UNIVERSITY OF CALIFORNIA SAN DIEGO

Hidden diversity in our estuaries: Undescribed and cryptic trematode parasite species from the California horn snail, *Cerithideopsis californica* (Gastropoda: Potamididae)

A thesis submitted in partial satisfaction of the requirements
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by

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The thesis of Alexandria P. Nelson is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Hidden diversity in our estuaries: Undescribed and cryptic trematode parasite species from the California horn snail, *Cerithideopsis californica* (Gastropoda: Potamididae)

by

Alexandria P. Nelson

Master of Science in Marine Biology

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Professor Ryan Hechinger, Chair

The California horn snail, *Cerithideopsis californica*, serves as first intermediate host to one of the best studied guilds of trematode parasites. This guild, and the individual species that comprise it, have been used as a model system for many ecological studies (see references herein). These marine parasite species have multiple-host complex life cycles and are an important part of salt marsh food webs (Lafferty & Kuris 2009; Kuris et al. 2008). Despite the trematodes' widespread presence throughout the literature, several members of the guild have not been accurately identified, formally described, or had their life cycles resolved. Proper

taxonomic naming and identification is important, particularly because we know that some of the guild members are composed of several cryptic species (e.g. Huspeni 2000). In this thesis, I provide formal descriptions of the colony demographics, parthenitae, and cercariae of *Cloacitrema michiganensis* (Philophthalmidae), *Cloacitrema kurisi n.sp.* (Philophthalmidae), *Parorchis catoptrophori* (Philophthalmidae), *Parorchis laffertyi n.sp.* (Philophthalmidae), *Cercocyathocotylida intexens* n.gen. n.sp. (Cyathocotylidae), and *Probolocoryphe uca* (Microphallidae). I also note the presence of an unrecognized *Probolocoryphe* (Microphallidae) species in California wetlands and provide genetic confirmation that the undescribed "small microphallid" (see Hechinger 2019) belongs in the genus *Maritrema* (Microphallidae). When possible, I describe the metacercariae and adults of these species obtained via experimental infections. Through analyses of their morphology and genetic data (at the CO1 and 28S loci) I elucidate the life cycles and assign proper taxonomic names to these species to enable them to be appropriately included into food webs and future ecological studies.

INTRODUCTION

Cerithideopsis californica, the California horn snail, serves as first intermediate host to one of the best studied guilds of trematode parasites. This guild and its individual species have been used as model systems for several community ecology (e.g. Hechinger et al. 2008), dispersal ecology (e.g. Fingerut et al. 2003), evolutionary ecology (e.g. Lafferty 1993), ecological parasitology (e.g. Buck et al. 2017; Hechinger & Lafferty 2005), and sociobiological (e.g. Hechinger et al. 2011a) studies. These marine parasites have multiple-host complex life cycles and are an important part of salt marsh food webs (e.g. Lafferty & Kuris 2009; Kuris et al. 2008). Despite the trematodes' prominent presence in the literature, several members of the guild have not been accurately identified, formally described, or had their complex life cycles resolved.

Proper taxonomic naming and identification is important, particularly because we know that some of the guild members are actually composed of several unidentified cryptic species (Huspeni 2000; Miura et al. unpublished). Numerous studies have documented how cryptic trematode species vary in ecology, with potential differences in their cercarial activity (Mouahid & Theron 1987; Theron & Combes 1988; Chasse & Theron 1988; Chapter 2 of this thesis), second intermediate host preferences (Nguyen et al. 2015; Chapter 4 of this thesis), and could possibly even vary in their competitive interactions with other trematodes. Cryptic species may also differ in colony demographics in their first intermediate host and use of final host species (Chapter 1 & 2 of this thesis). Therefore, recent pleas for more cryptic species awareness (Pérez-Ponce de León & Nadler 2010; Poulin 2011) should not be ignored.

Additionally, while the number of helminth species descriptions has grown exponentially over time, the number of taxonomic publications attempting to elucidate helminth life cycles or describe the "larval" life stages has grown disproportionately smaller (Blasco-Costa & Poulin 2017). In fact, it is believed that life cycles have been determined for less than 5% of all known marine helminth parasites (Poulin et al. 2016). This is unfortunate for several reasons, one of which is that the inclusion of parasites has been shown to increase diversity and connectance in salt marsh food webs (Lafferty et al. 2006). Parasite species are typically ignored by ecologists to begin with, so the exclusion of full life cycles and cryptic species by parasite taxonomists is counterproductive and serves to perpetuate the exclusion of parasites in ecology.

Finally, in these parasites' multiple-host life cycles, very different life stages use very different hosts. Most research is focused on a particular host and therefore a specific parasite life stage, creating a disconnect between the adult and various larval forms. Additionally, trematode species are typically only described in their sexual adult stage, without attention paid to earlier larval stages. There is a strong need to describe the different life stages of these organisms to permit connecting the life stages together as one unambiguous parasite species. The inclusion of previously undocumented cryptic species into long-term studies of California estuarine biodiversity (which began in 2001) will further our understanding of their food web structure. Carefully describing the "players" in this guild will facilitate future research on these parasites and enhance our general understanding of estuarine biodiversity.

The general life cycle for trematodes in the California horn snail guild is as follows: 1) in the snail first intermediate host there is a colony composed of parthenitae that asexually produce dispersive offspring known as cercariae, 2) a cercaria seeks out and infects a second intermediate host invertebrate or vertebrate and forms a metacercaria, and 3) metacercariae infect final host

birds when they eat second intermediate hosts. In this thesis, I provide formal descriptions for 6 trematode species, all of which have never had their larval stages described, and 4 of which have not had adults described. I describe the first intermediate host stages (parthenitae and cercariae) from wild-infected California horn snail hosts for all 6 species. When possible, I describe the metacercariae of these species obtained via collection of wild-infected crab second intermediate hosts, experimental infections of fish second intermediate hosts, or direct encystment of the cercariae as ectometacercariae. I describe adult specimens obtained through experimental infection of final host chickens and ducks. I also note the presence of an unrecognized *Probolocoryphe* species in California wetlands and provide information on the undescribed "small microphallid" (see Hechinger 2019).

The specific chapters are arranged as: Chapter 1. *Cloacitrema michiganensis* and *Cloacitrema kurisi* n.sp. (Philophthalmidae); Chapter 2. *Parorchis catoptrophori* and *Parorchis laffertyi* n.sp. (Philophthalmidae); Chapter 3. *Cercocyathocotylida intexens* (Cyathocotylidae); Chapter 4. *Probolocoryphe uca* and *Probolocoryphe* sp. with notes on "small microphallid" (Microphallidae).

To confirm the identity and relationships to known taxa for each of my species, I couple the abovementioned morphological and life cycle work with genetic analyses at the CO1 and 28S loci. I analyze the genetic data of several colonies (likely clones) for each species at the CO1 locus to screen for potential cryptic species. My analysis of the species' genetic data at the 28S locus reveals relationships to other known trematode taxa, links my focal species to adults already described in the literature, and permits identification of adult specimens collected from wild birds by my lab during a prior field expedition. Along with analyses of their morphological attributes and genetic data I assign formal taxonomic names to these species to facilitate

association between their adult and larval stages, enabling them to be appropriately included into food webs and future ecological studies. This thesis is an excellent example of why future taxonomic work should include genetic analyses in addition to traditional morphological methods to completely elucidate parasite life cycles and unveil cryptic species complexes.

Following the International Commission on Zoological Nomenclature (article 8.2), we disclaim the nomenclatural acts in this thesis for the purposes of formal zoological nomenclature. We are reserving said acts for future intended publication.

CHAPTER 1

Description of *Cloacitrema kurisi* n.sp. and redescription of *Cloacitrema michiganensis* (Trematoda: Digenea: Philophthalmidae)

INTRODUCTION

Herein, we describe the colony demographics, rediae, cercariae, metacercariae, and adults of *Cloacitrema kurisi*, n.sp., which was previously unrecognized among the trematodes infecting as first intermediate host the California horn snail, *Cerithideopsis californica* (Haldeman 1840). We also redescribe the colony demographics, rediae, cercariae, metacercariae, and adults of *Cloacitrema michiganensis* McIntosh 1938 originating from first intermediate infections in the same host snail.

The family Philophthalmidae was erected by Looss in 1899. There are currently 4 recognized subfamilies: Philophthalminae Looss 1899, Ommatobrephinae Poche 1926, Echinostephillinae Yamaguti 1958, and Cloacitrematinae Yamaguti, 1958. The Cloacitrematinae contains 2 genera, *Cloacitrema* and *Pygorchis*, the adults of which reside in bird cloacas. *Cloacitrema* serves as the type-genus, with the type-species being *Cloacitrema ovatum* Yamaguti 1935. There were 9 previously described *Cloacitrema* species. Details regarding their type host, locality, and morphology can be found below.

Cloacitrema michiganensis was originally described from adult stages naturally infecting the migratory spotted sandpiper, *Actitis macularia*, in Michigan, USA and a black-necked silt, *Himantopus mexicanus*, in Florida, USA (McIntosh 1938). Two preliminary reports (Robinson

1952; Martin 1972) stated that *C. michiganensis* was in California, using the California horn snail as first intermediate host. Later, LeFlore et al. (1985) described the life cycle of *C. michiganensis* in California, describing adults experimentally obtained from hatchery-raised chicks, metacercariae encysted on laboratory finger bowls, and rediae and cercariae from naturally infected California horn snails.

Our analysis of the DNA sequences of cercariae originating from over 20 infections revealed the presence of 2 different *Cloacitrema* species infecting the California horn snail as first intermediate host. Our subsequent life cycle and morphological investigation further confirmed that both *C. michiganensis* and a previously undescribed species are members of the California horn snail trematode guild. Here, we describe the life cycle of the new species and redescribe the life stages of *C. michiganensis*, as our data indicate that the original *C. michiganensis* description (LeFlore et al. 1985) included a mixture of the 2 species.

MATERIALS AND METHODS

Life cycle work and description techniques

Following Hechinger et al. (2011), we refer to the mass of sporocysts in a first intermediate host as a "colony". We collected California horn snails by hand from the intertidal mudflats of the University of California Kendall-Frost Natural Reserve in San Diego, California. We first identified philophthalmid colonies and collected material for barcoding by "shedding" cercariae from living colonies (infected snails). To shed cercariae, we placed live snails individually into compartments with sea water under warm lights in the laboratory. We assigned snails infected with philophthalmid trematodes a unique number identifier and used LOCTITE

brush-on glue to super-glue the number IDs onto the shell of the snails. To keep the snails alive until we could dissect them to examine parthenitae, we held them in outdoor, artificial tide, flow-through seawater mesocosms for a maximum of 6 months.

To obtain a metacercaria time series of development, we re-shed infected snails into individual glass fingerbowls labelled with their unique barcode ID. The cercariae encysted on the walls and floor of the fingerbowls within 12 hours post-shedding. We observed and chemically excysted metacercariae on days 1, 2, 3, 5, 7, and 12 post-encystment. It became necessary to slightly modify the chemical excystation protocol from LeFlore and Bass (1983) to obtain maximum excystment. We gently scraped the metacercariae off the glass fingerbowls and placed them in a solution comprised of: 2x DI water, 8x NaCl, 15x $NaHCO_3$, 8x ox bile, 7.5x trypsin, 0.0025x HCl, and 8x L-Cysteine. Maximum excystment occurred after incubation at 42 C for 2 hours. Chemical excystment success was typically ~90% for both *C. michiganensis* and *C. kurisi*. To obtain morphometrics for the excysted worms, we heat-killed 5 – 10 individuals each day post-encystment and preserved them in 10% formalin.

To experimentally obtain sexual adult stage worms, we infected 3 day-old Cornish cross chickens, *Gallus domesticus*, and Pekin ducks, *Anas platyrhynchos domesticus*. In May 2020, we infected 2 chickens and 2 ducks per cloaca (Allison 1943) with 25 chemically excysted metacercariae from single *Cloacitrema* colonies. One clone of each *Cloacitrema* species went to both a single chick and a single duck. To increase the probability of obtaining sexually active adults, we infected an additional 2 chicks and 3 ducks with multiple clones of *C. michiganensis*. All birds had a single exposure event, except for the *C. kurisi* chick, which had 2 exposures 6 days apart. We sacrificed the birds between 27 – 46 days post-exposure. No worms were recovered from the ducks, so they were not used in the subsequent round of infections. In August

2020, we infected 5 chickens with single clones of *C. michiganensis*. To increase our parasite yield we infected summer birds with 2 separate exposure events, which took place 10 days apart. Each of these 5 birds were exposed to a total of 50 metacercariae. We infected an additional 14 chicks with multiple clones of *C. michiganensis* and 2 chicks with multiple clones of *C. kurisi*. These 16 birds endured 3 separate exposure events over the course of 3 consecutive days. We sacrificed the chickens between 20 – 41 days post-initial exposure.

The presence of a soldier caste within trematode colonies was first documented in Hechinger et al. (2011). Since then, soldier castes have been documented to occur within philophthalmid trematodes, including members of the genera *Cloacitrema* and *Parorchis* parasitizing the California horn snail as a first intermediate host (Garcia-Vedrenne et al. 2016). We therefore provide separate descriptions for reproductive and soldier rediae for both of our species. We distinguish the redia morphs using the following criteria: reproductives contain developing cercariae, are large and less active, and have a pharynx that is small relative to their body size; soldiers lack free-floating germinal material, are small and active, and have a pharynx that is large relative to their body size (Hechinger et al. 2011a; Garcia-Vedrenne et al. 2016).

Morphological descriptions are based on live and formalin-fixed samples. All morphometrics were taken using a compound microscope ocular micrometer. We obtained measurements from 10 individual reproductive rediae, 10 soldier rediae, and 10 cercariae from each of 5 colonies for each species (n = 50 for each life stage). Developing embryos inside of reproductive rediae can be distinguished from developed cercariae by their translucent body, small tail lacking an invaginated tip, decreased mobility, and lack of a clear excretory system and digestive ceca. For the parthenitae and cercariae, we obtained 5 voucher specimens for each species from 5 naturally infected *C. kurisi* and 5 naturally infected *C. michiganensis* colonies.

Colony members of these types were used for our morphometric data and were included in our CO1 and 28S phylogenetic analyses. Ten individual cercariae from 3 separate voucher colonies were observed to obtain the species' flame cell formula.

We obtained metacercaria measurements from 4 to 5 live encysted individuals on days 1, 2, 3, 5, 7, and 12 post-encystment (total n = 25 to 30). Excysted metacercaria measurements are from 4 to 5 formalin-fixed individuals from each day post-encystment (total n=25 to 30).

Adult morphometrics are based on 7 *C. kurisi* and 18 *C. michiganensis* individuals. Adult worms were stained with acetocarmine and temporarily mounted in glycerin. The following adult worms were used to collect morphometric data. For *C. kurisi*, 5 immature worms were recovered 20 days post-infection and 2 fully developed worms were recovered 34 – 40 days post-infection. For *C. michiganensis*, 6 developing worms were recovered 13 – 23 days post-infection, 13 developing worms were recovered 23 – 36 days post-infection, and 6 developed worms were recovered 23 – 36 days post-infection. The largest, most mature worms were selected as our paratype and type specimens. Adult measurements are listed as the mean of means for the different age groups, followed by the haplotype's measurements and the total range. See Table 1.4 for comparison to other described species of *Cloacitrema*.

We also had the opportunity to examine adult specimens that were collected and saved in our lab by Dr. Kate Sheehan as part of a project examining the parasites of California estuarine birds (from the same birds used in Hechinger et al. 2019).

DNA sequencing and phylogenetic analysis

We isolated DNA from a single shed-cercaria shed from a live first intermediate host infection to permit connecting the cercaria DNA sequences to parthenitae and metacercariae later examined but originating from the same colony. We washed live cercariae through 3 wells of DI water and placed individuals into 7.9 uL proteinase K lysis buffer (5x Q solution, 2.5x PCR buffer, 0.4x proteinase K). The solution was placed in a thermocycler at 60 C for 1 hour and 95 C for 15 minutes.

For CO1, PCRs were 25 uL in volume and contained: 1 – 2 uL DNA template, 0.5 uL 25 mM $MgCl_2$, 2.5 uL 2.5 mM DNTPs, 2.5 uL 10x PCR buffer, 2.5 uL of 0.5 uM of each primer (DICE 1F and DICE 11R from Steenkiste et al. 2015), 0.2 uL 500 U Taq polymerase, and 14.8 – 15.8 uL molecular grade water. A touchdown protocol was used with the following thermocycler conditions: denaturation at 95 C for 2 min; annealing for 3x of 94 C for 40 sec, 51 C for 40 sec, 72 C for 1 min; 5x of 94 C for 40 sec, 50 C for 40 sec, 72 C for 1 min; 35 x of 94 C for 40 sec, 45 C for 40 sec, 72 C for 1 min; and extension at 72 C for 5 min.

For 28S, PCRs were 25 uL in volume and contained: 1 uL DNA template, 2.5 uL 2.5 mM DNTPs, 2.5 uL 10x PCR buffer, 2.5 uL of 0.5 uM of each primer (dig12 and 1500R from Tkach et al. 2003), 0.2 uL 500 U Taq polymerase, and 16.3 uL molecular grade water. Thermocycler conditions were the same as in Tkach et al. (2016). We ran PCR products on 1x TBE agarose gels for visualization and sent all successful PCR products to ETON Biosciences in San Diego, California for Sanger Sequencing.

We replicated the above 28S methods for Dr. Sheehan's subset of unidentified *Cloacitrema* adults collected from naturally infected birds along the coast of California and Baja California from 2014 – 2017. For DNA isolation, we carefully removed a small anterior portion

of the worm (~1/10th of the body, far from diagnostic organs) and placed this into 1 uL of our proteinase K lysis buffer to permit reexamination of the worm post-sequencing.

We performed CO1 and 28S phylogenetic analyses in the following manner. After trimming the ends of each sequence by eye using MEGA X (Stecher et al. 2020), we exported the sequences into Mesquite (version 3.61, Maddison & Maddison 2019), where we retrieved additional published echinostomoid sequences from Genbank (Table 1.1). We applied a MAFFT alignment (Katoh et al. 2002) to the data and adjusted by eye when needed. To mask the resulting alignment we used the GBLOCKS (Castresana 2000). To determine the appropriate evolutionary model (GTR+I+G) to apply to the masked alignment we used jModelTest (Guindon & Gascuel 2003; Darriba et al. 2012). For our maximum likelihood (ML) analysis, we executed the model in RaxML (Stamatakis 2014) with 1000 thorough bootstrap replicates. For Bayesian Inference (BI), we ran Markov chain Monte Carlo (MCMC) with 10,000,000 generations, sample frequency of 1000, and a burnin fraction of 10% with software from MrBayes (version 3.2.6, Ronquist & Huelsenbeck 2003) and Tracer (version 1.7.1, Rambaut et al. 2018). We used the outgroup *Microparyphium facetum* based on the analysis from Tkach et al. (2016). We used FigTree (version 1.4.4) to visualize the resulting BI and ML trees.

Table 1.1: List of Genbank sequences used in our 28S phylogenetic analysis along with their reference information.

Taxa	Genbank Num	Reference
Acanthoparyphium spinulosum Johnston 1917	KT956939	Tkach et al. 2016
Caballerotrema sp.	KT956941	Tkach et al. 2016
Cloacitrema michiganensis McIntosh 1938	KT956948	Tkach et al. 2016
Cloacitrema narrabeenensis Howell & Bearup 1967	AY222248	Olson et al. 2003
Microparyphium facetum Dietz 1909	KT956933	Tkach et al. 2016
Parorchis avitus Linton 1914	KT956949	Tkach et al. 2016
Parorchis sp. CG	KF451929	Tkach et al. (unpublished)
Parorchis sp. NZ	KJ868209	O'Dwyer et al. 2014
Philopthalmus gralli Mathis & Leger 1910	JQ246435	Church et al. 2013
Typhlocoelum sp.	KT956960	Tkach et al. 2016

Statistical analysis

We used separate 2 sample t-tests to evaluate how cercariae body length, reproductive rediae body length, and soldier rediae body length varied between the 2 species. All analyses were completed using R statistical software (version 3.5.1, R Development Core Team, 2009). The "stats" package was used to generate t-tests (R core Team, 2019). The "ggplot2" package was used to plot the data (R core Team, 2019).

RESULTS

Cloacitrema kurisi reproductive rediae and cercariae were significantly larger than *C. michiganensis* reproductive rediae and cercariae (p-values both < 0.000). There was no significant difference between the body lengths of *C. kurisi* soldier rediae and *C. michiganensis*

soldier rediae (p-value = 0.585). See Table 1.2 for reproductive redia, soldier redia, and cercaria morphometrics.

As expected for ectometacercariae, there was no significant growth in inner cyst length or excysted worm length from 1 day post-encystment to 12 days post-encystment for either species. See Table 1.3 for metacercaria morphometrics.

DESCRIPTION

Diagnosis for *Cloacitrema* adults (emended from Kanev, Radev, & Fried 2005 and Yamaguti 1935, emendments bolded)

Body elongate with rounded ends and bilaterally symmetrical constrictions at level of ventral sucker. Ventral sucker oval, well developed, post-equatorial **or equatorial**. Oral sucker and pharynx well developed. Pre-pharynx **absent or** very short. Esophagus **absent or** very short. Intestinal bifurcation **immediately or** just post-pharyngeal. Ceca simple, end near posterior extremity. Testes **simple,** oval, smooth **or slightly lobed**, symmetrical between cecal ends. External seminal vesicle present. Cirrus-sac short, not reaching ventral sucker. Genital pore bifurcal or just post-pharyngeal. Ovary oval to elliptical**, on the dextral side of the body anterior to the right testis or centered in the body symmetrical between the testes**. Uterus intra-cecal **or extra-cecal**, pre-testicular **or post-testicular** with coils reaching anterior margin of ventral sucker. Metraterm well differentiated. Vitelline fields follicular, pretesticular; anterior part of field lateral to caeca. In cloaca of birds; Japan, **Russia, Australia, Philippines, the Middle East,** North and South America.

Diagnosis for *Cloacitrema* cercariae

Develop in large rediae in estuarine gastropods. Body widest just above the ventral sucker, laterally constricts just posterior to midpoint of ventral sucker. Large gland cells present throughout entire body, potentially obscuring flame cell formula. Tegumental spines, collar, and collar spines absent. Eye spots absent. Oral sucker, pharynx, and ventral sucker well developed. Pre-pharynx present; esophagus short or absent. Ceca branch far above the ventral sucker; ventral sucker equatorial or post-equatorial. Excretory bladder typically visible. Tail widest at attachment point to body and tapers off, with invaginated tip. Encyst as oval-shaped "ectometacercariae" (metacercariae that encyst outside of their hosts) on hard surfaces and develop into adults of the genus *Cloacitrema*. Definitive host birds.

***Cloacitrema kurisi* n.sp. Nelson & Hechinger** (to be formally named in subsequent peer-reviewed publication)

Synonyms: None

Figs. 1.1 – 1.2

Diagnosis

Adults with the general characteristics of the genus and the following specific combination of key traits: pre-pharynx absent; pharynx large relative to oral sucker; esophagus absent; ventral sucker equatorial, comparatively small; seminal receptacle with dextral extension; ceca end at the excretory bladder; genital pore just post-pharyngeal; ovary elliptical, located just

above the right testis; uterus extra-cecal, post-testicular with coils reaching anterior margin of ventral sucker. Cercariae with the general characteristics of the genus and the following specific combination of key traits: body size relatively large; pre-pharynx moderately long; esophagus very short, ~1/6th the length of the pharynx.

Description

Colony: Colony locus mainly in the gonadal region of the first intermediate host, where rediae are usually densely packed, with some rediae (~10 – 20) often infiltrating the digestive gland and basal visceral mass; total reproductive count 270 – 620 (355 ± 116.0, n = 11) in snails collected during fall and spring, 290 – 430 (335 ± 35.3, n= 4) in snails collected during winter; total soldier count 190 – 1150 (468 ± 291.6, n = 11) in snails collected during fall and spring, 270 – 650 (305 ± 49.5, n = 4) in snails collected during winter.

Reproductive rediae: Body elongate (average length:width = 6:1); body wall opaque; pharynx ovoid; birth pore near body's anterior end, ventrad to the pharynx; gut extends 1/3 to 1/2 the body length and contains orange-brown granules; posterior appendages 2, not pronounced; developing cercariae show no clear spatial polarization; reproductives contain 1 – 13 (7 ± 2.9, n = 50) fully developed cercariae in summer, 3 – 12 (8 ± 2.94, n = 20) in winter; average ratio of developed cercariae to germinal balls and embryos in the summer 7 : 8 (n = 50), in winter 8 : 7 (n = 20).

Soldier rediae: Body elongate (average length:width = 5:1), active; posterior appendages 2, pronounced, used with "tail" for movement; mouth leading to muscular pharynx; pharynx

large relative to body size, leading to gut; gut extends slightly over ¾ of the body length and contains orange-brown granules; germinal material absent.

Cercariae (shed from snails): Body widest just anteriad of ventral sucker, laterally constricted just posterior to midpoint of ventral sucker; tegumental spines and collar spines absent; oral sucker subterminal; cystogenous glands present throughout entire body, sometimes obscuring other structures, appear dark with transmitted light; cerebral ganglion obscures majority of pre-pharynx; pre-pharynx moderately long; pharynx ovoid; esophagus nearly non-existent; ceca branch at a length of 49.5 – 190 (105 ± 23.96, n = 50) above the ventral sucker and terminate at the excretory bladder; ventral sucker slightly post-equatorial; flame cell formula 2[(3+3+3) + (3+3+3)]=36; excretory bladder sac-like, typically longer than wide, with excretory duct entering and bifurcating in tail to exit laterally close to tail base; tail invaginated, widest at attachment point to body and tapers off, reported tail width is from the widest point.

Metacercariae: Cyst ovoid, longer than wide or tall, with thin flat layer attaching the rest of the cyst to the second intermediate "host"; cyst layers number ~ 4 and are formed over a period of ~ 20 minutes; innermost cyst 240 – 270 (255 ± 6.59, n = 24) long, 210 – 240 (220 ± 8.06, n = 24) wide; oral sucker subterminal; pre-pharynx present; pharynx ovoid; esophagus short; ventral sucker slightly post-equatorial, wider than long; excretory system stenostomate; excretory bladder sac-like, typically longer than wide; worm much more active on days 1 – 3 post-encystment than on days 5 – 12 post-encystment, very active when excysted.

Adults: Body 1381 (1825, 1000 – 1825) long, 928 (1250, 700 – 1250) wide, and 398 (420, 370 – 420) tall; oral sucker subterminal, 262 (380, 170 – 380) long, 303 (300, 280 – 340) wide, and 166 (200, 130 – 200) tall; pre-pharynx nonexistent; pharynx 220 (280, 180 – 280) long, 233 (280, 180 – 280) wide, and 165 (210, 100 – 210) tall; pharynx to oral sucker ratio 1:1.3

(1:1.2, 1:1.2 – 1:1.3); esophagus nonexistent; ceca branch at a length of 100 (180, 50 – 180) above the ventral sucker; ventral sucker equatorial, 495 (610, 400 – 610) long, 607 (720, 470 – 720) wide, and 350 (400, 300 – 400) tall; sucker ratio 1:1.9 (1:2, 1:1.8 – 1:2.3); excretory bladder not always visible, but sac-like, wider than long, 68 (40, 40 – 90) long, 218 (200, 150 – 260) wide, with weak and irregularly shaped walls; genital pore ventral and just posterior to pharynx; cirrus pouch with large seminal receptacle; vas deferens passes dorsad along the left margin of the ventral sucker; cirrus sac elongate, 75 (110, 40 – 100) long, 37 (40, 30 – 40) wide; vitellaria irregularly globoid clusters of follicles, 5 to 6 on each side for fully developed adults, number 4 to 5 on each side for younger adults; testes ovoid, smooth, slightly touching, left testis 138 (150, 120 – 150) long and 173 (220, 130 – 220) wide, right testis 152 (190, 110 – 190) long and 172 (200, 120 – 200) wide; ovary located directly above and touching the right testis, 100 (110, 80 – 130) long and 127 (170, 80 – 170) wide; uterus extra-cecal from just below the ventral sucker to the posterior end of the testes; eggs thin-shelled, yellow colored, 67 (55 – 80) long and 35 (25 – 50) wide, for 20 day-old individuals eggs number 24 – 65, for 34 to 40 day-old individuals eggs number 95 – 100+, few eggs (8 – 15) contain miracidia with eyespots in 34 to 40 day-old individuals, development shows spatial polarization with more developed eggs in anterior portion of uterus.

First intermediate host: *Cerithideopsis californica*

Location in first intermediate host (reproductive rediae): gonadal region with some infiltration of basal visceral mass

Second intermediate “hosts”: crab exoskeletons, snail opercula

Final host: *Gallus domesticus* (experimental)

Location in final host: cloaca and bursa

Final host infection success: 3 of 3 exposed

Intensity in final host: 1 – 5

Type Locality: UC Kendall-Frost Nature Reserve, San Diego, California, USA (32.8°N, 117.2°W)

Habitat: Estuaries (intertidal flats, pans, channels); type from channels

Dates of collection: April - September 2020 (type collected March 2020)

Deposited material & Genbank accession numbers: TBA

Etymology: The specific epithet is a Latinized singular noun in the genitive case that honors Dr. Armand Kuris, for his substantial contribution to the ecological understanding of the larval trematode guild to which the new species belongs.

Behavior / other biology: Parthenitae parasitically castrate host snails, as evidenced by the total replacement of active gonadal tissue of snails with developed parasite colonies. Reproductive and soldier rediae use their appendages for locomotion (as is standard and mentioned in Garcia-Vedrenne et al. 2017). Cercariae swim by ventrally folding body with tail extended to form an S-shape and are not clearly phototactic or geotactic. Cercariae form "ectometacercaria" cysts by attaching to a hard surface with ventral sucker, dropping their tail, and swaying their entire body left and right at a ~30º angle from a fulcrum point just anterior to their ventral sucker, while excreting the contents of their cystogenous glands. Metacercariae have been observed encysting on crab exoskeletons and snail opercula, and readily and quickly encyst on glass or plastic in the lab. Adults from experimental infections were typically found in the

bursa or cloaca, and not in the colon or transition zone between colon and cloaca. Adults were tightly attached to host tissue with their ventral sucker, making them difficult to remove. Once removed, a round indentation from the ventral sucker was visible on the soft cloaca tissue.

Past literature did not recognize the existence of 2 disparate *Cloacitrema* species parasitizing the California horn snail as a first intermediate host, so reports on this species are likely a combination of *C. kurisi* and *C. michiganensis*. This includes reports on the proportion of the host that the colony composes (~24% from Hechinger et al. 2009), how invasion effects snail body growth rate (causes growth ~2x faster than uninfected snails from Hechinger 2010), and details of the composition of parthenitae pigmentation (Nadakal 1960). However, these 2 species appear to be very similar in their overall use of host space and in their pigmentation. In a study on their social organization (Garcia-Vedrenne et al. 2016), it is clear that "*C. michiganensis*" colonies 1 and 2 are representatives of the true *C. michiganensis*, while "*C. michiganensis*" colonies 3, 4, and 5 are representatives of *C. kurisi*. This is apparent from the reproductive parthenita body sizes, which can be found in the supplementary material.

Geographic distribution: Although our samples are exclusively from San Diego, the species is almost certainly wide ranging, potentially at least throughout the entire range of their first intermediate host, the California horn snail, from San Francisco, California to Baja California, Mexico. However, this species was pseudo-cryptic to *C. michiganensis*, which has been reported from first intermediate host *Cerithideopsis* species from California to Panama (e.g. Torchin et al. 2015), and in birds from Michigan (McIntosh 1938), Florida (McIntosh 1938), and North Dakota (Tkach et al. 2016). The latter 3 localities were in migratory birds that were likely infected in coastal wetlands from metacercariae originating from infections in Atlantic horn snails.

Remarks

The 28S molecular results (see below) support the placement of *C. kurisi* within *Cloacitrema* and justified our emendment of the diagnosis for *Cloacitrema*.

Cloacitrema kurisi larval stages can be easily distinguished from those of *C. michiganensis* by the larger size of their reproductive rediae (1.8x length), cercariae (1.4x length), and metacercariae (1.3x length) (Figure 1.7). *Cloacitrema kurisi* colonies have fewer total numbers of rediae than *C. michiganensis*: reproductive rediae (winter averages: 335 vs. 528; fall and spring averages: 355 vs. 454), soldier rediae (winter averages: 468 vs. 694; fall and spring averages: 305 vs. 706). *Cloacitrema kurisi* cercariae have a moderately long pre-pharynx, while in *C. michiganensis* cercariae the pre-pharynx is nearly non-existent. *Cloacitrema kurisi* metacercariae have a very short esophagus (~1/6th the length of their pharynx), while *C. michiganensis* metacercariae have a longer esophagus (~1/4th the length of their pharynx). *Cloacitrema kurisi* adults differ from *C. michiganensis* in their dextral ovary position, larger pharynx relative to their oral sucker (ratio of 1:1.3 vs. 1:1.5), complete absence of an esophagus, smaller ventral sucker relative to their oral sucker (ratio of 1:1.9 vs. 1:2.7), extra-cecal and post-testicular uterus, and the dextral extension of the seminal receptacle.

Cloacitrema kurisi adults differ from the closely related *Cloacitrema narrabeenensis* (see molecular results below) in the positions of their ovary and ceca. In *C. kurisi*, the ovary is dextral and touching the right testis while in *C. narrabeenensis* the ovary is centered at a distance well above the testes. The ceca of *C. kurisi* branch more anteriorly (50 – 180 from the ventral sucker) than the ceca of *C. narrabeenensis* (30 – 40 from the ventral sucker). *C. kurisi* adults have inter-testicular space, while the testes of *C. narrabeenensis* individuals are touching. While the sucker ratios of the 2 species are essentially equivalent (1:1.9 vs. 1:2), the pharynx to oral sucker ratio of

C. narrabeenensis adults is much larger than that of *C. kurisi* adults (*C. kurisi* 1:1.3 vs. *C. narrabeenensis* 1:1).

Cloacitrema kurisi adults can be distinguished from *C. deltoida*, *C. dubaiensis*, *C. marilae*, *C. oswaldoi*, *C. ovatum*, and *C. pharyngeata* by the dextral position of the ovary. *C. kurisi* individuals differ from *C. marilae*, *C. ovatum*, and *C. philippinum* adults in the extra-cecal and post-testicular uterus. The sucker ratio of *C. kurisi* (1:1.9) is larger than the sucker ratios of *C. deltoida* (1:2.6), *C. marilae* (1:2.4), and *C. oswaldoi* (1:4). Additional comparison to other described species can be found in Table 1.4.

Cloacitrema kurisi has likely previously been included in previous research focused on trematodes using the California horn snail as first intermediate host but was mixed in with *Cloacitrema michiganensis*. *Cloacitrema kurisi* should be distinguished in future studies of the California horn snail trematode guild.

