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Bolbophorus damnificus n. sp. (Digenea:
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Molecular Data

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***Bolbophorus damnificus* n. sp. (Digenea: Bolbophoridae) from the channel catfish *Ictalurus punctatus* and American white pelican *Pelecanus erythrorhynchos* in the USA based on life-cycle and molecular data**

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Abstract

The common pathogenic prodiplostomulum metacercaria in the flesh, mostly near the skin, of pond-produced channel catfish *Ictalurus punctatus* has been demonstrated to be *Bolbophorus damnificus* Overstreet & Curran n. sp. The catfish acquires the infection from the snail *Planorbella trivolvis*, the only known first intermediate host, and the species is perpetuated through the American white pelican *Pelecanus erythrorhynchos*, as confirmed by experimental infections with nestling and dewormed adult pelican specimens in conjunction with molecular data. It differs from the cryptic species *Bolbophorus* sp., also found concurrently in the American white pelican, by having eggs 123–129 μm rather than 100–112 μm long and consistent low values for nucleotide percentage sequence similarity comparing COI, ITS 1/2, 18S rRNA and 28S rRNA fragments. *Bolbophorus* sp. is comparable but most likely distinct from *B. confusus* (Kraus, 1914), which occurs in Europe and has eggs 90–102 μm long. Its intermediate hosts were not demonstrated. The adults of neither of the confirmed North American species of *Bolbophorus* were encountered in any bird other than a pelican, although several shore birds feed on infected catfish, and *B. damnificus* can survive but not mature when protected in the mouse abdominal cavity. *B. ictaluri* (Haderlie, 1953) Overstreet & Curran n. comb., a species different from *B. damnificus*, is considered a *species inquirenda*.

Introduction

A prodiplostomulum metacercarial stage consistent with that reported for species of *Bolbophorus* Dubois, 1935 infected the channel catfish *Ictalurus punctatus* (Rafinesque) from commercial ponds in Louisiana and the delta region in northwestern Mississippi. This prodiplostomulum infected the musculature of all sizes of channel catfish, but it was especially pathogenic to young fish. Fingerling catfish frequently became infected and experienced high mortality rates (e.g. Terhune et al., 2002). Also, commercial processors reject heavily infected surviving mature catfish

(Venable et al., 2000). Consequently, catfish farmers have been anxious to find out the identity of the species and the various hosts in its life-cycle so that they can manage infections. We attribute high mean intensity of the parasite to several factors, including the presence of the American white pelican *Pelecanus erythrorhynchos* Gmelin, high numbers of the planorbid marsh rams-horn *Planorbella trivolvis* (Say), which we (Venable et al., 2000; unpublished) previously determined to serve as the first intermediate host, and water temperatures above about 24 °C (Terhune et al., 2002; personal observations).

Shoop (1989) created the term 'prodiplostomulum' for metacercariae intermediate between a 'neascus' and 'diplostomulum'. Members of the *Bolbophoridae* Shoop, 1989, a family containing four genera, have prodiplostomulum metacercaria characterised by pseudosuckers and a true cyst with a thin, transparent, inner layer of parasitic origin and a thicker, opaque, outer layer of host origin. According to Shoop (1989), neascus metacercariae do not have pseudosuckers but encyst, and diplostomula have pseudosuckers and do not encyst. Hoffman (1960) and Dubois (1970) had previously considered *Bolbophorus* in the *Diplostomidae* Poirier, 1886 (as 'Diplostomatidae'). The superfamily name *Diplostomoidea* Poirier, 1886 was introduced to include this group of families by Gibson (1996), as it is an older family-group name than the more familiar *Strigeoidea* Railliet, 1919.

Bolbophorus confusus (Krause, 1914) is known to parasitise pelicans in Europe, and it has been reported from the American white pelican in North America (e.g. McNeil, 1949; Fox, 1965; Dubois, 1970; Pasnik, 1999; Venable et al., 2000). In North America, *B. confusus* has also been reported from the reddish egret *Egretta rufescens* (Gmelin) in Florida and Texas (Conti et al., 1986), and from the brown pelican *Pelecanus occidentalis* Linnaeus in Texas (Dronen et al., 1999). Considering those reports, we originally suspected that the common muscular prodiplostomulum present in catfish in Louisiana and Mississippi was probably that of *B. confusus*. However, upon investigating specimens from the American white pelican, we obtained molecular evidence supporting the presence of two species of *Bolbophorus* and then determined that adults of the two had different sized eggs, with those of one and maybe both being larger than those reported for *B. confusus*.

This study presents results from a long term study undertaken: (1) to identify the species of *Bolbophorus* represented by the prodiplostomulum in the channel catfish present in Louisiana and Mississippi, using both a feeding experiment and molecular analysis; (2) to determine whether the catfish prodiplostomula represented one or more species of *Bolbophorus* by sequencing nuclear DNA (nDNA), using an internal transcribed spacer region (ITS-1, 5.8S rRNA and ITS-2 ≈ 1030 bps), a portion of the small subunit 18S and a portion of the large subunit 28S rRNA as well as the mitochondrial DNA (mtDNA) protein coding gene cytochrome c oxidase I (COI); (3) to identify the two adult forms found in wild American white pelicans in the area; and (4) to identify the specimens obtained

from the feeding experiment. Early attempts to address these objectives were only partly successful. One of us (LP) maintained the prodiplostomulum for several days *in ovo* (chicken) and several weeks in tissue culture, but development did not proceed to the adult stage. We report here the same results administering the prodiplostomulum to domestic day-old chicks, mice and nestling American white pelican chicks as well as a successful feeding experiment with adult, wild caught, dewormed individuals of the American white pelican.

Materials and methods

Specimens of a prodiplostomulum consistent with those described for species of *Bolbophorus* were collected from catfish in commercial ponds in (1) St. Martin Parish, Louisiana, between 1994 and 2000, (2) Franklin Parish, Louisiana, in 1998 and (3) Holmes County, Mississippi, in 2000 and 2001. They were removed from the catfish along with some intact flesh surrounding the cyst and placed live in physiological saline for feeding studies. Representative specimens were removed from the cyst. Some of these were killed with hot water and immediately placed in 5% neutral buffered formalin. Others were placed immediately in 95% ethanol or SED buffer (saturated NaCl [3.42 M]; 250 mM EDTA, tetrasodium salt, pH 7.5; 20% DMSO; Amos & Hoelzel, 1991; Proebstel et al., 1993) for molecular studies. Some representative catfish tissues from representative experimental and natural infections were also fixed in Bouin's fixative or 10% formalin and sectioned.

Nestling pelican, domestic chick and mouse experiments

We obtained eggs laid by the American white pelican at Chase Lake National Wildlife Refuge, North Dakota, in June, 1998. They were hand-carried to Mississippi in a warmed ice-chest in the passenger section of a commercial airline. After incubation in a humidity-controlled unit initially at 40.0 °C but progressively reduced to 37.5 °C while being rolled and sprayed with distilled water, five birds hatched, and, over a 2–3 day period, three 4- or 11-day-old individuals were each fed 80–270 metacercariae presumed to be a species of *Bolbophorus* from the tissue of catfish obtained from St. Martin Parish, Louisiana.

Unfed day-old domestic chicks (black australorps) on two occasions were administered 100–300 metac-

ercariae either by mouth or gavigated into the anus with a no. 15 needle with point filed and smoothed fitted to a 0.25 ml syringe. Representatives were necropsied irregularly from 1 to 14 days. CrI:CD[®]-1 (ICR)BR Swiss mice were either administered a few metacercariae each in dialysis tubing filled with physiological saline or gavigated orally with 60. The 1-cm lengths of tubing were tied off and surgically implanted in the abdominal cavity and examined at 24, 48 or 167 hrs. All metacercariae were from St. Martin Parish.

