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# EXPERIMENTAL ELUCIDATION OF THE LIFE CYCLE OF *DREPANOCEPHALUS SPATHANS* (DIGENEA: ECHINOSTOMATIDAE) WITH NOTES ON THE MORPHOLOGICAL PLASTICITY OF *D. SPATHANS* IN THE UNITED STATES

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**KEY WORDS**

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*Ictalurus punctatus*
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Marsh rams-horn snail
*Phalacrocorax auritus*
Double-crested cormorant
Trematode

**ABSTRACT**

The echinostomatid *Drepanocephalus spathans* (syn. *Drepanocephalus auritus*) parasitizes the double-crested cormorant *Phalacrocorax auritus*. In North America, the marsh rams-horn snail *Planorbella trivolvis* and ghost rams-horn snail *Biomphalaria havanensis* serve as snail intermediate hosts, both of which inhabit catfish aquaculture ponds in the southeastern United States. Studies have demonstrated *D. spathans* exposure can be lethal to juvenile channel catfish *Ictalurus punctatus*. Two studies were undertaken to elucidate the life cycle of *D. spathans* to establish a developmental time line. In both studies, *D. spathans* cercariae collected from naturally infected *P. trivolvis* individuals were used to infect channel catfish fingerlings, which were then fed to double-crested cormorants (DCCOs) that had been pharmaceutically dewormed. In study 1, laboratory-reared *P. trivolvis* and *B. havanensis* individuals were placed in aviary ponds with experimentally infected DCCO and examined bi-weekly for release of cercariae. Trematode eggs were observed in the feces of exposed birds 3 days post-infection. Birds were sacrificed 18 days post-exposure (dpe), and gravid adults morphologically and molecularly consistent with *D. spathans* were recovered. Snails from the aviary pond were observed shedding *D. spathans* cercariae 18–54 dpe. In study 2, trematode eggs were observed in the feces of exposed DCCOs beginning 8 dpe. Once eggs were observed, birds were allowed to defecate into clean tanks containing naïve laboratory-reared *P. trivolvis* individuals. Additionally, eggs from experimental DCCO feces were recovered by sedimentation and placed in an aquarium housing laboratory-reared *P. trivolvis* individuals. Birds in study 2 were sacrificed after 60 days, and gravid *D. spathans* specimens were recovered. Snails from the experimental DCCO tanks shed *D. spathans* cercariae 89–97 dpe. Lastly, trematode eggs were isolated and observed for the hatching of miracidia, which emerged on average after 16 days at ambient temperatures. No *D. spathans* adults were observed in control birds fed non-parasitized fish. This is the first experimental confirmation of the *D. spathans* life cycle, resolving previously unknown developmental time lines. In addition, the effects of fixation on adult trematode morphology were assessed, clarifying reports of pronounced morphological plasticity for *D. spathans*.

The double-crested cormorant *Phalacrocorax auritus* is a piscivorous migratory bird widely distributed throughout North America (Dorr et al., 2014). Migrating populations often overwinter in the southeastern United States and forage on commercial aquaculture ponds in the Mississippi Delta and Blackland Prairie regions of Mississippi (Stickley and Andrews, 1989; Johnsgard, 1993; King, 1996; Dorr et al., 2004; Glahn and King, 2004; King et al., 2010). While foraging, double-crested cormorants injure and consume large amounts of catfish. Their behavior also disrupts catfish feeding patterns, which can reduce growth and production efficiency on catfish aquaculture farms (Wywialowski, 1999).

* Deceased 26 September 2016.

Studies have revealed that double-crested cormorants are definitive hosts for a variety of trematodes, many infecting fish, which serve as second intermediate hosts (Threlfall, 1982; Fedynich et al., 1997; Flowers et al., 2004; Wagner et al., 2012; O'Hear et al., 2014; Sheehan et al., 2016, 2017). The echinostomatid *Drepanocephalus spathans* was commonly found during these surveys, with prevalence ranging from 84 to 91% across the Mississippi Delta region, Saskatchewan, Canada, and the eastern United States (Wagner et al., 2012; O'Hear et al., 2014; Sheehan et al., 2017).

The genus *Drepanocephalus* Dietz, 1909 currently consists of three species, with adult stages reported primarily from New World cormorants (Dietz, 1909, 1910; Kostadinova et al., 2002; Hernández-Cruz et al., 2018). Although the history and faunistic accounts of *Drepanocephalus* spp. are rich and varied (Ostrowski de Nuñez, 1966; Salgado-Maldonado and Kennedy, 1997; Kostadinova et al., 2002; Flowers et al., 2004; Violante-González et al., 2011; Wagner et al., 2012; O'Hear et al., 2014; Pinto et al., 2014), few studies have included morphological data to support species identity (Dietz, 1910; Rietschel and Werding, 1978; Kostadinova et al., 2002; Kudlai et al., 2015; Pinto et al., 2016; Alberson et al., 2017; Hernández-Cruz et al., 2018). The systematics of the genus were convoluted when Kudlai et al. (2015) proposed *Drepanocephalus auritus* as a novel species, distinct from *Drepanocephalus spathans* and other congeners based on incongruence of general body morphological features, specifically increased spatial separation between the testes (Kudlai et al., 2015). Comparisons of molecular data supplementing their description with historical accounts of *D. spathans* specimens (larval and adult) supported claims that previous records of a North American *D. spathans* haplotype/morphotype were actually the newly proposed *D. auritus* (Kudlai et al., 2015).

The matter was complicated, as the original description and type material of *D. spathans* were based on only a single specimen collected from a Neotropical cormorant *Phalacrocorax brasilianus* from Brazil (Dietz, 1909, 1910), which was morphologically inconsistent with the specimens described by Kudlai et al. (2015). The erection of *D. auritus* has been countered by Hernández-Cruz et al. (2018), who cited *D. auritus* as a synonym of *D. spathans* based on archived specimens from an extensive survey of cormorants from Mexico. Hernández-Cruz et al. (2018) revealed that *D. spathans* displays a wide range of morphological variability, including morphotypes consistent with specimens documented by Kudlai et al. (2015). While the work of Hernández-Cruz et al. (2018) was thorough, the study involved temporally and geographically disparate isolates archived using various methods of fixation. Their study was further limited by the inclusion of molecular data from paragenophore vouchered specimens rather than hologenophores (sensu Pleijel et al., 2008). The latter more accurately couples molecular data to morphologically characterized specimens, which would have more resolutely clarified the extreme morphological plasticity reported for geographically disparate *D. spathans* isolates. Still, Hernández-Cruz et al. (2018) presented sufficient morphological and molecular evidence to collapse *D. auritus*, leaving the following species as valid: *Drepanocephalus spathans* (synonymized with *D. auritus*), *Drepanocephalus mexicanus* Lamothe-Argumedo and Perez Ponce de Leon, 1989, and *Drepanocephalus olivaceus* Nasir and Marval, 1968.

In laboratory challenges, *D. spathans* infection causes mortality in juvenile channel catfish within 7 days post-exposure (dpe) (Griffin et al., 2012, 2014; Alberson et al., 2017). In catfish aquaculture, natural infections of *D. spathans* have been documented in snail first intermediate hosts *Planorbella trivolvis* and *Biomphalaria havanensis* from Mississippi (Griffin et al., 2012; Alberson et al., 2017) and *Biomphalaria straminea* from Brazil (Pinto et al., 2016). While the definitive and intermediate hosts have been identified, the developmental time lines associated with the *D. spathans* life cycle remain unknown. Further, the current taxonomic identities of *Drepanocephalus* species in the Americas remain controversial (Kostadinova et al., 2002; Griffin et al., 2012, 2014; Kudlai et al., 2015; Pinto et al., 2016; Hernández-Cruz et al., 2018). The purpose of this work was to determine whether the channel catfish could serve as an intermediate host in the *D. spathans* life cycle, establish a developmental time line for *D. spathans* in all hosts, and briefly comment on the current taxonomic identity and purported morphological plasticity of *Drepanocephalus* spp. present in the United States.

## MATERIALS AND METHODS

### General procedures

*Snail collection:* In the summers of 2015 and 2016, *Planorbella trivolvis* individuals (n = 11,440) were collected from catfish aquaculture ponds in Lowndes County, Mississippi, and brought back to the Aquatic Parasitology Laboratory at the Mississippi State University College of Veterinary Medicine (Mississippi State, Mississippi). Snails were rinsed with reverse osmosis water and placed in 25-ml plastic vials (Diluvial, Elkay Laboratory Products, Hampshire, U.K.) containing 10 ml of autoclaved natural spring water (Kroger®, Cincinnati, Ohio). Snails were held at ambient temperatures (~25–27 C) for 48 hr and examined daily for the presence of cercariae with an Olympus SZ60 stereomicroscope (Olympus Optical, Tokyo, Japan). Pools of cercariae morphologically consistent with *D. spathans* (Griffin et al., 2012) were collected from infected snails (n = 131) and stored in 70% molecular-grade ethanol for molecular analysis.

*Molecular identification:* To expedite the initial identification process before fish challenges, a *Drepanocephalus* spp.–specific PCR was designed, amplifying a 189-bp region of the cytochrome *c* oxidase subunit 1 (*COI*) gene. Amplification was carried out using the forward primer 828F1 (5′-CCG CAC CAC CTA TCA TAC TTA AC-3′) and the reverse primer 1017R (5′-GTG ACT TCT CAC GGG ATA ATT ATG A-3′). Approximately 10 cercariae were isolated from each pool and washed 3 times with molecular-grade, nuclease-free water to remove residual ethanol. Genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, California). The PCR consisted of 13 µl of EconoTaq PLUS GREEN 2X Master Mix (Lucigen Corporation, Middleton, Wisconsin), 10 µM of each primer, 5 µl of template DNA, and nuclease-free water to volume. The thermocycling profile consisted of an initial denaturation of 2 min at 94 C, followed by 40 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 45 sec, and a final extension for 5 min at 72 C. Amplification products were visualized under ultraviolet light in a 1.2% agarose gel containing ethidium bromide (0.5 µg/ml) and run concurrently with a molecular weight ladder (HyperLadder™ 50 bp; Bioline, London, U.K.) to confirm the presence of the

**Table I.** Primers used in sequencing of the cytochrome *c* oxidase subunit 1 (*COI*) gene.

| Primer | Sequence (5′-3′) | Reference |
|---|---|---|
| cox11 | CACCTTAATACCCGTCGGAAT | Pinto et al. (2016) |
| cox820 | AATATTATTTCCCCGGRAGTYT | Pinto et al. (2016) |
| cox1_schist5′ | TCTTTRGATCATAAGCG | Lockyer et al. (2003) |
| acox650r | CCAAAAAACCAAAACATATGCTG | Kudlai et al. (2015) |

appropriately sized band. The remaining gDNA was archived at –20 C for *COI* sequencing.