Table 1.2: Morphometrics for reproductive rediae, soldier rediae, and cercariae of *Cloacitrema kurisi* n.sp. and *Cloacitrema michiganensis*. All values represent the range followed by mean ± SD. Units are in microns for all except cercariae count. Sample size is 50 for all stages for each species.

stage	character	*Cloacitrema kurisi*	*Cloacitrema michiganensis*
reproductive redia	body L	1225 - 2525 (1850 ± 339.9)	640 - 1625 (1031.6 ± 233.1)
	body W	230 - 460 (332.4 ± 57.7)	150 - 410 (235.8 ± 64.7)
	body H	200 - 450 (294 ± 55.5)	90 - 400 (198.8 ± 78.9)
	pharynx L	40 - 80 (54.15 ± 8.3)	50 - 80 (62.6 ± 7.8)
	pharynx W	30 - 60 (43.85 ± 6.1)	30 - 60 (47.2 ± 8.6)
	pharynx H	30 - 50 (42.8 ± 5.0)	20 - 60 (42.8 ± 12.1)
	cercariae count	1 - 13 (6.88 ± 2.9)	0 - 10 (2.96 ± 1.9)
soldier redia	body L	200 - 380 (286.4 ± 44.3)	160 - 330 (262 ± 38.5)
	body W	40 - 70 (52.8 ± 6.1)	40 - 70 (52 ± 8.1)
	body H	40 - 60 (49.4 ± 5.1)	20 - 60 (46.2 ± 8.8)
	pharynx L	30 - 60 (42.6 ± 5.3)	30 - 60 (45.4 ± 6.8)
	pharynx W	30 - 40 (33.8 ± 4.9)	30 - 50 (35.2 ± 7.1)
	pharynx H	30 - 50 (33.2 ± 5.1)	10 - 50 (31.2 ± 10.0)
cercaria	body L	400 - 760 (559 ± 70.5)	280 - 520 (394.2 ± 52.8)
	body W	180 - 250 (222.2 ± 16.3)	90 - 190 (148.8 ± 16.0)
	body H	60 - 110 (80 ± 13.4)	50 - 90 (68 ± 10.5)
	tail L	250 - 580 (407.8 ± 67.9)	140 - 410 (290.2 ± 50.4)
	tail W	50 - 90 (64.4 ± 9.9)	30 - 60 (44.6 ± 6.1)
	tail H	40 - 90 (57.8 ± 8.6)	30 - 50 (42 ± 6.4)
	oral sucker L	70 - 80 (72.8 ± 4.5)	50 - 70 (59.4 ± 4.2)
	oral sucker W	60 - 80 (68.6 ± 6.1)	40 - 60 (53.6 ± 5.6)
	oral sucker H	50 - 80 (63.8 ± 6.7)	30 - 60 (49.8 ± 8.4)
	ventral sucker L	70 - 110 (95 ± 7.6)	50 - 90 (69 ± 7.4)
	ventral sucker W	100 - 120 (106.6 ± 5.6)	60 - 80 (75.2 ± 5.4)
	ventral sucker H	60 - 100 (79.2 ± 10.1)	30 - 80 (62.8 ± 11.1)
	pre-pharynx L	7.5 - 57.5 (26.45 ± 12.4)	0 - 40 (17.85 ± 10.5)
	pharynx L	37.5 - 52.5 (44.1 ± 3.7)	27.5 - 35 (31.1 ± 2.0)
	pharynx W	27.5 - 42.5 (32.25 ± 2.7)	20 - 27.5 (22.2 ± 2.3)
	pharynx H	20 - 35 (30.1 ± 3.4)	10 - 25 (20.04 ± 3.8)
	esophagus L	0 - 45 (7 ± 7.4)	0 - 25 (8.65 ± 5.9)

Table 1.3: Morphometrics for metacercariae of *Cloacitrema kurisi* n.sp. and *Cloacitrema michiganensis*. All values represent the range followed by mean ± SD and sample size. Units are in microns. Morphometrics are combined for days 1 – 12 post-encystment. Cyst morphometrics are based on the outermost cyst layer.

stage	character	*Cloacitrema kurisi*	*Cloacitrema michiganensis*
metacercaria	cyst L	260 - 290 (275 ± 6.6, n= 24)	190 - 220 (201 ± 9.2, n= 31)
	cyst W	230 - 250 (237 ± 7.0, n= 24)	140 - 170 (156 ± 6.1, n= 31)
	cyst H	190 - 210 (199 ± 6.5, n= 24)	90 - 110 (101 ± 7.0, n= 31)
	excysted body L	340 - 570 (449 ± 76.1, n= 21)	300 - 420 (330 ± 35.4, n= 23)
	excysted body W	130 - 190 (164 ± 16.3, n= 21)	80 - 140 (117 ± 13.6, n= 23)
	excysted body H	40 - 60 (53 ± 6.4, n= 21)	40 - 60 (55 ± 5.9, n= 23)
	oral sucker L	42.5 - 57.5 (51 ± 3.7, n= 24)	35 - 50 (38 ± 3.2, n= 31)
	oral sucker W	72.5 - 87.5 (79 ± 4.0, n= 24)	40 - 65 (52 ± 6.2, n= 31)
	pharynx L	42.5 - 55 (49 ± 3.3, n= 24)	22.5 - 35 (29 ± 3.1, n= 31)
	pharynx W	22.5 - 40 (31 ± 4.0, n= 24)	17.5 - 27.5 (21 ± 2.1, n= 31)
	ventral sucker L	72.5 - 100 (85 ± 6.8, n= 24)	50 - 65 (57 ± 4.7, n= 31)
	ventral sucker W	100 - 120 (110 ± 5.5, n= 24)	67.5 - 92.5 (79 ± 5.4, n= 31)

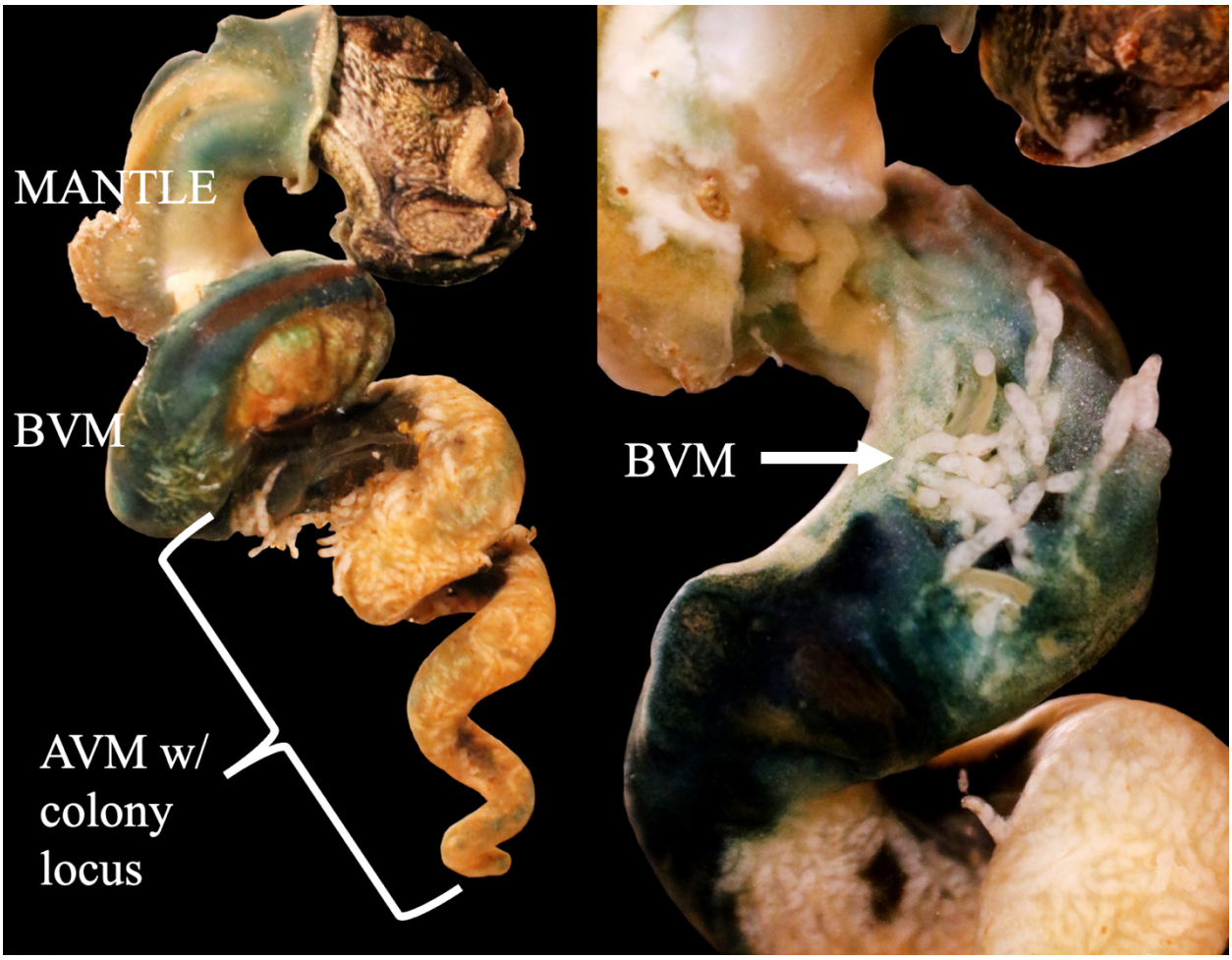

Figure 1.1: *Cloacitrema kurisi* n.sp. colony overview photos. (1) Entire California horn snail removed from its shell. (2) close-up view of *C. kurisi* rediae infiltrating host snail basal visceral mass. AVM = apical visceral mass. BVM = basal visceral mass. DG = digestive gland.

The following abbreviations are used to label morphology:

A = appendages	E = esophagus	O = ovary
BC = body cavity	EB = excretory bladder	P = pharynx
BP = birth pore	ET = excretory tubules	PP = pre-pharynx
C = ceca	G = gut lumen	SR = seminal receptacle
CeG = cerebral ganglion	Gl = glands	T = testes
CyGl = cystogenous glands	GP = genital primordium	U = uterus
CP = cirrus pouch	M = mouth	V = vitellaria

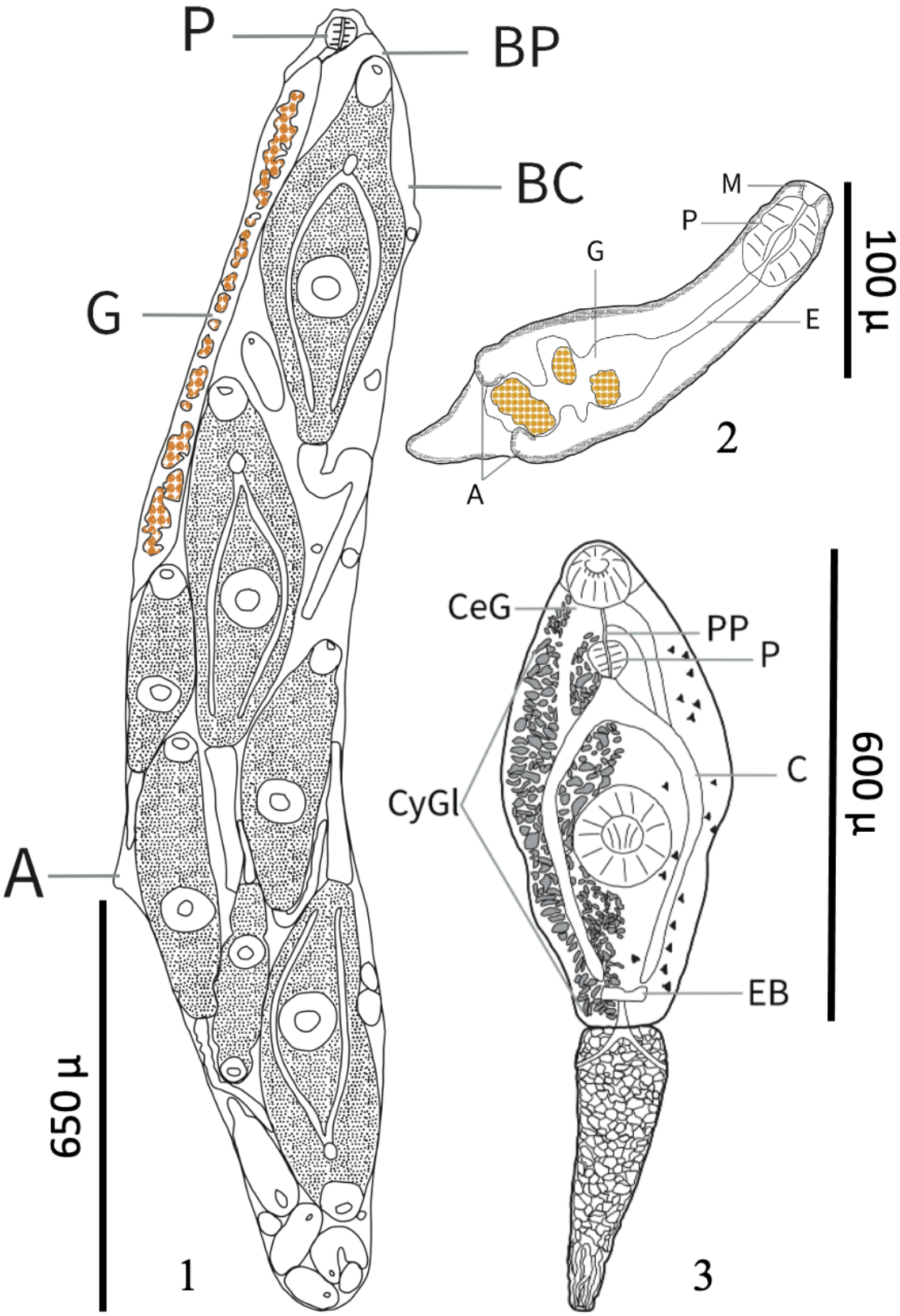

Figure 1.2A: *Cloacitrema kurisi* n.sp. reproductive redia (1), soldier redia (2), and cercaria (3). See above for morphology codes.

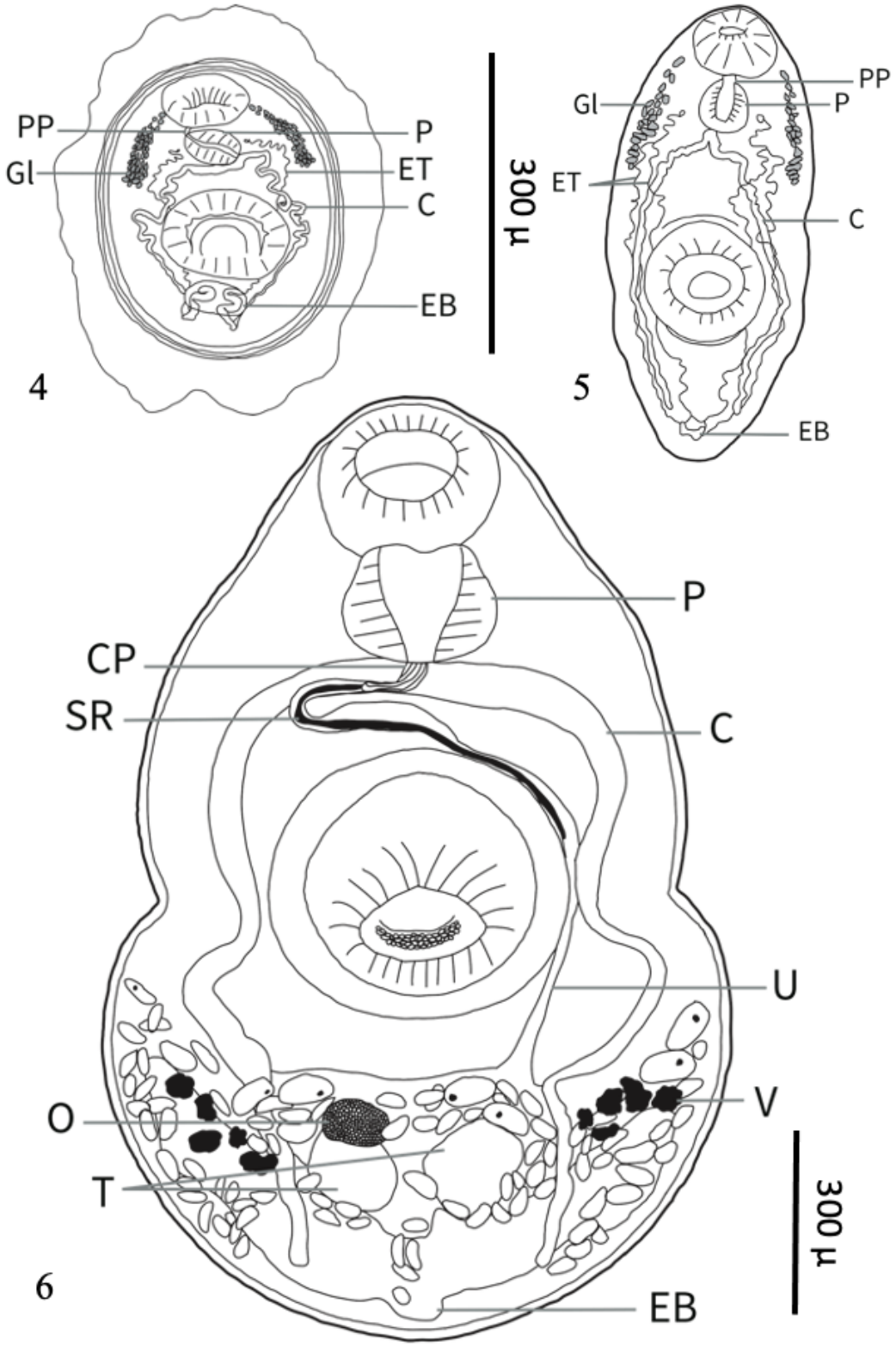

Figure 1.2B: *Cloacitrema kurisi* n.sp. encysted metacercaria (4), contracted excysted metacercaria (5), and adult (6). See above for morphology codes.

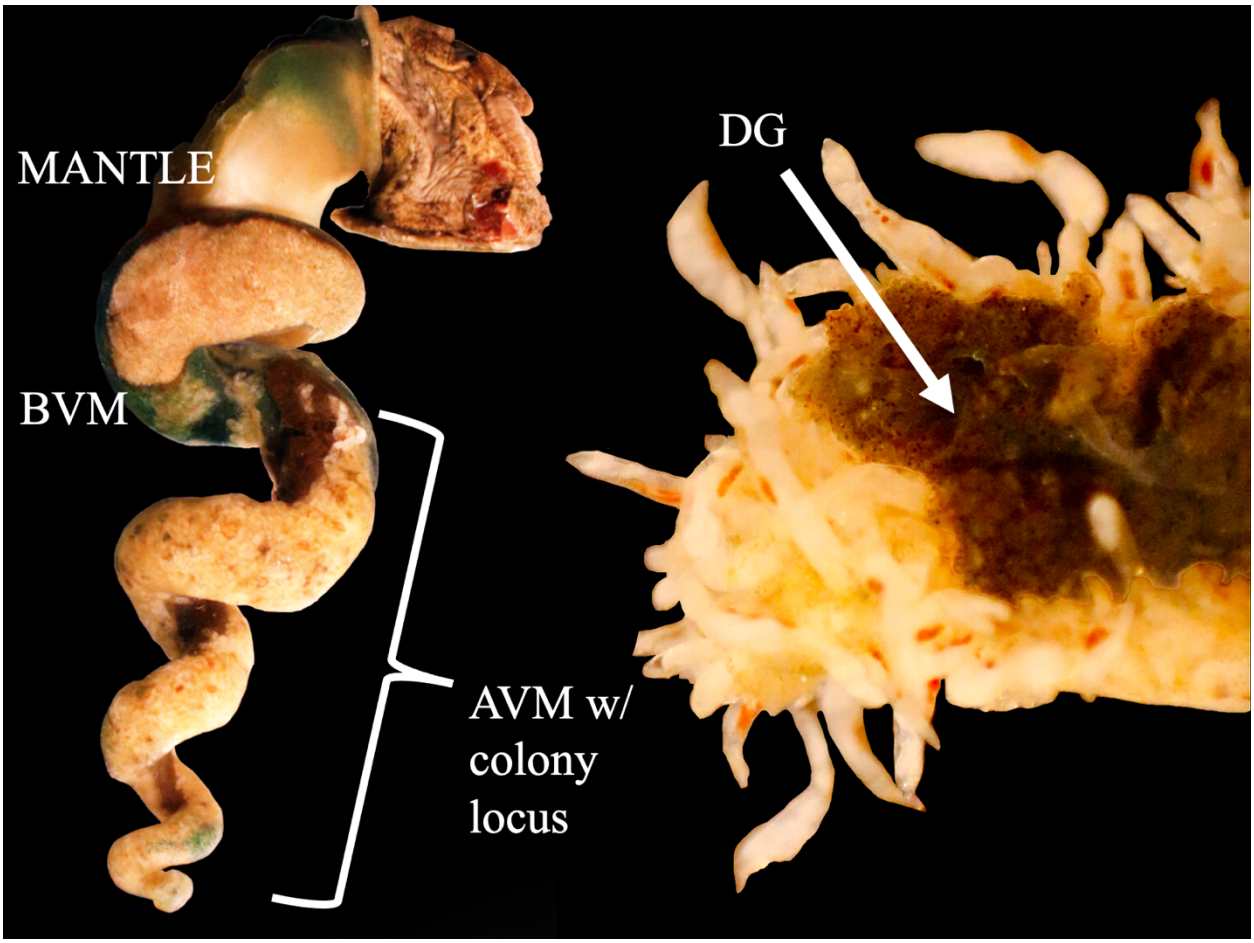

Figure 1.3: *Cloacitrema michiganensis* colony overview photos. (1) Entire California horn snail removed from its shell. (2) close-up view of *C. michiganensis* rediae in host snail tissue. AVM = apical visceral mass. BVM = basal visceral mass. DG = digestive gland.

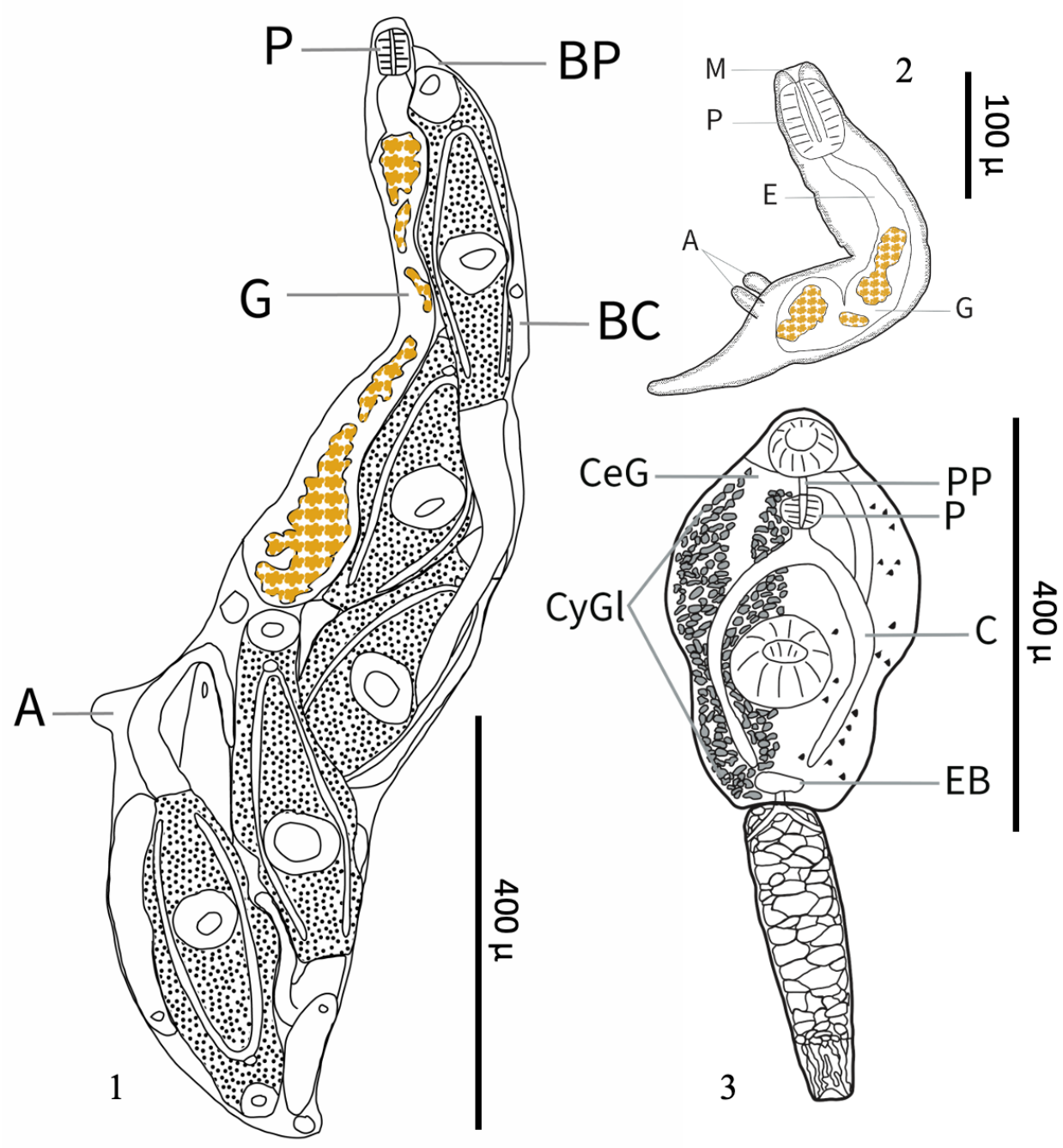

Figure 1.4A: *Cloacitrema michiganensis* reproductive redia (1), soldier redia (2), and cercaria (3). See above for morphology codes.

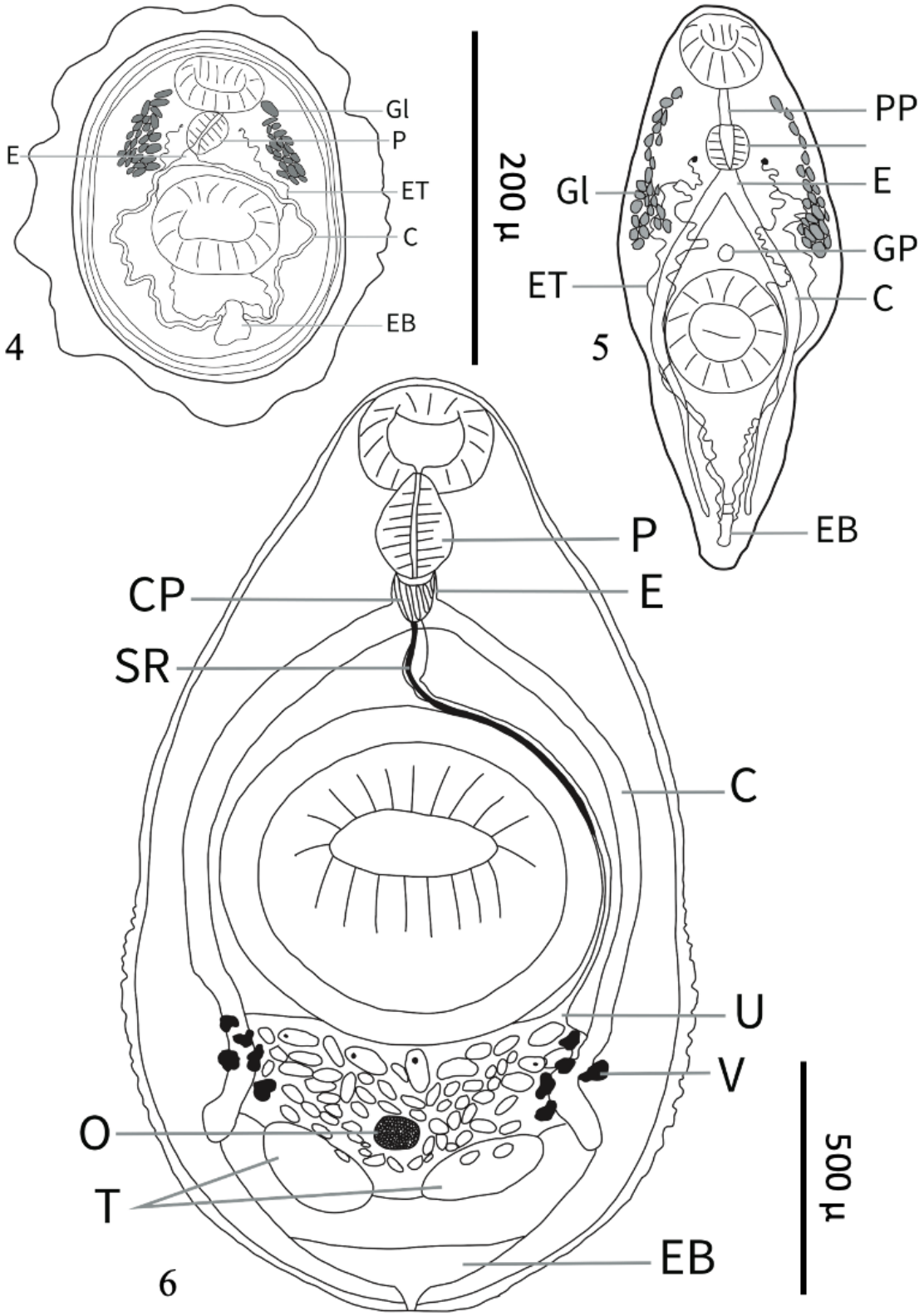

Figure 1.4B: *Cloacitrema michiganensis* encysted metacercaria (4), stretched excysted metacercaria (5), and adult (6). See above for morphology codes.

***Cloacitrema michiganensis* McIntosh 1938**

Synonyms: None

Figs. 1.3 – 1.4

Diagnosis

Adults with the general characteristics of the genus and the following specific combination of key traits: pharynx small relative to oral sucker; esophagus short; ventral sucker equatorial, relatively large; ceca end just posterior to anterior margin of excretory bladder; genital pore just post-pharyngeal; ovary elliptical, symmetrical between but not touching the testes; uterus intra-cecal, pre-testicular with coils reaching anterior margin of ventral sucker. Cercariae with the general characteristics of the genus and the following specific combination of key traits: body size relatively small; pre-pharynx nonexistent to short; esophagus short, ~1/4th the length of their pharynx.

Redescription

Colony: Colony locus mainly in the gonadal region of the first intermediate host, where reproductive rediae are usually densely packed, with some reproductive rediae (~20 – 40) often infiltrating the digestive gland and basal visceral mass; total reproductive count 230 – 830 (454 ± 187.0, n = 16) in snails collected during fall and spring, 260 – 1190 (528 ± 223.8, n = 18) in snails collected during winter; total soldier count 160 – 1140 (694 ± 357.7, n =16) in snails

collected during fall and spring, 380 – 1540 (706 ± 279.3, n =18) in snails collected during winter.

Reproductive rediae: Body elongate (average length:width = 4:1) with opaque body wall; pharynx ovoid; birth pore below the pharynx near the anterior end of the body; gut extends 1/3 to 1/2 the body length and contains orange-brown granules; posterior appendages 2, not pronounced; reproductives contain 0 – 10 (3 ± 1.9, n = 50) fully developed cercariae in summer, 0 – 16 (8 ± 3.6, n=85) in winter; developing cercariae show no clear spatial polarization; average ratio of developed cercariae to germinal balls and embryos in the summer 3 : 10 (n = 50), in winter 7 : 9 (n = 85).

Soldier rediae: Body elongate (average length:width = 5:1), active; posterior appendages 2, pronounced, use posterior appendages and "tail" for movement; mouth leading to muscular pharynx; pharynx large relative to body size, leading to gut; gut extends slightly over ¾ of the body length and contains orange-brown granules; germinal material absent.

Cercariae (shed from snails): Body widest just above the ventral sucker, constricts just posterior to midpoint of ventral sucker; tegumental spines and collar spines absent; oral sucker subterminal; cystogenous glands present throughout entire body, sometimes obscuring other structures, appear dark with transmitted light; cerebral ganglion obscures portion of pre-pharynx; pre-pharynx non-existent to short; pharynx ovoid; esophagus nearly non-existent; ceca branch at a length of 45 – 100 (71.7 ± 15.57, n = 50) above the ventral sucker and terminate at the excretory bladder; ventral sucker slightly post-equatorial; flame cell formula is 2[(3+3+3) + (3+3+3)] = 36; excretory bladder sac-like, typically longer than wide, with excretory duct entering and bifurcating in tail to exit laterally close to tail base; tail invaginated, widest at attachment point to body and tapers off, reported tail width is from the widest point.

Metacercariae: Cyst ovoid, longer than wide or tall, with a thin flat cyst layer attaching the rest of the cyst to the second intermediate "host"; cyst layers number ~ 4 and are formed over a period of ~ 20 minutes; innermost cyst 170 – 210 (189 ± 11.76, n = 31) long, 130 – 160 (147 ± 7.48, n = 31) wide; oral sucker subterminal; pre-pharynx short; pharynx ovoid; esophagus short; ventral sucker slightly post-equatorial, wider than long; excretory system stenostomate; excretory bladder sac-like, typically longer than wide; genital primordium clear at 3 days post-encystment; genital primordium small (~10 microns), only apparent in 7 – 12 day old excysted worms; worms much more active on days 1 – 3 post-encystment than on days 5 – 12 post-encystment, very active when excysted.

Adults: Body 1520 (2300, 1050 – 2300) long, 878 (1005, 640 – 1005) wide, and 417 (500, 360 – 500) tall; oral sucker subterminal, 212 (250, 160 – 260) long, 244 (260, 210 – 290) wide, and 193 (200, 170 – 210) tall; pre-pharynx nonexistent; pharynx oval 173 (200, 130 – 200) long, 144 (150, 110 – 180) wide, and 92 (100, 70 – 100) tall; pharynx to oral sucker ratio 1:1.5 (1:1.5, 1:1.1 – 1:1.8); esophagus short, 17 (80, 0 – 80) long; ceca branch at a length of 156 (200, 90 – 210) above the ventral sucker; ventral sucker equatorial, 567 (700, 410 – 700) long, 674 (830, 520 – 830) wide, and 318 (350, 280 – 350) tall; sucker ratio 1:2.7 (1:3, 1:2.2 – 1:3.6); excretory bladder not always visible, but sac-like, wider than long, 129 (200, 40 – 270) long and 302 (170, 50 – 500) wide, with weak and irregularly shaped walls; genital pore found just posterior to pharynx; seminal receptacle clear, extending from the genital pore, dorsal to the ventral sucker; vitellaria irregularly globoid clusters of follicles, 5 to 6 on each side for fully developed adults, number 4 to 6 on each side for younger adults; vas deferens passes dorsad along the left margin of the ventral sucker; cirrus sac elongate, not visible in immature adults, 77 (70, 50 – 130) long and 49 (50, 30 – 80) wide; testes smooth, not touching, ovoid; left testis 131

(170, 60 – 170) long and 173 (210, 90 – 250) wide; right testis 136 (160, 70 – 190) long and 169 (190, 90 – 220) wide; ovary centered between the 2 testes, 94 (100, 50 – 110) long and 112 (120, 65 – 150) wide; uterus intra-cecal and pre-testicular in developing adults, slightly extra-cecal and pre-testicular in developed adults; eggs thin-shelled and yellowish-brown in color, on average 64 (40 – 85) long and 31 (20 – 40) wide, number 0 – 35 for developing individuals and 26 – 64 for fully developed individuals, development shows spatial polarization, with more developed eggs in anterior portion of uterus.

