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The Study of the Life Cycle of Bolbophorus Damnificus and its Pathology in the Channel Catfish (Ictalurus Punctatus)

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THE STUDY OF THE LIFE CYCLE OF *BOLBOPHORUS DAMNIFICUS* AND ITS PATHOLOGY IN THE CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

By

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In channel catfish (*Ictalurus punctatus*), the digenetic trematode *Bolbophorus damnificus*, causes morality and reduced growth. Previous research has documented that the hosts for *B. damnificus* are: the American white pelican (*Pelecanus erythrorhynchos*), the Ram's horn snail (*Planorbella trivolvis*) and the channel catfish. The goals of this research were to confirm the life cycle of *B. damnificus* in a single life cycle study, determine if the snail *Biomphalaria havanensis* could serve as host for *B. damnificus*, and examine the pathology of *B. damnificus* in channel catfish.

American white pelicans (AWPE) were artificially infected with *B. damnificus* metacercariae which matured to the adult stage in four days and shed ova. The ova hatched in 12- 53 days, releasing miracidia which infected *P. trivolvis* and developed into *B. damnificus* cercariae which were shed in 23 days, used to infect catfish and matured into metacercariae in the superficial muscle in 23 days. Infected catfish were fed to

AWPE, and the metacercariae matured to patent adults in seven days and shed ova, thus completing the life cycle.

A second study was done to determine if the snail *B. havanensis* found in commercial catfish ponds, could serve as an intermediate host for *B. damnificus*. Parasite free *B. havanensis* exposed to *B. damnificus* ova shed cercariae that were molecularly identified as *B. damnificus*; confirming *B. havanensis* as a potential intermediate host for *B. damnificus*.

A third study examined the pathology associated with *B. damnificus* infections in channel catfish. Cercariae, confirmed by PCR to be *B. damnificus*, were used to infect fingerling catfish at 0, 25, 50, 100 and cercariae/ fish. The fish were euthanized 3, 4, 5 and 6 days post-infection, gross observations were noted and tissues were collected for histology. Mortalities of 20-100% occurred by day 6 post-infection in fish challenged with 200 cercariae. At day 6 post-infection, fish challenged with 100-200 cercariae had loss of hepatocyte vacoulation and lymphoid depletion in the spleen. Metacercariae were not only present in the subcutaneous muscle but were also in the dermis, behind the skull, within the muscular layers of the urinary bladder and around the heart.

DEDICATION

I would like to dedicate this research and dissertation to my grandparents Pommey, Pop Pop, Grandma, Papa and my Mississippi Pop Pop. They were not able to see this dream finally come to fruition, but I know they are looking down and smiling at me with pride.

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INTRODUCTION

Channel catfish industry

Commercial catfish production is the leading aquaculture industry in the United States, generating 482 million dollars (USDA 2006) and accounting for over 46% of the entire aquaculture industry (Tucker et al. 2004). Consumption of commercially farm-raised catfish (*Ictalurus punctatus*) has doubled since 1985 making catfish the fifth most popular fish in the United States (Robinson and Avery 2000). The largest number of catfish farms are located in the southeast United States, primarily in Alabama, Arkansas, Louisiana and Mississippi, with Mississippi producing 70% of the catfish in the US (Robinson and Avery 2000). Commercial catfish ponds occupy approximately 175,000 acres of land (Harvey 2005) with many farmers dependent on catfish as a source of income. In order to be economically competitive, intensive management practices have been implemented and high stocking densities of 5,000 to 10,000 fingerlings per acre are common (Robinson and Avery 2000). With these intensively managed operations, pathogens introduced into the pond by other fish or wildlife species can result in major fish losses.

As the catfish industry has expanded, the number of fish eating birds, including the American white pelican (*Pelecanus erythrorhynchos*, AWPE), great blue heron (*Ardea herodias*), great egret (*Ardea alba*) and double-crested cormorant (*Phalacrocorax uritus*), foraging on these ponds has also steadily risen. While feeding and loafing on the ponds, these birds have introduced several digenetic trematodes into the catfish population. Digenetic trematodes such as *Diplostomum spathaceum* and *Clinostomum complanatum* have traditionally been a concern for the catfish industry (Terhune et al. 2002). More recently the digenetic trematode, *Bolbophorus damnificus*, has been implicated in high mortalities resulting in severe economic losses for the commercial catfish industry (Overstreet et al. 2002).

Bolbophorus damnificus effects on channel catfish industry

Bolbophorus damnificus was initially isolated in farm-raised catfish in 1994 in Louisiana (Venable et al. 2000) and was first reported in Mississippi in 1999 (Avery et al. 2001). Economic losses have steadily risen due to deaths associated with *B. damnificus* infection. Approximately 1.5- 5.6% of the submissions to the Aquatic Diagnostic Laboratory at the Thad Cochran Warmwater Aquaculture Center in Stoneville, MS from 1999- 2002 were attributed to trematodes (Thad Cochran National Warmwater Aquaculture Center, 2004). The actual prevalence of infection in Mississippi may be much higher, as many losses go unreported once farmers learn to self-diagnose their fish. Since most farmers are aware there are no treatments for this parasite, farmers often fail

to submit trematode infected fish. However, even this low number reflects the significant negative economic impact of *B. damnificus* infections on catfish.

Natural infections of *B. damnificus* in channel catfish have been shown to damage the kidneys and liver of fish with younger fish being more susceptible to infection. In these infections, catfish had reduced feed intake and growth rates as well as small hemorrhagic or white raised cysts on all areas of the body, but often concentrated in the peduncle region. Distended abdomens and fluid filled body cavities often accompany severe infections in young fish and may mimic channel catfish virus disease or enteric septicemia of catfish (Avery et al. 2001). Older catfish may survive infection but are not marketable due to metacercariae cysts found under the skin and within the musculature.

In light infections, *Bolbophorus* spp. may not be the direct source of catfish mortality, but these infections may leave fish more susceptible to other pathogens. Catfish fingerlings challenged concomitantly with *Bolbophorus* spp. and *Edwardsiella ictaluri* had significantly higher mortalities than catfish challenged with *E. ictaluri* alone. However, fully developed *Bolbophorus* spp. metacercariae did not increase susceptibility to *E. ictaluri*. It has been hypothesized that the initial penetration site of the cercariae provides a portal of entry for bacterial and other opportunistic pathogens (Labrie et al. 2004).

Infections of *B. damnificus* have been shown to result in reduced fish weight per acre, thus negatively affecting net returns. A field study examined the economic impact of *Bolbophorus* spp. infection on a forty-acre commercial catfish farm (Wise et al. 2006). In this study, fish (20-30 per pond) from 40 production ponds were grossly examined for

metacercariae. Using a scoring system of light (1-33% fish with metacercariae), moderate (34-66% fish with metacercariae) and heavy (67-100% fish with metacercariae), researchers found 17 ponds with no trematodes, 6 ponds with light infections, 6 ponds with moderate infections and 11 ponds with heavy infections. The economic impact of *Bolbophorus* spp. infections was determined by correlating the number of ponds with these various levels of infection, revenue produced (pounds of fish produced, etc.) and variable costs (feed price, amount of feed used, etc.). Food consumption decreased with the increasing level of *Bolbophorus* infections. Fish in negative ponds consumed an average of 73.4 lbs/acre/day, while fish with light, moderate and heavy infections consumed 62.2 lbs/acre/day, 47.5 lbs/acre/day and 47.2 lbs/acre/day, respectively. Trematode infected ponds also produced fewer pounds of fish per acre than did the negative ponds. Light, moderate and heavy ponds produced 13.8%, 36.0% and 40.5% less pounds of fish per acre, respectively. There was also a corresponding decrease in profit in trematode infected ponds compared to negative ponds. Light infections had an 80.8% reduction in net returns whereas moderate and heavily infected ponds did not produce sufficient net returns to cover production costs. This study demonstrated the potential negative economic impact of *Bolbophorus* spp. infections, even in ponds with few infected fish and without the associated mortalities observed in severe outbreaks. Often losses due to mild to moderate infections, resulting from reduced feed conversion, are more subtle. These losses could go undetected for months but have as great or a greater economic impact than more severe infections.

Different species of *Bolbophorus*

Comparison of morphometric data indicates there are potentially three different species of *Bolbophorus*; *B. damnificus*, *B. confusus* (Fox 1965) and a third *Bolbophorus* sp. (Type 2; Table 1.1) Molecular data confirmed that *B. damnificus* and *Bolbophorus* sp. (Type 2) were different species. However, since there were no archived samples of *B. confusus* from the 1965 life cycle study (Fox 1965), molecular data was not available to confirm that *B. confusus* was a distinct species from *B. damnificus* and *Bolbophorus* sp. (Type 2). It was postulated that *B. confusus* is found only in Europe, that *B. damnificus* and *Bolbophorus* sp. (Type 2) are found in North America, and that the *B. confusus* reports in North America were actually *B. damnificus* (Overstreet et al. 2002). Current knowledge about the *B. damnificus* life cycle is based on morphological and molecular data on the different stages of *Bolbophorus* spp. collected in various artificial and natural infections rather than from a single well controlled life cycle study.

Table 1.1:

A comparison of morphometric differences between *Bolbophorus confusus*, *Bolbophorus damnificus* and *Bolbophorus* spp. (Type 2).

	Bolbophorus confusus	*Bolbophorus damnificus*	*Bolbophorus* spp. (Type 2)
Egg Size	115- 125 x 67- 82µm[a]	123-129 x 50- 89µm[c]	90- 102 x 55- 72µm[c]
Egg Development	12-13 days at 32.2˚C, 12- 15 days at 26.7- 29.4˚C, 16- 21 days at 21.1- 23.9˚C, 65 days at 20˚C[a]	*	*
Miracidium	150- 190 x 30- 40µm[a]	*	*
Cercariae Emergence	30- 34 days PI (21.1-23.9˚C)[a]	*	*
Cercariae Body Length	257 µm (232- 279 µm)[a]	186 µm (145- 200 µm)[d]	217 µm (196- 231 µm)[d]
Cercariae Tail Stem Length	280 µm (261- 309 µm)[a]	222 µm (208- 238µm)[d]	209 µm (178- 240µm)[d]
Cercariae Furcae Length	258 µm (247- 267 µm)[a]	201 µm (188- 213 µm)[d]	201 µm (165- 220µm)[d]
Cercariae Oral Sucker Length	50 µm[a]	46 µm (43- 49 µm)[d]	63 µm (59- 68 µm)[d]
Shedding Rate	1000 cercariae in 24 hours (21.1- 23.9˚C)[a]	*	*
Shedding Length	*	*	*
Metacercariae Length	2120 µm (1830- 2540 µm)[a]	1011- 1375 µm[c]	*
Metacercariae Width	860 µm (725- 930 µm)[a]	758- 1039 µm[c]	*
Adults present	3 days PI[a]	3 days PI[c]	*
Adult Length	2.53 mm (1.96- 3.25 mm)[a]	2.6- 3.2 mm[c] average legnth 2.3 mm[d]	average lengnth 1.02 mm[e]

[a]Fox, 1965, [b]Days post-infection, [c]Overstreet et al., 2002 , [d]Flowers et al., 2005, [e]Levy et al., 2002, *measurements unknown.

In order to confirm the molecular difference between these species, oligonucleotide primers that easily differentiated between *B. damnificus* and *Bolbophorus* sp. (Type 2) were developed. The single subunit rRNA from *B. damnificus* and *Bolbophorus* sp. (Type 2) was amplified using conserved trematode primers developed after examining trematode sequences in Genbank. The PCR products were sequenced, compared to trematode sequences in Genbank using BLAST, and species specific oligonucleotide primers were developed (Levy et al. 2002). These primers were used in PCR with several other trematodes that infect fish species with negative results proving they will not amplify other common trematodes. Amplicons produced from the primers for *B. damnificus* and *Bolbophorus* sp. (Type 2) are 820 kb and 420 kb, respectively.

Artificial and natural infections in the AWPE with *Bolbophorus* spp. demonstrated that, based on morphometric and molecular differences, there are several *Bolbophorus* spp. infecting these hosts (Overstreet et al. 2002). Adult trematodes morphologically identified as *Bolbophorus* in AWPEs fall into two different groups based on egg size and molecular data. Large (123-129 x 50-89 μm) and small trematode eggs (90-102 x 55-72 μm) are laid by the two different *Bolbophorus* species infecting AWPEs. Molecular analysis demonstrated that the gene sequence of the large eggs are identical to the gene sequence of metacercariae isolated from naturally infected catfish and identified as *B. damnificus*. Furthermore, the morphology of adults differ, with *B. damnificus* having vitellaria that does not reach the anterior of the ventral sucker and a vertically shaped tribocytic organ as compared to *Bolbophorus* sp. (Type 2) which has a circular triboctytic organ (Levy et al 2002; Overstreet et al. 2002).

Bolbophorus damnificus life cycle

Although it is confirmed that *B. damnificus* is prevalent in commercial catfish and causes significant economic losses to the catfish industry, there are still gaps in our understanding of this parasite. The first extensive life cycle studies pertaining to *Bolbophorus* species were in 1965 (Fox 1965). This initial work was done with *B. confusus*, which has been shown to be distinct from *B. damnificus* (Overstreet et al. 2002). Although Fox described *B. confusus*, it may in fact have been *B. damnificus*, but there are no archived samples to confirm this. While the identity of Fox's *Bolbophorus* sp. cannot be confirmed, information generated from his artificial life cycle studies with *B. confusus* can be used as a basis for the study of the *Bolbophorus* species found in catfish in the Southeast United States.

Fox (1965) laboratory studies with *Bolbophorus confusus*, the typical six life stages of digenetic trematodes were described; egg, miracidium, sporocyst, cercaria, metacercaria and adult. In these studies, *B. confusus* eggs were collected from fresh fecal material obtained from a naturally infected AWPE. The trematode eggs measured 119 µm x 72 µm (range 115 x 67 µm to 125 x 82 µm). Development of the embryo and hatching of the eggs was found to be temperature dependent. Eggs kept at 2- 4°C demonstrated no development; only a few eggs hatched in 65 days at 16-20 °C, with the remaining hatching in 16-21 days at 21-24 °C, 14-18 days at 24-27 °C, 12-15 days at 27-29 °C and in 12-13 days at 32 °C (Fox 1965).

Miracidia emerging from these eggs were 150-190 μm in length and 30-40 μm in width. They swam in a straight line for approximately 12 hours when held at 24-27 °C (75-80 °F) and it was hypothesized that contact with the snail intermediate host occurred by chance since miracidia did not appear to be phototrophic or stimulated by the presence of the snail (Fox 1965).

Fox (1965) demonstrated that *Planorbella trivolvis* served as the snail intermediate host for *B. confusus*. After exposure to miracidia, mother sporocysts and daughter sporocysts were found singly in the snail mantle. Numerous daughter sporocysts (n > 1000) were found encapsulating the digestive gland of the snail (Fox 1965).

Cercariae emerged from *P. trivolvis* 30-34 days after exposure of snails to miracidia at 21-24 °C. Most snails died 15-30 days after emergence of cercariae (Fox 1965). However, other studies showed that the rate of cercariae shedding from *P. trivolvis* decreased with decreasing temperatures. *Bolbophorus* spp infected *P. trivolvis* initially held at 23-25 °C then lowered to 15 °C showed a decreased cercaria shedding rate, but returned to the original shedding rate when temperatures were elevated to 25 °C (Terhune et. al 2002).

The level of infection and subsequent metacercariae development in fish were also shown to be temperature dependent. When rainbow trout were challenged with *B. confusus* at 9-12 °C, 13-18 °C or 19-29 °C the average number of metacercariae were 0.8 (range 0-6), 9.7 (range 1-28) and 35.9 (range 1-125), respectively (Olsen 1966).

Rainbow trout (*Oncorhynchus mykiss*) artificially exposed to cercariae developed metacercariae in the musculature three days post-exposure to cercariae; however *B.*

confusus was also found in the brain, mouth and behind the eyes of numerous fish species. Metacercariae were observed in rainbow trout artificially infected with *Bolbophorus confusus* cercariae seven to ten days post-infection when held at 21 °C. These metacercariae had a thin inner cyst wall, a second outer host cyst wall and petechial hemorrhaging was observed in the musculature of infected fish. The host cyst wall increased in thickness between 15 to 20 days post-infection and by 20 to 30 days post-infection the cyst containing the metacercariae developed a black pigmentation. Metacercariae were mature at 30-34 days post-infection (Fox 1965).

Bolbophorus damnificus

Bolbophorus damnificus metacercariae

Metacercariae of *B. damnificus* characteristically encyst under the dermis in the superficial muscle and are primarily located in the caudal dorsal regions with some present deeper in the muscle and on fins (Overstreet et al. 2002). Most *B. damnificus* metacercariae are elliptical in shape (Overstreet et al. 2002) and measure 1011- 1375µm x 758- 1039µm (Table 1.1). These metacercariae are enclosed in a double-walled cyst; the parasite derived cyst is transparent, surrounded by a milky white thicker walled cyst of host origin (Overstreet et al. 2002), and are grossly visible at seven days post-infection. At this time, stellate fibroblasts, myofiber fragments and eosinophilic debris can be seen microscopically surrounding the cyst. Fibroblasts begin to flatten around the cyst at 11 days post-infection and by 12 days post-infection, the characteristic thin

hyaline membrane surrounding the metacercariae is present. Thirteen to 18 days post-infection, the parasite capsule is highly organized with increasing amounts of collagen and decreasing numbers of fibroblasts present. Through the course of metacercarial development, no significant inflammatory response is evident; however, the presence of the metacercaria resulted in atrophy and coagulative necrosis of surrounding muscle fibers (Labrie et al. 2004).

***Bolbophorus damnificus* definitive host**

Recently, molecular data have verified the AWPE is a definitive host for *B. damnificus* (Levy et al. 2002; Overstreet et al. 2002; Table 1.2). The adults of *B. damnificus* and *B. confusus* are found in the anterior portion of the small intestine and are associated with the mucosa. In artificial infections with *B. confusus*, patent trematodes were present three days post-infection and survived for five months (Fox 1965). Similarly, artificial infections with *B. damnificus* have also resulted in patent adult trematodes at three days post-infection (Overstreet et al. 2002).

Table 1.2:

First, second and definitive hosts reported for *Bolbophorus* spp.

	B. damnificus	Type 2	*B. confusus*
First intermediate host	*Planorbella trivolvis*[a]	*Planorbella trivolvis*[a, b]	*Planorbella trivolvis*[e]
Second intermediate host	*Ictalurus punctatus*[b, c], *Pimephales promelas*[d]		Wide range of fish species[e]
Definitive host	*Pelecanus erythrorhynchos*[c]	*Pelecanus erythrorhynchos*[c]	*Pelecantus onocrotalus*[f]

[a]Flowers et al., 2005, [b]Levy et al., 2002, [c]Overstreet et al., 2002, [d]Mitchell et al., 2006, [e]Fox, 1965, [f]Dzikowski et al., 2003.

The limited *B. damnificus* studies indicate that the AWPE may be the only definitive host for this species. However, with the increasing number of pelicans and their migratory routes encompassing much of the areas of intensive catfish aquaculture in the US, the potential for spread of this parasite is significant. AWPEs have been reported in Mississippi, Louisiana, Texas (King and Michot 2002), Arkansas (King 1997), Nebraska (Sidle et al. 1990) and Georgia (Humphries 1986), with unusual sightings in Delaware (Hess 1986) and Indiana (Carey 1983). The largest breeding colony of AWPEs is at

Chase Lake, North Dakota (King 1997). Other breeding colonies are found in Wyoming (Findholt 1986, Findholt and Diem 1988), Oregon (Paulin et al. 1988), and during favorable years, small breeding colonies can be found in Montana and South Dakota (Sidle et al. 1985).

While the Brown Pelican (*Pelecanus occidentalis*, BRPE) is not a confirmed host, *B. confusus* was reported in a BRPE in Galvaston, Texas. However this species determination was based solely on morphological data (Dronen et al. 1999). Molecular analysis was not performed to confirm the species and samples were not archived, thus this record is in question. Brown pelicans are commonly found in Florida (Nesbitt et al. 2002) and may serve as the definitive host in that area. Both brown and white pelicans are found in North Carolina; however, they are usually present in coastal areas, and are therefore less likely to visit aquaculture farms located more inland (J. Flowers, North Carolina State University, personal communication).

***Bolbophorus* spp.**

***Bolbophorus* spp. fish hosts**

A broad range of fish hosts have been reported for *Bolbophorus* spp. Natural infections have been reported in mountain whitefish (*Prosopium williamsoni*), steelhead (*Salmo gairdneri*), brown trout (*Salmo trutta*), longnose sucker (*Catostomus catostomus*), white sucker (*Catostomus commersoni*) (Olsen 1964), artic grayling (*Thymallus arcticus*), Utah chub (*Gila atraria*) (Fox 1965), northern pike (*Esox lucius*), common

bream (*Abramis brama*), white-eye bream (*Abramis sapa*), rudd (*Scardinius erythrophthalmus*) (Dubois 1938), white bream (*Blicca bjoerkna*), leaping grey mullet (*Mugil saliens*), ide (*Leuciscus idus*), European perch (*Perca fluviatilis*) (Ciurea 1930), yellow perch (*Perca flavescens*), brook stickelback (*Culaea inconstans*) (Holloway and Hagstrom 1981) and fathead minnow (*Pimephales promelas*) (Mitchell et al. 2006). Artificial infections demonstrated that brook trout (*Salcelinus fontinalis*), fathead chub (*Hybopsis gracilis*), longnose dace (*Rhinichthys balteatus*), bluehead mountain sucker (*Pantosteus platyrhynchus*), channel catfish (*Ictalurus punctatus*), mosquitofish (*Gambusia affinis*), bluegill (*Lepomis macrochirus*), mottled sculpin (*Cottus bairdi*) (Olsen 1966), Northern squawfish (*Ptychocheilus oregonensis*), redside shiner (*Richardsonius balteatus*) and guppy (*Lebistes reticulates*) (Fox 1965) could be infected. Development of metacercariae was evident in all fish after exposure to cercariae, indicating *Bolbophorus* spp. have a wide range of fish hosts. However, with the exception of the specimens found in *Pimephales promelas* (Mitchell et al. 2006), metacercariae were identified only by morphology. None of these metacercariae were available for molecular analysis to confirm specific identity. Consequently, without the molecular data to confirm the species of *Bolbophorus* reported in these various fish hosts, there is current debate about their specific identity (Overstreet et al. 2002).

Bolbophorus sp. (Type 2) does not appear to infect the channel catfish, but has proven lethal to guppies (*Poecilia reticulata*), green swordtails (*Xiphophorus helleri*), hybrid striped bass (striped bass *Morone saxatilis* x white bass *Morone chrysops*) and mosquitofish (*Gambusia affinis*) in artificial infections. Mortality in these fish was

observed before the complete maturation of metacercariae (Levy et al. 2002; Overstreet and Curran 2004) indicating that these fish may not represent natural second intermediate hosts for *Bolbophorus* sp. (Type 2).

Bolbophorus spp. mollusk hosts

The range of mollusk species that can serve as intermediate hosts for *B. damnificus* is not well studied. The only confirmed first intermediate host for this trematode is the Ram's horn snail, *Planorbella trivolvis* (Overstreet et al. 2002). Although *P. trivolvis* is the only reported snail host for *B. damnificus* (Fox 1965), there are several other snail species present in catfish ponds. *Planorbella trivolvis* and *Physa gyrina* are both routinely found in catfish ponds and an exotic snail, *Biomphalaria havanensis* has recently been reported in the Mississippi Delta (Yost et al. 2008). The time of introduction and prevalence of this species into the Mississippi Delta is currently unknown. The potential for the spread of *B. damnificus* is present in other parts of the United States where pelicans and *Planorbella* species are present. Areas of concern include North Carolina where catfish and hybrid striped bass ponds are abundant with large populations of *P. trivolvis* and *Planorbella duryi* snails and pelican populations (J. Flowers, North Carolina State University, personal communication). Another potential snail host, *Planorbella trivolvis subcrenatum* is found in areas of North Dakota that serve as breeding grounds to AWPEs during the summer months.

Although *P. gyrina* and *Biomphalaria* spp. have, as yet, not been confirmed as intermediate hosts in the *B. damnificus* life cycle, they are hosts to several digenetic

trematodes. *Physa gyrina* has been shown to be naturally infected with the digenetic trematodes *Halipegus eccentricus*, *Haematoloechus complexus*, *Glypthelmins quieta*, *Echinostoma trivolvis*, *Strigeiid spp.* and *Megalodiscus temperatus* (Snyder and Esch 1993), *Diplostomulum spathaceum* (Hendrickson 1978) and experimentally infected with *Ornithodiplostomum ptychocheilus* (Hendrickson 1986), *Cyclocoelum vanelli* (Taft 1974), *Posthodiplostomum minimum* (Palmieri 1976) *Ophthalmophagus singularis* (Taft 1986), *Echinostoma trivolvis* (Beaver 1937) and *Gigantobilharzia huronensis* (Sauer et al. 1975). The only above listed trematode that infects catfish is *D. spathaceum* which infects the lens of the eye (Overstreet and Curran 2004).

Biomphalaria havanensis, type locality in Cuba (Young et al. 1997), is also found in Mexico, Central America, the Antilles region (Malek 1985), the Dominican Republic (Malek 1969), and Southern Idaho (Bowler and Frest 1992). It is an intermediate host for the medically important trematode *Schistosoma mansoni* (Cram et al. 1945; Michelson 1976). A very similar, if not identical mollusk, *Biomphalaria obstructa* is also an intermediate host for *S. mansoni* and has been documented in South Carolina (Dillon and Dutra-Clarke 1992) and Louisiana (Malek 1969). *Biomphalaria obstructa* has also been found in California, however this strain of *B. obstructa* was refractory to infection with *S. mansoni* (Basch et al. 1975). *Biomphalaria havanensis* and *B. obstructa* can not be differentiated by shell characteristics, instead, various parts of the genitalia must be examined. While molecular data is being generated for this snail species, the taxonomy remains unclear and it is often referred to in the literature as *Biomphalaria havanensis* complex (Durand et al. 1998).

Planorbella trivolvis is not only an intermediate host for *B. damnificus* and *Bolbophorus* sp. (Type 2), but can serve as an intermediate host for *Echinostoma trivolvis* (Pennsylvania strain of *P. trivolvis*) (Anderson and Fried 1987; Fried 1985; Fried et al. 1987), *Ophthalmophagus singularis* (Taft 1986), *Alaria marcinanae* (Johnson 1968), *Cephalogonimus brevicirrus* (Brooks and Welch 1976), *Cyclocoelum vanelli* (Taft 1974), *Cyclocoelum oculeum* (Taft 1972), *Cyclocoelum mutabile* (McKindsey and McLaughlin 1995) and is a competent second intermediate host for *Echinostoma coalitum* (Krull 1935) and *Echinostoma trivolvis* (Colorado *P. trivolvis* strain; Schmidt and Fried 1996). *Planorbella trivolvis* has been found naturally infected with *Cephalogonimus vesicaudus* (Dronen and Underwood 1977), *Cephalogonimus salamandrus* (Dronen and Lang 1974), *Uvulifer ambloplitis* (Lemly and Esch 1984), *Petasiger nitidus* (Shostak 1993), *Alloglossidium macrobdellensis* (Corkum 1975) *Haplometrana intestinalis* (Current 1975) and *Echinostoma trivolvis* (Beaver 1937). The presence of these trematode species in catfish ponds and their impact on the epidemiology of *B. damnificus* has not been documented.

***Bolbophorus damnificus* control**

In order to interrupt the life cycle of *B. damnificus*, one solution would be to control the bird host. American white pelicans not only introduce *B. damnificus* ova into commercial catfish farms in the Delta, but also consume large amounts of catfish from ponds (Glahn and King 2004). Harassment strategies include pyrotechnics, live ammunition, propane gas exploders, and bird distress calls (Mott and Brunson 1997).

Even though these techniques are often used by Delta catfish farmers, some are more effective than others. In addition, bird harassment is expensive, costing on average $7,400.00 per year per farm (Stickley and Andrews 1989). These techniques also become less effective over time as birds habituate to control measures (Mott and Brunson 1997).

A more effective and practical control for *Bolbophorus* spp. is through elimination of the snail host. Several chemicals have been tested for efficacy and various biological controls have also been attempted. A mixture of 589 g copper sulfate and 58.9 g citric acid applied along 10 m of the pond perimeter in a 2 m swath has proved lethal to snails in vegetation around the pond perimeter. Using this treatment, snail survival was 2.2% and 0% compared with sham treatment survival of 63.3% and 77.8% (Mitchell 2002).

Increased levels of copper sulfate administered to an entire catfish pond is effective in killing *P. trivolvis* and causes minimal damage to catfish. In a field study comparing the efficacy of application of copper sulfate applied at 2.5 and 5.0 ppm to 0.25-acre and10-acre experimental catfish ponds, resulted in mortalities of 92% to 100%. Fish mortality was observed in the 0.25- acre ponds, but no mortality occurred in the 10-acre ponds. Fish mortality may have occurred in fish confined to a small area before the chemical was completely dissolved. This study was completed in the fall when temperatures were low (20-24 °C) and algae bloom density was below the average found in summer months. Therefore this treatment would need to be evaluated at different temperatures and alkalinities before use to ensure copper sulfate toxicity did not occur in resident catfish during treatment (Wise et al. 2005).

Hydrated lime, used dry or as a slurry, is also an effective molluscicide, but can only be used in well buffered waters (total alkalinity > 50 ppm) and as a spray 3-4 feet from the pond margin. Application of dry hydrated lime to ponds has been shown to be partially effective when used at a rate of 50 lbs. every 75-100 feet of pond bank or as a slurry, at 4.0-4.7 lbs. of lime per gallon of water and applied to the pond margin at a rate of 20 gallons per 100 feet of levee (Avery et al. 2001).

Natural products, such as vulgrone B from the *Artemisia douglasiana* plant and steam-distilled oil from the *Erigeron speciosus* plant, also have molluscicidal activity against *P. trivolvis*. Although these compounds are lethal to *P. trivolvis* snails, research on fish and mammalian toxicity must be completed before they can be used in commercial catfish ponds (Meepagala et al. 2002; Meepagala et al. 2004).

Biological control has also been attempted to eliminate snails. Black carp (*Mylopharyngodon piceus*) are mollusk- eating cyprinids that have been used as mollusk control in Israel and Russia and more recently in commercial catfish ponds in Mississippi. Ponds averaging 4.48 ha stocked with 62 black carp have been shown to reduce snail densities. None of the fish in these ponds experienced any trematode associated mortality, whereas ponds without black carp experienced substantial trematode associated mortality (Venable et al. 2000). Laboratory experiments have also been undertaken to compare the efficacy of native fish species in the control of snail populations with the black carp. Triploid black carp, redear sunfish (*Lepomis microlophus*) and blue catfish (*Ictalurus furcatus*) were placed in aquaria with Rams-horn snails to compare the number of snails consumed by the different fish species. Black carp

consumed the most snails (86%), whereas redear sunfish and blue catfish consumed 46.7% and 26.7%, respectively, indicating that native fish were not as effective as the exotic black carp in reducing the snail population (Ledford and Kelly 2006). Field studies with these more desirable native fish species must be undertaken before they are ruled out as potential biological control agents.

Attempts have also been made to control snails by increasing pond salinity. Field studies in 4.54 ha ponds examined salinities of 2.5 ppt, 1.25 ppt and 0.25 ppt. These experiments demonstrated that 2.5 ppt salinity inhibited snail growth, survival, reproduction and suppressed snail egg development (Venable et al. 2000).

Conclusions

Bolbophorus damnificus has been extensively studied since its appearance in the Mississippi Delta, but significant gaps in our knowledge pertaining to this parasite remain. It is confirmed that *P. trivolvis* serves as a first intermediate host in the life cycle, but it is not known if any of the other mollusk species present in catfish ponds can also serve as first intermediate hosts. It is now established that this parasite can cause mortalities and morbidity in the fish second intermediate host, but the mechanisms associated with this pathology are poorly understood. Several studies have examined the effects of mixed *Bolbophorus* spp. infection but pathology associated with pure *B. damnificus* infections has not been documented. With the recent development of *B. damnificus* specific PCR primers (Levy et al. 2002), it is now possible to perform pathology studies with pure *B. damnificus* challenges. Moreover, these primers can also

be used to study all of the life stages in each host of *B. damnificus* in a single life cycle study thus confirming the life cycle of the species and fulfilling Koch's postulates.