Further molecular confirmation was performed by PCR amplification and sequencing of the partial *COI* gene from archived gDNA using previously established primers and protocols (Table I). Products were visualized as described above. Amplification products were purified directly using the QIAquick PCR Purification Kit (QIAGEN) and sequenced with the same forward and reverse primers used in the initial amplification (Eurofins MWG Operon, Huntsville, Alabama). Contiguous sequences were assembled using Geneious software version 9.1.5 (Biomatters Ltd., Auckland, New Zealand) with ambiguous base calls manually annotated. Gene sequences generated from all specimens were compared to *Drepanocephalus* spp. sequence data in the National Center for Biotechnology Information non-redundant nucleotide database (NCBI nr/nt) using a Blastn search for somewhat similar sequences (Altschul et al., 1990).

*Channel catfish infection:* For each challenge, fresh cercariae (<24 hr old) were collected and pooled in a graduated beaker of autoclaved spring water and gently stirred with a magnetic stir bar. Ten 100-µl aliquots were collected, and the number of cercariae in each aliquot was counted using a stereomicroscope (Olympus) to obtain the approximate challenge dose. Channel catfish fingerlings (~3–5 cm) reared for disease research at the Thad Cochran National Warmwater Aquaculture Center (NWAC) in Stoneville, Mississippi, were placed individually into 1-L plastic containers containing 300 ml of dechlorinated municipal water with constant aeration. Individual fish were exposed to ~150 cercariae for 4 hr by immersion, after which water was decanted, and fish were placed in discrete recirculating aquaria according to challenge date. Fish were housed at ambient temperatures (~25–27 C) with constant aeration until they could be fed to experimental double-crested cormorants (DCCOs).

*Histology:* Fish were euthanized with an overdose of tricaine methanesulfonate (Western Chemical, Ferndale, Washington). The coelomic cavity was opened via sharp incision, and fish were placed into 10% neutral buffered formalin (Leica Biosystems, Richmond, Illinois) for a minimum of 24 hr. Fish were subsequently decalcified using CalEx® (ThermoFisher Scientific, Waltham, Massachusetts) until calcified tissues were pliable. Fish were then rinsed with running water for at least 3 hr before trimming. Approximately 3–5 mm cross sections were made through the fish starting rostrally and moving caudally, with small adjustments in thickness to ensure sections of the major organs. Based on previous work, multiple thinner sections were made through the head region, where *D. spathans* metacercariae are typically located. Tissue sections were processed routinely, embedded in paraffin, cut into 4-µm sections, and mounted. Slides were stained with hematoxylin and eosin (H&E) and examined using an Olympus BX-50 microscope (Olympus Optical), with representative images captured with an Olympus DP72 camera and DP2-TWAIN–cellSens software (Olympus Optical).

*Double-crested cormorant collection and care:* Birds were captured under Federal Collection Permit MB019065-0 using soft-catch leg-hold traps or rocket net as previously described (King et al., 1998). Post-capture, birds were transported live in an air-conditioned, enclosed trailer to the National Wildlife Research Center Mississippi Field Station avian test facility, located on the campus of Mississippi State University (SOP AC 006.00). Birds were placed on an enclosed research pond for acclimation. After an initial acclimation period, individuals were weighed, equipped with individually numbered leg bands, and housed indoors in individual $3 \times 6 \times 2$-m pens. Each pen was equipped with a 1,000-L dechlorinated municipal water flow-through tank equipped with a ramp. Each bird was fed a daily diet of 600 g of channel catfish obtained from local producers and observed for general health and body conditioning. Fecal samples were collected daily and examined for trematode eggs using a modified fecal sedimentation method (Foreyt, 2001). Fecal samples from each bird were weighed, homogenized, and placed into individual 15-ml polypropylene conical-bottom graduated centrifuge tubes (CELLTREAT Scientific Products, Pepperell, Massachusetts). A 1% soap solution, prepared using distilled water and liquid dish soap, was added to each tube, mixed with the feces, and allowed to sit undisturbed for 5 min, after which the supernatant was removed. This process was repeated until the supernatant was clear. Once cleared, a final wash was performed using autoclaved spring water. After sitting for an additional 5 min, the supernatant was removed 1 final time and diluted with 10 ml of autoclaved spring water. To estimate the number of eggs in each sample, samples were stirred thoroughly, and 1-ml aliquots were pipetted into a lined Petri dish and viewed using a stereomicroscope (Olympus SZ60, Olympus Optical).

After 10 days of acclimation to the indoor pens, each DCCO received 34 mg/kg per os praziquantel (Droncit® 34; Bayer Corporation, Shawnee Mission, Kansas) to clear any naturally occurring platyhelminth infections. Feces were collected daily, and egg counts were performed as above. Beginning on the day of praziquantel administration, birds were fed a diet of 600 g parasite-free catfish reared indoors for disease research at NWAC.

*Double-crested cormorant necropsy*: DCCOs were euthanized by carbon dioxide asphyxiation, and the complete gastrointestinal tract was removed from the esophagus to the cloaca. The stomach was separated from the intestines, and both were processed individually. The stomach and intestines were opened longitudinally, and contents were rinsed into a 75-µm brass sieve using dechlorinated water. Additionally, the mucosal lining of each organ was manually scraped by hand and rinsed 3 times to ensure

trematodes were dislodged, and the contents were washed into the sieve. Screened contents were examined in a lined Petri dish under a stereomicroscope, and adult trematodes were collected and enumerated.

*Drepanocephalus spathans staining and identification*: Recovered adult trematodes were initially relaxed in slightly boiling 0.09% saline. A sub-sample of trematodes from each DCCO was fixed in 70% molecular-grade ethanol for molecular analysis. The remaining trematodes were fixed in 10% neutral buffered formalin. A sub-sample of formalin-fixed adults was stained with acetocarmine, destained in 1% acidic ethanol, dehydrated in an ethanol series, cleared, and mounted in Canada balsam. Photomicrographs and measurements of stained specimens were obtained using an Olympus BX-53 compound microscope (Olympus Optical) equipped with an Olympus DP-74 camera and the accompanying CellSens software (Olympus).

The following measurements were obtained from stained gravid specimens according to Kudlai et al. (2015): body length (BL), body width at ventral sucker (BWVS), body width at anterior testis (BWAT), collar length (CL), collar width (CW), oral sucker length (OSL), oral sucker width (OSW), prepharynx length (PPL), pharynx length (PL), pharynx width (PW), esophagus length (OL), cirrus sac length (CSL), cirrus sac width (CSW), length of anterior portion of seminal vesicle (SV1L), width of anterior portion of seminal vesicle (SV1W), length of posterior portion of seminal vesicle (SV2L), width of posterior portion of seminal vesicle (SV2W), ventral sucker length (VSL), ventral sucker width (VSW), ovary length (OVL), ovary width (OVW), Mehlis' gland length (MEL), Mehlis' gland width (MEW), anterior testis length (ATL), anterior testis width (ATW), posterior testis length (PTL), posterior testis width (PTW), egg length (EL), egg width (EW), forebody length (FORE), distance between ovary and posterior margin of ventral sucker (OVAR), and distance between the posterior margin of the posterior testis and the posterior extremity of the body (TEND). Additionally, the following proportions expressed as percentages were calculated according to Kostadinova (2005): maximum body width as a proportion of body length (BW), length of the forebody as a proportion of body length (FO), length of the uterine field posterior to the ventral sucker (U), and the length of the post-testicular region as a proportion of body length (T).

*Examination of archived specimens:* In addition to adult worms collected from experimental infections, archived specimens were examined to clarify the purported morphological plasticity reported for *D. spathans* (Hernández-Cruz et al., 2018). Adult trematodes consistent with the morphology of *D. auritus* as described by Kudlai et al. (2015) were collected from a DCCO captured in Lowndes County, Mississippi, in June 2016. These specimens were relaxed in boiling saline and fixed in 70% molecular-grade ethanol. Comparably, 6 archived specimens (ethanol fixed) consistent with the alternate *D. spathans* morphology as described by Hernández-Cruz et al. (2018), taken from a DCCO collected from Leflore County, Mississippi, were examined from the parasite collection of the late Dr. Linda Pote (Mississippi State University, College of Veterinary Medicine).

In addition to these archived specimens, 4 live specimens collected from a DCCO from Lowndes County, Mississippi, in 2018 were placed directly in 70% molecular-grade ethanol by T. G. Rosser without heat relaxation. All live and archived specimens were treated as follows: Latero-posterior sections of the body were excised with sterile scalpel blades and placed into 1.5-ml microcentrifuge tubes, and genomic DNA was extracted as detailed above. Hologenophore specimens were subsequently stained using Van Cleave's hematoxylin, dehydrated in an increasing ethanol series, cleared in methyl salicylate, and mounted in Canada balsam. The *COI* gene was amplified and sequenced as above to obtain a molecular identification.

## 2015 life cycle study

*Bird exposures:* Five DCCOs were live-captured from 3 different sampling sites in eastern Mississippi and western Alabama. The DCCOs were selected arbitrarily to serve as experimental (n = 3; 10R, U91, U66) or control (n = 2; U67, U96) birds. Fish exposures were performed over 4 days to produce *D. spathans*–infected fish for bird challenge. Twenty fish from challenge days 1 and 2, and 10 fish from challenge days 3 and 4 were collected and euthanized with an overdose of tricaine methanesulfonate (Western Chemical) at 5 dpe for histological examination, and the number of metacercariae per fish was estimated.

Catfish fingerlings infected with *D. spathans* (~5–6 metacercariae/fish) were pooled and fed to 3 experimental birds 8–12 dpe. Each experimental bird received 45 infected fish in the a.m. and 46 infected fish in the p.m., resulting in an estimated inoculum of ~550 metacercariae/bird. Two DCCOs were fed parasite-free catfish to serve as controls. Experimental and control DCCOs were observed eating the infected fish via a video monitoring system. Fecal samples from each bird were collected daily, and sedimentations were performed as above. To confirm successful infection, a single experimental bird was euthanized 8 dpe and necropsied, while others (2 experimental, 2 control) were euthanized 18 dpe.

*Snail exposures (aviary ponds):* Two separate infectivity trials were undertaken to clarify the timing and water temperature involved with snail infection by *D. spathans*. In the first trial, laboratory-reared *P. trivolvis* (n = 30) and *B. havanensis* (n=30) individuals were housed in discrete cricket cages (Challenge Plastic Products, Incorporated, Edinburgh, Indiana) and placed into an outdoor experimental pond inhabited by DCCOs at the NWRC Avian Testing Facility. Water temperatures ranged from 20 to 29 C (mean: 24.6 ± 2.7 C). Snails were taken out of the pond at ~14-day intervals and examined for the presence of cercariae. After 48 hr observation, snails were returned to the pond, and the procedure repeated. After 53 days, snails were removed from ponds and housed indoors in individual plastic vials (Dilu vials, Fisher Scientific, Waltham, Massachusetts) containing autoclaved spring water and observed daily for cercariae.

The second infectivity trial involved staggered exposures of small groups of snails. Group 1 snails (n = 10) were housed in a cricket cage and placed in an experimental pond with water temperatures ranging from 28 to 34 C (mean: 31.3 ± 2.0 C). The second group of snails (group 2) was placed in the pond 7 days after group 1. Each snail group was checked every 14 days for the presence of cercariae. After the 48 hr observation period was complete, snails were returned to the pond. This procedure was repeated until cercariae were observed.