First intermediate host: *Cerithideopsis californica*

Location in first intermediate host (reproductive rediae): gonadal region with some infiltration of basal visceral mass

Second intermediate "hosts": crab exoskeletons, snail opercula

Final host: *Gallus domesticus* (experimental)

Location in final host: cloaca and bursa

Final host infection success: 20 of 22 exposed

Intensity in final host: 1 - 15

Type Locality: UC Kendall-Frost Nature Reserve, San Diego, California, USA (32.8°N, 117.2°W)

Habitat: Estuaries (intertidal flats, pans, channels); type from channels

Dates of collection: April - September 2020 (type collected March 2020)

Deposited material & Genbank accession numbers: TBA

Etymology: This species was named by McIntosh (1938), who used the epithet michiganensis to refer to the collection locality of the type specimen (Michigan, USA).

Behavior / other biology: Parthenitae parasitically castrate host snails, as evidenced by the total replacement of active gonadal tissue of snail with parasite parthenitae. Reproductive and soldier rediae use their appendages for locomotion (as is standard and mentioned in Garcia-Vedrenne et al. 2017). Cercariae swim by ventrally folding body with tail extended to form an S-shape, not clearly phototactic or geotactic. Cercariae form "ectometacercaria" cysts by attaching to a hard surface with ventral sucker, dropping their tail, and swaying their entire body left and right at a ~30º angle from a fulcrum point just anterior to their ventral sucker, while excreting the contents of their cystogenous glands. Metacercariae have been observed encysting on crab exoskeletons and snail opercula, readily and quickly encyst on glass or plastic in the lab. Adults from experimental infections were typically found in the bursa or cloaca, rather than in the colon or transition zone between colon and cloaca. Adults were tightly attached to host tissue with their ventral sucker, making them difficult to remove. Once removed, a round indentation from the ventral sucker was visible on the soft cloaca tissue.

As previously mentioned, reports on this species from the California horn snail are likely a combination of *C. kurisi* and *C. michiganensis*, but the species are similar in many studied traits. With this in mind, *C. michiganensis* has been documented to occupy ~24% of its snail first intermediate host's body (Hechinger et al. 2009) and cause infected snails to grow ~2x faster than uninfected snails (Hechinger 2010). Details of the composition of parthenita pigmentation (Nadakal 1960) are also likely true for both species. In a study on their social organization (Garcia-Vedrenne et al. 2016), it is clear that "*C. michiganensis*" colonies 1 and 2 are representatives of the true *C. michiganensis* (see above).

Geographic distribution: Although our samples are exclusively from San Diego, *C. michiganensis* can likely be found throughout the entire range of their first intermediate host, the California horn snail, from San Francisco, California to Baja California, Mexico. Further, *C. michiganensis* colonies in first intermediate host snails have been identified from California to Panama (e.g. Torchin et al. 2015). *Cloacitrema michiganensis* adults have been observed in Michigan (McIntosh 1938), Florida (McIntosh 1938), and North Dakota (Tkach et al. 2016). In our 28S analysis (see below) we identified *C. michiganensis* adults in a naturally infected bird from Santa Barbara, California.

Remarks

Cloacitrema michiganensis fits well within the *Cloacitrema* adult diagnosis emended from Yamaguti 1935 (see above). The 28S molecular results (see below) support the placement of *C. michiganensis* within *Cloacitrema*. These results, along with our morphometric data, confirm that this species is the same *C. michiganensis* originally described by McIntosh (1938) from migratory birds from central and southeastern United States. We suspect that the inland birds were infected in Atlantic estuaries.

This description of *C. michiganensis* differs from that of LeFlore et al. (1985) and is more closely aligned with the original *C. michiganensis* description from McIntosh (1938). We believe that the following attributes of the LeFlore et al. (1985) description are erroneous and are a result of confusing some *C. kurisi* as *C. michiganensis*: the relatively large pharynx, the relatively small ventral sucker, the positioning of the ovary above the right testis, and the post-

testicular extension of the uterus. Our reported pharynx to oral sucker ratio, sucker ratio, and centered position of the ovary in relation to the testes agree with McIntosh (1938).

See above for morphological comparison to *C. kurisi*. Adults of *C. michiganensis* can be distinguished from *C. deltoida, C. dubaiensis, C. narrabeenensis, C. oswaldoi,* and *C. pharyngeata* by their intra-cecal and pretesticular uterus. Unlike *C. michiganensis*, the ovary of *C. deltoida, C. marilae,* and *C. ovatum* adults are touching both testes. The pharynx of *C. michiganensis* is relatively smaller (pharynx to oral sucker ratio 1:1.5) than the pharynx of all other described species of *Cloacitrema* (ratios between 1:1.3 and 1:1). The sucker ratio of *C. michiganensis* (1:2.7 – 1:3.3) is smaller than the sucker ratios of *C. dubaiensis* (1:1.8), *C. marilae* (1:2.4), *C. narrabeenensis* (1:2), *C. ovatum* (1:1.6), *C. pharyngeata* (1:1.9), and *C. philippinum* (1:1.9). Additional comparison to other described species can be found in Table 1.4.

Molecular results

The CO1 alignment was 244 base pairs in length. Although CO1 sequences were not available for other *Cloacitrema* species, *C. michiganensis* and *C. kurisi* clearly formed 2 distinct monophyletic clades in the CO1 tree (Fig 1.5). While some *C. michiganensis* colonies had single base pair mutations (maximum divergence of 1.2%), this is within normal intraspecies variation for the CO1 locus of philophthalmids (Miura et al. 2005). The minimum pairwise divergence value between *C. michiganensis* and *C. kurisi* was 14.5%. This is consistent with other reported ranges of interspecies divergence (6.0 – 21%) recorded for the CO1 locus of flatworm parasites within the same genus (Bowels & McManus 1993; Bowels & McManus 1994; Okamoto et al. 1995; Bowels et al. 1995; Morgan & Blair 1998). The BI analysis produced a tree congruent to the maximum likelihood analysis.

The 28S alignment was 1027 base pairs in length. The 28S phylogeny included our 2 study species and an additional 12 sequences from Genbank (see Table 1.1). *Cloacitrema michiganensis* and *C. kurisi* each formed distinct monophyletic clades with high bootstrap support (Fig 1.6). The minimum pairwise divergence value between *C. michiganensis* and *C. kurisi* was 1.3%. Minimum sequence divergence between our California *C. michiganensis* and midwestern *C. michiganensis* (from Tkach et al. 2016) was 0.0%. One of the worms collected from our wild birds (a marbled godwit, *Limosa fedoa* collected from the UC Carpinteria Salt Marsh Reserve in Santa Barbara, California) matched *C. michiganensis* with 0.0% divergence. *Cloacitrema michiganensis* forms a sister clade to *C. narrabeenensis* with high bootstrap support (93), which is greater than the branch support reported in the Tkach et al. (2016) analysis (84). *Cloacitrema kurisi* groups closely with the Australian *C. narrabeenensis*, with 28S minimum divergence of 0.9%. This is not uncommon for interspecies 28S data (Tkach et al. 2016). Their groupings within the 28S dataset confirm that these 2 species belong within the genus *Cloacitrema.* The BI analysis produced a tree congruent to the maximum likelihood analysis.

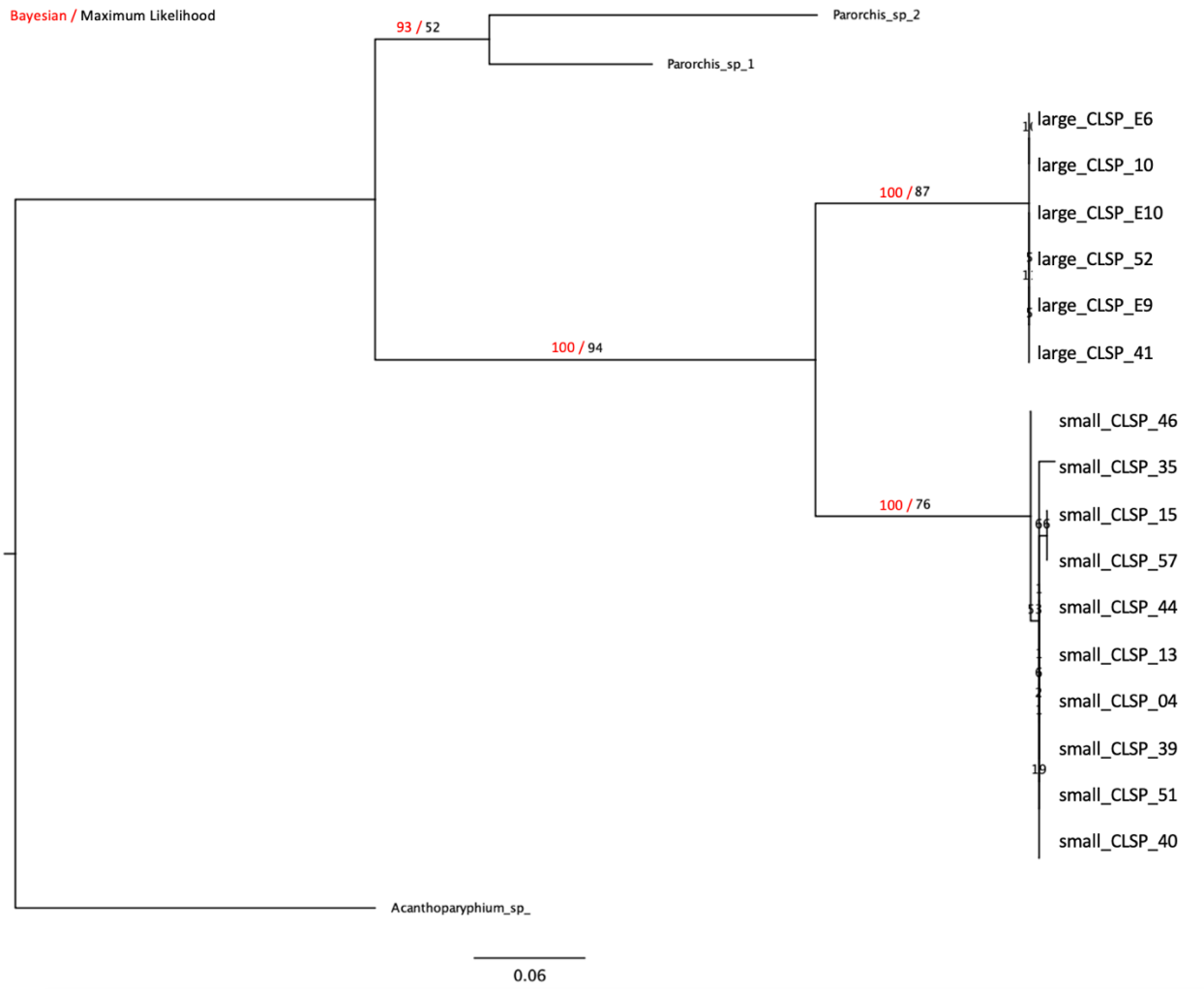

Figure 1.5: CO1 phylogenetic tree for *Cloacitrema michiganensis* (small_CLSP in the tree) and *Cloacitrema kurisi* n.sp. (large_CLSP in the tree) individual colonies. Red values represent Bayesian Inference branch lengths and black values represent Maximum Likelihood branch lengths. Numbers after species names designate unique colonies from which the sequences derive.

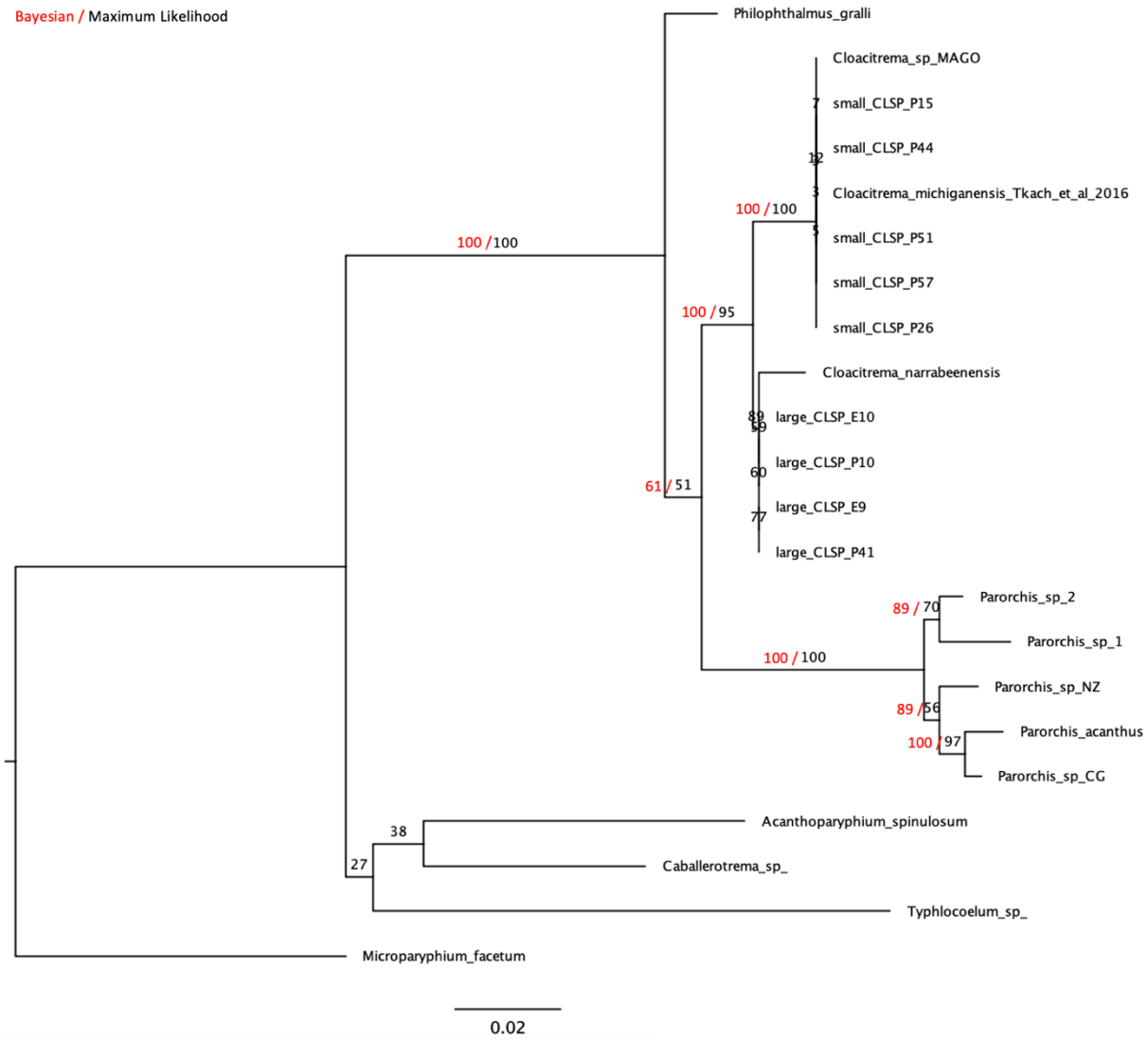

Figure 1.6: 28S phylogenetic tree for *Cloacitrema michiganensis* (small_CLSP in the tree) and *Cloacitrema kurisi* n.sp. (large_CLSP in the tree) individual colonies. Red values represent Bayesian Inference branch lengths and black values represent Maximum Likelihood branch lengths. Numbers after species names designate unique colonies from which the sequences derive.

DISCUSSION

We described the colony demographics, reproductive rediae, soldier rediae, cercariae, metacercariae, and adults of *Cloacitrema michiganensis* and *Cloacitrema kurisi*. The most obvious morphological difference between these 2 species is the disparity in size of the

reproductive redia, cercaria, and metacercaria stages. With practice, these life stages of these 2 species are readily distinguishable from one another with a compound microscope. Their morphological differences are further exaggerated in the adult stage, where they differ in pharynx to oral sucker ratio, sucker ratio, esophagus length, ovary position, uterus extension, and dextral extension of the seminal receptacle. The CO1 and 28S sequence data further confirms that these are 2 distinct species of *Cloacitrema*. It is extremely likely that previous reports on *C. michiganensis* from the California horn snail unknowingly included individuals of the cryptic species *C. kurisi*. This species can now be readily recognized in future studies on the California horn snail trematode guild.

The fact that *C. kurisi* is more closely related to the Australian *C. narrabeenensis* than it is to *C. michiganensis*, indicates that the ancestors of the 2 horn snail species diverged long ago and their decedents have colonized different host species throughout the world. CO1 data for *C. narrabeenensis* would certainly shed additional light on its relatedness with *C. kurisi*, but the 0.9% difference in the 28S sequence data and differences in their adult morphology indicates that they are different species. Additional genetic data on the genus would likely reveal interesting patterns of host use and geographic distribution.

ACKNOWLEDGMENTS

Chapter 1, in full, is currently being prepared for submission for publication of the material. Nelson, Alexandria; Metz, Daniel; Hechinger, Ryan. The thesis author was the primary investigator and author of this material.

Table 1.4: Morphometric data and associated information on all described species of *Cloacitrema*. We present both descriptions of *C. michiganensis* from McIntosh (1938) and LeFlore, Bass, & Martin (1985) next to our new species to facilitate comparison. For "life stages described" letters indicate the following life stages were included in the original description: P = parthenitae, C = cercariae, M = metacercariae, A = adults. All measurements are in microns. Reported values are the range presented by the original authors or only the type measurement where applicable. The 'pharynx to oral sucker ratio' and the 'sucker ratio' refer to the ratio of the sum of the length and width of the oral sucker to that of the pharynx or ventral sucker.

Species	*Cloacitrema michiganensis*	*Cloacitrema michiganensis*	† *Cloacitrema michiganensis*
References	Present study	McIntosh (1938)	LeFlore et al. (1985)
Type host	*Gallus domesticus*	*Actitis macularia*	*Gallus domesticus*
Other hosts	*Limosa fedoa*	*Himantopus mexicanus*	--
Locality	San Diego, California, USA	Michigan, USA Florida, USA	California, USA
Life stages described	P, C, M, A	A	P, C, M, A
Body Length	1050 – 2300	2670	1370 – 2700
Body Width	640 – 1005	1200	450 – 1100
Oral sucker L x W	160 – 260 x 210 – 290	225 x 240	140 – 330 x 220 – 450
Pharynx L x W	130 – 200 x 110 – 180	170 x 140	130 – 250 x 150 – 360
Pharynx to OS ratio	1:1.5	1:1.5	1:1.3
Esophagus L	0 – 80	130	10 – 90
Ventral sucker L x W	410 – 700 x 520 – 830	720 x 800	310 – 650 x 290 – 630
Sucker ratio	1:2.7	1:3.3	1:1.6
Num vitellaria on each side	5 – 6	5 – 6	5 – 7
Testes L x W	60 – 190 x 90 – 250	160 – 170 x 90 – 110	180 W
Ovary W	65 – 150	100	130
Eggs L x W	40 – 85 x 20 – 40	65 x 29	40 – 90 x 30 – 60

† Measurements very likely to be coming from both *Cloacitrema michiganensis* and *Cloacitrema kurisi*.

Table 1.4, Continued.

Species	*Cloacitrema kurisi* n.sp.	*Cloacitrema deltoida*	*Cloacitrema dubaiensis*
References	Present study	Mamaev (1959)	Shuster (2013)
Type host	*Gallus domesticus*	*Tringa incana*	*Phoenicopterus roseus*
Other hosts	--	--	--
Locality	San Diego, California, USA	Yakutia, Russia	Persian Gulf, Dubai
Life stages described	P, C, M, A	A	A
Body Length	1000 – 1825	2400 – 2600	3030 – 3800
Body Width	700 – 1250	1600 – 1870	1100 – 1200
Oral sucker L x W	170 – 380 x 280 – 340	270 – 290 x 350 – 360	300 – 370 x 400 – 500
Pharynx L x W	180 – 280 x 180 – 280	240 – 260 L	320 – 370 x 300 – 350
Pharynx to OS ratio	1:1.3	1:1.1*	1:1.2
Esophagus L	0	0	30 – 50
Ventral sucker L x W	400 – 610 x 470 – 720	800 x 880	650 – 800 x 650 – 800
Sucker ratio	1:1.9	1:2.6	1:1.8
Num vitellaria on each side	5 – 6	5 – 6	4 – 7
Testes L x W	110 – 190 x 120 – 220	240 x 210	200 – 300 x 150 – 250
Ovary W	80 – 170	160 – 190	140 – 220
Eggs L x W	55 – 80 x 25 – 50	73 – 90 x 36 – 40	118 – 125 x 68 – 72

* Where only one measurement is available, the ‘pharynx to oral sucker ratio’ and the ‘sucker ratio’ are calculated as the average oral sucker value to that of the average pharynx or ventral sucker value.

Table 1.4, Continued.

Species	*Cloacitrema marilae*	*Cloacitrema narrabeenensis*	*Cloacitrema oswaldoi*
References	Tzimbaliuk & Leonov (1963)	Howell & Bearup (1967)	Travassos (1940)
Type host	*Aythya marila*	*Larus novaehollandiae*	*Nyctanassa violacea*
Other hosts	--	--	--
Locality	Bering Sea, Russia	Eastern Australia	Eastern Brazil
Life stages described	A	P, C, M, A	A
Body Length	1600 – 2200	2200 – 3300	4200 – 5200
Body Width	770 – 1450	1000 – 1400	1300 – 1500
Oral sucker L x W	275 – 330 x 210 – 220	160 – 370 x 330 – 530	360 – 370 x 240 – 290
Pharynx L x W	190 – 198 x 200 – 242	330 – 400 x 270 – 500	200 – 260 W
Pharynx to OS ratio	1:1.2	1:1	1:1.2*
Esophagus L	30 – 35	5	80
Ventral sucker L x W	495 – 517 W	400 – 800 x 640 – 1000	1000 – 1100 W
Sucker ratio	1:2.4*	1:2	1:4*
Num vitellaria on each side	5 – 6	5 – 6	5 – 6
Testes L x W	--	200 – 600 x 200 – 300	400 W
Ovary W	100 – 110	110 – 150	200
Eggs L x W	--	120 x 67	82 x 36 - 41

* Where only one measurement is available, the ‘pharynx to oral sucker ratio’ and the ‘sucker ratio’ are calculated as the average oral sucker value to that of the average pharynx or ventral sucker value.

Table 1.4, Continued.

Species	*Cloacitrema ovatum*	*Cloacitrema pharyngeata*	*Cloacitrema philippinum*
References	Yamaguti (1935)	Belopol'skaja (1963)	Velasquez (1969)
Type host	*Bucephala clangula*	*Tringa glareola*	*Gallus domesticus*
Other hosts	--	--	*Anas platyrhynchos*
Locality	Japan	Bering Sea, Russia	Northern Philippines
Life stages described	A	A	P, C, M, A
Body Length	3180 – 3500	2210	1050 – 1490
Body Width	1800	928	330 – 1010
Oral sucker L x W	350 – 400 W	435 x 286	140 – 170 x 160 – 210
Pharynx L x W	270 – 290 W	380 x 365	130 – 210 x 110 – 238
Pharynx to OS ratio	1:1.3*	1:1	1:1
Esophagus L	--	134	--
Ventral sucker L x W	560 – 620 W	738 x 611	270 – 360 x 270 – 400
Sucker ratio	1:1.6*	1:1.9	1:1.9
Num vitellaria on each side	5 – 6	4 – 5	4 – 5
Testes L x W	270 – 380 x 230 – 280	55 x 26	270 – 360 x 270 – 400
Ovary W	100 – 130	--	90 – 130
Eggs L x W	32 – 33 x 17 - 18	--	40 – 55 x 20 – 25

* Where only one measurement is available, the 'pharynx to oral sucker ratio' and the 'sucker ratio' are calculated as the average oral sucker value to that of the average pharynx or ventral sucker value.

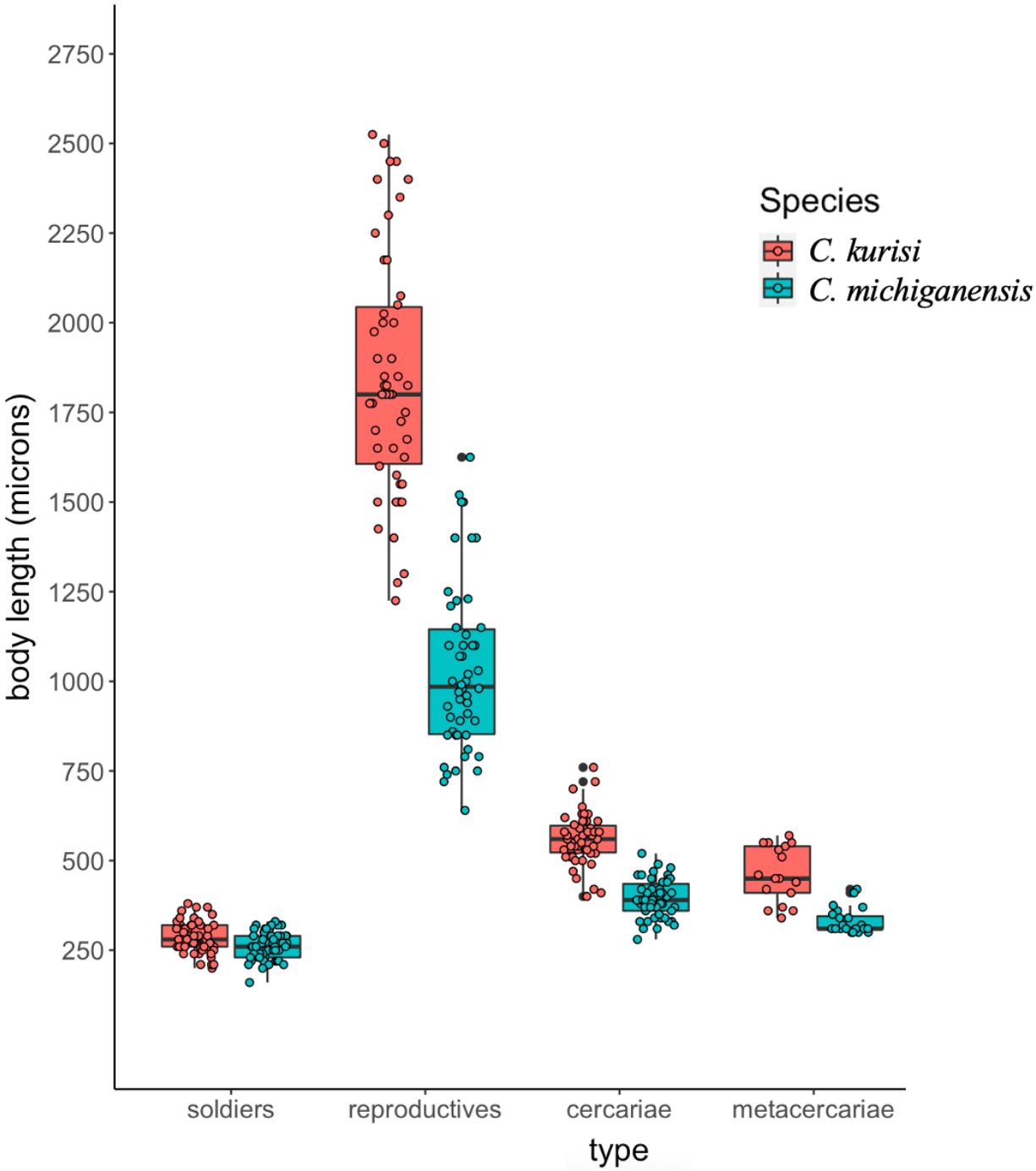

Figure 1.7: Body lengths of soldier rediae, reproductive rediae, cercariae, and excysted metacercariae for *Cloacitrema kurisi* n.sp. (red) and *Cloacitrema michiganensis* (blue).

CHAPTER 2

Description of *Parorchis catoptrophori* and *Parorchis laffertyi* n.sp. (Digenea: Trematoda: Philophthalmidae)

INTRODUCTION

Herein, we describe the rediae, cercariae, metacercariae, and adults of *Parorchis catoptrophori* Dronen & Blend 2008 which had been erroneously identified as *Parorchis acanthus* from the California horn snail, *Cerithideopsis californica* (Haldeman 1840). We also describe the rediae, cercariae, metacercariae, and an immature adult of *Parorchis laffertyi* n.sp., which was previously operationally unrecognized among the trematodes infecting as first intermediate host the California horn snail.

In 1907, Nicoll erected the genus *Parorchis* with the type species *Parorchis acanthus* (formerly *Zeugorchis acanthus*). Dronen and Blend (2008) provide a comprehensive history of the genus and separated its members into 2 distinct "*Parorchis* body types". These are: subtype 1 – smooth to slightly irregular testes, esophagus without diverticuli; subtype 2 – deeply lobed and irregular testes, esophagus with lateral diverticuli. Dronen and Blend (2008) additionally described 3 new species of *Parorchis*, while transferring 3 previously described species into a new genus, *Stenomesotrema*. Accounting for this taxonomic restructuring, there were 15 described *Parorchis* species prior to the present study. Details regarding their type-hosts, locality, and morphology can be found below.

In Martin's (1972) key to the California horn snail trematode guild, he reported (without substantiation) the presence of *Parorchis acanthus* in the eastern Pacific. Prior to the 21st century it was widely accepted that *P. acanthus* (later synonymized with *P. avitus*) was a cosmopolitan species found throughout North America, Asia, Australia, and Europe. However, a subsequent genetic analysis in an unpublished PhD thesis (Huspeni 2000) revealed the presence of at least 4 disparate species of *Parorchis* in North American snail first intermediate hosts. In his phylogenetic analysis of the CO1 locus, Huspeni (2000) found 2 distinct *Parorchis* species parasitizing *Cerithideopsis* spp. (formerly *Cerithidea* spp.) in the Pacific and the Gulf of Mexico, 1 *Parorchis* species parasitizing *Cerithideopsis scalariformis* in the Atlantic, and 1 *Parorchis* species parasitizing *Acanthina tyrianthina* and *Nucella* spp. from the Pacific and the Atlantic. Based on his morphological and geographic data, Huspeni (2000) concluded that the species from *C. scalariformis* was identical to *Cercaria carribea* V described from *C. costata* by Cable (1956), which was later determined to be *Parorchis holotestis* (Cable et al. 1960). The *Parorchis* species in *Acanthina tyrianthina* and *Nucella* spp. very likely correspond to the actual *P. avitus*, while the species infecting Pacific and Gulf of Mexico *Cerithideopsis* hosts represented 2 undescribed species (Huspeni 2000).

Huspeni (2000) was able to distinguish the 2 distinct *Parorchis* species infecting *Cerithideopsis californica* by cercariae size and referred to them as CE-SM (*Cerithidea*, now *Cerithideopsis*, small morphotype) and CE-LG (*Cerithidea* large morphotype). Additional possible morphological differences were noted in Hechinger's (2019) guide to the trematodes infecting the California horn snail. Hechinger (2019) noted that the cercaria of one morphotype appeared to have more prominent body spines and an esophagus that branches just anterior to the ventral sucker, while the other morphotype had less prominent spines with a shorter esophagus

branching approximately midway from pharynx to ventral sucker. Founded on these genetic and morphological differences, we decided to elucidate the life cycles of both "small *Parorchis*" and "large *Parorchis*" through experimental infection of final host chickens (*Gallus domesticus*) and ducks (*Anas platyrhynchos domesticus*), and additional CO1 and 28S genetic analyses. The "small *Parorchis*" was revealed to be *Parorchis catoptrophori,* originally described by Dronen & Blend 2008 from adults found in 4 naturally infected willets from Galveston, Texas. We describe the "large *Parorchis*" as a new species, *Parorchis laffertyi*.

MATERIALS AND METHODS

Life cycle work and description techniques

Following Hechinger et al. (2011), we refer to the mass of rediae in a first intermediate host as a "colony". We collected California horn snails by hand from the intertidal mudflats of the University of California Kendall-Frost Natural Reserve in San Diego, California. We first identified philophthalmid colonies and collected material for barcoding by "shedding" cercariae from living colonies (infected snails). To shed cercariae, we placed live snails individually into compartments with sea water under warm lights in the laboratory. We assigned snails infected with philophthalmid trematodes a unique number identifier and used LOCTITE brush-on glue to super-glue the number IDs onto the shell of the snails. To keep the snails alive until we could dissect them to examine parthenitae, we held them in outdoor, artificial tide, flow-through seawater mesocosms for a maximum of 6 months.

To obtain a metacercaria time series of development, we re-shed infected snails into individual glass fingerbowls labelled with their unique barcode ID. The cercariae encysted on the walls and floor of the fingerbowls within 12 hours post-shedding. We observed and chemically

excysted metacercariae on days 1, 3, 5, 7, and 12 post-encystment. It became necessary to slightly modify the chemical excystation protocol from LeFlore and Bass (1983) to obtain maximum excystment. We gently scraped the metacercariae off the glass fingerbowls and placed them in a solution comprised of: 2x DI water, 8x NaCl, 15x $NaHCO_3$, 8x ox bile, 7.5x trypsin, 0.0025x HCl, and 8x L-Cysteine. Maximum excystment occurred after incubation at 42 C for 2 hours. Chemical excystment success was typically ~75% for both *P. catoptrophori* and *P. laffertyi*. To obtain morphometrics for the excysted worms, we heat-killed 3 – 5 individuals from each day post-encystment and preserved them in 10% formalin.

To experimentally obtain sexual adult stage worms, we infected 3 day-old Cornish cross chickens, *Gallus domesticus*, and Pekin ducks, *Anas platyrhynchos domesticus*. In May 2020, we infected 2 chickens and 2 ducks per cloaca (Allison 1943) with 25 chemically excysted metacercariae from single *Parorchis* colonies. One clone of each *Parorchis* species went to both a single chick and a single duck. To increase the probability of obtaining sexually active adults, we infected an additional 3 chicks and 3 ducks with multiple clones of *P. catoptrophori.* All birds had a single exposure event. We sacrificed the birds between 33 – 39 days post-exposure. No worms were recovered from the ducks, so they were not used in the subsequent round of infections. In August 2020, we infected 5 chickens with single clones of *P. laffertyi.* To increase our parasite yield we infected summer birds with 2 separate exposure events, which took place 10 days apart. Each of these 5 birds were exposed to a total of 50 metacercariae. We infected an additional 14 chicks with multiple clones of *P. catoptrophori.* These 14 birds endured 3 separate exposure events over the course of 3 consecutive days. We sacrificed 1 chicken on day 12 post-initial exposure, and the remaining chickens between 29 – 41 days post-initial exposure.