Currently, there is no existing data on each life stage of *B. damnificus* or *Bolbophorus* (Type 2) in a single artificial infection study. This would aid in distintinguishing one species from the other and confirm each life stage morphologically and molecularly. Using the primers developed by Levy et al. (2002) it is now possible to challenge different fish species with pure cultures of different *Bolbophorus* spp. This will aid in determining the fish host range of *Bolbophorus* spp. Presently there is little known about the penetration of this parasite into the fish host, the pathology associated with each of the subsequent life stages in pure *B. damnificus* infections, and the final formation of the double-layered cysts.

Although the life cycle of *B. damnificus* has been completed and the adult, metacercariae, and cercariae stages have been described (Levy et al. 2002; Overstreet et al. 2002), these findings were not based on a single life cycle study in which each life stage was described. In addition, questions still remain pertaining to the intermediate and definitive host range and the pathology associated with this parasite in the catfish host.

Objectives

The goal of this research was to use morphometrics and molecular analysis to document each *Bolbophorus damnificus* life stage in a single life cycle study, examine other potential snail hosts, and describe the pathology of this parasite as it develops in the catfish. The main objectives were: 1) document the life cycle of *B. damnificus* in a single

life cycle study, 2) determine if *B. havanensis* can serve as an intermediate host for *B. damnificus*, and 3) determine the pathology of *B. damnificus* in channel catfish.

To achieve the first objective, parasite-free AWPEs, Ram's horn snails and channel catfish were identified. In a single study, AWPEs were infected with *B. damnificus* metacercariae, patent *B. damnificus* occurred and the adults shed trematode ova. The ova hatched releasing miracidia that infected *P. trivolvis*. Cercariae developed, were shed from the snail and infected *I. punctatus*. Mature metacercariae developed and were used to infect parasite-free AWPEs completing the *B. damnificus* life cycle. Each life stage was photographed and molecular analysis performed to confirm the species identity.

The second objective was based on finding the exotic snail, *B. havanensis*, in Mississippi catfish ponds. These snails were kept in the laboratory and confirmed to be free of *B. damnificus* infection. *Bolbophorus damnificus* ova were introduced into an aquarium with snails and snails were monitored for *B. damnificus* cercariae shedding.

To achieve the third objective, specific pathogen free channel catfish fingerlings were infected with varying numbers of *B. damnificus* cercariae. Samples of fish were euthanized at designated days post-infection, tissues were collected for routine histological preparation and examined microscopically for pathological changes.

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CHAPTER II

CONFIRMATION OF ALL LIFE STAGES IN THE *BOLBOPHORUS DAMNIFICUS* LIFE CYCLE USING MOLECULAR AND MORPHOMETRIC ANALYSIS

Abstract

A single life cycle study was done to confirm each life stage of *Bolbophorus damnificus* and compare it to the known life cycle of *Bolbophorus confusus*. American white pelicans (*Pelecanus erythrorhynchos*) were infected with *B. damnificus* metacercariae from infected channel catfish (*Ictalurus punctatus*). Patent *B. damnificus* infections were established four days post-infection; ova were collected and used to infect the snail host, *Planorbella trivolvis*. Ova hatched and miracidia emerged in 12 days at 30.0 °C and 53 days at ambient temperatures ranging from 13.5 to 35.7 °C. Snails began shedding cercariae 23-32 days post-exposure to the *B. damnificus* miracidia, with a shedding rate ranging from 0-2,547 cercariae per snail per day. These cercariae were used to infect parasite-free channel catfish. Metacercariae from these infected catfish were fed to trematode negative pelicans and patent infections were established at seven days post-infection. Birds were necropsied and *B. damnificus* were collected from the small intestine, completing the life cycle. Each life stage of this parasite was confirmed to be *B. damnificus* morphologically and using molecular techniques. This research confirmed each life stage of *B. damnificus* in a single infection using molecular analysis; provided

further morphological data on each life stage; and contributed additional information on the events occurring in this life cycle.

Introduction

As the catfish industry has expanded, the number of fish-eating birds such as the American white pelican (*Pelecanus erythrorhynchos*), great blue heron (*Ardea herodias*), great egret (*Ardea Alba*) and double-crested cormorant (*Phalacrocorax auritus*) foraging on these ponds has steadily risen (Glahn and King 2004). While feeding and loafing on the ponds, these birds have introduced several digenetic trematodes into the catfish population. Digenetic trematodes such as *Diplostomulum spathaceum* and *Clinostomum complanatum* have been a source of concern in the catfish industry for many years. However, recently the digenetic trematode, *Bolbophorus damnificus*, has been implicated as a cause of high mortalities in commercial catfish resulting in severe economic losses (Overstreet et al. 2002; Wise et al. 2006).

Bolbophorus damnificus was first isolated in farm-raised catfish in 1994 in Louisiana (Venable et al. 2000) and first reported in Mississippi in 1999 (Avery et al. 2001). Economic losses have steadily risen due to deaths associated with *B. damnificus*. Between 1.5-5.6% of the diagnostic cases submitted to the Aquatic Diagnostic Laboratory at the Thad Cochran Warmwater Aquaculture Center in Stoneville, MS from 1999-2002 were attributed to trematodes (Thad Cochran National Warmwater Aquaculture Center). Although the incidence has appeared to decline in recent years, many losses go unreported once farmers become familiar with signs of disease and begin

to diagnose their own fish. Incidence rates determined from diagnostic trematode submissions also do not account for production losses in unreported subclinical infections. Economic losses are also incurred as pond surveillance, pond treatment and snail control become increasingly common in affected ponds.

The first extensive life cycle studies pertaining to *Bolbophorus* species were conducted in 1965 (Fox 1965). In this initial work, Fox (1965) identified this species as *Bolbophorus confusus* which differs from the recent descriptions of *B. damnificus* morphologically, molecularly, and in the size of ova (Overstreet et al. 2002). Fox (1965) described *B. confusus* in his life cycle studies; however, there are no archived samples to confirm identification by molecular methods. It has been assumed that the data generated from Fox's artificial life cycle studies with *B. confusus* would be similar to the life cycle of the *Bolbophorus* species reported in catfish the southeast United States.

A more recent study in which American white pelicans (AWPE) were artificially infected with commercial catfish naturally infected with *B. damnificus* metacercariae resulted in patent *B. damnificus* adults which were confirmed molecularly (Overstreet et al. 2002). Molecular data from several studies have also confirmed that the snail *Planorbella trivolvis* is a host for *B. damnificus* and *Bolbophorus* sp. (Type 2) (Levy et al. 2002). When channel catfish were exposed to cercariae shed by these snails, the resulting metacercariae were identified molecularly as *B. damnificus*. Catfish have been found naturally infected with *B. damnificus* (Overstreet et al. 2002) and an artificial infection of catfish with *Bolbophorus*-like cercariae produced metacercariae confirmed by PCR to be *B. damnificus* (Labrie et al. 2004). Although there have been several studies

confirming the separate life stages in the *B. damnificus* life cycle, there have been no studies to date in which the *B. damnificus* life cycle was completed in a single infection with all three hosts, fulfilling Koch's postulate. This study describes each life stage in the *B. damnificus* life cycle in a continuous single infection in the bird host, *P. erythrorhynchos*, the snail, and the channel catfish confirming all of the life stages molecularly and using morphometrics.

Materials and methods

American white pelicans (AWPE)

Four adult AWPE were live captured southwest of Lake Chicot in Chicot Co, Arkansas. Pelicans were restrained using modified padded leghold traps (King et al. 1998) and transferred to Mississippi State University, College of Veterinary Medicine. Birds were weighed and housed in outdoor avian facilities specifically designed for long-term avian studies at the Mississippi Field Station of the National Wildlife Research Center, Mississippi State University, Mississippi (Glahn et al. 2000).

During the 44 day acclimation period (February 19, 2003- March 12, 2003), AWPE were individually housed in pens (304.8 cm x 304.8 cm x 182.9 cm) with 200 L diving pools. The pools were equipped with ramps for perching and provided continuous accessibility to water with a recirculating water system containing bio-filters and particulate waste filters. A misting system was supplied for pelicans during the summer

months which was a sprinkler attached to the top of the pen angled down, with a misting option set on a timer during the daylight period.

At the start of the study, fresh fecal material was collected from each to calculate the number of eggs shed per gram of feces per day (EPG) per pelican. After each collection the pens were thoroughly washed. The first fecal collection was prior to the praziquantel treatment on study day 0 (Table 2.1) and then again on study day 4 and daily thereafter for the duration of the trial. Fecals were processed daily using a modified sedimentation procedure to detect trematode ova (Foreyt 2001). Total fresh feces from each pelican were collected, individually mixed, a subsample was weighed (0.5 g), placed in a conical test tube and a 1% soap solution was added to a volume of ten mL. The fecal material and soap solution were mixed thoroughly and allowed to sit for five minutes then decanted without disturbing the pellet. This wash procedure was repeated until the sample was sufficiently clear. Reverse osmosis water was added after the final decant to a total volume of 10 mL. To enumerate eggs, a 1 mL aliquot was added to a Petri dish with grid lines 3 mm apart, diluted with reverse osmosis water and all ova were counted. Daily EPGs were calculated using the following formula:

$$[(\text{eggs in 1 mL})\ (10\ \text{mL})/\ (\text{weight (0.5 g) of feces}) \times 2]$$

Table 2.1:

Daily log of the *Bolbophorus damnificus* life cycle study

Event	Study day	Month	[A]Pelican Fecals (EPG)			
			AWPE 1	AWPE 2	AWPE 3	AWPE 4
Praziquantel treatment.	0	13-Mar	2060	300	160	40
	1		[B]NT	NT	NT	NT
	2		NT	NT	NT	NT
	3		NT	NT	NT	NT
	4		0	0	0	0
	5		0	0	0	0
	6		0	0	0	0
	7		0	0	0	0
AWPEs 1-2 given *B. damnificus* infected fish, AWPE 3 given metacercariae.	8			0	0	0
	9		0	0	0	0
	10		0	0	0	0
	11		0	0	0	0
Positive EPG (4 days post-infection).	12		23020	2080	20	0
	13		820	80	420	0
	14		3500	700	696	0
AWPEs placed on experimental ponds.	15		400	1580	220	0
	16		1180	2500	120	0
	17		[C]NM	680	280	0
	18		1460	NM	180	0
AWPEs taken off experimental ponds.	19	1-Apr	320	300	0	0
Gathered eggs for temperature studies from AWPE 3.	20		NM	10880	3000	0
	21		120	40	40	0
Eggs placed in experimental tank 3.	22		26320	20	0	0
Began looking at outside tank 3 eggs from AWPE 3 for developmental changes.	23		2280	0	100	0
	24		[D]W	W	W	W
	25		3060	1000	20	0
	26		260	100	0	0
AWPE 1, 2 and 4 fecal collection terminated.	27		1800	80	60	0

Table 2.1 (Continued)

	28		NT	NT	0	NT
	29		NT	NT	20	NT
	30		NT	NT	NM	NT
	31		NT	NT	1060	NT
	32		NT	NT	0	NT
	33		NT	NT	NM	NT
	34		NT	NT	0	NT
	35		NT	NT	0	NT
	36		NT	NT	200	NT
	37		NT	NT	160	NT
	38		NT	NT	0	NT
	39		NT	NT	200	NT
	40		NT	NT	20	NT
	41		NT	NT	60	NT
	42		NT	NT	W	NT
	43		NT	NT	20	NT
	44		NT	NT	0	NT
	45		NT	NT	20	NT
	46		NT	NT	0	NT
	47		NT	NT	60	NT
Added snails (n=30) to experimental tanks 2 and 3.	48		NT	NT	220	NT
	49	1-May	NT	NT	20	NT
	50		NT	NT	60	NT
	51		NT	NT	320	NT
	52		NT	NT	0	NT
	53		NT	NT	20	NT
	54		NT	NT	0	NT
	55		NT	NT	0	NT
	56		NT	NT	180	NT
	57		NT	NT	20	NT
	58		NT	NT	NM	NT
	59		NT	NT	260	NT
	60		NT	NT	40	NT
	61		NT	NT	40	NT
	62		NT	NT	0	NT
	63		NT	NT	0	NT
	64		NT	NT	0	NT
	65		NT	NT	0	NT
	66		NT	NT	0	NT
	67		NT	NT	0	NT

Table 2.1 (Continued)

Miracidium observed. Began checking snails in experimental tanks 2 and 3 for cercariae.	73		NT	NT	NT	NT
Miracidium observed. Began checking snails in experimental tanks 2 and 3 for cercariae.	75		NT	NT	NT	NT
	80	1-Jun	NT	NT	NT	NT
Snails in experimental tank 3 began shedding (21 days post- ova hatch). Artificial fish challenge 1.	96		NT	NT	NT	NT
Snails in experimental tank 2 began shedding (27 days post- ova hatch.	100		NT	NT	NT	NT
Challenge 1 (experimental tank 3) fish began dying.	104		NT	NT	NT	NT
	110	1-Jul	NT	NT	NT	NT
Challenge 1 (experimental tank 2) fish began dying.	113		NT	NT	NT	NT
Artificial fish challenge 2.	117		NT	NT	NT	NT
Challenge 2 (experimental tank 3) fish began dying.	130		NT	NT	NT	NT
Challenge 2 (experimental tank 2) fish began dying.	133		NT	NT	NT	NT
	141	1-Aug	NT	NT	NT	NT
Artificial fish challenge 3.	145		NT	NT	NT	NT
Challenge fish examined for metacercariae.	165		NT	NT	NT	NT
Pelican 2 given praziquantel.	166		NT	NT	NT	NT
	167		NT	0	NT	NT
Pelican 2 fed 30 artificially infected fish.	168		NT	0	NT	NT
	169		NT	NT	NT	NT
	170		NT	NT	NT	NT
	171		NT	0	NT	NT
	172	1-Sep	NT	0	NT	NT
	173		NT	0	NT	NT
	174		NT	0	NT	NT
	175		NT	16	NT	NT
Pelican 2 necropsied.	176		NT		NT	NT
Pelican 3 given praziquantel.	179		NT		0	NT
	180		NT		0	NT
	181		NT		0	NT

Table 2.1 (Continued)

	182		NT		0	NT
Pelican 3 fed 12 infected fish.	183		NT		NM	NT
	184		NT		NT	NT
	185		NT		NT	NT
	186		NT		0	NT
	187		NT		0	NT
	188		NT		0	NT
	189		NT		0	NT
	190		NT		6	NT
	191		NT		220	NT
	192		NT		W	NT
	193		NT		0	NT
	194		NT		NM	NT
Pelican 3 necropsied.	195		NT			NT

[A]Number of ova per 1.0 g of fecal material from AWPEs. [B]NT= no fecal samples taken, [C]NM= no fecal material available, [D]W= fecal samples could not be obtained due to inclement weather.

Praziquantel treatment

Pelicans were treated orally with a single dose of praziquantel (Droncit® 34, Bayer Corporation, Shawnee Mission, Kansas 66201), at 30 mg/ kg body weight to eliminate all trematodes (study day 0; Tables 2.1 and 2.2). Praziquantel tablets were placed in gel capsules and AWPEs were gavaged using manual manipulation to ensure passage down the esophagus into the crop. The gavage was followed with a water flush into the esophagus and the birds were observed for several hours to ensure the capsules were not regurgitated.

Table 2.2:

Major events in ova and cercariae study in outdoor experimental ponds

Event	Date	Study Date	Water Temp (°C)
Praziquantel treatment.	13-Mar	0	[A]NT
AWPEs Given infected fish.	21-Mar	8	9.0
EPG positive.	25-Mar	12	NT
AWPEs on experimental ponds.	28-Mar	15	19.3
AWPEs taken off experimental ponds.	1-Apr	19	17.8
AWPE 1 and 2 ova collected.	2-Apr	20	19.3
Ova placed in container in AWPE experimental pond.	4-Apr	22	21.5
Snails introduced.	30-Apr	48	21.5
Miracidium observed.	25-May	73	22
Begin snail/ cercariae checking.	27-May	75	22.3
Cercariae present.	17-Jun	96	26.6
Fish challenge 1.	17-Jun	96	26.6
Fish challenge 2.	8-Jul	117	28.2
Snail checking ends.	31-Jul	140	25.1
Fish challenge 3.	5-Aug	145	25

[A]NT= water temperature not obtained.

Artificial infection

Prior to the infection of AWPEs, metacercariae were collected from channel catfish from a commercial catfish pond in Mississippi (Leflore County) that had experienced a trematode outbreak. A subsample of ten catfish from the naturally infected population were sacrificed on study day 7 to confirm the trematode infections were *Bolbophorus* sp. morphologically (Figure 2.1) and molecularly with PCR (Levy et al 2002). Twenty-four hours later (study day 8), fish (n= 51) were sacrificed using ethyl 3-aminobenzoate methanesulfonate salt (Sigma, St. Louis, Missouri). Fish weight (g) and length (cm) measurements were made for each fish and *Bolbophorus* sp. metacercariae were excised and enumerated. The metacercariae were excised from the outer host cyst, ensuring each metacercariae remained intact in the parasite-derived inner cyst wall. Metacercariae were morphologically identified and placed live in physiological saline. Random subsamples of metacercariae were placed in 70% ethanol for molecular identification and staining to confirm they were *B. damnificus*.

On the same day (study day 8; Tables 2.1 and 2.2), all 4 AWPEs were challenged with infected fish or excised metacercariae, AWPEs 1 and 2 were each given *B. damnificus* positive channel catfish (n = 50 per bird) from the same fish population and allowed time to consume all fish (approximately 2 hours). Pelicans were observed after the 2 hours to confirm all catfish were consumed and checked again 24 hours later to confirm catfish had not been regurgitated.

Pelican 3 was intubated and given 179 excised metacercariae that had been excised from the same fish population used to challenge AWPEs 1 and 2. A subsample of

these metacercaria was kept for later staining and PCR. Pelican 3 was intubated using a silicone gastric duodenal Levine tube (Jorgensen Labs Inc. Loveland, Colorado). The tube was inserted into the esophagus past the trachea and the metacercariae suspended in physiological saline were introduced into the tube followed with a physiological saline wash using a sterile disposable pipette. Additional physiological saline was added to the top of the Levine tube using a 60 mL syringe to ensure metacercariae were washed down the length of the tube, the tube was removed and visually inspected to confirm no metacercariae were left inside the tube. Pelican 4 was intubated and given an unknown Diplostome-type metacercariae (n= 84) that had been found in this same population of fish but appeared morphologically different from *B. damnificus* metacercariae.

Beginning on study day 0, all AWPEs were checked daily and observations were recorded in a daily log. Prior to and after the fish challenge (study day 8), each AWPE was fed ± 1000 g of SPF fish per day until the termination of the trial. Air and water temperatures were measured and recorded daily from study days 15 to 175.

***Bolbophorus damnificus* ova study**

Pelicans (1- 4) were moved to experimental cages with diving pools seven days post-infection (study day 15; Tables 2.1 and 2.2). The experimental cages were identical to those previously described except the water in the diving pool was not flow-through. Pelicans remained in these experimental cages for four days, were allowed to freely defecate into the water tanks, then were returned to their original pens (study day 19;

Tables 2.1- 2.2). Experimental ponds used in the subsequent studies with snails and catfish corresponded to AWPEs 1- 4 and were designated experimental ponds 1-4.

In order to compare trematode egg development in field simulated ambient temperatures with egg development in a controlled temperature of 30.0 °C, trematode eggs were collected from the fecal material of AWPE 3, 24 hours after it had been returned to its original pen (study day 20; Tables 2.1- 2.2). The eggs were observed for developmental changes every 48 hours beginning 72 hours post-collection (study day 23) for 52 days (study day 75). To accomplish this, ova were collected using a sedimentation procedure on fecal material collected from AWPE 3. Ova were concentrated and 150 were placed in a cylindrical plastic container, suspended in the water of experimental tank 3 so the same ova could be observed over time. Ova were brought back to the lab every 48 hours, 10 ova were randomly selected, microscopically examined using an Olympus BX60F5 (Tokyo, Japan) with Image Pro-Plus software. Changes were documented by visual inspection and were recorded photograpically.

***Bolbophorus damnificus* cercariae study**

Planorbella trivolvis used in the study were offspring of parasite-negative snails laboratory-reared at the CVM-MSU snail facility. To further confirm the snail population was negative, juvenile to adult snails (5-10 mm) were randomly selected for testing. Snails (n= 9) were placed in test tubes containing 3 mL of sterile spring water and checked daily for 10 days to ensure there was no shedding of cercariae. After 10 days, all

snails were sacrificed and PCR was performed to confirm they were negative for any trematode life stages.

Snails (n= 30/ tank) from the confirmed negative population were introduced into experimental tanks 2 and 3 (AWPEs defecated in for 4 days at the beginning of the study) at day 40 post-infection of the AWPEs using a specifically designed container to house the snails (study day 48, Tables 2.1- 2.2). The housing consisted of PVC pipe (15.24 across x 101.6 cm long) placed upright in the middle of each tank. Each PVC pipe had large rectangular windows (21.1 x 67.6 cm) with a mesh screen (aperture size 1.53 mm) which allowed the free flow of water, miracidia and cercariae through the pipe into the surrounding water. Four snails were randomly selected three times weekly and checked for cercarial shedding. Snails were measured with digital calipers and placed in a 15 mL test tube with three mL of sterile spring water. The water was checked daily for the presence of cercariae for two days using a compound microscope (Olympus 2412PS-12W-B30). To enumerate cercariae, the contents of the test tube were thoroughly mixed and three aliquots of 0.1 mL each were added to three individuals wells of a concave microscope slide. Cercariae in each individual aliquot was counted and the following formula used to calculate total number of cercariae shed from each individual snail:

Average of 3 counts x 10 x 3 mls= Total cercariae shed in 24 hours

Cercariae collected from the snails were confirmed to be *B. damnificus* morphologically and then stored in 70% molecular grade ethanol for later molecular analysis. After snails were checked for two days, four new snails were taken out of the

PVC pipes and snails that had been previously observed were placed back in the PVC containers.

Bolbophorus damnificus metacercariae studies

To obtain *B. damnificus* positive fish, specific pathogen free catfish (approximately 12.7 cm) obtained from the Mississippi State University College of Veterinary Medicine hatchery were introduced into the outside experimental tanks 2 and 3 containing snails shedding confirmed *B. damnificus* cercariae from the cercariae study. Challenges 1, 2 and 3 began on study days 96, 117 and 145, respectively (Tables 2.1 and 2.3). Fish infected during challenge 1 (n= 30 in each tank) were left in experimental tanks 2 and 3 throughout the challenge (n= 22 days), fish in challenge 2 (n= 20 in each tank) were exposed for three hours and fish in challenge 3 (n= 40 in tank 2, n= 20 in tank 3) were exposed for one hour. Following challenges 2 and 3, all catfish were removed and placed in two flow-through tanks (8.33 L, dechlorinated charcoal filtered water) at the MSU- CVM corresponding to the experimental tank numbers designated at the time of challenge (experimental tanks 2 and 3). Fish were checked daily and all mortalities recorded. Fish were fed a standard floating feed ad libitum. All fish in challenge 1 and 85- 90% in challenge 2 died prior to metacercariae development (Table 2.3) Subsequently, fish from challenge 3 were used for all metacercariae descriptions and bird infection

Table 2.3

Fish exposed to *Planorbella trivolvis* snails shedding *Bolbophorus damnificus* cercariae: Challenges 1-3

Challenge Number	Tank Number	Study Date Fish Added	Fish Added Number	Cercariae Range/ Snail	Exposure Time	Water Temperature at Challenge (°C)	Water Temperture Range (°C)	Fish Mortality Start Date	Dead %	Mortality End Date	Dead %	Mean number of Metacercariae
1	2	96	30	0- 1330	22 days	26.6	22.8-29.5	17 days PI (study day 113)	30%	22 days PI (study day 118)	100%	7
1	3	96	30	0- 373	22 days	26.6	23.7	8 days PI (study day 104)	6.70%	34 days PI (study day 130)	100%	23
2	2	117	20	0- 1533	3 hours	28.2	N/ A	16 days PI (study day 133)	10%	19 days PI (study day 136)	85%	10
2	3	117	20	0- 240	3 hours	28.2	N/ A	13 days PI (study day 130)	5%	19 days PI (study day 136)	90%	3
2		145	40	Unknown	1 hour	25	N/ A	N/ A	0	N/ A	0	0.86
3		145	20	Unknown	1 hour	25	N/ A	N/ A	0	N/ A	0	Unknown

Twenty days post-exposure to cercariae (study day 165; Appendix D.3), seven artificially infected catfish were sacrificed from challenge 3. All viable metacercariae were excised, counted, and the average number of metacercariae per fish recorded. A subsample of metacercariae were saved for further morphological and molecular analysis.

Pelican reinfection

Pelicans 2 and 3 were selected to be challenged with fish from challenge 3. Pelican 2 was treated with praziquantel on study day 166, following the procedure outlined previously (Tables 2.1 and 2.4. To confirm AWPE 2 was negative for *B. damnificus*, feces were collected and EPG's were performed at 24 and 48 hours post-treatment and daily thereafter until the termination of the trial (study day 176). At 48 hours post-treatment (study day 168), AWPE 2 was fed live infected catfish (n= 30) as previously described using fish from challenge 3 (tank 2). These fish were a subset of the 40 that had been artificially infected with cercariae from the *B. damnificus* metacercariae studies (challenge 3, tank 2) 23 days previously (Tables 2.1, 2.3 and 2.4).

Table 2.4

A summary of events of AWPE 2 and 3 *Bolbophorus damnificus* reinfection.

	Event	Date	Study Date
Pelican 2	Praziquantel treatment	26-Aug	166
	Fish challenge	28-Aug	168
	EPG collected	26-Aug-4/Sep	171-175
	First positive EPGs (7 days post-infection)	4-Sep	175
	Necropsy date	5-Sep	176
Pelican 3	Praziquantel treatment	8-Sep	179
	Fish challenge	12-Sep	183
	EPG collected	13-Sep-23-Sep	186-193
	First positive EPGs (7 days post-infection)	19-Sep	190
	Necropsy date	24-Sep	195

Pelican 3 was retreated with praziquantel (study day 179) following the previously described protocol and EPGs were checked at 24 hours post-treatment and daily thereafter until the termination of the trial (study day 195). At 4 days post-treatment, AWPE 3 was fed live infected fish (n= 12; study day 183). These fish were 12 of the 20 fish that were artificially infected with cercariae from the *B. damnificus* metacercariae studies (challenge 3, tank 3) 38 days previously

Confirmation of patent infection

Pelicans 2 and 3 were necropsied 8 and 12 days post-reinfection, respectively (study days 176 and 195; Table 2.1), and all *B. damnificus* were collected using the fecal sedimentation procedure described previously. Pelicans were humanely euthanized with carbon dioxide (American Veterinary Medical Association), intestinal tracts were removed, opened longitudinally and all intestinal contents were collected and gently screened with distilled water through a sieve (0.75 μm aperture). Contents retained in the sieve were examined microscopically using a dissecting microscope and all parasites were collected for molecular analysis or staining.

Ova development laboratory study

One hundred and fifty eggs were collected from the feces of AWPE 2 and 4 to compare ova development at a controlled water temperature (30.0° C). Ova were isolated using the previously described fecal sedimentation procedure. Fifteen eggs each were placed in each of three wells of a 96-well plate and incubated at a constant temperature of

30.0° C. Eggs were examined using a confocal microscope (Lieca TCS NT, Bannockburn, Illinois) on days 1- 4, 8, 10, 12 and 15 and all observed developmental changes were photographed. For the field of view, the microscope was set to 10 X plain and for individual eggs, the microscope was set to 10 X zoom 4. Data were compared with development of ova at ambient temperatures.

***Planorbella trivolvis* infection laboratory study**

To compare cercarial shedding in snails infected at a constant water temperature of 24-27 °C with those at ambient water temperatures in the *B. damnificus* cercariae study, 30 parasite-negative *P. trivolvis* housed in a 95 L aquarium were exposed to *B. damnificus* ova collected from AWPE 2 (Table 2.1). Snails were housed at the snail colony at Mississippi State University College of Veterinary Medicine. Feces (15.1 g) collected from AWPE 2, containing approximately 2,114 ova (140 EPG x 15.1 g of feces), were added to the aquarium containing the snails. Ten days post-exposure, four snails were removed from the aquarium, measured and individually placed in test tubes with three mL of sterile spring water. Tubes were examined microscopically using an Olympus model 2412PS-12W-B30 for cercariae at 24, 48 and 72 hours. Cercariae were enumerated as previously described and stored in 70% molecular grade ethanol. After two to three days, four new snails were removed from the tank and the previously checked snails were returned to the appropriate tanks. This continued for the duration of the study (84 days post-exposure).

Mo**rphometric analysis of parasite life stages**

All *B. damnificus* from the small intestinal tracts of AWPEs 2 and 3 were directly transferred to 70% molecular grade ethanol until further molecular confirmation or heat fixed for staining. Specimens were stained using a modified technique as described by Pritchard and Kruse (1982). Specimens were regressively stained in acetocarmine, destained in acid alcohol (99 parts 70% ethanol, one part 1% HCl), then progressively dehydrated in an alcohol series, cleared in CitriSolv (Fisherbrand, Fisher Scientific, Fair Lawn, New Jersey), and mounted in permount.

Molecular analysis

A subsample of each life stage was collected and preserved in 70% molecular grade ethanol for molecular analysis. Samples were placed individually in microfuge tubes with 300 µL cell lysis solution and 1.5 µL proteinase K. DNA was extracted using the Puregene DNA purification kit (Gentra Systems, Minneapolis, Minnesota) following the manufacturer's protocol, except half reactions were performed. Genomic DNA from eggs were rehydrated in 25 µl hydration solution and genomic DNA from all other life stages were rehydrated in 50 µl hydration solution. *Bolbophorus damnificus* species-specific primers, P1-650F (5'-TCA GTT TCG AAC GAT GAT GA-3') and P1-14700R (5'-CGG TCT ACG GTT CCA CC-3') were used (Levy et al. 2002). Reaction volumes for PCR were 25 µL using 10X buffer, 2.0 µL template, 0.625 U *Taq* polymerase (Hot Start Taq; Takara Bio, Inc., Shiga, Japan), 200 nM of each primer and 200 µM of each deoxynucleotide triphosphate. Polymerase chain reaction conditions were a modification

of those described by Levy et al. (2002) Reaction times were shortened to 5.0 minutes at 92 ˚C, followed by 35 cycles of 94 ˚C for 1.0 minutes, 58 ˚C for 1.0 minute, 72 ˚C for 1.0 minute, and a final cycle of 72 ˚C for 5.0 minutes using a PTC-100 Peltier Thermal Cycler (MJ Research, Minnesota). PCR products and positive and negative controls were visualized on a 1.2 % agarose gel. The gel was post-stained with gelstar nucleic acid stain (Cambrex BioScience Rockland, Inc) and observed under ultraviolet light.

Results

Artificial metacercariae challenge of AWPEs

The subsample of ten naturally infected catfish (5.5 cm-7.7 cm) used to confirm the presence of *B. damnificus* averaged 8.4 (range: 0-20) metacercariae per fish (MPF; Table 2.5). Only one fish had no metacercariae (Appendix A.1). Molecular (n= 14) and morphologic analysis of the excised metacercariae confirmed these trematodes to be *B. damnificus* (Appendix A.1; Figure 2.1). Staining of the unknown diplostome-type metacercariae also present in this same fish population had morphological characteristics of a *Hysteromorpha* sp. (Figure 2.2).

