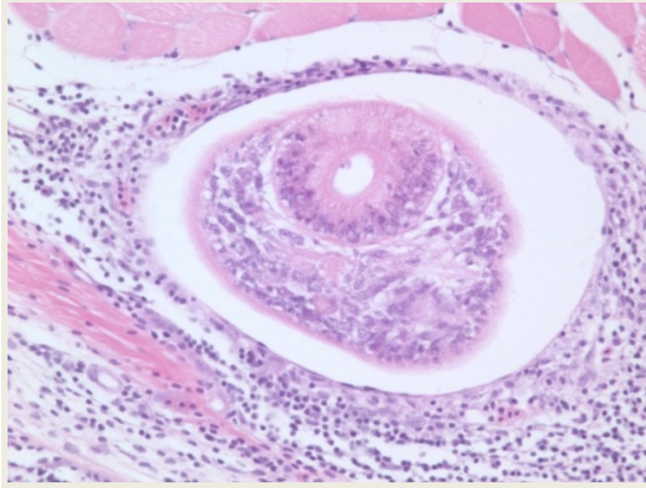

Figure 4.4 Cross Section of Fish Tissue at 21 dpi (Challenge Study 3), Showing Developing Metacercaria with Ventral Sucker.

Long-term Challenge (Study 1)

At 40 dpi (study 1), two of the fish necropsied in study 1 challenged with pooled type I and type II cercariae exhibited signs of infection. Fish 1 had pale gills and grossly visible subcutaneous metacercariae. Eight metacercariae were collected from the gill filaments, ocular cavity, and subcutaneous tissues near the oral cavity and vent. Fish 2 also had pale gills and grossly visible subcutaneous metacercariae. Nine metacercariae were recovered from the gill filaments, gill operculum, and the subcutaneous tissues near the vent. No other signs of pathology were noted. Histologically, at 40 dpi, the inflammatory component, if present, was more prominent. In addition to the inflammatory cells, there also appeared to be plump, spindle-shaped mesenchymal cells that may be fibroblasts. The sucker, if present, in the sections appeared to be more developed with a paler eosinophilic layer around the stoma, followed by a thick

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eosinophilic layer of myocytes in a radial pattern with smaller, less vesicular (when compared to 21 dpi) oval shaped nuclei. The sucker was located within a pale basophilic, poorly cellular parenchyma together with the developing cecum (Figure 4.5).

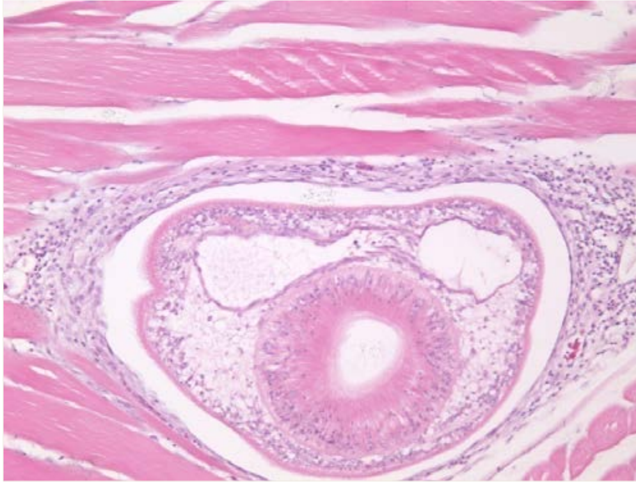

Figure 4.5 Cross Section of Fish Tissue at 40 dpi (Challenge Study 1), Showing Prominent Ventral Sucker in Metacercaria.

At 73 dpi, one fish died prematurely. Metacercariae were grossly visible in the subcutaneous tissues. A total of 24 metacercariae were recovered from the gill operculum (n=6), gill arch (n=2), ocular cavity (n=2), the musculature (n=7), and near the oral cavity (n=4) and vent (n=3). Five of the metacercariae were stained with acetocarmine and all other metacercariae were preserved in 70% molecular grade ethanol. The fish was preserved in 10% buffered formalin.

At 80 dpi, numerous metacercariae were present in the two fish challenged with the pooled type I and type II cercariae. In fish 1, 16 metacercariae were collected from the gill filament (n= 1), dorsal surface of the oral cavity (n=5), skeletal muscle (n=4), and the subcutaneous tissues near the oral cavity (n=2), abdomen (n=2), and vent (n=2). In fish 2, a total of 17 metacercariae were recovered from the gill operculum (n=3), gill arch

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(n=1), gill filament (n=1), ocular cavity (n=4), skeletal muscle (n=7), and the subcutaneous tissues near the vent (n=1). No other signs of gross pathology were observed. Histology (Figure 4.6) revealed that the inflammatory component was reduced and there was compression of the fibroblast, possibly indicating the formation of a host cyst wall. In some tissue sections, there was a markedly basophilic cellular structure that may be the excretory vesicle.

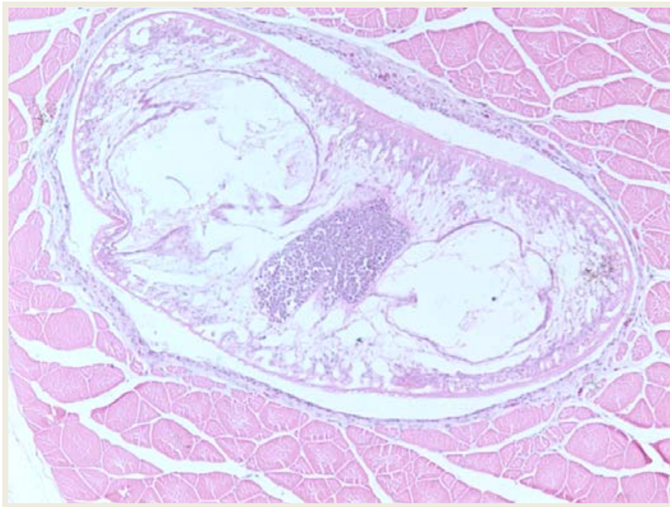

Figure 4.6 Cross Section of Fish Tissue at 80 dpi (Challenge Study 1), Showing Metacercaria with Basophilic Structure.

At 120 dpi, metacercariae were found in both of the fish that were examined grossly. In fish 1, a total of 20 metacercariae were found in the gill cavity (n=2), ocular cavity (n=3), skeletal muscle (n=6), and the subcutaneous tissues near the oral cavity (n=4), vent (n=2), and abdomen (n=3). Cysts were observed surrounding the metacercariae. No other signs of pathology were noted. A total of 20 metacercariae were recovered from fish 2 which were found in the gill cavity (n=5), ocular orbit (n=4), skeletal muscle (n=4), near the heart (n=2), and the subcutaneous tissues near the oral cavity (n=2), dorsal fin (n=1), and pelvic fin (n=2). Histologically, the development of a

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host-derived metacercarial cyst was noted, but no other signs of pathology were observed. At 120 dpi, the internal anatomy of the metacercariae was evident with an oral sucker, cecum, ventral sucker, excretory vesicle, and layer of muscle cells below the tegument (Figure 4.7).