The presence of a soldier caste within trematode colonies was first documented in Hechinger et al. (2011). Since then, soldier castes have been documented to occur within philophthalmid trematodes, specifically for members of the genera *Cloacitrema* and *Parorchis* parasitizing the California horn snail as a first intermediate host (Garcia-Vedrenne et al. 2016). We therefore provide separate descriptions for reproductive and soldier rediae for both of our species. We distinguish our redia morphs based on the following criteria: reproductive rediae are large and less active, have a pharynx that is proportionally small relative to their body size, and contain developing cercariae; soldier rediae are small and active, have a pharynx that is proportionally large relative to their body size, and do not contain any germinal material (Hechinger et al. 2011a; Garcia-Vedrenne et al. 2016).

Morphological descriptions are based on live and formalin-fixed samples. All morphometrics were taken using a compound microscope ocular micrometer. We obtained measurements from 10 individual reproductive rediae, 10 individual soldier rediae, and 10 individual cercariae from each of 5 colonies for *P. catoptrophori* (n = 50 for each life stage) and 3 colonies for *P. laffertyi* (n = 30 for each life stage). Developing embryos inside of reproductive rediae can be distinguished from developed cercariae by their translucent body, small tail lacking an invaginated tip, decreased mobility, and lack of a clear excretory system and digestive ceca. For the parthenitae and cercariae, we obtained 5 voucher specimens for *P. catoptrophori* from 5 naturally infected colonies and 3 voucher specimens for *P. laffertyi* from 3 naturally infected colonies. Colony members (assumed clone-mates) of these types were used for our morphometric data and were included in our CO1 and 28S phylogenetic analyses. Ten individual cercariae from 3 separate voucher colonies were observed to obtain the species' flame cell formula.

We obtained metacercariae measurements from 3 to 4 live encysted individuals on days 1, 3, 5, 7, and 12 post-encystment (total n= 15 to 20). Excysted metacercaria measurements are from 3 to 4 formalin fixed individuals from each day post-encystment (total n=15 to 20).

Adult morphometrics are based on 15 *P. catoptrophori* individuals. Adult worms were stained with acetocarmine and temporarily mounted in glycerin. The following adult worms were used to collect morphometric data. For *P. catoptrophori*, 3 immature worms were recovered 37 – 41 days post-infection and 12 fully developed worms were recovered 35 – 41 days post-infection. The largest, most mature worms were selected as our *P. catoptrophori* voucher specimens. For *P. laffertyi*, 1 immature worm was recovered 2 – 12 days post-infection. Adult measurements are listed as the mean of means of the developing worms and the fully developed worms, followed by the haplotype's measurements and the total range.

We also had the opportunity to examine adult specimens that were collected and saved in our lab by Dr. Kate Sheehan as part of a project examining the parasites of California estuarine birds (from the same birds used in Hechinger et al. 2019).

DNA sequencing and phylogenetic analysis

We isolated DNA from a single cercaria shed from a live first intermediate host infection to permit connecting the cercaria DNA sequences to parthenitae and metacercariae later examined but originating from the same colony. We washed live cercariae through 3 wells of DI water and placed individuals into 7.9 uL proteinase K lysis buffer (5x Q solution, 2.5x PCR buffer, 0.4x proteinase K). The solution was placed in a thermocycler at 60 C for 1 hour and 95 C for 15 minutes.

For CO1, PCRs were 25 uL in volume and contained: 1 – 2 uL DNA template, 0.5 uL 25 mM $MgCl_2$, 2.5 uL 2.5 mM DNTPs, 2.5 uL 10x PCR buffer, 2.5 uL of 0.5 uM of each primer (DICE 1F and DICE 11R from Steenkiste et al. 2015), 0.2 uL 500 U Taq polymerase, and 14.8 – 15.8 uL molecular grade water. A touchdown protocol was used with the following thermocycler conditions: denaturation at 95 C for 2 min; annealing for 3x of 94 C for 40 sec, 51 C for 40 sec, 72 C for 1 min; 5x of 94 C for 40 sec, 50 C for 40 sec, 72 C for 1 min; 35 x of 94 C for 40 sec, 45 C for 40 sec, 72 C for 1 min; and extension at 72 C for 5 min.

For 28S, PCRs were 25 uL in volume and contained: 1 uL DNA template, 2.5 uL 2.5 mM DNTPs, 2.5 uL 10x PCR buffer, 2.5 uL of 0.5 uM of each primer (dig12 and 1500R from Tkach et al. 2003), 0.2 uL 500 U Taq polymerase, and 16.3 uL molecular grade water. Thermocycler conditions were the same as in Tkach et al. (2016). We ran PCR products on 1x TBE agarose gels for visualization and sent all successful PCR products to ETON Biosciences in San Diego, California for Sanger Sequencing.

We replicated the above 28S methods for Dr. Sheehan's subset of unidentified *Parorchis* adults collected by our lab from naturally infected birds along the coast of California and Baja California from 2014 – 2017. For DNA isolation, we carefully removed a small anterior portion of the worm (~$1/10^{th}$ of the body, far from diagnostic organs) and placed this into 1 uL of our proteinase K lysis buffer to permit reexamination of the worm post-sequencing.

We performed CO1 and 28S phylogenetic analyses in the following manner. After trimming the ends of each sequence by eye using MEGA X (Stecher et al. 2020), we exported the sequences into Mesquite (version 3.61, Maddison & Maddison 2019), where we retrieved additional published echinostomoid sequences from Genbank (Table 2.1). We applied a MAFFT alignment (Katoh et al. 2002) to the data and adjusted by eye when needed. To mask the

resulting alignment we used the GBLOCKS (Castresana 2000). To determine the appropriate evolutionary model (GTR+I+G) to apply to the masked alignment we used jModelTest (Guindon & Gascuel 2003; Darriba et al. 2012). For our maximum likelihood (ML) analysis, we executed the model in RaxML (Stamatakis 2014) with 1000 thorough bootstrap replicates. For Bayesian Inference (BI), we ran Markov chain Monte Carlo (MCMC) with 10,000,000 generations, sample frequency of 1000, and a burnin fraction of 10% with software from MrBayes (version 3.2.6, Ronquist & Huelsenbeck 2003) and Tracer (version 1.7.1, Rambaut et al. 2018). We used the outgroup *Microparyphium facetum* based on the analysis from Tkach et al. (2016). We used FigTree (version 1.4.4) to visualize the resulting BI and ML trees.

Table 2.1: List of Genbank sequences used in our 28S phylogenetic analysis along with their reference information.

Taxa	Genbank Num	Reference
Acanthoparyphium spinulosum Johnston 1917	KT956939	Tkach et al. 2016
Caballerotrema sp.	KT956941	Tkach et al. 2016
Cloacitrema michiganensis McIntosh 1938	KT956948	Tkach et al. 2016
Cloacitrema narrabeenensis Howell & Bearup 1967	AY222248	Olson et al. 2003
Microparyphium facetum Dietz 1909	KT956933	Tkach et al. 2016
Parorchis avitus Linton 1914	KT956949	Tkach et al. 2016
Parorchis sp. CG	KF451929	Tkach et al. (unpublished)
Parorchis sp. NZ	KJ868209	O'Dwyer et al. 2014
Parorchis sp. TH	LC438643	Han et al. 2019
Philopthalmus gralli Mathis & Leger 1910	JQ246435	Church et al. 2013
Typhlocoelum sp.	KT956960	Tkach et al. 2016

Statistical analysis

We used separate 2 sample t-tests to evaluate how cercariae body length, reproductive rediae body length, and soldier rediae body length varied between the 2 species. All analyses

were completed using R statistical software (version 3.5.1, R Development Core Team, 2009). The "stats" package was used to generate t-tests (R core Team, 2019). The "ggplot2" package was used to plot the data (R core Team, 2019).

RESULTS

Parorchis laffertyi reproductive rediae and cercariae were significantly larger than *P. catoptrophori* reproductive rediae and cercariae (p-values 0.010 and 0.015, respectively). There was no significant difference between the body lengths of *P. laffertyi* soldier rediae and *P. catoptrophori* soldier rediae (p-value = 0.279). See Table 2.2 for reproductive rediae, soldier rediae, and cercariae morphometrics.

As expected for ectometacercariae, there was no significant growth in inner cyst length or excysted worm length from 1 day post-encystment to 12 days post-encystment for either species. See Table 2.3 for metacercariae morphometrics.

DESCRIPTION

Diagnosis for *Parorchis* adults (emended from Kanev, Radev, & Fried 2005, emendments bolded)

Body oval, elliptical to elongate, spinose, with collar-like thickening at anterior end, which **bears spines on collar-margin**, and enlarged rounded posterior end. Ventral sucker well developed, **pre-equatorial** or equatorial. Oral sucker smaller than ventral sucker, subterminal. Pre-pharynx short. Pharynx small. Esophagus long. Intestine bifurcates anteriorly to ventral sucker. Ceca simple, ending blindly near posterior extremity. Testes oval. Genital pore just post-bifurcal and anterior to ventral sucker. Ovary oval, equal in size to testes. Uterine seminal

receptacle present. Uterine coils reach posterior margin of ventral sucker, extend laterally to ceca and posteriorly to testes. Vitelline fields with many follicles lateral to ceca, pre-testicular, posterior to ventral sucker. In bursa Fabricii of birds; Asia, Australia, **Europe,** and **North America**. Type-species *P. acanthus* (Nicoll, 1906) Nicoll 1907.

Diagnosis for *Parorchi*s cercariae

Develop in large rediae in marine gastropods. Body is widest just above the ventral sucker, constricts at midpoint of ventral sucker. Large gland cells present throughout entire body, potentially obscuring flame cell formula. Tegumental spines, collar, and collar spines present. Eye spots absent. Oral sucker, pharynx, and ventral sucker well developed. Pre-pharynx present; esophagus long. Ceca branch just above the ventral sucker; ventral sucker equatorial or post-equatorial. Excretory bladder typically visible. Tail widest at attachment point to body and tapers off, with invaginated tip. Encyst as oval-shaped "ectometacercariae" (metacercariae that encyst outside of their hosts) on hard surfaces and develop into adults of the genus *Parorchis*. Definitive host birds.

***Parorchis catoptrophori* Dronen & Blend 2008**

Synonyms: None

Figs. 2.1 – 2.2

Diagnosis

Adults with the general characteristics of the genus and the following specific combination of key traits: ~80 spines arranged in a single row on the collar-margin; pharynx

large relative to oral sucker, bipartite; esophagus long, with ~10 lateral diverticuli; ventral sucker pre-equatorial, comparatively small; testes deeply lobed, inter-testicular space present; ovary centered at a distance above the testes; uterine coils terminate at mid-level of testes. Cercariae with the general characteristics of the genus and the following specific combination of key traits: body size relatively small; tegumental spines pronounced; pre-pharynx nonexistent to short.

Description

Colony: Colony locus mainly in the gonadal region of the first intermediate host, where rediae are usually densely packed, usually with some rediae (~10 – 20) infiltrating the digestive gland and basal visceral mass; total reproductive count 60 – 610 (280 ± 129.70, n = 24) in snails collected during fall and spring, 110 – 390 (242 ± 84.48, n = 7) in snails collected during winter; total soldier count 100 – 1110 (509 ± 233.68, n = 24) in snails collected during fall and spring, 340 – 770 (437 ± 80.16, n = 7) in snails collected during winter.

Reproductive rediae: Body elongate (average length:width = 3.6:1); body wall opaque; pharynx ovoid; bird pore near body's anterior end, ventrad to the pharynx; gut extends ~1/3 the body length and contains orange-brown granules; posterior appendages 2, relatively pronounced; developing cercariae show no clear spatial polarization; reproductives contain 2 – 14 (7 ± 2.5, n = 50) fully developed cercariae in summer, 0 – 11 (6 ± 2.8, n = 30) in winter; average ratio of developed cercariae to germinal balls and embryos 7: 5 (n = 50) in summer and 6 : 12 (n = 35) in winter.

Soldier rediae: Body elongate (average length:width = 4.3:1), active; posterior appendages 2, pronounced, used with "tail" for movement; mouth leading to muscular pharynx;

pharynx large relative to body size, leading to gut; gut extends slightly over ¾ of the body length and contains orange-brown granules; germinal material absent.

Cercariae (shed from snails): Body widest just above the ventral sucker, laterally constricted at or just posterior to midpoint of ventral sucker; tegumental spines pronounced; collar spines present but difficult to see even at compound scope; collar pronounced; oral sucker subterminal; cystogenous glands present throughout entire body, sometimes obscuring other structures, appear dark with transmitted light; cerebral ganglion obscures portion of pre-pharynx; pre-pharynx nonexistent to short; pharynx ovoid; esophagus long; ceca branch at a length of 10 – 50 (19.7 ± 8.65, n = 50) above the ventral sucker and terminate at the excretory bladder; ventral sucker slightly post-equatorial; flame cell formula 2[(3+3+3) + (3+3+3)] = 36; excretory bladder sac-like, typically longer than wide, with excretory duct entering and bifurcating in tail to exit laterally close to tail base; tail invaginated, widest at attachment point to body and tapers off, reported tail width is from the widest point.

Metacercariae: Cyst ovoid viewed from above, with thin flat base attached to hard surface including second intermediate hosts, longer than wide or tall; cyst layers number ~ 5 and are formed over a period of ~ 20 minutes; innermost cyst 220 – 230 (222.5 ± 4.44, n = 20) long, 170 – 200 (184.5 ± 6.86, n = 20) wide; tegumental spines pronounced; oral sucker subterminal; pre-pharynx short; pharynx ovoid; esophagus long; ventral sucker slightly post-equatorial, wider than long; excretory system stenostomate; excretory bladder sac-like, typically longer than wide; worm much more active on days 1 – 3 than on days 5 – 12.

Adults: Body pyriform, 2899 (3050, 2550 – 3375) long, 1171 (1650, 875 – 1750) wide, and 596 (525, 450 – 775) tall; tegumental spines present throughout entire body, most pronounced anterior to ventral sucker; head collar well developed, 666 (690, 580 – 750) wide;

collar spines arranged in single row, number 80 – 83, 20 (15, 15 – 30) long and 8.9 (10, 7.5 – 10) wide; oral sucker subterminal, 303 (290, 270 – 350) long, 336 (355, 280 – 410) wide, and 189 (250, 125 – 300) tall; pre-pharynx short, 9 (0, 0 – 50) long; pharynx bipartite, composed of muscular anterior primary pharynx, ovoid, 198 (180, 180 – 240) long, 142 (140, 120 – 190) wide, and 68 (75, 50 – 100) tall, followed by a smaller, posterior, non-muscular secondary pharynx extending ~70 long; pharynx to oral sucker ratio 1:1.8 (1:2, 1:1.6 – 1:2.2); esophagus with at least 10 clear lateral diverticuli, 466 (420, 330 – 600) long; ceca branch at a length of 93 (110, 60 – 130) above the ventral sucker, terminate blindly near the excretory bladder; ventral sucker pre-equatorial, 725 (730, 680 – 820) long, 732 (900, 650 – 900) wide, and 479 (500, 400 – 600) tall; sucker ratio 1:2.3 (1:2.5, 1:2.1 – 1:2.5); excretory tubules singular on each side of the body, extend from the level of the oral sucker to the excretory bladder; excretory bladder vertically elongate, 240 (310, 180 – 310) long and 50 (120, 20 – 130) wide; genital pore immediately anterior to the ventral sucker, 28 (27.5, 20 – 40) long and 34 (57.5, 20 – 57.5) wide; external seminal vesicle large, dorsad, extends posterior to the ventral sucker, leading to seminal chamber 186 (410, 100 – 410) long and 65 (80, 30 – 110) wide; cirrus sac rounded, immediately anterior to the ventral sucker and posterior to branching of the ceca, 89 (100, 70 – 130) long and 117 (135, 90 – 190) wide; vitelline follicles in fields extending from level of posterior fourth of the ventral sucker to the midline of the testes; testes deeply lobed, not touching, inter-testicular space ~ 120, left testis 212 (250, 150 – 300) long and 193 (300, 120 – 300) wide, right testis 216 (240, 170 – 330) long and 197 (320, 120 – 320) wide, post-testicular space ~450; ovary centered above but not touching the testes, ovoid, 96 (120, 60 – 150) long and 103 (140, 70 – 150) wide; uterus fills hindbody from midlevel of testes to level of ventral sucker, extra-cecal; eggs elliptical, brownish to dark red in color, on average 59 (42 – 67) long and 30 (39 – 22) wide, not

yet present in developing individuals, number 49 – 100+ in developed individuals, few eggs (1 – 10) contain miracidia with eyespots that measure ~ 90 x 45 in developed individuals, development shows spatial polarization with more developed eggs in anterior portion of uterus.

First intermediate host: *Cerithideopsis californica*

Location in first intermediate host (reproductive rediae): gonadal region with some infiltration of basal visceral mass

Second intermediate "hosts": crab exoskeletons, snail opercula

Final host: *Gallus domesticus* (experimental)

Location in final host: colon, cloaca, and bursa

Prevalence in final host: 15 / 19

Intensity in final host: 1 – 15

Locality: UC Kendall-Frost Natural Reserve, San Diego, California, USA (32.8°N, 117.2°W)

Habitat: Estuaries (intertidal flats, pans, channels); type from channels

Dates of collection: April – September 2020 (type collected March 2020)

Deposited material & Genbank accession numbers: TBA

Etymology: This species was named by Dronen & Blend (2008), who used the epithet catoptrophori in reference to the genus of the type host bird, *Catoptrophorus*.

Behavior / other biology: Parthenitae parasitically castrate host snails, as evidenced by the total replacement of active gonadal tissue of snail with parasite parthenitae. Reproductive and soldier rediae use their appendages for locomotion (as is standard and mentioned in Garcia-Vedrenne et al. 2017). Cercariae swim by ventrally folding body with tail extended to form an S-shape, not clearly phototactic or geotactic. Cercariae form "ectometacercaria" cysts by attaching

to a hard surface with their ventral sucker, dropping their tail, and swaying their entire body left and right at a ~30º angle from a fulcrum point just anterior to their ventral sucker, while excreting the contents of their cystogenous glands. Metacercariae have been observed encysting on crab exoskeletons and snail opercula, readily and quickly encyst on glass or plastic in the lab. Adults from experimental infections were typically found in the transition zone between cloaca and colon, rather than at the entrance to the cloaca or in the bursa.

Past literature did not recognize the existence of 2 disparate *Parorchis* species parasitizing the California horn snail as a first intermediate host, so reports on this species are likely a combination of *P. catoptrophori* and *P. laffertyi.* This includes reports on the proportion of the host that the colony comprises (~26% from Hechinger et al. 2009), cercarial emergence patterns (Fingerut et al. 2003), details on the structure and development of the tegument for rediae and cercariae (Bils & Martin 1966), and details of the composition of parthenitae pigmentation (Nadakal 1960). However, these 2 species appear to be extremely similar in their overall use of host space and in their pigmentation. In a study on their social organization (Garcia-Vedrenne et al. 2016), it is not immediately clear which colonies are representative of *P. catoptrophori* or *P. laffertyi,* since the sizes of their parthenitae stages have major overlap.

Geographic distribution: Although our samples are exclusively from San Diego, the species is almost certainly wide ranging, potentially at least throughout the entire range of their first intermediate host, the California horn snail, from San Francisco, California to Baja California, Mexico. Further, *P. catoptrophori* colonies in first intermediate host snails have been identified from California to Panama (e.g. Torchin et al. 2015). Using CO1 sequences, Huspeni (2000) documented this species (as *Cerithidea* small) as occurring in the Pacific coast snails *C. californica* and *C. mazatlanica* at 9 localities from northern California (Bolinas Lagoon) to

southern Baja California (Bahia de Magdalena). Huspeni (2000) also found this species in Atlantic *C. pliculosa* snails from Galveston (Texas). The description of *P. catoptrophori* came later using adult worms in willets, *Catoptrophorus semipalmatus*, in Galveston, Texas (Dronen & Blend 2008). In our 28S analysis (see below) we identified *P. catoptrophori* adults in a naturally infected bird from Santa Barbara, California.

Remarks

Parorchis catoptrophori fits well within the *Parorchis* adult diagnosis emended from Kanev, Radev, & Fried (2005; see above). *Parorchis catoptrophori* adults fall within the "Parorchis body type" subtype 2 of Dronen and Blend (2008) given their deeply lobed testes and esophagus with clear lateral diverticuli. The 28S molecular results (see below) support the placement of *P. catoptrophori* within the genus *Parorchis* and further confirms that it is a distinct species from *P. acanthus* (now *P. avitus*).

We determined that our "small *Parorchis*" was actually *P. catoptrophori* through a combination of morphological and genetic evidence. They are similar to each other and unique from other *Parorchis* species in collar spine number (~80) and arrangement (~32 on the ventral side with the remainder on the dorsal side); sucker ratio (they are identical, 1:2.3); pharynx to oral sucker ratio (the pharynx of "small *Parorchis*" and *P. catoptrophori* adults are relatively larger than any other described species); possession of a bipartite pharynx (they only share this trait with *P. chauhani*, which is different from both in a number of ways, see Table 2.4); and presence of ~10 esophageal diverticuli and deeply lobed testes, rendering them members of the *Parorchis* body type, subtype 2 of Dronen and Blend (2008). They are also similar in the dimensions of their oral sucker (270 – 350 x 280 – 410 vs. 335 – 425 x 350 – 435), pre-pharynx

(0 – 50 vs. 20 – 50), pharynx (180 – 240 x 120 – 190 vs. 270 – 230 x 110 – 150), ventral sucker (680 – 820 x 650 – 900 vs. 820 – 975 x 820 – 1005), testes (150 – 330 x 120 – 320 vs. 170 – 385 x 175 – 350), inter-testicular space (~120 vs. ~130), post-testicular space (~450 vs. ~560), cirrus sac width (90 – 190vs. 105 – 225), eggs prior to miracidial eyespot development (42 – 67 x 22 – 38 vs. 57 – 65 x 33 – 43), and eggs with fully developed miracidia (90 x 45 vs. 83 – 91 x 44 – 53). Their morphometrics only differ in overall body size (2550 – 3375 x 875 – 1750 vs. 4225 – 5700 x 1325 – 2575), esophagus length (330 – 600 vs. 755 – 835), and ovary width (70 – 150 vs. 210 – 245) as our "small *Parorchis*" is smaller than *P. catoptrophori* in all of these aspects. However, these differences are very likely due to our use of inferior final hosts (experimental chickens), which may have limited the extent of adult development. Comparison to other described species of *Parorchis* can be found in Table 2.4.

Additionally, Huspeni (2000) confirmed via cercarial size data and his phylogenetic analysis at the CO1 locus that the "small *Parorchis*" infecting *Cerithideopsis californica* as a first intermediate host is identical to the "small *Parorchis*" infecting *Cerithideopsis pliculosa* as a first intermediate host in Galveston, Texas. The original *P. catoptrophori* description is based on adults recovered from willet final hosts in the same locality: Galveston, Texas (Dronen & Blend 2008).

Parorchis catoptrophori from the California horn snail has previously been included in the published and unpublished literature as *Parorchis acanthus* (Nadakal 1960; Martin 1955; Martin 1972; LeFlore & Bass 1983; LeFlore et al. 1985; Fingerut et al. 2003; Hechinger et al. 2007; Hechinger et al. 2009; Hechinger et al. 2011b; Garcia-Vedrenne et al. 2016), *Parorchis* sp. (Hechinger 2019), *Parorchis avitus* (Hunter 1942), and "*Cerithidea* small" or "CE-SM" (Huspini

2000). The cercaria morphometrics of *P. catoptrophori* collected for this study agree with the length and width measurements of Huspeni's (2000) "CE-SM".

Table 2.2: Morphometrics for reproductive rediae, soldier rediae, and cercariae of *Parorchis catoptrophori* and *Parorchis laffertyi* n.sp. All values represent the range followed by mean ± SD. Units are in microns for all except cercariae count and germball/embryo count. Sample size is 50 for all stages of *P. catoptrophori* and 30 for all stages of *P. laffertyi*.

stage	character	*Parorchis catoptrophori*	*Parorchis laffertyi*
reproductive redia	body L	920 - 1975 (1386.8 ± 250.1)	1050 - 2150 (1542.5 ± 256.1)
	body W	230 - 530 (380.8 ± 78.3)	260 - 520 (392.0 ± 57.4)
	body H	220 - 500 (362.8 ± 72.4)	250 - 480 (378.0 ± 52.2)
	pharynx L	40 - 70 (56.3 ± 7.9)	40 - 70 (57.2 ± 6.3)
	pharynx W	40 - 60 (46.1 ± 7.1)	40 - 60 (46.2 ± 6.3)
	pharynx H	40 - 60 (45.2 ± 5.7)	40 - 60 (44.7 ± 5.7)
	cercariae count	2 - 14 (6.6 ± 2.5)	1 - 7 (2.8 ± 1.4)
	germball/embryo count	2 - 13 (5.0 ± 2.6)	1 - 9 (4.0 ± 1.7)
soldier redia	body L	190 - 350 (254.4 ± 36.3)	210 - 400 (321.0 ± 42.5)
	body W	45 - 80 (58.0 ± 7.6)	50 - 80 (64.2 ± 6.2)
	body H	40 - 70 (55.4 ± 7.3)	50 - 80 (62.0 ± 6.5)
	pharynx L	30 - 60 (41.1 ± 5.3)	35 - 60 (47.8 ± 5.0)
	pharynx W	30 - 50 (35.3 ± 5.5)	30 - 45 (39.7 ± 2.9)
	pharynx H	30 - 50 (33.8 ± 5.3)	30 - 40 (39.3 ± 2.5)

Table 2.2, Continued.

stage	character	*Parorchis catoptrophori*	*Parorchis laffertyi*
cercaria	body L	350 - 660 (472.1 ± 70.6)	560 - 840 (698.0 ± 69.7)
	body W	140 - 260 (206.8 ± 23.1)	230 - 350 (288.3 ± 32.7)
	body H	80 - 100 (94.4 ± 5.4)	90 - 120 (108.3 ± 8.3)
	tail L	200 - 520 (304.0 ± 76.8)	300 - 710 (455.7 ± 109.9)
	tail W	40 - 70 (55.5 ± 9.0)	50 - 100 (64.2 ± 12.0)
	tail H	40 - 60 (53.4 ± 6.9)	50 - 90 (61.8 ± 9.9)
	collar W	100 - 130 (114.0 ± 7.0)	110 - 155 (130.7 ± 12.2)
	oral sucker L	60 - 75 (65.8 ± 4.8)	70 - 100 (87.3 ± 7.8)
	oral sucker W	50 - 70 (62.0 ± 3.8)	60 - 95 (83.3 ± 9.4)
	oral sucker H	50 - 60 (54.2 ± 5.0)	70 - 90 (73.8 ± 5.0)
	ventral sucker L	60 - 90 (81.1 ± 7.5)	90 - 120 (106.0 ± 11.0)
	ventral sucker W	70 - 95 (80.0 ± 7.6)	100 - 130 (114.3 ± 8.6)
	ventral sucker H	60 - 70 (66.0 ± 4.9)	90 - 100 (94.2 ± 4.7)
	pre-pharynx L	0 - 25 (4.3 ± 5.7)	12.5 - 40 (23.8 ± 7.7)
	pharynx L	27.5 - 37.5 (31.7 ± 1.9)	35 - 45 (38.3 ± 2.5)
	pharynx W	20 - 30 (22.5 ± 2.2)	22.5 - 30 (27.0 ± 2.1)
	pharynx H	20 - 25 (22.2 ± 2.5)	25 - 30 (26.2 ± 2.2)
	esophagus L	50 - 137.5 (91.6 ± 20.0)	97.5 - 157.5 (117.7 ± 11.4)

Table 2.3: Morphometrics for metacercariae of *Parorchis catoptrophori* and *Parorchis laffertyi* n.sp. All values represent the range followed by mean ± SD and sample size. Units are in microns. Morphometrics are combined for days 1 – 12 post-encystment. Cyst morphometrics are based on the outermost cyst layer.

stage	character	*Parorchis catoptrophori*	*Parorchis laffertyi*
metacercaria	cyst L	230 - 250 (240.5 ± 6.9, n= 20)	350 - 380 (367.8 ± 8.1, n= 18)
	cyst W	190 - 220 (200.0 ± 7.9, n= 20)	270 - 300 (286.1 ± 7.8, n= 18)
	cyst H	100 - 120 (113.5 ± 5.9, n= 20)	190 - 210 (203.9 ± 6.1, n= 18)
	excysted body L	410 - 600 (512.5 ± 52.2, n= 20)	600 - 840 (706.5 ± 68.3, n= 17)
	excysted body W	135 - 180 (154.3 ± 13.0, n= 20)	180 - 240 (221.8 ± 17.8, n= 17)
	excysted body H	50 - 80 (69.5 ± 7.6, n= 20)	70 - 100 (92.4 ± 8.3, n= 17)
	excysted collar W	110 - 130 (118.0 ± 6.2, n= 20)	130 - 155 (140.9 ± 8.5, n= 17)
	oral sucker L	42.5 - 52.5 (46.8 ± 2.5, n= 20)	50 - 67.5 (57.6 ± 4.6, n= 18)
	oral sucker W	62.5 - 77.5 (69.9 ± 5.3, n= 20)	72.5 - 105 (91.0 ± 9.3, n= 18)
	pharynx L	25 - 40 (31.6 ± 3.4, n= 20)	37.5 - 60 (44.7 ± 5.4, n= 18)
	pharynx W	17.5 - 27.5 (22.1 ± 3.4, n= 20)	25 - 40 (30.9 ± 3.5, n= 18)
	ventral sucker L	50 - 75 (59.1 ± 6.7, n= 20)	75 - 102.5 (84.1 ± 8.2, n= 18)
	ventral sucker W	75 - 92.5 (85.4 ± 6.1, n= 20)	117.5 - 145 (133.1 ± 7.2, n= 18)

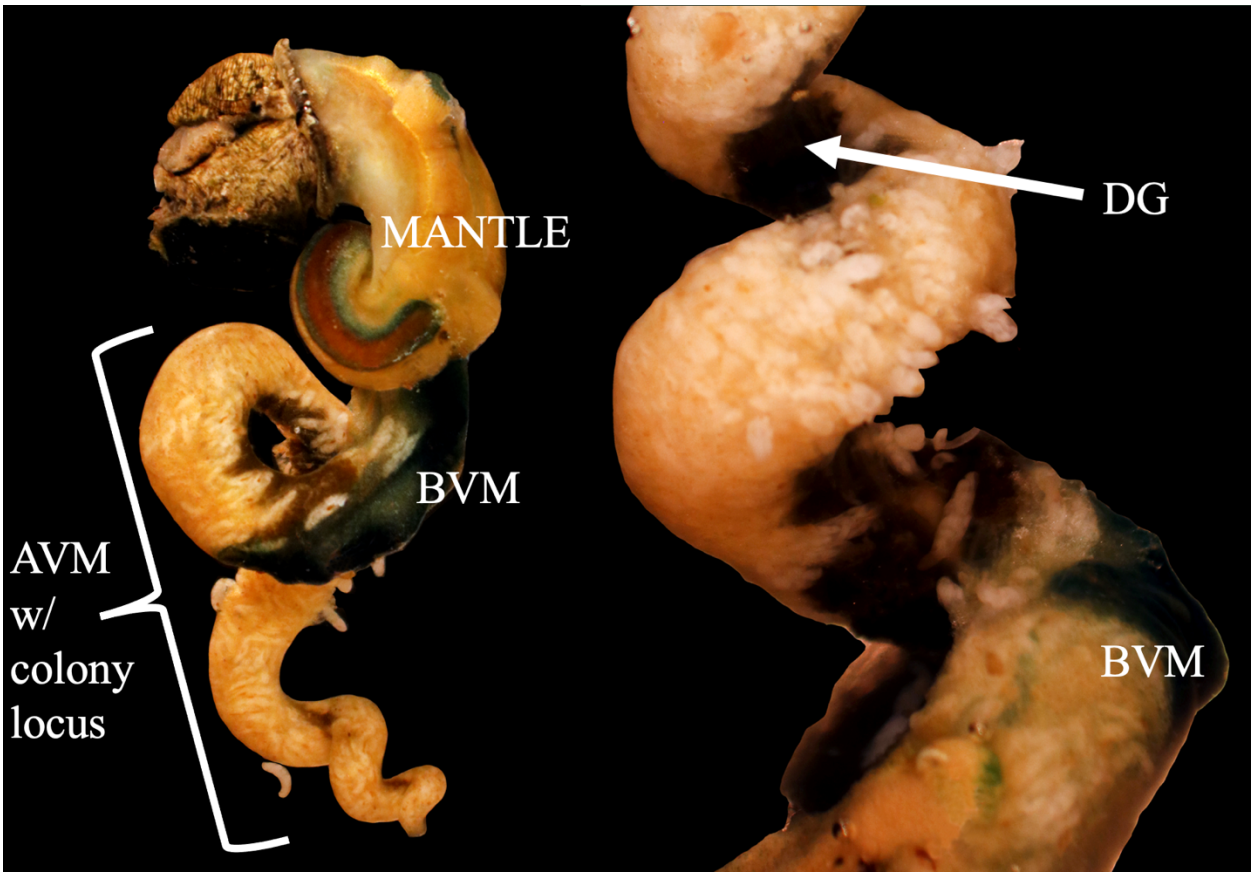

Figure 2.1: *Parorchis catoptrophori* colony overview photos. (1) Entire California horn snail removed from its shell. (2) close-up view of *P. catoptrophori* rediae in situ at the apical to basal visceral mass junction, highlighting infiltration of snail basal visceral mass. AVM = apical visceral mass. BVM = basal visceral mass. DG = digestive gland.