Table 2.5:

Subsample of *Bolbophorus damnificus* and *Hysteromorpha* sp. metacercariae used for artificial infection

Metacercariae Number	Study Day	Length (µm)	Width (µm)
B. damnificus 1	7	1686.6	550.4
B. damnificus 2	7	1583.4	547.6
B. damnificus 3	7	1536.8	615.2
Hysteromorpha sp. 1	7	727.6	409.2
Hysteromorpha sp. *2*	7	811.1	499.9
Hysteromorpha sp. 3	7	722.1	443.4
Hysteromorpha sp. 4	7	761.4	391.4

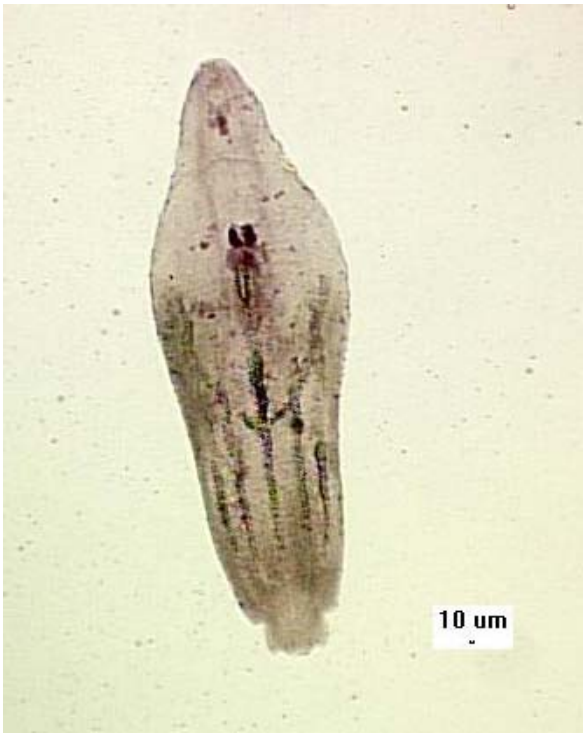


Figure 2.1:

Bolbophorus damnificus metacercariae from subsample of catfish used for artificial infection

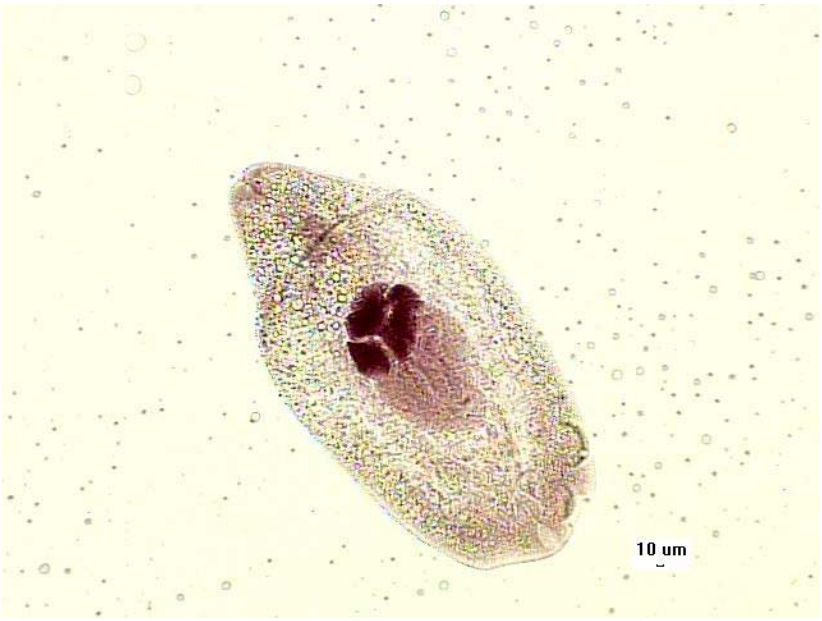


Figure 2.2:

Hysteromorpha-like metacercariae from subsample of catfish used for artificial infection

Catfish (n= 52) from the same naturally infected stock had an average of 8.4 MPF (range: 0-31) with three fish with no metacercariae (Appendix A.2). In 24 hours, AWPE 1 ate 50 infected catfish with an estimated 420 metacercariae (50 fish x 8.4 metacercariae) and AWPE 2 ate 48 fish with an estimated 403 metacercariae (48 fish x 8.4 metacercariae).

American White Pelican daily EPGs

On the day of praziquantel treatment (study day 0) EPGs for pelicans 1- 4 were 2060, 300, 160 and 40, respectively (Table 2.1; Figures 2.3-2.5). Pelicans were negative at four to seven days post-treatment, and remained negative until four days post-challenge (study day 12). At this time, AWPEs 1-3 began shedding ova (Figures 2.3-2.5),

while the EPG for AWPE 4 (given the diplostome-type metacercariae) was negative and remained negative for the duration of the trial. Pelican 1 shed ova continuously (except for study days 17, 20 and 24 when samples were not available) until fecal sampling was terminated, 19 days post-challenge (study day 28; Figure 2.3). The highest EPG (26,320 EPG) was 14 days post-infection (study day 22; Figure 2.3, Table 2.1). Pelican 2 EPGs were positive until the trial termination, 19 days post-challenge, except for a negative EPG on day 15 post-challenge (study day 23; Figure 2.4) and on study days when fecal material was not available (study days 18 and 24). The highest EPG (10,880 EPG) was 12 days post-infection (study day 20; Table 2.1, Figure 2.4). During this same period, AWPE 3 had positive EPGs (Figure 2.5) except for those days when samples could not be collected (study days 19, 26 and 24). Similar to AWPE 2, the highest EPG (3,000 EPG) in AWPE 3 was on day 12 post-infection (study day 20; Table 2.1, Figure 2.5). At 19 days post-infection (study day 27) fecal collection was discontinued for AWPE 1, 2 and 4 but was continued for AWPE 3 until 59 days post-infection (study day 67). During this time, intermittent shedding was observed in AWPE 3 (Figure 2.5; Table 2.1) with 19 days of negative EPGs (study days 19, 22, 26, 28, 32, 34, 35, 38, 44, 46, 52, 54, 55, 62, 63, 64, 65, 66 and 67). No samples were available on study days 30, 33 and 58. After 6 days of consecutive negative EPGs (59 days post-infection), fecal sampling was discontinued.

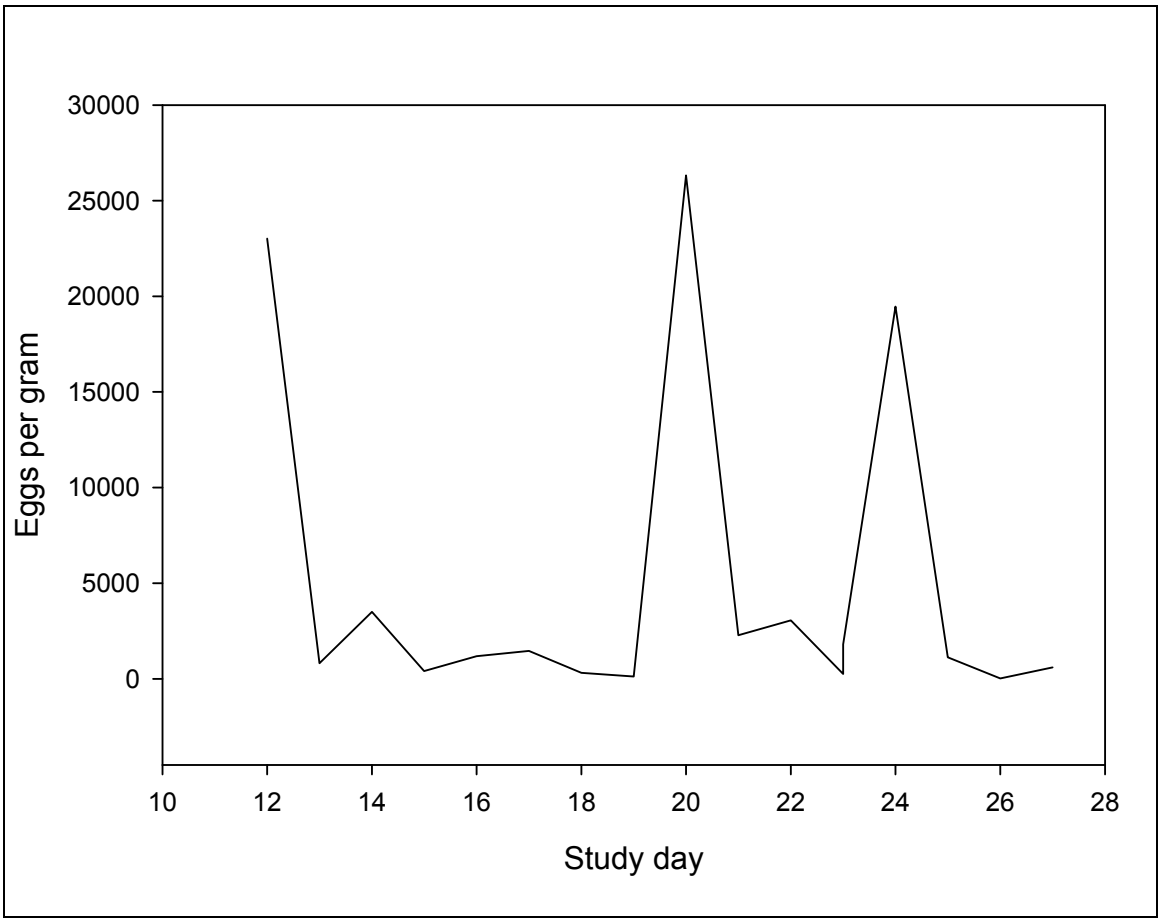


Figure 2.3:

Number of eggs shed per 1.0 gram of feces from AWPE 1 after initial *Bolbophorus damnificus* infection

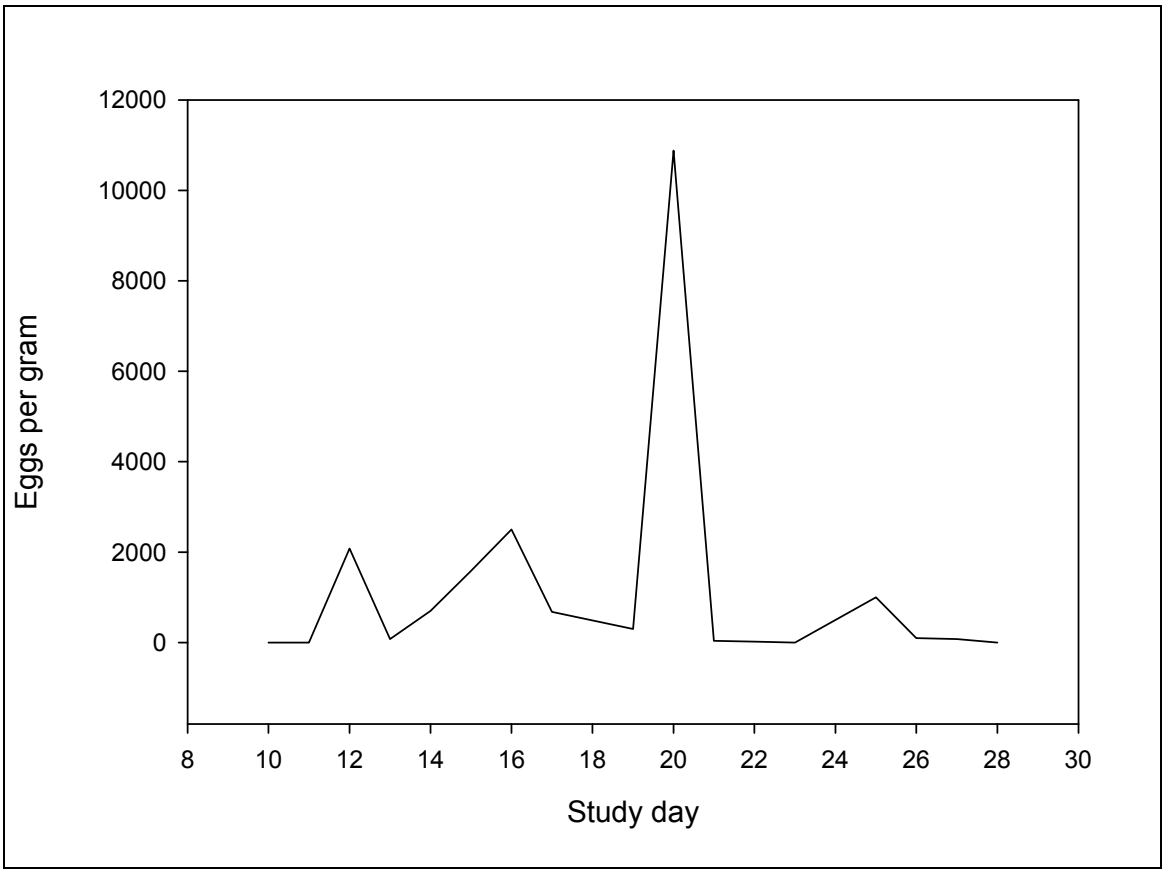


Figure 2.4:

Number of eggs shed per 1.0 gram of feces from AWPE 2 after initial *Bolbophorus damnificus* infection.

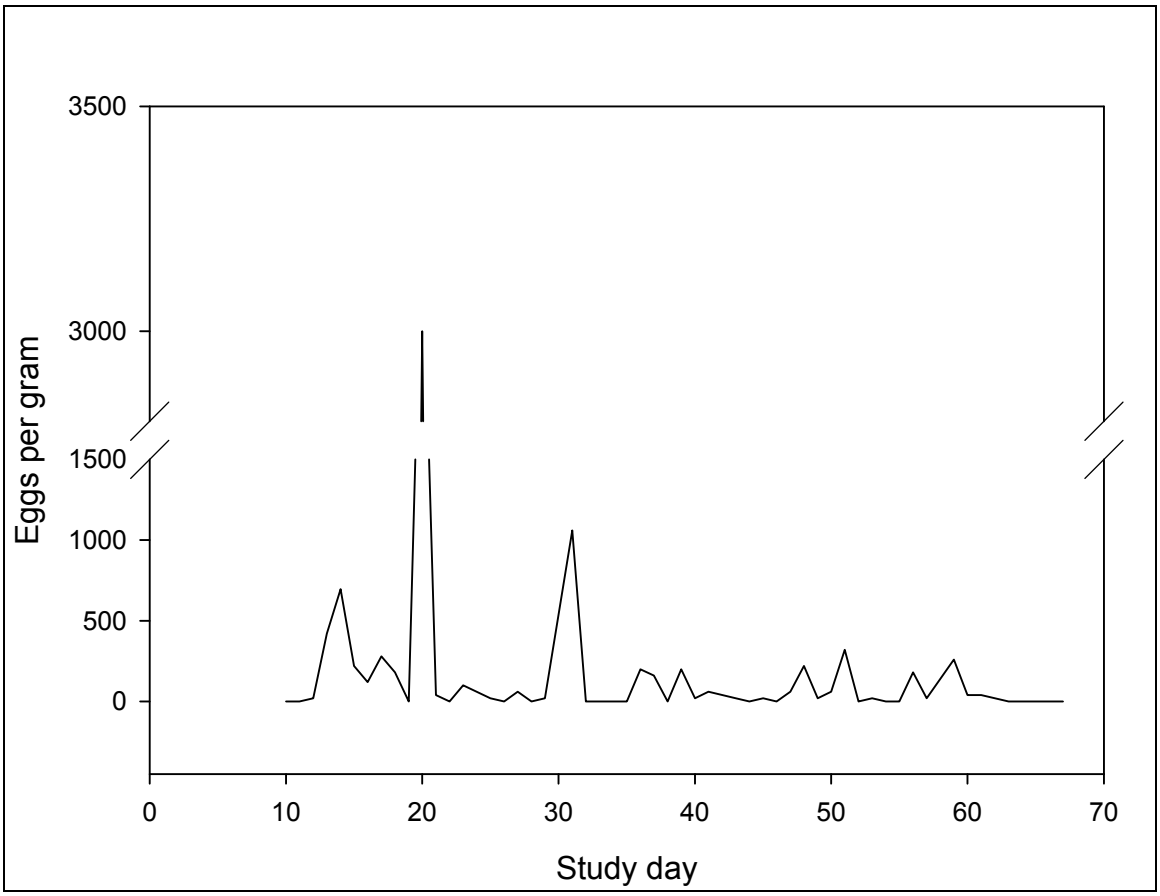
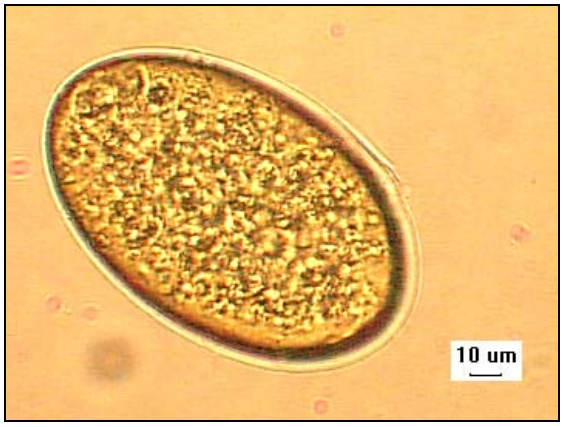


Figure 2.5:

Number of eggs shed per 1.0 gram of feces from AWPE 3 after initial *B. damnificus* infection.

Bolbophorus damnificus ova description

Bolbophorus damnificus eggs held for observation in experimental tank 3 had a yellow pigment (Figures 2.6- 2.22) and an average egg size (n= 8) of 119.0 µm x 72.0 µm (range: 104-126 µm x 44-79 µm; Appendix A.3) at the start of the study. Hatched eggs (Figures 2.14, 2.16 and 2.17) were observed at days 57, 59 and 61 post-collection (study days 77, 79 and 81) with an average egg size of 112.4 µm x 74.3 µm (range: 109.4 µm-115.9 µm x 65.8 µm-78.7 µm; Appendix A.4).



Figures 2.6-2.22:

Bolbophorus damnificus eggs and miracidium from experimental pond 3.

Fig. 2.6:

Three days after collection of *Bolbophorus damnificus* eggs (study day 23).

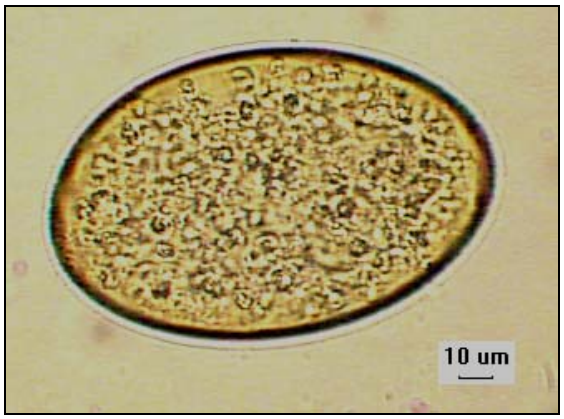


Fig. 2.7:

Five days after collection of *Bolbophorus damnificus* eggs (study day 25).

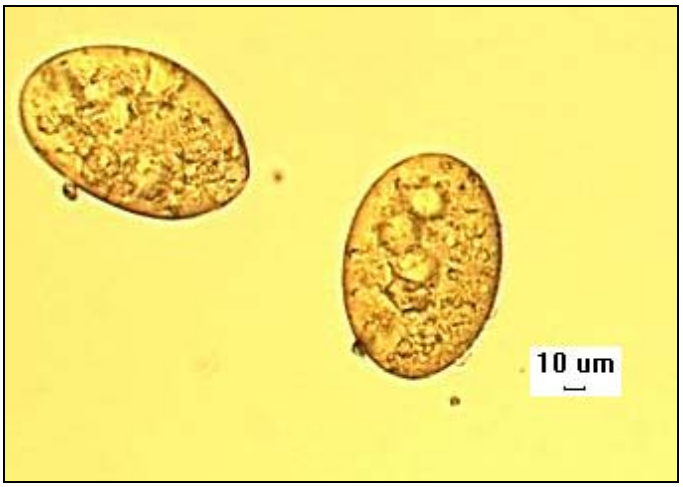


Fig. 2.8:

Six days after collection of *Bolbophorus damnificus* eggs (study day 26).

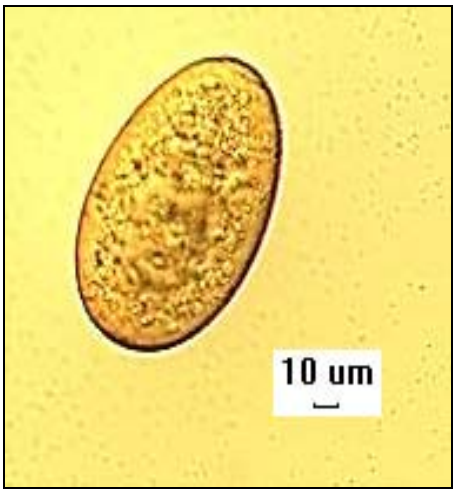


Fig. 2.9:

Eight days after collection of *Bolbophorus damnificus* eggs (study day 28).

Fig. 2.10:

Ten days after collection of *Bolbophorus damnificus* eggs (study day 30).

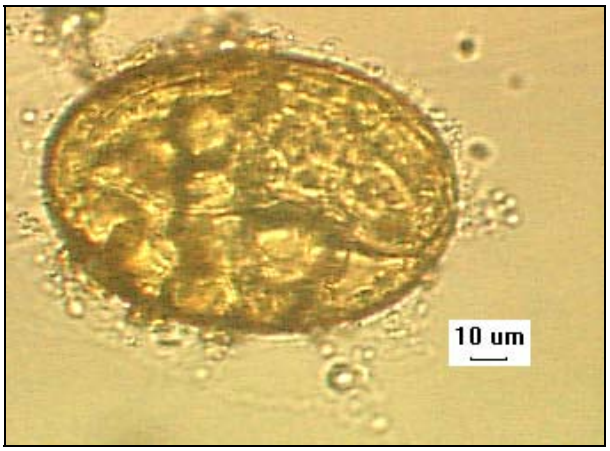


Fig. 2.11:

Forty-five days after collection of *Bolbophorus damnificus* eggs (study day 65).

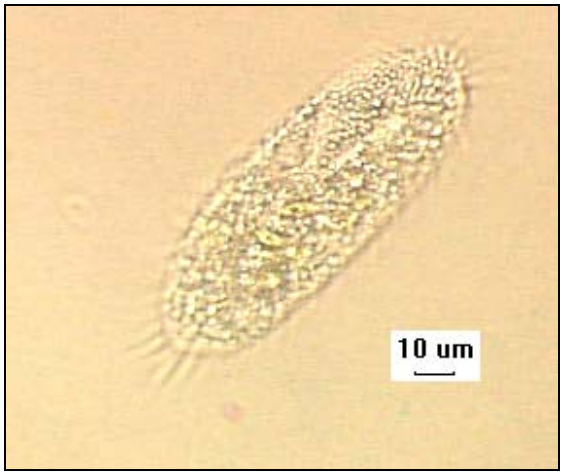


Fig. 2.12:

Miracidia observed fifty-three days after collection of *Bolbophorus damnificus* eggs (study day 73).

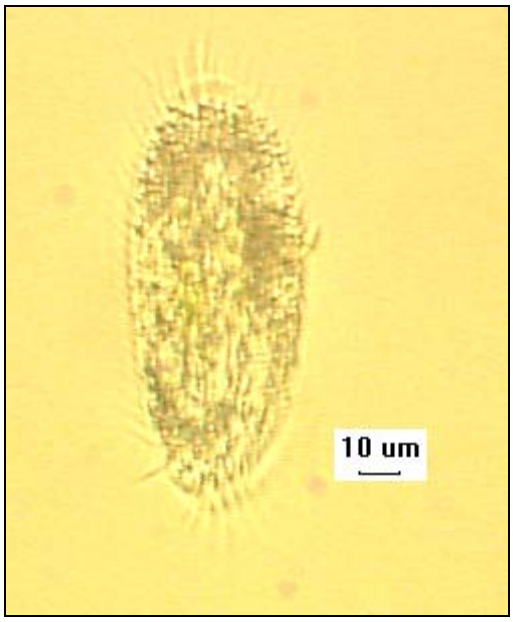


Fig. 2.13:

Miracidia observed fifty-five days after collection of *Bolbophorus damnificus* eggs (study day 75).

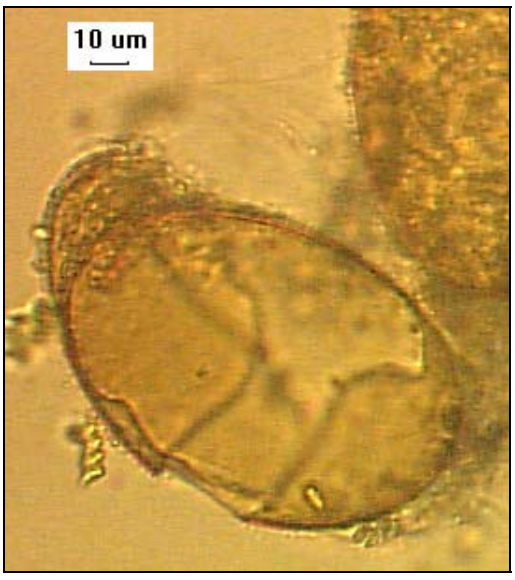


Fig. 2.14:

Hatched egg observed fifty-seven days after collection of *Bolbophorus damnificus* eggs (study day 77).

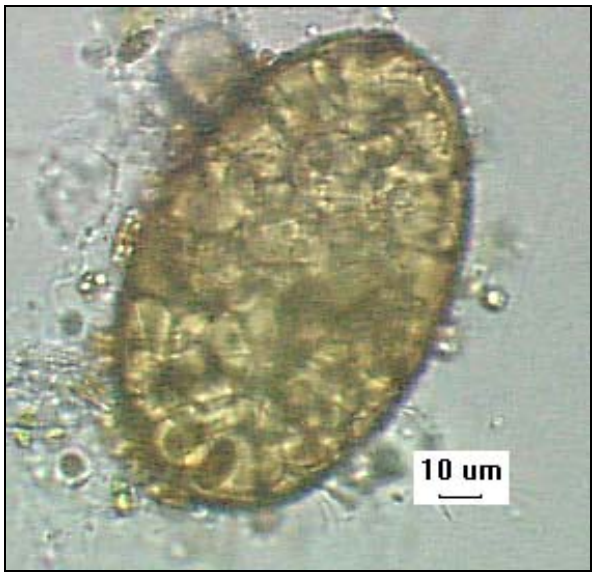


Fig. 2.15.

Fifty-nine days after collection of *Bolbophorus damnificus* eggs (study day 79).

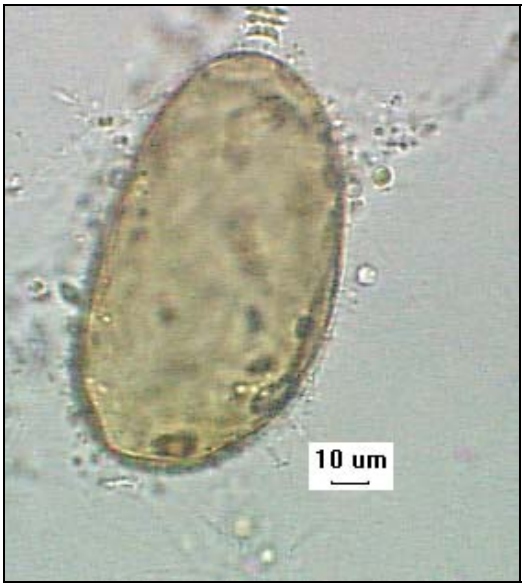


Fig. 2.16:

Hatched egg observed fifty-nine days after collection of *Bolbophorus damnificus* eggs (study day 79).

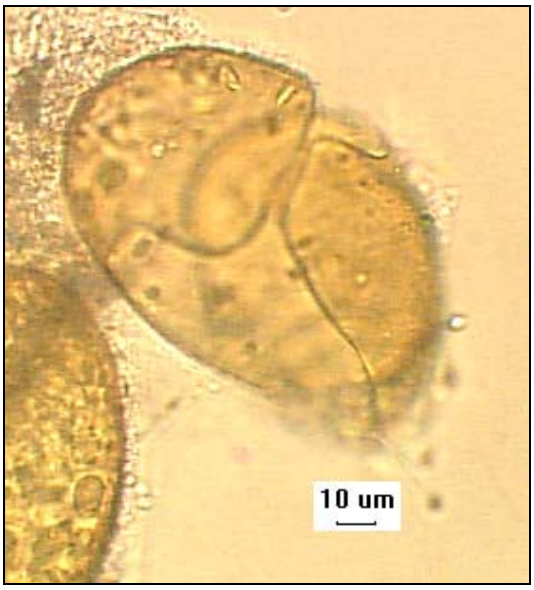


Fig. 2.17:

Hatched egg observed sixty-one days after collection of *Bolbophorus damnificus* eggs (study day 81).

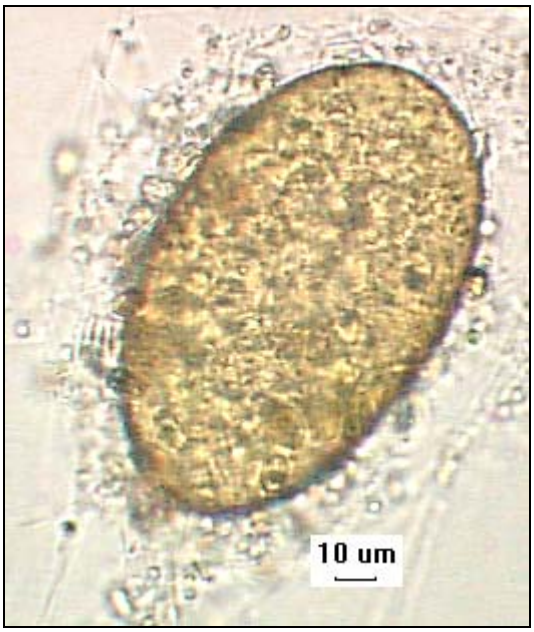


Fig. 2.18:

Sixty-seven days after collection of *Bolbophorus damnificus* eggs (study day 87).

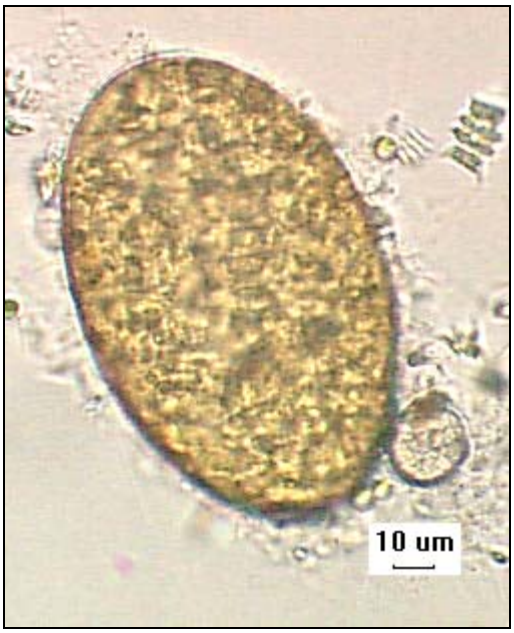


Fig. 2.19:

Sixty-nine days after collection of *Bolbophorus damnificus* eggs (study day 89).

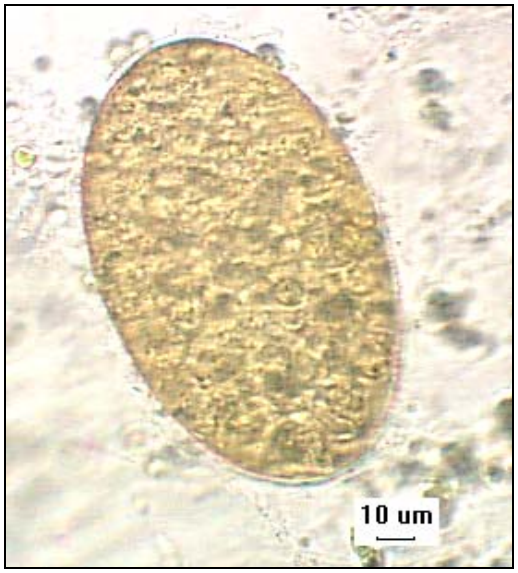


Fig. 2.20:

Seventy-one days after collection of *Bolbophorus damnificus* eggs (study day 91).

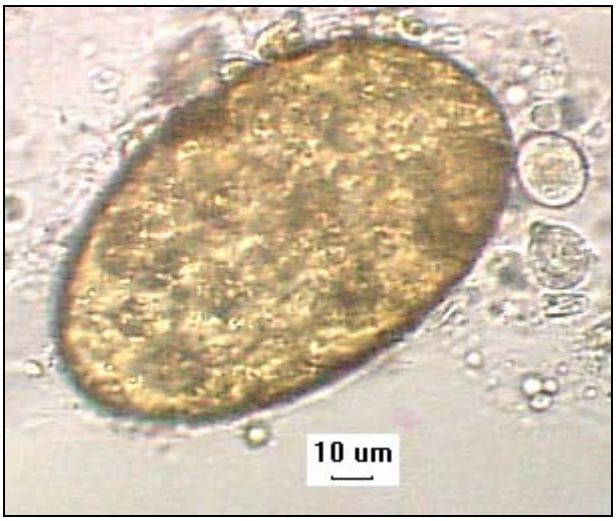


Fig. 2.21:

Seventy-two days after collection of *Bolbophorus damnificus* eggs (study day 92).