### 2016 life cycle study

*Bird exposures:* Four DCCOs were live-captured at Cat Island, Green Bay, Wisconsin. Two separate fish challenges were performed 12 days apart due to the small number of *D. spathans*–infected snails. A subsample of 10 fish from each challenge was collected at 5 days post-challenge for pepsin digest (n = 5) and histological examination (n = 5) to estimate the number of metacercariae per fish. Fish were euthanized as previously described. For pepsin digestion, the heads were removed via sharp dissection and placed into a 125-ml Erlenmeyer flask containing 25 ml of a 0.5% pepsin solution. Bodies were cut into thirds and placed into a 125-ml Erlenmeyer flask containing 50 ml of a 0.5% pepsin solution. Flasks were placed into a 35 C water bath, and fish were digested overnight. Once digestion was complete, the solution was poured into a lined Petri dish and examined for metacercariae using a stereomicroscope. Metacercariae from each fish were enumerated, collected, and archived in 70% molecular-grade ethanol for molecular analysis at a later date.

Due to fewer cercariae available for experimental infections in the 2016 trial, catfish fingerlings infected with *D. spathans* (~8 metacercariae/fish) were fed to 2 experimental DCCOs on 2 separate occasions. The first experimental bird (XR30) received 40 infected fish, and the second (XR44) received 38 infected fish, equating to ~350 metacercariae/bird (~8 metacercariae/fish). Two DCCOs were fed laboratory reared, non-parasitized catfish and served as controls. Again, experimental and control DCCOs were observed eating the fish using a video monitoring system, and fecal samples from each bird were collected daily and processed as above. All DCCOs (2 experimental, 2 control) were euthanized on study day 60 (XR30 = 43 dpe; XR44 = 31 dpe) and necropsied.

*Snail exposures (aviary tanks):* Once eggs were detected in experimental DCCO feces, experimental and control birds were moved to individual pens equipped with 1,000-L tanks containing clean water. Experimental and control DCCOs were allowed to defecate into individual tanks for 96 hr before being returned to their pens. Once birds were removed from the aviary tanks, 80 laboratory-reared *P. trivolvis* snails were placed into 4 separate cricket cages (20 snails/tube). Individual tubes were placed in each 1,000-L tank. Every 14 days, snails were examined for the presence of cercariae. After the 48 hr observation period, snails were returned to their tubes and respective tanks. Forty-four days after placement, all surviving snails were removed and housed in individual 177-ml glass containers holding ~60 ml of autoclaved spring water at ambient temperatures (~25–27 C) and observed daily for cercariae.

*Snail exposures (aviary ponds):* Ten laboratory-reared *P. trivolvis* individuals and 10 laboratory-reared *B. havanensis* individuals were housed in discrete cricket cages and placed in an outdoor experimental pond inhabited by 5 DCCOs at the NWRC Avian Testing Facility, with water temperatures ranging from 25 to 33 C (mean: 28.1 ± 1.5 C). Snails were removed from the pond weekly, checked for the presence of cercariae for 24 hr, and then returned to the pond. The second group of 10 *P. trivolvis* individuals was placed in the pond 40 days following the first group and treated similarly.

*Snail exposures (laboratory):* Two 19-L aquaria (1 experimental and 1 control) containing dechlorinated municipal water were maintained at ~23–25 C. Fifteen laboratory reared *P. trivolvis* snails were placed in each aquarium and fed a diet of blanched romaine lettuce ad libitum. Once trematode eggs were observed in fecal samples from experimental birds, eggs were collected from bird feces using the Flukefinder® (Visual Difference, Moscow, Idaho). Eggs were enumerated under a stereomicroscope (Olympus), and an estimated ~1,137 eggs were used to inoculate the experimental aquarium. Feces from control birds were treated similarly, and the filtrate was placed in the control aquarium. Snails were removed every 14 days, placed in 177-ml glass containers with autoclaved spring water, and observed for the presence of cercariae for 48 hr before being returned to their respective aquaria.

*Hatching of miracidia (laboratory): Drepanocephalus spathans* eggs (n = 100) were collected from DCCO feces as above and placed in a glass Petri dish containing autoclaved spring water (Ozarka, Nestlé Waters North America, Stamford, Connecticut), covered, and kept at ~23–25 C. Similarly, eggs collected from fresh and cold-stored (~4 C for 15 days) DCCO feces were placed individually into each well of a 24-well plate containing autoclaved spring water and kept at ~23–25 C. The glass Petri dish and 24-well plates were observed daily using a stereomicroscope for the development and hatching of miracidia.

## RESULTS

### Life cycle study 2015

*Bird exposures:* Histologically, metacercariae morphologically consistent with *D. spathans* (Griffin et al., 2012, 2014; Alberson et al., 2017) were present in 9 of the 10 fish (~5–6 metacercariae/fish). Metacercariae were usually present within the lateral line system (Fig. 1A) as well as within the tissues of the branchial cavity. Infiltrates of a moderate to a large number of mononuclear cells immediately surrounding the metacercariae were associated at these sites and also in adjacent tissues, which were expanded or swollen, with loss or obliteration of the lateral line architecture at these sites. Besides the typical sites, metacercariae were rarely observed in the base of the pelvic fin (Fig. 1B) and also caudal to the abdominal cavity. Numerous trematode eggs were observed in the fecal samples before treatment with praziquantel (Fig. 2). However, fecal samples were clear 4 days after praziquantel treatment.

Trematode eggs were observed in the feces of 1 experimental bird (U91) on 3, 4, 6, and 8 dpe. At 7 dpe, a single trematode egg was observed in a second experimental bird (10R). At 8 dpe, dead adult *D. spathans* individuals were observed in the feces of all experimental DCCOs, leading to the necropsy of 1 bird (U91). Live, adult *D. spathans* (n = 2,103) individuals were recovered from this bird, and the experiment was allowed to continue. Dead, adult *D. spathans* (n = 7) individuals were observed again in the feces of a single remaining experimental DCCO 14 dpe, along with a single egg at 17 dpe. At 18 dpe, all remaining DCCOs were euthanized. During necropsy, adult *D. spathans* individuals were recovered from the intestines of the 2 experimental birds (n = 64 from 10R; n = 186 from U66) (Fig. 3, 4). Sequence analysis of the *COI* gene from recovered adult specimens (n = 5/bird) revealed 98.0–100% similarity to published sequences of *D. spathans* (Griffin et al., 2012; O'Hear et al., 2014; Kudlai et al., 2015; Van Steenkiste et al., 2015; Pinto et al., 2016; Alberson et al., 2017; Hernández-Cruz et al., 2018). No eggs were observed in the feces

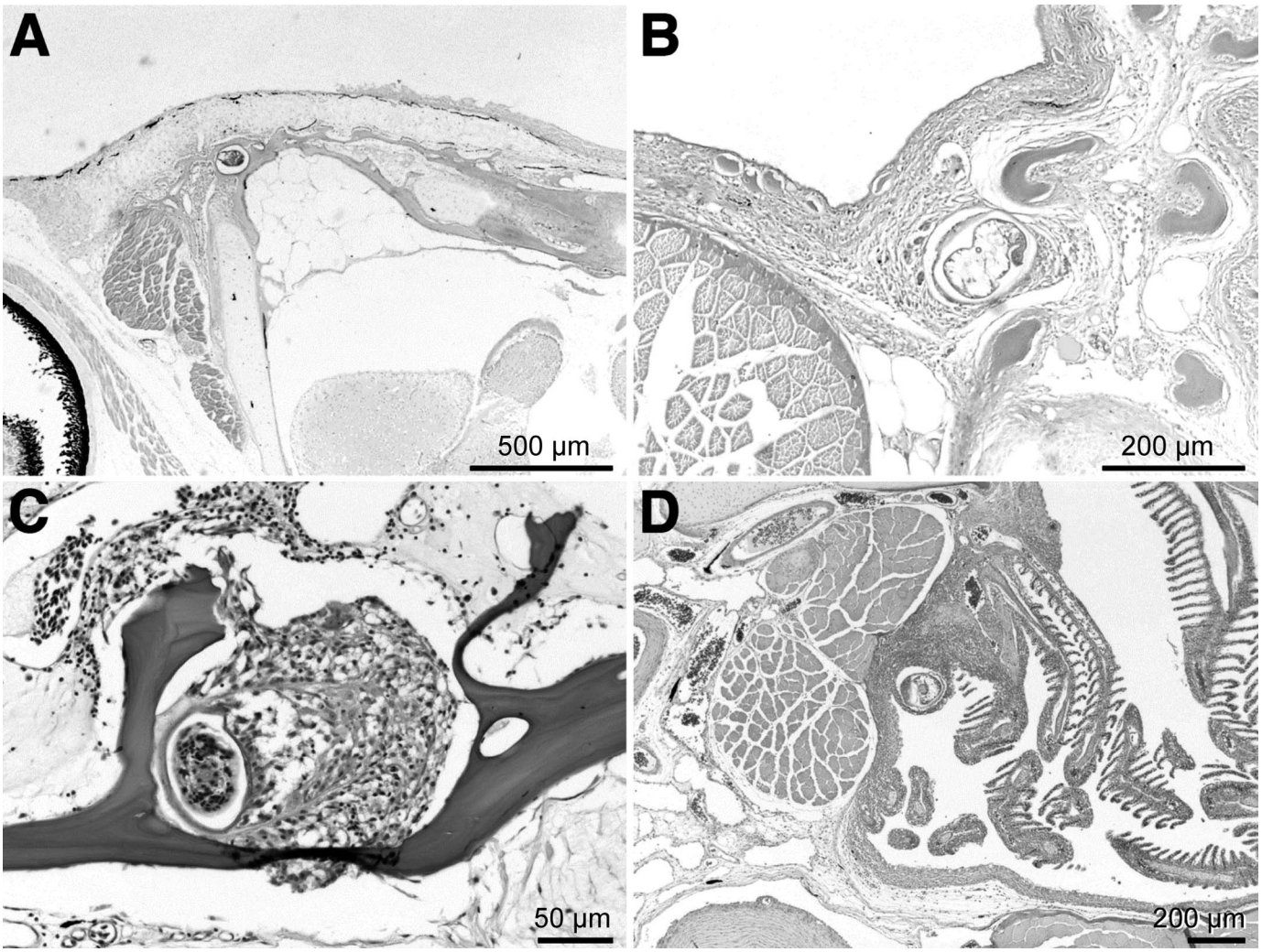


**Figure 1.** Histopathological development of *Drepanocephalus spathans* metacercariae in experimentally infected channel catfish *Ictalurus punctatus*. (**A**) Developing metacercaria in lateral line system surrounded by bone in the cranium. (**B**) Developing metacercaria at base of pelvic fin, an atypical site of development compared to previous studies. (**C**) Metacercaria present in the head with accompanying inflammation. (**D**) *Drepanocephalus spathans* metacercaria present in the base of the gill.

of either control bird, and no adult trematodes were recovered at necropsy.

Measurements for the 2015 study were based on 5 gravid specimens (Table II). All measurements are reported in micrometers with mean and SD in parentheses. Body elongate with the greatest width at the level of the anterior collar; BW = 14.9–17.7% (16.7% ± 1.1%)%; BL 4,215–5,395 (4,729 ± 425); BWVS 583–692 (639 ± 40); BWAT 446–488 (469 ± 17); FORE 868–1,069 (962 ± 74) long with small laterally placed spines; FO = 19.8–20.9% (20.4% ± 0.5%).