The following abbreviations are used to label morphology:

A = appendages	ED = excretory ducts	S = spines
BC = body cavity	ET = excretory tubules	SR = seminal receptacle
BP = birth pore	G = gut lumen	T = testes
C = ceca	GePr = genital primordium	U = uterus
CeG = cerebral ganglion	GP = genital pore	VF = vitelline fields
CP = cirrus pouch	M = mouth	
CyGl = cystogenous glands	O = ovary	
EB = excretory bladder	P = pharynx	

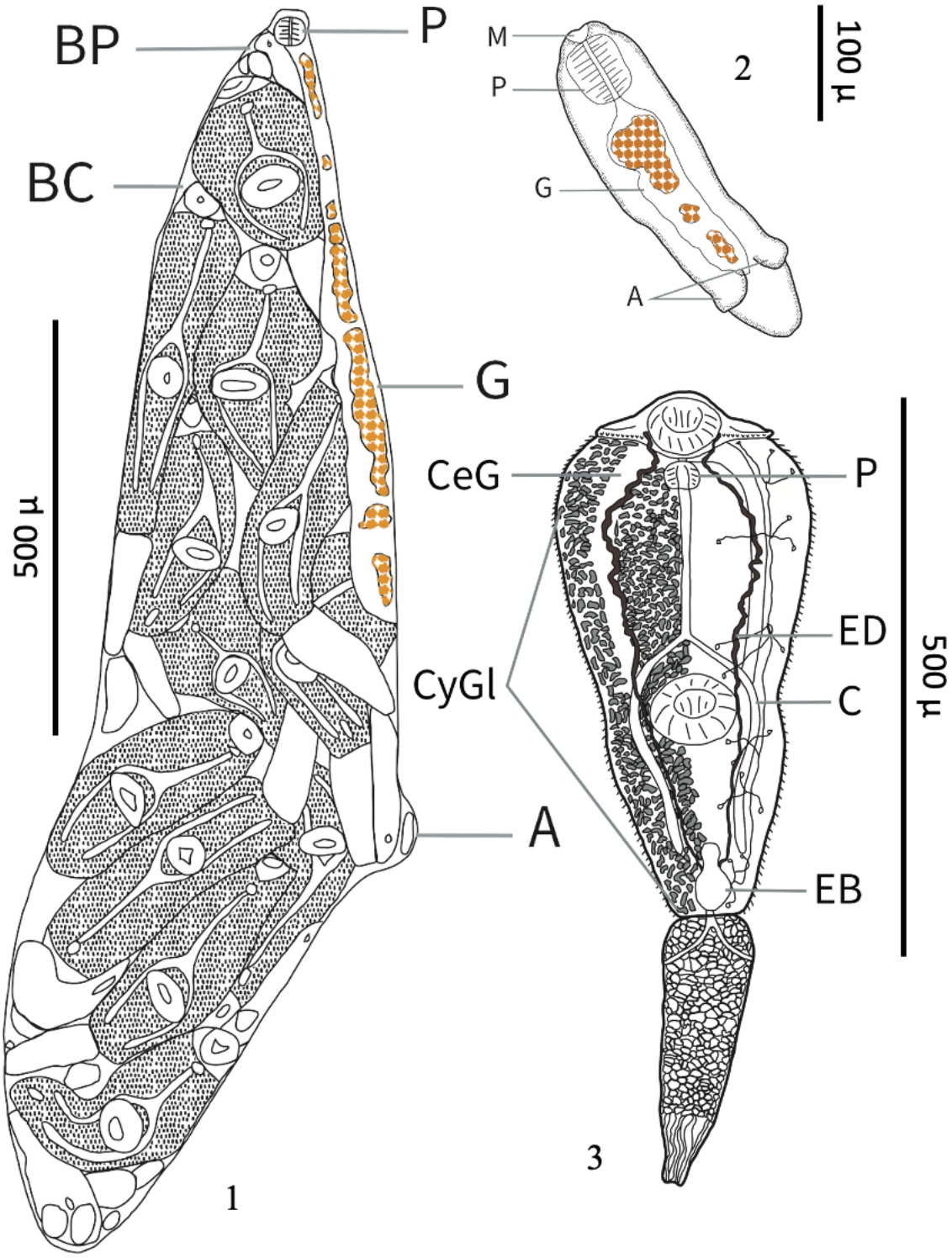

Figure 2.2A: *Parorchis catoptrophori* reproductive redia (1), soldier redia (2), and cercaria (3). See above for morphology codes.

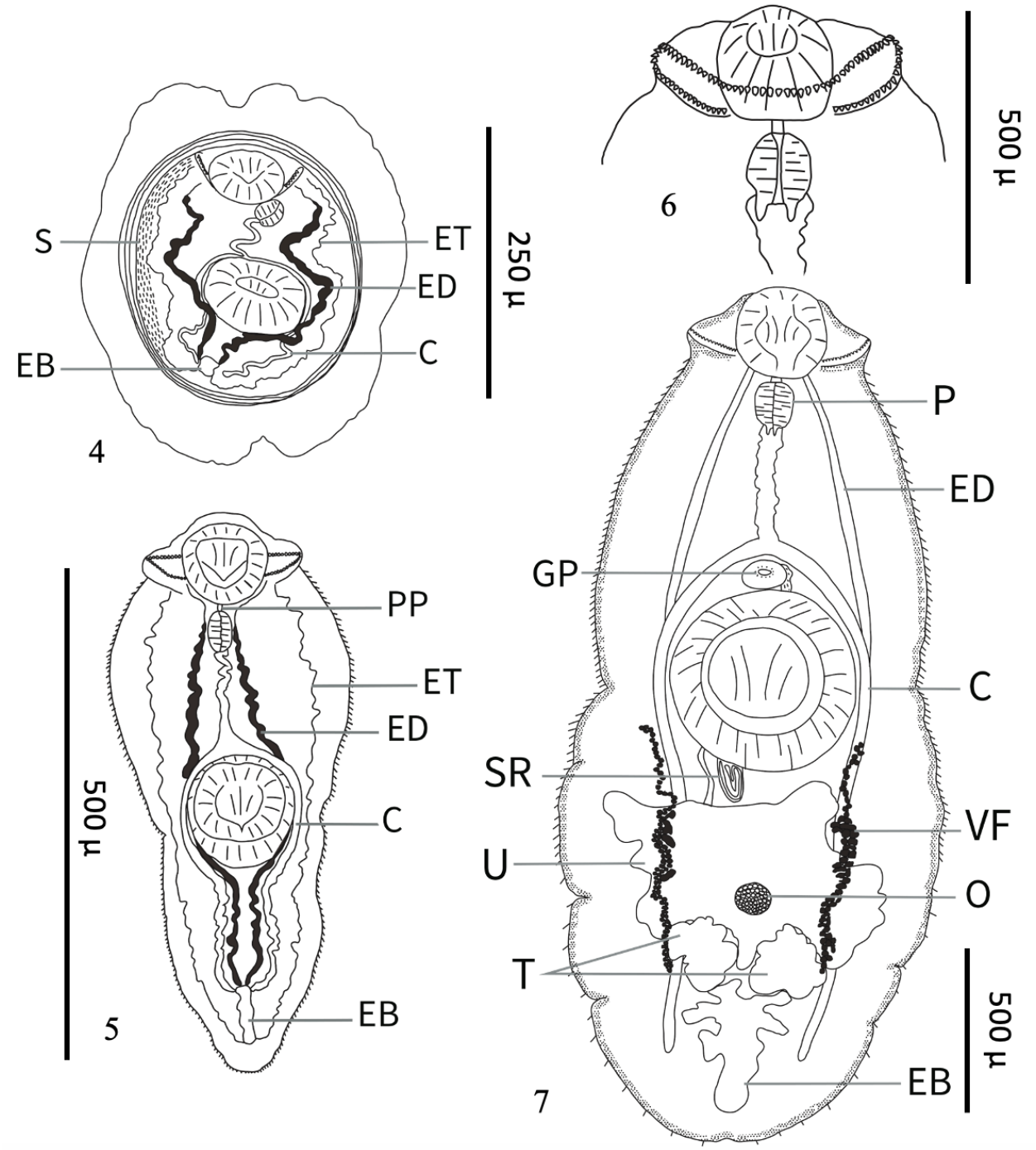

Figure 2.2B: *Parorchis catoptrophori* encysted metacercaria (4), excysted metacercaria (5), adult anterior end (6), and whole adult (7). See above for morphology codes.

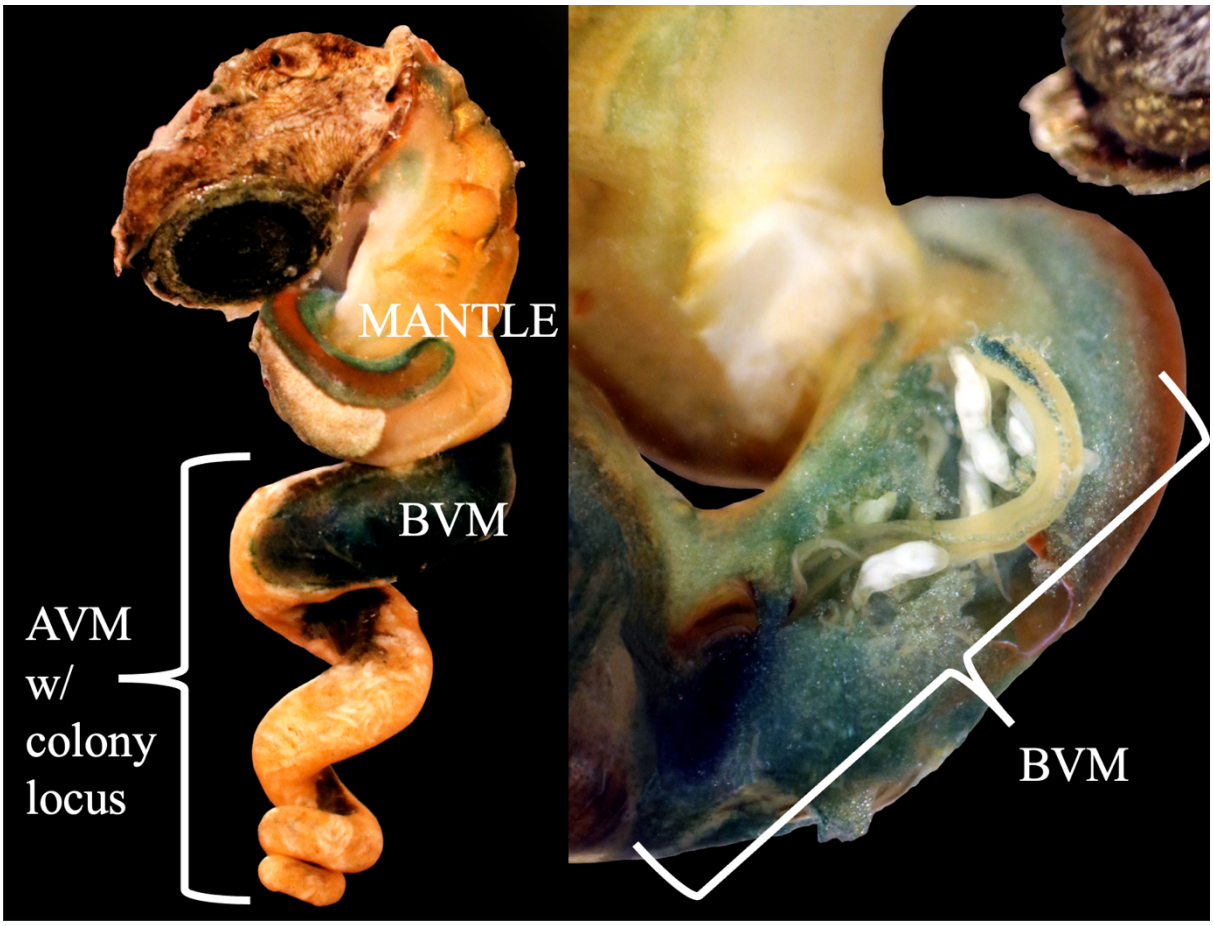

Figure 2.3: *Parorchis laffertyi* n.sp. colony overview photos. (1) Entire California horn snail removed from its shell. (2) close-up view of *P. laffertyi* rediae infiltrating host snail basal visceral mass. AVM = apical visceral mass. BVM = basal visceral mass.

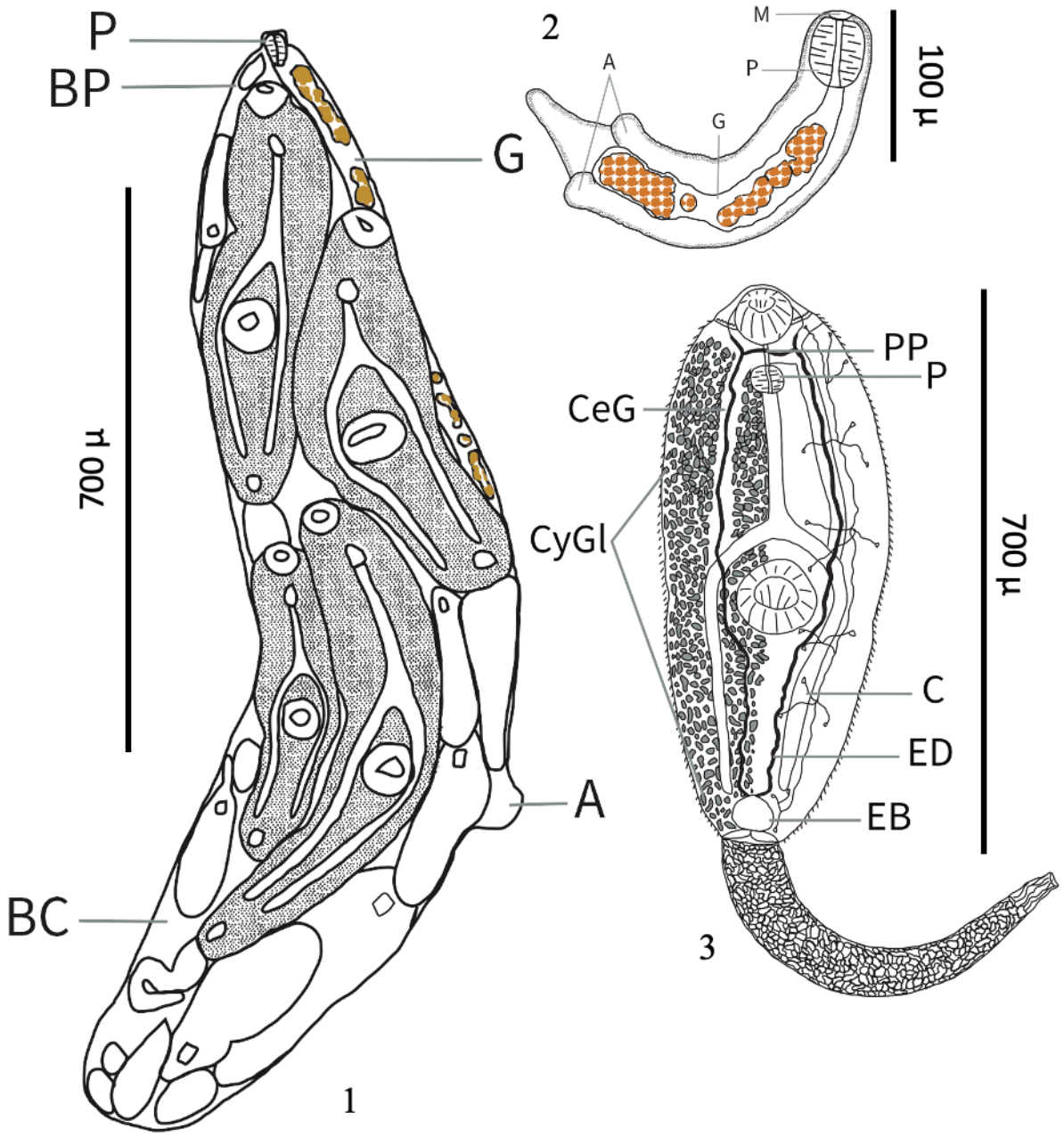

Figure 2.4A: *Parorchis laffertyi* n.sp. reproductive redia (1), soldier redia (2), and cercaria (3). See above for morphology codes.

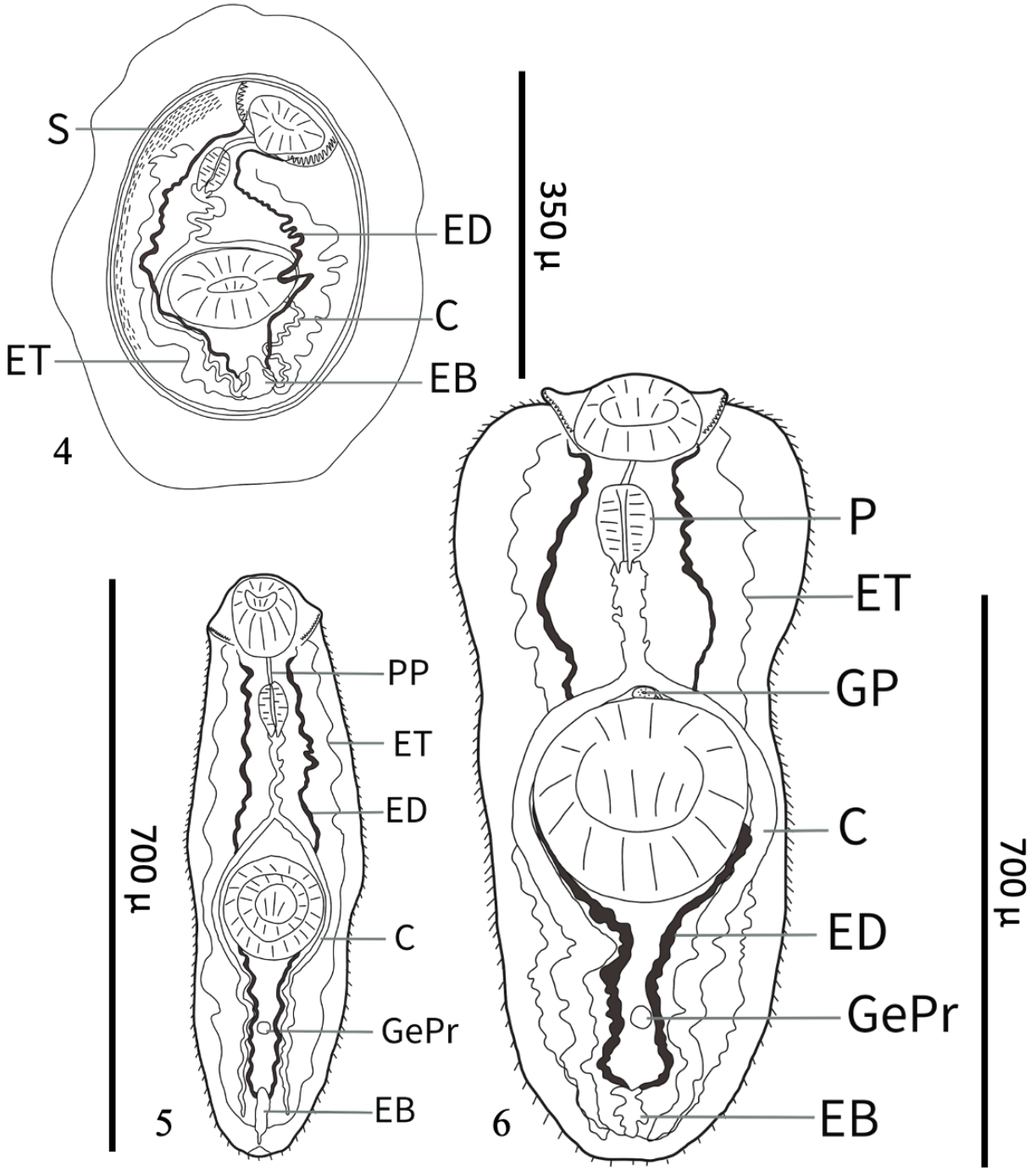

Figure 2.4B: *Parorchis laffertyi* n.sp. encysted metacercaria (4), excysted metacercaria (5), and immature adult (6). See above for morphology codes.

***Parorchis laffertyi* n.sp. Nelson & Hechinger** (to be formally named in subsequent peer-reviewed publication)

Synonyms: None

Figs. 2.3 – 2.4

Diagnosis

Adults with the general characteristics of the genus and the following specific combination of key traits: ~55 spines arranged in a single row on the collar-margin; pharynx large relative to oral sucker, bipartite; esophagus long, with ~4 lateral diverticuli; ventral sucker equatorial, comparatively small. Cercariae with the general characteristics of the genus and the following specific combination of key traits: body size relatively large; tegumental spines not pronounced; pre-pharynx nonexistent to short; ceca branch above the ventral sucker at a distance ~1/3 the length of the esophagus.

Description

Colony: Colony locus mainly in the gonadal region of the first intermediate host, where rediae are densely packed, with some rediae (~5 – 10) often infiltrating the digestive gland and basal visceral mass; total reproductive count 200 – 240 (220 ± 20, n=3) in snails collected during summer; total soldier count 220 – 320 (270 ± 50, n=3) in snails collected during summer, no winter data available.

Reproductive rediae: Body elongate (average length:width = 4:1); body wall opaque; pharynx ovoid; bird pore near body's anterior end, ventral to the pharynx; gut extends ~1/3 the body length and contains orange-brown granules; posterior appendages 2, relatively pronounced;

developing cercariae show no clear spatial polarization; reproductives contain 1 – 7 (2.8 ± 1.4, n = 30) fully developed cercariae in summer; average ratio of developed cercariae to germinal balls and embryos (3 : 4, n = 30) in summer, no winter data available.

Soldier rediae: Body elongate (average length:width = 5:1), active; posterior appendages 2, pronounced, used with "tail" for movement; mouth leading to muscular pharynx; pharynx large relative to body size, leading to gut; gut extends slightly over ¾ of the body length and contains orange-brown granules; germinal material absent.

Cercariae (shed from snails): Body widest just above the ventral sucker, constricts at midpoint of ventral sucker; tegumental spines present but not pronounced; collar spines present; collar pronounced; oral sucker subterminal; cystogenous glands present throughout entire body, sometimes obscuring other structures, appear dark with transmitted light; cerebral ganglion obscures portion of pre-pharynx; pre-pharynx nonexistent to short; pharynx ovoid; esophagus long; ceca branch at a length of 30 – 60 (45.7 ± 6.79, n = 30) above the ventral sucker and terminate at the excretory bladder; ventral sucker slightly post-equatorial; flame cell formula 2[(3+3+3) + (3+3+3)] = 36; excretory bladder sac-like, typically longer than wide, with excretory duct entering and bifurcating in tail to exit laterally close to tail base; tail invaginated, widest at attachment point to body and tapers off, reported tail width is from the widest point.

Metacercariae: Cyst ovoid viewed from above, with thin flat base attached to hard surface including second intermediate hosts, longer than wide or tall; cyst layers number ~ 3 and are formed over a period of ~ 20 minutes; innermost cyst 330 – 350 (345.56 ± 7.05, n = 18) long, 260 – 290 (267.8 ± 8.08, n = 18) wide; outermost cyst extremely thick, obscures anatomy unless removed; tegumental spines not pronounced unless outer cyst is removed; collar spines not visible unless outer cyst is removed, clearly larger on day 12 than on day 1; oral sucker

subterminal; pre-pharynx present; pharynx ovoid; esophagus long; ventral sucker slightly post-equatorial, wider than long; excretory system stenostomate; excretory bladder sac-like, typically longer than wide; genital primordium small (~10 microns), present in worms 7 – 12 days post-encystment; worms much more active on days 1 – 3 than on days 5 – 12, moderately active when excysted.

Immature adult: Body elongate, 1100 long, 370 wide, and 130 tall; tegumental spines present on entire surface of body, more pronounced anterior to the ventral sucker; head collar 180 wide; collar spines pronounced, in a single row, number ~55, 7.5 long and 2.5 wide; oral sucker subterminal, 105 long, 117.5 wide, and 70 tall; pre-pharynx 20 long; pharynx bipartite, 70 long, 55 wide, and 40 tall; pharynx to oral sucker ratio 1:1.8; esophagus with at least 4 clear lateral diverticuli, 200 long; ceca branch at a length of 60 above the ventral sucker, terminate on either side of the excretory bladder; ventral sucker equatorial, 210 long, 210 wide, and 170 tall; sucker ratio 1:1.9; genital primordium present, centered posterior to the ventral sucker, 50 long and 32.5 wide; excretory tubules in 2 pairs on each side of body, originating at level of the pre-pharynx, terminating at the excretory bladder; excretory bladder vertically elongate, 47.5 long and 12.5 wide; cirrus developing, spherical, located just anterior to the ventral sucker and just posterior to the branching of the ceca; testes not yet present; eggs not yet present.

First intermediate host: *Cerithideopsis californica*

Location in first intermediate host: gonadal region with some infiltration of basal visceral mass

Second intermediate “hosts”: crab exoskeletons, snail opercula

Final host: *Gallus domesticus* (experimental)

Location in final host: cloaca

Prevalence in final host: 1 / 7

Intensity in final host: 1

Type Locality: UC Kendall-Frost Natural Reserve, San Diego, California, USA (32.8°N, 117.2°W)

Habitat: Estuaries (intertidal flats, pans, channels); type from channels

Dates of collection: April 2020 – April 2021 (type collected March 2020)

Deposited material & Genbank accession numbers: TBA

Etymology: The specific epithet is a Latinized singular noun in the genitive case that honors Dr. Kevin Lafferty, for his substantial contribution to the ecological understanding of the larval trematode guild to which the new species belongs.

Behavior/other biology: In Huspeni's (2000) account of this species (as CE-LG), *P. laffertyi* was found with much lower prevalence than other *Parorchis* species. *Parorchis laffertyi* composed 12% - 40% of all *Parorchis* infections in the Pacific, and only 2 infected snails were found in Texas.

Parthenitae parasitically castrate host snails, as evidenced by the total replacement of active gonadal tissue of snail with parasite parthenitae. Reproductive and soldier rediae use their appendages for locomotion (as is standard and mentioned in Garcia-Vedrenne et al. 2017). Cercariae swim by ventrally folding body with tail extended to form an S-shape, not clearly phototactic or geotactic. Cercariae form "ectometacercaria" cysts by attaching to a hard surface with their ventral sucker, dropping their tail, and swaying their entire body left and right at a ~30º angle from a fulcrum point just anterior to their ventral sucker, while excreting the contents of their cystogenous glands. Metacercariae have been observed encysting on crab exoskeletons and snail opercula, readily and quickly encyst on glass or plastic in the lab.

Geographic distribution: Although our samples are exclusively from San Diego, the species is almost certainly wide ranging, potentially at least throughout the entire range of their first intermediate host, the California horn snail, from San Francisco, California to Baja California, Mexico. Further, *P. catoptrophori* colonies in first intermediate host snails have been identified from California to Panama, which likely included misidentifications of *P. laffertyi* (e.g. Torchin et al. 2015). Using CO1 sequences, Huspeni (2000) documented this species (as *Cerithidea* large) as occurring in the Pacific coast snails *C. californica* and *C. mazatlanica* from 5 localities from southern California (Carpinteria Salt Marsh) to southern Baja California (Laguna San Ignacio). Huspeni (2000) also found this species in Atlantic *C. pliculosa* snails from Galveston (Texas), although the cercariae from Texas showed moderate divergence at the CO1 locus.

Remarks

Although we were not able to observe the reproductive organs of *P. laffertyi* (eggs, ovary, uterus, testes, and seminal receptacle), our 28S molecular results (see below) and morphological data support its placement within the genus *Parorchis*. Our CO1 and 28S molecular results (see below) confirm that it is a distinct species from *P. catoptrophori*.

Although they qualitatively take up approximately the same amount of host space, *P. laffertyi* colonies have fewer total numbers of rediae than *P. catoptrophori*: reproductive rediae (average 220 vs. 280 in summer) and soldier rediae (average 270 vs. 509 in summer). *Parorchis laffertyi* can be easily distinguished from *P. catoptrophori* by its larger size in the reproductive rediae, soldier rediae, cercaria and metacercaria stages. The reproductive rediae, soldier rediae, cercariae and metacercariae of *P. laffertyi* are, on average, 1.1x, 1.2x, 1.5x, and 1.5x as large as

those of *P. catoptrophori*, respectively (Figure 2.7). *Parorchis catoptrophori* cercariae also have more prominent body spines and a relatively longer esophagus than *P. laffertyi* cercariae. The disparity in cercaria size at least partly explains why *P. laffertyi* reproductive rediae tend to produce fewer cercariae (average 3 developed cercariae per redia in summer) than *P. catoptrophori* (average 7 developed cercariae per redia in summer). *Parorchis laffertyi* reproductive rediae are only slightly larger than the reproductive rediae of *P. catoptrophori*, but the cercariae they produce are disproportionately larger.

The metacercariae of *P. laffertyi* are much larger (350 – 380 x 270 – 300) than the metacercariae of *P. avitus* (230 – 270 x 200 – 230; Stunkard & Shaw 1931).

The lack of developed reproductive organs in our *P. laffertyi* immature adult renders it difficult to compare to other described species of *Parorchis*, but the presence of esophageal diverticuli indicate that it belongs to the "*Parorchis* body type" subtype 2 category of Dronen & Blend (2008). Of the species that share this body type, *P. laffertyi* individuals resemble only *P. catoptrophori* and *P. chauhani* in their bipartite pharynx. However, *P. laffertyi* individuals differ from *P. catoptrophori* genetically (see below) and morphologically in their larval stages. *Parorchis laffertyi* individuals differ from both *P. catoptrophori* and *P. chauhani* in pharynx to oral sucker ratio (1:1.8 vs. 1:2.3 vs. 1:2) and sucker ratio (1:1.9 vs. 1:2.3 vs. 1:2.4).

It is possible that the pharynx to oral sucker ratio and the sucker ratio of *P. laffertyi* may be subject to change with development. While we cannot be sure of the true extent of their organ growth, we can infer what is possible given the ratio ranges of developing *P. catoptrophori* (see above), and 2 other cloaca-dwelling philophthalmids, *Cloacitrema michiganensis* and *Cloacitrema kurisi* (see Chapter 1 of this thesis). For *P. catoptrophori, C. michiganensis,* and *C. kurisi* adults, their oral sucker increased in size relative to their pharynx by 38%, 64%, and 8%,

respectively and their ventral sucker increased in size relative to their oral sucker by 19%, 64%, and 28%, respectively. These values are conservative since they are derived from comparison of the minimum ratios to the maximum ratios for each species. *Parorchis laffertyi* adults would need their oral sucker to increase by 11% relative to their pharynx and their ventral sucker to increase by 26% relative to their oral sucker to have ratios comparable to *P. chauhani*. Based on the data from *P. catoptrophori, C. michiganensis,* and *C. kurisi,* it is likely that the pharynx to oral sucker ratio and sucker ratio of *P. laffertyi* will grow to be much smaller than those of *P. chauhani*. That is, *P. laffertyi* will likely have a relatively larger oral sucker and a relatively larger ventral sucker.

Another key difference between *P. laffertyi* and *P. chauhani* is their great geographic distance; they have been documented on opposite sides of the Pacific (western USA and Northern Borneo, respectively) and *P. chauhani* is only known from *Calidris ruficollis,* a migratory bird that has not been recorded in the southwestern United States. We therefore feel the above-stated differences from *P. chauhani* in pharynx to oral sucker ratio, sucker ratio, and geographic range are sufficient to establish *P. laffertyi* as a new species.

In the 2 – 12 days it had to mature in the final host, the immature *P. laffertyi* worm clearly grew, as it is larger than the excysted metacercariae in body length (1100 vs. 600 – 840), body width (370 vs. 180 – 240), oral sucker size (105 x 117.5 vs. 50 – 67.6 x 72.5 – 105), and ventral sucker size (210 x 210 vs. 75 – 102.5 x 117.5 – 145). There are also clear developing reproductive organs (cirrus, more extensive genital primordium) that are not visible in the excysted metacercariae. The recovered worm was extremely active, which is surprising considering that no worms were recovered past 12 days of development.

Reports on *Parorchis* larvae from the California horn snail (erroneously referred to as *Parorchis acanthus* or *Parorchis avitus*) were undoubtedly composed of a combination of *P. laffertyi* and *P. catoptrophori* individuals (e.g. Hunter 1942; Nadakal 1960; Martin 1955; Martin 1972; LeFlore & Bass 1983; LeFlore et al. 1985; Fingerut et al. 2003; Hechinger et al. 2007; Hechinger et al. 2009; Hechinger et al. 2011b; Garcia-Vedrenne et al. 2016). The cercaria morphometrics of *P. laffertyi* collected for this study are in agreement with the cercaria length and width measurements of *Cerithidea* large from Huspeni (2000). *Parorchis laffertyi* should be incorporated into future studies on the California horn snail trematode guild, although additional genetic work or successful final host experimental infections are needed to accurately describe the adults of this species.

Molecular results

The CO1 alignment was 245 base pairs in length. In the CO1 phylogeny, *P. catoptrophori* and *P. laffertyi* form distinct monophyletic clades (Fig 2.5). While some *P. catoptrophori* colonies had single base pair mutations (maximum divergence of 0.8%), this is within normal intraspecies variation for *Parorchis* at the CO1 locus (Huspeni 2000). The minimum pairwise divergence value between *P. catoptrophori* and *P. laffertyi* was 17.6%. This is consistent with other reported ranges of congeneric divergences (6.0 – 21%) for the CO1 locus of flatworm parasites (Bowels & McManus 1993; Bowels & McManus 1994; Okamoto et al. 1995; Bowels et al. 1995; Morgan & Blair 1998). The BI analysis produced a tree congruent to the maximum likelihood analysis.

The 28S alignment was 1001 base pairs in length. In the 28S phylogeny, *P. catoptrophori* and *P. laffertyi* form distinct monophyletic clades with high bootstrap support (Fig 2.6). The

minimum pairwise divergence value between *P. catoptrophori* and *P. laffertyi* was 1.6%. Minimum sequence divergence between *P. catoptrophori* and midwestern *P. avitus* (collected from Mississippi by Tkach et al. 2016) was 2.4%. Minimum sequence divergence between *P. laffertyi* and the same *P. avitus* was 1.8%. One of the worms collected from our wild birds (a least sandpiper, *Calidris minutilla* collected from the UC Carpentaria Salt Marsh Reserve in Santa Barbara, California) matched to *P. catoptrophori* with 0.0% divergence. However, this worm was immature, so it is excluded from our morphometric data (see above). *Parorchis catoptrophori* and *P. laffertyi* form sister clades, indicating that they are more closely related than they are to *P. avitus* or the undescribed *Parorchis* variants. Their groupings within the 28S dataset confirm that these 2 species belong within the genus *Parorchis.* The BI analysis produced a tree congruent to the maximum likelihood analysis.