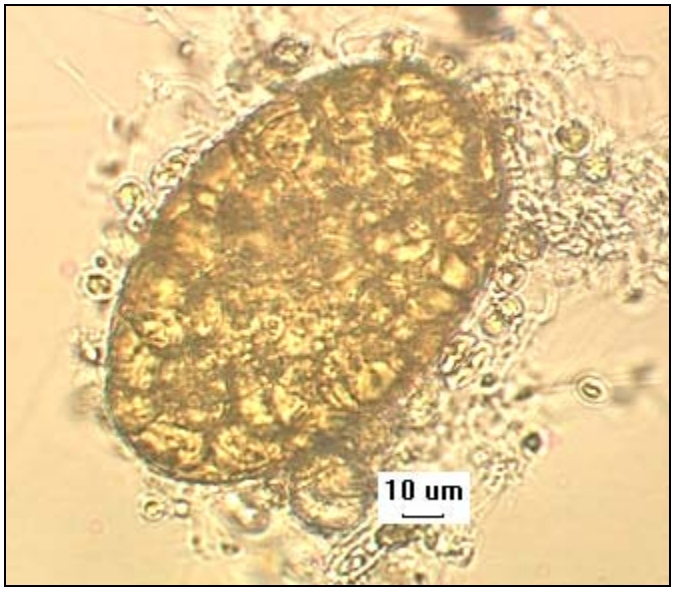


Fig. 2.22:

Eighty-one days after collection of *Bolbophorus damnificus* eggs (study day 101).

Hatching of *Bolbophorus damnificus* miracidia

In this same ova population, miracidia (Figures 2.12 and 2.13) were observed hatching 53 and 55 days after the *B. damnificus* eggs were collected from AWPE 2 and 3 (study day 73 and 75). Water temperatures at hatch were 22.0 °C and 22.3 °C, respectively. Temperatures had ranged from 10.8-27.6 °C during the period prior to hatching. Although miracidia were only observed emerging on these days, empty hatched eggs were observed 57, 59 and 61 days after egg collection (study days 77, 79 and 81) at water temperatures of 25.4 °C, 26.6 °C and 25.4 °C, respectively. The two miracidium measured were elongate, surrounded by cilia, and measured 104.1 x 37.3 µm and 101.5 x 43.4 µm (Figures 2.12 and 2.13).

Shedding of *Bolbophorus damnificus* cercariae

Cercariae were first observed being shed by snails exposed to trematode ova isolated from AWPE 2 and 3 on days 23 and 27 post-miracidia emergence (study days 96 and 100), respectively, (Appendices A.5 and A.6; Tables 2.1 and 2.2) at water temperatures of 26.6 and 26.8 °C (range: 22.0-27.3° C). Snails continued to shed cercariae 63 days (tank 2, study day 138) and 75 days (tank 3, study day 40) after ova hatched at an average rate of 327 (range: 0-1533) and 171 (range: 0-693) cercariae per day, respectively (Figures 2.23 and 2.24). The highest cercariae count observed for a single snail in each tank during the trial was 1,560 cercariae per day on study day 126 in tank 2 and 693 cercariae per day on study day 107 in tank 3.

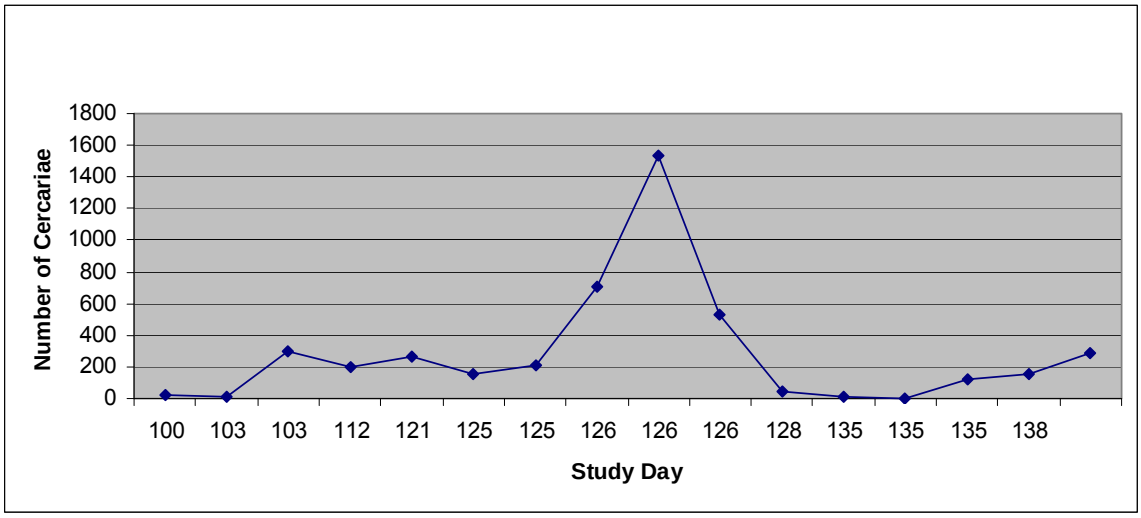


Figure 2.23:

Total number of cercariae shed from snails in experimental tank 2 in 24 hour periods after tubing.

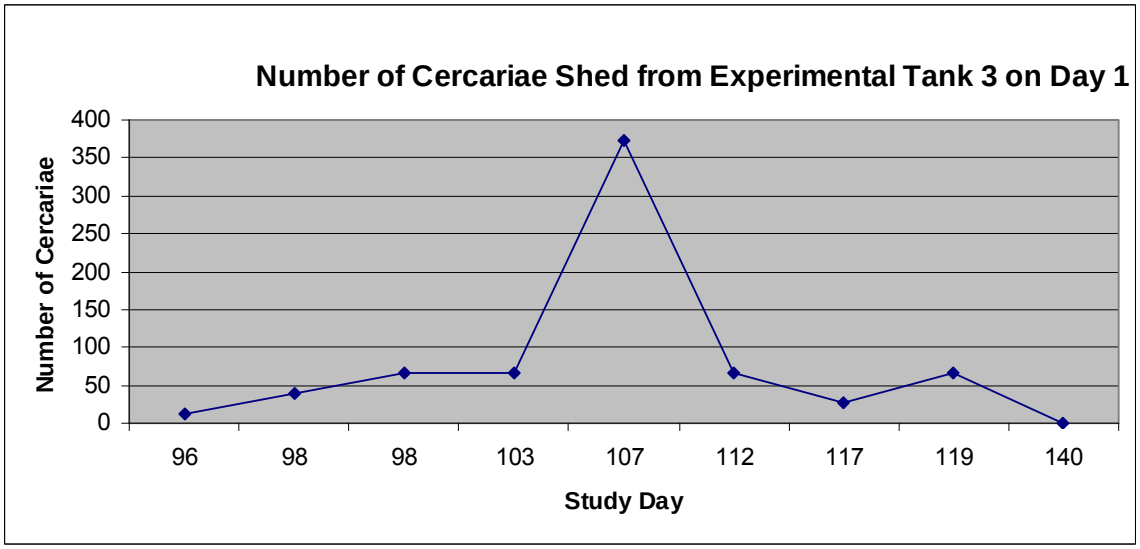


Figure 2.24:

Total number of cercariae shed from snails in experimental tank 3 in 24 hours after tubing.

***Bolbophorus damnificus* metacercariae study**

Catfish exposed to cercariae in fish challenge 1- tank 2 (22 day continuous exposure) began dying on day 17 post-infection (study day 113) with 100% mortality at 22 (Appendix A.8) days post-infection (study day 118). Those catfish that were not decomposed (n= 3) had an average count of 7 metacercariae (range: 2-16) per fish. Catfish in challenge 1- tank 3 (22 day continuous exposure) began dying on day 8 post-infection (study day 104) with 100% mortality at 34 days (study day 130) post-infection (Appendix A.9). An average of 23 MPF were observed (range: 1-36) in the fish (n= 9) necropsied.

In fish challenge 2- tank 2 (3 hour exposure), 17 of the 20 fish exposed to cercariae died between 16-19 days post-infection (study days 133-136) with an average count of ten MPF (range: 0-23; Appendix A.9). Eighteen of the 20 fish in challenge 2- tank 3 (3 hour exposure) died between 13- 19 days post-infection (Table 2.3; study days 30-136) with an average count of three MPF (range: 0-21). Due to the high mortalities in these two challenges, too few fish remained for the subsequent AWPE reinfection study.

All catfish in challenge 3- tanks 2 and 3 (1 hour exposure) survived and were used as the source of metacercariae for the pelican reinfection study. A subsample of these fish (n= 7) were necropsied prior to the reinfection and found to average 0.86 metacercariae per fish (range: 0-2), with four out of the seven fish being infected with metacercariae (Appendix A.10).

***Bolbophorus damnificus* metacercariae description**

When reinfecting AWPEs with experimentally infected catfish, AWPE 2 consumed 30 metacercariae positive catfish (study day 168) from catfish challenge 3, or approximately 25 metacercariae (30 fish x 0.86 metacercariae/ fish= 25; Table 2.4). Pelican 3 consumed 12 metacercariae positive fish (study day 183) from catfish challenge 3 or approximately ten metacercariae (12 fish x 0.86 metacercariae/ fish= 10). PCR analysis and morphometrics confirmed these trematodes to be *B. damnificus* (Figure 2.25).

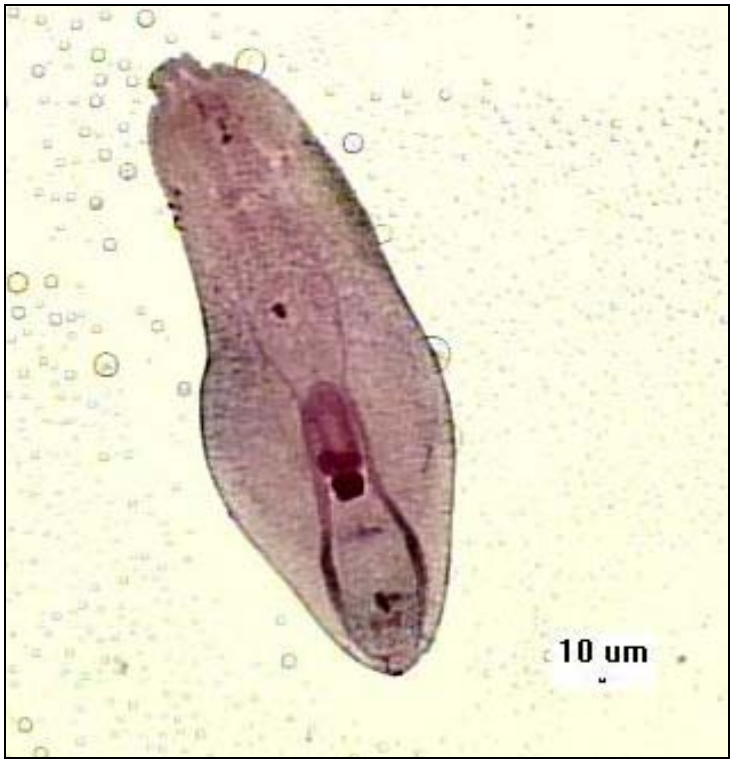


Figure 2.25:

Bolbophorus damnificus metacercariae from artificially infected catfish used for pelican reinfection.

Detailed measurements were obtained on two *B. damnificus* metacercariae; one each from the AWPE initial infection and one from the reinfection study. Morphometric results were: Metacercariae from initial infection: pseudosuckers 85.7 µm x 57.0 µm and 84.2 µm x 58.2 µm, oral sucker 59.3 µm x 54.0 µm, pharynx 30.1 µm x 32.8 µm, ventral sucker 52.1 µm x 47.4 µm, acetabulum 227.3 µm x 131.1 µm, ovary 61.3 µm x 47.5 µm, anterior testis irregular shaped measuring 82.6 µm x 53.9 µm and posterior testis dumbbell shaped, left lobe 76.1 µm x 55.4 µm and right lobe 57.9 µm x 78.7 µm with a total length of 165.6 µm.

Metacercariae from the AWPE reinfection: pseudosuckers 53.6 µm x 32.0 µm and 61.9 µm x 33.0 µm, oral sucker 43.3 µm x 42.3, pharynx 31.2 µm x 22.4 µm, ventral sucker 53.1 µm x 43.0 µm, acetabulum 208.2 µm x 88.1 µm, ovary 47.7 µm x 16.8 µm, anterior testis irregular shaped measuring 34.1 µm x 15.1 µm and posterior testis dumbbell shaped, left lobe 31.4 µm x 19.4 µm, right lobe 20.4 µm x 39.5 µm with a total length of 67.0 µm.

***Bolbophorus damnificus* adults**

In the reinfection study, patent infections were observed in both AWPE 2 and AWPE 3 on day seven post-infection (Tables 2.1 and 2.3). Necropsies were performed on AWPEs 2 and 3 on days 8 and 12 post-challenge, respectively. Three adult *B. damnificus* were recovered from AWPE 2 and five from AWPE 3. PCR analysis and morphology confirmed that these were adult *B. damnificus*.

Detailed measurements were made on two wholemount preperations (Figure 2.26) of *B. damnificus* stained with carmine to facilitate observation of internal organs.

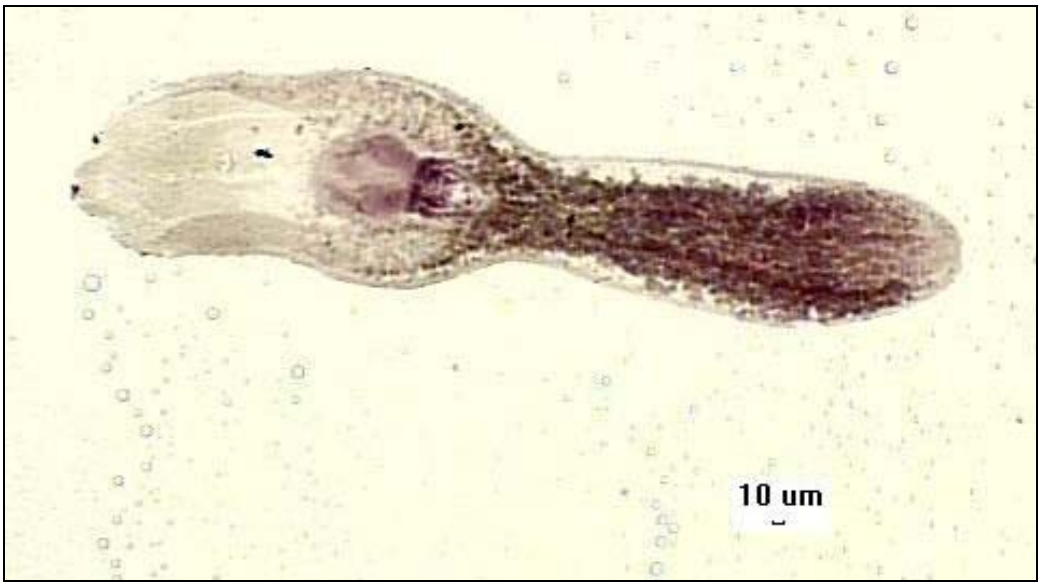


Figure 2.26:

Adult *Bolbophorus damnificus* from AWPE reinfection.

Adult 1: forebody spoon-shaped 1185.2 µm x 394.5 µm, hindbody 1091.1 µm, pseudosuckers 87.7 µm x 42.3 µm and 90.6 µm x 45.0 µm, oral sucker 39.0 µm x 41.4 µm, pharynx 29.4 µm x 36.2 µm, esophagus 33.6 µm, ventral sucker 14.7 µm x 24.5 µm, acetabulum compromised of two lobes surrounded posterior and anterior by two glandular masses, left lobe 136.4 µm x 69.2 µm and 134.7 µm x 59.7 µm, ovary 65.7 µm x 87.7 µm, anterior testis irregular shaped measuring 130.7 µm x 152.2 µm and posterior

testis H shaped with left lobe measuring 246.3 µm x 64.6 µm, right lobe 326.0 µm x 64.7 µm with middle section 48.7 µm x 50.6 µm.

Adult 2: forebody spoon-shaped 1042.9 µm x 489.3 µm, hindbody 1015.6 µm, pseudosuckers 65.4 µm x 33.6 µm and 75.6 µm x 37.2 µm, oral sucker 44.5 µm x 29.6 µm, pharynx 44.5 µm x 39.5 µm, esophagus 39.5 µm, ventral sucker 46.5 µm x 54.8 µm and acetabulum comprised of 2 lobes surrounded by posterior and anterior glandular masses with left lobe measuring 152.2 µm x 62.4 µm and right lobe 131.2 µm x 65.7 µm. Ovary and testes not discernable on *B. damnificus* adult specimen 2.

Ova development at a constant temperature

Bolbophorus damnificus eggs held at a constant temperature of 30 °C in the laboratory were observed to hatch 12 days after collection from AWPEs (Figures 2.27-2.38). Observed development was as follows:

Figures 2.27- 2.38: Development of *Bolbophorus damnificus* eggs maintained at approximately 30 °C.

Fig. 2.27:

One day after collection of *Bolbophorus damnificus* eggs (study day 95).

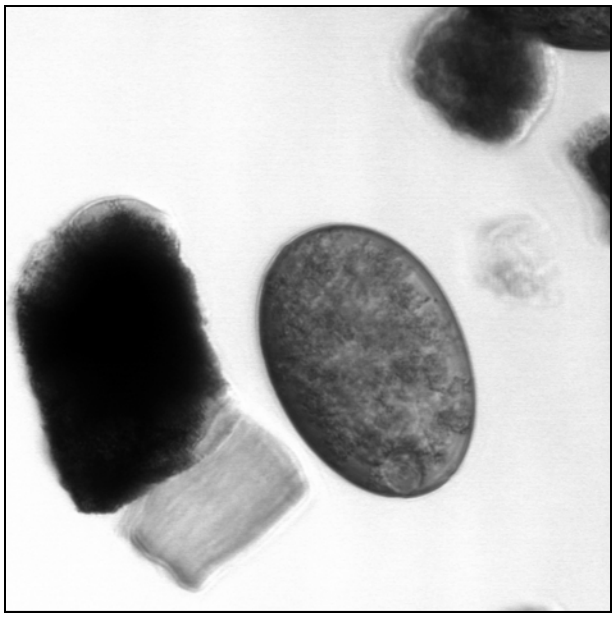


Fig. 2.28:

One day after collection of *Bolbophorus damnificus* eggs (study day 95).

Fig. 2.29:

Two days after collection of *Bolbophorus damnificus* eggs (study day 96).

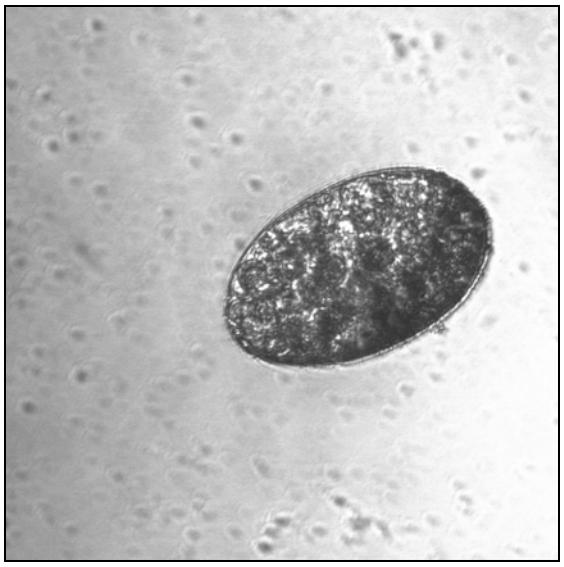


Fig 2.30:

Three days after collection of *Bolbophorus damnificus* eggs (study day 97).

Fig. 2.31:

Four days after collection of *Bolbophorus damnificus* eggs (study day 98).

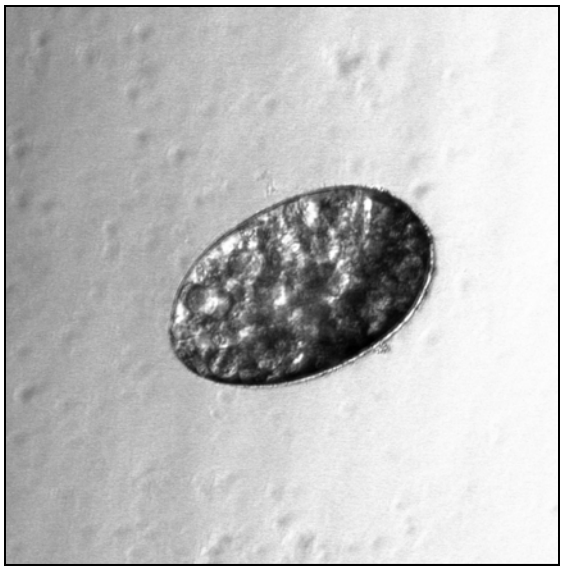


Fig. 2.32:

Eight days after collection of *Bolbophorus damnificus* eggs (study day 102).

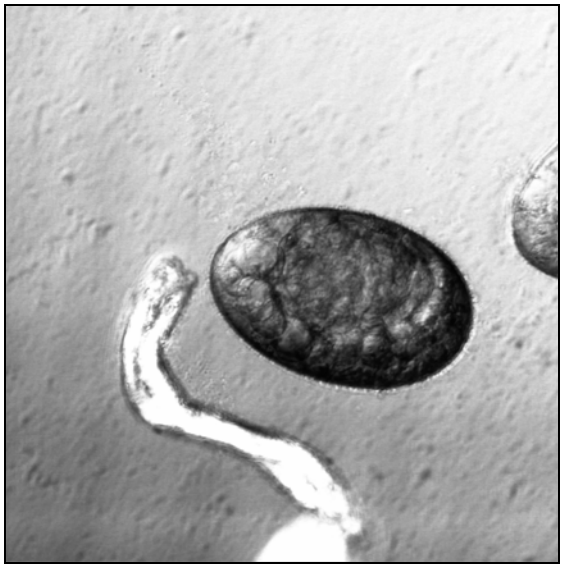


Fig. 2.33:

Ten days after collection of *Bolbophorus damnificus* eggs (study day 104).

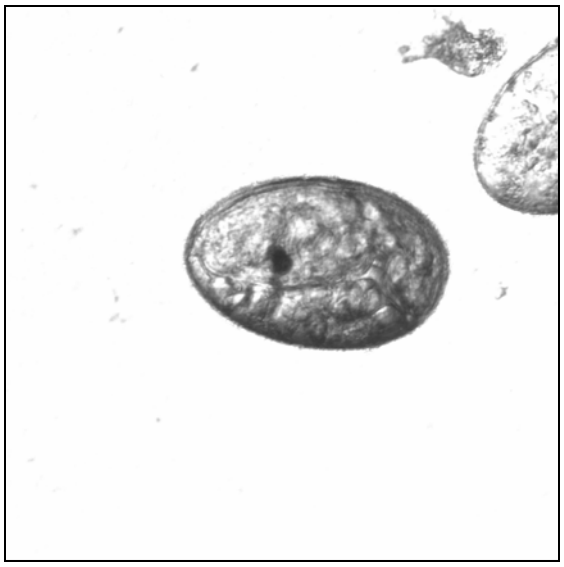


Fig 2.34:

Ten days after collection of *Bolbophorus damnificus* eggs (study day 104).

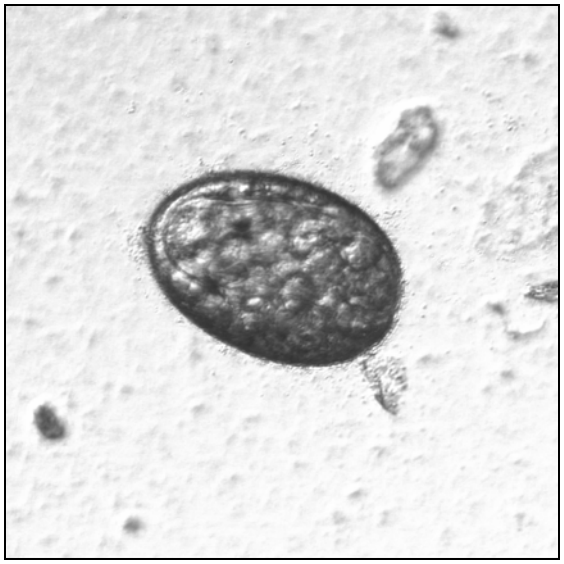


Fig. 2.35:

Twelve days after collection of *Bolbophorus damnificus* eggs (study day 106).

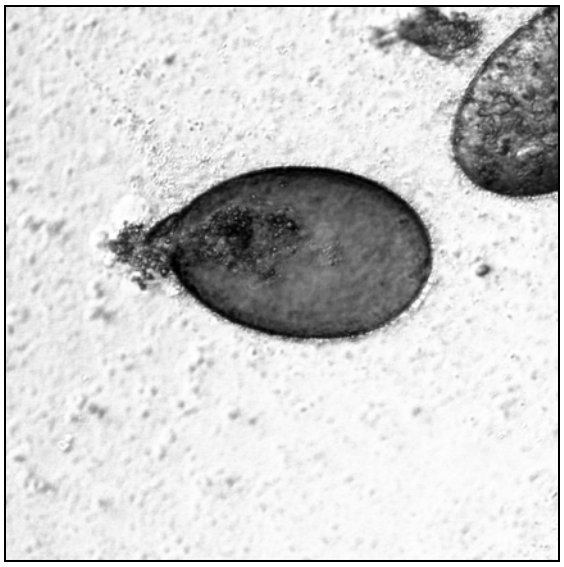


Fig. 2.36:

Twelve days after collection of *Bolbophorus damnificus* eggs (study day 106).

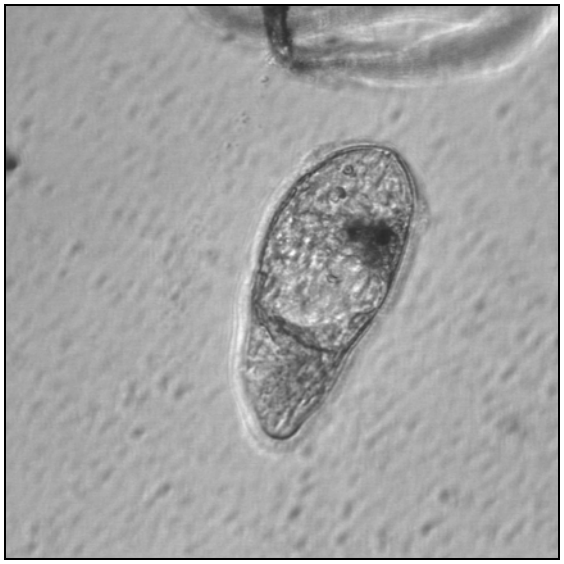


Fig. 2.37:

Miracidia observed fifteen days after collection of *Bolbophorus damnificus* eggs (study day 109).

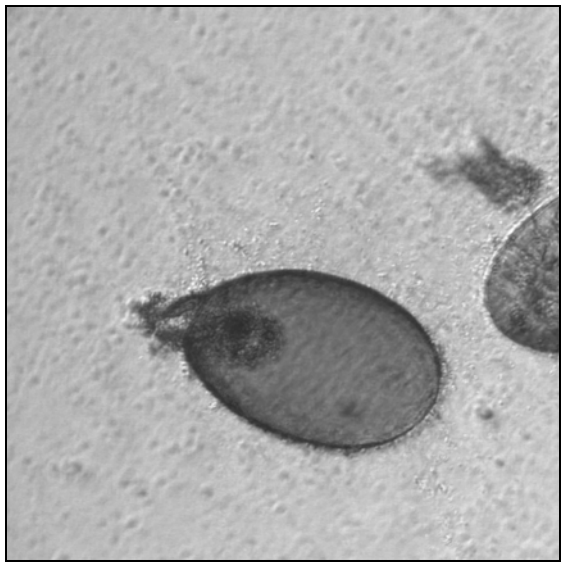


Fig. 2.38:

Hatched egg observed fifteen days after collection of *Bolbophorus damnificus* eggs (study day 109).

Day 3: Several large globules in many eggs.

Day 4: Operculated eggs had multiple cells present.

Day 6: Continued differentiation of cells occurred in most eggs. Some eggs looked undeveloped.

Day 8: Almost all eggs had cell differentiation. A few cells had a large vacular space with cells surrounding the space. Slight outline of miracidia present in some eggs.

Day 10: Most eggs had a slight "depression" in them with cells pushed against the egg wall around the "depression".

Day 12: One egg observed hatching. Eyespots were visible in most eggs. Miracidium movement inside eggs observed in many cases.

Day 15: Many empty hatched eggs visible. The only eggs left unhatched were the ones that failed to develop. A single miracidium was observed swimming among hatched eggs.

Planorbella trivolvis infection in laboratory study

Planorbella trivolvis held at a constant water temperature of 23.9 to 26.7 °C and exposed to *B. damnificus* ova, were first observed shedding cercariae 32 days after exposure to ova (Figure 2.39; Appendix A.7). The highest cercariae count observed in a 24 hour period for a single snail was 2,547 cercariae, which occurred 40 days after ova exposure (Appendix A.7). Snails continued to shed cercariae until 84 days post-ova exposure, when all snails had died and the trial was terminated.

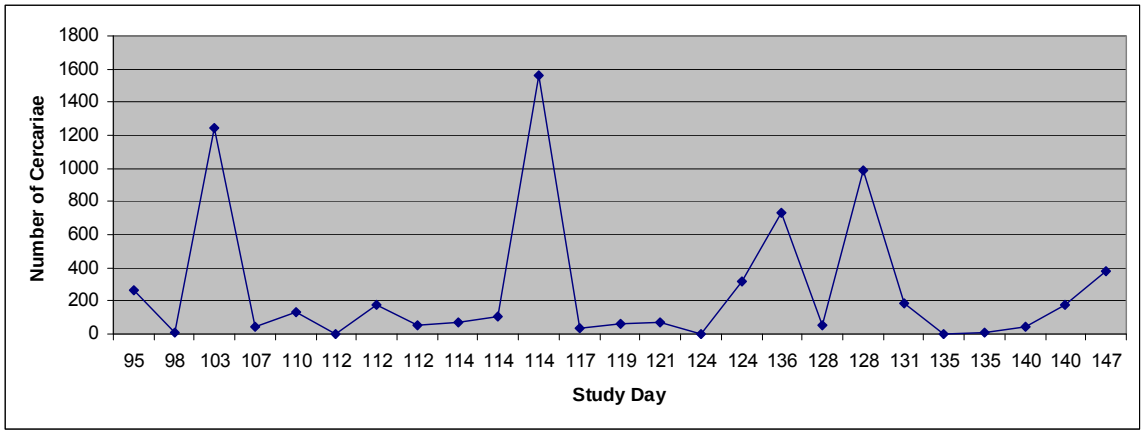


Figure 2.39:

Number of cercariae shed from *Planorbella trivolvis* exposed to *Bolbophorus damnificus* eggs and held at 30 °C. Numbers are counts 24 hours after tubing.

Discussion

This is the first report of a life cycle study with *B. damnificus* in which each life stage was documented in a single infection thus fulfilling Koch's postulates. Several life stages of *B. damnificus* have been previously reported in their respective hosts, adults in AWPEs (Overstreet et al. 2002), cercariae in *P. trivolvis* (Levy et al. 2002) and metacercariae in catfish (Overstreet et al. 2002, Labrie et al. 2004). However, none of these reports replicated successive stages of the *B. damnificus* life cycle through their natural hosts. This research confirms that the AWPE is a true definitive host for *B. damnificus*, with each life stage verified morphologically and with molecular analysis. Patent adults were present in the AWPEs 4-7 days post-infection with *B. damnificus* metacercariae (Table 2.1; Figure 2.1). Ova were shed by these adults (4-7 days post-infection); miracidia hatched from these ova (Figures 2.12 and 2.13) 12 to 53 days post-infection at a constant temperature of 30 °C or ambient temperatures ranging from 10.8 to 27.6 °C degrees respectively. These snails began shedding cercariae 23 days post-exposure to the hatched miradidium; cercariae infected the catfish, developed into viable metacercariae as early as 23 days post-infection and were able to infect AWPEs establishing patent infections, thus completing the *B. damnificus* life cycle.

