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Marlena Yost
*Mississippi State University*

Linda Pote
*Mississippi State University*

David J. Wise
*Mississippi Agricultural and Foresq Extension Service, Thad Cochran National Warmwater Aquaculture Research Center, Post Ofice Box 197, Stoneville, Mississippi*

Brian Dorr
*USDA-APHIS-Wildlife Services*, brian.s.dorr@aphis.usda.gov

Terry Richardson
*University of North Alabama, Department of Biology*

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[Communication]

# *Biomphalaria havanensis* Identified as a Potential Intermediate Host for the Digenetic Trematode *Bolbophorus damnificus*

MARLENA C. YOST AND LINDA M. POTE*
*College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi 39762, USA*

DAVID J. WISE
*Mississippi Agricultural and Forestry Extension Service, Thad Cochran National Warmwater Aquaculture Research Center, Post Office Box 197, Stoneville, Mississippi 38776, USA*

BRIAN S. DORR
*U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, Post Office Box 6099, Mississippi State, Mississippi 39762, USA*

TERRY D. RICHARDSON
*Department of Biology, University of North Alabama, Box 5212, Florence, Alabama 35632, USA*

*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 ram's horn snail *Planorbella trivolvis*. Recently, the exotic snail *Biomphalaria havanensis* has been isolated in several channel catfish ponds in the Mississippi Delta. The aim of this study was to determine whether this invasive snail species could also serve as a first intermediate host in the *B. damnificus* life cycle. *Bolbophorus damnificus* ova were collected from an American white pelican *Pelecanus erythrorhynchos* that had been artificially infected with *B. damnificus* metacercariae; the ova were then introduced into an aquarium with parasite-negative *B. havanensis*. *Bolbophorus damnificus* cercariae (confirmed by polymerase chain reaction) were detected in *B. havanensis* 45 d after exposure to *B. damnificus* ova. The snails continued to shed *B. damnificus* cercariae throughout the 80-d postexposure examination period. Before this research, the only reported natural *B. damnificus* infection of snails involved *P. trivolvis*. This study indicates that the range of snail hosts is broader than previously suspected. A survey should be implemented to identify additional snail species that can serve as hosts for *B. damnificus*. Further research should determine whether *B. havanensis* is a natural host for *B. damnificus*, and if so the prevalence of *B. havanensis* in commercial channel catfish ponds should be assessed.

The constant presence of fish-eating birds and the presence of appropriate snail host species fulfill the life cycle requirements of some digenetic trematodes in ponds used for commercial production of channel catfish *Ictalurus punctatus* in the southeastern United States (Overstreet and Curran 2004). Consequently, an increase in diagnosed digenetic trematode infections in channel catfish has been observed. The 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 marsh 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 attributed to *Bolbophorus confusus* (Overstreet et al. 2002), were first reported in farm-raised channel catfish in 1994 in Louisiana (Labrie et al. 2004) and later were observed in Mississippi in 1999 (Terhune et al. 2002). Since these first reports, economic losses associated with *B. damnificus* have steadily risen due to mortality and morbidity. Between 1.1% and 5.6% of the channel catfish submissions to the Aquatic Diagnostic Laboratory (Thad Cochran National Warmwater Aquaculture Center, Stoneville, Mississippi) from 1999 to 2005 were diagnosed with these trematodes. The highest incidence was in 2000, when *B. damnificus* accounted for 5.6% of diagnostic submissions. The lowest incidence was in 2003, when only 1.1% of submissions were attributed to *B. damnificus*. Since 2003, the incidence has steadily increased; *B. damnificus* accounted for 2.6% of diagnostic submissions in 2004 and 3.4% in 2005 (Aquatic Diagnostic Laboratory 2004, 2005). These continual infections have been attributed to the increasing number of wintering and migratory American white pelicans in the Mississippi Delta region, where channel catfish ponds provide a readily available food source. In addition, certain

* Corresponding author: lpote@cvm.msstate.edu

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management practices may have also contributed to the steady increase of *B. damnificus* in channel catfish since 2003; for example, new farm personnel may lack experience in detecting *B. damnificus* in fish, and staff at farms that are known to have *B. damnificus* infections may assume that the problem is under control and thus are less vigilant in detecting the parasite (Avery et al. 2004).

Although the reported incidence of *B. damnificus* is lower than that of 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 channel catfish farmers are aware of available treatments for *B. damnificus*, they often do not submit trematode-infected fish to laboratories; 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 can substantially decrease production and profitability (Wise et al. 2006).