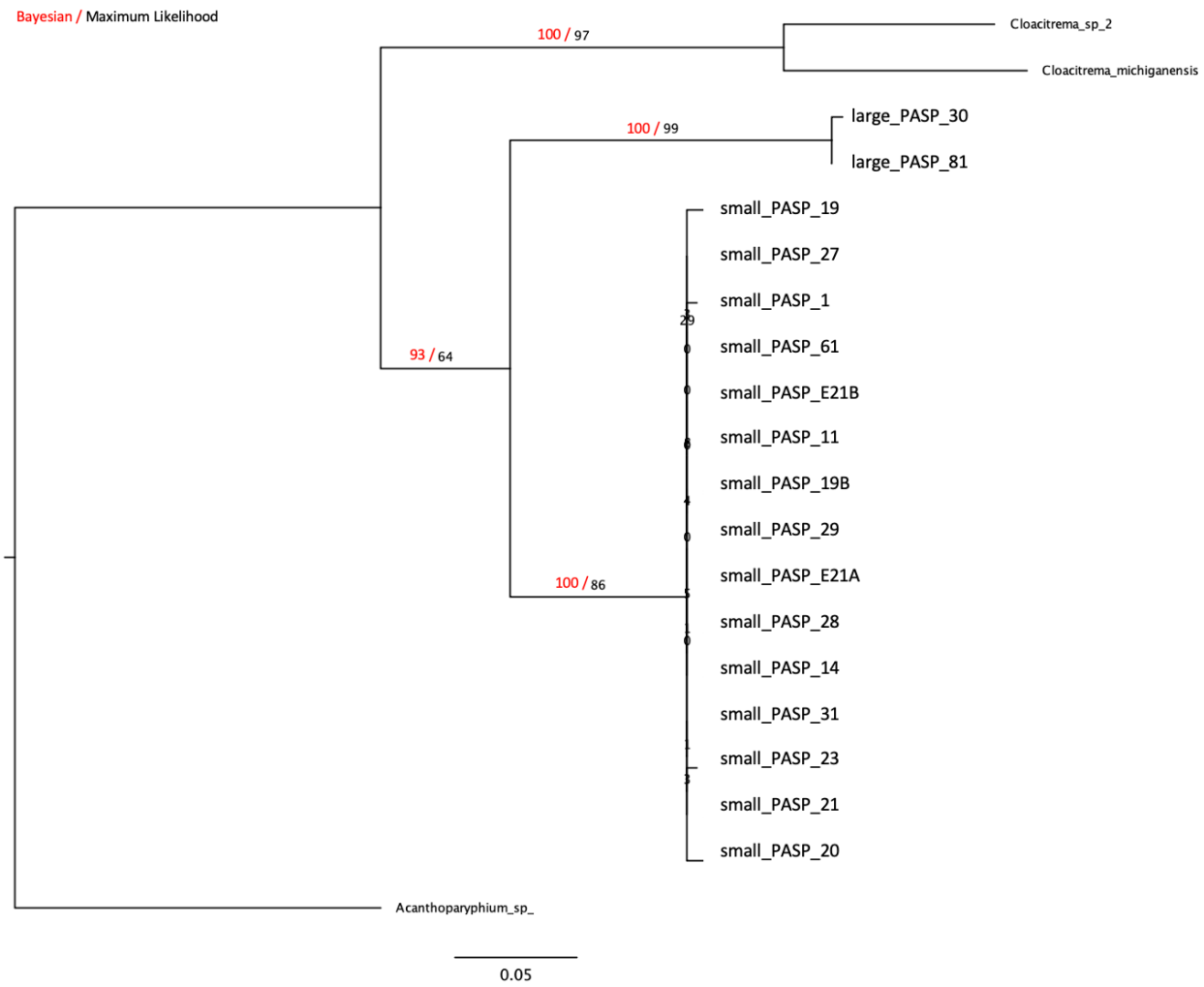

Figure 2.5: CO1 phylogenetic tree for *Parorchis catoptrophori* (small_PASP in the tree) and *Parorchis laffertyi* (large_PASP in the tree) individual colonies. Red values represent Bayesian Inference branch lengths and black values represent Maximum Likelihood branch lengths. Numbers after species names designate unique colonies from which the sequences derive.

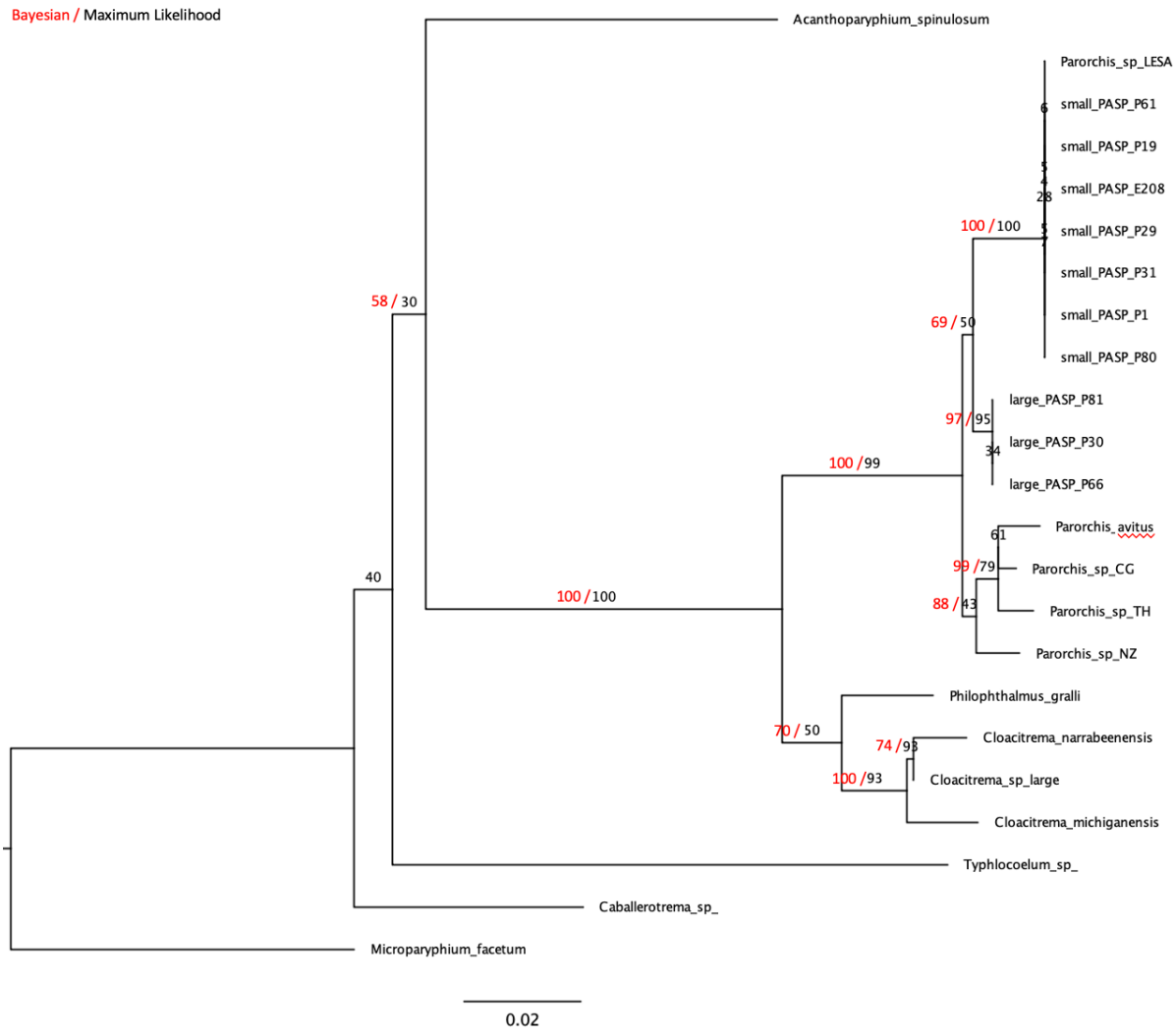

Figure 2.6: 28S phylogenetic tree for *Parorchis catoptrophori* (small_PASP in the tree) and *Parorchis laffertyi* (large_PASP in the tree) individual colonies. Red values represent Bayesian Inference branch lengths and black values represent Maximum Likelihood branch lengths. Numbers after species names designate unique colonies from which the sequences derive.

DISCUSSION

Here, we describe the colony demographics, reproductive rediae, soldier rediae, cercariae, metacercariae, and adults of *Parorchis catoptrophori* and the larval stages of *Parorchis laffertyi*. The most remarkable morphological difference between these 2 species is the disparity in size in the cercariae and metacercariae stages. With practice, these 2 species are

readily distinguishable from one another with a dissecting microscope. Although we were not able to obtain mature *P. laffertyi* adults, we know they are similar to *P. catoptrophori* in their large bipartite pharynx and esophageal diverticuli. The CO1 and 28S sequence data further confirms that these are 2 distinct species of *Parorchis*. It is extremely likely that previous reports on *P. catoptrophori* (erroneously referred to as *Parorchis acanthus*) from the California horn snail unknowingly included individuals of the cryptic species *P. laffertyi*. It is essential that *P. laffertyi* be incorporated into future studies on the California horn snail trematode guild to improve our understanding of ecological differences between these 2 species. Our inability to obtain *P. laffertyi* adults from experimental chickens suggests that the 2 species likely have different ecological requirements from their final hosts.

Dronen and Blend (2008) provided brief descriptions of several *Parorchis* adult specimens in the United States National Parasite Collection (USNPC) that were erroneously identified as *Parorchis acanthus*. Two of these specimens were originally collected from wild birds in California. Both individuals belong to the *Parorchis* body type subtype 2, so it is very likely that 1 may be representative of *P. laffertyi*. Yet, it is impossible to confirm this without obtaining *P. laffertyi* adults or genetic data from the museum specimens. The defining characteristic of these individuals is their contiguous testes (Dronen & Blend 2008), but we do not know the state of the testes of *P. laffertyi*.

The cercariae of *P. avitus* have been reported from Australia (Angel 1954), England (Lebour 1914), the United States (in Florida by Holliman 1961; in California by Martin 1972; in Massachusetts by Stunkard & Cable 1932; in Mississippi and Texas by Cooley 1957), Canada (Ching 1978), Japan (Harada & Suguri 1989), and Ireland (Prinz et al. 2010). In addition to their widespread geographical range, *P. avitus* larvae have also been reported from first intermediate

host snails spanning 3 separate Orders. The lack of apparent morphological differences between the cercariae of different *Parorchis* species has clearly led to erroneous identifications across the globe. The work of Huspeni (2000) and the present study emphasizes the importance of including genetic vouchers with new species records and the necessity of elucidating trematode life cycles to truly uncover patterns in species' ecology and geographic range.

ACKNOWLEDGEMENTS

Chapter 2, in full, is currently being prepared for submission for publication of the material. Nelson, Alexandria; Metz, Daniel; Hechinger, Ryan. The thesis author was the primary investigator and author of this material.

Table 2.4: Morphometric data and associated information on 10 described species of *Parorchis* that belong in the *Parorchis* body type, subtype 2 of Dronen & Blend (2008, see above). For "life stages described" letters indicate the following life stages that have been described: P = parthenitae, C = cercariae, M = metacercariae, A = adults. All measurements are in microns. Reported values are the range presented by the original authors or only the type measurement where applicable. The 'pharynx to oral sucker ratio' and the 'sucker ratio' refer to the ratio of the sum of the average length and average width of the oral sucker to that of the pharynx or ventral sucker.

Species	*Parorchis catoptrophori*	*Parorchis catoptrophori*	†*Parorchis avitus* (syn. *P. acanthus*)
References	Present study	Dronen & Blend (2008)	Nicoll (1907), Linton (1914), Stunkard & Shaw (1931), Stunkard & Cable (1932)
Type host	*Gallus domesticus*	*Catoptrophorus semipalmatus*	*Larus argentatus*
Other hosts	*Calidris minutilla*	--	*Larus canus*
Locality	San Diego, California, USA	Texas, USA	Scotland, UK Massachusetts, USA
Life stages described	P, C, M, A	A	P, C, M, A
Body L	2550 – 3375	4225 – 5700	1800 – 6200
Body W	875 – 1750	1325 – 2575	1200 – 4000
Collar W	580 – 750	760 - 970	820 – 1200
Num of collar spines	80 – 83	80 – 85	~ 60 – 68
Collar spine L x W	15 – 30 x 7.5 – 10	21 – 26 x 11 – 18	37 L
Oral sucker L x W	270 – 350 x 280 – 410	335 – 425 x 350 – 435	300 – 440 x 236 – 490
Pre-pharynx L	0 – 50	20 – 50	110
Pharynx L x W	180 – 240 x 120 – 190	170 – 230 x 110 – 150	180 – 240 x 100 – 240
Pharynx to OS ratio	1:1.8	1:2.3	1:1.9
Esophagus L	330 – 600	755 – 835	420 – 720
Ventral sucker L x W	680 – 820 x 650 – 900	820 – 975 x 820 – 1005	710 – 1350 x 433 – 1260
Sucker ratio	1:2.3	1:2.3	1:2.5
Testes L x W	150 – 330 x 120 – 320	170 – 385 x 175 – 350	550 – 600 W
Ovary W	70 – 150	210 – 245	250
Eggs L x W	42 – 67 x 22 – 38	83 – 91 x 44 – 53	81 – 120 x 40 – 70

Table 2.4, Continued.

Species	*Parorchis chauhani*	*Parorchis longivesiculus*	*Parorchis magnus*
References	Fischthal & Kuntz (1975)	Dronen & Blend (2008)	Belopol'skaja (1963), Dronen & Blend (2008)
Type host	*Calidris ruficollis*	*Larus atricilla*	*Numenius madagascariensis*
Other hosts	--	--	--
Locality	Northern Borneo	Texas, USA	Amur Basin, Russia
Life stages described	A	A	A
Body L	2795	4110 – 4950	7500 – 7800
Body W	1298	1660 – 2125	1470 – 1620
Collar W	--	590 – 690	865 – 907
Num of collar spines	61	64 – 66	--
Collar spine L x W	--	20 – 39 x 13 – 31	30 W
Oral sucker L x W	240 W	290 – 350 x 350 – 400	464 – 485 W
Pre-pharynx L	--	0 – 20	105 – 211
Pharynx L x W	118 W	180 – 210 x 130 – 140	232 – 253 x 260 – 274
Pharynx to OS ratio	1:2*	1:2.1	1:1.8*
Esophagus L	--	350 – 500	886 – 949
Ventral sucker L x W	580 W	850 – 975 x 875 – 975	865 – 907 W
Sucker ratio	1:2.4*	1:2.6	1:1.9*
Testes L x W	--	420 – 680 x 370 – 700	168 – 189 W
Ovary W	240	150 – 250	--
Eggs L x W	61 – 72 x 26 – 32	75 – 91 x 39 – 47	80 –105 x 35 – 46

Table 2.4, Continued.

Species	*Parorchis parvicollis*	*Parorchis proctobium*	*Parorchis ralli*
References	Belopol'skaja (1963)	Travassos (1918) Shtrom (1927)	Dronen & Blend (2008)
Type host	*Squatarola squatarola*	*Nyctanassa violacea*	*Rallus longirostris*
Other hosts	*Numenius phaeopus*	--	--
Locality	Amur Basin, Russia	Brazil	Texas, USA
Life stages described	A	A	A
Body Length	2530 – 4500	5000 – 7000	5680 – 5960
Body width	527 – 1360	3000 – 4000	2175 – 2925
Collar width	527	--	750 – 900
Num of collar spines	54	--	60 – 63
Collar spine L x W	26 W	35 L	16 – 25 x 8 – 13
Oral sucker L x W	350 – 400 x 286 – 316	530 W	365 – 400 x 420 – 475
Pre-pharynx L	--	0	2 – 15
Pharynx L x W	168 – 253 x 211 – 274	260 x 190	180 – 205 x 110 – 130
Pharynx to OS ratio	1:1.5	1:2.8*	1:2.7
Esophagus L	232 – 485	520	770 – 980
Ventral sucker L x W	1010 x 584 – 907	1000 – 1159 W	960 – 1075 x 990 – 1250
Sucker ratio	1:2.6	1:2*	1:2.6
Testes L x W	633 – 1150 W	600 – 870 W	460 – 630 x 430 – 680
Ovary W	340	400	187 – 260
Eggs L x W	51 – 68 x 30 – 35	134 – 142 x 71	70 – 86 x 33 – 47

* Where only one measurement is available, the 'pharynx to oral sucker ratio' and the 'sucker ratio' are calculated as the average oral sucker value to that of the average pharynx or ventral sucker value.

Table 2.4, Continued

Species	*Parorchis schachtachtinskoi*	*Parorchis snipis*
References	Dadasheva & Filimonova (1978)	Lal (1936)
Type host	*Numenius phaeopus*	*Totanus hypoleucos*
Other hosts	--	--
Locality	Azerbaijan	Northern India
Life stages described	A	A
Body Length	4448	2580
Body width	1408	1100
Collar width	--	--
Num of collar spines	--	~ 60**
Collar spine L x W	--	25 L
Oral sucker L x W	--	240 x 270
Pre-pharynx L	--	70
Pharynx L x W	240 x 210	120 x 180
Pharynx to OS ratio	--	1:1.7
Esophagus L	--	190
Ventral sucker L x W	1080 W	580 x 550
Sucker ratio	--	1:2.2
Testes L x W	--	350 – 360 x 225 – 270
Ovary W	--	140
Eggs L x W	--	50 – 60 x 25

† Reported morphometrics are those from Linton's (1914) *Parorchis avitus,* Nicoll's (1907) *Parorchis acanthus,* and Stunkard & Cable's (1932) *P. avitus*. The name *P. avitus* takes priority, as suggested by Dronen & Blend (2008). Stunkard & Shaw (1931) described the parthenitae and cercariae, while Stunkard & Cable (1932) described the metacercariae and experimentally obtained adults.

** The number of collar spines is not specified in the original description. This number comes from the author's drawing and is subject to error.

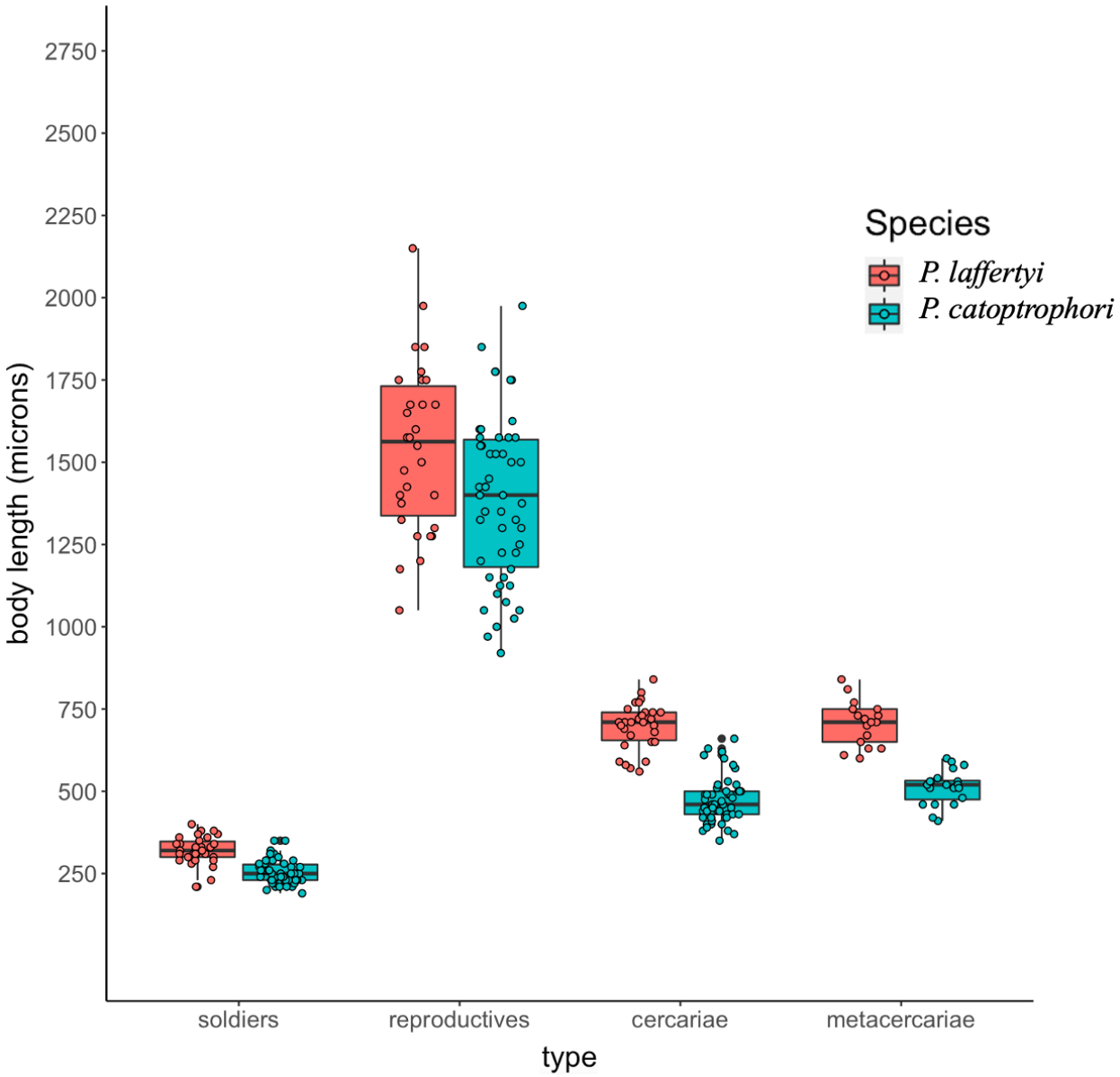

Figure 2.7: Body lengths of soldier rediae, reproductive rediae, cercariae, and excysted metacercariae for *Parorchis catoptrophori* (blue) and *Parorchis laffertyi* (red).

CHAPTER 3

Description of *Cercocyathocotylida intexens* n.gen. n.sp. (Digenea: Trematoda: Cyathocotylidae)

INTRODUCTION

For over 60 years, researchers studying the larval trematodes of the California horn snail, *Cerithideopsis californica* (Gastropoda, Potamididae), have recognized and included an undescribed cyathocotylid trematode in their work, most often referred to as "Small Cyathocotylid" (see Hechinger 2019 and references therein). Hence, concerning first intermediate host infections, information is available for this undescribed species' prevalence (Martin 1955; Sousa 1993), competitive relationships with the other trematodes (Kuris 1990; Sousa 1992; Lafferty et al. 1994; Hechinger et al. 2008), parthenita colony mass (Hechinger et al. 2009), and influence on the snail host's growth rate (Hechinger 2010). Further, unpublished experimental infections confirming the appearance and host use of its metacercariae in second intermediate host fish (RFH, unpub. obs.) permitted researchers to examine how the parasite influences second intermediate host fish behavior (Hernandez 2019 unpub. thesis) and to include the metacercariae, alongside the parthenitae and cercariae, in the food webs for 3 coastal estuaries of California and Baja California (Lafferty et al. 2006; Hechinger et al. 2011b). Although Hechinger (2019) includes a provisional morphological diagnosis for the sporocysts and cercariae, we lack a thorough and formal description for this species.

This manuscript presents DNA sequence data and morphological descriptions of the daughter sporocysts, cercariae, and metacercariae for this cyathocotylid species. In an attempt to

place the species into a known genus, given a lack of adult traits in the metacercariae, we used analyses of 28S and CO1 DNA sequence data. To characterize the general development and appearance of metacercariae, we experimentally infected second intermediate host fish. We also conducted experimental exposures of final host birds, *Gallus domesticus* and *Anas platyrhynchos domesticus*. We conducted a series of chemical excystment trials with recipes varying in medium, acidity, basicity, enzyme content, and temperature on the metacercariae to describe the excysted worms.

To avoid the taxonomic ambiguity associated with the traditional collective-group name used for larval trematodes (namely, *Cercaria* xxx), we erect a new collective-group name for larval cyathocotylid trematodes that cannot be placed into a known genus.

MATERIALS AND METHODS

Life cycle work and description techniques

Before describing the species, we refer to it as SMCY, the most commonly used code for this "small cyathocotylid" species (Hechinger 2019). Following Hechinger et al. (2011), we refer to the mass of sporocysts in a first intermediate host as a "colony". We collected California horn snails by hand from the intertidal mudflats of the UC Kendall-Frost Nature Reserve in San Diego, California. We first identified SMCY colonies and collected material for barcoding by examining "shed" cercariae from living colonies (infected snails). To shed cercariae, we placed live snails individually into compartments with sea water under warm lights in the laboratory. We assigned infected snails a unique code and used LOCTITE brush-on glue to attach a sticker with the code onto the snail shell. To keep the snails alive until we could dissect them to examine

parthenitae, we held them in outdoor, artificial tide, flow-through seawater mesocosms for a maximum of 6 months.

To obtain second intermediate hosts for experimental infections, we collected arrow gobies (*Clevelandia ios* Jordan & Gilbert 1882) from the University of California Kendall-Frost Natural Reserve during the spring and summer of 2020. By locating burrows in the sediment, we were able to collect gobies with a shrimp-gun by slurping sediment into the gun and depositing the contents in a clean tub for inspection. To obtain uninfected fish, we chose a collection site far from California horn snails. We conducted preliminary dissections of 5 fish in spring and 5 fish in summer to ensure a lack of previous SMCY infection. We stored fish in several seawater tanks with 16 to 20 fish per tank, for an acclimation period of 5 days before exposure to SMCY. We fed the fish a diet of defrosted bloodworms and brine shrimp every other day, but not on parasite exposure days. During the red tide of April and May 2020 it became necessary to siphon and clean the tanks twice a day. Only 1 fish mortality occurred during the red tide period.

In April 2020, we infected spring arrow gobies with SMCY. We shed SMCY cercariae using the aforementioned methods for 3 hours prior to fish exposures. To infect each fish, we placed individuals into 500 mL Erlenmeyer flasks containing 200 mL of fresh seawater and an air stone. We pipetted approximately 100 SMCY cercariae into each flask, where the fish remained for 1 – 3 hours post-exposure. Each fish had 3 separate exposure events, each of which occurred 5 days apart. In June 2020, we infected summer arrow gobies. We replicated the above methods, but to increase the number of retrievable metacercariae, approximately 250 SMCY cercariae were pipetted into each flask and fish remained in the flask for 6 – 8 hours post-exposure before being returned to their housing tanks. In summer, we infected a subset of fish only once, to permit observation of metacercariae development over time.

To allow metacercariae to mature before final host infections, we housed fish in the laboratory for approximately 37 days. Throughout the maturation process, we periodically dissected the single-exposure fish to obtain a metacercaria developmental time series. We observed and manually excysted metacercariae on days 3, 8, 13, 18, 24, 29 and 36 post-encystment. We additionally performed a series of chemical excystment trials with 10 metacercariae pooled into a single well per trial (see Table 3.1 for conditions). The conditions of each trial are based on the experiments of Fried (1994). The 1st stage conditions were meant to replicate the chemistry of a bird stomach, while the 2nd stage conditions were meant to replicate the chemistry of a bird intestine. We checked the incubating metacercariae every 1/2 hour over a period of 3 hours.

In an effort to experimentally obtain sexual adult stage worms, we infected 3 day-old experimental Cornish cross chickens, *Gallus domesticus*, and Pekin ducks, *Anas platyrhynchos domesticus*. In May 2020, we fed 2 chickens and 2 ducks 7 experimentally infected *C. ios* each. Preliminary fish dissections suggest that each bird was exposed to approximately 50 metacercariae. We sacrificed the hosts 8 days post-exposure. In August 2020, we fed 4 chickens and 4 ducks 2 experimentally infected *C. ios* each. This second round of fish infections was more successful and preliminary fish dissections suggest that each bird was exposed to approximately 400 metacercariae. We sacrificed summer birds on days 1, 2, 4, 6, 22, and 34 post-initial exposure.

Morphological descriptions are based on live and formalin-fixed samples. We obtained measurements from 10 individual daughter sporocysts and 10 individual cercariae from each of 5 colonies (total n = 50 for each life stage). Cercariae measurements are from shed cercariae, not those that we dissected from sporocysts. We designate 5 type specimens from 5 SMCY colonies

(1 holotype and 4 paratypes). Each type is a single formalin-fixed cercaria mounted on a slide along with ~10 cercariae from the same infection (colony-mates). Colony-mates (presumed clone-mates) of these types were used for our morphometric data and for our CO1 / 28S phylogenetic analyses. Ten individual cercariae from 3 separate paratype colonies were observed to obtain the flame cell formula and the excretory duct pattern.

Cercariae shed from 10 of our CO1-confirmed SMCY colonies were used to successfully infect second intermediate host arrow gobies for the metacercaria developmental time series. We obtained metacercaria measurements from 4 to 5 live encysted individuals during each time point (3, 8, 13, 18, 24, 29, and 36 days post-encystment, total n=30). Excysted metacercaria measurements are from 1 to 3 individuals each day post-encystment (total n= 11). Throughout the fall, winter, and spring we dissected snails infected with SMCY to understand how seasonal variation affects colony demographics.

Table 3.1: SMCY metacercaria chemical excystment treatment trials. All treatments were performed on 10 metacercariae in a single well at a time. Each condition was prepared in 10 mL quantities.

Treatment Num	1st Stage Conditions	2nd Stage Conditions	Incubation Temp
1	0.5% pepsin in Hanks BSS + 5 drops 2 M HCl for 1 hr	0.2% bile salts + 0.5% trypsin in saline + sodium bicarbonate until pH=8	37º C
2	0.5% pepsin in Hanks BSS + 5 drops 2 M HCl for 1 hr	0.8% saline	37º C
3	none	0.8% saline	37º C
4	0.5% pepsin in Hanks BSS + 5 drops 2 M HCl for 1 hr	0.5% trypsin in saline + sodium bicarbonate until pH=8	37º C
5	0.5% pepsin in Hanks BSS + 5 drops 2 M HCl for 1 hr	0.2% bile salts in saline + sodium bicarbonate until pH=8	37º C
6	0.5% pepsin in Hanks BSS + 5 drops 2 M HCl for 1 hr	0.5% trypsin in Hank's BSS + sodium bicarbonate until pH=8	37º C
7	none	0.2% bile salts + 0.5% trypsin in saline + sodium bicarbonate until pH=8	37º C
8	none	0.5% trypsin in Hank's BSS + sodium bicarbonate until pH=8	37º C
9	saline wash, then 0.5% pepsin in Hanks BSS for 1 hr	0.2% bile salts + 0.5% trypsin in Hank's BSS + sodium bicarbonate until pH = 8	40º C
10	saline wash, then 0.5% pepsin in Hanks BSS + 1 drop 2 M HCl for 1 hr	0.2% bile salts + 0.5% trypsin in Hank's BSS + sodium bicarbonate until pH = 8	40º C
11	saline wash, then 0.5% pepsin in Hanks BSS + 3 drops 2 M HCl for 1 hr	0.2% bile salts + 0.5% trypsin in Hank's BSS + sodium bicarbonate until pH = 8	40º C
12	0.5% pepsin in Hanks BSS for 1 hr	0.2% bile salts + 0.5% trypsin in Hank's BSS + sodium bicarbonate until pH = 8	40º C
13	0.5% pepsin in Hanks BSS + 1 drop 2 M HCl for 1 hr	0.2% bile salts + 0.5% trypsin in Hank's BSS + sodium bicarbonate until pH = 8	40º C
14	0.5% pepsin in Hanks BSS + 3 drops 2 M HCl for 1 hr	0.2% bile salts + 0.5% trypsin in Hank's BSS + sodium bicarbonate until pH = 8	40º C

DNA sequencing and phylogenetic analysis

We isolated DNA from a single cercaria shed from a live first intermediate host infection to permit connecting the cercaria's DNA sequence to sporocysts and metacercariae later examined that originated from the same colony. Hence, our types serve as "isogenophores" (sensu Pleijel et al. 2008) for our DNA sequence data. We washed live cercariae through 3 wells of DI water and placed individuals into 1 uL proteinase K lysis buffer (5x Q solution, 2.5x PCR

buffer, 0.4x proteinase K). The solution was placed in a thermocycler at 60 C for 1 hour and 95 C for 15 minutes.

For CO1, PCRs were 25 uL in volume and contained: 1 uL DNA template, 0.5 uL 25 mM $MgCl_2$, 2.5 uL 2.5 mM DNTPs, 2.5 uL 10x PCR buffer, 2.5 uL of 0.5 uM of each primer (DICE 1F and DICE 11R from Steenkiste et al. 2015), 0.2 uL 500 U Taq polymerase, and 15.8 uL molecular grade water. A touchdown protocol was used with the following thermocycler conditions: denaturation at 95 C for 2 min; annealing for 3x of 94 C for 40 sec, 51 C for 40 sec, 72 C for 1 min; 5x of 94 C for 40 sec, 50 C for 40 sec, 72 C for 1 min; 35 x of 94 C for 40 sec, 45 C for 40 sec, 72 C for 1 min; and extension at 72 C for 5 min.

For 28S, PCRs were 25 uL in volume and contained: 1 uL DNA template, 2.5 uL 2.5 mM DNTPs, 2.5 uL 10x PCR buffer, 2.5 uL of 0.5 uM of each primer (dig12 and 1500R from Tkach et al. 2003), 0.2 uL 500 U Taq polymerase, and 16.3 uL molecular grade water. Thermocycler conditions were the same as in Tkach et al. (2016). We ran PCR products on 1x TBE agarose gels for visualization and sent all successful PCR products to ETON Biosciences in San Diego, California for Sanger Sequencing.

We performed CO1 and 28S phylogenetic analyses in the following manner. After trimming the ends of each sequence by eye using MEGA X (Stecher et al. 2020), we exported the sequences into Mesquite (version 3.61, Maddison & Maddison 2019), where we retrieved additional published cyathocotylid sequences from Genbank (Table 3.2). We applied a MAFFT alignment (Katoh et al. 2002) to the data and adjusted by eye when needed. To mask the resulting alignment we used the GBLOCKS (Castresana 2000). To determine the appropriate evolutionary model to apply to the masked alignments we used jModelTest (Guindon & Gascuel 2003; Darriba et al. 2012), which picked GTR+I+G for both loci. For maximum likelihood (ML)

analysis, we executed the model in RaxML (Stamatakis 2014) with 1000 thorough bootstrap replicates. For Bayesian inference (BI), we ran Markov chain Monte Carlo (MCMC) with 10,000,000 generations, sample frequency of 1000, and a burnin fraction of 10% with software from MrBayes (version 3.2.6, Ronquist & Huelsenbeck 2003) and Tracer (version 1.7.1, Rambaut et al. 2018). We used the outgroup *Crocodillicola pseudostoma* based on the analysis from Achatz et al. (2019). We used FigTree (version 1.4.4) to visualize the resulting BI and ML trees.

Table 3.2: List of Genbank sequences used in our cyathocotylid 28S phylogenetic analysis along with their reference information.

Taxa	Genbank Num	Reference
Braunina cordiformis Wolf 1903	KM258670	Fraija-Fernandez et al. (2015); Blasco-Costa and Locke (2017)
Braunina sp.	MK650438	Achatz et al. (2019)
Crocodillicola pseudostoma Willemoes-Suhm 1870	MF398328	Hernández-Mena et al. (2017)
Cyathocotyle bushiensis Khan 1962	MK650440	Achatz et al. (2019)
Cyathocotyle prussica Muhling 1896	MH521249	Locke et al. (2018)
Cyathocotylidae sp.	MH257776	Huston, Cutmore, & Cribb (2018)
Gogatea mehri Mehra 1947	MK650441	Achatz et al. (2019)
Holostephanoides ictaluri Vernberg 1952	MK650443	Achatz et al. (2019)
Holostephanus dubinini Vojtek & Vojtkova 1968	MK650444	Achatz et al. (2019)
Mesostephanus cubaensis Alegret 1941	MK650445	Achatz et al. (2019)
Mesostephanus microbursa Caballero et al. 1953	MK650446	Achatz et al. (2019)
Mesostephanus microbursa Caballero et al. 1953	MF398325	Hernández-Mena et al. (2017)
Neogogatea sp.	MK650447	Achatz et al. (2019)
Suchocyathocotyle crocodile Yamaguti 1954	MK650450	Achatz et al. (2019)

RESULTS

Chemical excystation of metacercariae

All 14 chemical excystment trials were unsuccessful for different reasons. The acid pepsin 1st stage treatment with 5 drops of 2 M HCl was detrimental to metacercariae; all of the worms were dissolved in the acid solution. In contrast, metacercariae survived acid pepsin 1st stage treatments with 1 – 3 drops of 2 M HCl, but these worms did not excyst. Metacercariae that

underwent a saline wash prior to an acid pepsin 1st stage treatment had a higher survival rate than those placed straight into the 1st stage treatment solution. Several metacercariae that did not undergo a saline wash dissolved during the 40 C 2nd stage treatment, but all metacercariae survived the 37 C treatments.