Head collar falciform, muscular, 421–494 (455 ± 31) × 730–834 (789 ± 38); ventral lappets present. Collar spines large, 27 in a single row (Table III). Four angle spines, each in 2 pairs on each ventral lappet, larger than marginal spines. Each pair having a single ventral and dorsal spine; ventral spines smaller than dorsal spines. First pair: ventral spine 174–196 (183 ± 7) × 37–41 (39 ± 1); dorsal spine 179–200 (191 ± 8) × 36–45 (39 ± 3). Second pair: ventral spine 149–188 (166 ± 11) × 33–38 (36 ± 2); dorsal spine 141–168 (154 ± 10) × 29–35 (32 ± 2); first pairs larger than second pairs. Lateral spines in a single row, 4 on each lappet; first lateral spine 75–91 (84 ± 5) × 20–25 (23 ± 2). Dorsal spines in a single row, 11, smaller than lateral spines, decreasing in size toward collar midline.

Oral sucker subspherical, subterminal, 174–215 (198 ± 15) × 171–201 (186 ± 11). Prepharynx indistinct, 39–73 (57 ± 12). Pharynx, highly muscular, elongate-ovoid 197–217 (208 ± 8) × 125–141 (132 ± 7). Esophagus, elongate 403–465 (436 ± 23); bifurcating at the level of anterior margin of ventral sucker. Ceca extending almost to the posterior of body.

Testes tandem, elongate-ovoid, variably and deeply lobed. Anterior testis just posterior to equatorial region of body, with 4–6 distinct lobes, 290–398 (332 ± 40) × 204–256 (231 ± 21); posterior testis slightly more elongate, with 5–7 distinct lobes, 332–397 (363 ± 32) × 225–248 (239 ± 9); DBT = 92–270 (185 ± 90). Post-testicular region 1,610–2,051 (1,725 ± 184) long; T = 34.9–38.2% (36.5% ± 1.5%). Cirrus sac, elongate-ovoid, 267–296 (278 ± 12) × 144–205 (169 ± 25), antero-dorsal to ventral sucker. Internal seminal vesicle, bipartite; anterior portion 123–168 (138 ±

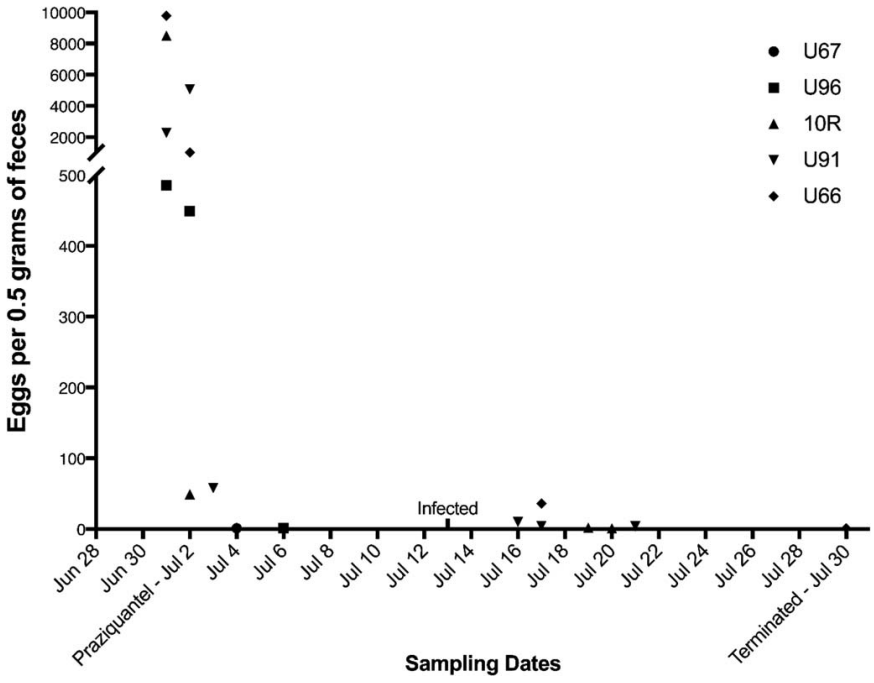


**Figure 2.** Fecal egg counts from 2015 trial for experimental (n = 3; 10R, U91, U66) and control (n = 2; U67, U96) birds. The day birds were fed infected fish is annotated on the x-axis (infected).

17) × 85–138 (112 ± 19); posterior portion 174–225 (202 ± 22) × 127–151 (135 ± 10). Genital pore, medial, just posterior to ceca bifurcation.

Ovary dextral, small, elongate-ovoid, 108–142 (125 ± 15) × 73–105 (91 ± 15); U = 3.5–6.8% (5.5% ± 1.2%). Mehlis' gland median, contiguous with ovary, transversely ovoid, 102–200 (125 ± 19) × 96–145 (125 ± 19). Metraterm muscular. Few eggs, ovoid, 65–90 (80 ± 9) × 41–54 (53 ± 1). Vitellarium in 2 fields of follicles along the lateral margins of the body, beginning at posterior margin of ventral sucker and ending in body posterior. Excretory pore terminal.

*Snail exposures (aviary ponds):* In the first infectivity trial, one *B. havanensis* specimen and one *P. trivolvis* specimen each were observed shedding cercariae morphologically consistent with *D. spathans* (Griffin et al., 2012) 54 days after being placed in the experimental pond. In the second infectivity trial, one snail from group 1 and 1 snail from group 2 began shedding *D. spathans* cercariae 26 and 18 days after being placed into the pond, respectively.

## Life cycle study 2016

*Bird exposures:* The number of metacercariae per fish was estimated from histological sections of a subset of parasitized fish. In the absence of visible parasites, infection was assumed based on the presence of inflammation in relevant areas. When present, parasites were observed in the lateral line system, particularly near the head, with accompanying inflammation (Fig. 1C). Meanwhile, parasites present in the gills, often near the base of the gill filament (Fig. 1D), tended to lack inflammation, which when present was composed largely of macrophages and lymphocytes. Metacercariae were also recovered by pepsin digest from 4 out of 5 fish from the first fish exposure and 5 of 5 fish from the second exposure. All metacercariae were PCR positive by *Drepanocephalus* spp.–specific PCR, and the *COI* sequence from 1 metacercariae was a 99–100% match to published *D. spathans COI* sequences (Griffin et al., 2012; Kudlai et al., 2015; Pinto et al., 2016; Alberson et al., 2017; Hernández-Cruz et al., 2018).

Numerous trematode eggs were observed in feces collected from DCCOs before praziquantel administration (Fig. 5). After receiving praziquantel, DCCO fecal samples were cleared of trematode eggs within 3 days. The first experimental DCCO (XR30) began shedding trematode eggs 16 dpe (n = 619 eggs in 0.4 g feces), while the second (XR44) began shedding eggs 8 dpe (n = 9 eggs in 0.1 g feces). The largest number of eggs was observed 13 dpe from bird XR44, with 2,383 eggs in 0.3 g of feces. No eggs were observed in feces collected from any control birds after deworming. Upon necropsy, adult *D. spathans* specimens were recovered from intestines of both experimental DCCOs (29 worms from XR30; 20 from XR44). No *D. spathans* individuals were observed in any control birds. Ten adult worms (n = 5 per experimental bird) were PCR positive using primers targeting the ~189-bp region of the *COI* gene of *D. spathans*, which was confirmed by *COI* sequencing revealing 99–100% homology to published *D. spathans COI* sequences (Griffin et al., 2012; Kudlai et al., 2015; Pinto et al., 2016; Alberson et al., 2017; Hernández-Cruz et al., 2018).

Measurements for the 2016 study were based on 25 gravid specimens (Table II). All measurements are reported in micrometers with mean and SD in parentheses. Body elongate with greatest width at the level of the anterior collar; BW = 14.4–17.5% (15.6% ± 0.9%). Body length 6,914–8,995 (7,928 ± 507); BWVS 724–1,046 (949 ± 82); BWAT 636–914 (791 ± 60); FORE 1,227–1,486 (1,351 ± 75) long with small laterally placed spines; FO = 15.1–19.4% (17.1% ± 0.9%).

Head collar falciform, muscular, 583–784 (689 ± 50) × 1,095–1,401 (1,235 ± 81); ventral lappets present. Collar spines, 27, large, in a single row. Four angle spines, 2 pairs on each ventral lappet, larger than marginal spines. Each pair having a single ventral and dorsal spine; ventral spines smaller than dorsal. First

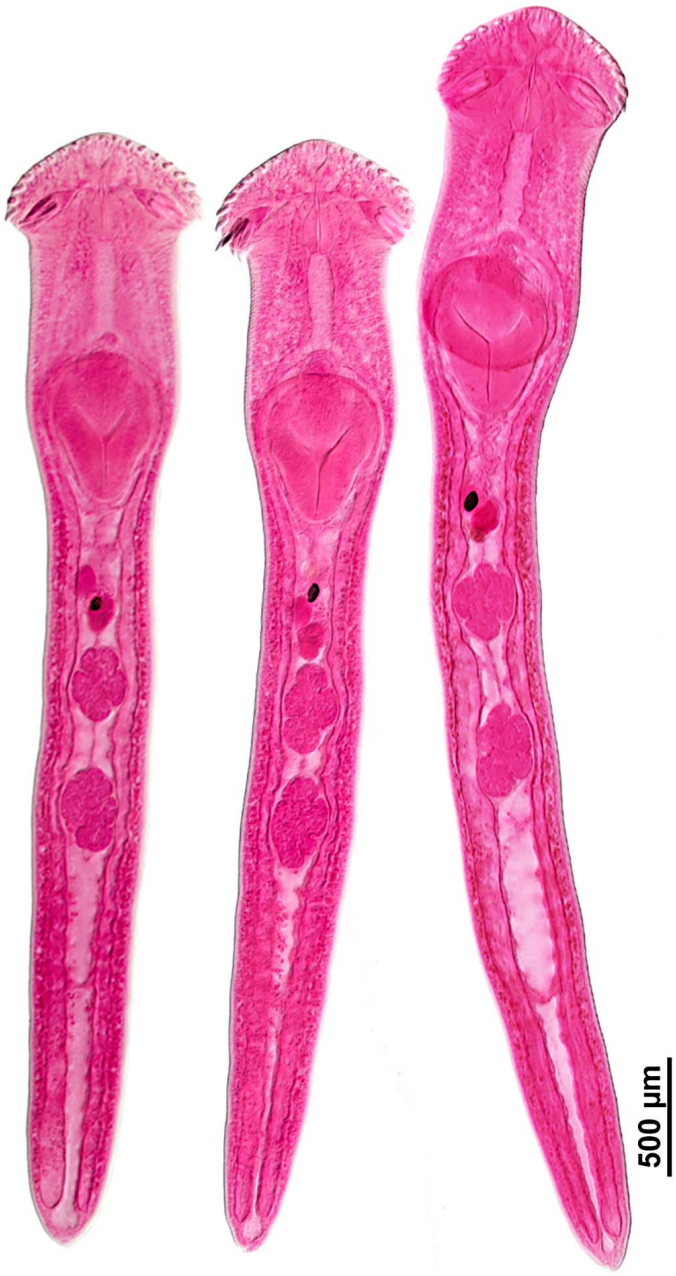


**Figure 3.** Semichon's acetocarmine–stained, gravid *Drepanocephalus spathans* echinostomatid recovered from the intestine of double-crested cormorants at 17 days post-exposure of the first experimental trial. Color version is available online.

pair: ventral spine 213–322 (276 ± 23) × 59–76 (67 ± 4); dorsal spine 243–351 (303 ± 24) × 53–74 (67 ± 4). Second pair: ventral spine 195–324 (245 ± 21) × 48–69 (59 ± 5); dorsal spine 185–272 (230 ± 20) × 47–57 (52 ± 3); first pairs larger than second pairs. Lateral spines in a single row, 4 on each lappet; first lateral spine 94–146 (121 ± 13) × 30–42 (36 ± 3), and remaining lateral spines 101–148 (125 ± 14) × 36–46 (41 ± 3). Dorsal spines in a single row, 11, smaller than lateral spines, decreasing in size toward collar midline.