Adult pelican feeding experiment

For feeding adult pelicans, we used farm-raised fingerling catfish from Holmes County, each naturally infected with approximately five metacercariae known to belong in *Bolbophorus*. Approximately 100 of these metacercariae with surrounding intact parasite and host layers plus physiological saline were placed in each of three gelatin capsules within a period of 3 hrs of removal from the fish. These capsules were promptly fed to three caged pelicans as indicated below. Twenty of the remaining infected catfish were fed live in their entirety to each of the three additional pelicans. A total of six American white pelicans was captured using Softcatch[®] leg hold traps (King et al., 1998) in Mississippi for this feeding experiment, with one from near Midnight, Humphreys County, in February, 2001 and five from near Morgan City, Leflore County, in March, 2001. All of the birds were individually housed in specially designed 3.0 × 6.0 × 1.5 m cages containing a 1,000-litre water tank at the National Wildlife Research Center (NWRC) in Mississippi State, Mississippi, and maintained on live, uninfected, laboratory-spawned and -reared channel catfish. Before and during the experiment, faecal samples from each bird were examined daily for eggs of digeneans and other helminths using Sheather's sugar flotation and a faecal sedimentation technique. Every bird initially had a natural digenean infection. Before initiating the experimental feeding, we administered each bird a single 204 mg dose of praziquantel per os. By the second day, no digenean egg was found in the faeces of any treated bird, and each remained free of digenean eggs for a period of 1 week except for single empty eggshells in a few cases. At that time, three pelicans were each fed a single gelatin capsule containing 100 live encysted prodiplostomula. Also, the remaining three pelicans were each fed 20 live catfish, or approximately 100 prodiplostomula. After 72 hrs, necropsies were conducted on all birds given metacercariae in capsules and one provided them in live fish.

Because a sufficient number of mature worms were obtained from those four birds and because of the sensitive status of the birds, the last two were donated to the Jackson Zoo in Jackson, Mississippi. All animals were killed in accordance with accepted scientific practices, with the birds being administered carbon dioxide in a chamber by a university veterinarian.

Bird collections, for this experiment and other aspects of the study, were conducted under the following authorities and permits: US Fish and Wildlife Scientific Collecting permits PRT-681207, MB681207 and MB019065-1, Special Use Permit 98-018, and permits issued to commercial farms plus wildlife and fisheries permits from Mississippi and Louisiana (e.g., LNHP-98-057 and 99-005). Research was conducted with these permits and approved under the Institutional Animal Care and Use Committee (IACUC) of NWRC.

Digenean specimens for examination and molecular studies

Adult specimens of species of *Bolbophorus* were collected from both experimentally infected specimens of the American white pelican as well as wild individuals, which were shot, from Louisiana at Grand Terre Island, Jefferson Parish, in April, 1999 at a commercial farm in St. Martin Parish in May, 1999 and from Mississippi at Stack Island, about 5 km south-southwest of Mayersville, Issaquena County, in April, 1999. Once removed from the anterior portion of the pelican intestine, these adult specimens were first placed in physiological saline and then placed in either 95% ethanol or SED buffer for molecular studies or fixed in near boiling water and pipetted into 5% neutral buffered formalin for morphological studies. Several cormorants, herons and egrets from near catfish ponds were also shot and examined for any infection with a species of *Bolbophorus*.

Adults and prodiplostomula were prepared as wholemounts, and adults were sectioned. Those for wholemounts were transferred from the formalin solution to distilled water and stained overnight in Van Cleave's haematoxylin with additional Ehrlich's haematoxylin. After being transferred from the stain to a graded ethanol series, they were buffered with enough lithium chloride and butylamine at 70% ethanol to produce a basic solution and then completely dehydrated to absolute ethanol. Finally, they were cleared in clove oil and mounted in Canada balsam. Histological sections were prepared from seven representative adult specimens from the feeding experiment and wild birds as well as metacercariae

from infected catfish tissues that were transferred to distilled water, dehydrated through a graded ethanol series, cleared in xylene and embedded in Paraplast[®]. The material was cut at 4 μm intervals. Sections were stained in Gill's haematoxylin and eosin and mounted with a synthetic commercially available mounting medium. Descriptive measurements are in micrometres and illustrations were prepared with the aid of a drawing tube.

Molecular analysis

Total DNA was extracted from individual samples using a procedure described by Taggart et al. (1992), with modifications described by Garber (2001). PCR-amplifications were conducted in an Applied Biosystems GeneAmp 2400 Thermal Cycler. Reaction volumes were 25 μl and contained 1.0 μl template (50 ng), 1.5 mM MgCl_2 , 200 μM each of dNTP (Promega), 0.3 μM each primer, and 1.75 U Taq DNA polymerase (Amersham Pharmacia & Biotech). PCR-conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 0.75 min, 52 °C for 1.0 min, and 72 °C for 2.0 min, with a final cycle of 72 °C for 7.0 min.

Initially, primers from other studies were used in an attempt to obtain sequence data. They are as follows: 28S rRNA - LSU5' and LSU3' (Lenaers et al., 1989) with polylinkers based on those by Medlin et al. (1988) that amplify from approximately the last 65 bps of ITS-2 through the D1–D3 regions of 28S rRNA; 18S rRNA - 143F and 145R (Kim & Abele, 1990; Lo et al., 1996) that amplify bp-positions 352–1,200 spanning the V3–V5 regions of decapods; cytochrome c oxidase I (COI) - JB3 and JB4.5 (Garey & Wolstenholme, 1989) that amplify bp-positions 2,575–3,021; and ITS-1, 5.8S rRNA, ITS-2 (ITS-1/2) - universal forward primer Br (5'-GTAGG TGAAC CTGCA GG-3') designed by Colomban de Vargas, University of Geneva, and the digenean-specific reverse primer dig11 (Tkach et al., 2000) that anneal in 18S rRNA and 28S rRNA, respectively, and amplify the entire region. After sequence data were obtained, the following species-specific primers were then designed and employed for the remainder of the study: 28SrRNA - Bolbo28S-F (5'-CTAGT AACTG CGAG TGAAG-3') and Bolbo28S-R (5'-CCTAT ACTCA CGTTA GACG-3'); 18s rRNA - Bolbo18S - F (5'-TGATC TGCCT ACCAT GGTGA T-3') and Bolbo18S-R (5'-CGTCA GTTGG CATCG TTTAT G-3'); COI - BolboCOI-F (5'-CAGGT TTTGG AATGA TTAGT C-3') and BolboCOI-R (5'-ACAAA TCAAG TGTCA TGAA- 3'); and ITS-1/2 - BolboITS-F (5'-TATTC

ACGTC TGATC CGAGG T-3') and BolboITS-R (5'-CTAGC AATTG CGTTG GTCTG-3').

Appropriate PCR-products were gel-purified (Qiagen Gel Extraction Kit), quantified using fluorescence spectrophotometry (Gallagher, 1994) and cloned into Promega's pGEM[®]-T Easy Vector System utilising blue/white selection (Sambrook et al., 1989). Plasmids from white colonies were purified with Promega's Wizard[®] Plus Minipreps DNA Purification System and screened for inserts by EcoRI-digestion (Promega) followed by agarose gel electrophoresis. Clones were quantified as above, and then they were sequenced on a LI-COR NEN Global IR2 DNA Sequencer using the Epicentre[®] SequiTherm EXCEL[™] II DNA Sequencing Kit-LC (for 66-cm gels), according to the manufacturer's instructions. Sequences were edited and aligned, using the default settings, with AlignIR[™] alignment and assembly software (LI-COR, Inc.).