Bolbophorus damnificus eggs (n= 8) at the beginning of the study were similar in length and width (104-126 x 44-79 µm) to measurements previously reported for *B. confusus* (115-125 µm x 67-82 µm) but were shorter in length when compared to *B. damnificus* (123-129 µm x 50-89 µm), respectively (Fox 1965; Overstreet et al. 2002). Seven of the eight eggs were in the size range of 119-126 x 71-79 µm, while one egg was

much smaller (104 x 44 µm). Had this egg been excluded, the average ova measurements would have been 76-121 µm which would still have been smaller than the egg length reported for *B. damnificus* (Overstreet et al. 2002). The time required for eggs to hatch in this study was comparable to times observed in previous studies. In the present study eggs hatched in 12 days at 30.0 °C and in 53 days at ambient temperatures of 13.5 to 35.7 °C . Results of this study confirmed previous studies in which ova hatched in 12-13 days at 32.2 °C and in 65 days at 20.0 °C (Fox 1965), with delayed hatching with decreasing temperature (Table 1.1).

Miracidia in this study were shorter (88-140 µm) and wider (42-52 µm) than previously reported for *B. confusus* (150-190 µm x 30-40 µm; Table 1.1). However, because only two miracidia were available, it is unclear whether their measurements represent true differences in the size of miracidia between the two species.

Cercariae emerged from snails in the present study 23 days after miracidia first hatched (Table 2.2), which was 7-11 days earlier than observed for *B. confusus* (Fox 1965). However, snails in the present study were held at ambient temperatures of 22.0-27.3 °C versus 21.1-23.9 °C in the *B. confusus* study. In addition, at peak shedding, 2,547 cercariae were counted versus 1000 for *B. confusus* (Fox 1965; Appendix A.7). Adult *B. damnificus* in this study showed a one to three day delay in patency, which occurred at four to seven days in this study versus three days reported for both *B. confusus* (Fox 1965) and *B. damnificus* (Overstreet et al 2002; Table 1.1). Adult stages of *B. damnificus* were shorter (2.05-2.25 mm) compared to findings of 2.53 mm and 2.6-3.2 mm by Fox (1965) and Overstreet et al. (2002).

EPG results indicated that patent *B. damnificus* infections occurred four days post-infection with metacercariae (Table 2.1). This patency range was 1-3 days later than previously reported for *B. damnificus* (Overstreet et al. 2002) and *B. confusus* (Fox 1965). Several factors may have contributed to this delay in patency. In the first challenges with AWPEs 1-3, the level of challenge was higher, 403-420 versus 10-25 metacercariae in the subsequent reinfection. The initial EPG counts were higher in the first infection, with the exception of AWPE 3 given metacercariae directly, rather than infected fish. It is possible that because the initial EPGs were so low after reinfection, ova may have been missed on day four to six, but AWPE 3 had similarly low counts in the first challenge and ova were detectable on day four post-infection. Another scenario is that eggs were present in the fecal material, however their numbers were less than the detection threshold for the sedimentation technique used. This study indicated that once an AWPE is infected, AWPEs begin to shed ova four to seven days post-infection and can continue for at least 53 days post-infection. During this period, trematode ova production is continual, but intermittent, with occasional consecutive days of negative EPGs and with peak ova shedding at days 12-14 post-infection (study days 20-22; Tables 2.1-2.3).

Data from this study can be used to estimate the potential number of *B. damnificus* ova that can be introduced into a pond by a single *B. damnificus* infected AWPE. In AWPE reinfection, AWPE 3 was found to be infected with eight patent *B. damnificus* adults at necropsy. The maximum EPG observed for those trematodes was 220 EPG at 8 days post-infection (Table 2.1). Assuming an average 27 ova/ per

trematode (220 EPG / eight trematodes present at necropsy) it is possible that a single AWPE could release high numbers of trematode eggs while feeding on a catfish pond. Compound this with reports of flocks of 250 AWPE/ flock routinely observed in the Mississippi Delta (King and Michot 2002) and their constant presence from November to May (Glahn and King 2004); an ideal scenario is created for the continual perpetuation of *B. damnificus* in commercial catfish ponds . Additionally, this does not take into consideration that in naturally infected AWPEs often more than eight adult *B. damnificus* are present (C. Doffitt, personnel communication).

In addition, this study indicates that AWPE's may be repeatedly reinfected with *B. damnificus*, suggesting they may not mount a significant immune response to this parasite. The AWPEs in this study had natural trematode infections as indicated by the presence of *B. damnificus* ova when captured (Table 2.1). Trematodes were eliminated with praziquantel as evidenced by repeated negative EPGs after treatments (Table 2.1). In both reinfections, AWPEs became infected by ingesting *B. damnificus* metacercariae, and shed *B. damnificus* eggs 4-7 days after each reinfection, suggesting they do not mount a protective immune response and can potentially become repeatedly infected after ingestion of fish positive for *B. damnificus* metacercariae. And according to these findings, with a single feeding of *B. damnificus*-infected catfish, AWPEs could begin shedding *B. damnificus* ova in 4-7 days and continue to shed these ova for at least 53 days (Table 2.1, Figure 2.3). Knowing that *B. damnificus* positive fish are widely distributed in Delta operations, it is probable that AWPEs in this region are constantly

being reinfected and are continually shedding parasite ova, perpetuating this life cycle in catfish ponds.

Several factors may play an important role in the viability and hatching of *B. damnificus* ova, once introduced into a pond. Data indicated that temperature affects egg development, hatching, and the release of miracidia. Miracidia hatched in 11 days at a constant temperature of 30.0 °C. However, hatching was delayed when the same ova population was held at ambient temperatures of 10.8 °C to 27.6 °C in March and April. Average water temperatures in catfish ponds have been reported in January-12.6 °C, February-11.7 °C, March-19.1 °C, April-19.5 °C and May-24.9 °C (Thad Cochran National Warmwater Aquaculture Center). This study suggests that trematode eggs deposited in catfish ponds when AWPEs are present in January-March probably remain dormant when pond temperatures are low, but rapidly undergo development as temperatures increase in the spring, with rapid hatching occurring in 11 days. These increasing water temperatures in April and May, would coincide with the emergence of host snails (B. George, personnel communication) and provide an explanation as to why large populations of infected *P. trivolvis* are present in the spring and summer months, after AWPEs have migrated north in March.

Although this would imply elevated water temperature decreases the time required for development of cercariae in the snail host, this did not appear to be the case. In the snail study, *P. trivolvis* were kept at 23.9 to 26.7 °C and cercariae were observed 32 days post-exposure to *B. damnificus* ova. If 12 days are required for eggs to hatch, this would indicate that cercariae maturation occurred in 22 days. When snails and ova were

held at ambient temperatures of 10.8 to 27.6 °C, ova began hatching in 53 days and snails began shedding cercariae 23 days later, or 76 days post-exposure. This ova development indicates that the development from the penetration of the miracidium into the snail, through the asexual stages, to the final release of mature cercariae takes 21-23 days. Temperature plays a role in the timing of initial infection of the snail but does not appear to effect the development of this parasite once it is in the snail host.

The number of cercariae shed per snail was not affected when infected snails were held indoors at temperatures of 23.9-26.7 °C or in ambient outdoor temperatures of 20.6-34.4 °C (water temps 20.4-27.6 °C) for 52 days (May- August; study days 48-100). Since these snails were not held for longer than 48 hours, this was probably not enough time to draw any conclusions about the relationship of temperature and patterns of cercarial shedding. Cercariae shedding did not appear to increase after 48 hours in the laboratory at 21.1-23.9 °C, with 60-78% of the snails shedding more cercariae at the 48 hour count. Peak shedding of *B. damnificus* cercariae by a single snail was 2,547 cercariae/ day (Appendix C.1) which is higher than the cercariae/ day observed by Fox (1965) of 1000 cercariae/ day and Terhune et al. of 414-891 (2002; Table 2.5), respectively. Positive snails held at 23.9-26.7 °C shed cercariae for 38-44 days but the number of cercariae shed fluctuated.

In the study at ambient temperatures, positive snails were first detected 81 days post-exposure to AWPE feces containing *B. damnificus* ova or 23 days after ova were first observed hatching. Since ova were not collected from AWPE 3 until 5 days after the AWPEs were introduced, it is possible that ova may have matured 5 days earlier,

shortening the time for snail infection to cercariae emergence to 27-32 days in experimental tank 2 and 23-28 days in experimental tank 3. These findings are in agreement with observations by Fox (1965) of 30-34 days for *B. confusus*, when snails were held at 21.1-23.9 °C.

Of the three challenge models used to obtain catfish with viable *B. damnificus* metacercariae, only the third model was successful. Specific pathogen free catfish constantly exposed to cercariae positive snails (challenge 1) in experimental tanks 2 and 3, as well as those fish exposed to snails for 3 hours (challenge 2) resulted in 100% mortality in both challenges. In challenge 3, fish were exposed for 1 hour to this same infected snail population, with no resulting mortality. This study indicates that a lethal challenge of cercariae for naïve fish could occur with as few as 30 snails shedding up to 2,547 cercariae/ snail. When considering the potentially high number of snails present from May to October (B. George, personnel communication), with each shedding up to 2,547 cercariae/ day, there is tremendous potential for acute infections to occur rapidly in these ponds. A recent study confirmed that fish (fingerlings/ 5 cm) exposed to 200 *B. damnificus* cercariae caused mortality (Chapter IV). Combining fish mortality information with a shedding rate of 2,547 cercariae shed/ snail, this would mean that a single snail could potentially shed enough cercariae in a day to kill 12-13 fingerling catfish (5 cm or less).

Although mortalities occurred in fish in challenges 1 and 2, the estimated average MPF in challenge 1 was only seven and 23 in experimental tanks 2 and 3, respectively. In challenge 2 the estimated average metacercariae in dead fish was ten and three in

experimental tanks 2 and 3, respectively. While in challenge 3 the average was one metacercariae in challenge 3, tank 2. Catfish from metacercariae challenge 1 and 2 produced results similar to ponds experiencing mortalities indicative of severe natural infections, whereas challenge 3 simulated mild infections. These results are similar to a previous study where 100% mortality occurred in channel catfish fingerlings 2-29 days post-infection (Levy et al. 2002). However, in this previous study, molecular confirmation of the *Bolbophorus* species used was not done and fish may have been challenged with a mixture of *B. damnificus* and *Bolbophorus* sp. (Type 2) cercariae. In this research (Chapter IV), exposing catfish to 200 *B. damnificus* cercariae for 2 hours resulted in 100% mortalities starting on day 5 post-infection.

This study indicates that it takes at least 23 days for metacercariae to become infective to the AWPEs. Metacercariae were also found to remain infective in the tissue for at least 38 days. Fox (1965) demonstrated that it took 30-34 days *Bolbophorus confusus* to mature and be infective for the fish host.

It is estimated that AWPE 2 ingested an estimated 25 metacercariae and three of these (12%) were confirmed to have developed into adults at necropsy. In contrast, 50% of the metacercariae ingested by AWPE 3 developed into adults. Although the AWPE 2 reinfection did not result in a high percentage of trematode adults as was observed in reinfection of AWPE 3, both of these resulted in higher percentage than other researchers have generated. In a similar study, 182 *B. damnificus* metacercariae were given to each of 2 AWPEs. Both AWPEs were necropsied and only 1 and 5 *B. damnificus* adults were recovered, respectively (Doffitt et al., unpublished data).

The efficacy of the trematode challenge varied with the two methods used to artificially infect AWPEs. Patent *B. damnificus* infections occurred in all AWPEs at day 4 post-infection. However, AWPE 3, which had been challenged with excised metacercariae shed a lower number of *B. damnificus* ova throughout the study versus the other two birds fed live infected fish. This indicated that the muscle surrounding the metacercariae could have served as protection for the encysted stage as it enters the AWPE's stomach and proceeds to the upper small intestine. While naturally infected fish appeared to be the most efficacious method to infect the AWPE, one drawback is that there may be more than one species of trematode naturally infecting the fish. This occurred in this study in which *Hysteromorpha*-like metacercariae were present in the fish used for the initial AWPE challenge. In order to confirm that AWPE could not serve as a host for this *Hysteromorpha*-like diplostome, AWPE 4 was intubated with these unknown diplostomes during this study. A patent infection did not occur which was verified by the continual negative EPGs for the duration of the study. There have been no reports of the AWPE serving as a host for this parasite and this research further substantiated this. Previous reports have shown definitive hosts of *Hysteromorpha tribola* to be several species of cormorants (*Phalacrocorax* sp.) and the bittern (*Botaurus stellaris*) (Huggins 1954).

This study also demonstrated that praziquantel at the dose of 30 mg/ kg body weight in a single dose is efficacious against *B. damnificus* infections in AWPEs. In all trials, prior to treatment, the pelicans had positive EPGs (Table 2.1) and became negative

post-treatment and remained negative until patent infections were established 4-7 days post-infection.

The EPGs were consistently lower in AWPE 1 and 2 after reinfection compared to the EPGs observed in AWPEs after the first challenge. These lower counts were probably due the lower number of *B. damnificus* adults present in AWPEs (n= 3 and 5 trematodes) versus the number present in the first challenge. Since the AWPEs were not necropsied following the first challenge, the number of trematodes present in these AWPEs is unknown. Greater numbers of metacercariae were also used in the first challenges in pelicans 1-3 (420, 403, 179, respectively) versus the reinfection in which an estimated 25 and 10 were ingested by AWPEs 2 and 3, respectively. Assuming that 12-50% of these metacercariae established infection, it is possible that these AWPEs could have been harboring 50-210 adult trematodes/ AWPE.

It has been postulated that the *Bolbophorus* species from Montana studied by Fox (1965) could have actually been *B. damnificus* (Overstreet et al. 2002). However, this study found differences in the morphology of life stages, the development of these stages and the pathology associated with this parasite. The mortality seen in catfish challenges 1 and 2, and the subsequent *B. damnificus* metacercariae formation in channel catfish in this study did not concur with past reports of *B. confusus* infections. Previous reports of *B. confusus* concluded that *I. punctatus* was an aberrant host for the parasite since investigators did not find any metacercariae when *I. punctatus* were challenged with 1000 and 1,200 *B. confusus* cercariae (Olsen 1966) and no mortalities were observed.

The production of commercial catfish and the ecosystem in which it lives has proven to be an ideal environment for the perpetuation of the *B. damnificus* life cycle. In this habitat, the American white pelicans have been provided with a readily available year-round food source in the commercial catfish ponds as they migrate and overwinter in the Southeastern United States. In addition to this constant source of catfish, they also have the potential to constantly ingest large numbers of *B. damnificus* metacercariae with ingestion of infected fish. Within 4 days post-infection the *B. damnificus* metacercariae can mature to adults. Patent adults present in the AWPE begin laying large number of trematode eggs as the pelican eats and defecates these trematode ova into the pond. As pond temperatures begin to increase in the spring, *B. damnificus* eggs, that have been dormant, begin hatching rapidly releasing motile miracidium. These miracidium infect the ubiquitous and abundant *P. trivolvis* increasing in numbers as water temperatures rise in the catfish ponds. Less than a month later, these infected snails can begin to shedding large numbers of cercariae (up to 2,547/ day) continually for at least two months. Ideally, for perpetuation of the trematode, enough cercariae are shed to infect catfish at a subclinical level, thus providing the AWPE a metacercariae population that has matured in less than one month, is infective, and rapidly develops to the adult stage after ingestion by the AWPE. This would be expected in natural fish populations, but is not always the case in the artificial environment of catfish ponds, thus clinical outbreaks with high mortalities are frequent in the commercial setting.

This study not only confirmed all of the life stages and hosts in the *B. damnificus* life cycle, it also provides information that may prove to be useful in the control of this

parasite. This study indicated that pelicans can become infected, the trematode ova can remain dormant in the ponds and then hatch rapidly as water temperatures increase. Additionally, it takes very low numbers of positive snails to supply enough cercariae for a continual subclinical infection in the fish population. Because the American white pelican is protected by state and federal regulation, there are limitations in the control of this host. Therefore, it is important in the control of this parasite to target the snail population in the early spring before water temperatures begin to rise and they become easily infected and. The trematode ova can remain dormant in the ponds and then hatch rapidly as water temperatures increase requiring a very low number of infected snails to produce enough cercariae to establish subclinical infections in the fish population. Ideally, a strategy to control this parasite would be to eradicate the snail population as it emerges in the spring and becomes infected before this population begins shedding cercariae. Equally important is the persistent and continual monitoring of the snail and fish population in each pond, as this study indicates once this parasite is introduced by the AWPE and if the snail population is present, this parasite quickly establishes itself in the catfish pond.

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CHAPTER III

BIOMPHALARIA HAVANENSIS IDENTIFIED AS A POTENTIAL INTERMEDIATE HOST IN THE *BOLBOPHORUS DAMNIFICUS* LIFE CYCLE

Abstract

The digenetic trematode *Bolbophorus damnificus* has been associated with mortalities in commercial channel catfish *Ictalurus punctatus* in the Mississippi Delta. In the life cycle of *B. damnificus*, the only confirmed first intermediate host is the rams-horn snail *Planorbella trivolvis*. Recently, an exotic snail species, *Biomphalaria havanensis* has been isolated in several catfish ponds in the Mississippi Delta. The aim of this study was to determine if this newly emerging snail species could also serve as a first intermediate host in the *B. damnificus* life cycle. *Bolbophorus damnificus* ova, collected from an American white pelican *Pelecanus erythrorhynchos* artificially infected with *B. damnificus* metacercariae, were introduced into an aquarium with parasite-negative *B. havanensis*. *Bolbophorus damnificus* cercariae, confirmed by PCR, were detected in these *B. havanensis* 45 days post-exposure to the *B. damnificus* ova. These *B. havanensis* continued to shed *B. damnificus* cercariae for 80 days post-exposure at which time the study was terminated. Prior to this research, the only report of *B. damnificus* naturally infecting snails has been with *P. trivolvis*. This study indicates that the range of snail hosts may be broader than previously suspected. A survey of all potential snail hosts should be implemented to ensure there are not additional snail species that can serve as

hosts for this parasite. Further research needs to be done to determine if this snail host is a natural host for this parasite and if so, the prevalence of this snail host in commercial catfish ponds.

Introduction

The constant presence of fish-eating birds along with the appropriate snail species fulfills the life cycle requirements of some digenetic trematodes in commercial catfish ponds in the southeastern United States (Overstreet and Curran 2004). Consequently, an increase in diagnosed digenetic trematode infections in channel catfish *Ictalurus punctatus* has been observed. A newly described digenetic trematode, *Bolbophorus damnificus* has been associated with mortalities and economic losses in farm-raised channel catfish (Overstreet et al. 2002). The life cycle of this parasite involves the ram's horn snail *Planorbella trivolvis* as the first intermediate host, the channel catfish as the second intermediate host, and the American white pelican *Pelecanus erythrorhynchos* as the definitive host.

Bolbophorus damnificus infections, initially published as *Bolbophorus confusus* (Overstreet et al. 2002), were first reported in farm-raised catfish in 1994 in Louisiana (Labrie et al. 2004) and later in Mississippi in 1999 (Terhune et al. 2002). Since these first reports, economic losses associated with *B. damnificus* have steadily risen. Between 1.1-5.6% of the catfish submissions to the Aquatic Diagnostic Laboratory at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, Mississippi (Aquatic diagnostic laboratory) were diagnosed with these trematodes from 1999-2005. The

highest incidence was in 2000 with *B. damnificus* accounting for 5.6% of diagnostic submissions. The lowest incidence was in 2003 with only 1.1% of submissions attributed to *B. damnificus*. Since the low in 2003, the incidence has steadily increased with *B. damnificus* accounting for 2.6% in 2004 and 3.4% in 2005 of diagnostic submissions (2005 Annual Case Summary Report Aquatic Diagnostic Laboratory). These continual infections have been attributed to the increasing number of wintering and migratory American white pelicans in the Delta region of Mississippi where catfish ponds serve as a readily available food source. In addition, certain management practices may have also contributed to the steady increase of *B. damnificus* in catfish since 2003. This could have been due to new farm personnel lacking experience in the detection of *B. damnificus* in fish and farms that had *B. damnificus* infections may have been less vigilant in detecting *B. damnificus*, assuming the problem was under control (Avery et al. 2004).

Although the reported incidence of *B. damnificus* is low compared to other fish pathogens such as *Flavobacterium columnare* and *Edwardsiella ictaluri*, occurrence may go unreported due to on-farm diagnosis by the producer. Furthermore, given that most catfish farmers are aware that there are no available treatments for this parasite in the catfish host, farmers often do not submit trematode infected fish, therefore the incidence of *B. damnificus* may be greater than diagnostic reports suggest. In addition to clinical disease and mortality, production losses occur due to subclinical infections. Ponds with subclinical infections may go unrecognized by producers, as there is no extensive fish mortality. These infections may persist for years and have been shown to decrease production and profitability substantially (Wise et al. 2006).

The only confirmed first intermediate host for this trematode is the ram's horn snail *P. trivolvis* (Overstreet et al. 2002). Although this is the only reported snail to transmit *B. damnificus* (Fox 1965), other snail species are present in catfish ponds. *Planorbella trivolvis* and *Physella gyrina* are both routinely found in catfish ponds (Overstreet and Curran 2004) and recently an exotic snail, *Biomphalaria havanensis*, was found in commercial catfish ponds in the Delta region of Mississippi (B. George, Mississippi State University, personal communication). It is not known when this snail first appeared in the Mississippi Delta, how it was introduced or its current prevalence.

Biomphalaria havanensis, type locality in Havana, Cuba (Young et al. 1997), is also found in Mexico, Central America, the Antilles region (Malek 1985), and the Dominican Republic (Malek 1969), and can serve as an intermediate host for other digenetic trematodes (Cram et al. 1945; Michelson 1976). Presently there are only sporadic reports of *B. havanensis* in the United States; the Middle Snake River in Idaho (Bowler and Frest 1992), on the east coast of Florida (Thompson 1984), and in South Carolina (Dillon and Dutra-Clarke 1992).

In order to develop control strategies for *B. damnificus*, it is important to determine which mollusks can serve as first intermediate hosts for this parasite. Although *Biomphalaria* snails can serve as an intermediate host for other trematodes, it is not known if it can serve as a host for *B. damnificus*. The purpose of this study was to determine whether *Biomphalaria* snails could serve as an intermediate host for *B. damnificus*.

Materials and methods

Snails morphologically identified as *B. havanensis* (Burch, 1989) were collected from a commercial catfish pond in the Mississippi Delta and reared in our laboratory for at least one year. Offspring of this original population were tested in our laboratory and demonstrated to be *B. damnificus* negative by PCR. To further confirm the snail population used in this study was negative for *B. damnificus* infection, PCR was performed on a subsample of this snail population. Juvenile and adult snails (5 mm to 10 mm in size) were randomly selected for testing. Snails (n=10) were placed in test tubes containing three mL of sterile spring water. Prior to the beginning of the study, snails were checked daily for a period of ten days to ensure that none were shedding cercariae. After ten days, the subsample of snails were sacrificed and PCR completed to further confirm they were negative for *B. damnificus* life stages. A subsample of snails was also sent to the University of New Mexico for molecular analysis to confirm snail species.

Molecular analysis of snails and cercariae

Biomphalaria bodies or individual cercariae were placed individually in microfuge tubes with 300 µL cell lysis solution and 1.5 µL proteinase K. DNA was extracted using the Puregene DNA purification kit (Gentra Systems, Minneapolis, Minnesota) following their protocol, only performing half reactions. *Bolbophorus damnificus* species-specific primers, P1-650F (5'-TCA GTT TCG AAC GAT GAT GA-3') and P1-14700R (5'-CGG TCT ACG GTT CCA CC-3') were used (Levy et al. 2002) to amplify DNA from cercariae. Reaction volumes for PCR were 25 µL using 10X

buffer, 2.0 µl template, 0.625 U *Taq* polymerase (Hot Start Taq; Takara Bio, Inc., Shiga, Japan), 200 nM of each primer and 200 µM of each deoxynucleotide triphosphate. Polymerase chain reaction conditions were those described by Levy et al. (2002) except the reaction times were shortened and were 92 ˚C for 5 min followed by 35 cycles of 94 ˚C for 1.0 min, 58 ˚C for 1.0 min, and 72 ˚C for 1.0 min, with a final cycle of 72 ˚C for 5.0 min. This was carried out on a MJ Research PTC-100 Peltier Thermal Cycler. PCR products were visualized on a 1.2 % agarose gel along with positive and negative controls. The gel was post-stained with gelstar nucleic acid stain (Combrex BioScience Rockland, Inc) and observed under ultraviolet light.

The *B. damnificus* ova used in this study were collected from an American white pelican that had been artificially infected with *B. damnificus* as part of another study. The bird was captured in Chicot County, Arkansas and housed in outdoor avian facilities specifically designed for live captured pelicans at the Mississippi Field Station of the National Wildlife Research Center, Mississippi State University, Mississippi (Glahn et al. 2000). The pelican was treated orally with a single dose of praziquantel (Droncit® 34, Bayer, Corporation, Shawnee Mission, Kansas), at 30 mg/kg body weight to eliminate trematode infections resulting in repeated negative fecals, and then artificially infected with *B. damnificus* metacercariae (confirmed by PCR) as previously described (Chapter II).

In order to isolate *B. damnificus* ova, pelican fecal material was collected after a patent *B. damnificus* infection was confirmed. Fecal sedimentation was performed on the fecal material using a modified sedimentation technique as described in Foreyt (2001) to

detect trematode eggs. Briefly, total feces from the pelican was collected, homogenized thoroughly, weighed and 0.5 g was used for the sedimentation procedure. Fecal material was added to a 15 mL test tube and 1 % soap solution added. The fecal material and soap solution were mixed thoroughly and allowed to sit for at least five minutes. The soap solution was carefully decanted as not to disturb the pellet. Fecal material was repeatedly washed with the soap solution to remove debris. When the sample was sufficiently clear, it was diluted to 10 mLs in reverse osmosis water. To enumerate eggs, a one-milliliter aliquot was added to a Petri dish with grid lines three mm apart, diluted with reverse osmosis water and all ova were counted. The number of eggs per gram (EPG) were calculated.

Thirty parasite-negative *B. havanensis* snails were added to a 19 L aquarium and maintained at room temperature (18- 23°C). Feces collected from the adult American white pelican artificially infected with *B. damnificus* were used as a source of *B. damnificus* eggs. These feces were collected for four days and a fecal sedimentation as described previously was performed on the pooled sample to enumerate and isolate *B. damnificus* ova. Fecal ova counts indicated there were approximately 1,100 eggs per g (EPG) of feces. Feces (13.5 g) containing approximately 14,850 ova (1,100 EPG x 13.5 g of feces) were added to the aquarium. A subsample of these ova was analyzed as previously described using PCR primers specific for *B. damnificus* to confirm they were *B. damnificus* ova (Levy et al. 2002).

Snails were monitored for the presence of cercariae beginning 45 days post *B. damnificus* ova exposure. At this time, four snails were removed from the aquarium,

measured and placed in test tubes with three mL of sterile spring water. Tubes were examined for cercariae under a dissecting microscope at 24 and 48 hours. Cercariae were enumerated and stored in 70 % molecular grade ethanol for DNA analysis. Three times weekly, four new snails were removed from the tank and the previously checked snails were returned to the aquarium. Following this procedure, four snails were randomly selected three times per week for the duration of the study. Additionally, all remaining live snails (n= 24) were checked for cercariae shedding on day 47 and returned to the aquaria. The last sampling date was 80 days post-exposure. After this date, no living snails remained.

If a snail was found to be positive for cercariae, a subsample (n= 5) of cercariae was collected. Using the protocol previously described, each subsample was confirmed by PCR to be *B. damnificus*.

Results

Microscopic examination of snails shedding cercariae began 45 days post-exposure to the *B. damnificus* ova. Four snails collected at that time were confirmed by PCR to be shedding *B. damnificus* cercariae. *Biomphalaria* snails continued shedding for the duration of the study, day 80 (Figure 3.1). Of the remaining live snails (n= 24) examined on 47 day post-exposure, five specimens were confirmed to be infected with *B. damnificus*. Cercariae shed by all positive snails for the duration of the trial were confirmed by PCR to be *B. damnificus*. The highest number of cercariae shed by a single snail per day was 640 cercariae which occurred on day 45 post-exposure (Figure 3.2)

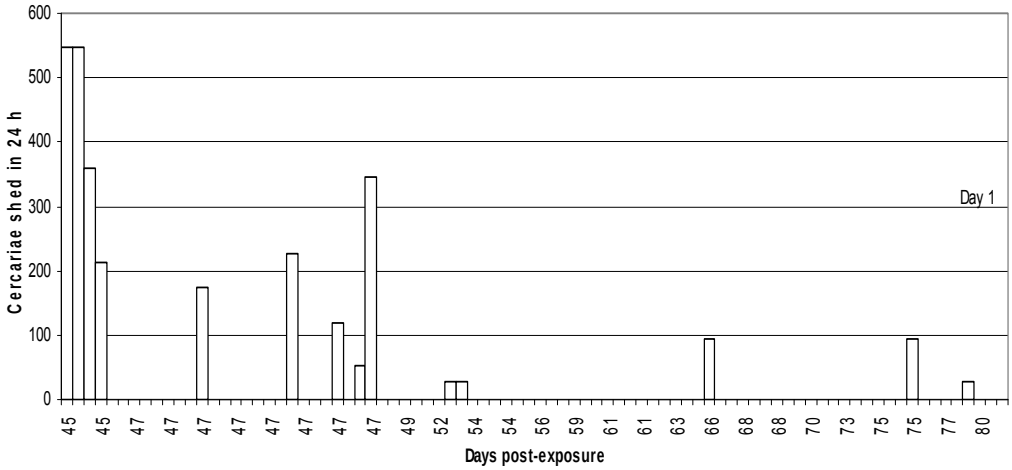

Figure 3.1:

Number of *Bolbophorus damnificus* cercariae shed 24 hours after removal from aquarium by *Biomphalaria havanensis* exposed to *B. damnificus* ova. (Each bar represents an individual snail)

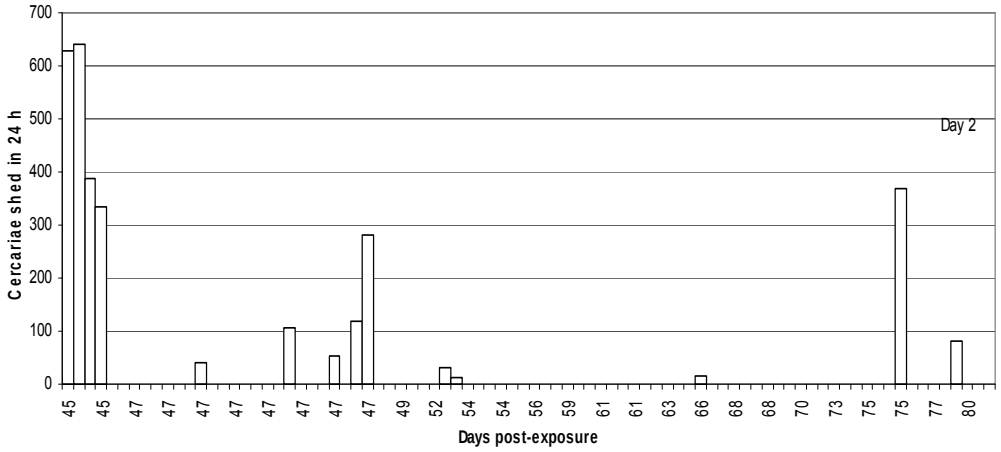

Figure 3.2:

Number of *Bolbophorus damnificus* cercariae shed 48 hours after removal from aquarium by *Biomphalaria havanensis* exposed to *B. damnificus* ova. (Each bar represents an individual snail)

Two snails were used for molecular identification of snail species at the University of New Mexico. Two slightly different partial 16S sequences were obtained by using PCR primers (DeJong et al. 2001). Examination of the sequence data confirmed that the species were *Biomphalaria* with 99% matches to *Biomphalaria obstructa*, *Biomphalaria havanensis* and *Biomphalaria temascalensis*. Because the sequence data failed to distinguish the specimens and due to the continued confusion over species in this group, morphological characteristics were relied upon for positive identification of *B. havanensis*.

Discussion

This study demonstrates that an alternative snail, *B. havanensis*, can serve as an intermediate host for *B. damnificus*. This snail species has been collected from several commercial catfish ponds by our laboratory. However, none of these snails have been found to be naturally infected with *B. damnificus*. In order to determine if this infection occurs naturally, a more methodical survey needs to be completed. Although there have not yet been any *B. havanensis* confirmed to be naturally infected with *B. damnificus*, this study demonstrates that this snail has the potential to serve as a host for this parasite. The high concentration of *P. erythrorhynchos* ingesting catfish, loafing and defecating *B. damnificus* ova in high numbers near the pond perimeter provides the ideal scenario to perpetuate the *B. damnificus* life cycle in a susceptible molluscan species such as *B. havanensis*.