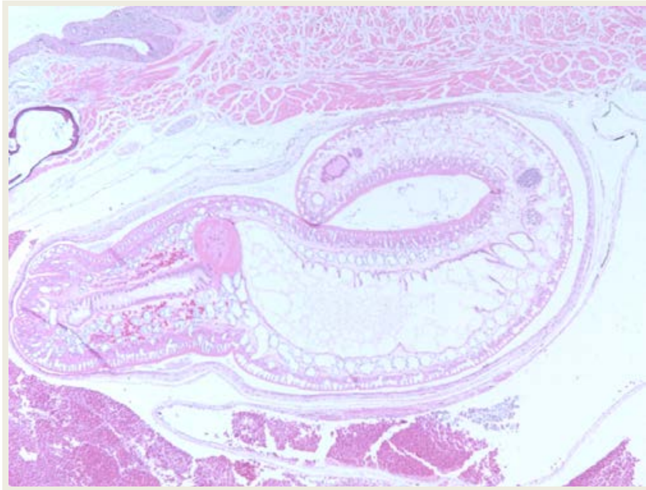

Figure 4.7 Cross Section of Fish Tissue at 120 dpi (Challenge Study 1) Showing a Well-Developed Metacercaria.

There were no histological lesions or pathology associated with the other tissues of any of the challenged or control fish, including the liver, spleen, or kidney. The histopathology due to the developing type I metacercariae was limited to the metacercarial site and its cercarial migration tract. There appeared to be a limited mild inflammatory component with no hemorrhage evident at anytime of the development from seven to 120 dpi. The parasitic cyst separating the metacercariae from the host cells only had a host component and no parasitic component. No histological lesions, gross lesions, or pathology was observed in the control fish.

Over the course of study 1, one fish died before its scheduled termination date at 73 dpi (study 1, treatment group 1). Gross examination revealed that the catfish was

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infected with 24 metacercariae recovered from the gill arch (n=2), gill operculum (n=3), ocular cavity (n=2), in the skeletal muscle (n=7), and in the subcutaneous tissues near the oral cavity (n=4), gill operculum (n=3), and the vent (n=3).

Description of Cercariae

The type I cercariae, used in the challenge studies, were brevifurcous, pharyngeate, lophocercous, and oculate. The body region measured an average of 134.1 x 35.5 µm. The trunk was an average of 233.2 x 27.6 µm. The two furcae each measured an average of 79.8 x 14.7 µm (Table 4.5). The total length of the cercariae was an average of 447.2 µm. Visible morphological characters, from the anterior body region to the posterior body region were the penetration organ and glands, two eyespots, and the ventral sucker analogue. A dorso-median finfold that spanned the entire length of the body was present (Figure 4.8). The caudal excretory canal extended down the length of the trunk and both furcae (Figure 4.9). When alive, the movement of the cercariae was one of swimming with frequent, brief periods of rest during which the cercariae remained suspended in the water column.

The type II cercariae were longifurcate and pharyngeate. The body region was an average of 170.8 x 43.9 µm and the trunk was an average of 78.3 x 27.2 µm. The furcae measured an average of 117.9 x 14.3 µm. The total length of the cercariae averaged 366.9 µm (Table 4.5). Visible morphological characters, from the anterior body region to the posterior body region were the penetration organ and glands, and the ventral sucker. No eyespots or dorso-median finfolds were evident (Figure 4.10). Living cercariae swam consistently, without any rest periods.

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Table 4.5 Morphometrics of Unknown Type I and Type II Cercariae.

Type I	Mean[1]	St Dev	N	Min	Max
body length	134.1	12.6	11	115.5	153.2
body width	35.5	4.7	11	29.7	44.8
trunk length	233.2	29.5	11	198.0	279.4
trunk width	27.6	4.0	11	21.7	34.7
furcae length	79.9	10.4	11	60.8	91.9
furcae width	14.7	1.4	11	11.9	16
penetration gland length	44.2	5.6	11	38.9	57.2
ventral sucker length	NA	NA	NA	NA	NA
ventral sucker width	NA	NA	NA	NA	NA
total length	447.2	37.7	11	389.3	494.4

Type II	Mean	St Dev	N	Min	Max
body length	170.8	9.3	11	149.3	185.0
body width	43.9	4.1	11	38.0	51.6
trunk length	78.3	5.3	11	72.2	87.8
trunk width	27.2	2.6	11	22.2	31.0
furcae length	117.9	11.0	11	97.1	132.3
furcae width	14.3	1.7	11	12.1	17.6
penetration gland length	49.8	7.1	11	41.7	68.1
ventral sucker length	20.9	2.6	11	16.1	24.8
ventral sucker width	22.1	2.2	11	18.8	25.9
total length	366.9	14.5	11	341.6	387.1

[1]Measurements in µm.

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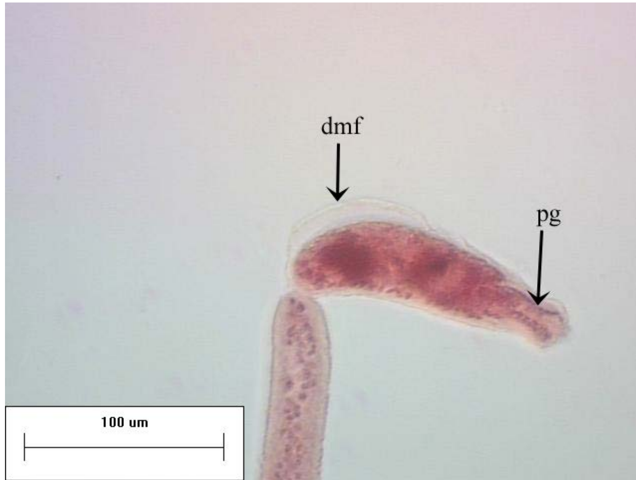

Figure 4.8 Type I Cercaria. Detail of Body, Showing Penetration Glands (pg), Eye Spots, Ventral Sucker Analogue, and Dorso-median Finfold (dmf)

Figure 4.9 Type I Cercaria. Whole Body, Showing Body Region, Trunk, Furcae, and Caudal Excretory Canal.

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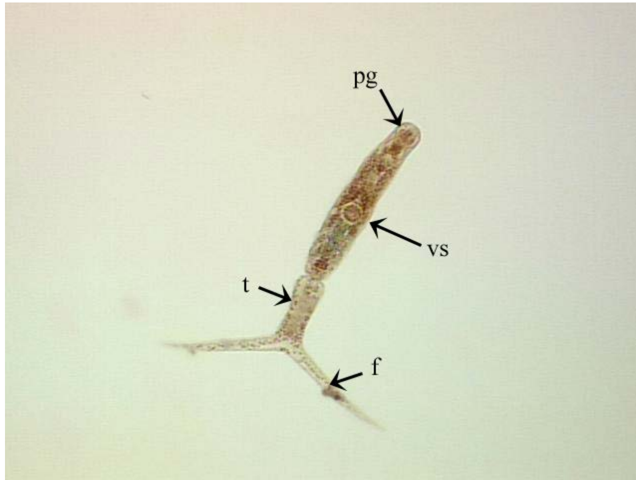

Figure 4.10 Strigeid-type II Cercaria. Whole body, Showing Body Region, Trunk (t), Furcae (f), Penetration Gland (pg), and Ventral Sucker (vs).