Specimens were deposited in the British Museum (Natural History) collection (BMNH) at The Natural History Museum, London, UK and the Harold W. Manter Laboratory (HWML) of University of Nebraska State Museum, Lincoln, Nebraska, and all sequences were submitted to GenBank and assigned accession numbers AF470538–AF470615. Alignments of these were assigned the following accession numbers by WEBin-Align: COI gene alignment: ALIGN_000293; 18S gene alignment: ALIGN_000294; ITS 1 & 2 + 5.8S gene alignment: ALIGN_000295; and 28S gene alignment: ALIGN_000297.

Results

All prodiplostomula identifiable as '*Bolbophorus*' appeared morphologically and molecularly to represent the same species. Those in catfish from different localities (St. Martin Parish and Holmes County) at different times (May, 1999 and April, 2001) expressed DNA sequence similarity values of 98.9–99.7%, depending on the gene (Tables 1,2). In contrast, randomly selected adult specimens collected from the same individual wild American white pelican captured at both Belzoni and Stack Island clustered into two groups differing molecularly (Tables 1,2, which also give details of transitions, transversions and indels). When genetic analyses of three specimens with large eggs from Stack Island were compared with those of three with relatively small eggs from the same bird, they dif-

Table 1. DNA sequences of *Bolbophorus damnificus* (■) and *Bolbophorus* sp.(□) sorted by stage, source and location.

| Species | Life-Stage | Collection Site | 28S rRNA | 18S rRNA | ITS 1/2 | COI |
|------------------------|--------------------------|-----------------------|----------------|----------|------------------|------------------|
| <i>B. damnificus</i> | metacercaria, experiment | Holmes County, MS | ■ | ■ | ■ | ■ |
| <i>B. damnificus</i> | metacercaria, experiment | Holmes County, MS | ■ | ■ | ■ | ■ |
| <i>B. damnificus</i> | metacercaria, experiment | Holmes County, MS | ■ | ■ | ■ | ■ |
| <i>B. damnificus</i> | metacercaria, pond | St. Martin Parish, LA | ■ ¹ | ■ | ■ | ■ |
| <i>B. damnificus</i> | metacercaria, pond | St. Martin Parish, LA | ■ ¹ | ■ | ■ | ■ |
| <i>B. damnificus</i> | metacercaria, pond | St. Martin Parish, LA | ■ ¹ | ■ | ■ | ■ |
| <i>B. damnificus</i> | adult, experiment | Holmes County, MS | ■ | ■ | ■ | --- ³ |
| <i>B. damnificus</i> | adult, experiment | Holmes County, MS | ■ | ■ | ■ | ■ |
| <i>B. damnificus</i> | adult, experiment | Holmes County, MS | ■ ¹ | ■ | ■ | ■ |
| <i>B. damnificus</i> | adult, large body | Belzoni, MS | ■ | ■ | ■ | ■ |
| <i>Bolbophorus</i> sp. | adult, large body | Belzoni, MS | □ ² | □ | □ | □ |
| <i>B. damnificus</i> | adult, large body | Belzoni, MS | ■ | ■ | ■ | ■ |
| <i>B. damnificus</i> | adult, random | Stack Island, MS | ■ | ■ | --- ³ | ■ |
| <i>Bolbophorus</i> sp. | adult, random | Stack Island, MS | □ ² | □ | □ | --- ³ |
| <i>Bolbophorus</i> sp. | adult, random | Stack Island, MS | □ ² | □ | □ | --- ³ |
| <i>Bolbophorus</i> sp. | adult, small-egged | Stack Island, MS | □ ² | □ | □ | □ |
| <i>Bolbophorus</i> sp. | adult, small-egged | Stack Island, MS | □ ² | □ | □ | □ |
| <i>Bolbophorus</i> sp. | adult, small-egged | Stack Island, MS | □ ² | □ | □ | □ |
| <i>B. damnificus</i> | adult, large-egged | Stack Island, MS | ■ | ■ | ■ | ■ |
| <i>B. damnificus</i> | adult, large-egged | Stack Island, MS | ■ | ■ | ■ | --- ³ |
| <i>B. damnificus</i> | adult, large-egged | Stack Island, MS | ■ | ■ | ■ ⁴ | --- ³ |

¹missing first 65 base pairs; ²does not contain 16 base pair deletion; ³no sequence obtained; ⁴missing last 64 base pairs

ferred (86.4–95.2 DNA percentage sequence similarity, depending on the gene, Table 2), but that large-egged group was essentially the same as all specimens of the evaluated metacercaria (96.8–97.6%, Table 2) from catfish and the material that developed in pelicans fed the catfish metacercaria. There was no evidence that the metacercaria of the small-egged species infected the catfish. Of the three pelicans fed the capsules, all had eight to ten specimens, including mature ones, and the bird fed live fish had 95 such specimens. These large-egged specimens were consistent morphologically with specimens collected from pelicans locally over several years as well as those reported as *B. confusus* by Fox (1965) but different from *B. confusus* (*sensu stricto*); they are described below as a new species.

Of the three nestling pelican chicks fed metacercariae from under the skin of the catfish, only one produced an infection, and there were 29 partly developed individuals present when it died 30 hrs after being fed the worms. The other two birds had no specimen recovered at days 6 or 7.

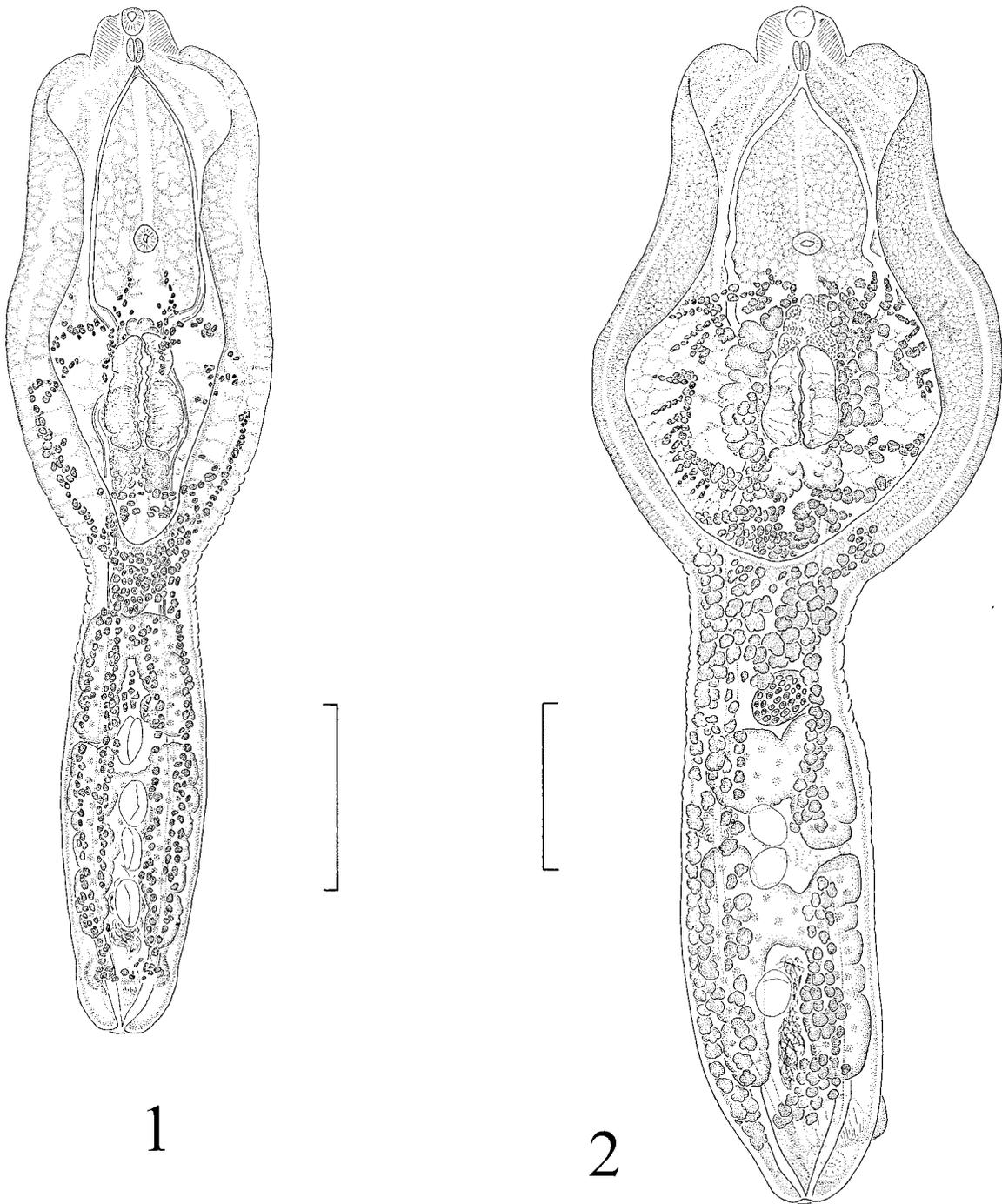
No specimen was recovered from the intestine of either domestic chicks or mice at any time other than a couple of individuals at 24 hrs; worms from the abdominal cavity of mice survived for at least 7 days when in dialysis tubing. By that time, however, some specimens apparently had been dead for a long period and some were beginning to undergo necrosis. Neither these nor the live seemingly healthy specimens had matured, but they had undergone minimal development.