Second intermediate host infections

After the spring *C. ios* experimental infections, preliminary dissections revealed that 5 fish had SMCY metacercaria intensities between 3 – 20. Preliminary dissections after the summer experimental infections revealed that 5 fish had SMCY metacercaria intensities between 130 – 270.

Final host infections

No adult worms were recovered from the exposed 6 chickens and 6 ducks. Close inspection of the duodenum, jejunum, ileum, ceca, and colon showed no obvious gross tissue pathology.

DESCRIPTION

***Cercocyathocotylida* n. gen. Nelson & Hechinger** (to be formally named in subsequent peer-reviewed publication)

Type species

Collective groups lack type species (ICZN, Art. 42.3.1), although otherwise are treated as genera (ICZN, Art. 42.2.1).

Diagnosis (from Cable's 1956 diagnosis for cyathocotylid cercariae, changes in bold)

Furcocercous **cercariae** developing in elongate sporocysts in freshwater and marine gastropods. Oral sucker, pharynx, and intestinal ceca well developed; ventral sucker embryonic or lacking. Tail with long slender stem and furcae, stem with long setae, furcal fins present or absent. Tail attached dorsally near posterior end of body. The larva rests suspended in water, typically with tail stem bent near its middle. Body spinose, with sensory papillae and setae, concave ventrally. Eyespots absent. **Cystogenous glands countable and present along outer edges of the body**. Excretory system diagnostic. From thin-walled vesicle at base of tail, 4 tubules extend anteriorly as a median and a lateral pair. The median pair converges and fuses to form a single tubule connected anteriorly with the lateral tubules by a cross-commissure. On each side, just posterior to that level, a recurrent collecting tubule joins each lateral tubule, extends posteriorly a short distance, and receives an anterior and posterior collecting tubule, each typically with 3 groups of flame cells, although other patterns have been described. Posterior flame-cell group on each side almost always in tail stem. Encyst in fishes or, rarely, in the molluscan host. Definitive hosts, birds; less often, mammals; and, rarely, fishes.

Etymology

"Cerco" is the transliterated Greek stem for tail (which underlies Cercaria) with the standard connecting vowel, o, while "cyathocotylida" is the Latin nominative singular form of the family name, Cyathocotylidae, to which the new species belongs. As is typical for Latin first declension nouns, the gender for *Cercocyathocotylida* should be female.

Remarks

Cercocyathocotylida is a collective group for trematodes described from cercariae that can be placed in the Family Cyathocotylidae, but not within a known genus. Collective groups have been recognized by the ICZN for over 100 years (see Poinar and Welch, 1981), and the current edition of the code uses the collective group *Cercaria* O. F. Müller 1873 as an example (ICZN, Art. 67.14). We erect this new collective group name to reap the benefits of having a formal, ICZN-regulated name while providing more taxonomic resolution than that provided by *Cercaria*.

Cercocyathocotylida intexens **n.sp. Nelson & Hechinger** (to be formally named in subsequent peer-reviewed publication)

Synonyms: None formal, but 2 provisional descriptions and names: "Cercaria cerithidia 17" of Hunter (1942), "small cyathocotylid" of multiple authors this century (see Hechinger, 2019).

Figs. 1 – 2

Diagnosis

Furcocercous cercariae developing in elongate sporocysts residing in the gonadal and digestive gland regions of the gastropod first intermediate host. Body size relatively small; embryonic ventral sucker lacking; furcal fins on tail present. Excretory system typical of cyathocotylids, but with abnormal flame cell formula: 2[(2+2) + (2+2)] with 2 pairs of flame cells in the tail stem. Encyst in fishes. Definitive host likely birds.

Description

Colony: Colony locus in the apical half of the visceral mass of first intermediate host, with clear vesicular snail tissue partly filling the former gonadal space; sporocysts sparsely distributed, primarily in the former gonadal region (at most only residual host gonadal tissue observed in developed infections), with substantial infiltration of the digestive gland and a little infiltration of the basal visceral mass; total sporocyst count 120 – 530 (265 ± 109.4, n = 25) in snails of lengths 21 – 32 mm collected during fall and spring, 60 – 310 (167 ± 52.5, n=35) in snails of lengths 23 – 30 mm from winter.

Daughter sporocysts: Body extremely elongate (average length:width = 20:1), translucent white, thin walled; inactive; anterior end narrows to a point, posterior end rounded; birth pore located ~40 microns from the anterior tip; cercariae of varying degree of development show no obvious spatial polarization; sporocysts contain 3 – 21 (8 ± 3.6, n = 50) fully developed cercariae in summer, only 0 – 18 (1 ± 2.20, n = 160) in winter; average ratio of developed cercariae to germinal balls and embryos in the summer 8 : 36 (n = 50) compared to winter 1 : 45 (n=160). Developed cercariae in sporocysts can be distinguished from developing embryos by the presence of a well-developed tail, clear ceca and oral sucker, and their frequent movement within the sporocyst.

Cercariae (shed from snails): Furcocercous; body nearly oval in dorsal view, slightly narrower at anterior end, translucent colorless, non-oculate, length ~100 when contracted in life, ~200 when stretched in life; sensory papillae present along entire body and tail; cystogenous glands number 20 on each side of body, distributed from outer edges of oral sucker posteriad to ~25 microns from body posterior end, each side of body has 2 pairs of 8 anterior glands followed posteriorly by 3 single glands, with 1 additional gland next to pharynx (2(8) + 3 + 1 = 20); oral

sucker as “modified anterior organ”, longer than wide; penetration glands originate at anterior end of the body and are contained within the oral sucker, number 16 in total with 2 sets of 4 distinct glands on the ventral side and 2 sets of 4 glands on the dorsal side (2(4) + 2(4) = 16); minute spines visible at 400x, only present along oral sucker; pre-pharynx absent; pharynx simple, longer than wide; esophagus of moderate length, leading to plump ceca; ventral sucker absent; ceca terminate on either side of the excretory bladder, slight undulations may appear more pronounced when body is majorly contracted; flame cell formula 2[(2 + 2) + (2 + 2)] = 16, with 6 pairs in the body and 2 pairs in the tail stem; excretory system typical, with 2 ducts originating on either side of the posterior end of the pharynx, ducts extend laterally and converge medially to form 3 branches, lateral branches run along the length of the ceca and converge into the excretory bladder, medial branch furcates at a point 2/3 the length of the ceca, the 2 medial ducts converge into the excretory bladder; excretory bladder horizontally elongate, emptying into 2 ducts at the entrance of the tail, the ducts merge into a single tube which runs along the midline of the tail and splits in 2 once again just before the furcae, with 1 duct leading to the end of each furca; forked tail approximately 2.5x as long as body, with dorso-ventral fins beginning just below the point of bifurcation (~10 microns), tail total length reported in Table 3.3, length of the tail from the posterior end of the body to the point of bifurcation 160 – 220 (194.8 ± 12.66, n = 50).

Metacercariae: Outer cyst nearly circular, translucent, comprised of ~3 layers, surrounds a dark nearly circular inner cyst; inner and outermost cysts separated by a distance of ~10 microns (3 – 8 days old). Inner cyst becomes slightly more elliptical, longer than wide; distance between inner and outermost cysts grows larger to ~20 microns (13 – 18 days old). Outer cyst transparent, nearly circular, comprised of > 6 layers, surrounds elliptical dark inner cyst; inner

and outermost cysts separated by a distance of ~30 microns (24+ days old). Inner and outer cysts grow longer and narrower (24 – 36 days old). Outer cyst grew ~ 45 microns longer from day 3 to day 36. Inner cyst appears to be filled with tightly packed circular granules that do not resemble any identifiable organs (all time periods). Excysted worms teardrop in shape, longer than wide; oral sucker (anterior organ) subterminal, longer than wide, visible in only a few encysted individuals, easier to visualize in excysted individuals. Worm volume did not drastically change throughout development, but outer cyst volume increased ~ 2x from day 3 to day 36.

Table 3.3: Morphometrics for *Cercocyathocotylida intexens* n.gen. n.sp. daughter sporocysts and cercariae. All values represent the range followed by mean ± SD. Units are in microns for all except cercariae count and germ ball/embryo count. Sample size is 50 for both stages.

stage	character	morphometrics
daughter	body L	1275 - 3800 (2944.5 ± 467.6)
sporocyst	body W	90 - 220 (150.8 ± 30.6)
	body H	80 - 220 (147.2 ± 30.2)
	cercaria count	3 - 21 (7.9 ± 3.6)
	germ ball/embryo count	14 - 85 (35.48 ± 14.1)
cercaria	body L	125 - 160 (144.2 ± 9.3)
	body W	60 - 80 (67.1 ± 5.4)
	body H	40 - 70 (53 ± 6.1)
	tail L	300 - 420 (356.2 ± 28.5)
	tail W	30 - 40 (33.9 ± 4.7)
	tail H	20 - 40 (30.6 ± 3.7)
	oral sucker L	37.5 - 45 (39.9 ± 2.0)
	oral sucker W	27.5 - 35 (31.05 ± 1.5)
	oral sucker H	25 - 30 (29.8 ± 1.0)
	pharynx L	12.5 - 20 (14.95 ± 2.2)
	pharynx W	10 - 15 (12.4 ± 0.7)
	pharynx H	12.5 - 12.5 (12.5 ± 0.0)
	esophagus L	10 - 22.5 (15.6 ± 2.5)
	ceca L	32.5 - 62.5 (47.75 ± 6.6)

Type host: *Cerithideopsis californica* (first intermediate host)

Location in first intermediate host (daughter sporocysts): gonadal, digestive gland, and basal visceral mass regions

Second intermediate hosts: estuarine fish

Type Locality: UC Kendall-Frost Nature Reserve, San Diego, California, USA (32.8°N, 117.2°W)

Habitat: Estuaries (intertidal flats, pans, channels); type from channels

Dates of collection: April – September 2020

Deposited material / Genbank accession numbers: TBA

Etymology: The specific epithet *intexens* is a Latin present active participle in the nominative case that means "to be weaving (into)". It refers to how the extremely long sporocysts of this species are interwoven throughout the host's visceral mass tissues.

Behavior / other biology: The colony undergoes severe regression from early December to late March, when sporocyst distribution throughout the snail becomes increasingly sparse and cercariae do not shed. Sporocysts are difficult to remove from snail tissue, but it is possible with dissecting needles. *Cercocyathocotylida intexens* has a relatively low dominance rank (10.5) within the California horn snail guild (Hechinger et al. 2009). Mature colonies comprise ~17% of the soft-tissue weight of an infected snail (Hechinger et al. 2009). Snails infected with *C. intexens* parthenitae grow over 2x faster than uninfected snails (Hechinger 2010).

Cercariae swim in quick bursts by rapidly whipping their tail, typically moving in a circular pattern. Swimming bursts are followed by a long rest and sinking in the water column. In their rest period, cercariae are positioned with their tail bent anterior to its furcation, forming a U-shape. Cercariae swimming can be triggered by stimulation (vibration or touching). There is no sign of cercariae being geotactic, but they are positively phototactic.

The worms are completely inactive when encysted. They primarily encyst in the fins and musculature of fish hosts. *Cercocyathocotylida intexens* act as a generalist metacercariae, infecting over 10 species of estuarine fish. Common second intermediate hosts include *Clevelandia ios, Fundulus parvipinnis, Gillichthys mirabilis,* and *Atherinops affinis* (Hechinger et al. 2011b). It is possible that *C. intexens* infects every estuarine teleost throughout its geographic distribution.

Geographic distribution: Although our samples are exclusively from San Diego, the species is almost certainly wide ranging, potentially at least throughout the entire range of their first intermediate host, the California horn snail, from San Francisco to Baja California. *Cercocyathocotylida intexens* life stages have been observed in at least 5 estuaries ranging from southern California to Baja California (Martin 1955; Martin 1972; Hechinger et al. 2007; Hechinger et al. 2009; Hechinger et al. 2011b).

Remarks

Cercocyathocotylida intexens cercariae well fit the diagnosis for cyathocotylid cercaria. The molecular results (see below) confirm that this is a species of cyathocotylid. *Cercocyathocotylida intexens* cercariae possess characteristics that belong to both the "Vivax" and "Testis" sub-types of furcocercous cercariae. In accordance with the "Vivax" type, the furcal rami have fin folds that are continuous around the ends and extend the entire length of the tail (Sewell 1922). Yet, the absence of a rudimentary ventral sucker and the simplicity of the excretory system (~5 pairs of flame cells in the body and ~2 pairs in the tail, as opposed to ~12 pairs in the body and ~3 pairs in the tail) are characteristic of the "Testis" type (Sewell 1922).

Of the other trematode species that infect *Cerithideopsis californica*, *C. intexens* is readily distinguished from the only other cyathocotylid, *Mesostephanus appendiculatus*. Daughter sporocysts can be easily distinguished from those of *M. appendiculatus* by their occupation of the gonadal and digestive gland regions, as opposed to the mantle. Additionally, *M. appendiculatus* sporocysts are extremely active and have a much lower length to width ratio than *C. intexens* sporocysts. *Cercocyathocotylida intexens* cercariae can also be readily distinguished from *M. appendiculatus* even at the dissecting scope, by their smaller size (body size of 145 vs. body size of 220), lack of pronounced ceca undulations, diverging anterior blind excretory ducts (and excretory formula), and more posterior position of gut branching (see Hechinger 2019).

Based on our molecular results (see below), *C. intexens* is most closely related to members of the subfamily Prohemistominae. However, of the 5 existing prohemistomine genera, *C. intexens* differs from members of *Mesostephanus*, *Paracoenogonimus*, *Prohemistomum,* and *Linstowiella* in flame cell formula. *Cercocyathocotylida intexens* also differs from members of *Prohemistomum* in the absence of a rudimentary ventral sucker and members of *Linstowiella* in the presence of furcal fin folds. The cercariae of *Mesostephanoides burmanicus,* the only member of its genus, have not been described and the adults are only known to infect reptile final hosts. Additional comparison of *C. intexens* cercariae to members of this group can be found in Table 3.5 and in the discussion below.

In size, *C. intexens* cercariae most closely resemble *Cercaria ariformis* Khan 1962, *Cercaria balthica* Szidat 1933, *Cercaria hirsuticauda* Probert 1966*,* Cercariae Indicae XXXIII of Sewell 1922*, Cercaria kasenyi* Fain 1953, *Cercaria kuwaitae* II of Abdul-Salam & Sreelatha 1993, Cercaria Levantina 14 of Lengy & Gold 1978, and *Cercaria vivacis* Iles 1959 (Table 3.5).

In flame cell formula, *C. intexens* is atypical from most described cyathocotylids and only resembles *Cercaria kasenyi, Cercaria yankapinensis* Goodman 1951, *Cercaria schoutedeni* Fain 1953, Cercariae Indicae XXXIII, and *Cercaria kuwaitae* II in arrangement. However, *C. intexens* is unique from the aforementioned species (other than *Cercaria kuwaitae* II and *Cercaria kasenyi*, which are distinguished below) in the presence of flame cells in the tail stem, presence of furcal fin folds, and the origin of the furcal fin folds from the point of bifurcation to a contiguous extension around the tail rami.

The cercariae of *Cercaria kuwaitae* II were described from the Persian Gulf from *Prenella cingulata* (form. *Cerithidea cingulata*), an estuarine snail confamilial with the California horn snail. *Cercaria kuwaitae* II are identical to *C. intexens* in flame cell formula, excretory duct morphology, presence and morphology of furcal fin folds, spination, ciliation, and absence of a rudimentary ventral sucker. The sporocysts of *Cercaria kuwaitae* II resemble *C. intexens* in general morphology and occupation of the hepatopancreas of the first intermediate host. However, the cercariae of *Cercaria kuwaitae* II are ~40% larger (average body length 187 vs. 145; body width 95 vs. 67; oral sucker length 45 vs. 40; tail stem 212 vs. 195) and the sporocysts are much larger (average body length 5741 vs. 2945; body width 354 vs. 151; number of mature cercariae 35 – 95 vs. 3 – 21) than *C. intexens*. *Cercaria kuwaitae* II is also very geographically distant from *C. intexens*, further indicating they are not the same species. The morphology of the metacercariae and adults of *Cercaria kuwaitae* II is unknown. Based on the larval morphology and host use, it is very likely that *C. intexens* and *Cercaria kuwaitae* II are related, but additional genetic and/or life cycle data is needed to confirm this suspicion.

The cercariae of *Cercaria kasenyi* were described from *Melanoides tuberculata* from Uganda, Africa. The cercariae are identical to *C. intexens* in flame cell formula, excretory duct

morphology, presence of furcal fin folds, spination, ciliation, and absence of a rudimentary ventral sucker. However, their sporocysts are larger than *C. intexens* (max length 5000 vs. 3800). While the description of *Cercaria kasenyi* is rather short, additional differences between this species and *C. intexens* can be inferred from the detailed drawing. *Cercaria kasenyi* appears to have a body length that is equal to the length of the tail stem, while the body length of *C. intexens* cercariae is ~1/2 the length of the tail stem. There are only 14 cystogenous glands drawn on *Cercaria kasenyi,* with no sign of a cystogenous gland located near the pharynx. There is also a short pre-pharynx present, while *C. intexens* has none. Lastly, the furcal fin folds on *Cercaria kasenyi* appear to be very thin (~1/5 the width of the furcae), while in *C. intexens* the fin folds are large (~2x the width of the furcae). Based on the larval morphology it is possible that *C. intexens* and *Cercaria kasenyi,* are related, but genetic data and additional descriptive work on *Cercaria kasenyi* is needed.

The lack of apparent internal structures renders the metacercariae of *C. intexens* difficult to compare to other described species. Of the Prohemistominae, *C. intexens* metacercariae differ from *Linstowiella szidati*, *Linstowiella viviparae*, *Paracoenogonimus ussuriensis*, *Mesostephanus appendiculatus,* and *Mesostephanus milvi* in their elliptical shape (as opposed to circular). *Paracoenogonimus ovatus* and *Prohemistomum chandleri* metacercariae are elliptical in shape but are > 3x longer than *C. intexens* (length 470 – 570 and 606 vs. 130 – 150) and have a clear oral sucker, pre-pharynx, pharynx, ventral sucker, and genital primordium. *Cercocyathocotylida intexens* has the smallest metacercariae out of all of the aforementioned species. However, the metacercariae of *L. viviparae, P. ovatus, P. ussuriensis* and *Pro. chandleri* are similar to *C. intexens* in their large inter-cyst space between the innermost and outermost cyst

layers (~30 – 100 microns). The metacercariae of the genus *Mesostephanoides* have not been described.

Cercocyathocotylida intexens cercariae have been provisionally described by Hunter (1942 unpub. dissertation) as Cercaria cerithidea 17. A separate provisional description of the cercariae and sporocysts was provided in Hechinger's (2019) guide to the trematodes infecting the California horn snail. Hechinger (2019) used the provisional name "small cyathocotylid" that has been used widely throughout the literature (e.g. Hechinger et al. 2007; Hechinger et al. 2009; Hechinger et al. 2011b). These cercariae have also been recorded as "small strigeid" (Martin 1955), "strigeid cercaria" (Martin 1972), and "unidentified cyathocotylid" (Lafferty et al. 2006). While the "Furcocercous Cercaria" of Maxon and Pequegnat (1949) is most certainly *M. appendiculatus* (Hechinger 2019), it is possible that they erroneously included some *C. intexens* cercariae in their morphometric data, as the lower bound of their reported body length (105) is much too small to be *M. appendiculatus*.

Table 3.4: Morphometrics for *Cercocyathocotylida intexens* n.gen. n.sp. metacercariae throughout the developmental time series. All values represent the range followed by mean ± SD. Units are in microns. Sample size is 4 – 5 for cyst measurements and 1 – 3 for excysted measurements. Sample size is 1 where SD is unavailable.

	day post-encystment		
character	3	8	13
inner cyst L	80 - 100 (87.5 ± 9.6)	100 - 120 (105.0 ± 10.0)	105 - 120 (111.7 ± 7.6)
inner cyst W	60 - 90 (72.5 ± 12.6)	90 - 100 (92.5 ± 5.0)	85 - 130 (101.7 ± 24.7)
outer cyst L	90 - 110 (95.0 ± 10.0)	110 - 130 (115.0 ± 10.0)	120 - 120 (120.0 ± 0.0)
outer cyst W	80 - 100 (85.0 ± 10.0)	100 - 110 (102.5 ± 5.0)	100 - 135 (111.7 ± 20.2)
cyst H	80 - 100 (86.7 ± 11.5)	100 - 100 (100.0 ± 0.0)	90 - 100 (96.7 ± 5.8)
excysted body L	155 - 155 (155.0 ± N/A)	170 - 170 (170.0 ± N/A)	155 - 157.5 (156.3 ± 1.8)
excysted body W	100 - 100 (100.0 ± N/A)	110 - 110 (110.0 ± N/A)	105 - 112.5 (108.8 ± 5.3)
excysted body H	70 - 70 (70.0 ± N/A)	60 - 60 (60.0 ± N/A)	60 - 70 (65.0 ± 7.1)
oral sucker L	42.5 - 42.5 (42.5 ± N/A)	45 - 45 (45.0 ± N/A)	40 - 42.5 (41.3 ± 1.8)
oral sucker W	35 - 35 (35.0 ± N/A)	30 - 30 (30.0 ± N/A)	35 - 37.5 (36.3 ± 1.8)

Table 3.4, Continued.

	day post-encystment			
character	18	24	29	36
inner cyst L	100 - 120 (108.0 ± 8.4)	100 - 105 (101.3 ± 2.5)	100 - 115 (109.0 ± 5.5)	100 - 120 (111.0 ± 7.4)
inncer cyst W	80 - 120 (94.0 ± 15.2)	60 - 70 (63.8 ± 4.8)	55 - 70 (60.0 ± 6.1)	50 - 60 (58.0 ± 4.5)
outer cyst L	120 - 140 (126.0 ± 8.9)	130 - 150 (135.0 ± 10.0)	130 - 150 (138.0 ± 8.4)	130 - 150 (139.0 ± 7.4)
outer cyst W	100 - 130 (110.0 ± 11.7)	100 - 120 (112.5 ± 9.6)	100 - 110 (104.0 ± 5.5)	100 - 110 (108.0 ± 4.5)
cyst H	90 - 100 (98.0 ± 4.5)	100 - 110 (105.0 ± 5.8)	100 - 110 (104.0 ± 5.5)	90 - 110 (102.0 ± 8.4)
excysted body L	160 - 160 (160.0 ± 0.0)	135 - 165 (150.0 ± 15.0)	175 - 175 (175.0 ± N/A)	165 - 170 (167.5 ± 3.5)
excysted body W	100 - 100 (100.0 ± 0.0)	90 - 115 (105.0 ± 13.2)	120 - 120 (120.0 ± N/A)	110 - 120 (115.0 ± 7.1)
excysted body H	60 - 60 (60.0 ± 0.0)	60 - 80 (70.0 ± 10.0)	60 - 60 (60.0 ± N/A)	60 - 70 (65.0 ± 7.1)
oral sucker L	37.5 - 40 (38.8 ± 1.8)	35 - 37.5 (36.3 ± 1.8)	42.5 - 42.5 (42.5 ± N/A)	42.5 - 42.5 (42.5 ± 0.0)
oral sucker W	32.5 - 35 (33.8 ± 1.8)	30 - 30 (30.0 ± 0.0)	30 - 30 (30.0 ± N/A)	32.5 - 35 (33.8 ± 1.8)

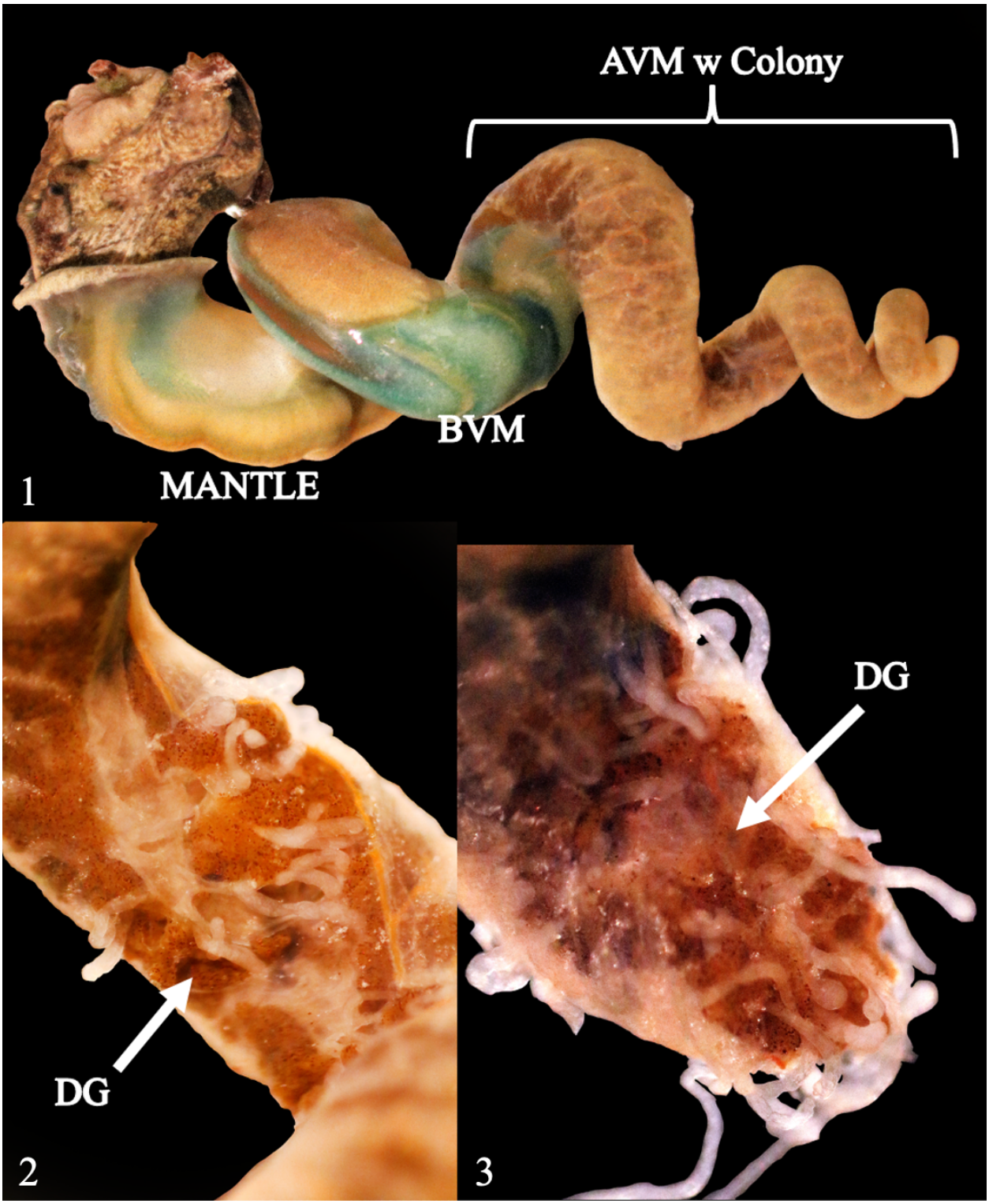

Figure 3.1: *Cercocyathocotylida intexens* n.gen. n.sp. colony overview photos from type colony. (1) Entire California horn snail removed from its shell. (2 & 3) Close-up view of *C. intexens* sporocysts in host snail tissue. AVM = apical visceral mass. BVM = basal visceral mass. DG = digestive gland.

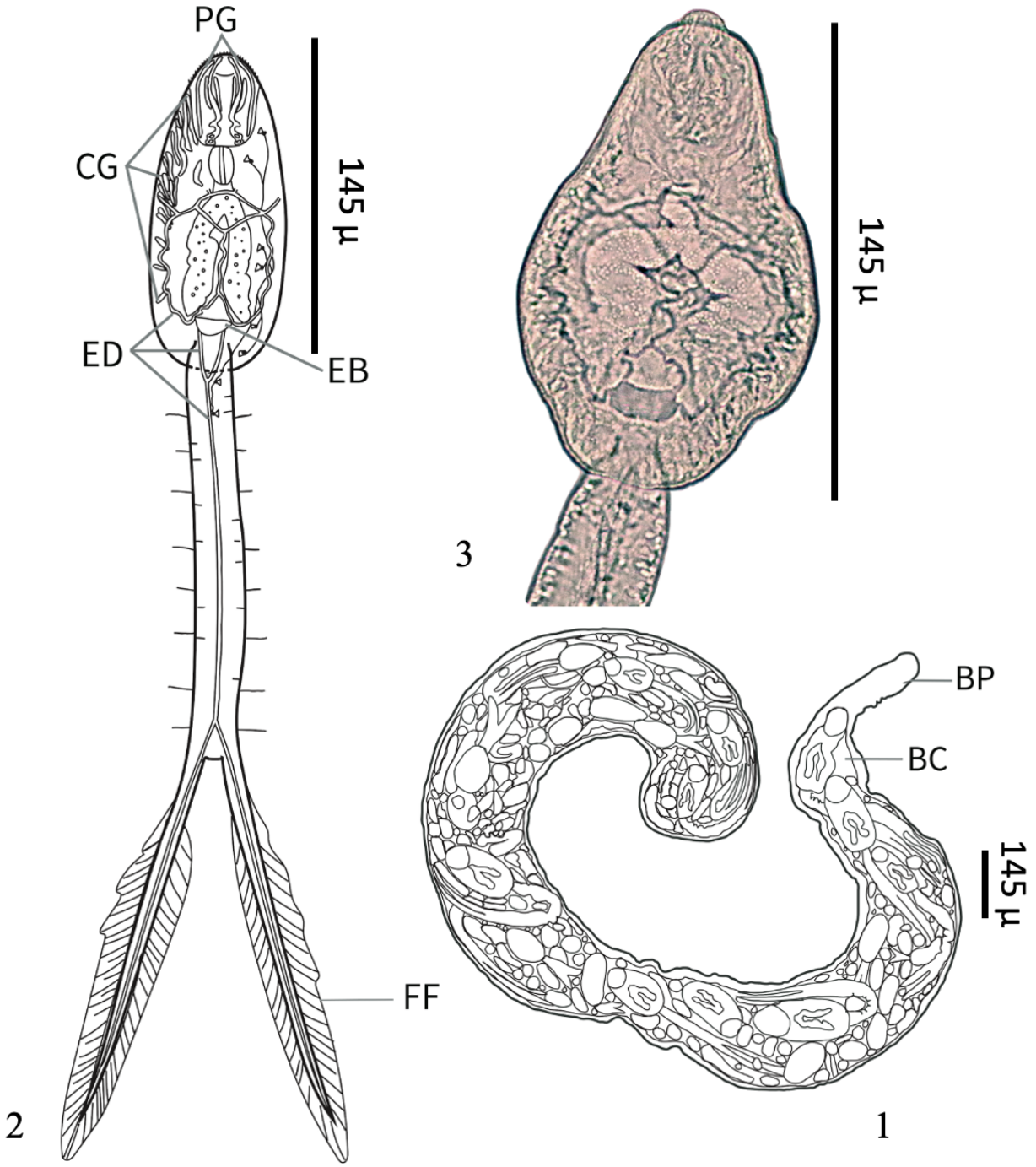

Figure 3.2: *Cercocyathocotylida intexens* n.gen. n.sp. daughter sporocyst that is short and contracted (1) and ventral view of *C. intexens* cercaria (2 and 3). Drawings are based on individuals from the type colony. Abbreviations: BP = birth pore, BC = body cavity, CG = cystogenous glands, EB = excretory bladder, ED = excretory ducts, FF = fin folds, and PG = penetration glands.

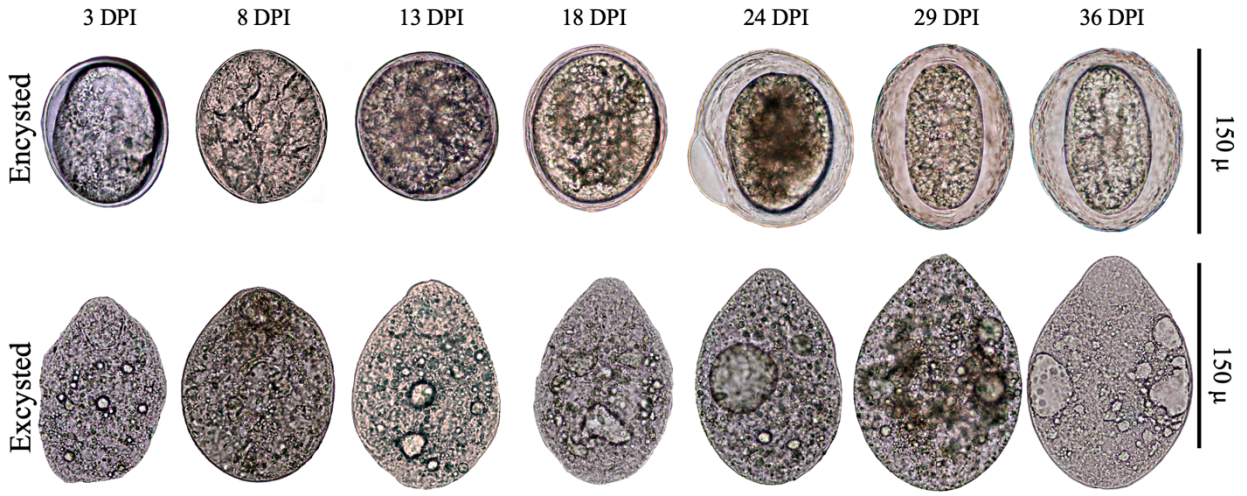

Figure 3.3: *Cercocyathocotylida intexens* n.gen. n.sp. metacercariae developmental time series. Photos of live encysted and manually excysted metacercariae observed with transmitted light on a compound microscope. Specimens were not stained. Scale bar is 150 microns.

Molecular results

The CO1 alignment was 400 base pairs in length. Due to a scarcity of previous cyathocotylid COI sequences available on Genbank, our tree includes only *C. intexens* and *Mesostephanus appendiculatus* from the California horn snail (Figure 3.4). *Cercocyathocotylida intexens* forms a distinct monophyletic clade, with most (12/17) individuals from separate colonies having identical sequences and a few (5) showing between 1 and 3 single base pair mutations (maximum divergence of 0.76%). This low amount of CO1 variability is within normal intraspecies variation characterizing digenean trematodes (e.g. Locke et al. 2010). *Cercocyathocotylida intexens* exhibited 15.97% - 19.1% CO1 sequence divergence with *M. appendiculatus*, a level of divergence that is in the high range of that characterizing different digenean genera (e.g. Bowels & McManus 1993; Bowels & McManus 1994; Okamoto et al. 1995; Bowels et al. 1995; Morgan & Blair 1998). The BI analysis produced a tree congruent to the maximum likelihood analysis.

The 28S rDNA alignment was 976 base pairs in length, and we were able to include sequences from 15 other species available on GenBank. In the 28S phylogeny (Figure 3.5) *C. intexens* forms a distinct monophyletic clade with high bootstrap support. It groups sister to Cyathocotylidae sp. 'Heron Island' from Huston et al. (2018) with a divergence value of 8.9%. In our 28S analysis, the same relationships between taxa were recovered as in Achatz et al. (2019), but the addition of *C. intexens* in the phylogenetic tree increased the branch support of the *Mesostephanus*/Cyathocotylidae sp. clade from 50 to 82. The BI analysis produced a tree congruent to the maximum likelihood analysis.