Description of Metacercariae

Descriptions of growth and development were based on type I metacercariae stained at 21, 31, 40, 42, 73, 80, 110, and 120 dpi. No morphological descriptions of metacercariae at days 7 and 14 post infection were available because the metacercariae were not developed enough for gross detection.

At 21 dpi, only one metacercaria was recovered, so measurements and descriptions were based on a sample of one. The metacercaria was small (225 x 201 µm) (Table 4.6). The oral sucker was terminal and measured 66 x 32 µm. The ventral sucker was located towards the center or posterior end of the metacercariae and was relatively large (101 x 95 µm). Two eyespots were located on both sides of and posterior to the oral sucker. A small structure, presumably the developing gonads, could be seen in the hindbody (Figure 4.11).

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Table 4.6 Morphometrics of Clinostomoid-type I Metacercariae.

21 dpi	Mean[1]	St dev	N	Min	Max
oral sucker length	66	NA	1	NA	NA
oral sucker width	32	NA	1	NA	NA
ventral sucker length	101	NA	1	NA	NA
ventral sucker width	95	NA	1	NA	NA
forebody length	201	NA	1	NA	NA
hindbody length	22	NA	1	NA	NA
total length	225	NA	1	NA	NA
width	201	NA	1	NA	NA
anterior testis length	NA	NA	NA	NA	NA
anterior testis width	NA	NA	NA	NA	NA
posterior testis length	NA	NA	NA	NA	NA
posterior testis width	NA	NA	NA	NA	NA
uterus length	NA	NA	NA	NA	NA
uterus width	NA	NA	NA	NA	NA

31 dpi	Mean	St dev	N	Min	Max
oral sucker length	61.8	6.2	4	55	70
oral sucker width	84.5	17.1	4	61	102
ventral sucker length	157.8	31.4	4	118	192
ventral sucker width	166.0	25.7	4	138	198
forebody length	161.5	42.1	4	127	218
hindbody length	101.8	62.7	4	20	170
total length	411.3	58.6	4	332	470
width	246.8	21.8	4	225	277
anterior testis length	NA	NA	NA	NA	NA
anterior testis width	NA	NA	NA	NA	NA
posterior testis length	NA	NA	NA	NA	NA
posterior testis width	NA	NA	NA	NA	NA
uterus length	NA	NA	NA	NA	NA
uterus width	NA	NA	NA	NA	NA

40 dpi	Mean	St dev	N	Min	Max
oral sucker length	47	20.0	3	34	70
oral sucker width	57.7	26.4	3	40	88
ventral sucker length	115.3	65.7	3	73	191
ventral sucker width	119.0	46.0	3	90	172
forebody length	256.3	129.7	3	176	406
hindbody length	348.7	140.4	3	189	453

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Table 4.6 (Continued)

total length	603.7	246.4	3	370	861
width	202.0	92.7	3	145	309
anterior testis length	NA	NA	NA	NA	NA
anterior testis width	NA	NA	NA	NA	NA
posterior testis length	NA	NA	NA	NA	NA
posterior testis width	NA	NA	NA	NA	NA
uterus length	NA	NA	NA	NA	NA
uterus width	NA	NA	NA	NA	NA

42 dpi	**Mean**	**St dev**	**N**	**Min**	**Max**
oral sucker length	71.0	13.7	4	52	82
oral sucker width	115.3	50.3	4	72	188
ventral sucker length	204.5	35.5	4	166	252
ventral sucker width	254.0	47.9	4	195	306
forebody length	200.5	22.9	4	173	229
hindbody length	274.8	184.3	4	0	393
total length	666.0	171.1	4	410	763
width	237.0	14.0	4	220	254
anterior testis length	NA	NA	NA	NA	NA
anterior testis width	NA	NA	NA	NA	NA
posterior testis length	NA	NA	NA	NA	NA
posterior testis width	NA	NA	NA	NA	NA
uterus length	NA	NA	NA	NA	NA
uterus width	NA	NA	NA	NA	NA

73 dpi	**Mean**	**St dev**	**N**	**Min**	**Max**
oral sucker length	184.8	16.2	4	163	202
oral sucker width	249.5	17.1	4	232	272
ventral sucker length	391.5	40.0	4	359	447
ventral sucker width	440.8	22.3	4	409	461
forebody length	517.3	76.4	4	457	629
hindbody length	1457.0	256.2	4	1093	1657
total length	2385.3	291.0	4	1985	2647
width	519.8	56.4	4	466	587
anterior testis length	114.3	38.4	4	67	160
anterior testis width	150.0	26.1	4	127	187
posterior testis length	106.0	33.3	4	68	149
posterior testis width	160.8	28.6	4	140	203

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Table 4.6 (Continued)

uterus length	121.5	47.1	4	73	185
uterus width	182.3	35.5	4	143	228

80 dpi	Mean	St dev	N	Min	Max
oral sucker length	165.7	13.8	3	150	176
oral sucker width	168.3	23.2	3	147	193
ventral sucker length	444.3	25.5	3	418	469
ventral sucker width	458.3	30.0	3	425	483
forebody length	952.3	58.4	3	888	1002
hindbody length	1614.7	66.3	3	1542	1672
total length	2565.3	65.5	3	2501	2632
width	807.0	103.7	3	700	907
anterior testis length	76.0	41.9	3	38	121
anterior testis width	147.7	61.6	3	78	195
posterior testis length	76.3	24.0	3	49	94
posterior testis width	173.7	95.0	3	67	249
uterus length	91.0	20.1	3	68	105
uterus width	148.7	49.2	3	92	180

110 dpi	Mean	St dev	N	Min	Max
oral sucker length	178.5	21.6	10	146	218
oral sucker width	249.5	20.0	10	213	274
ventral sucker length	389.6	32.6	10	335	440
ventral sucker width	424.2	40.4	10	359	475
forebody length	543.5	77.1	10	407	665
hindbody length	1623.9	305.1	10	1177	2031
total length	2544.8	395.3	10	1953	3078
width	586.1	76.1	10	493	699
anterior testis length	101	22.8	10	70	150
anterior testis width	157.4	28.2	10	113	205
posterior testis length	91.4	24.6	10	51	129
posterior testis width	163.1	28.4	10	113	203
uterus length	99.2	24.9	10	51	127
uterus width	134	27.0	10	77	180

120 dpi	Mean	St dev	N	Min	Max
oral sucker length	216.3	44.9	4	150	249
oral sucker width	234.8	36.7	4	188	276
ventral sucker length	558.0	110.7	4	412	654

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Table 4.6 (Continued)

ventral sucker width	606.5	102.6	4	462	696
forebody length	1274.3	274.5	4	893	1513
hindbody length	2517.5	585.8	4	1657	2897
total length	3766.8	811.5	4	2550	4207
width	1077.5	220.7	4	793	1268
anterior testis length	155.3	25.5	4	120	179
anterior testis width	252.5	42.2	4	214	292
posterior testis length	143.0	41.0	4	88	186
posterior testis width	245.0	25.9	4	210	267
uterus length	178.8	50.6	4	113	228
uterus width	228.8	47.1	4	173	288

[1]All measurements in µm.