Bolbophorus damnificus Overstreet & Curran n. sp.

Description (Tables 1–2; Figures 1–4)

Adult

Description measurements based on 6 wholemounts and other aspects on 7 sectioned specimens (5 sagittal and 2 frontal). Bolbophoridae Shoop, 1989. Body strongly bipartite, spatulate, 2.6–3.2 mm long; anterior segment (forebody of some authors) 1,349–1,572 long, 589–1,067 wide at maximum width be-



Figures 1–2. *Bolbophorus damnificus* Overstreet & Curran n. sp. from experimentally infected American white pelican. 1. Adult, ventral, holotype. 2. Adult, ventral. Scale-bars: 1,2, 500 μ m.

Table 2. Nucleotide sequence comparisons for stages of *Bolbophorus damnificus* (■) and *Bolbophorus* sp.(□).

| 28S rRNA | | | | |
|---|------------|-------------|---------------|--------|
| ■ = 1170 bp; □ = 1249 bp; ■ vs. □, all = 1253 bp; ■ vs. □, adults = 1252 bp; metacercaria = 1234 bp | | | | |
| Alignment | % Identity | Transitions | Transversions | Indels |
| ■ | 97.0 | 20 | 9 | 3 |
| □ | 99.1 | 8 | 2 | 0 |
| ■ vs. □, all | 90.6 | 66 | 24 | 22 |
| ■ vs. □, adults | 90.6 | 66 | 22 | 23 |
| ■ metacercaria | 99.7 | 2 | 2 | 0 |
| 18S rRNA 798 bp except □ = 795 bp | | | | |
| ■ | 97.6 | 10 | 5 | 4 |
| □ | 99.4 | 4 | 0 | 1 |
| ■ vs. □, all | 94.6 | 27 | 8 | 8 |
| ■ vs. □, adults | 95.2 | 25 | 5 | 8 |
| ■ metacercaria | 99.4 | 2 | 3 | 0 |
| ITS 1/2 ■ = 1023 bp; □ = 1024 bp; ■ vs. □, all = 1036 bp; ■ vs. □, adults = 1036 bp; ■ metacercaria = 1023 bp | | | | |
| Alignment | % Identity | Transitions | Transversions | Indels |
| ■ | 96.8 | 22 | 8 | 1 |
| □ | 99.4 | 5 | 1 | 0 |
| ■ vs. □, all | 86.1 | 76 | 35 | 24 |
| ■ vs. □, adults | 86.5 | 71 | 36 | 24 |
| ■ metacercaria | 99.4 | 5 | 1 | 0 |
| COI 376 bp | | | | |
| Alignment | % Identity | Transitions | Transversions | Indels |
| ■ | 97.9 | 3 | 3 | 2 |
| □ | 99.7 | 0 | 0 | 1 |
| ■ vs. □, all | 85.4 | 21 | 31 | 3 |
| ■ vs. □, adults | 86.4 | 19 | 29 | 3 |
| ■ metacercaria | 98.9 | 2 | 2 | 0 |

tween middle and posterior thirds of segment, spoon-shaped, with ventral surface concave, with anterior blunt protuberance; posterior segment (hindbody of some authors) elongate, cylindrical, 1,123–1,600 long, 360–561 wide at middle region. Ratio of posterior to anterior body segment length 1: 1.0–1.3. Tegument devoid of spines. Oral sucker 75–85 × 63–83, terminal, with subterminal mouth, flanked by pseudosuckers, or lappets; pseudosuckers 2, 122–159 × 56–73, muscular, containing aligned usually swollen glandular ducts; ducts consisting of 2 kinds with different secretory products (presumably attachment and extra-

corporel digestive), with collecting ducts extending well posterior into anterior body segment and some into posterior segment; outer-most series of terminal swollen ducts more deeply staining of 2 with carmine, confined to distal half; dorsal series longer, also occurring throughout, more swollen of 2. Prepharynx very short or appearing as absent. Pharynx 63–85 × 40–57, oval. Oesophagus 6–31 long. Caeca diverging in anterior portion of segment, converging at lateral margins of holdfast organ, terminating blindly near posterior end of body. Ventral sucker 48–63 × 48–71, always narrower than oral sucker. Ratio of oral

sucker to ventral sucker width 1: 0.76–0.89. Distance between anterior end and anterior margin of ventral sucker 517–600 long, with body in that region usually containing fewer subtegumentary gland-cells than in *Prodiplostomulum*; subtegumentary gland-cells located dorsal and ventral from anterior end to level of ventral sucker; internal gland-cells of 2 types in parenchyma, with glandular products typically irregular shaped, often approximately 17×10 (ducts often empty in older specimens from wild hosts), with associated ducts (some from posterior segment) leading to pseudosuckers. Holdfast organ (tribocytic or adhesive organ of some authors), $256\text{--}476 \times 159\text{--}334$, oval, situated medially in posterior third of anterior segment, 1.0–2.8 ventral sucker widths posterior to that sucker, protruding slightly from ventral surface, with medial longitudinal slit, with anterior and posterior dorsal glandular masses; anterior mass eosinophilic in section; posterior mass basophilic; combined masses $502\text{--}530 \times 201\text{--}335$, difficult to discern in most wholemounts.

Testes 2, tandem in dorsal portion of posterior segment; anterior testis $223\text{--}407 \times 229\text{--}335$, lobed, somewhat inversely U-shaped; posterior testis $430\text{--}597 \times 285\text{--}435$, approximately H-shaped. Vas deferens ventro-medial to testes; vasa efferentia 2, medial, each emanating from anterior ventral margin of testis. Seminal vesicle sinuous, leading to simple ejaculatory duct. Cirrus-sac absent.