Oral sucker subspherical, subterminal, 287–333 (314 ± 13) × 260–344 (298 ± 19). Prepharynx indistinct, 23–95 (56 ± 24). Pharynx, highly muscular, elongate-ovoid, 239–339 (302 ± 26) × 161–255 (198 ± 20). Esophagus, elongate, 567–754 (671 ± 49), bifurcating at the level of anterior margin of ventral sucker. Ceca extending almost to the posterior of body.

Testes tandem, elongate-ovoid, variably and deeply lobed. Anterior testis just posterior to equatorial region of body, with 4–6 distinct lobes, 448–694 (556 ± 57) × 381–540 (455 ± 42); posterior testis slightly more elongate, with 5–7 distinct lobes, 553–803 (639 ± 65) × 374–554 (455 ± 48); DBT = 198–522 (347 ± 102). Post-testicular region 1,216–3,398 (2,790 ± 397) long; T = 15.2–38.4% (35.2% ± 4.4%). Cirrus sac, elongate-ovoid, 345–503 (434 ± 43) × 256–389 (324 ± 37), antero-dorsal to ventral sucker. Internal seminal vesicle, bipartite; anterior portion 71–225 (162 ± 35) × 67–174 (111 ± 23); posterior portion 308–444 (370 ± 42) × 155–287 (222 ± 33). Genital pore, medial, just posterior to ceca bifurcation.

Ovary dextral, small, elongate-ovoid, 221–304 (261 ± 21) × 163–286 (210 ± 24); U = 4.3–9.2% (7.4% ± 1.4%). Mehlis' gland median, contiguous with ovary, transversely ovoid 199–323 (236 ± 30) × 229–325 (261 ± 22). Metraterm muscular. Few eggs, ovoid, 68–99 (87 ± 9) × 44–68 (54 ± 6). Vitellarium in 2 fields of follicles along the lateral margins of the body, beginning at posterior margin of ventral sucker and ending in body posterior. Excretory pore terminal.

*Snail exposures (aviary tanks):* The first group of *P. trivolvis* individuals held in aviary tanks coincided with experimental bird XR30 and control bird CR18, while the second group coincided with experimental bird XR44 and control bird CR41. A single snail from the XR30/CR18 group began shedding cercariae morphologically consistent with *D. spathans* 97 days after exposure to tank water containing DCCO feces. No cercariae were observed in snails placed in control tanks.

Five *P. trivolvis* snails from the first exposure group shed cercariae consistent with *D. spathans* at 37 dpe (n = 1), 44 dpe (n = 3), and 51 dpe (n = 1). A single *P. trivolvis* from the second group was observed shedding cercariae consistent with *D. spathans* at 43 dpe. Cercariae were not observed from any *B. havanensis* snail held in the aviary pond housing 5 DCCOs. Sequencing of the *COI* gene revealed cercariae were a 99–100% match to published *D. spathans COI* sequences (Griffin et al., 2012; Kudlai et al., 2015; Pinto et al., 2016; Alberson et al., 2017; Hernández-Cruz et al., 2018).

*Snail exposures (laboratory):* A single snail exposed to trematode eggs in the laboratory was observed shedding *D. spathans* cercariae 89 dpe. The identity of observed cercariae was confirmed by *COI* sequencing.

*Hatching of miracidia (laboratory):* Two miracidia were observed 16 days post-inoculation in the glass Petri dish inoculated with 100 *D. spathans* eggs and maintained at ambient temperatures (~23–25 C) (Fig. 6). The remaining eggs were checked daily for another 18 days, but neither miracidia development nor hatching was observed, and eggs began to deteriorate.

For fresh feces, miracidia were observed from eggs 15 days (n = 3), 17 days (n = 1), and 21 days (n = 1) after being placed in a 24-well plate and held at ambient temperatures (~23–25 C). Similarly, 1 egg recovered from feces kept at ~4 C for 15 days prior to sedimentation hatched 15 days after removal from cold storage and incubation at ambient temperatures.

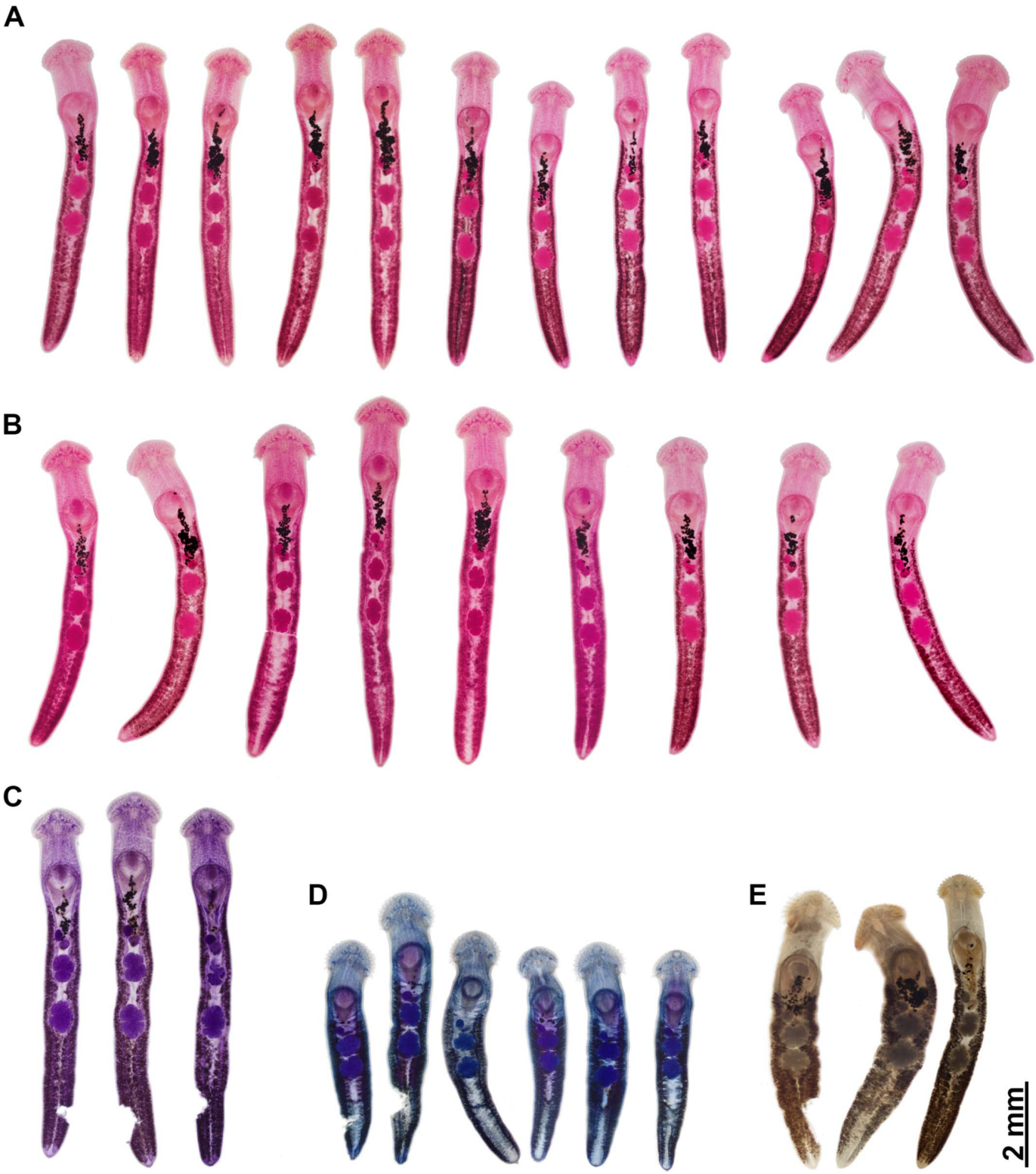


**Figure 4.** Semichon's acetocarmine– and Van Cleave's hematoxylin–stained gravid *Drepanocephalus spathans* echinostomatid from the intestine of experimentally infected double-crested cormorants (**A–B**) and specimens from natural infections exhibiting varying morphologies resulting from (**C**) heat relaxation or (**D–E**) direct placement in 70% ethanol. Semichon's acetocarmine–stained specimens at (**A**) 43 days and (**B**) 31 days post-exposure during the second trial. (**C**) Van Cleave's hematoxylin–stained specimens previously relaxed with slightly boiling saline from a naturally infected double-crested cormorant from Lowndes County, Mississippi. (**D**) Contracted, Van Cleave's hematoxylin–stained specimens from the archived collection of L. M. Pote and (**E**) specimens directly placed in 70% ethanol by T. G. Rosser. Color version is available online.