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

The type locality of *B. havanensis* is in Havana, Cuba (Young et al. 1997), and these snails are also found in Mexico, Central America, the Antilles region (Malek 1985), and the Dominican Republic (Malek 1969). These snails are known to serve as intermediate hosts for other digenetic trematodes (Cram et al. 1945; Michelson 1976). Sporadic reports of the species' presence in the United States have originated from the middle Snake River in Idaho (Bowler and Frest 1992), the east coast of Florida (Thompson 1984), and South Carolina (Dillon and Dutra-Clarke 1992).

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

## Methods

*Biomphalaria havanensis* were collected from a commercial channel catfish pond in the Mississippi Delta. Snails were identified to species based on morphological characteristics according to Burch (1989). To verify snail species, a subsample of snails ($n = 2$) was subjected to polymerase chain reaction (PCR) analysis using primers 16Sar (5′-TCGCCTGTTTATCAAAAACAT-3′) and 16Sbr (5′-TCCGGTCTGAACTCAGATCACGT-3′; DeJong et al. 2001). Snails were reared in the snail colony at MSU for at least 1 year. Offspring of this original population were tested in the laboratory and were found to be PCR negative for *B. damnificus*. To further confirm that the population used in this study was negative for *B. damnificus* infection, PCR was performed on a subsample of the population. Juvenile and adult snails (5–10 mm in size) were randomly selected for testing. Snails ($n = 10$) were placed into test tubes containing 3 mL of sterile spring water. Before the start of the experiment, snails were checked daily for 10 d to ensure that none were shedding *B. damnificus* cercariae. After 10 d, the subsampled snails were sacrificed and PCR was completed to further confirm that the snails were negative for *B. damnificus* life stages.

*Molecular analysis of snails and cercariae.*—*Biomphalaria havanensis* or individual cercariae of *B. damnificus* were placed individually in microfuge tubes containing 300 μL of cell lysis solution and 1.5 μL of proteinase K (enzyme number 3.4.21.64; IUBMB 1992). The DNA was extracted with the Puregene DNA purification kit (Gentra Systems, Minneapolis, Minnesota) via their protocol; only half-reactions were performed. *Bolbophorus damnificus*-specific primers, P1-650F (5′-TCAGTTTCGAAC-GATGATGA-3′) and P1-14700R (5′-CGGTCTA-CGGTTCCACC-3′), were used (Levy et al. 2002). Reaction volumes for PCR were 25 μL using 10× buffer, 2.0 μL of template, 0.625 units of *Taq* polymerase (2.7.7.7; Takara Bio, Inc., Shiga, Japan; Hot Start Taq), 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: 92°C for 5 min; 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final cycle of 72°C for 5 min. This was carried out on a PTC-100 Peltier Thermal Cycler (MJ Research, Inc., St. Bruno,

Quebec). The PCR products were visualized on a 1.2% agarose gel along with positive and negative controls. The gel was poststained with GelStar nucleic acid stain (Cambrex Bio Science Rockland, Inc., Rockland, Maine) and was observed under ultraviolet light.

The *B. damnificus* ova used in this study were collected from an American white pelican that was 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 American white pelicans at the Mississippi Field Station of the National Wildlife Research Center (NWRC; U.S. Department of Agriculture [USDA]) located in Mississippi State (Glahn et al. 2000). The American white pelican was treated orally with a single dose of praziquantel (Droncit 34; Bayer Corporation, Shawnee Mission, Kansas) at 30 mg/kg of body weight to eliminate trematode infections and then was artificially infected with *B. damnificus* metacercariae (confirmed by PCR) as previously described. All procedures used in this study were approved by the NWRC's Institutional Animal Care and Use Committee under protocol QA-1009.

To isolate *B. damnificus* ova, American white pelican fecal material was collected after a patent *B. damnificus* infection was confirmed. Fecal sedimentation was performed using a modified technique described by Foreyt (2001) for detection of trematode eggs. Briefly, total feces from the American white pelican were collected, homogenized thoroughly, and weighed; 0.5 g was used for the sedimentation procedure. Fecal material was added to a 15-mL test tube, and a 1% soap solution was then added. The fecal material and soap solution were mixed thoroughly and allowed to sit for at least 5 min. The soap solution was carefully decanted so 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 mL in water purified by reverse osmosis. To enumerate eggs, a 1-mL aliquot was added to a Petri dish with grid lines (3-mm spacing) and was diluted with the purified water. All ova were counted, and the number of eggs per gram was calculated.