In this study, *B. havanensis* were shedding *B. damnificus* cercariae at the first observation period, day 45. Previous reports with a closely related species, *Bolbophorus confusus* demonstrated cercarial shedding by *P. trivolvis* snails between 30- 51 days after exposure to trematode eggs when kept at 21- 24°C (Fox 1965). In order to ensure the maximum exposure time to the hatching of *B. damnificus* ova, *B. damnificus* ova and *B. havanensis* snails were kept at 18- 23°C and day 45 was chosen to be the first observation period. Now that it is confirmed that *B. havanensis* can serve as an intermediate host, further life cycle studies need to be done to determine when cercariae shedding first occurs, the number of cercariae shed per day and the duration of shedding.

Bolbophorus damnificus control efforts have focused on the eradication of the snail intermediate host, *P. trivolvis*. Shore-line chemical treatments with copper sulfate-citric acid combinations and slurried hydrated lime have shown to be efficacious against *P. trivolvis* (Mitchell 2002; Mitchell et al. 2007). These treatments efficiently eradicate *P. trivolvis*, but whether these same treatments are efficacious against *B. havanensis* is currently unknown.

The propagation of digenetic trematodes relies on their ability to move from host to host. Parasites vary from one another in their host specificity. Thus far, the demonstrated definitive avian host for *B. damnificus* is the American white pelican (Overstreet et al. 2002). Recently it has been demonstrated that not only are *B. damnificus* metacercariae found in channel catfish but the fathead minnow can also serve as an intermediate host (Overstreet et al. 2002; Mitchell et al. 2006). This study indicates

that *B. damnificus* may also have a broader range of molluscan hosts than previously suspected.

Although there is very little information on the prevalence of *B. havanensis* in catfish ponds at this time, the implications of this study warrant further research into the life cycle of this parasite and its molluscan hosts. This study confirms that *B. havanensis* can serve as an intermediate host for *B. damnificus* in an artificial infection, however an extensive survey needs to be done to determine if this phenomenon occurs naturally. Further life cycle studies are needed to be done to determine the initial duration of *B. damnificus* cercariae shedding and the number of cercariae shed per day. If it is found that *B. havanensis* is a natural host for *B. damnificus*, additional studies need to be done on the life cycle of *B. damnificus* in this snail and potential measures to control this snail host.

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CHAPTER IV

HISTOPATHOLOGY OF *BOLBOPHORUS DAMNIFICUS* IN

CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) FINGERLINGS

Abstract

Natural infections of *Bolbophorus damnificus* in commercial channel catfish (*Ictalurus punctatus*) have been associated with mortalities and reduction in growth and feed intake. Preliminary research in our laboratory using artificial infections have demonstrated that mortalities can occur on day 5 post-infection (5 cm fingerlings) when fish are challenged with 200 *B. damnificus* cercariae/ fish. In order to better understand this infection and the subsequent pathology associated with this parasite over the course of 6 days, a titration study was done. Cercariae, confirmed by PCR to be *B. damnificus*, were collected from snails *(Planorbella trivolvis*), pooled and enumerated. Fingerling catfish (n=20/group) were individually exposed to 0, 25, 50, 100 or 200 cercariae for 2 hours. After exposure, catfish were rinsed and held in flow-through tanks. Fish were necropsied at 3, 4, 5 and 6 days post-infection. Mortalities were first observed on day 5 in fish exposed to 200 (13%) and day 6 in fish exposed to 200 (49%), 100 (33% mortality) and 50 (7% mortality) with associated ascites in each group (53, 31 and 2% respectively). The trial was terminated on day 6 post-challenge, gross lesions were recorded and organs and muscle samples were collected for histopathology. Histopathology results showed a

gradual loss of hepatocellular vacoulation in the liver and decreased number of lymphocytes present in the spleen as the infective dose of cercariae increased.

Introduction

Infections with the digenetic trematode *Bolbophorus* spp. in channel catfish (*Ictalurus punctatus*) have been associated with morbidity and mortality in commercial catfish. In the life cycle of *Bolbophorus* sp., the first intermediate host is the ram's horn snail, *Planorbella trivolvis*, the second intermediate host is the channel catfish and the definitive host is the American white pelican, *Pelecanus erythrorhynchos*. This trematode, now identified as *Bolbophorus damnificus* (Overstreet et al. 2002) was first documented in Louisiana in 1994 (Labrie et al. 2004) and later in Mississippi (Terhune et al. 2002). Since the first documented *B. damnificus* reports, the presence of this parasite in commercial catfish appears to have steadily increased. A field survey of 821 channel catfish ponds demonstrated that 262 (31.9%) of these ponds had catfish with *B. damnificus* metacercariae (Terhune et al. 2002).

A more recent field survey demonstrated that even at subclinical levels of infection, this parasite had significant economic impact in infected ponds (Wise et al. 2006). In this study, Wise et al. (2006) evaluated a catfish farm with 40 food fish ponds reporting *B. damnificus* infections. Twenty to thirty fish from each pond were sampled and metacercariae enumerated using a scoring system of light (1 -33% fish with metacercariae), moderate (34-66% fish with metacercariae) and heavy (67- 100% fish with metacercariae). Results from this survey found only 43% ponds (17/ 40 ponds) were

negative for trematodes while 15% (6/ 40 ponds) had light infections, 15% (6/ 40 ponds) with moderate infections and 27.5% (11/ 40 ponds) had heavy infections. Combining these findings with revenue produced (pounds of fish produced, etc.), and variable costs (feed price, amount of feed used, etc.), the economic impact of *Bolbophorus* spp. infections was determined. It was also found that feed consumption decreased with the increasing level of *Bolbophorus* infections and ponds with scores of light, moderate, and heavy infections produced 13.8%, 36.0% and 40.5% less pounds of fish per acre, respectively. This resulted in an 80.8% reduction in net return in ponds with light infection and negative net returns in those ponds scored with moderate and heavy infections (Wise et al 2006).

Although the adverse effects of the trematode to catfish production have been documented, the pathogenesis of this parasitic disease is poorly understood. To date only two artificial challenge studies have documented histopathological lesions associated with *Bolbophorus* spp. infections (Terhune et al. 2002; Labrie et al. 2004). However, while both used *Bolbophorus*- type cercariae, they were not molecularly confirmed as *B. damnificus* cercariae. Terhune et al. (2002) reported severe fish loss in several commercial catfish operations. Snails infected with *Bolbophorus* spp. from those ponds were collected and specific pathogen free (SPF) catfish were exposed to random numbers of shed cercariae. Reported pathological changes included subdermal nodules surrounded by granulomatous inflammation and renal tubular necrosis (Terhune et al. 2002).

Labrie et al. (2004) documented the pathologic effects of concurrent infections of *B. damnificus* and *Edwardsiella ictaluri*. In this study, SPF catfish fingerlings were

introduced to tanks with naturally infected *P. trivolvis* (n= 14) shedding approximately 770 *Bolbophorus* spp. cercariae/ 24 hours. After the 24 hour exposure, fish were exposed to a virulent strain of *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC). Fish were sampled daily and mortality was noted. Labrie et al. (2004) found mortalities did not occur in the fish exposed to *Bolbophorus* spp.-only treatment group, but were observed in the *Bolbophorus* spp.- ESC treatment group (84%) and the ESC-only treatment (46%). Twenty-four hours post-exposure, there was no histological evidence of cercariae penetration or metacercariae development (Labrie et al 2004). Seven days post-challenge, metacercariae were grossly visible on dead fish and histopathologically there were stellate fibroblasts, myofiber fragments, eosinophilic debris and macrophages surrounding the developing metacercariae (Labrie et al 2004). Prior to day 9 post-infection, bacterial colonies were noted within the metacercariae capsule of those exposed and those not exposed to *E. ictaluri* (Labrie et al 2004). At 11 days post-infection, fibroblasts in the capsule began to become more flattened and by day 12 post-infection, the hyaline membrane (parasite cyst wall) surrounding the metacercariae was evident (Labrie et al 2004). At days 13-18 post-infection there was further organization of the metacercariae capsule and occasionally mixed inflammatory infiltrates were present. Changes to host tissue surrounding the metacercariae were noted throughout this experiment but lesions in internal organs were not described (Labrie et al. 2004). Although this study did confirm molecularly that metacercariae excised from challenged fish were *B. damnificus*, the cercariae used in the study were not molecularly confirmed to be *B. damnificus* prior to the challenge.

Gross and histopathological observations in channel catfish caused by *Bolbophorus* spp. have been previously documented. Metacercariae cysts were approximately 1 mm in diameter or less, typically occurring near the caudal peduncle, but also noted in internal organs in heavy infections. Ascites was often seen and histologically a granulomatous reaction was observed surrounding the parasitic cyst and renal tubule necrosis was detected (Hawke and Khoo 2004). *Bolbophorus damnificus* metacercariae in naturally infected catfish were also been briefly described by Overstreet et al. (2002). The metacercariae were surrounded by a transparent cyst of parasite origin encased by a smooth-walled host cyst and were typically elliptical and found immediately under the dermis (Overstreet et al. 2002).

Cultured fathead minnows collected from a baitfish pond in the Mississippi Delta were recently found to be naturally infected with both *B. damnificus* and *Bolbophorus* sp. (Type 2). Histological examination showed most of the metacercariae were surrounded by a thin cyst wall, but had little or no accompanying inflammatory response. A few encysted metacercariae that appeared to elicit an inflammatory response were surrounded by macrophages. Results from PCR confirmed both *Bolbophorus* species were present in the metacercariae excised from these minnows, but the species of *Bolbophorus* metacercariae in the histological lesions were not confirmed with molecular analysis (Mitchell et al. 2006).

Although these studies are indicative of tissue changes associated with *Bolbophorus* spp. infections, none of these reports confirmed with molecular analysis that these findings could only be attributed to *B. damnificus* infections. There are currently

several known species of *Bolbophorus* found in the Mississippi Delta, *B. damnificus* and an unnamed species referred to as *Bolbophorus* sp. (Type 2; Levy et al. 2002). The ram's horn snail is a host to both of these species and often dual infections occur in the same snail (Flowers et al. 2005). Species specific PCR primers are used to confirm the identity of cercariae (Levy et al. 2002). Although preliminary studies indicate that *Bolbophorus* sp. (Type 2) may not form mature metacercariae in the channel catfish, it is still unknown whether this species can penetrate, infect the channel catfish, and cause subsequent pathologic changes.

While past studies provide some insight into histopathological lesions associated with *Bolbophorus* species infections, the question remains whether pathological changes could solely be attributed to *B. damnificus*. Additionally, the pathology associated with this parasite as the infection progresses in the catfish is unknown. To address these questions, artificial challenge studies with molecularly confirmed *B. damnificus* need to be completed. This study will examine the pathologic changes associated with molecularly confirmed *B. damnificus* infections in catfish over the course of the infection and the effects the level of challenge has on the severity of this pathology.

Materials and methods

Planorbella trivolvis snails were collected from catfish ponds with *Bolbophorus* spp. infections and transported in pond water to the Parasitology Lab at the College of Veterinary Medicine, Mississippi State University. Twenty-four hours post-collection, each snail was individually placed in plastic dilu-vials with 10 mL of dechlorinated water. Snails were examined microscopically at 24, 48 and 36 hours. Snails that did not

shed cercariae after 36 hours were transferred to aquaria in the snail colony at Mississippi State University College of Veterinary Medicine.

Snails shedding *Bolbophorus*-type cercariae were assigned a number. Cercariae were collected from each of these snails and placed in individual 15 mL test tubes making sure the cercariae population from each snail remained separate. Each test tube containing cercariae was placed on ice water for approximately 30-60 minutes allowing cercariae to settle to the bottom of the tube. A volume of 300 µL from the bottom of each tube was collected and transferred to individual microfuge tubes to which were added 300 µL cell lysis solution and 1.5 µL proteinase K. DNA was then extracted using the Purgene DNA purification kit (Gentra Systems, Minneapolis, Minnesota) following the manufacturers protocol, but performing half reactions. *Bolbophorus damnificus* species-specific primers, P1-650F (5'-TCA GTT TCG AAC GAT GAT GA-3') and P1-14700R (5'-CGG TCT ACG GTT CCA CC-3') were used (Levy et al. 2002). Reaction volumes for PCR were 25 µL using 10X buffer, 2.0 µL template, 0.625 U *Taq* polymerase (Hot Start Taq; Takara Bio, Inc., Shiga, Japan), 200 nM of each primer, and 200 µM of each deoxynucleotide triphosphate. Polymerase chain reaction conditions were those described by Levy et al. (2002) except that the reaction times were shortened and were 92 °C for 5 min followed by 35 cycles of 94 °C for 1.0 min, 58 °C for 1.0 min, and 72 °C for 1.0 min, with a final cycle of 72 °C for 5.0 min. This was carried out on a MJ Research PTC-100 Peltier Thermal Cycler. Polymerase chain reaction products were visualized on a 1.2% agarose gel along with positive and negative controls. The gel was post-stained

with gelstar nucleic acid stain (Combrex BioScience Rockland Inc.) and observed under ultraviolet light.

Snails identified by PCR to be positive for only *B. damnificus* were used as a source for the artificial infections. Specific pathogen free (SPF) channel catfish (approximately 5 cm in length) obtained from the Mississippi State University College of Veterinary Medicine hatchery were placed in individual containers containing 250 mL of dechlorinated water with continual aeration. Prior to challenge, cercariae from the *B. damnificus* positive snails were collected, pooled and enumerated using a previously described standard protocol (Yost et al. 2008). A subsample of cercariae used in each challenge was placed in 70% ethanol for further molecular analysis to confirm that each fish challenge was pure *B. damnificus* cercariae.

This study consisted of three identical experiments, with variable sampling times (Table 4.1). In each experimental group, 20 fish were challenged with 25, 50, 100, 200 cercariae/fish for two hours. Negative controls were exposed to water only. Two hours post-exposure, fish from the same challenge group were pooled together, rinsed three times with dechlorinated water and housed in 10 gallon flow-through tanks for the duration of the study. Fish were checked twice daily and fed a standard commercial catfish floating fed ad libitum.

Table 4.1:

Experimental design for experiments 1, 2 and 3.

	Experiment 1	Experiment 2	Experiment 3
Cercariae doses	0, 25, 50, 100, 200	0, 25, 50, 100, 200	0, 25, 50, 100, 200
Number fish per dose	20	20	20
Sampling times post-infection	Day 3, Day 6	Day 5, Day 6	Day 4, Day 6
Number fish necropsied	Day 3= 5 Day 6= 11[A]	Day 5= 5 Day 6= 15	Day 4= 5 Day 6= 14[B]

[A]Only 11 fish available at day 6 post-challenge sampling due to four mortalities on day 5 post-challenge. [B]Only 14 fish available at day 6 post-challenge due to the loss of one fish.

Fish (n= 5) from each challenge group were sampled at days 3 (experiment 1), 4 (experiment 3) and 5 (experiment 2) post-infection (Table 4.1). The remaining fish (n= 15) from each group were terminated and necropsied at 6 days post-infection in each experiment, unless fish died prior to day 6. At each necropsy, fish were euthanized with ethyl 3-aminobenzoate methanesulphonate salt (Sigma, St. Louis, Missouri) and gross lesions recorded. Coelomic cavities were opened ventrally and fish were placed in 10% neutral buffered formalin for a minimum of 24 hours. Fish were trimmed into 4 sections (at the level of the eye, at the dorsal commisure of the operculum, just caudal to the pectoral girdle and anterior to the dorsal fin, and between the pelvic fin and the anal fin). Sections were chosen to ensure that the major organs could be examined and to reflect

areas where the heaviest concentrations of *B. damnificus* metacercariae typically occur. Spleens were removed by sharp dissection to ensure they were included in the sections. 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 6 µm. Prepared slides were then stained with hematoxylin and eosin (Luna 1968).

Results

Fish observations

Fish mortality (n= 4) was first observed at day 5 post- infection in fish exposed to 200 cercariae (experiment 1). On day 6 post- infection, mortalities occurred in all experiments in the fish groups challenged with 200 cercariae/ fish with losses of 100%, 87% and 20% in experiments 1, 2 and 3, respectively (Tables 4.2- 4.4). Losses also occurred at dose levels of 100 cercariae/ fish in experiment 1 (100%) and in 7% of the fish challenged with 50 cercariae/ fish in experiment 2 (Tables 4.2- 4.4). No mortalities occurred in any of the groups challenged with 25 cercariae/ fish in any of the experiments.

Table 4.2:

Experiment 1: Post-mortem observations 6 days post-challenge.

Cercariae/ fish	% mortalities prior to termination	% ascites	% hemorrhagic foci on ventral head region	% right side hemorrhagic foci (Average, Range)	% left side hemorrhagic foci (Average, Range)	% ventral hemorrhagic foci (Average, Range)	% hemorrhagic foci in oral cavity	% exophthalmia
200*	100% (15/15)	73% (8/11)	91% (10/11)	100% (6.9, 2- 11)	100% (5.4, 1- 9)	100% (5.0, 2- 10)	0	9.0% (1/11)
100	100% (15/15)	80% (12/15)	47% (7/15)	6.4 (2- 12)	4.0 (0- 7)	5.4 (2- 9)	7% (1/15)	7% (1/15)
50	0	7% (1/15)	73% (11/15)	100% (3.1, 0- 11)	100% (3.8, 1- 7)	100% (3.7, 0- 8)	0	0
25	0	0	0	87% (1.13, 0- 6)	100% (1.27, 0- 5)	93% (0.67, 0- 3)	0	0
Negative Control	0	0	0	0	0	0	0	0

Table 4.3:

Experiment 2: Post-mortem observations 6 days post-challenge.

Cercariae/ fish	% mortalities prior to termination	% ascites	% hemorrhagic foci on ventral head region	% right side hemorrhagic foci (Average, Range)	% left side hemorrhagic foci (Average, Range)	% ventral hemorrhagic foci (Average, Range)	% hemorrhagic foci in oral cavity	% exophthalmia
200	87% (13/15)	60% (9/15)	93% (14/15)	100% (6.6, 1- 12)	100% (7.1, 1- 10)	100% (8.2, 1- 15)	20% (3/15)	13% (2/15)
100	0	7% (1/15)	33% (5/15)	67% (2.33, 0- 6)	40% (1.6, 0- 6)	87% (4.67, 0- 9)	13% (2/15)	7% (1/15)
50	7% (1/15)	0	20% (3/15)	67% (1.27, 0- 5)	33% (0.8, 0- 4)	33% (1.2, 0- 6)	0	0
25	0	0	0	27% (0.27, 0- 1)	20% (0.2, 0- 1)	7% (0.07, 0- 1)	0	0
Negative Control	0	0	0	0	0	0	0	0

Table 4.4:

Experiment 3: Post-mortem observations 6 days post-challenge.

Cercariae/ fish	% mortalities prior to termination	% ascites	% hemorrhagic foci on ventral head region	% right side hemorrhagic foci (Average, Range)	% left side hemorrhagic foci (Average, Range)	% ventral hemorrhagic foci (Average, Range)	% hemorrhagic foci in oral cavity	% exophthalmia
200	20% (3/15)	26% (4/15)	87% (13/15)	87% (5.2, 0-11)	93% (3.8, 0-8)	100% (6.27, 2- 11)	0	13% (2/15)
100	0	7% (1/15)	13% (2/15)	33% (0.6, 0-3)	33% (0.6, 0-4)	80% (2.33, 0- 5)	0	13% (2/15)
50	0	0	0	14% (0.07, 0- 1)	14% (0.14, 0- 1)	20% (0.43, 0- 3)	0	0
25*	0	0	0	0	0	0	0	0
Negative Control	0	0	0	0	0	0	0	0

Gross observations

On day 6 post-infection in experiments 1- 3, minimal to mild abdominal distension was observed in 73%, 60% and 26% of fish challenged with 200 cercariae in experiments 1-3, respectively. Similar findings occurred in 80%, 7% and 7% of fish challenged with 100 cercariae in experiments 1-3, respectively. Distention was only observed in 7% of fish challenged with 50 cercariae in experiment 1 (Tables 4.2- 4.4) and none of the fish challenged with 25 cercariae. The distension was caused by small amounts (usually less than 1 mL) of a yellow tinged ascitic fluid. Exopthalmia was only observed in fish challenged with 200 cercariae (n=5) and 100 cercariae (n=4).

Papular lesions with associated petechial and ecchymotic hemorrhages were observed especially on ventral surfaces of the head posterior to the dentary bone, between the articular bones and around the pectoral girdle, hereafter referred to as the ventral head region. Similar hemorrhagic lesions were also present on the right, left and ventral surface of the trunk in all groups challenged with 100 or 200 cercariae/ fish (Tables 4.2- 4.4; Figures 4.1- 4.3). Lesions in the ventral head region were present in 87% to 93% of fish challenged with 200 cercariae in experiments 3 and 2, respectively (Tables 4.2 and 4.4; Figures 4.1 and 4.3); in 13% to 47% of fish challenged with 100 cercariae from experiments 3 and 1, respectively and in 20% to 73% of the fish challenged with 50 cercariae from experiments 2 and 1 (Figures 4.1 and 4.3). There were no lesions in fish exposed to 25 cercariae/ fish.

Figure 4.1:

Fish challenged with 200 cercariae exhibiting hemorrhagic foci on the left side, ventrally and on ventral head region (experiment 1).

Figure 4.2:

Fish challenged with 200 cercariae exhibiting hemorrhagic foci and unilateral exopthalmia (experiment 1).

Figure 4.3:

Fish challenged with 200 cercariae exhibiting hemorrhagic foci on ventral head region (experiment 1).

The papular lesions with petechial hemorrhages (hereafter referred to as hemorrhagic foci) were present on the right and left side and ventrally in 100% of the fish challenged with 200 cercariae in experiments 1 and 2 (Tables 4.2- 4.3; Figures 4.1- 4.2). It was also observed in experiments 1- 3 in fish challenged with 100 and 50 cercariae/ fish and in those fish challenged with 25 cercariae/ fish in experiments 1 and 2 (Tables 4.2- 4.4).

The average number of hemorrhagic foci observed on the right and left side and ventrally were the highest in fish challenged with 200 cercariae with the average number of hemorrhages ranging from 5.2 (experiment 3) to 6.9 (experiment 1) on the right side; 3.8 (experiment 3) to 7.1 (experiment 2) on the left side and 5.0 (experiment 1) to 8.2

(experiment 2) ventrally (Tables 4.2- 4.4). Hemorrhagic foci were also observed in these areas in fish challenged with 50 and 100 cercariae in experiments 1- 3; but only in experiments 1 and 2 in fish challenged with 25 cercariae.

Hemorrhagic foci in the oral cavity were only present in three fish in experiment 2 challenged with 200 cercariae/ fish, and 1 and 2 fish challenged with 100 cercariae/ fish in experiments 1 and 2, respectively (Tables 4.2- 4.4). None were observed in the mouths of any fish challenged with 25 or 50 cercariae. Exophthalmia (bilateral or unilateral) was observed in fish challenged with 200 cercariae/ fish in experiments 1- 3 in one, two and two fish respectively, and in fish challenged with 100 cercariae/ fish in experiments 1- 3 in 1, 1 and 2 fish, respectively (Tables 4.2- 4.4; Figure 4.2).

Histopathology results

Consistent histopathological changes were mainly limited to the liver, spleen and musculoskeletal tissues. In the liver, there appeared to be a gradual loss of the typical hepatocellular vacuolation, resulting from lipid and glycogen stores, with increasing dose of cercariae (Figures 4.4- 4.6). The livers of control fish had moderate, diffuse, microvesicular vacuolation (Figure 4.7). Hepatocytes were large with pale eosinophilic cytoplasm, numerous non-discrete small clear vacuoles and often a peripherially displaced nucleus. These hepatocytes were in close apposition to each other and sinusoidal spaces were not readily apparent. Even by day 3 post-challenge, fish challenged with 200 cercariae had hepatocytes that were darker with a more condense cytoplasm, less vacoulation and more prominent sinusoidal spaces (Figure 4.4). By day 6

post-challenge changes were more pronounced, hepatocytes had little or no vacuolation, and the sinusoidal spaces were often very prominent with distinct, wide clear spaces separating the branching laminae of hepatocytes (Figures 4.5 and 4.6). Absence of digesta in the gastrointestinal tract of the infected fish usually corresponded with these hepatocellular changes. Fish infected at lower doses also had these fairly consistent hepatocellular changes although they tended to be less marked. Congestion in the liver was also present in some infected fish but there was not a consistent finding or trend.

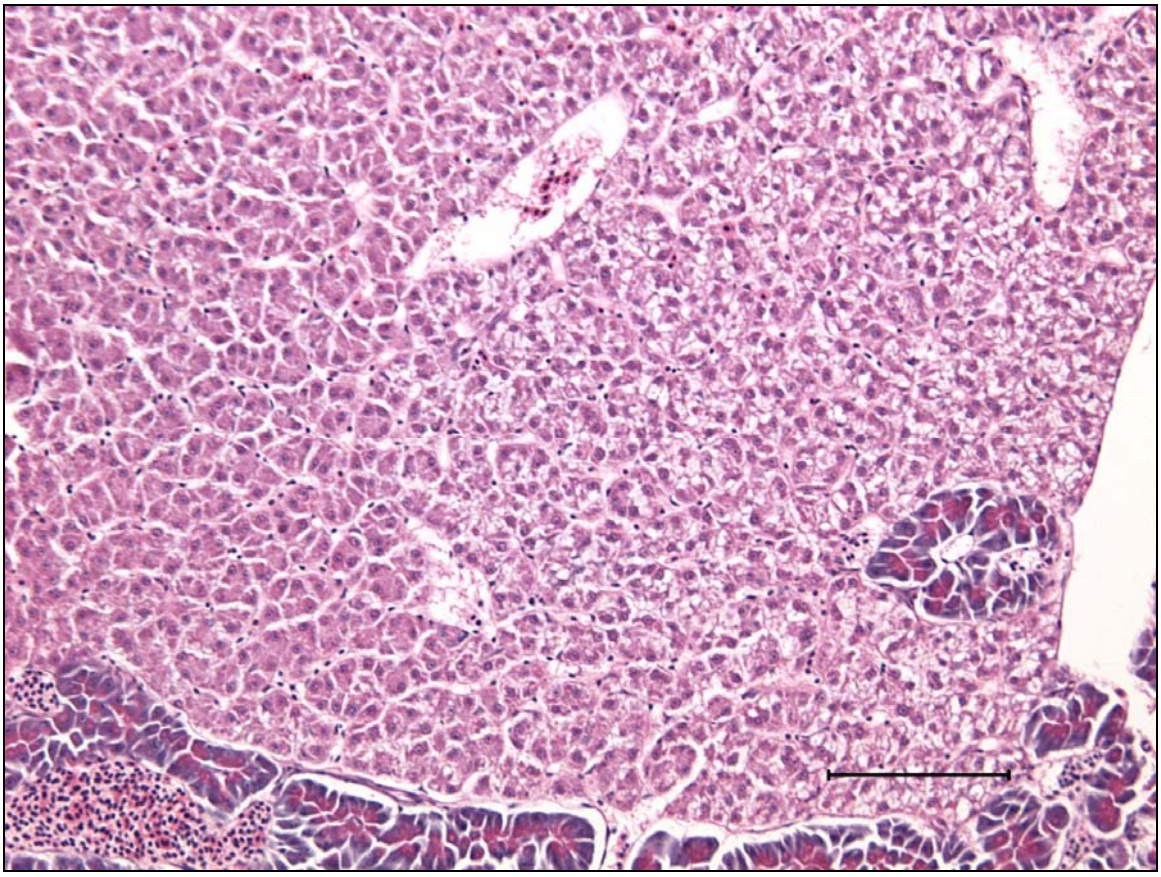

Figure 4.4:

Liver of channel catfish 3 days post-challenge with 200 cercariae (experiment 1) revealing decreased hepatocellular vacuolation (H & E; Bar ≈ 100µm; 200X).

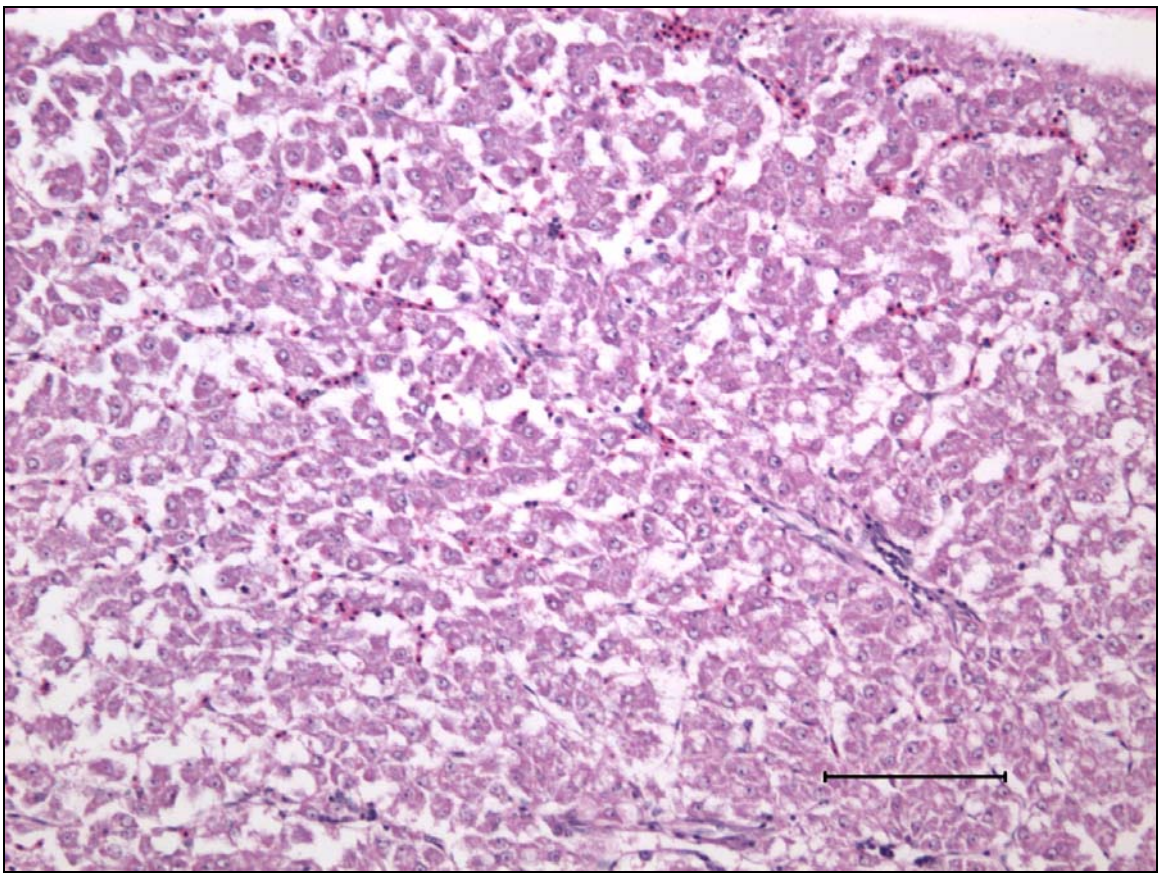

Figure 4.5:

Liver of channel catfish 6 days post-challenge with 100 cercariae (experiment 1) revealing decreased hepatocellular vacuolation and increased space between the hepatocyte laminae (H & E; Bar ≈100µm; 200X).