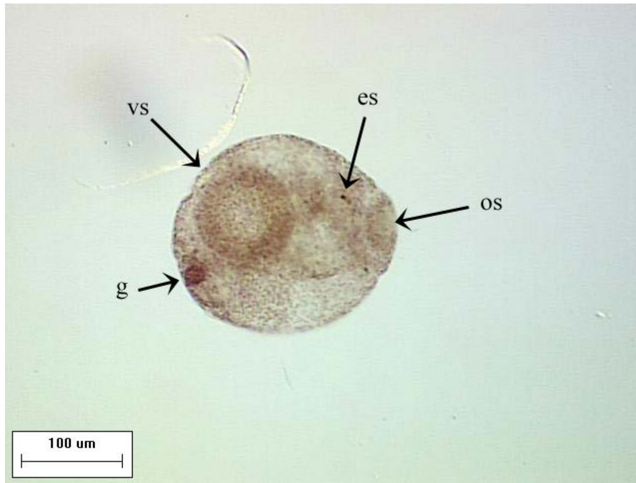

Figure 4.11 Type I Metacercaria, Showing Oral Sucker (os), Eye Spots (es), Ventral Sucker (vs), and Immature Gonad (g) at 21 dpi in Study 3.

At 31 dpi, metacercariae, although still small (average 411.3 x 246.8 µm) (Table 4.6) had almost doubled in length. The oral sucker was terminal and measured an average of 61.6 x 84.5 µm. The ventral sucker was located towards the center or posterior end of the metacercariae and was relatively large (average = 157.8 x 166.0 µm). Two eyespots

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were located on either side of and posterior to the oral sucker. In most specimens, a small structure, likely immature gonads, could be seen in the hindbody (Figure 4.12).

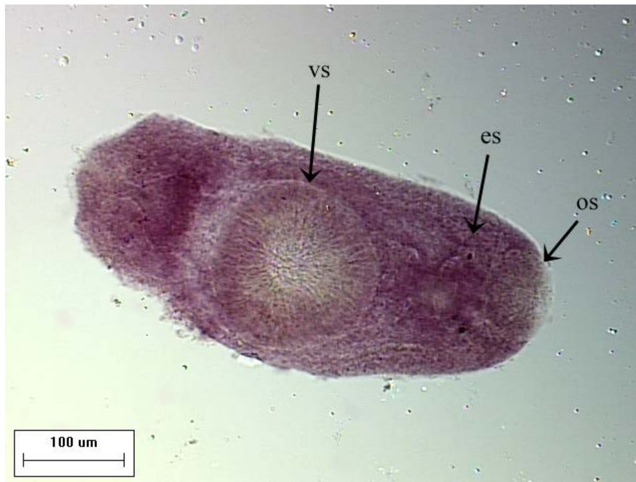

Figure 4.12 Type I Metacercaria, Showing Oral Sucker (os), Eye Spots (es), and Ventral Sucker (vs) at 31 dpi in the Preliminary Study.

At 40 dpi, metacercariae were generally larger and more developed. One prominent change was the proportion of the hindbody length compared to the total length. The average whole body length was 603.7 µm and the mean hindbody length was 348.7 µm. The oral sucker measured an average of 47.0 x 57.7 µm and the ventral sucker was an average of 115.3 x 119.0 µm (Table 4.6). The ventral sucker was located in the center or anterior most third of the body. Two developing gonads were visible and located in the posterior third of the body. Two eyespots were located on either side of the pharyngeal bulb (Figure 4.13).

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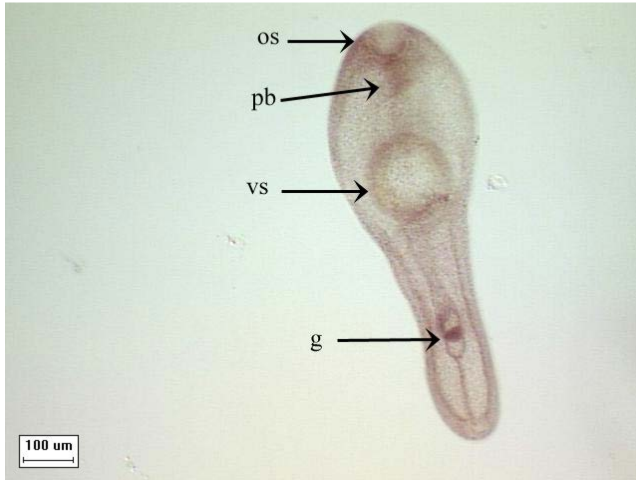

Figure 4.13 Type I Metacercaria, Showing Oral Sucker (os), Pharyngeal Bulb (pb), Ventral Sucker (vs), and Developing Gonads (g) at 40 dpi in Challenge Study 1.

At 42 dpi, metacercariae generally resembled those at 40 dpi. The average whole body length was 666.0 µm and the mean hindbody length was 274.8 µm. The oral sucker measured an average of 71.0 x 115.6 µm and the ventral sucker was an average of 204.5 x 254.0 µm (Table 4.6). The ventral sucker was located in the center or anterior most third of the body. Two developing gonads were visible and located in the posterior third of the body. The pharyngeal bulb was present, with two eye spots located on either side (Figure 4.14).

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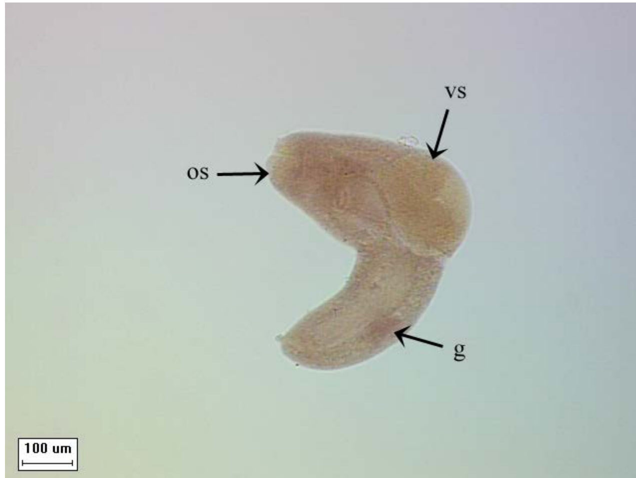

Figure 4.14 Type I Metacercaria, Showing Oral Sucker (os), Ventral Sucker (vs), and Developing Gonads (g) at 42 dpi in the Preliminary Study.