Thirty parasite-negative *B. havanensis* were added to a 19-L aquarium and maintained at room temperature (18–23°C). Feces collected from the infected adult American white pelican were used as a source of *B. damnificus* eggs. Feces were collected for 4 d, and fecal sedimentation was performed on the pooled sample to enumerate and isolate *B. damnificus* ova. Fecal ova counts indicated that there were approximately 1,100 eggs/g of feces. Feces (13.5 g) containing approximately 14,850 ova (1,100 eggs/g × 13.5 g of feces) were added to the aquarium. A subsample of these ova was analyzed as previously described using *B. damnificus*-specific PCR primers to confirm that they were *B. damnificus* ova (Levy et al. 2002).

Beginning at 45 d after exposure to *B. damnificus* ova, *B. havanensis* were monitored for the presence of cercariae. At that time, four snails were removed from the aquarium, measured, and placed into test tubes with 3 mL of sterile spring water. Tubes were examined for cercariae under a dissecting microscope at 24 and 48 h. Cercariae were enumerated and stored in molecular-grade 70% ethanol for DNA analysis. Three times weekly for the duration of the study, four new snails were randomly selected from the tank and the previously checked snails were returned to the aquarium. Additionally, all remaining live snails ($n$ = 24) were checked for cercariae shedding on day 47 and were returned to the aquaria. The last sample was taken at 80 d postexposure. 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 PCR protocol previously described, each subsample was confirmed to be *B. damnificus*.

## Results

Two slightly different partial 16S sequences were obtained from snails by use of PCR primers (DeJong et al. 2001). The sequence data were inconclusive and failed to distinguish snails from catfish ponds from a complex containing: *B. obstructa*, *B. temascalensis*, and *B. havanensis*. Because of the continued confusion over species in this group, morphological characters were used for positive identification of ghost ram's horn snails.

Microscopic examination of snails shedding cercariae began at 45 d postexposure to the *B. damnificus* ova. Four snails collected at that time were confirmed by PCR to be shedding *B. damnificus* cercariae. *Biomphalaria havanensis* continued shedding for the duration of the study, which ended on day 80 (Figure 1). Of the remaining live snails ($n = 24$) examined on day 47 postexposure, five specimens were confirmed to be infected with *B. damnificus*. Cercariae that were shed by all positive snails for the duration of the trial were confirmed as *B. damnificus* based on PCR. The highest number of cercariae shed by a single snail within a single day was 640 cercariae at 45 d postexposure (Figure 1).

## Discussion

This study demonstrates that *B. havanensis* can serve as an alternative intermediate host for *B. damnificus*. This snail species has been collected from several