**Table II.** Morphological data* of *Drepanocephalus spathans* and *Drepanocephalus auritus* isolates from this and previous studies. Measurements are reported in micrometers. When reported, mean and SD are presented in parentheses.

| Identity | *Drepanocephalus spathans* | | *Drepanocephalus spathans* | |
|---|---|---|---|---|
| Host | *Phalacrocorax auritus* | | *Phalacrocorax auritus* | *Phalacrocorax brasilianus* |
| Locality | Mississippi, United States | | Mexico | |
| Reference | Present study, 2015 trial (n = 5) | Present study, 2016 trial (n = 25) | Hernández-Cruz et al. (2018) | Hernández-Cruz et al. (2018); Violante-González et al. (2011) |
| BL | 4,215–5,395 (4,729 ± 425) | 6,914–8,995 (7,928 ± 507) | 4,459–9,181 (6,692 ± 1,591) | 6,826–7,543 (7,185 ± 507) |
| BWVS | 583–692 (639 ± 40) | 724–1,046 (949 ± 82) | 917–1,439 (1,109 ± 136) | 764–1,150 (957 ± 273) |
| BWAT | 446–488 (469 ± 17) | 636–914 (791 ± 60) | 734–1,512 (1,009 ± 243) | 687–1,233 (960 ± 386) |
| CL | 421–494 (455 ± 31) | 583–784 (689 ± 50) | 646–995 (769 ± 102) | 531–909 (720 ± 267) |
| CW | 730–834 (789 ± 38) | 1,095–1,401 (1,235 ± 81) | 1,044–1,794 (1,383 ± 170) | 1,062–1,509 (1,286 ± 316) |
| OSL | 174–215 (198 ± 15) | 287–333 (314 ± 13) | 232–362 (279 ± 34) | 191–266 (229 ± 53) |
| OSW | 171–201 (186 ± 11) | 260–344 (298 ± 19) | 256–403 (315 ± 42) | 233–299 (266 ± 47) |
| PPL | 39–73 (57 ± 12) | 23–95 (56 ± 24) | 33–74 (49 ± 15) | 29–45 (37 ± 11) |
| PL | 197–217 (208 ± 8) | 239–339 (302 ± 26) | 206–363 (300 ± 43) | 229–289 (259 ± 42) |
| PW | 125–141 (132 ± 7) | 161–255 (198 ± 20) | 149–240 (187 ± 25) | 171–199 (185 ± 20) |
| OL | 403–465 (436 ± 23) | 567–754 (671 ± 49) | 522–742 (637 ± 64) | 669–745 (707 ± 54) |
| CSL | 267–296 (278 ± 12) | 345–503 (434 ± 43) | 250–555 (376 ± 85) | 255† |
| CSW | 144–205 (169 ± 25) | 256–389 (324 ± 37) | 210–460 (264 ± 62) | 216† |
| SV1L | 123–168 (138 ± 17) | 71–225 (162 ± 35) | 82–134 (101 ± 14) | 65† |
| SV1W | 85–138 (112 ± 19) | 67–174 (111 ± 23) | 58–161 (101 ± 30) | 79† |
| SV2L | 174–225 (202 ± 22) | 308–444 (370 ± 42) | 101–265 (197 ± 47) | 129† |
| SV2W | 127–151 (135 ± 10) | 155–287 (222 ± 33) | 82–183 (131 ± 30) | 129† |
| VSL | 607–666 (635 ± 27) | 796–1,113 (986 ± 109) | 814–1,118 (940 ± 110) | 729–991 (860 ± 185) |
| VSW | 480–582 (528 ± 37) | 729–917 (829 ± 54) | 611–1,066 (768 ± 130) | 525–896 (711 ± 262) |
| OVL | 108–142 (125 ± 15) | 221–304 (261 ± 21) | 176–308 (252 ± 43) | 153–312 (233 ± 112) |
| OVW | 73–105 (91 ± 15) | 163–286 (210 ± 24) | 182–270 (222 ± 23) | 136–261 (199 ± 88) |
| MEL | 102–200 (125 ± 19) | 199–323 (236 ± 30) | 142–302 (209 ± 54) | 199–327 (263 ± 91) |
| MEW | 96–145 (125 ± 19) | 229–325 (261 ± 22) | 206–423 (297 ± 74) | 216–366 (291 ± 106) |
| ATL | 290–398 (332 ± 40) | 448–694 (556 ± 57) | 396–626 (510 ± 69) | 108–600 (354 ± 348) |
| ATW | 204–256 (231 ± 21) | 381–540 (455 ± 42) | 425–911 (594 ± 141) | 107–637 (372 ± 375) |
| PTL | 332–397 (363 ± 32) | 553–803 (639 ± 65) | 385–717 (549 ± 100) | 124–609 (367 ± 343) |

**Table II.** Extended.

| *Drepanocephalus spathans* | *Drepanocephalus auritus* | *Drepanocephalus spathans* | | *Drepanocephalus spathans* | |
|---|---|---|---|---|---|
| *Phalacrocorax brasilianus* | *Phalacrocorax auritus* | *Phalacrocorax brasilianus* | *Phalacrocorax auritus, Sula leucogaster* | *Phalacrocorax brasilianus* | |
| Mexico | United States | Paraguay | Colombia | Argentina | Brazil |
| Hernández-Cruz et al. (2018); Ramos-Ramos (1995) | Kudlai et al. (2015) | Kostadinova et al. (2002) | Rietschel and Werding (1978) | Ostrowski de Núñez (1966) | Dietz (1909; 1910) |
| 8,132–11,004 (9,389 ± 1,469) 8,210–11,109 | 6,584–9,129 (8,123) | 4,384–4,946 | 4,800–6,660 | 5,106–9,990 | 7,250 |
| 1,338–1,592 (1,492 ± 135) 1,368–1,626 | 779–1,186 (944) | – | 990–1,110 | 925–1,369 | 340–1,400 |
| 1,138–1,184 (1,162 ± 23) – | 531–974 (719) | 932–1,284 | – | – | – |
| 808–1,012 (907 ± 102) – | 558–797 (638) | 884–963 | – | – | – |
| 1,511–1,579 (1,545 ± 48) 1,481–1,819 | 991–1,416 (1,165) | 1,210–1,482 | 1,260–1,470 | 1,110–1,739 | – |
| 296–421 (355 ± 63) 273–418 | 195–336 (274) | 247–301 | 160–212 | 221–351 | 350† |
| 281–451 (360 ± 86) 322–354 | 212–319 (264) | 235–333 | 235–310 | 221–390 | |
| 0–101 0–144 | 17–106 (73) | 0–7 | 23–103 | 0–91 | 46† |
| 359–412 (391 ± 28) 483–490 | 212–372 (287) | 247–281 | 259–263 | 249–390 | 354† |
| 256–295 (274 ± 20) 241–273 | 124–212 (171) | 161–198 | 164–226 | 143–221 | 261† |
| 601–888 (702 ± 161) 611–885 | 53–761 (599) | 411–685 | 684–780 | 351–715 | 430† |
| 480–567 (524 ± 62) 434–563 | 320–578 (440) | 408† | – | 286–455 | – |
| 354–435 (395 ± 57) 354–450 | 212–331 (257) | 231–272 | – | 182–325 | – |
| 329† – | 88–159 (109) | 197† | – | – | – |
| 234† – | 66–154 (104) | 163† | – | – | – |
| 92† – | 278–498 (361) | 291† | – | – | – |
| 204† – | 146–265 (193) | 202† | – | – | – |
| 1,011–1,218 (1,115 ± 146) 1,046–1,223 | 797–1,115 (914) | 766–1,110 | 684–912 | 845–1,235 | 950† |
| 765–857 (823 ± 51) 1,040–1,223 | 566–867 (692) | 671–973 | 780–864 | 611–884 | 770† |
| 257–271 (264 ± 10) 225–257 | 150–283 (225) | 116–171 | 180–216 | 130–312 | 308† |
| 247–266 (257 ± 13) 241–338 | 124–248 (186) | 192–253 | 132–264 | 234–273 | 231† |
| 270–344 (307 ± 52) – | 106–306 (182) | 86–164 | – | 130–416 | 280–350 |
| 302–346 (324 ± 31) – | 195–372 (252) | 222–288 | – | 221–325 | |
| 639–757 (712 ± 64) 627–805 | 389–708 (560) | 247–363 | 300–456 | 325–728 | 740† |
| 585–701 (661 ± 66) 547–692 | 283–549 (421) | 593–685 | 552–600 | 312–715 | 690† |
| 652–804 (746 ± 82) 644–901 | 443–689 (579) | 358–582 | 384–540 | 299–884 | – |

**Table II.** Continued.

| Identity | *Drepanocephalus spathans* | | *Drepanocephalus spathans* | |
|---|---|---|---|---|
| Host | *Phalacrocorax auritus* | | *Phalacrocorax auritus* | *Phalacrocorax brasilianus* |
| Locality | Mississippi, United States | | Mexico | |
| Reference | Present study, 2015 trial (n = 5) | Present study, 2016 trial (n = 25) | Hernández-Cruz et al. (2018) | Hernández-Cruz et al. (2018); Violante-González et al. (2011) |
| PTW | 225–248 (239 ± 9) | 374–554 (455 ± 42) | 425–894 (590 ± 129) | 107–649 (378 ± 383) |
| EL | 65–90 (80 ± 9) | 68–99 (87 ± 9) | 65–91 (80 ± 7) | 59–80 (73 ± 12) |
| EW | 41–54 (53 ± 1) | 44–68 (54 ± 6) | 37–61 (48 ± 7) | 36–59 (49 ± 12) |
| FORE | 868–1,069 (962 ± 74) | 1,227–1,486 (1,351 ± 75) | 1,031–1,627 (1,325 ± 186) | 1,360–1,468 (1,414 ± 76) |
| OVAR | 149–319 (264 ± 67) | 344–801 (591 ± 116) | 101–655 (408 ± 203) | 354–518 (436 ± 116) |
| TEND | 1,610–2,051 (1,725 ± 184) | 1,216–3,398 (2,790 ± 397) | 1,178–3,751 (2,504 ± 809) | 2,500–2,975 (2,738 ± 336) |
| BW (%) | 14.9–17.7 (16.7 ± 1.1) | 14.4–17.5 (15.6 ± 0.9) | 3.6–8.5 (6.1 ± 1.8) | 6.6–8.9 (7.8 ± 1.7) |
| FO (%) | 19.8–20.9 (20.4 ± 0.5) | 15.1–19.4 (17.1 ± 0.9) | 3.4–6.7 (5.1 ± 1.1) | 5.0–5.2 (5.1 ± 0.1) |
| U (%) | 3.5–6.8 (5.5 ± 1.2) | 4.3–9.2 (7.4 ± 1.4) | 8.2–19.7 (13.2 ± 4.2) | 10.0† |
| T (%) | 34.9–38.2 (36.5 ± 1.5) | 15.2–38.4 (35.2 ± 4.4) | 2.3–3.8 (2.8 ± 0.4) | 2.5–2.7 (2.6 ± 0.1) |

* Abbreviations: body length (BL), body width at ventral sucker (BWVS), body width at anterior testis (BWAT), collar length (CL), collar width (CW), oral sucker length (OSL), oral sucker width (OSW), prepharynx length (PPL), pharynx length (PL), pharynx width (PW), esophagus length (OL), cirrus sac length (CSL), cirrus sac width (CSW), length of anterior portion of seminal vesicle (SV1L), width of anterior portion of seminal vesicle (SV1W), length of posterior portion of seminal vesicle (SV2L), width of posterior portion of seminal vesicle (SV2W), ventral sucker length (VSL), ventral sucker width (VSW), ovary length (OVL), ovary width (OVW), Mehlis' gland length (MEL), Mehlis' gland width (MEW), anterior testis length (ATL), anterior testis width (ATW), posterior testis length (PTL), posterior testis width (PTW), egg length (EL), egg width (EW), forebody length (FORE), distance between ovary and posterior margin of ventral sucker (OVAR), and distance between posterior margin of posterior testis and posterior extremity of body (TEND). Additionally, the following proportions expressed as percentages were calculated according to Kostadinova (2005): maximum body width as a proportion of body length (BW), length of the forebody as a proportion of body length (FO), length of the uterine field posterior to the ventral sucker (U), and the length of the post-testicular region as a proportion of body length (T).
† Character reported as a single measurement.