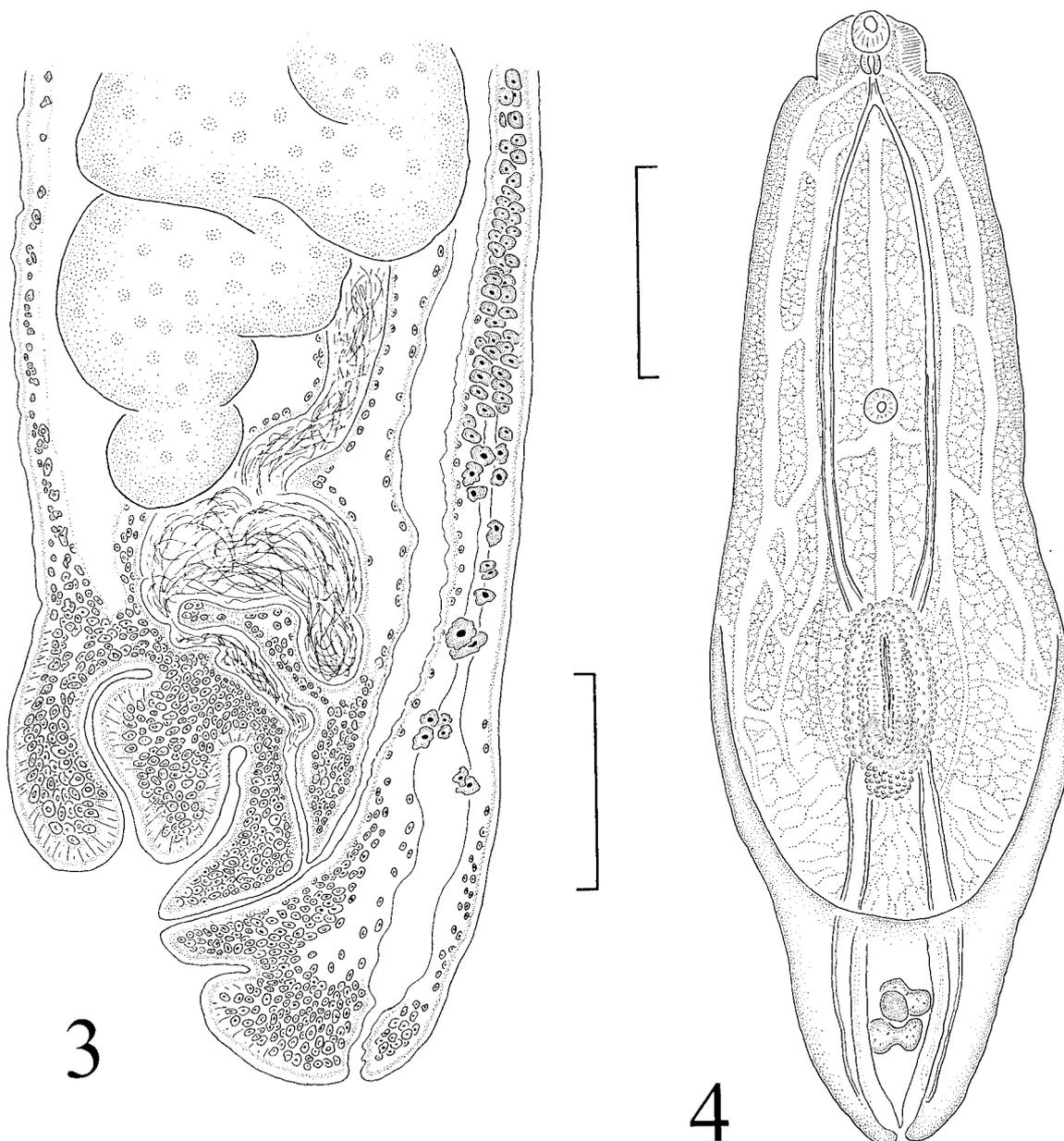
Ovary $102\text{--}123 \times 112\text{--}162$, pretesticular, medial or submedian at anterior end of posterior body segment. Oviduct running posteriorly, dorsal to anterior testis, leading to oötype; oötype between testes. Laurer's canal simple, branching from oviduct dorsal to anterior testis, opening on dorsal surface at level of anterior testis. Mehlis' gland surrounding oötype, consisting of at least 2 cell types. Vitelline reservoir tear-drop-shaped, adjacent to Mehlis' gland between testes. Vitellarium a field of ventral follicles in posterior segment, extending into anterior segment, reaching to approximately same level as anterior margin of cells associated with holdfast organ, 34–100 from posterior margin of ventral sucker in recently matured individuals (in some additional specimens from wild hosts frozen or fixed without heat, follicles extended anteriorly slightly anterior to level of ventral sucker), typically with ventral follicles larger than dorsal ones. Uterus thin-walled, tubular, running anteriorly from oötype to level of ovary, turning and running directly to posterior region of body, dorsal to vitelline follicles, ventral to vas deferens, joining with ejaculatory

duct to form hermaphroditic duct in posterior end of body; hermaphroditic duct short (73 in 2 sectioned worms), running through genital cone, opening in genital atrium; genital atrium with dorsal genital bulb. Eggs 1–8 in number (in 3-day-old specimens, with as many as 25 eggs in specimens from wild pelican), $123\text{--}129 \times 50\text{--}89$, with indistinct operculum (often popped open in older specimens from wild hosts).

Excretory vesicle V-shaped, with arms passing ventral to testes and ovary into anterior segment of body; paranephridial system ('reserve bladder system' of some authors) becoming diffuse in region between anterior and posterior body segments, differentiating into 5 longitudinal channels in anterior segment (1 medial and 2 lateral pairs), with channels each uniting in pharyngeal region, with transverse commissures and numerous anastomosing small diverticula as in *metacercaria*; pore ventro-terminal.

Prodiplostomulum

Description based on 7 wholemounts. Encysted in elliptical or in rare cases nearly spherical transparent cyst of parasite origin, typically in muscles immediately under dermis, with most individuals usually concentrated in caudal portion and dorsal surface; cyst (fixed) $1,011\text{--}1,375 \times 758\text{--}1,039$, surrounded by smooth-walled encapsulating layer of host origin (as distinguished from host response); host layer milky white, $1,263\text{--}1,684 \times 926\text{--}1,291$. Body chalky white in life, $1,740\text{--}2,133 \times 561\text{--}758$, divided into 2 distinct segments; anterior segment $1,380\text{--}1,853$ long, pyriform, with ventral surface concave, with rows of minute spines (about 1 long) dorsally and ventrally, with abundance of subtegumentary glands; glands approximately 7×5 , located dorsally and ventrally, ranging posteriorly from anterior end to ventral sucker (slightly posterior to sucker in some specimens), opening by short duct at tegumental surface; posterior segment $280\text{--}393$ long, conical. Oral sucker $61\text{--}78 \times 67\text{--}78$, terminal. Pseudosuckers $100\text{--}125 \times 35\text{--}61$, muscular, containing ducts. Prepharynx absent. Pharynx $40\text{--}56 \times 30\text{--}47$ wide. Oesophagus $16\text{--}39$ long. Caeca located same as in adult, with basophilic glandular cells associated with oesophagus and anterior portion. Ventral sucker $48\text{--}60 \times 43\text{--}60$, $595\text{--}753$ or about a third of body length from anterior end. Ratio of oral sucker to ventral sucker width 1:0.60–0.86. Holdfast organ $268\text{--}318 \times 140\text{--}207$, 3.6–7.4 ventral sucker widths posterior to that sucker, with elongate, slit-like opening, with 2 distinct dorsal glandular masses posteriorly; anterior of 2 masses somewhat U- or



Figures 3-4. *Bolbophorus damnificus* Overstreet & Curran n. sp. 3. Adult terminal genitalia, lateral, composite from sagittal sections, from experimentally infected American white pelican. 4. Prodiplostomulum, ventral, from channel catfish. Scale-bars: 3, 100 μm ; 4, 400 μm .

kidney-shaped, usually larger than posterior one, usually lighter staining than posterior one; posterior-most mass subspherical or subcuboidal.