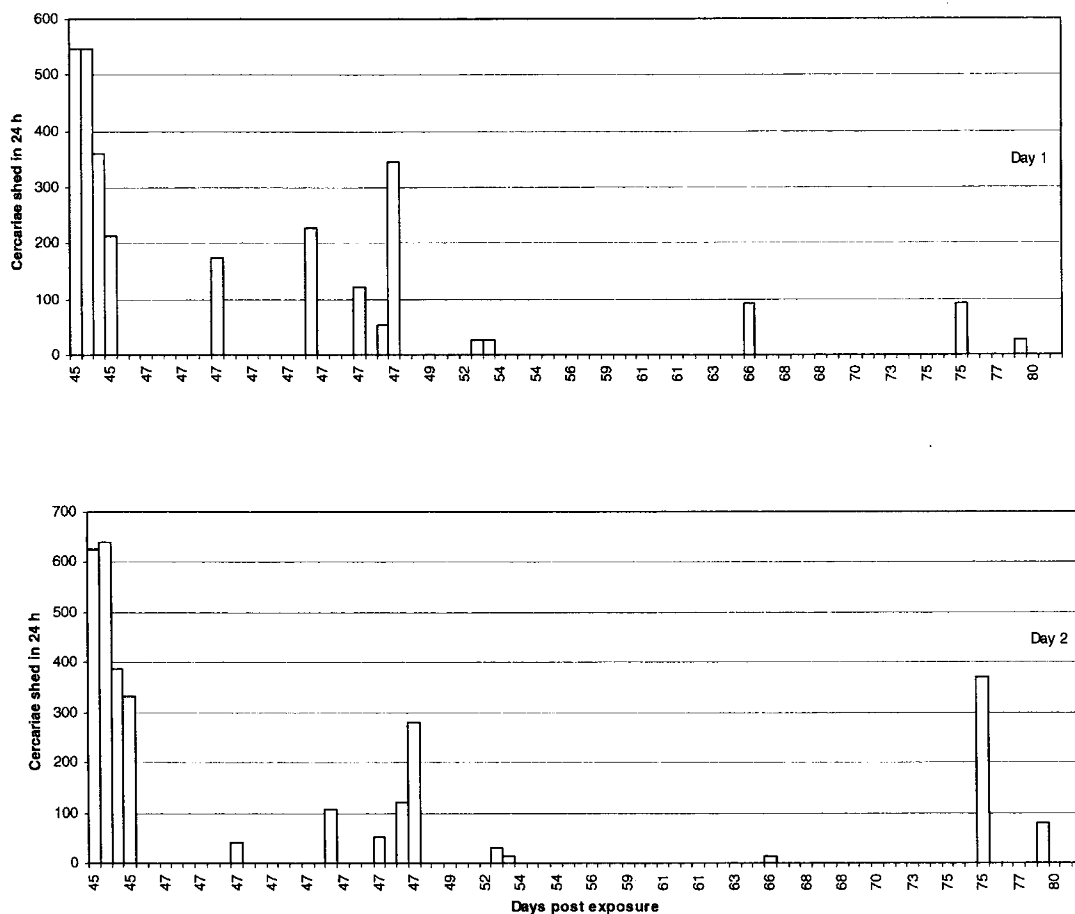


FIGURE 1.—Number of *Bolbophorus damnificus* cercariae shed by ghost ram's horn snails within the first 24 h (day 1) and second 24 h (day 2) after the snails were removed from the aquarium and placed individually into test tubes. Snails were exposed to *B. damnificus* ova and were selected randomly three times weekly from 45 to 80 d postexposure for monitoring of cercarial shedding. Each bar represents an individual snail.

commercial channel catfish ponds. However, none of these snails was found to be naturally infected with *B. damnificus*. To determine whether the infection occurs naturally, a more methodical survey must be completed. Although there have been no confirmed natural *B. damnificus* infections in *B. havanensis*, this study demonstrates that these snails have the potential to serve as hosts for the parasite. The high concentration of American white pelicans ingesting channel catfish and defecating *B. damnificus* ova in high numbers near pond perimeters (Overstreet et al. 2002) provides the ideal scenario for perpetuating the *B. damnificus* life cycle in a susceptible mollusk species like *B. havanensis*.

In this study, *B. havanensis* were shedding *B. damnificus* cercariae on the first observation date at 45 d postexposure. A previous study of a closely related trematode, *Bolbophorus confusus*, demonstrated cercarial shedding by *P. trivolvis* from 30 to 51 d postexposure to trematode eggs at 21–24°C (Fox 1965). To ensure the maximum exposure time to the hatching *B. damnificus* ova, the ova and *B. havanensis* snails were kept at 18–23°C and observations began at 45 d postexposure. Confirmation that *B. havanensis* can serve as an intermediate host suggests that further life cycle studies should be done to determine when cercariae shedding first occurs, the number of cercariae that are shed each day, and the duration of shedding.

*Bolbophorus damnificus* control efforts have focused on the eradication of the intermediate host, the *P. trivolvis*. Shoreline chemical treatments with copper sulfate–citric acid combinations and slurried hydrated lime are effective against *P. trivolvis* (Mitchell 2002; Mitchell et al. 2007), but whether these same

treatments are capable of eradicating *B. havanensis* is currently unknown.

The propagation of digenetic trematodes relies on their ability to move from host to host. Parasites vary 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 in addition to channel catfish, fathead minnow *Pimephales promelas* can also serve as second intermediate hosts to *B. damnificus* metacercariae (Overstreet et al. 2002; Mitchell et al. 2006). The present study indicates that *B. damnificus* may also have a broader range of mollusk hosts than previously suspected.

Although little information currently exists on the prevalence of *B. havanensis* in channel catfish ponds, the implications of our study warrant further research into the life cycle of *B. damnificus* and its mollusk hosts. This study confirms that the *B. havanensis* can serve as an intermediate host for *B. damnificus* after artificial infection; however, an extensive survey should be done to determine whether this phenomenon occurs naturally. Further life cycle studies should determine the initial duration of *B. damnificus* cercariae shedding and the number of cercariae shed per day. If *B. havanensis* is found to be a natural host for *B. damnificus*, then additional studies on the life cycle of *B. damnificus* in this host and the potential measures to control this snail will be needed.

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