### Relaxation effects on morphological plasticity of adult *D. spathans* specimens

Adult specimens collected from the experimentally infected birds of both trials exhibited morphological consistencies with specimens of Kudlai et al. 2015 (see figure 1 within Kudlai et al., 2015) and the elongate specimens of Hernández-Cruz et al., 2018 (see figure 5c within Hernández-Cruz et al., 2018). Live worms killed and relaxed with nearly boiling saline displayed greatly separated testes (Fig. 4A–C). In the case of the archived specimens and those placed directly into 70% ethanol without heat relaxation, the resultant morphology (Fig. 4D–E) was consistent with historical accounts of *D. spathans* (see figure 2a within Rietschel and Werding, 1978; figure 1 within Kostadinova et al., 2002; figure 1b within Hernández-Cruz et al., 2018), in which the separation of testes is not as pronounced (9–194 μm vs. 198–522 μm).

Measurements based on 10 gravid specimens fixed in 70% ethanol without heat relaxation: Body compact and slightly elongate with greatest width at the level of the anterior collar; BW = 15.3–22.7% (20.5% ± 2.4%); BL 5,123–7,096 (6,015 ± 756); BWVS 858–1,166 (975 ± 106); BWAT 784–1,266 (976 ± 149); FORE 923–1,568 (1,176 ± 203) long with small, laterally placed spines; FO = 17.4–23.9% (19.5% ± 1.8%).

Head collar falciform, muscular, 590–925 (773 ± 121) × 1,029–1,492 (1,223 ± 154); ventral lappets present. Collar spines, 27, large, in a single row. Four angle spines, 2 pairs on each ventral lappet, larger than marginal spines. Each pair having a single ventral and dorsal spine; ventral spines smaller than dorsal. First pair: ventral spine 193–387 (244 ± 47) × 45–78 (55 ± 10); dorsal spine 235–360 (280 ± 35) × 49–80 (60 ± 9). Second pair: ventral spine 161–317 (223 ± 38) × 37–70 (48 ± 9); dorsal spine 183–337 (256 ± 35) × 42–80 (54 ± 9); first pairs larger than second pairs. Lateral spines in a single row, 4 on each lappet; first lateral spine 118–180 (138 ± 18) × 25–54 (33 ± 8); remaining lateral spines 134–229 (156 ± 29) × 25–54 (33 ± 8). Dorsal spines in a single row, 11, smaller than lateral spines, decreasing in size toward collar midline.

Oral sucker subspherical, subterminal, 217–324 (273 ± 36) × 225–335 (281 ± 36). Prepharynx indistinct, 0–73 (33 ± 32).

**Table II.** Continued, Extended.

| *Drepanocephalus spathans* | *Drepanocephalus auritus* | *Drepanocephalus spathans* | | *Drepanocephalus spathans* | |
|---|---|---|---|---|---|
| *Phalacrocorax brasilianus* | *Phalacrocorax auritus* | *Phalacrocorax brasilianus* | *Phalacrocorax auritus, Sula leucogaster* | *Phalacrocorax brasilianus* | |
| Mexico | United States | Paraguay | Colombia | Argentina | Brazil |
| Hernández-Cruz et al. (2018); Ramos-Ramos (1995) | Kudlai et al. (2015) | Kostadinova et al. (2002) | Rietschel and Werding (1978) | Ostrowski de Núñez (1966) | Dietz (1909; 1910) |
| 626–679 (647 ± 28) | 319–549 (410) | 480–685 | 540–684 | 364–715 | – |
| 623–756 | | | | | |
| – | 74–99 (88) | 73–91 (85 ± 5) | 61–80 | 70–95 | 61–65 |
| 80† | | | | | |
| – | 42–61 (52) | 35–54 (46 ± 6) | 43–56 | 36–56 | 50–53 |
| 48† | | | | | |
| 1,645–2061 (1,793 ± 233) | 1,239–1,628 (1,459) | 980–1,028 | – | – | – |
| – | | | | | |
| 615† | 531–1,530 (809) | 0–41 | – | – | – |
| – | | | | | |
| 2,839–4,000 (3,582 ± 645) | 2,372–3,264 (2,857) | 1,507–1,987 | – | – | – |
| – | | | | | |
| 5.9–6.9 (6.2 ± 0.6) | 10.6–12.0 (11.6) | 18.9–30.7 | – | – | – |
| – | | | | | |
| 4.9–5.5 (5.2 ± 0.3) | 15.6–20.0 (18.1) | – | – | – | – |
| – | | | | | |
| 4.1–8.4 (6.9 ± 2.4) | 8.1–16.8 (10.0) | 0–0.9 | – | – | – |
| – | | | | | |
| 2.3–2.9 (2.7 ± 0.3) | 34.0–36.0 (35.2) | 32.2–40.2 | – | – | – |
| – | | | | | |

Pharynx highly muscular, elongate-ovoid, 203–349 (265 ± 51) × 150–260 (198 ± 37). Esophagus, elongate, 517–865 (644 ± 102), bifurcating at the level of anterior margin of ventral sucker. Ceca extending almost to the posterior of body.

Testes tandem, ovoid, variably and deeply lobed. Anterior testis just posterior to equatorial region of body, with 4–6 distinct lobes, 403–610 (521 ± 72) × 439–659 (550 ± 76); posterior testis slightly more elongate, with 5–7 distinct lobes, 527–749 (616 ± 90) × 427–769 (571 ± 96); DBT = 9–194 (74 ± 68). Post-testicular region 1,904–2,749 (2,262 ± 270) long; T = 33.4–41.2% (37.8% ± 3.0%). Cirrus sac and internal seminal vesicle immeasurable and obscured by ventral sucker and compacted egg-filled uterus. Genital pore, medial, just posterior to ceca bifurcation.

Ovary dextral, small, elongate-ovoid, 206–301 (246 ± 33) × 188–275 (230 ± 30); U = 0.6–4.8% (2.0% ± 1.4%). Mehlis' gland median, contiguous with ovary, transversely ovoid, 99–191 (120 ± 28) × 230–417 (291 ± 56). Metraterm muscular. Eggs ovoid, 85–93 (88 ± 4) × 52–55 (53 ± 1). Vitellarium in 2 fields of follicles along the lateral margins of the body, beginning at posterior margin of ventral sucker and ending in body posterior. Excretory pore terminal.

## DISCUSSION

In experimental infections, *D. spathans* cercariae penetrated juvenile catfish, causing mortalities within the first 7 dpe (Griffin et al., 2012, 2014; Alberson et al., 2017). In surviving fish, metacercariae seen at 7 dpe were mostly in the cranial region (Griffin et al., 2012, 2014; Alberson et al., 2017). However, the infection began to resolve as early as 7–21 dpe (Griffin et al., 2014), raising the question of whether channel catfish are an intermediate host in the *D. spathans* life cycle. At present, there have been no reported natural infections in channel catfish. Doffitt et al. (2009) reported a single adult trematode, morphologically identified as *Drepanocephalus spathans*, in a DCCO fed channel catfish collected from production ponds naturally infected with *Bolbophorus damnificus*. It was speculated this was the result of birds ingesting catfish naturally infected with *D. spathans* or an unsuccessful clearing of gastrointestinal parasites before their experimental inoculation (Doffitt et al., 2009). Moreover, Pinto et al. (2016) attempted to complete the life cycle, successfully infecting the guppy *Poecilia reticulata* with *D. spathans* and extracting metacercariae from the caudal fin and gills. Metacercariae were fed to chicks *Gallus gallus domesticus* and mice *Mus musculus*, but adult trematodes were not recovered in hosts euthanized at 5 dpe.

This current work confirms the previous speculation and demonstrates channel catfish can serve as an intermediate host in the life cycle of *D. spathans*. This work also offers support to previous studies suggesting that crowding can impact parasite size and maturation. The adults recovered in the 2015 study were smaller than those reported for *D. spathans* by Kudlai et al. (2015). It is suspected these adults were smaller in size due to crowding, a phenomenon first reported by Read (1951) in cestodes, wherein parasite size decreases as the number of parasites increases. This crowding effect has also been reported in trematodes. Fried and Freeborne (1984) observed decreased body area of *Echinostoma revolutum* found in crowded sites versus

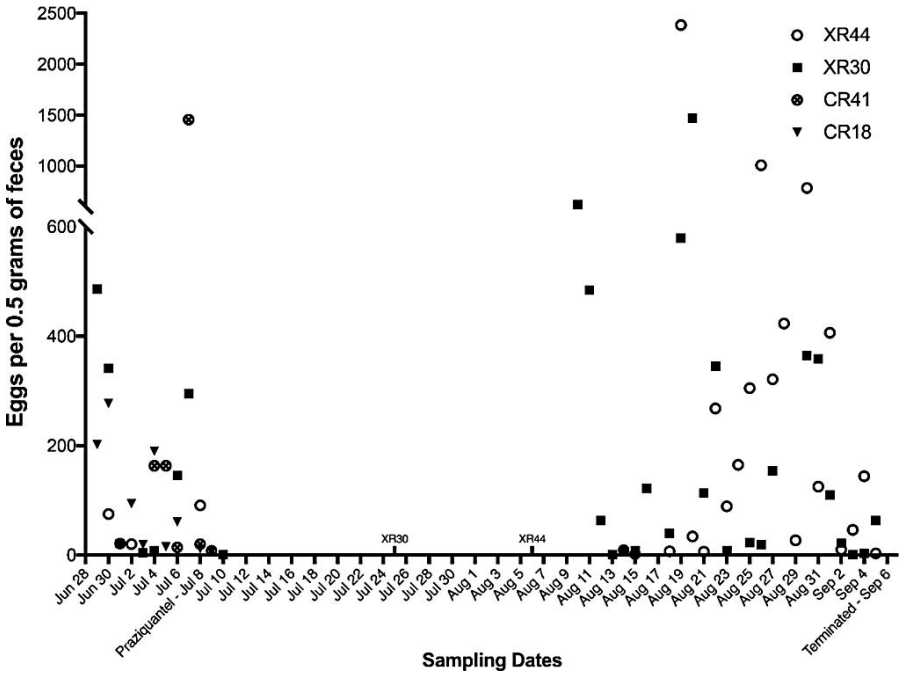


**Figure 5.** Fecal egg counts from 2016 trial for experimental (n = 2; XR30, XR44) and control (n = 2; CR18, CR41). The day each bird was fed *Drepanocephalus spathans*–infected fish is annotated on the x-axis.