At 73 dpi, the metacercariae continued to grow in size and further development of organs was evident. The average whole body length was 2385.3 µm, with the hindbody accounting for most of the length (average = 1457.0 µm), versus the forebody (average = 517.3 µm). The oral sucker (average = 184.8 x 249.5 µm) was developing and the oral collar was present. The ventral sucker was an average of 391.5 x 440.8 µm (Table 4.6) and was consistently located in the anterior third of the body. Eyespots were present, but were less prominent and were located on either side of the pharyngeal bulb. The ceca were developed and extended on either side of the body to the level of the excretory pore. However, the diverticula of the ceca were not fully developed and no substance was evident within the ceca. The excretory vessel was developed and terminated at the excretory pore. Reproductive organs were prominent. The testes and uterus were evident, as well as the ovary, which was situated towards the right side of the body and superior to the uterus. The genital pore and cirrus were visible and located towards the right side of the body (Figure 4.15).

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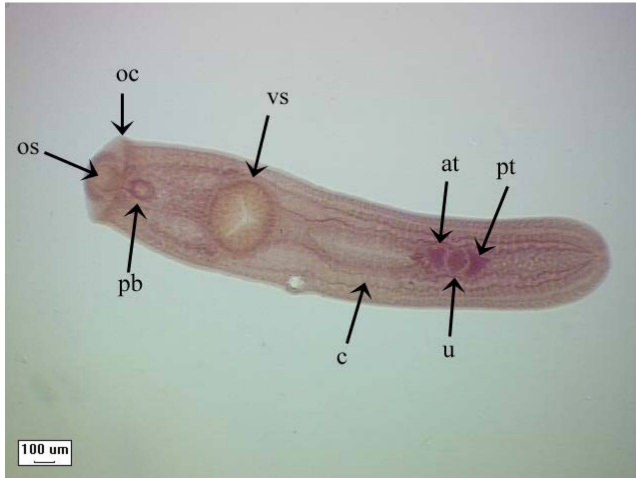

Figure 4.15 Type I Metacercaria, Showing the Oral Sucker (os), Oral Collar (oc), Pharyngeal Bulb (pg), Ceca (c), Ventral Sucker (vs), Anterior Testis (at), Posterior Testis (ts) and Uterus (u) at 73 dpi in the Preliminary Study.

At 80 dpi, the average whole body length was 2565.3 µm, with the hindbody accounting for most of the length (average = 1614.7 µm), versus the forebody (average = 952.3 µm). The oral sucker (average = 165.7 x 168.3 µm) was developing and the oral collar was present. The ventral sucker was an average of 444.3 x 458.3 µm and was situated in the anterior third of the body (Table 4.6). Eyespots were on each side of the pharyngeal bulb. The ceca were less prominent than at 73 dpi, but that is likely an artifact of staining. Reproductive organs were continuing to develop. The testes and uterus were evident and the genital pore and cirrus were visible and located towards the right side of the body (Figure 4.16).

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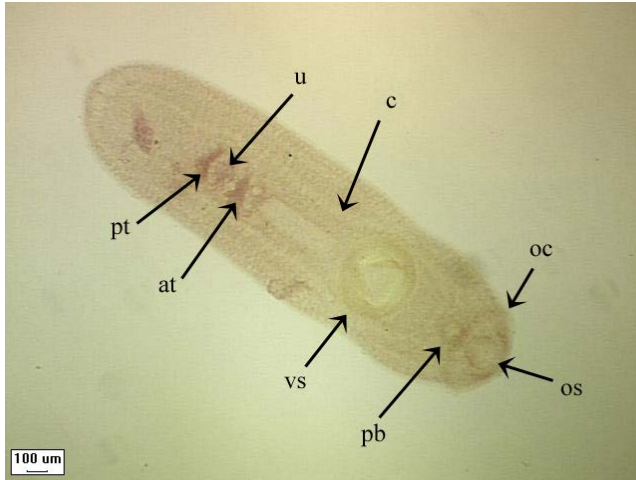

Figure 4.16 Type I Metacercaria, Showing Oral Sucker (os), Oral Collar (oc), Pharyngeal Bulb (pb), Ventral Sucker (vs), Ceca (c), Anterior Testis (at), Posterior Testis (pt), and Uterus (u) at 80 dpi in Challenge Study 1.

At 110 dpi, there was little increase in total body length, forebody and hindbody length, or width (Table 4.6) from 80 dpi. The size and relative location of the oral and ventral suckers were similar (Figure 4.17). The oral sucker and collar were prominent (Figure 4.18). The relative position of the anterior testis, posterior testis, and uterus in the body was unchanged. The uterine sac was prominent and was located anterior to the anterior testis and was connected to the uterus by a small tube on the left side of the body. The cirrus pouch was evident (Figure 4.19). The two eyespots were still present, but difficult to visualize. The digestive system continued to develop, with the diverticula being more prominent and a dark-staining substance present within the ceca. Vitellaria were evident in some of the specimens and extended from the posterior most end of the body to the level of the ventral sucker or oral sucker.

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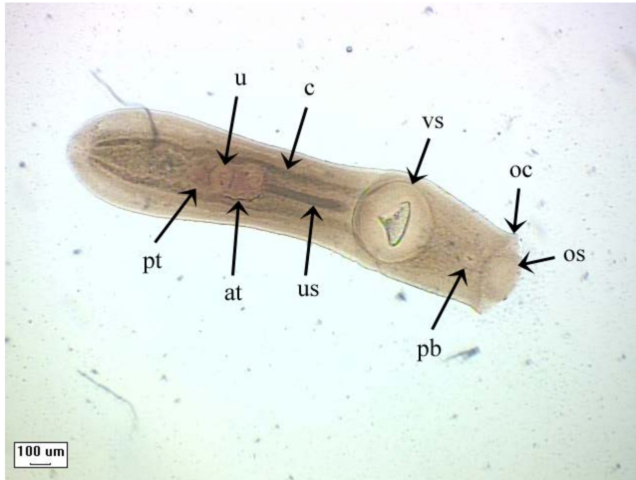

Figure 4.17 Type I Metacercaria, Showing Oral Sucker (os), Oral Collar (oc), Pharyngeal Bulb (pb), Ventral Sucker (vs), Ceca (c), Anterior Testis (at), Posterior Testis (pt), Uterus (u), and Uterine Sac (us) at 110 dpi in Challenge Study 1.