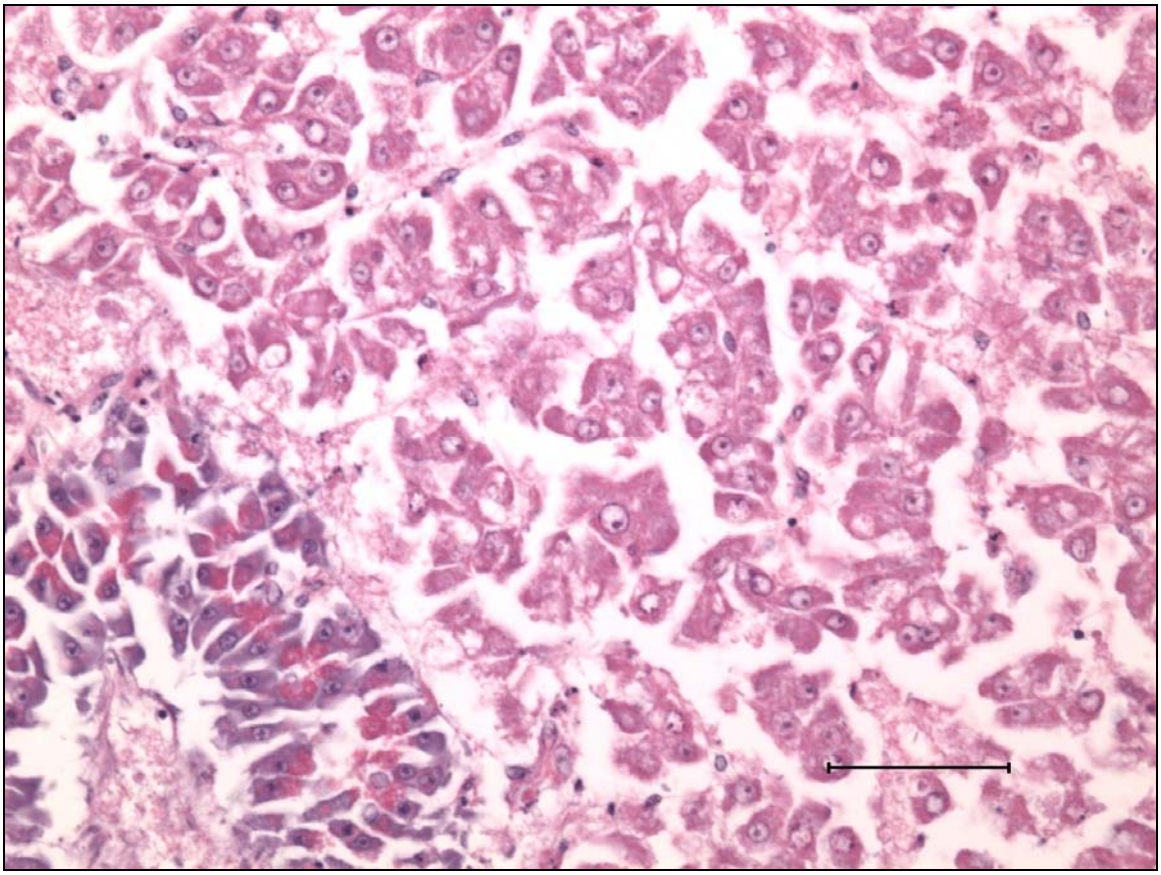

Figure 4.6:

Higher magnification of the liver of channel catfish 6 days post-challenge with 200 cercariae (experiment 1) revealing decreased hepatocellular vacuolation and increased intralaminar space (H & E; Bar ≈ 50µm; 400X).

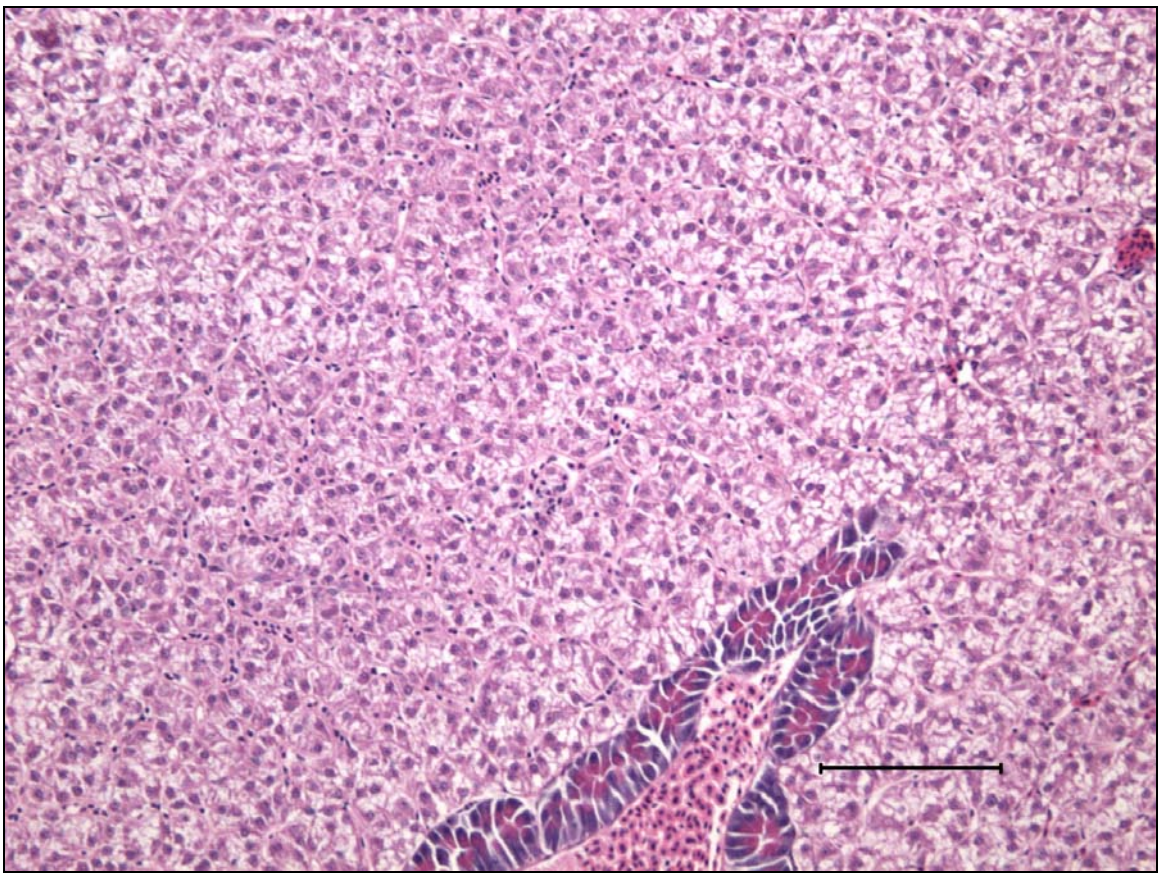

Figure 4.7:

Liver of a negative control fish (experiment 1). Note the diffuse, prominent hepatocellular vacuolation (H & E; Bar ≈ 100µm; 200X).

Changes in the spleen were less consistent than the liver. However, observable changes tended to correlate with the severity of the infective dose and time post-challenge (Figures 4.8- 411). Infected fish often had mildly to moderately congested spleens depleted of periarteriolar lymphocytes. Lymphoid depletion occurred with increasing cercariae dose and time, resulting in increased palor of periarteriolar lymphoid

sheaths with increased prominence of the reticular framework surrounding the arterioles. Changes were most significant in fish that had succumbed to the parasitic infection.

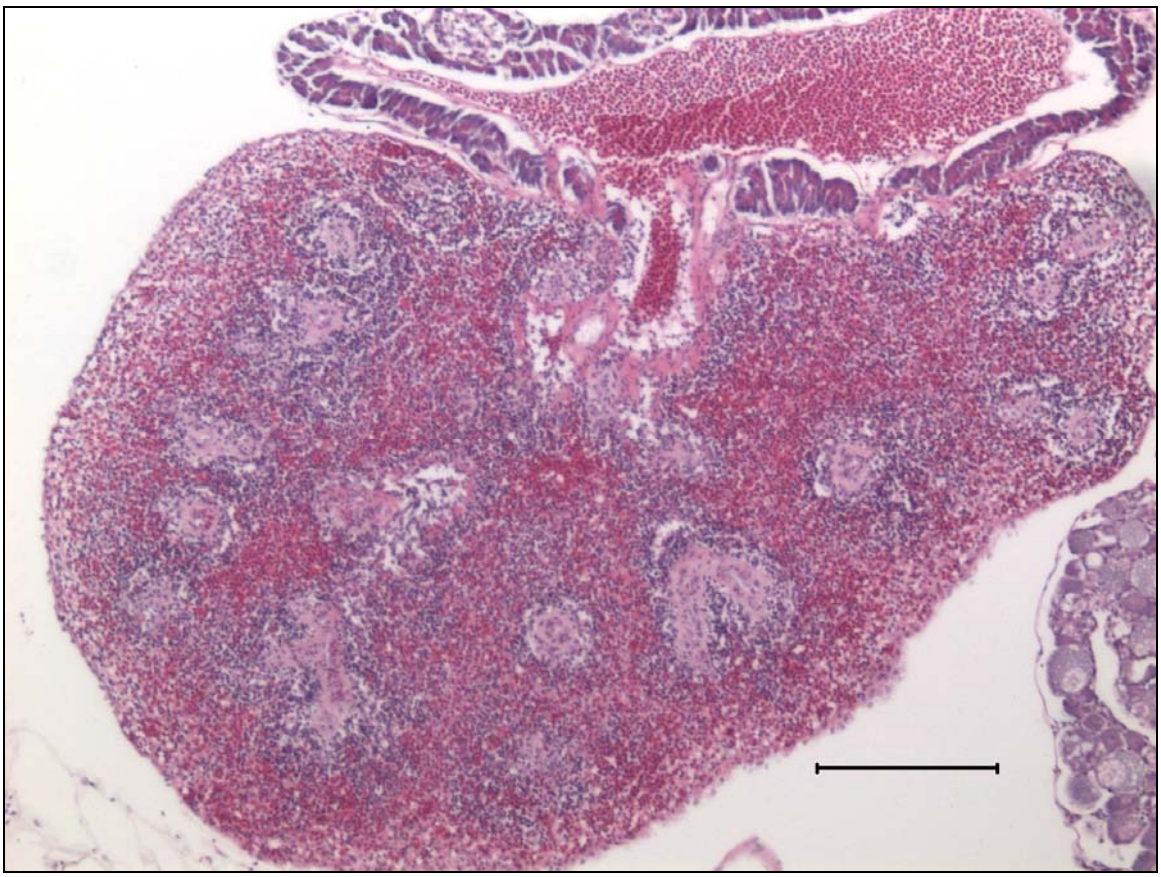

Figure 4.8:

Section of spleen from a channel catfish 3 days post-challenge with 200 cercariae (experiment 1). Note the congestion and relatively small numbers of lymphocytes present (H & E; Bar ≈ 200μm; 100X).

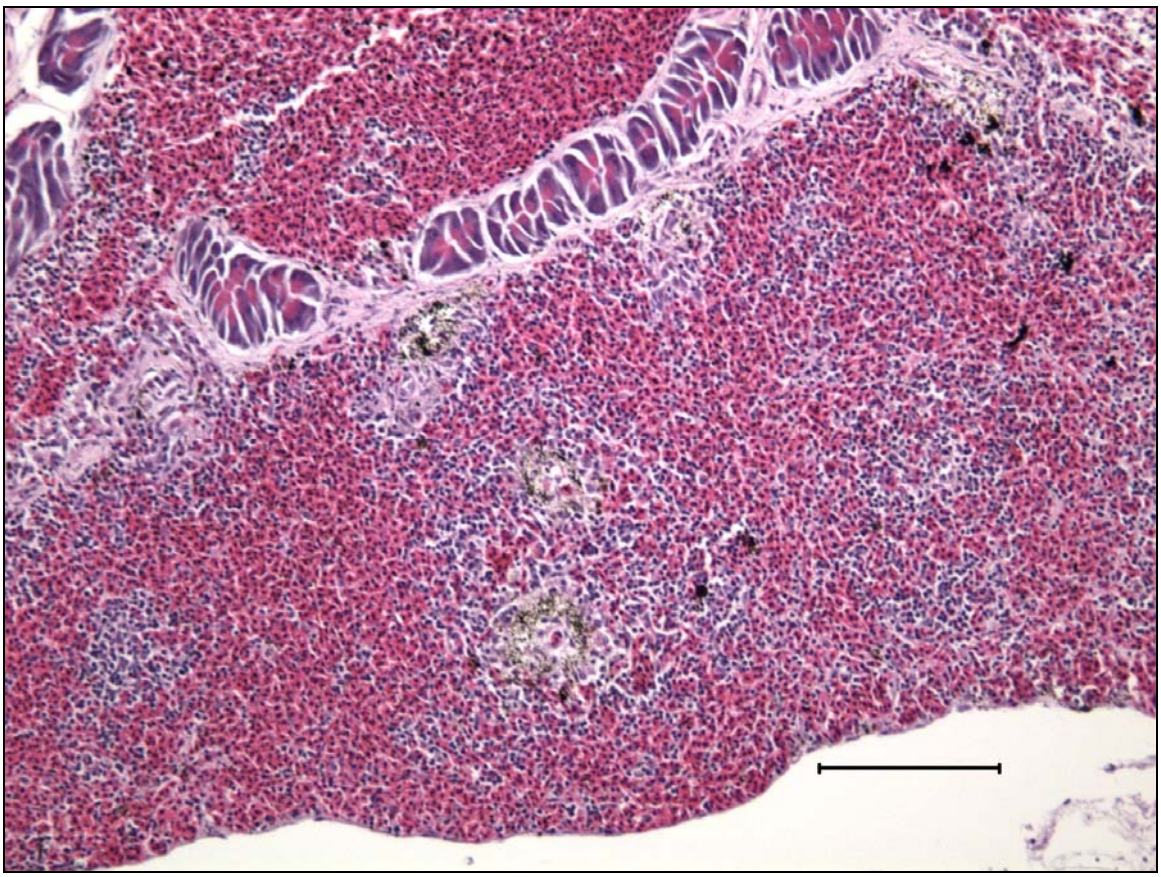

Figure 4.9:

Higher magnification of a section of spleen from a channel catfish 4 days-post challenge with 200 cercariae (experiment 3). Note the congestion and relatively small numbers of lymphocytes present (H & E; Bar ≈ 100μm; 200X).

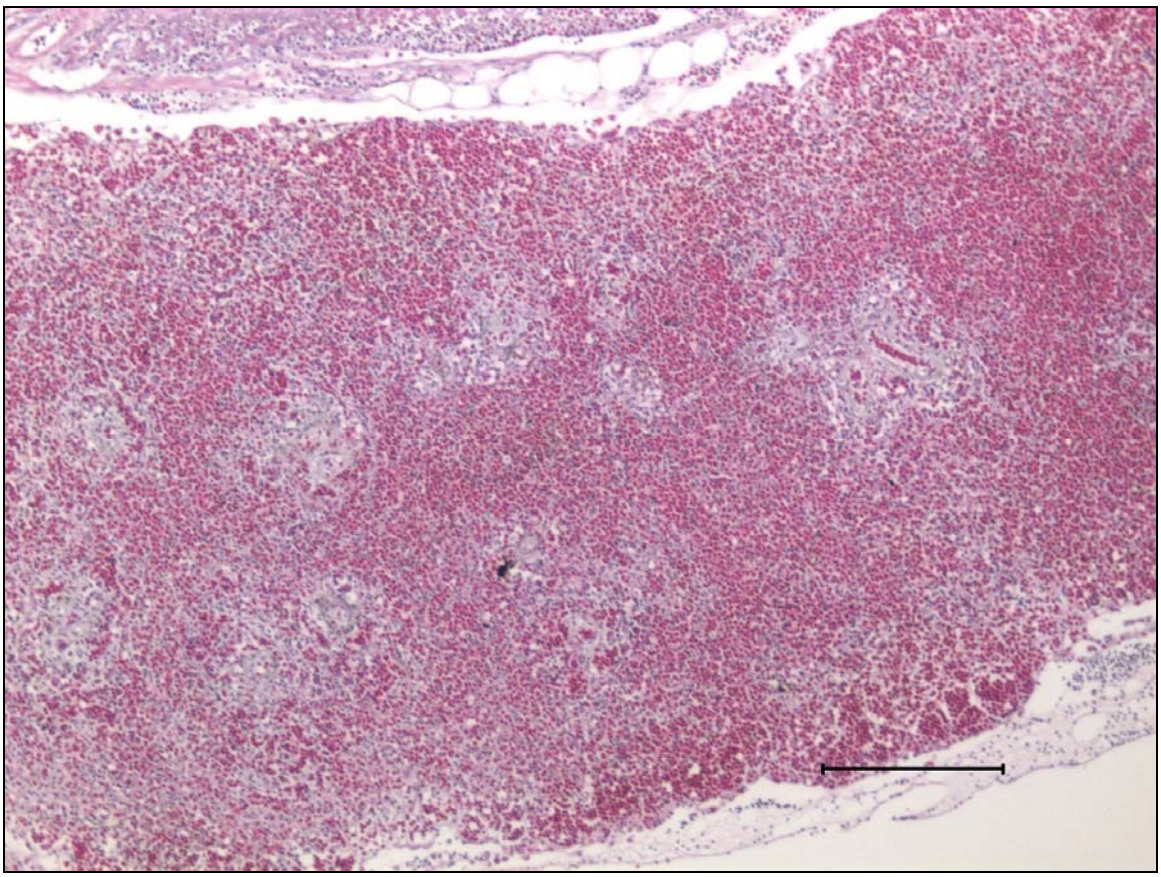

Figure 4.10:

Section of spleen from a channel catfish 6 days post-challenge with 100 cercariae (experiment 1). Note the congestion and relatively small numbers of lymphocytes present and increased prominence of the reticular framework and the increased pallor around the splenic arterioles (H & E; Bar ≈ 200µm; 100X).

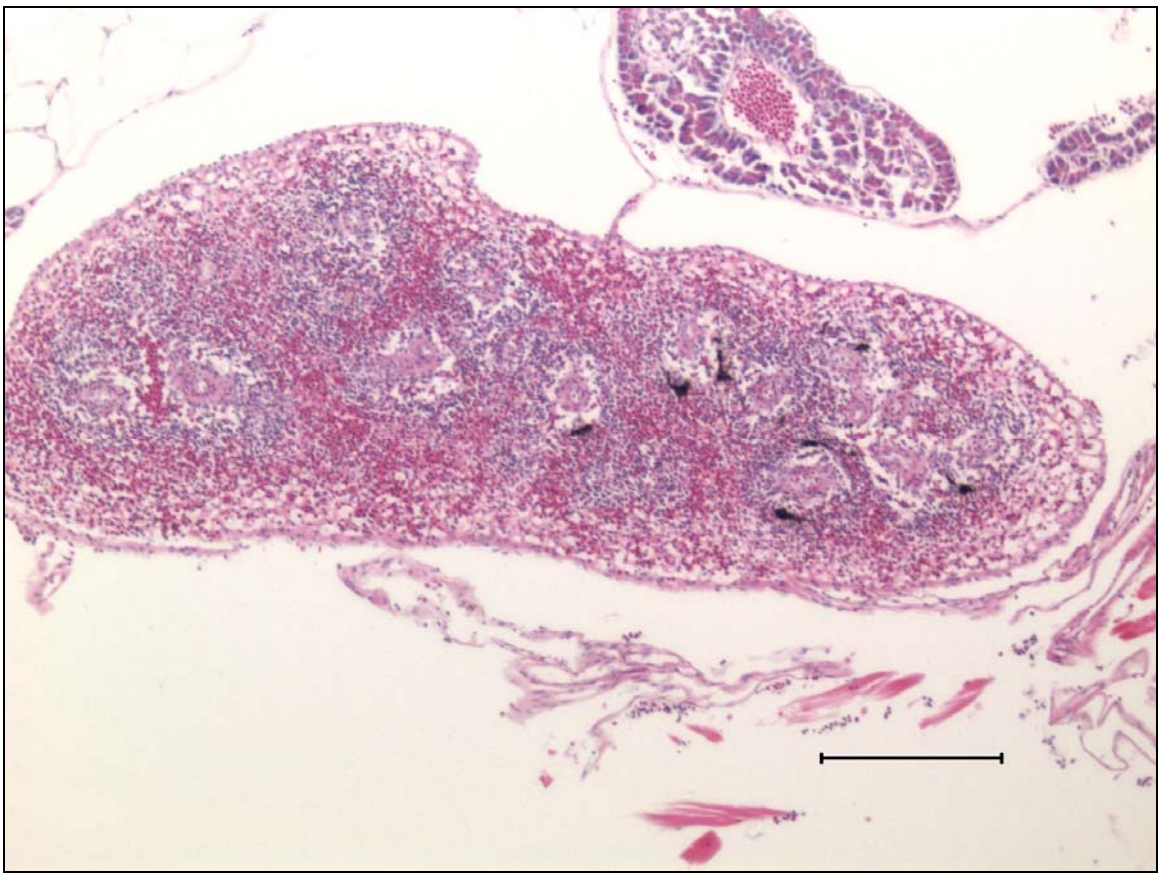

Figure 4.11:

Section of spleen from a negative control channel catfish (experiment 1). Note the basophilic cuffs of lymphocytes around the arterioles (H & E; Bar ≈ 200μm; 100X).

Significant histopathological findings were also present in skeletal muscle at sites of metacercarial encystment. In most cases, metacercariae were located in the superficial subcutaneous musculature. However, a few were found deeper in muscle, around the pharyngeal teeth, pectoral girdle, bordering the gills, around the heart, and within the muscular layers of the urinary bladder.

Although numerous metacercariae were examined, determining the point of entry and lesions associated with penetration of cercariae was difficult. In most instances, there was a discernable tract marked by accompanying proliferation of plump, spindloid fibroblasts, a few red blood cells, and rare mononuclear inflammatory cells. In only one fish exposed to 200 cercariae (6 day post-challenge), could changes be correlated between the point of cercarial penetration and a subadjacent developing metacercaria (Figures 4.12 and 4.13). There was a distinct depression in the epidermis and dermis, with loss of integrity of the epidermal layer. Empty spaces were created within the epidermis due to loss of normally prominent alarm cells. Several alarm cells adjacent to these clear spaces were shrunken, hypereosinophilic, with pyknotic nuclei indicative of necrosis. There was also mild mononuclear inflammatory cell infiltrate within the epidermis and dermis. In other sections, there was no depression in the epidermis and dermis although there were mild mononuclear infiltrates and similar but less severe changes to alarm cells.

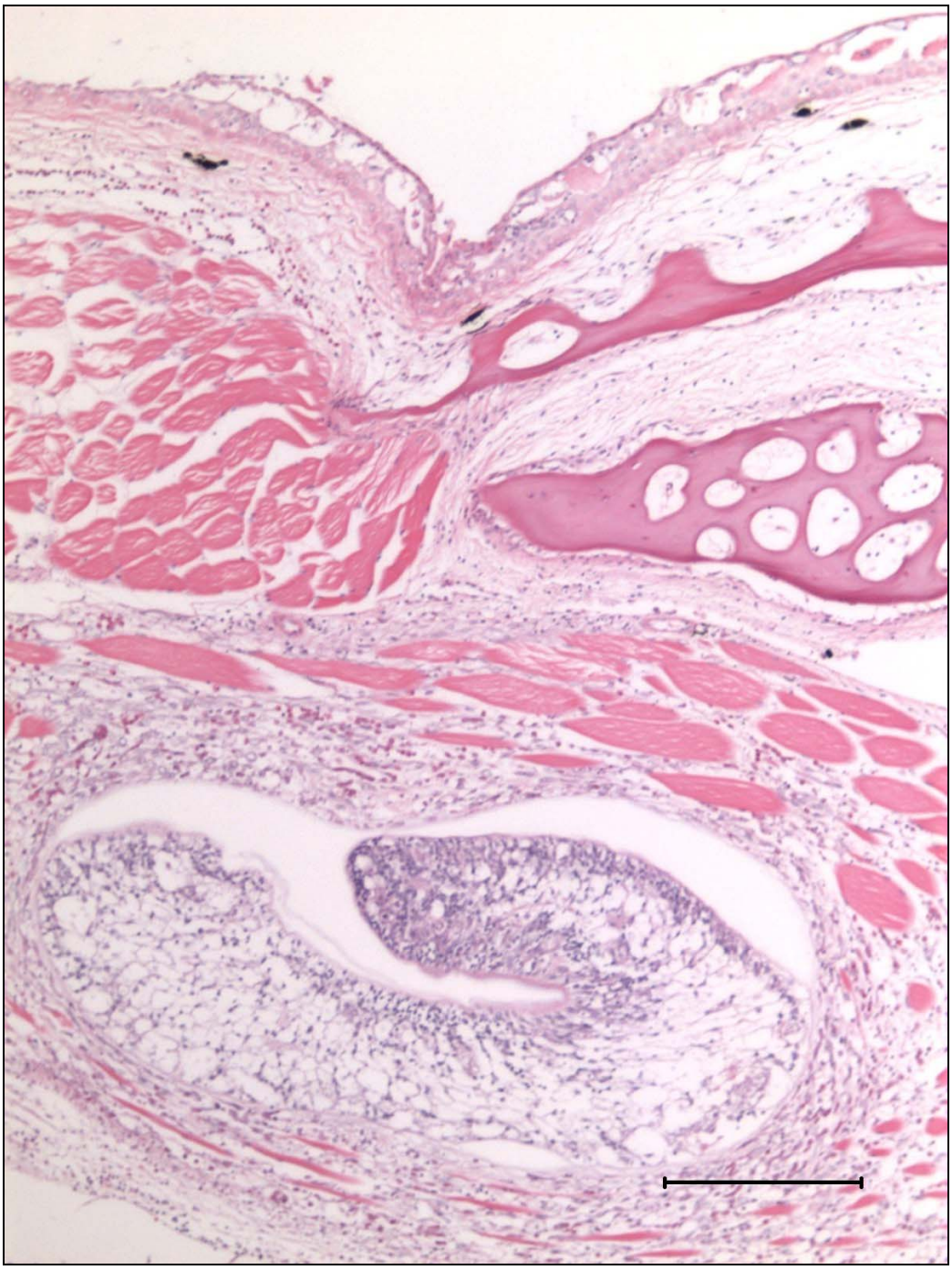

Figure 4.12:

Section of lateral body wall from a channel catfish 6 days post-challenge with 200 cercariae (experiment 1). Note the possible entry site of the cercaria (depression in the epidermis and dermis), the loss (clear spaces) of alarm cells in the epidermis and the developing metacercaria in the underlying muscle (H & E; Bar ≈ 200µm; 100X).

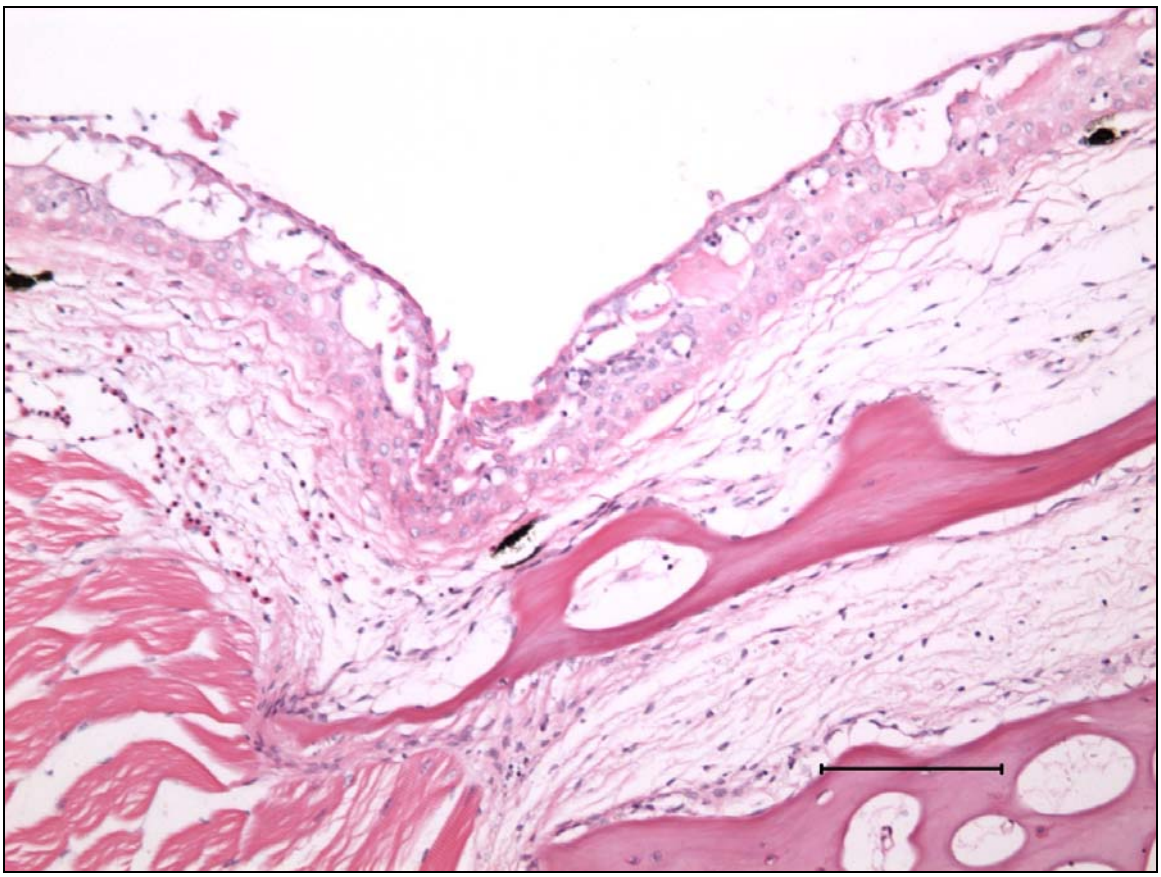

Figure 4.13:

Higher magnification of Figure 4.12 revealing the defect in the epidermis at the possible site of entry. Note that most of the alarm cells in the immediate surrounding area are lost and the few that remain appear to be necrotic (hypereosinophilic) (H & E; Bar ≈ 100µm; 200X).

Histologic changes immediately surrounding developing metacercariae were the same regardless of cercariae dose. At day 3 post-challenge, developing metacercariae were surrounded by its own cyst wall, which in most sections was very thin and eosinophilic. A clear space separated the organism from the parasitic wall. Plump spindloid cells arranged haphazardly or in bundles, or linear tracts surrounded the cyst

wall at day 3 post-challenge. Red blood cells were also linearly arranged between the cells and resembled blood filled nascent capillaries. Interspersed among these cells were also a few mononuclear inflammatory cells and a few red blood cells (Figure 4.14). Surrounding muscle bundles were often smaller and compressed. The cellular infiltrate described above was sometimes observed dividing and separating individual muscle fibers immediately surrounding the parasite. By day 6 post-challenge, this cellular infiltrate appeared more organized and formed concentric rings around the parasitic cyst wall (Figures 4.15- 4.16). The spindle cells were more flattened and their nuclei thinner. Fewer red blood cells were present and the inflammatory infiltrate remained relatively bland. Depending on the sections examined, there was sometimes marked variation in the thickness and relative composition of this developing fibrous capsule surrounding the parasite (Figure 4.17).

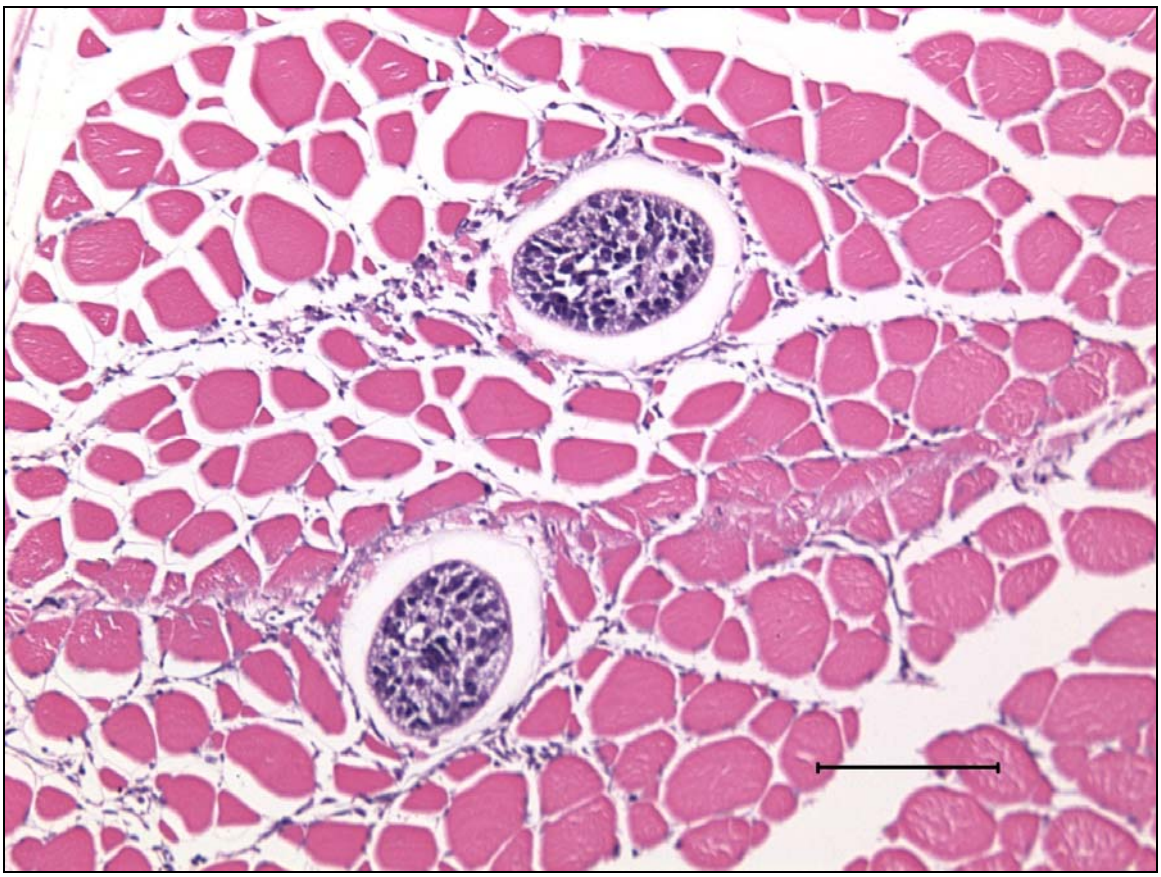

Figure 4.14:

Cross section of body wall from a channel catfish 3 days post-challenge with 200 cercariae containing a developing metacercariae (experiment 1). Note the relatively light cellular infiltrates highlighting the track of that the parasite took as well as around the parasite. The muscle bundles surrounding the developing metacercaria are smaller and mildly compressed (H & E; Bar ≈ 100µm; 200X).