**Table III.** Morphological data of collar spines of *Drepanocephalus spathans* and *Drepanocephalus auritus* isolates from this and previous studies. Measurements are reported in micrometers, with mean and SD in parentheses.

| Identity | *Drepanocephalus spathans* | | *Drepanocephalus spathans* | *Drepanocephalus auritus* |
|---|---|---|---|---|
| Host | *Phalacrocorax auritus* | | *Phalacrocorax brasilianus* | *Phalacrocorax auritus* |
| Locality | Mississippi, United States | | Mexico | United States |
| Reference | Present study, 2015 trial (n = 5) | Present study, 2016 trial (n = 25) | Hernández-Cruz et al. (2018) | Kudlai et al. (2015) |
| Angle Spines | | | | |
| First ventral pair | | | | |
| Length | 174–196 (183 ± 7) | 231–322 (276 ± 23) | 172–312 (265 ± 42) | 219–320 (274 ± 28) |
| Width | 37–41 (39 ± 1) | 59–76 (67 ± 4) | 49–74 (59 ± 6) | 49–66 (55 ± 6) |
| First dorsal pair | | | | |
| Length | 179–200 (191 ± 8) | 243–351 (303 ± 24) | 200–338 (268 ± 38) | 246–384 (324 ± 32) |
| Width | 36–45 (39 ± 3) | 53–74 (67 ± 4) | 48–74 (59 ± 7) | 58–72 (64 ± 5) |
| Second ventral pair | | | | |
| Length | 149–188 (166 ± 11) | 195–324 (245 ± 21) | 152–293 (241 ± 35) | 205–297 (247 ± 25) |
| Width | 33–38 (36 ± 2) | 48–69 (59 ± 5) | 40–70 (52 ± 8) | 39–51 (44 ± 4) |
| Second dorsal pair | | | | |
| Length | 141–168 (154 ± 10) | 185–272 (230 ± 20) | 153–324 (244 ± 42) | 256–402 (302 ± 47) |
| Width | 29–35 (32 ± 2) | 47–57 (52 ± 3) | 40–77 (55 ± 10) | 43–78 (55 ± 11) |
| Lateral spines | | | | |
| First | | | | |
| Length | 75–91 (84 ± 5) | 94–146 (121 ± 13) | 83–170 (124 ± 26) | 142–197 (175 ± 19) |
| Width | 20–25 (23 ± 2) | 30–42 (36 ± 3) | 28–44 (36 ± 6) | 31–47 (37 ± 5) |
| Remaining lateral spines | | | | |
| Length | 73–83 (78 ± 4) | 101–148 (125 ± 14) | 60–176 (122 ± 31) | 148–243 (190 ± 27) |
| Width | 24–26 (25 ± 1) | 36–46 (40 ± 3) | 29–48 (40 ± 4) | 22–47 (37 ± 5) |
| Dorsal spines | | | | |
| Length | – | – | 59–165 (95 ± 24) | 135–225 (171 ± 27) |
| Width | – | – | 20–47 (37 ± 7) | 25–40 (35 ± 5) |

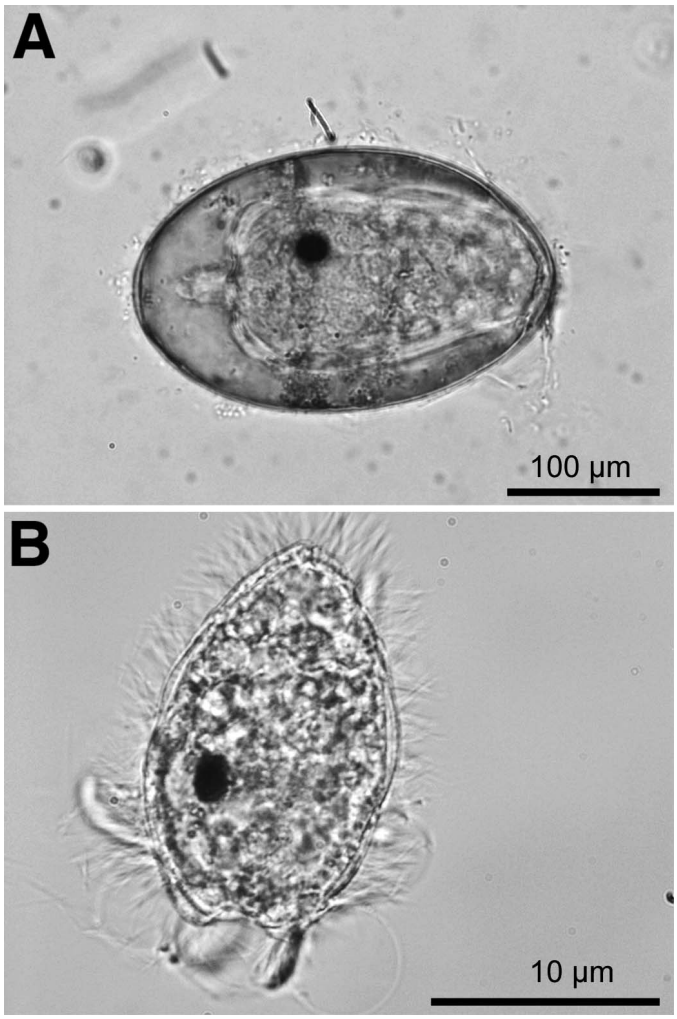


**Figure 6.** Developing *Drepanocephalus spathans* miracidia. (**A**) Miracidium present within an egg collected from experimentally infected double-crested cormorant feces. (**B**) Miracidium emerged from an egg recovered from double-crested cormorant feces.

non-crowded sites in intestines of chickens. Franco et al. (1988) also investigated the crowding effect of *E. revolutum*, employing a golden hamster (*Mesocricetus auritus*) model. They found intraspecific crowding, extended maturation time, and reduced overall body size. Similarly, Valero et al. (2006) studied the effects of crowding on growth, pre-patent period, and the release of eggs of *Fasciola hepatica* in Wistar rats, and found adults were smaller when there were higher numbers of parasites. Similarly, they reported that pre-patent period and egg production both decreased as parasite numbers increased. Along those lines, Stillson and Platt (2007) looked at the effects of crowding on morphometric variability of *Echinostoma caproni* in mice and found reductions in size to organs, body measurements, muscular structures, and eggs. During their study, Stillson and Platt (2007) recovered low numbers of adult *E. caproni* trematodes from a mouse infected with 300 metacercariae and suggested the expulsion of juveniles may be due to an inflammatory response, indicating this response could play a factor in the crowding effect. It is also possible the worms were smaller simply due to sampling at an early developmental stage (8 dpe).

In the 2015 study, estimations of metacercariae numbers in fish suggested the possibility of DCCOs ingesting up to ≥550 metacercariae with no ill effect. Once *D. spathans* adults were observed in the feces of experimental DCCOs at 8 dpe, the first bird was sacrificed, revealing >2,000 *D. spathans* individuals, indicating histology is not an accurate method of metacercariae estimation. In the 2016 life cycle study, it was estimated that each experimental bird received ~350 metacercariae, which was thought to be a more accurate estimate, as they were largely derived from pepsin digests. At no time during the 2016 study were adults seen in the feces; at necropsy, many fewer *D. spathans* individuals were recovered. Furthermore, adults recovered in the 2016 study were noticeably larger than those recovered in the 2015 study, either from a lack of crowding or because they were sampled later. Regardless, worms sampled in the 2016 study were morphologically congruous with the morphotype specimens reported by Kudlai et al. (2015).

Similarly, eggs measured from stained gravid adult *D. spathans* specimens from both the 2015 and 2016 studies were within the range reported by Kudlai et al. (2015). In the 2015 study, eggs were first observed in the feces of experimental DCCOs 3 dpe. Eggs continued to be observed on days 4, 6, 7, 8, and 17 dpe. During the 2016 study, eggs were not observed until 16 dpe in the first DCCO infected and 8 dpe in the second DCCO. It is speculated these early eggs observed in fecal samples in the 2015 study were from residual feces leftover after the daily cleaning process and did not represent a patent infection, while later eggs, observed on days 8 and 17, were consistent with the 2016 study and more likely shed by gravid *D. spathans* individuals.

The *D. spathans* miracidia first emerged from eggs collected from fresh DCCO feces 15 days after being placed in water held at ambient temperatures (~23–25 C), with the last miracidium emerging at 21 days. Miracidia also emerged from eggs at 15 days after being removed from cold storage (4 C). Emergence of miracidia from eggs kept at these lower temperatures was congruous with emergence from freshly deposited eggs held at ambient temperatures. It is believed this represents a dormant phase during cool temperatures. It is speculated that eggs can remain dormant, yet viable, in catfish ponds throughout the winter months, when water temperatures are <10 C, and miracidia emergence corresponds with increasing water temperatures in the spring, which likely coordinates with the emergence of the snail host.

At warmer water temperatures (~27–30 C), cercariae were released from *P. trivolvis* snails as early as 18 days after being placed into experimental ponds. Comparatively, cercariae emergence took upwards of 51 days when water temperatures were <27 C or >30 C, suggesting an optimal range for intramolluscan development. In laboratory infections at ambient temperatures, it took 89 days from egg exposure to the release of cercariae (~23–25 C). Based on the observations reported here, where miracidia hatched within 21 days at these temperatures, this would suggest an arrested development of 60–70 days compared to the 18-day incubation observed at ~27–30 C.

This study also demonstrated the capacity for large numbers of *D. spathans* eggs to be introduced into catfish production systems. O'Hear et al. (2014) reported intensities in DCCOs as high as 346 adult worms per bird. The shedding rates observed here for birds with much milder infections (20–29 adult worms per bird) still exceeded 1,000 eggs/g of feces. According to studies by Brugger

(1993) and Marion et al. (1994), cormorants can defecate up to 30 g dry weight of feces daily. During a DCCO depredation study on catfish ponds in the Delta region of Mississippi, Stickley et al. (1992) reported as many as 85 cormorants on a pond in a single day. Extrapolated from the results of the present study, an estimated 30,000 eggs can be shed by a single DCCO daily, and a flock of 50 DCCOs can shed up to 1.5 million eggs in a single day. Estimates of DCCO populations in the Delta region of Mississippi range from 30,000 to >80,000 birds over a year (Glahn and Stickley, 1995; Glahn et al., 1996, 2000; Dorr et al., 2008, 2012). When one considers these estimates, the potential number of *D. spathans* eggs introduced to catfish ponds is alarming.

While the main goal of this work was to experimentally replicate the life cycle of *D. spathans*, access to adult specimens at known developmental time points and in a single species infection offered opportunities to comment on the morphological plasticity previously cited in this species. This study revealed the morphological discrepancies cited by Kudlai et al. (2015) and Hernández-Cruz et al. (2018) are likely an artifact of differences in fixation. The effects of heat relaxation versus direct placement of the specimens into 70% ethanol were obvious, with directly fixed worms appearing to be contracted. Although this effect has been subjected to limited formal evaluation, it has been reported for other trematodes, including *Clinostomum complanatum* (Kurashvili, 1957) and *Phyllodistomurn umbalae* (Bakke, 1988). Many surveys of helminths mention a variety of methods used for the relaxation of trematodes for morphological studies. Most report the use of heated solutions, often water, saline, or formalin; however, these studies do not typically report an exact temperature used, and the morphological variability associated with partially relaxed or contracted specimens remains uncertain. Furthermore, molecular data from the hologenophore specimens support the identity of the specimens as conspecifics and corroborates the synonymy of *D. auritus* and *D. spathans* proposed by Hernández-Cruz et al. (2018).

Herein, each step of the *D. spathans* life cycle was completed, from natural and experimental infections in snail hosts to successful recovery of gravid adults in the DCCO definitive host. Observations from the miracidia emergence study raise the possibility that trematode eggs can remain dormant in catfish ponds during the winter months in Mississippi, with miracidia hatching somewhat synchronously once pond water temperatures increase in the spring. It is speculated that this temperature-induced hatching corresponds with snail emergence, making control of the snail hosts extremely important, since controlling the DCCO definitive host is challenging. The information gathered from this research is useful to commercial catfish producers and fish health professionals, as data regarding the timing of cercariae release and miracidia emergence can be used to implement best management practices to minimize impacts of digenetic trematode infection in catfish aquaculture operations.

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