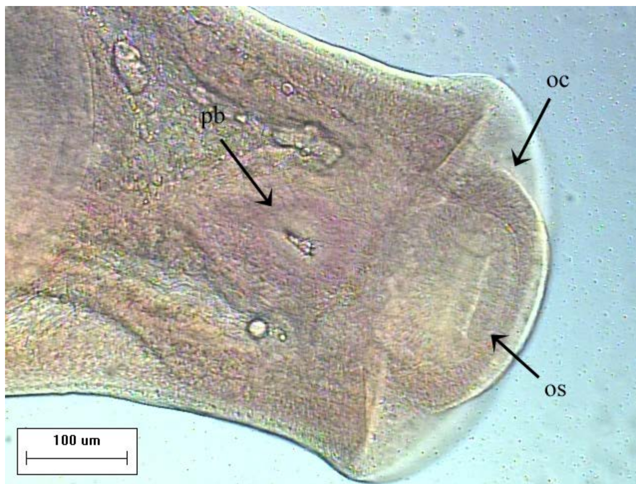

Figure 4.18 Type I Metacercaria Showing Oral Sucker (os), Oral Collar (oc), and Pharyngeal Bulb (pb) at 110 dpi in Challenge Study 1.

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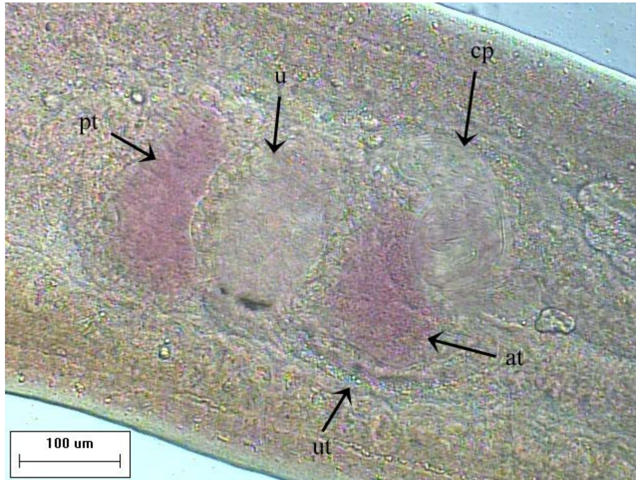

Figure 4.19 Type I Metacercaria , Showing Anterior Testes (at), Posterior Testis (pt) Uterus (ut), Uterine Sac (ut) and Cirrus Pouch (cp) at 110 dpi in the Preliminary Study 1.

At 120 dpi, there was an increase in total body length, forebody and hindbody length, and width (Table 4.6) from 110 dpi. The location of the oral and ventral suckers was similar to those at 110 dpi. The oral sucker, and collar were prominent (Figure 4.20) and the relative position of the testes and uterus in the body was unchanged. The uterine sac was prominent (Figure 4.21) and was superior to the anterior testis. It was connected to the uterus by a small tube on the left side of the body.

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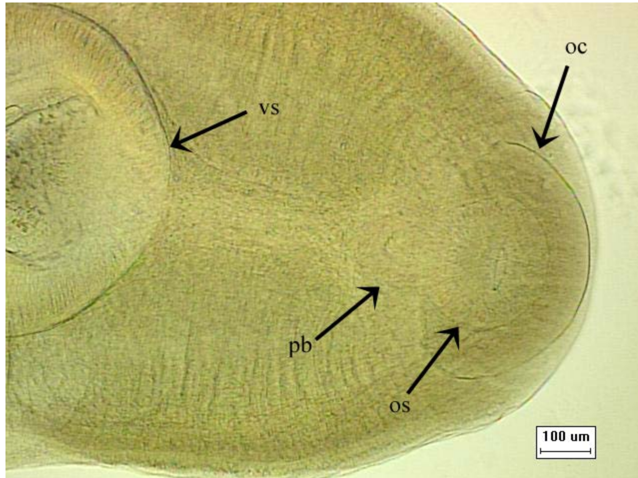

Figure 4.20 Type I Metacercaria , Showing Oral Sucker (os), Oral Collar (oc), Pharyngeal Bulb (pb), and Ventral Sucker (vs) at 120 dpi in Challenge Study 1.

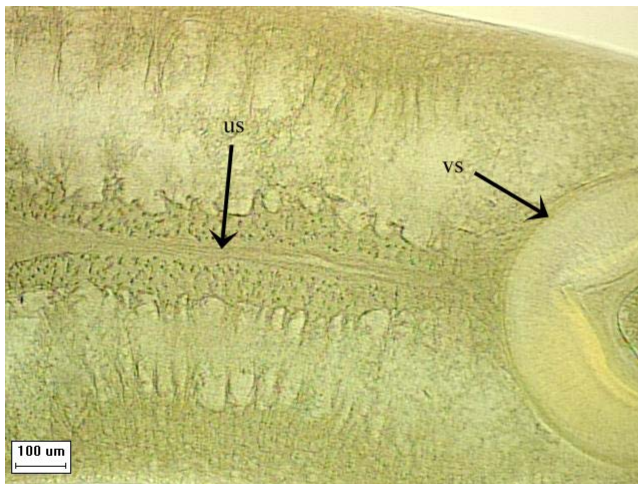

Figure 4.21 Type I Metacercaria , Showing the Uterine Sac (us) and Ventral Sucker (vs) at 120 dpi in Challenge Study 1.

Morphological Comparisons

When compared to the few published descriptions of *Clinostomum marginatum*, the clinostomoid-type I in this study shared many morphological characters and basic body form, but both the cercariae and metacercariae of the clinostomoid-type I species were distinctly smaller. Most descriptions of *C. marginatum* in the published literature

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focus on the metacercariae and use only descriptive terms, instead of measurements, making a direct comparison of morphological features difficult between the clinostomoid type-I species in this study and *C. marginatum*.

The live clinostomoid-type I cercariae that were collected from Belzoni, MS, when observed using a dissecting microscope, resembled (basic body shape), and behaved (swimming pattern) as *C. marginatum*, when compared to previous observations of *C. marginatum* cercariae collected during routine cercariae surveys. The characteristics that distinguished the two were size, with the clinostomoid-type cercariae being approximately half the length of *C. marginatum*, and cercariae productivity, with the clinostomoid-type cercariae being more prolific in the snail host than *C. marginatum* (Table 4.4). Closer examination of the two unknown types I and type II cercariae, using a compound light microscope, revealed distinct morphological differences between the two cercariae types. Type I possessed pigmented eyespots and lacked a ventral sucker, while type II lacked eyespots and possessed a ventral sucker (Figures 4.9 and 4.10).

Morphologically, metacercariae of the clinostomoid-type I very closely resembled descriptions of *C. marginatum* (Hopkins, 1933; Osborn, 1912). Therefore, the following comparisons focused on differences between the two species in this study. The clinostomoid-type I metacercariae had a smaller body size at an average length of 3.8 mm at 120 dpi, compared to *C. marginatum* metacercariae at 4.1 mm (Osborn, 1912) or 3.5-6.6 mm (Cort, 1913). Clinostomoid-type I produced small, white subdermal cysts, rather than larger, yellowish cysts reported for *C. marginatum* (Hawke and Khoo, 2004). The cysts of *C. marginatum* can be found free or encapsulated in subdermal and muscle tissues, on the fins and gills, and in the visceral organs (Overstreet and Curran, 2004; Plumb and Rogers, 1990). The metacercariae of clinostomoid-type I were found in the

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dermis, musculature, gills, and ocular cavity, but not in the visceral organs, with the exception of fish 2 at 120 dpi that had two metacercariae located near, but not in, the heart. The vitellaria in the clinostomoid-type I metacercariae extended from the hindbody to the level of the ventral sucker or the oral sucker, while in *C. marginatum*, the vitellaria reach only to the level of the ventral sucker (Osborn, 1912). The ventral sucker was reported to be 0.7 x 0.7 mm (Osborn, 1912), compared with 0.56 x 0.61 mm in clinostomoid-type I. The oral sucker was reported to be 0.28 x 0.25 mm (Osborn, 1912), compared with 0.22 x 0.23 mm in this study.