Ovarian anlage 30-73 \times 30-50, subspherical, lying ventral to anterior testis in posterior body segment. Anterior testis 56-73 \times 67-89, irregular-shaped. Posterior testis 50-70 \times 84-122, dumbbell-shaped.

Excretory vesicle V-shaped in posterior body segment, with branches extending anteriorly into anterior body segment where branching and somewhat obscured; paranephridial system divided into 5 longitudinal channels in anterior segment (1 medial and 2 lateral pairs); channels each uniting in pharyngeal region, with transverse commissures (1 with medial channel and 3 with lateral channels when consider-

ing anterior segment) and numerous anastomoses at various locations (see Figure 4), with channels and commissures containing excretory corpuscles ('lime-bodies', most readily observed in live and carmine-stained specimens); excretory pore terminal.

Material studied

Type-host: *Pelecanus erythrorhynchos* Gmelin, American white pelican (Pelecanidae). *Type-locality:* Thornton, Holmes County, Mississippi, USA; other confirmed localities with natural infections in Issaquena County, Mississippi, and Jefferson and St. Martin parishes, Louisiana, USA.

Site: Anterior intestine.

Intermediate hosts: First intermediate host: *Planorbella trivolvis* (Say), marsh rams-horn (Planorbidae); second intermediate host: *Ictalurus punctatus* (Rafinesque), channel catfish (Ictaluridae).

Site in catfish: In experimental and natural infections usually encysted immediately under dermis in caudal and dorsal regions but occasionally deeper in muscle, in fins, near maxilla, in isthmus, along inside of operculum and inside skull.

Material deposited: Holotype, HWML 16630; paratypes, adult, HWML 16631 and prodiplostomulum, HWML 16632. BMNH paratypes 2002.1.2.1-2 (adult), prodiplostomulum 2002.1.2.3.

Etymology: The Latin adjectival name *damnificus*, meaning 'injurious', refers to the harmful nature of this species to catfish in confined ponds.

Discussion

Wholemounds and sectioned material of adult worms from experimental and natural infections confirmed the presence of a genital cone and a genital bulb, indicating that the material belongs in *Bolbophorus*. The presence of a true cyst wall around the metacercaria corroborates this generic identification. According to Krause (1914), the European species, *B. confusus*, type for the genus and named by him (1914), was initially misidentified by Brandes (1888a,b) as *Hemistomum trilobum* (Rudolphi, 1819) Diesing, 1850 from the Dalmatian pelican *Pelecanus crispus* Bruch in Europe. The species was later reported also from the eastern (great) white pelican *P. onocrotalus* Linnaeus in Romania and Syria (Ciurea, 1930). Dubois (1935) erected *Bolbophorus* for *H. confusum* Krause, 1914 and listed the above two pelicans as hosts as well as *P. erythrorhynchos* from Minnesota, USA.

B. confusus has since been reported from a variety of other pelicans from around the world (e.g. Dubois, 1970). The record from Minnesota appears to be from one specimen collected by Professor Gustav Swanson of the University of Minneapolis for which Dubois (1935) presented little data and no egg size. He later (1970) provided the range for the species of up to 112 μm , based on that specimen; we measured an egg 117 μm (Dubois no. J-80), and the vitellarium reached anteriorly almost to the ventral sucker.

The North American material described here, *B. damnificus* n. sp. from pelicans that had been fed infected catfish, compared well with numerous adult specimens collected over several years from wild specimens of the American white pelican in Mississippi and Louisiana. It is very similar to *B. confusus* (*sensu stricto*), but it differs morphologically at least by having larger eggs (123–129 \times 50–89 compared with 90–102 \times 55–72 μm). Our molecular data from all the genes comparing two North American species of *Bolbophorus* strengthen the importance of difference in egg size. As expected, use of the relatively variable nuclear ITS-1/2 and mitochondrial COI gene fragments of *B. damnificus* and the sympatric species exhibited more differences than those of 18S and 28S rRNA alignments. The nuclear genomic spacers had 85.9 or 86.1 DNA percentage sequence similarity of total mutations in the alignments between the two species, depending on whether the lack of the last 64 base pairs in one of three specimens of *B. damnificus* from Stack Island are included (Table 1). The taxonomic status of the cryptic species sympatric in the white pelican hereafter referred to as *Bolbophorus* sp. will be discussed later in this paper. This divergence inherent in the ITS-region of digeneans corresponds with that in the ITS-1 study by Bartoli et al. (2000). Those authors matched the sporocyst and adult of the digenean *Monorchis parvus* Looss, 1902, which differed by two transition events, and then differentiated that species with the morphologically similar *M. monorchis* (Stossich, 1890) by the presence of 74–76 substitutions in the 1,017-bp-fragment (7.3–7.5%). That study accepted on one hand nominal species that several authors considered conspecific and on the other hand individual phenotypic variability among the same species in different hosts. Another study by Jousson & Bartoli (2000), also using the ITS-1 fragment, showed genetic differences not apparent in the phenotypes of two species of *Opecoeloides* Odhner, 1928. *O. furcatus* (Bremser in Rudolphi, 1819) has been reported from six host fishes in the Mediterranean Sea, but Jousson

& Bartoli (2000) demonstrated that at least the specimens in two of the hosts represented two separate species. They determined that an amplified 60-bp-long fragment occurred once in the total 840-bp fragment in both the adult of *O. columbellae* (Pagenstecher, 1863) Jousson & Bartoli, 2000 in the fish and the cercaria in a mollusc, but there was a repeat of the 60-bp fragment plus 11 nucleotide substitutions (2.6%) in both the adult and the cercaria of what they considered *O. furcatus* (*sensu stricto*) from a different fish and mollusc. The latter differs by 8.5% in the same gene from *Poracanthium furcatum* Dollfus, 1948 (see Jousson et al., 1999). These relative differences are less than the 13.9–14.1% that we report here.

Even though the mitochondrial genome is typically considered less conservative than the nuclear one, the COI gene codes for a core protein and is most likely more conservative than most mitochondrial genes. Nevertheless, the COI alignments demonstrated a 14.6% difference in total mutations between the two species of *Bolbophorus*. That difference compared similarly and in some cases better than related species in other studies. For example, Morgan & Blair (1998) found a range from 6.3 to 10.6% difference in base pairs among five digenean species of *Echinostoma* Rudolphi, 1809 with 37 collar spines and 11.4 and 14.1% when they added two additional species without 37 spines. No difference existed between isolates of one of those species. In a similar study when examining the COI 372 bp-sequences of three species of *Schistosoma* Weinland, 1858, Bowles et al. (1993) found *S. japonicum* Katsurada, 1904 differed from the two other species at 13 and 24% of the nucleotide positions but did not differ in any of its nucleotide positions among its various geographical isolates. We note in our data that there are more transitions than transversions for all three sequenced nuclear genes (Table 1), but just the opposite biases were true for COI, suggesting an ancient separation of the two species. There was one deletion in COI of one of the four sequenced individuals of *Bolbophorus* sp., and it may have been an artifact. In the case of the translated amino acids in the COI gene among the 12 individuals of *B. damnificus*, we noted three synonymous and four non-synonymous substitutions, not counting one insertion and one deletion.