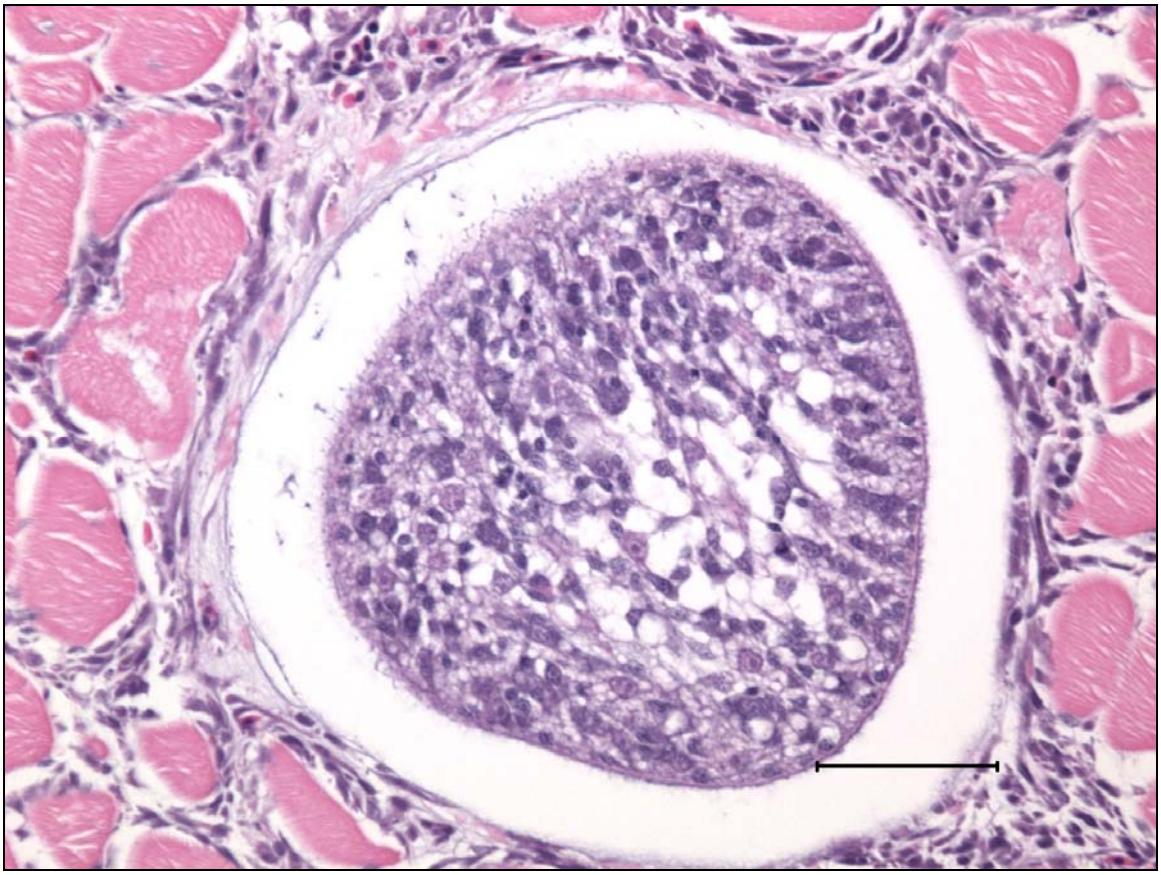

Figure 4.15:

Cross section of body wall from a channel catfish 4 days post-challenge with 200 cecariae containing a developing metacecaria (experiment 3). Note the increased number of plump spindle cells arranged both haphazardly and in bundles (H& E; Bar ≈ 50µm; 400X).

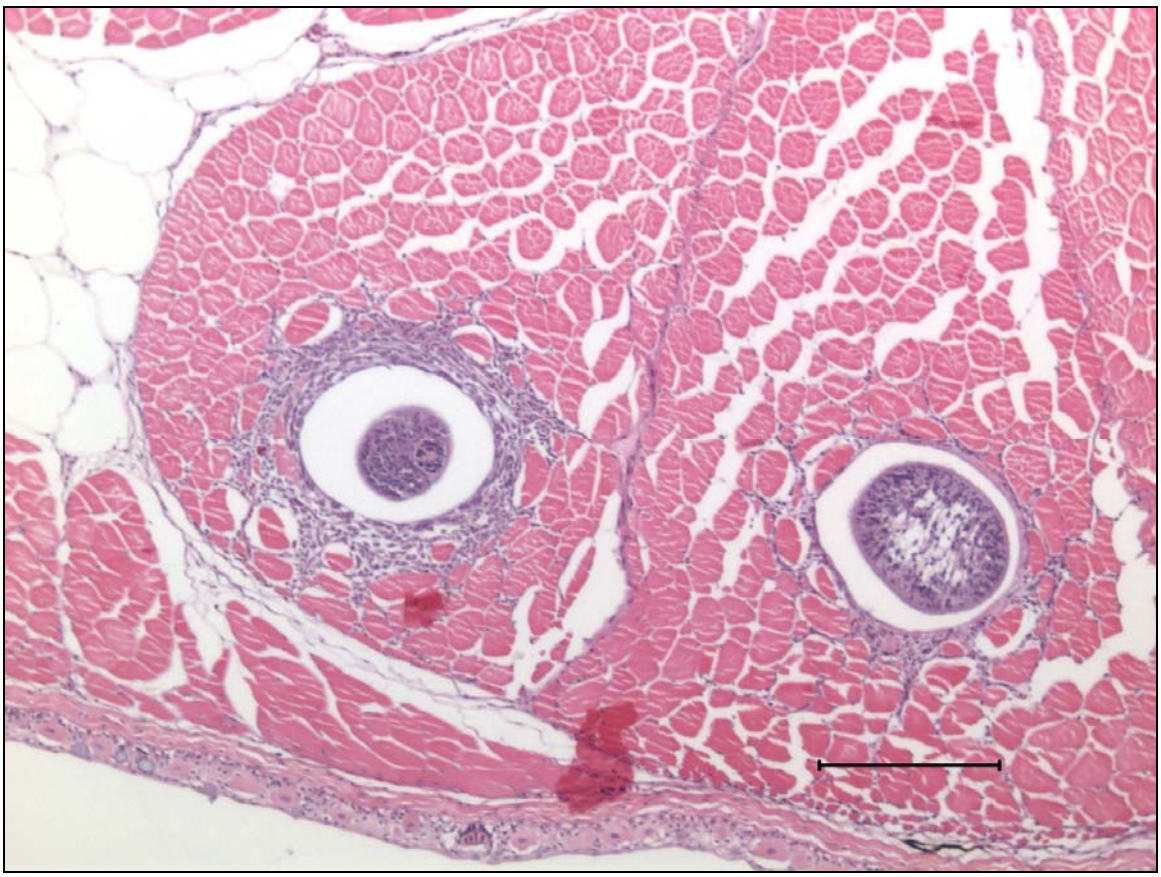

Figure 4.16:

Cross section of body wall from a channel catfish 5 days post-challenge with 100 cecariae containing a developing metacecariae (experiment 2). Note the difference in the infiltrate between the two developing metacecariae (H& E; Bar ≈ 200µm; 100X).

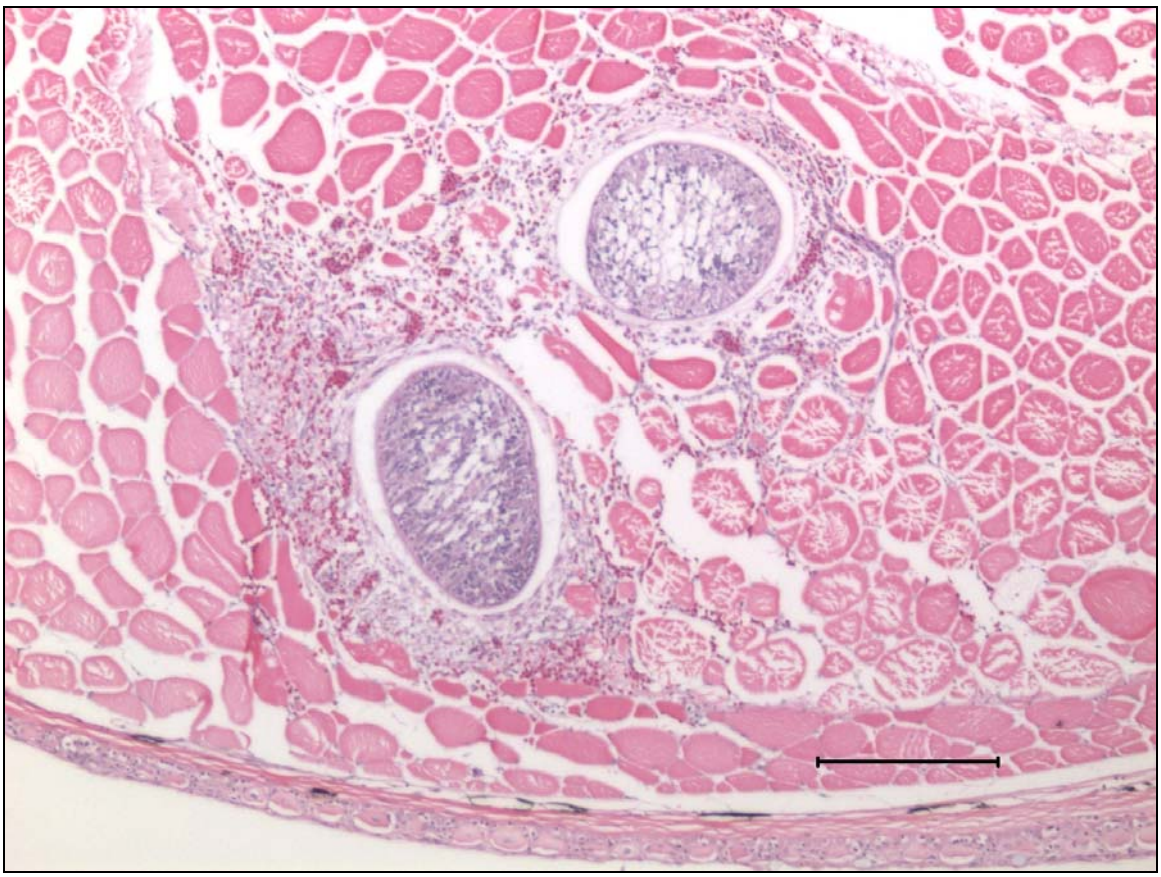

Figure 4.17:

Cross section of body wall from a channel catfish 6 days post-challenge with 200 cecariae containing a developing metacecariae (experiment 1). Note the more developed cellular infiltrates around the metacecariae compared to Figure 4:15 (H& E; Bar ≈ 200µm; 100X).

Discussion

Severe outbreaks of disease and major economic losses have been attributed to *B. damnificus* infection in commercial catfish. Diagnostic case reports confirm the presence *Bolbophorus* spp. metacercariae in these fish which are usually the only evidence of a known pathogenic agent. Often it is assumed that the presence of this parasite is the main cause of the pathology and mortality observed; an assumption that is not substantiated with much scientific data confirming these observations.

While diagnostic case reports and early experimental trials (Labrie et al. 2004) using *Bolbophorus* spp. challenges have provided evidence that this parasite results in mortality and pathologic changes, thus far all diagnostic reports and studies in which pathology has been described are based on the presence of *Bolbophorus*- type metacercariae or challenges in which *Bolbophorus*- type cercariae were used. There are currently no studies in which *Bolbophorus* infections were confirmed to be the result of pure *B. damnificus* cercariae, despite the knowledge that at least two types of *Bolbophorus* spp. cercariae are present in *P. trivolvis* snails in commercial catfish ponds (Levy et al. 2002). Additionally, in diagnostic cases and previous experimental trials, although mortality and pathology is documented, these finding were based on unknown levels of parasite challenge, variation in fish size/ age and the possibility of concurrent infections; all variables that could contribute to the pathology associated with this parasite.

To better understand and describe the pathology of *B. damnificus* infections, controlled experimental trials were carried out in channel catfish fingerlings. This set

of experiments provides the first baseline data on the pathology of molecularly confirmed *B. damnificus* infections in fingerling catfish individually challenged with known numbers of cercariae and at various exposure times.

The findings of this study provide proof that *B. damnificus* alone can cause mortality in channel catfish. Previous research with mixed *Bolbophorus* spp. challenges documented mortalities (n= 10 fish) at 2 days post- challenge at a dose of 29 *Bolbophorus* spp. cercariae/ fish and 100% mortality at day 29 post-challenge (Levy et al. 2002). Labrie et al. (2004) reported no mortality with *Bolbophorus* spp. infections, but mortality in fish exposed to concurrent infections of *Bolbophorus* spp. and ESC starting at day 7 post-challenge with 86% mortality by day 28 post-challenge. In the present study (using smaller fish) the first mortalities began at day 5 post-challenge in fish challenged with 200 cercariae with 100% of the fish (experiment 1) dead by day 6 post-challenge. By day 6 post-challenge, 100% mortalities also occurred in fish challenged with 100 cercariae/ fish (experiment 1) and 7% of the fish (experiment 2) exposed to 50 cercariae. All of these deaths were attributed to the *B. damnificus* infection. These findings and previous reports (Levy et al. 2002) indicate that infection with this parasite does cause mortality, however the mechanism in which this parasite kills the host and the reason for the 5- 7 day delay in mortality is still not clear.

This study provides some insight into the pathologic effects caused by *B. damnificus* cercariae in channel catfish fingerlings. At early sampling times (3 days post-challenge) inflammatory infiltrates were often observed in the epidermis in areas

that could have been entry points for *B. damnificus* cercariae. At that time, there was not a significant host response around the encysting metacercariae and internal fish organs appeared normal. From 4 to 5 days post-challenge, mesenchymal cells were recruited to the periphery of the developing metacercariae. Also during this time, loss of hepatocellular glycogen stores and depletion of splenic periarteriolar lymphocytes were observed. At 6 days post-challenge, when the majority of mortality is beginning to occur, the liver has lost all vacoulation and there was moderate splenic lymphoid depletion. Perhaps, at this time in the *B. damnificus* infection, the infected fish has lost the energy stores typically found in the liver and lymphocytes have been recruited to the epidermis when cercariae initially invaded the fish. It is unknown whether the changes described above can be attributed directly to the parasite or represent secondary changes associated with anorexia and stress induced lymphopenia.

Previous research has documented a marked inflammatory response adjacent to metacercariae and renal tubular necrosis both of which were not observed in this study. Kidney sections were not evident in some slides, but when observed, necrosis was not evident. Previous results were observed in naturally infected *Bolbophorus* spp. catfish. The *Bolbophorus* infection may weaken the immune system of the infected fish, as suggested by Labrie et al. (2004) and/ or provide a portal of entry for other pathogens present in catfish ponds. Concurrent infections with these potential pathogens could have contributed to the observed inflammation surrounding the metacercariae and the renal changes in these reported field cases.

Mortalities and severity of lesions differed when comparing the same challenge levels between experiments. The only variation between experiments 1- 3 was that each experiment was performed a week apart, with experiment 1 occurring in the first week. At the time of experiment 3, the snails had been held in the lab for four weeks post-collection. For the duration of all three experiments, the snails were held at 21.1- 23.9 °C, their water was changed daily, and they were fed continuously. Although only viable, motile cercariae were counted and used for each challenge, the viability, virulence or their ability to infect fish may have been affected by holding snails under laboratory conditions for a prolonged period of time. Over the course of the experiment, the cercariae shed by these snails appeared viable and motile, but the number of cercariae shed/ day fluctuated, with some snails shedding intermittantly. Whether this only occurs under laboratory conditions or is a natural phenomenon in the field is not known. It is also possible that even in natural infections as the infection progresses; subsequent generations of cercariae may be less viable or lose their virulence. Additionally, it is not known whether there are *B. damnificus* strains that may be more virulent than others, thus the cercariae population shed by one snail *P. trivolvis* could be more virulent than the population shed by another *P. trivolvis*.

This study provides evidence that *B. damnificus* causes mortalities in controlled laboratory trials as have been reported with natural infections. There is also significant pathology associated with this parasite, which does appear to increase with increased parasite challenge as was apparent with these experiments. However, although pathology was evident, the pathology observed did not explain the

mortalities 5- 6 days post-challenge, nor did the pathologic changes appear to be severe enough to cause these mortalities. While this study provided some necessary baseline information on pathology of this parasite in catfish fingerlings, it also indicated that there is still much research needed to further understand the events that occur with this parasite from the time of initial infection to the death of the catfish host.

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CHAPTER V

CONCLUSION

In this research, all stages in the life cycle of *Bolbophorus damnificus* were confirmed molecularly in a single life cycle study. A new snail host, *Biomphalaria havanensis* was reported for *B. damnificus*; and the pathologic changes associated with *B. damnificus* infections in the channel catfish (*Ictalurus punctatus*) were documented.

American white pelicans (AWPE) were artificially infected with *B. damnificus* metacercariae which matured to the adult stage and shed ova 4 days post- infection. The ova hatched in 11 days at 30.0 °C and in 53 days at ambient temperatures ranging from 13.5-35.7 °C, releasing miracidia that infected snails, *Planorbella trivolvis*. These infected snails began shedding *B. damnificus* cercariae 23 days post-infection at water temperatures ranging from 20.6-34.4 °C. These cercariae were used to infect specific pathogen free catfish and they matured to infective metacercariae in the superficial muscle of catfish in 23 days post-infection and were fed to AWPEs. Metacercariae matured to patent adults and shed ova seven days post-infection, completing the *B. damnificus* life cycle and fulfilling Koch's postulates for this organism.

The channel catfish and the ponds in which they are raised have provided an ideal environment for the *B. damnificus* life cycle. American white pelicans are present in the Mississippi Delta from November-March, infected with adult *B. damnificus*. This

research has shown that a pelican artificially infected with *B. damnificus* has the potential to introduce thousands of trematode eggs per day. Once these eggs are introduced, this research indicates that egg development can be delayed at lower water temperatures. Thus in theory, in a pond setting, these ova would remain dormant at the pond bottom while temperatures in the ponds are low. As pond temperatures increase in the spring, ova could begin to hatch within 12 days according to this research, releasing miracidia. Miracidia would infect *P. trivolvis* snails present in catfish ponds in the spring and summer months, undergo development, and as this research indicates, could begin to shed cercaria 23 days post-infection at the rate of 2,547 cercariae/ snail/ 24 hours and could continue this shedding for months. In ponds with numerous *B. damnificus* infected snails, this research indicates that massive mortalities could begin as early as five days post-infection. If these acute infections mimic these findings, exposure of 100-200 cercariae could cause 100% fish mortality five to six days post-infection with fish displaying exopthalmia, ascites, petechial hemorrhages on the body surface, loss of liver vacoulation and lymphoid depletion in the spleen. In chronic infections, simulated by the pathology studies in this research in which fish were challenged with 50 cercariae/fish, mortalities would not occur, but instead there would be a continual supply of fish with infective metacercariae for the pelican host to consume. This study not only indicated that these consumed metacercariae would develop rapidly 4-7 days post-infection to egg-laying adults, but this could occur repeatedly as this research indicated.

The continual and growing presence of the final bird host, the AWPE; the ideal snail habitat the catfish ponds provide, and the large concentrated population of a highly

susceptible fish host all contribute to the propagation of this parasite. Currently, there are no efficacious methods to practically treat this parasite in any of its hosts. While there are chemicals efficacious in controlling the snail host, in order to implement practical management schemes, information is needed about all of the hosts in the *B. damnificus* life cycle. This research provided vital information on these hosts. In the bird host this research showed: AWPEs are the only known definitive hosts, it takes 4-7 days for an AWPE to become infected and shed thousands of *B. damnificus* ova in catfish ponds, and the AWPE can become repeatedly reinfected. In the snail host this study demonstrated that: trematode ova remain dormant, hatch in less than two weeks and once snails are infected, they can shed 2,547 cercariae/day, remain infected for months and *B. damnificus* can infect several snail hosts. In the fish host this research showed: high mortalities can occur six days post-infection with as few as 100 cercariae/fish and in subclinical infections fish have infective metacercariae by 23 days post-infection. This research provided further justification for the necessity to devise management schemes that aggressively target each *B. damnificus* host; the AWPE on the ponds and all snail and infected fish populations in the ponds. It also provides evidence of the importantance of constant vigilance in monitoring this parasite and the necessity for continued research such as this, studying the dynamics of this parasite in all of its hosts.

APPENDIX A

BOLBOPHORUS DAMNIFICUS LIFE CYCLE DATA

Table A. 1:

Subsample of *Bolbophorus damnificus* naturally infected catfish used for artificial infection.

Fish Number	Weight (g)	Size (cm)	Number of Metacercariae	Number used for PCR
8	3.6	7	9	n=2
9	2.3	6	5	n=1
10	2.4	6	8	n=2
11	4.1	7	7	n=1
12	2.3	5.5	10	n=2
13	1.8	5.5	0	n=0
14	2.4	6	9	n=2
15	5.5	7.6	20	n=2
16	5.5	8	3	n=1
17	5.1	7.75	13	n=1
Average	3.5	6.6	8.4	Total: 14

Table A. 2:

Fifty-one catfish; their size, weight and metacercariae per fish determined for artificial infection.

Fish Number	Weight (g)	Size (cm)	Number of Metacercariae
18	8.2	9.4	3
19	7.1	8.7	7
20	6.9	8.9	6
21	5.5	9.0	4
22	5.5	8.0	3
23	5.0	8.0	6
24	4.3	7.3	16
25	5.0	8.0	4
26	5.5	8.0	5
27	4.3	7.5	2
28	7.1	9.0	6
29	9.5	10.0	3
30	4.2	7.4	11
31	5.0	8.0	3
32	4.5	7.5	6
33	4.4	7.5	7
34	2.4	6.0	4
35	4.3	7.4	0
36	5.0	8.2	8
37	9.0	9.4	27
38	4.8	7.5	5
39	2.7	6.0	5
40	10.3	9.5	3
41	3.4	7.0	0
42	13.1	11.0	4
43	6.0	7.8	9
44	2.6	6.5	12
45	2.7	6.5	6
46	4.4	7.5	13
47	3.1	6.9	25
48	3.1	6.5	17
49	4.9	7.6	7
50	2.2	6.5	5
51	5.4	8.2	10
52	6.7	8.5	6
53	4.3	7.5	17
54	4.8	7.5	3
55	4.3	7.0	31

Table A.2 (Continued)

56	3.6	7.1	17
57	5.7	7.5	4
58	13.1	11.0	6
59	8.1	9.0	6
60	2.8	7.5	7
61	8.7	9.0	6
62	3.4	7.0	16
63	4.9	7.8	30
64	4.4	7.0	2
65	3.4	7.0	1
66	5.1	8.0	16
67	4.8	8.0	0
68	3.4	7.0	16
69	5.7	7.5	2
Average	5.4	7.9	8.4

Table A.3:

Measurements of *Bolbophorus damnificus* eggs collected from experimental pond 3.

Study Day	Days after eggs collected	Length (µm)	Width (µm)
21	1	120.4	78.7
		119.4	78.8
		119.6	76.3
		126.4	78.5
		121.3	74.8
		103.5	44.5
		119.3	71.3
		123.8	76.2
23	3	128.3	79.6
		104.8	79.2
		122.4	80.0
25	5	131.9	84.4
		114.9	81.1
26	6	130.7	76.5
		127.1	77.2
		127.8	70.7
		114.2	77.2

Table A.3 (Continued)

		123.1	78.6
		114.6	77.9
		120.8	73.2
		101.9	77.7
		118.8	74.8
		122.5	70.6
27	7	134.8	86.0
		131.9	90.0
		125.2	89.1
28	8	118.9	73.2
		118.2	72.5
		128.1	69.4
		122.3	72.6
		116.3	78.6
		122.2	71.6
		120.0	72.9
		116.5	79.5
		118.1	74.2
		127.0	71.7
29	9	129.3	71.1
		115.2	73.2
		118.0	70.6
		126.0	71.9
		118.2	73.8
		122.3	70.1
		124.7	70.9
		119.0	79.2
		117.4	73.4
30	10	127.6	69.9
		112.7	72.5
		121.4	71.1
		123.0	73.0
		123.4	75.6
		129.9	70.8
		132.7	76.7
		117.0	74.0
		113.6	68.5
		117.2	75.0
		123.1	72.0
32	12	131.1	87.6
		127.7	85.1
		134.7	87.5
33	13	140.4	88.3

Table A.3 (Continued)

		132.4	83.7
35	15	132.3	83.1
		129.8	73.9
		129.5	87.6
39	19	123.7	76.6
41	21	113.4	76.3
		122.3	76.4
		120.7	77.9
43	23	118.5	76.0
		118.9	72.3
		116.9	81.6
45	25	117.7	80.6
		109.0	77.5
47	27	115.0	79.8
		117.7	73.7
49	29	113.6	75.7
		127.3	76.0
51	31	116.2	76.3
		123.1	74.7
53	33	118.9	72.5
		108.8	73.6
55	35	113.3	73.7
		118.0	75.8
57	37	116.9	78.2
		114.7	77.9
59	39	116.1	76.5
		123.6	72.7
61	41	120.5	74.2
		119.1	76.8
63	43	117.6	74.0
		116.0	73.5
65	45	116.8	79.9
		111.3	76.3
		112.5	89.9
67	47	119.9	78.4
		118.8	75.2
69	49	112.2	76.8
		111.6	75.9
		108.3	80.0
71	51	108.8	75.3
		121.5	76.1
73	53	109.0	75.2
		125.6	74.7

Table A.3 (Continued)

		124.5	71.6
75	55	120.4	85.1
		106.8	75.7
77	57	95.1	80.6
		112.0	76.6
		114.4	77.2
79	59	100.6	71.8
		120.6	71.2
81	61	115.2	78.4
		114.4	71.2
		111.5	75.7
83	63	107.6	73.5
		121.8	74.2
85	65	125.1	71.0
		116.7	74.8
87	65	121.9	76.0
		116.8	76.9
89	69	120.9	74.9
		121.4	75.3
91	71	116.3	77.5
		127.3	77.4
92	72	116.9	72.8
		125.6	80.5
95	75	110.8	72.6
		111.3	70.1
		120.4	73.4
97	77	108.5	71.5
		110.9	77.9
99	79	105.0	72.7
		110.5	75.1
		110.1	75.6
101	81	123.1	78.5
Average		119.1	76.0

Table A.4:

Measurements of *Bolbophorus damnificus* hatched eggs from experimental pond 3.

Study Day	Days after eggs collected	Length (µm)	Width (µm)
77	57	109.4	76.5
77	57	111.9	76.2
79	59	115.9	65.8
81	61	112.4	78.7
Average		112.4	74.3

Table A.5:

Number of *Bolbophorus damnificus* cercariae shed in outdoor experimental pond 2. Snails were checked three times weekly; negative days not shown.

Study Day	Month	Measurement (mm)	Number of Cercariae Shed	
			Day 1	Day 2
100 (27 days post-hatch)	June	3.44	20	0
103	June	3.49	10	30
103	June	7.94	293	1330
112	July	7.77	200	267
121	July	8.94	267	28
125	July	5.54	160	67
125	July	6.11	213	120
126	July	7.66	707	840
126	July	8.00	1533	1400
126	July	6.98	533	1253
128	July	7.40	40	100
135	July	6.64	13	267
135	July	8.40	0	507
135	July	5.14	120	187
138	July	4.01	160	160
Average		6.50	285	437

Table A.6:

Number of *Bolbophorus damnificus* cercariae shed in outdoor experimental pond 3. Snails were checked three times weekly; negative days not shown.

Study Day	Month	Measurement (mm)	Number of Cercariae Shed	
			Day 1	Day 2
96 (23 days post-hatch)	June	10.93	13	93
98	June	11.06	40	80
98	June	11.81	67	40
103	June	11.86	67	373
107	June	10.98	373	693
112	July	12.12	67	367
117	July	12.21	27	20
119	July	12.03	67	240
140	July	7.30	0	450
Average		11.14	80	262

Table A.7:

Number of *Bolbophorus damnificus* cercariae shed from *Planorbella trivolvis* snails during experimental *Planorbella trivolvis* infection at 21.9 °C- 26.7 °C. Snails were checked three times weekly; negative days not shown.

Study Day	Month	Measurement (mm)	Number of Cercariae Shed	
			Day 1	Day 2
95	June	9.36	266	653
98	June	9.20	13	707
103	June	9.57	1240	2547
107	June	9.59	40	133
110	July	8.44	133	107
112	July	9.67	0	317
112	July	7.31	175	200
112	July	8.07	53	0
114	July	8.79	67	300
114	July	9.91	105	120
114	July	7.41	1560	390
117	July	10.24	35	60
119	July	9.23	60	310
121	July	7.25	67	130
124	July	9.15	0	27
124	July	7.55	320	213
136	July	8.07	733	1173
128	July	6.87	53	13
128	July	6.19	987	1070
131	July	6.75	187	213
135	July	7.17	0	5
135	July	7.12	13	400
140	July	7.20	40	0
140	July	7.48	173	200
147	August	10.43	380	170
Average		8.32	268	378

Table A.8:

Mortality of artificially infected catfish during metacercariae fish challenge 1. Attempts were made to recover all metacercariae, however some fish were too autolyzed to excise metacercariae.

Study Day	Days PI	Tank 2	Metacercariae recovered	Tank 3	Metacercariae recovered
96		30 fish added		30 fish added	
104	8			2 dead	
109	14			4 dead	N=5, 16, 1, 1
111	15			8 dead	N= 28, 34, 51
112	16			6 dead	n= 36
113	17	10 dead		4 dead	
115	19	12 dead	n= 2, 2	3 dead	n= 36
116	20	2 dead			
117	21	3 dead	n= 16		
118	22	3 dead			
122	26			1 dead	
123	27			1 dead	
130	34			1 dead	

Table A.9:

Mortality of artificially infected catfish during metacercariae fish challenge 2. Attempts were made to recover all metacercariae, however some fish were too autolyzed to excise metacercariae.

Study Day	Days PI	Tank 2	Metacercariae Recovered	Tank3	Metacercariae Recovered
117		20 fish added, 3 hour exposure		20 fish added, 3 hour exposure	
130	13			1 dead	n= 9
133	16	2 dead	n= 10, 2	7 dead	n= 21, 0, 0, 17, 0, 0
134	17	5 dead	n= 13, 9, 9, 6, 14, 9	6 dead	N= 0, 0, 0, 0
135	18	6 dead	n= 11, 23, 9, 0, 0	4 dead	n= 1, 1, 0, 0, 0
136	19	4 dead	n= 15, 12, 15, 6	2 dead	n= 0, 0

Table A.10:

Number of metacercariae in artificially infected catfish from fish metacercariae challenge 3 for pelican reinfection.

Fish Number	Experimental Tank	Number of Metacercariae	Length (cm)	Weight (g)
F189	2	2	10.5	11.7
F190	2	0	10.0	13.5
F191	3	0	10.0	11.7
F192	2	1	9.5	8.3
F193	2	1	9.0	9.9
F194	2	0	8.5	8.0
F195	2	2	11.0	11.9