Molecular Analysis

All specimens used in the molecular analysis were sampled from the preliminary study. Type I cercariae (n=3) and type II cercariae (n=4) were collected from the same snail population that was used in study 1. The metacercariae (n=5) were sampled from the metacercariae excised in the preliminary study at 42 dpi. When edited and aligned consensus sequences for the 18S rRNA gene region in cercariae (types I and II) and metacercariae (type I) were compared to other 18S rRNA sequences in GenBank BLAST, Type 1 cercariae were a 99% match with *Clinostomum* sp. (AY222095.1) and a 99% match with *Clinostomum marginatum* (AY245760.1). Type 2 cercariae were a 99% match with *Apharyngostrigea cornu* (AY222092.1). Metacercariae recovered following the experimental challenges were a 99% match with *Clinostomum* sp. (AY222095.1) and a 99% match with *C. marginatum* (AY245760.1). Neither of the two COI barcode nor the ITS1 + 5.8S +ITS2 gene regions produced PCR products suitable for sequencing.

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Discussion

Gross examinations and histopathological analysis indicated that channel catfish are refractory to infection by strigeid-type II cercariae. This was evident when fish were challenged with pure strigeid-type II cercariae in challenge studies 2 and 3 and the challenged catfish had no detectable gross or histopathological lesions and no metacercariae were recovered from them. Channel catfish challenged with clinostomoid-type I cercariae, however, were highly susceptible to infections and showed signs of pathology. In study 1, when a pooled inoculum of both type I and type II was used as a challenge, metacercaria development was seen. These metacercariae were considered to be the result of clinostomoid-type I cercariae, because no evidence of infection was noted in the later challenge studies in treatment groups exposed to only strigeid-type II, while fish exposed to a pure clinostomoid-type I inoculum (studies 2 and 3) did become infected.

The growth and development of the clinostomoid-type I parasite in channel catfish appears to be relatively slow, especially when compared to *B. damnificus,* under comparable conditions. *Bolbophorus damnificus* are fully developed at 23 dpi in channel catfish (Yost, 2008). However, the clinostomoid-type I metacercariae at 21 dpi were immature in their development, as was evident by the lack of mature morphological structures, including the gonads and ceca (Figure 4.11). Development of metacercariae was detectable with histological analysis at 14 dpi, but they were not grossly visible until 21 dpi. By 73 and 80 dpi, most internal organs seemed to be developing. From 80 dpi to 120 dpi, most of the organs present changed little in their general body position, but most structures increased in size (Table 4.6). However, at 110 and 120 dpi, the uterine sac was

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more prominent compared to metacercariae at 80 dpi and most internal structures were in place.

Other than the presence of metacercariae, there appeared to be little pathology associated with this parasite. Most fish appeared normal throughout the study. Grossly, pale gills were observed 40 dpi. Mild levels of inflammatory infiltrates and fibroblasts were seen at the histological level at 40 dpi. Furthermore, this parasite seemed to cause little to no mortality, since only three fish died prematurely during the study (preliminary study at 31 and 110 dpi; study 1 at 73 dpi). No reported comparable challenge studies using clinostomoid species exist; however, comparable studies with the digenetic trematode, *B. damnificus*, in which catfish were also challenged with 200 cercariae/fish for two hours, resulted in nearly 80-100% mortality by seven dpi (Yost, 2008).

There is a lack of thorough descriptions of the cercariae and metacercariae of *Clinostomum* species reported in the published literature. Most descriptions use only descriptive terms, instead of measurements, confounding direct morphological comparisons of this clinostomoid-type I with other *Clinostomum* species. At 120 dpi, the clinostomoid-type I metacercariae resembled those described previously for *C. marginatum* (Cort, 1913; Hopkins, 1933; Osborn, 1912); therefore, comparisons between these two organisms will focus on differences. Clinostomoid-type I metacercariae appeared as small, white subdermal cysts, while *C. marginatum* metacercariae encyst in larger, yellowish cysts. The vitellaria usually spanned from the hindbody to the ventral sucker, but, in some specimens, the vitellaria reached the oral sucker. In *C. marginatum*, the vitellaria reached only to the level of the ventral sucker (Osborn, 1912). The average length of *C. marginatum* metacercariae is 4.1 mm (Osborn, 1912) and 3.5-6.6 mm (Cort, 1913), while an average length of 3.7 mm was described in this study. The ventral sucker

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was reported to be 0.7 x 0.7 mm (Osborn, 1912), compared with 0.558 x 0.601 mm. The oral sucker was reported to be 0.28 x 0.25 mm (Osborn, 1912), compared with 0.22 x 0.23 mm in this study.

The molecular data was inconclusive in determining if clinostomoid-type I cercariae and metacercariae are conspecific with *C. marginatum*. While the 18S rRNA sequences did provide information at the genus level, suggesting that clinostomoid-type I cercariae and the resultant metacercariae may be members of the genus *Clinostomum* and that the strigeid-type II cercariae may be *Apharyngostrigea* sp., it did not provide information at the species level. The 18S rRNA gene region is highly conserved and is useful for determining phylogenetic relationships at higher levels of taxonomy, but not for determining species level distinctions (Nolan and Cribb, 2005). Therefore, these organisms cannot be accurately identified to species using this gene region. The genus *Clinostomum* is currently in a phylogenetic upheaval (Dzikowski et al., 2004), with researchers moving away from using the more conserved 18S rRNA gene region to less conserved regions, such as ITS and COI. The ITS1 + 5.8S + ITS2 and COI barcode gene regions are better for determining species-level distinctions (Moszczynska et al., 2009; Nolan and Cribb, 2005); however, attempts to amplify those genes in the present study were unsuccessful. The molecular differences between clinostomoid-type I and strigeid-type II cercariae confirm the morphological differences observed, providing further evidence that these two cercariae types represent two different species.