Although the similarity in typically conservative 18S rRNA alignments (94.6%, total alignment mutations) between *B. damnificus* and *Bolbophorus* sp. also compares favorably with other differentiating genes for distinguishing closely related species, 18S se-

quences are typically used for studying higher levels of phylogeny (e.g. Lumb et al., 1993; Littlewood et al., 1999; Cribb et al., 2001). Lumb et al. (1993) reported only four or five bp-differences in relatively long fragments that could be unambiguously resolved when comparing sequences of closely related lepecreidiid and fellodistomid digenean species and recommended using the 18S for family-level relationships. The interpretation of our similarity value for 28S rRNA (90.6%) is not as clear. The sequences for *B. damnificus* contained a 16-bp deletion and were missing the first 65 bps in some of the metacercariae of *B. damnificus* from St. Martin Parish and in one of three adults resulting from feeding metacercariae from Holmes County but not evident in those adults of *Bolbophorus* sp. No comparable arrangement occurred in the sequences of other genes, and such deletions could represent artifacts related to the techniques. León-Règagnon et al. (1999) used 883 bps of 28S to assess seven digenean species of *Haematoloechus* Looss, 1899 from frogs and determined that 15.4% of the sites were variable. Those authors were able to show that some of the seven species were molecularly more closely related than assumed earlier on the sole basis of morphological features and that two were conspecific.

A complex of North American species of *Bolbophorus* exists as well as a larger complex worldwide. In North America, we believe we have two species in this study, *B. damnificus*, which can serve as a useful molecular standard, and *Bolbophorus* sp. Both infect the American white pelican in the southern USA. The second species may or may not be conspecific with *B. confusus*, but we do not think it is. Based on egg length (100–112 vs 90–102 μm), it may represent another new species, but molecular analyses of European material from pelicans should settle that question. We assume that the material in Montana is *B. damnificus*, but we cannot be positive without molecular analyses because it may consist of more than one species. The egg size reported by Fox (1965) overlaps with that of *B. damnificus* (115–125 \times 67–82 compared with 123–129 \times 50–89 μm), and, considering our molecular data on two species and the fact that the birds from Mississippi migrate to and from western Canada and western USA (Turcotte & Watts, 1999), we wonder if there are not two species in Montana. Fox (1965) mentioned the difference in egg size between his material and that of *B. confusus*, but he attributed the difference to methods. He measured only eggs that had been obtained from the host faeces rather than *in utero* from wholemounted spec-

imens, an explanation with which we disagree, if he measured fixed eggs. We examined six specimens in the Dubois Collection (W-39) collected from the pelican in Saskatchewan that were represented by five specimens over 3 mm long with eggs typically 124–136 μm long and one with most eggs 112 μm long; these probably represent the same two species that we report here. The prodiplostomulum that Haderlie (1953) described as *Diplostomulum ictaluri* Haderlie, 1953 appears to be a species of *Bolbophorus*, but it is different from *B. damnificus* in that the large fully-developed metacercaria was smaller (1.4–1.7 vs 1.7–2.1 mm long), had a smaller inner cyst size (0.8–1.0 vs 1.0–1.4 mm long), an oral sucker about the same size but with a ventral sucker about 0.04 mm wide rather than 0.10–0.13 mm, or half the width of the oral sucker rather than about 1.5 times larger. More importantly, the ventral sucker is less than one ventral sucker length anterior to the holdfast organ rather than 2.5–3.1 times the sucker width. It was collected from Clear Lake, Lake County, California, in 1949 from *Ameiurus catus* (Linnaeus) (as *Ictalurus catus*) and has not been reported since. It may be conspecific with *Bolbophorus* sp. from Mississippi, but we know neither the morphological features of the latter's metacercaria nor what fish or snail it infects. It may be a junior synonym of *B. confusus* (*sensu stricto*), if the latter has or had been introduced into North America. If it does not represent the metacercarial stage of the three mentioned above, then *B. ictaluri* (Haderlie, 1953) Overstreet & Curran n. comb. may be one of a complex of four North American species. In any event, we consider it a *species inquirenda*.

Bolbophorus damnificus differs from all previously described species of *Bolbophorus* as well as the cryptic *Bolbophorus* sp. by egg size and the vitellarium not reaching anterior to the holdfast organ, at least in well-fixed young adults. It has eggs 123–129 \times 50–89 μm compared to 90–102 \times 55–72 μm in *B. confusus* [in 1970, Dubois increased the length from his earlier papers (e.g. 1935) to 112 μm , possibly based on literature of non-European specimens and not the European specimens reported by Krause (1914) or Ciurea (1930)], 98–112 \times 58–68 μm in *B. levantinus* Paperna & Lengy, 1963 [Dubois (1970) provided those measurements and stated that the measurements of 169 \times 92 μm for that species by Paperna & Lengy (1963) were given in error; we measured well-developed eggs (Dubois T-35) as 107–112 \times 50–57 μm in a 1.74 mm specimen], 89–99 \times 50–64 μm in *B. indianus* Mehra, 1962, 81–93 \times 52–76 μm in

B. deodhari Ali & Karyakarte, 1971 (from *Pelecanus onocrotalus* in India) and 100–112 μm long in *Bolbophorus* sp. A close relationship among all species of *Bolbophorus* is apparent from their relative morphological similarity. This especially close affinity between the two North American forms and *B. confusus* (*sensu stricto*) of Europe is also evident by their restriction to pelican definitive hosts and perhaps their range in teleost intermediate hosts. The report of *B. confusus* from the reddish egret *Egretta rufescens* from Florida, USA, by Conti et al. (1986) is a misidentification. We examined the four specimens on one slide deposited by those authors (USNPC No. 78084, all in poor condition) and determined that the species lacked a genital bulb, a feature diagnostic for *Bolbophorus*, and we consider it to represent a species of *Diplostomum* von Nordmann, 1832. To our knowledge, neither *B. damnificus* nor *B. confusus* is known from a bird other than a pelican. Dronen et al. (1999) reported *B. confusus* from one of four specimens of the brown pelican in coastal Texas, but Courtney et al. (1974) did not report it from 113 specimens of that pelican in Florida and Louisiana. We have searched for *B. damnificus* and other species of *Bolbophorus* without success by examining at least a few specimens of each of the following birds: *Phalacrocorax auritus* (Lesson) (double-crested cormorant), *P. brasilianus* (Gmelin) (Neotropic cormorant, previously considered the olivaceous cormorant *P. olivaceus* (Humboldt)), *Ardea herodias* Linnaeus (great blue heron), *Ardea alba* Linnaeus (great egret), *Egretta thula* (Molina) (snowy egret), *E. caerulea* (Linnaeus) (little blue heron), *E. tricolor* Müller (tricolored heron), *Nycticorax nycticorax* (Linnaeus) (black-crowned night-heron) and *Nyctanassa violacea* (Linnaeus) (yellow-crowned night-heron). All the birds were from or near catfish ponds and had presumably fed on infected catfish and defecated in the ponds.

Pelicans seem to be the only host for *B. damnificus*. Development progressed some by 30 hrs in a nestling pelican and reached maturity with eggs by day 3 in the experimentally fed adult pelican. Fox (1965) determined it took three days to develop and survived for five months, suggesting he was also dealing with *B. damnificus*. In contrast, when we fed metacercaria to domestic day-old chicks and mice and a series was examined over several days, we obtained a low recovery of specimens and at about one day only. When in the mouse's abdominal cavity, they survived for a week or so, but they developed little.

The reason that two of the three nestling pelicans did not have worms could have been from a shortage of enough of the appropriate lipids in their fish diet. Since the third died 30 hrs after being fed the metacercariae, there was not enough time for the worm to mature.

All species of *Bolbophorus* do not infect pelicans. *B. levantinus* was originally described as a subspecies of *B. confusus* from a single experimentally infected purple heron (*Ardea purpurea* Linnaeus) from Tel-Aviv University in Israel, but they had also seen it in the wild purple heron. It was accepted as a species by Dubois (1970), and we agree. Paperna & Lengy (1963) considered it as a subspecies different from *B. confusus confusus* by having vitelline follicles reaching to about the pharynx rather than to the holdfast organ, relatively smaller pseudosuckers, and, perhaps, a relationship of the anterior divided by posterior body portion equal to 0.9 rather than about 1.4–1.5. Eggs from the heron's faeces were reported as operculate and $169 \times 92 \mu\text{m}$, a size considered as erroneous by Dubois (1970, see above). The miracidium infected the planorbid *Bulinus truncatus* (Audouin) in the laboratory but not four other local snails, and the cercaria developed in *Tilapia nilotica* (Linnaeus) but not *T. zilli* (Gervais), a cyprinid, or *Gambusia affinis* (Baird & Girard) (see Paperna & Lengy, 1963). *Ardea purpurea* (experimental and wild) serves as the only known host for this species. *Bolbophorus indianus*, the only other nominal species not from a pelican, was described from the Australian darter *Anhinga melanogaster* Pennant in India. Other than by having small eggs, that species differs from *B. confusus* by having an indistinctly bisegmented body and by having a vitellarium that reaches anteriorly to the ventral sucker (Dubois, 1970).

No evidence exists that *B. confusus (sensu stricto)* occurs in North America. Hoffman (1970) speculated that what he considered to be *B. confusus* was introduced to North America from Europe by a stray European pelican. If *B. confusus sensu stricto* does occur in North America, we find this scenario highly unlikely, considering the migratory patterns of European pelicans (Johnsgard, 1993) and the west-to-east movement of storms. If an introduction occurred, a more believable colonisation scenario might be an inadvertent one of the early stages of *B. confusus* in fishes or snails. Introductions from Europe into North America are not common, but they have occurred. Some introductions occurred in states such as California and Montana. California (Dill & Cordone, 1997) ranks second to Florida as receiving the largest num-

ber of introduced species of fish in the USA, with at least 90 species and subspecies, including the host of *B. ictaluri*. With some of those fish or other imported aquatic life could have come (at some time in the past) infected snails or fish. If not introduced, *B. confusus* may have a natural distribution in North America. Some species of freshwater trematodes, including *B. confusus*, have been reported to have a Holarctic distribution [e.g. *Aspidogaster conchicola* von Baer, 1827, *Plagioporus angusticolis* (Housmann, 1896), *Allocreadium isosporum* (Looss, 1895), *Bunodera luciopercae* (Müller, 1776), *Crepidostomum auriculatum* (Wedl, 1858), *C. farionis* (Müller, 1874) and *Diplostomum spathaceum* (Rudolphi, 1819)], but the vast majority of Holarctic freshwater trematode species have either a Nearctic or Palaearctic distribution (see Manter, 1963 for discussion of the distribution of species in genera found in both North America and Eurasia). We, however, question the Holarctic distribution of at least the two diplostomoids. Furthermore, the others infect clam or fish hosts that have a history of being introduced. Regardless of these hypothetical scenarios for the presence of *B. confusus* in North America, we contend that it is just as plausible that species of *Bolbophorus* have diverged separately in North America and Eurasia. Furthermore, as indicated above, *B. confusus* has not been confirmed from North America.

In North America, what has been considered *B. confusus* has been reported on several occasions since 1935 (McNeil, 1949; Huggins, 1956; Fox, 1962; Fox & Olson, 1965; Fox, 1965). McNeil (1949) and Huggins (1956) provided minimal information about the specimens that they obtained, all from the American white pelican. Fox (1962) reported a prodiplostomulum from Montana tentatively identified as *B. confusus* in the rainbow trout *Oncorhynchus mykiss* (Walbaum) (as *Salmo gairdneri*), and brown trout, *Salmo trutta* Linnaeus. In an abstract, Fox & Olson (1965) reported that they experimentally infected fishes with material from the planorbid snail *Planorbella trivolvis* (as *Helisoma trivolvis*), which they had infected with eggs from adults in the American white pelican. In his dissertation, Fox (1965) reported details of the life-cycle and thoroughly described the adult of what they considered to be *B. confusus* (probably *B. damnificus*). Fox (1965) and later Olson (1966) assessed the host-specificity for the same, plus, as we indicated above, perhaps an additional cryptic or introduced species. They found natural infections in Montana in the salmonids *Prosopium*

williamsoni (Girard), *S. trutta*, *O. mykiss* and *Thymallus arcticus* (Pallas), in the catostomids *Catostomus catostomus* (Forster) and *C. commersoni* (Lacepède) and in the cyprinid *Gila atraria* (Girard). Experimentally, they infected the trouts *P. williamsoni*, *S. trutta*, *O. mykiss* and *Salvelinus fontinalis* (Mitchill), the cyprinids *Pimephales promelas* Rafinesque, *Platygo bio gracilis* (Richardson), *Ptychocheilus oregonensis* (Richardson), *Rhinichthys cataractae* (Valenciennes) and *Richardsonius balteatus* (Richardson), the centrarchid *Lepomis macrochirus* Rafinesque, the cottid *Cottus bairdi* Girard, the ictalurid *I. punctatus* and the poeciliids *Gambusia affinis* and *Poecilia reticulata* Peters. Olson (1966) considered *I. punctatus* an abnormal host for the trematode because he obtained no encysted metacercaria in six fish exposed at 18 °C and just one in a single exposed fish at 22 °C. We know that temperature influences infections, at least in the southern states. No evidence supports the presence of *Bolbophorus* sp. or any other prodiplostomulum in *I. punctatus* in the southern USA, but such an infection may occur.

In Europe, *B. confusus* is reported to develop in a variety of fish species, including many cyprinids, *Liza saliens* (Risso) (Mugilidae, listed as *Mugil*), *Perca fluviatilis* Linnaeus (Percidae) and *Esox lucius* Linnaeus (Esocidae) (e.g. Bykhovskaya-Pavlovskaya, 1964; Dubois, 1970). The snail host there has not been reported. More importantly, the adult has been reported from a variety of pelicans from Australia, the Philippines, Vietnam (as Indochina), India, Israel, the Nile River Valley and Central Africa (Dubois, 1970). We suspect a comparative molecular analysis will reveal some of those represent additional new species in the *B. confusus*-complex.

The extant species of *Bolbophorus* likely diverged from a diplostomid-like ancestor during the Triassic (about 250 mya) when continents were still together. The various speciation events probably occurred as the result of vicariance when Europe, North America, India, Australia and Africa separated, isolating the host and digenean populations. We suspect further evidence for vicariate speciation among species of *Bolbophorus* will become available when molecular evaluations and intermediate hosts of non-North American species are determined.

This study has implications for catfish farmers in the USA. The catfish may be an abnormal host, as suggested by Olson (1966), but we recognise an abundance of infections in Mississippi and Louisiana. The infections may be related to exposure to a high number

of cercariae in aquaculture conditions. Nevertheless, the pathogenic effect in the southern USA may reflect an atypical host. Occasionally, we observed heavy infections, and they often had haemorrhaging associated with both the cercaria and metacercaria, muscle necrosis surrounding some cysts, kidney tubule necrosis and inflammation in the absence of the digenean in the kidney, and numerous poorly developed individuals. Death, usually in fingerlings, probably resulted from a combination of heavy infection plus an effect of the agent on the catfish. In sections from three fish, we noted no extensive systemic bacterial infection, but secondary infections probably occur. Fish typically died within a few hours when experimentally exposed to over 500 cercariae for a couple of hours and from two to seven days when exposed to moderate numbers of cercariae (RMO, personal observations). Infections in the catfish appear to have resulted from infections in the snail *P. trivolvis* only and transmitted by pelicans only. The American white pelican feeds extensively on catfish in commercial ponds, as do several other birds (e.g. King, 1997), and those losses of fish from predation by the pelican are intensified by the introduction of the parasite from the pelican into the snail-inhabited ponds. Management of the infected snail with 2.5 ppt NaCl and black carp both appear to control infections and benefit the catfish industry (Venable et al., 2000).

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