Strigeid-type II cercariae may belong to the genus *Apharyngostrigea*, based on 18S rRNA analysis. *Apharyngostrigea* spp. adults have been reported in great blue herons (*Ardea herodias*) in North Carolina (Flowers et al., 2004), great egrets (*Ardea alba*) in Florida (Sepulveda et al., 1999), and little blue herons (*Egretta caerulea*) in Texas

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(Dronen and Chen, 2002). Metacercariae have been reported in amphibians (Goldberg et al., 2001) and many fish, including the rainbow cichlid (*Herotilapia multispinosa*) in Nicaragua (Aguirre-Macedo et al., 2001). It has not been reported in channel catfish (*I. punctatus*). *Planorbella trivolvis* has not been reported as a host of *Apharyngostrigea* spp., but other planorbids have been reported as first intermediate hosts, including *Biomphalaria peregrine* in Brazil (Dias et al., 2002) and *Anisus contortus* in Russia (Ginetsinskaya, 1998).

This study has shown that two previously unreported cercariae inhabit some commercial catfish ponds. Clinostomoid-type I is infective to channel catfish, while strigeid-type II is not. The development of clinostomoid-type I metacercariae is relatively slow when compared to other digenetic trematodes that infect catfish. Infection by these metacercariae appear to have few direct pathogenic effects on the catfish host, and those effects are primarily caused by the presence of the metacercariae themselves. While these metacercariae share many characteristics with *C. marginatum*, some differences were noted. In clinostomoid-type I metacercariae, the cyst is white and smaller than those of *C. marginatum*, which are larger and yellow. The vitellaria in *C. marginatum* extends from the posterior end of the body to the level of the ventral sucker, while in clinostomoid-type I metacercariae, the vitellaria extend to the ventral sucker or to the oral sucker. Overall, the size of the body and most structures are smaller in clinostomoid-type I metacercariae, than in *C. marginatum*. Molecular analysis of clinostomoid-type I cercariae and metacercariae and strigeid-type II cercariae was inconclusive, so these organisms were not identified to species. Continued molecular research on these cercariae and metacercariae is necessary.

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Because clinostomoid-type I is a previously unreported parasite, many questions remain to be answered and investigations into the epidemiology and life history of this parasite need to be done. Now that it is known that these two unknown cercariae types are different species, more molecular and pathology studies need to be done to determine the species and confirm pathology. After a species-level identification has been made and confirmed molecularly, the prevalence of this parasite in commercial catfish production ponds needs to be determined. It is currently not known whether this parasite has been present in catfish at subclinical levels causing little pathology, or whether this parasite has been overlooked or misdiagnosed and may be contributing to subtle economic losses in the commercial catfish industry. In either case, all of the hosts in the life cycle of this parasite need to be identified so that management schemes can be developed to control this parasite.

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CHAPTER V

CONCLUSION

This research has provided further evidence that American white pelicans (*Pelecanus erythrorhynchos*) serve as definitive hosts for the channel catfish (*Ictalurus punctatus*) parasite, *Bolbophorus damnificus.* Double-crested cormorants (*Phalacrocorax auritus*), great blue herons (*Ardea herodias*), and great egrets (*Ardea alba*) were refractory to *B. damnificus* in natural and experimental infections, indicating that it is unlikely that they are natural definitive hosts for this parasite. Two studies were performed to make this determination. The first study was an experimental challenge (Study objective 1) to determine the potential of these birds to be definitive hosts and the second study was a helminthological survey (Study objective 2) of these birds to not only confirm natural infections of *Bolbophorus damnificus,* but also to determine the levels of infection.

In the first study, two American white pelicans, two double-crested cormorants, two great blue herons, and two great egrets were captured from channel catfish production intensive areas of the Mississippi Delta and treated with praziquantel to remove all existing trematode and cestode infections. They were fed channel catfish infected with *Bolbophorus damnificus* metacercariae. Patent infections of *B. damnificus*, which developed in both American white pelicans at 3 days post infection (dpi), were confirmed by the presence of trematode ova in the feces. Mature *B. damnificus* trematodes were recovered from the intestines of both pelicans at 21 dpi. No evidence of

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B. damnificus infections were observed in the other bird species studied. Using this information, catfish producers can focus their avian control efforts on the American white pelican when attempting to reduce the damage caused by *B. damnificus*, rather than targeting the other bird species.

In the second study, a total of 33 American white pelicans, 34 double-crested cormorants, 35 great blue herons, and 32 great egrets were collected monthly (January to March or April) for two years in areas of the Mississippi Delta with concentrated production ponds. The prevalence of this *B. damnificus* in American white pelicans was 93.9% (31/33 birds infected), with an average of 158 *B. damnificus* found per bird (range 0-681). *Bolbophorus damnificus* was not found in any of the other bird species sampled. This study provided further evidence, molecularly and morphologically, that the American white pelican is the only demonstrated host for *B. damnificus*. The *B. damnificus* prevalence data collected in this study suggests that the mean abundance of this parasite in a population of pelicans may increase over a season as they feed on *B. damnificus* infected catfish, contributing to further increases in the number of ova that are introduced into the pond system. This study demonstrated that not only are many of these birds heavily infected, but also these infections may persist in these birds from season to season, indicating that they are a constant source of *B. damnificus* infections in commercial catfish. This study provides further evidence of the importance of the implementation of persistent efforts to control American white pelicans on commercial catfish ponds.

The third study (Study objective 3) focused on two unknown cercariae types (clinostomoid-type I and strigeid-type II) that were infective to the rams-horn snail (*Planorbella trivolvis*). A series of challenge studies using specific pathogen-free (SPF)

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channel catfish that were exposed to both type I and type II cercariae were performed. The catfish were later necropsied at days 7, 14, 21, 31, 40, 42, 73, 80, 110, and 120 days post infection (dpi). The studies showed that type I was infective to channel catfish, while type II was not. Histologically, the first evidence of clinostomoid-type I metacercariae was at 14 dpi, but metacercariae could not be seen grossly until 21 dpi. The development of clinostomoid-type I metacercariae continued until the last termination date at 120 dpi, when both gross and histological analyses suggested that the metacercariae had reached maturation. The development of clinostomoid-type I metacercariae was relatively slow and caused little pathology in the catfish host. No evidence of infection was observed in catfish challenged with strigeid-type II cercariae in any of the studies. Molecular analysis of the 18S rRNA gene region suggested that the clinostomoid-type I cercariae and metacercariae may belong in the genus *Clinostomum*, and strigeid-type II cercariae may be a species of *Apharyngostrigea*. While this research confirms that there is another digenetic trematode infecting commercial catfish, there are still gaps in knowledge about its identification, pathology, and life cycle.

Overall, this research has shown that double-crested cormorants, great blue herons, and great egrets are incapable of transmitting *B. damnificus* to channel catfish and that a new trematode is capable of infecting channel catfish. Future research should focus on determining if the American white pelicans, double-crested cormorants, great blue herons, great egrets or other fish-eating birds inhabiting commercial catfish ponds are involved in the transmission of other digenetic trematodes, such as the newly described clinostomoid-type I species reported in this research. Further research is necessary to not only confirm the identification of this parasite, complete its life cycle, and study the pathology of this new clinostomoid species, but determine if there are other digeneans

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capable of infecting commercial catfish. This research and further studies such as these will provide commercial catfish producers with useful information that they can use to implement strategies in the control of these digeneans in commercial catfish ponds.

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