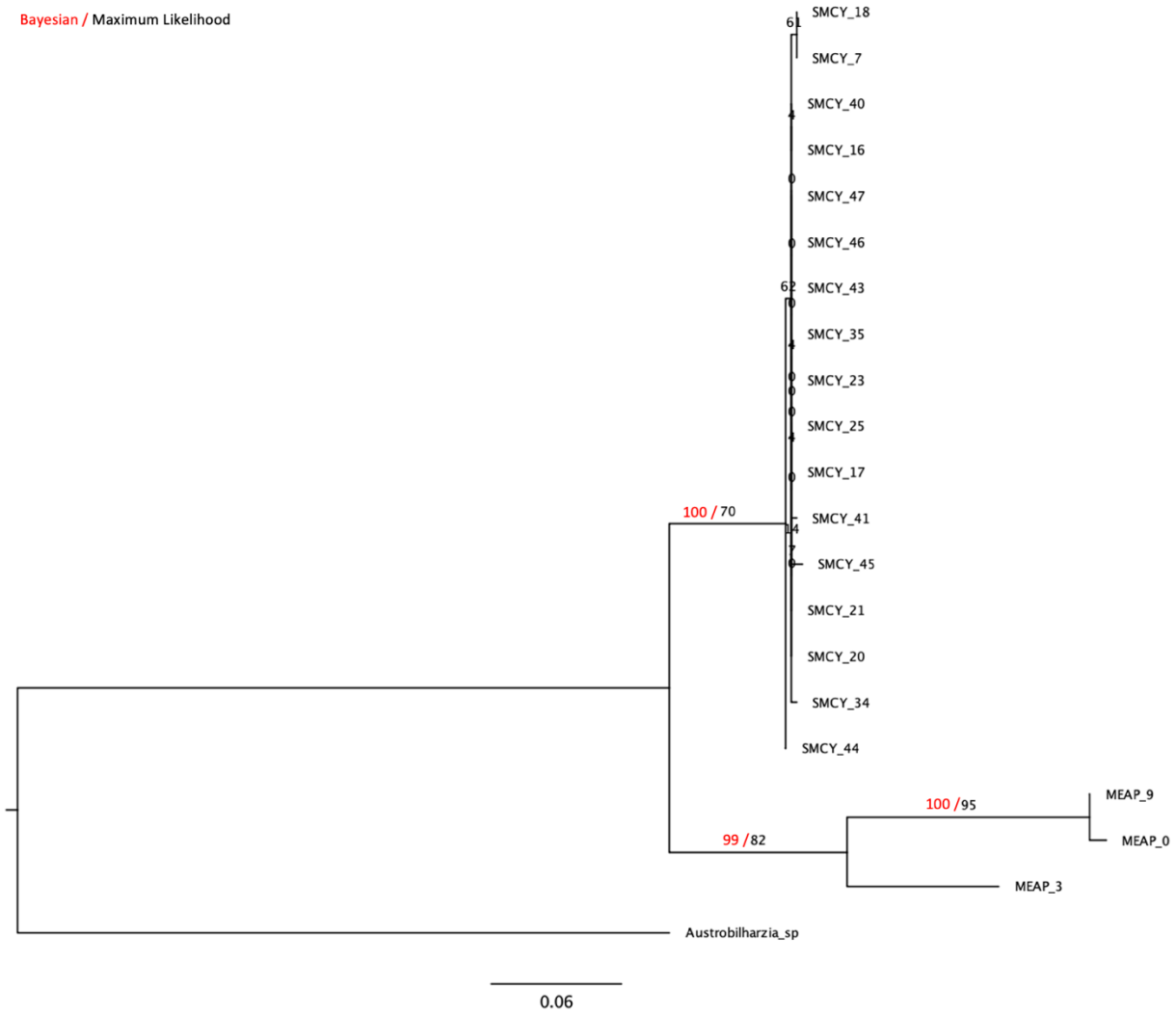

Figure 3.4: CO1 phylogenetic tree for *Cercocyathocotylida intexens* n.gen. n.sp. (SMCY in the tree) individual colonies. Red values represent Bayesian Inference branch lengths and black values represent Maximum Likelihood branch lengths. Numbers after species names designate unique colonies from which the sequences derive.

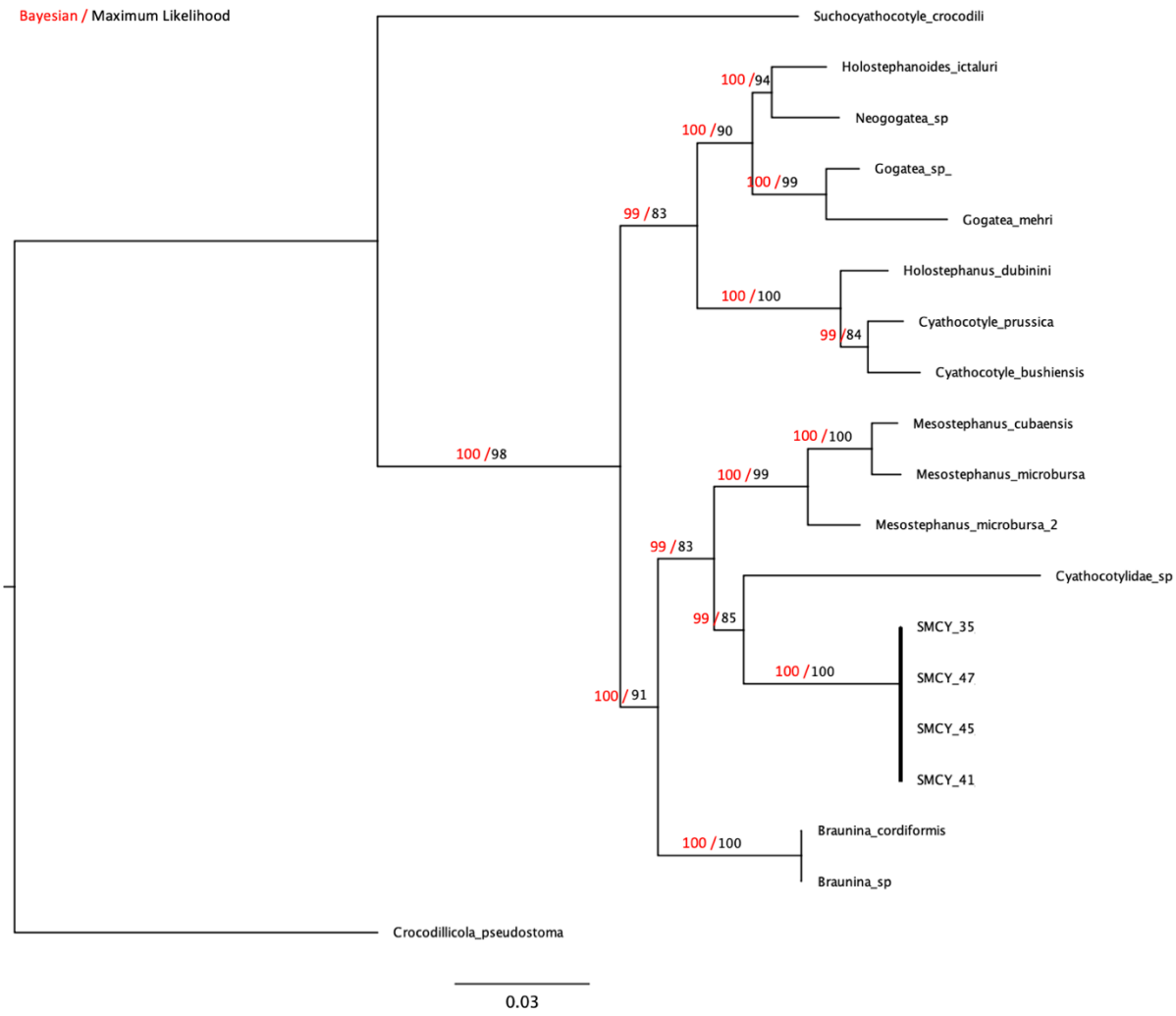

Figure 3.5: 28S phylogenetic tree for *Cercocyathocotylida intexens* n.gen. n.sp. (SMCY in the tree) individual colonies. Red values represent Bayesian Inference branch lengths and black values represent Maximum Likelihood branch lengths. Numbers after species names designate unique colonies from which the sequences derive.

DISCUSSION

Here, we describe *C. intexens* daughter sporocysts and cercariae from wild-infected California horn snails. We obtained and analyzed genetic data from 17 unique *C. intexens* colonies to produce CO1 and 28S phylogenetic trees. We successfully infected second intermediate host arrow gobies with *C. intexens* to obtain a metacercaria developmental time series. Our metacercariae chemical excystation trials and our final host experimental infections

were unsuccessful, but we are able to provide a new taxonomically informative name for the species.

Although our chemical excystment trials failed, we are able to provide recommendations for future experiments. Given higher mortality under certain conditions, we recommend that future trials consist of a saline wash, no or a low-acid initial treatment, and incubation at a temperature no higher than 40 C.

Although our final host experimental infections failed, we have reason to believe that *C. intexens* adults infect estuarine birds. We postulate that *C. intexens* is a member of the family Prohemistominae (see below and molecular results), members of which typically infect bird final hosts (Niewiadomska 2002). Birds are also the most common consumers of fish in California estuaries, so they are the most logical final host, given *C. intexens*' second intermediate hosts (estuarine fish). Additionally, domestic chickens and ducks are less than ideal hosts when compared to natural estuarine birds, as seen with previous experimental exposures with bird-using prohemistomines (e.g. Szidat 1933; Yamaguti 1975). In 1 case, infections of only newly hatched chicks was successful, while infections of 2-day old chicks failed (Yamaguti 1975). To abide by health and safety regulations, we were not able to infect newly hatched birds, so it is possible that after a 3-day acclimation period these birds were unable to be infected with *C. intexens* metacercariae.

Our CO1 analysis from 17 different *C. intexens* colonies suggests that there may not be a species cryptic to *C. intexens* in the California horn snail. Our data agrees with an unpublished study that found no evidence of cryptic species in 21 different *C. intexens* colonies from Southern California to Nayarit, Mexico (Osamu Miura, pers. comm.).

Cercocyathocotylida intexens forms a sister clade to Cyathocotylidae sp. 'Heron Island', a cyathocotylid cercaria found in the Australian cerithiid gastropod *Clypeomorus batillariaeformis* (Huston et al. 2018). Neither Huston et al. (2018), nor Achatz et al. (2019) were able to place Cyathocotylidae sp. 'Heron Island' into a described subfamily. However, given the relationship of *C. intexens* and Cyathocotylidae sp. 'Heron Island' to other taxa in our 28S phylogenetic tree, we can eliminate 4 out of the 7 subfamilies within the Cyathocotylidae (these are Cyathocotylinae, Szidatiinae, Suchocyathocotylinae, and Braunininae). Given the likelihood that *C. intexens* parasitizes bird final hosts, we can tentatively rule out the 2 cyathocotylid subfamilies unrepresented in our phylogenetic analysis (these are Prosostephaninae and Muhlinginae). Thus, it is likely that *C. intexens* and Cyathocotylidae sp. 'Heron Island' are representatives of the single remaining cyathocotylid subfamily, Prohemistominae, due to their close taxonomic relationship with the genus *Mesostephanus* and their highly probable use of bird final hosts.

It is unlikely that *C. intexens* is already described as an adult. There are 5 genera within the subfamily Prohemistominae: *Mesostephanus*, *Paracoenogonimus*, *Prohemistomum, Linstowiella,* and *Mesostephanoides* (Achatz et al. 2019). First, from our 28S phylogenetic tree, it is clear that *C. intexens* is not a member of *Mesostephanus*. Second, *C. intexens* is likely not a member of *Mesostephanoides*, as the only member of this genus, *Mesostephanoides burmanicus*, develops in reptiles (Niewiadomska 2002). The larval stages of *M. burmanicus* have not been described, so we cannot compare them to *C. intexens* morphologically. Third, *C. intexens* is likely not a member of *Prohemistomum*, as members of this genus possess a developing ventral sucker as cercariae and possess a group of 3 flame cells in the tail stem (Vernberg 1952). Finally, the cercariae of the remaining 2 genera, *Paracoenogonimus* and *Linstowiella,* lack the

combination of the following *C. intexens* traits: absence of a clear developing ventral sucker, presence of furcal fin folds, relatively small body size (the cercariae are 2x – 4x larger than *C. intexens*), and the excretory formula of 2[(2+2) + (2+2)]. Additional differences between described cyathocotylid cercariae and *C. intexens* can be found in Table 3.5. It is therefore likely that *C. intexens* is a representative of a new genus, but additional molecular work on the Prohemistominae is needed to confirm this suspicion.

ACKNOWLEDGEMENTS

Chapter 3, in full, is currently being prepared for submission for publication of the material. Nelson, Alexandria; Hechinger, Ryan. The thesis author was the primary investigator and author of this material.

Table 3.5: Morphological attributes of named furcocercous cyathocotylid-like cercariae. *Cercocyathocotylida intexens* of this manuscript is bolded. Described cercariae that have been proven to be genetically distant from *C. intexens* (e.g. cercariae of *Holostephanus, Cyathocotyle*, etc.) have been excluded. *The column header "VS pres?" refers to presence of a rudimentary ventral sucker noted by the authors, not a developed ventral sucker.

trematode name	†host species	sporocysts develop in	length	width	pre-phar pres?	*VS pres?	fin folds pres?	excretory formula
Cercaria agstaphensis 37 Manafov 2011	*Melanopsis praemorsa*	N/A	220 - 248	99 - 110	y	y	n	2[(3+3+3) + (3+3)+(3)]
Cercaria agstaphensis 38 Manafov 2011	*Melanopsis praemorsa*	N/A	196 - 216	106 - 120	y	y	y	2[(3+3+3) + (3+3)+(3)]
Cercaria ariformis Khan 1962	*Bithynia tentaculata*	digestive gland	93 - 200	83 - 143	n	n	y	2[(2+2+2+2)]
Cercaria balthica Szidat 1933	*Bithynia tentaculata*	N/A	140	75	n	n	n	2(5+2)
Cercaria caribbea L Cable 1956	*Cerithium algicola*	N/A	254 - 260	117 - 120	n	y	y	2[(3+3+3) + (3+3+3)]
Cercaria caribbea LI Cable 1956	*Cerithium literaium / Cerithium algicola*	branchial region	199 - 219	82 - 92	n	y	y	2[(3+3+3) + (3+3+3)]
Cercaria caribbea LVIII Cable 1963	*Cerithium variabile*	branchial region	248 - 270	103 - 118	y	y	y	2[(3+3+3) + (3+3+3)]
Cercaria curonensis Szidat 1933	*Bithynia tentaculata*	N/A	200	100	y	n	y	2(5+2)
Cercaria dorsocauda Tubangui 1928	*Ampullaria lagunaensis*	repro glands	520 - 640	210 - 400	n	y	y	2[12+(3)]
Cercaria hirsuticauda Probert 1966	*Bithynia tentaculata*	digestive gland	140 - 180	100 - 140	n	n	y	2[(2) + (4) + (2)]
Cercariae Indicae XV of Sewell 1922	*Melanoides tuberculata / M. lineatus*	N/A	316 - 368	72 - 158	y	y	y	2[12+(3)]
Cercariae Indicae LVIII of Sewell 1922	*Paludomus baculus*	N/A	351 - 368	140 - 158	y	n	y	2[12+(3)]
Cercariae Indicae XXXIII of Sewell 1922	*Paludomus baculus*	N/A	140 - 213	70 - 89	y	n	n	2(5+2)
Cercaria indicae LXXXII Sultana 1982	*Viviparus bengaiensis*	N/A	N/A	N/A	y	y	n	2[(3+3+3) + (3+3+3)]
Cercaria indicae LXXXIII Sultana 1982	*Viviparus bengaiensis*	N/A	N/A	N/A	y	n	n	2[(3+3+3) + (3+3+3)]
Cercaria kasenyi Fain 1953	*Melanoides tuberculata*	N/A	80 - 210	20 - 80	y	n	y	2[(2+2) + (2+2)]

Table 3.5, Continued.

trematode name	†host species	sporocysts develop in	length	width	pre-phar pres?	*VS pres?	fin folds pres?	excretory formula
Cercaria kuwaitae I Abdul-Salam & Sreelatha 1993	*Cerithidea cingulata*	branchial region	340 - 470	150 - 190	y	y	y	2[(3+3+3) + (3+3+3)]
Cercaria kuwaitae II Abdul-Salam & Sreelatha 1993	*Cerithidea cingulata*	hepatopancreas	145 - 245	72 - 115	y	n	y	2[(2+2) + (2 + 2)]
Cercaria leighi Holliman 1961	*Cerithidea scalariformis*	branchial region	204 - 219	92 - 99	y	y	y	2[(3+3+3) + (3+3+3)]
Cercaria leptoderma Faust 1922	*Viviparus quadratus*	liver	350	150	n	n	n	2[(3+3+3) + (3+3+3)]
Cercaria Levantina 14 of Lengy & Gold 1978	*Bithynia sidoniensis*	hepatopancreas	90 - 200	44 - 100	n	n	y	2[(2) + (4) + (2)]
Cercaria notopalae Johnston & Beckwith 1945	*Notopala hanleyi*	liver	229 - 328	114 - 155	y	n	n	2[(3+3+3) + (3+3+3)]
Cercaria ogatai Ito 1956	*Cerithidea cingulata*	midgut gland	206 - 286	90 - 127	n	y	y	2[(3+3+3) + (3+3+3)]
Cercaria papillosoma Khan 1962	*Bithynia tentaculata*	digestive gland	256 - 316	100 - 166	y	n	y	2[(3+3) + (3+3) + (3)]
Cercaria pendulata Baugh 1954	*Vivipara bengalensis*	gonads	264 - 330	148 - 205	y	n	n	2[15(2) + 3(2)]
Cecaria "R" of McCoy 1928	*Cerithium litteratum*	N/A	N/A	N/A	n	y	y	"at least 12 on each side of body"
Cercaria rhionica XII Galaktionov et al. 1980	*Melanopsis praemorsa*	hypobranchial gland	310 - 380	110 - 180	y	y	y	2[(3+3+3) + (3+3+3)]
Cercaria schoutedeni Fain 1953	*Melanoides tuberculata*	N/A	150 - 300	40 - 90	y	n	n	2[(2+2) + (2+2)]
Cercaria spatulate Probert 1966	*Bithynia tentaculata*	digestive gland	220 - 280	130 - 330	y	n	n	2[(2+2)+(2+2+2+(2))]
Cercaria tatei Johnston & Angel 1940	*Plotiopsis tatei*	N/A	184 - 242	108 - 125	n	y	y	2[(3+3+3) + (3+3+3)]
Cercaria vivacis Iles 1959	*Bithynia tentaculata*	digestive gland	130 - 150	60 - 90	y	y	y	2[(3+3+3)]
Cercaria yankapinensis Goodman 1951	*Physa gyrina*	N/A	275 - 300	N/A	N/A	n	n	2[(2+2+2)+(2+2+2)]
Cyathocotylidae sp. 'Heron Island' of Huston et al. 2018	*Clypeomorus batillariaeformis*	N/A	262 - 352	131 - 205	y	n	y	N/A
***Cercocyathocotylida intexens* of this ms**	***Cerithideopsis californica***	**gonad / digestive gland**	**125 - 160**	**60 - 80**	**n**	**n**	**y**	**2[(2 + 2) + (2 + 2)]**

Table 3.5, Continued.

trematode name	†host species	sporocysts develop in	length	width	pre-phar pres?	*VS pres?	fin folds pres?	excretory formula
Mesostephanus appendiculatoides Lutz 1935 by Hutton & Sogandares-Bernal 1960	*Cerithium muscarum*	N/A	N/A	N/A	n	y	y	2[(3+3+3) + (3+3+3)]
Mesostephanus appendiculatus Lutz 1935 by Martin 1961	*Cerithidea californica*	digestive gland	180 - 258	78-115	y	y	y	2[(3+3+3) + (3+3+3)]
Mesostephanus milvi Yamaguti 1939 by Pike 2007	*Cleopatra aegyptiaca*	N/A	200 - 216	80 - 88	y	y	y	N/A
Mesostephanus haliasturis Tubangui & Masilungan 1941 by Cribb et al. 1995	*Melanoides tuberculata*	N/A	141 - 172	66 - 84	n	y	y	N/A
Mesostephanus indicum Mehra 1947 by Sheena et al. 2007	*Bellamya bengalensis*	hepatopancreas	408 - 500	246 - 308	y	y	n	2[(3+3+3)+(3+3)+(3)]
Paracoenogonimus ussuriensis Besprozvannykh & Ermolenko 2009	*Amuropaludina praerosa* / *Cipangopaludina ussuriensis*	liver	270 - 350	84 - 110	n	n	y	2[(3+3+3) + (3+3+3)]
Paracoenogonimus ovatus (Komiya 1938)	*Viviparus viviparus*	"midgut gland"	314 - 352	130 - 157	y	n	n	2[(3+3+3) + (3+3+3)]
Linstowiella viviparae (Szidat 1933)	*Viviparus viviparus*	"midgut gland"	225	120	n	n	n	2[(3+3) + (3+[3])]
Linstowiella szidati (Anderson 1944)	*Campeloma sp.*	mantle	386 - 495	172 - 248	y	n	n	2[(3+3) + (3+[3])]
Prohemistomum chandleri Vernberg 1952	*Pleurocera acuta*	digestive gland	304 - 321	195 - 218	y	y	y	2[(3+3+3) + (3+3+3)]
Prohemistomum vivax (Loos 1896, Sonsino 1892)	*Cleopatra bulimoides* / *Melanopsis praemorsa*	visceral sac	280	N/A	N/A	y	y	2(?+3)

†Host species names are included as in original description. These scientific names may not be current.

CHAPTER 4

Description of the larval stages of *Probolocroyphe uca* (Digenea: Trematoda: Microphallidae) with observations of an undocumented cryptic species of *Probolocoryphe* and an undescribed species of *Maritrema* (Digenea: Trematoda: Microphallidae)

INTRODUCTION

The existence and ecology of *Probolocoryphe uca* (Sarkisian 1957) Heard & Sikora 1969 (Trematoda: Microphallidae) in the California horn snail (*Cerithideopsis californica* Gastropoda: Potamididae) trematode guild has been studied for over 60 years (see Hechinger 2019 and references therein). Since the description of the precocious metacercaria by Sarkisian (1957), researchers have examined the basic biology of first intermediate host infections (Nadakal 1960; Hechinger et al. 2009) and how the parasite may influence second intermediate host fiddler crab behavior (Mora 2013).

Although Hechinger (2019) includes a provisional morphological diagnosis for the sporocysts and cercariae of *P. uca*, we lack a thorough description for these life stages in the California horn snail. Additionally, the inability of Sarkisian (1957) to establish experimental infections in sympatric grapsid shore crabs, despite being able to readily infect local fiddler crabs, suggests that there may be undocumented cryptic species in play, because there have been numerous observations of supposed *P. uca* metacercariae in wild-infected shore crabs (see host list in Hechinger et al. 2011).

This manuscript presents DNA sequence data and morphological descriptions of the colony demographics, daughter sporocysts, and cercariae for *P. uca*. To screen for cryptic species that may be present in first intermediate host infections, wild infected shore crabs, and fiddler crabs and to unveil relationships between *P. uca* and known microphallid taxa, we used a combination of 28S rDNA and CO1 mtDNA genetic analyses. In our 28S analysis we included sequences from unidentified microphallid adults previously collected by our lab from estuarine birds (see methods below) and first intermediate host infections of the undescribed species "small microphallid" from the California horn snail (see Hechinger 2019). We sought to obtain *P. uca* adults, which have never been described, through experimental infections of final host chicks (*Gallus domesticus*) and ducks (*Anas platyrhynchos domesticus*). We compare the morphometrics of *P. uca* metacercariae from naturally infected crabs to those in the original species description (Sarkisian 1957). Additionally, our genetic and morphological analyses of the metacercariae confirm the presence of a previously unrecognized cryptic *Probolocoryphe* species infecting estuarine shore crabs as a second intermediate host. We postulate that this cryptic species may be *Probolocoryphe lanceolata* and compare the metacercariae morphometrics to those of *P. uca*, but further genetic work is needed to confirm the species' identity.

MATERIALS AND METHODS

Life cycle work and description techniques

Following Hechinger et al. (2011) and Hechinger (2019), we refer to the mass of sporocysts in a first intermediate host as a "colony". We collected California horn snails by hand from the intertidal mudflats of the University of California Kendall-Frost Natural Reserve in San Diego, California. We first identified microphallid colonies and collected material for barcoding

by examining "shed" cercariae from living colonies (infected snails). To shed cercariae, we placed live snails individually into compartments with sea water under warm lights in the laboratory. We assigned snails infected with microphallid trematodes a unique number identifier and used LOCTITE brush-on glue to super-glue the number IDs onto the shell of the snails. To keep the snails alive until we could dissect them to examine parthenitae, we held them in outdoor, artificial tide, flow-through seawater mesocosms for a maximum of 6 months.

To obtain second intermediate hosts for experimental infections, we collected fiddler crabs (*Leptuca crenulata*) and shore crabs (*Pachygrapsus crassipes*) from the University of California Kendall-Frost Natural Reserve during the summer of 2020. By locating burrows in the sediment, we were able to collect crabs with a hand-trowel. We placed crabs into individual wells of large compartment boxes with holes drilled into all sides for transport to the lab. In the lab, we placed the compartment boxes on constant flow-through water tables for a maximum of 2 weeks prior to dissections. We conducted preliminary dissections to ensure crabs were infected with *P. uca* and to collect metacercariae for CO1 sequencing (see below). We fed crabs a diet of dissolvable algae pellets every other day.

To experimentally obtain sexual adult stage worms, we infected 3 day-old experimental Cornish cross chickens, *Gallus domesticus*, and Pekin ducks, *Anas platyrhynchos domesticus*. In August 2020, we fed 7 chickens and 6 ducks various "treatments" of wild-infected crabs. Our feeding treatments were as follows: 1) the digestive gland from a single shore crab, 2) the thoracic musculature from a single shore crab, or 3) the digestive gland and thoracic musculature from 5 fiddler crabs. Preliminary crab dissections suggest that each bird was exposed to approximately 100 – 500 metacercariae each. We fed each bird 1 of the aforementioned

treatments 2 times on 2 consecutive days. Chickens and ducks were sacrificed on days 1, 2, 3, 4, 7, 8, 9, 21, 22, 30, and 31 post-exposure.

Morphological descriptions are based on live and formalin-fixed samples. We obtained measurements from 10 individual daughter sporocysts and 10 individual cercariae from each of 5 colonies (total n = 50 for each life stage). Cercariae measurements are from shed cercariae, not those that we dissected from sporocysts. For the parthenitae and cercariae, we obtained 5 voucher specimens from 5 naturally infected *P. uca* colonies. Colony members (assumed clone-mates) of these types were used for our morphometric data and were included in our CO1 and 28S phylogenetic analyses. Ten individual cercariae from 3 separate voucher colonies were observed to obtain the species' flame cell formula. See Table 4.2 for sporocyst and cercariae morphometrics.

We obtained metacercaria measurements from 5 fixed excysted individuals from each of 3 wild-infected fiddler crabs and 4 wild-infected shore crabs (total n = 35). We excysted metacercariae by incubation in Hank's Bile Salt Solution at 40 C for 1/2 hour. Excystment success was consistently 100%. Throughout the winter we dissected snails infected with *P. uca* to understand how seasonal variation affects colony demographics.

We also had the opportunity to examine adult specimens that were collected and saved in our lab by Dr. Kate Sheehan as part of a project examining the parasites of California estuarine birds (from the same birds used in Hechinger et al. 2019). We included these specimens in our 28S phylogenetic analysis, along with "small microphallid" (see Hechinger 2019) cercariae shed from 5 naturally-infected California horn snails from the Kendall-Frost Natural Reserve.

DNA sequencing and phylogenetic analysis

We isolated DNA from 5 *P. uca* and 5 "small microphallid" cercariae at a time shed from a live first intermediate host infection to permit connecting the cercaria DNA sequences to sporocysts later examined but originating from the same colony. We isolated DNA from single *Probolocoryphe* excysted metacercariae collected from wild-infected second intermediate hosts. We washed live worms through 3 wells of DI water and placed individuals into 1 uL proteinase K lysis buffer (5x Q solution, 2.5x PCR buffer, 0.4x proteinase K). The solution was placed in a thermocycler at 60 C for 1 hour and 95 C for 15 minutes.

For CO1, PCRs were 25 uL in volume and contained: 1 uL DNA template, 0.5 uL 25 mM $MgCl_2$, 2.5 uL 2.5 mM DNTPs, 2.5 uL 10x PCR buffer, 2.5 uL of 0.5 uM of each primer (DICE 1F and DICE 11R from Steenkiste et al. 2015), 0.2 uL 500 U Taq polymerase, and 15.8 uL molecular grade water. A touchdown protocol was used with the following thermocycler conditions: denaturation at 95 C for 2 min; annealing for 3x of 94 C for 40 sec, 51 C for 40 sec, 72 C for 1 min; 5x of 94 C for 40 sec, 50 C for 40 sec, 72 C for 1 min; 35 x of 94 C for 40 sec, 45 C for 40 sec, 72 C for 1 min; and extension at 72 C for 5 min.

For 28S, PCRs were 25 uL in volume and contained: 1 uL DNA template, 2.5 uL 2.5 mM DNTPs, 2.5 uL 10x PCR buffer, 2.5 uL of 0.5 uM of each primer (dig12 and 1500R from Tkach et al. 2003), 0.2 uL 500 U Taq polymerase, and 16.3 uL molecular grade water. Thermocycler conditions were the same as in Tkach et al. (2016). We ran PCR products on 1x TBE agarose gels for visualization and sent all successful PCR products to ETON Biosciences in San Diego, California for Sanger Sequencing.

We replicated the above 28S methods for Dr. Sheehan's subset of unidentified microphallid adults collected by our lab from naturally infected birds along the coast of

California and Baja California in 2014 – 2017. For DNA isolation, we placed single entire worms into 1 uL of our proteinase K lysis buffer.

We performed CO1 and 28S phylogenetic analyses in the following manner. After trimming the ends of each sequence by eye using MEGA X (Stecher et al. 2020), we exported the sequences into Mesquite (version 3.61, Maddison & Maddison 2019), where we retrieved additional published sequences from Genbank (Table 4.1). We applied a MAFFT alignment (Katoh et al. 2002) to the data and adjusted by eye when needed. To mask the resulting alignment we used the GBLOCKS (Castresana 2000). To determine the appropriate evolutionary model to apply to the masked alignments we used jModelTest (Guindon & Gascuel 2003; Darriba et al. 2012), which picked GTR+I+G for both loci. For maximum likelihood (ML) analysis, we executed the model in RaxML (Stamatakis 2014) with 1000 thorough bootstrap replicates. For Bayesian Inference (BI), we ran Markov chain Monte Carlo (MCMC) with 10,000,000 generations, sample frequency of 1000, and a burnin fraction of 10% with software from MrBayes (version 3.2.6, Ronquist & Huelsenbeck 2003) and Tracer (version 1.7.1, Rambaut et al. 2018). We used the outgroup *Lecithodendrium linstowi* based on the analysis from Tkach et al. (2003). We used FigTree (version 1.4.4) to visualize the resulting BI and ML trees.

Table 4.1: List of Genbank sequences used in our microphallid 28S phylogenetic analysis along with their reference information.

Taxa	Genbank Num	Reference
Candidotrema loossi (Africa 1930)	AY220621	Tkach et al. 2003
Lecithodendrium linstowi Dollfus 1931	AF151919	Tkach et al. 2000
Maritrema arenaria Hadley & Castle 1940	AY220629	Tkach et al. 2003
Maritrema brevisacciferum Shimazu & Pearson 1991	KT355818	Kudlai, Cutmore & Cribb 2015
Maritrema corai Hernandez-Orts et al. 2016	KT880221	Hernandez-Orts et al. 2016
Maritrema deblocki Presswell, Blasco-Costa, & Kostadinova 2014	KJ144173	Presswell, Blasco-Costa, & Kostadinova 2014
Maritrema eroliae Yamaguti 1939	JF826247	Al-Kandari, Al-Bustan, & Alnaqeeb 2011
Maritrema heardi (Kinsella & Deblock 1994)	AY220632	Tkach et al. 2003
Maritrema neomi Tkach 1998	AF151927	Tkach et al. 2000
Maritrema novaezealandense Martorelli et al. 2004	KT355820	Kudlai, Cutmore & Cribb 2015
Maritrema oocysta Lebour 1907	AY220630	Tkach et al. 2003
Maritrema poulini Presswell, Blasco-Costa, & Kostadinova 2014	KJ144174	Presswell, Blasco-Costa, & Kostadinova 2014
Maritrema prosthometra Deblock & Heard 1969	AY220631	Tkach et al. 2003
Maritrema sp. (*Cercaria queenslandae* V)	MH257771	Huston, Cutmore, & Cribb 2018
Maritrema subdolum Jagerskiold 1909	AF151926	Tkach et al. 2000
Microphallus abortivus Deblock 1974	AY220626	Tkach et al. 2003
Microphallus basodactylophallus (Bridgman 1969)	AY220628	Tkach et al. 2003
Microphallus primas Jagerskiold 1908	AY220627	Tkach et al. 2003
Microphallus simillis Jagerskiold 1900	AY220625	Tkach et al. 2003
Prosthogonimus ovatus (Rudolphi 1803)	AF151928	Tkach et al. 2000

RESULTS

Our 28S genetic analysis revealed the presence of a second *Probolocoryphe* species that is cryptic to *P. uca* in the metacercaria stage in wild-infected shore crab second intermediate hosts (Figure 4.5). We do not redescribe the metacercariae of *P. uca*, as the original description is unambiguously of the same species that infects fiddler crab second intermediate hosts. We do however, provide a brief explanation of the morphological differences between the metacercariae of *P. uca* and the cryptic *Probolocoryphe* sp. and provide morphometric data for both (Table 4.3; Figure 4.3).

Final host infections

No adult worms were recovered from the 7 chicken and 6 duck final hosts. Close inspection of the duodenum, jejunum, ileum, ceca, and colon showed no sign of tissue damage.

DESCRIPTION

Diagnosis for *Probolocoryphe* adults (from DeBlock 2008)

Body medium-sized, fusiform. Oral sucker longer than ventral, with short anterodorsal rostellum. Digestive tract short, in forebody, anterior to cirrus-sac. Testes symmetrical, post-ovarian, post-vitelline, in mid-hindbody. Cirrus-sac thin-walled, arcuate, transverse, anterior to ventral sucker; evaginated cirrus filiform, unarmed. Genital atrium minute. Genital pore sinistrolateral to ventral sucker. Ovary dextral to ventral sucker. Anterior region of uterus with two dorsal symmetrical coils, anterior to caeca, ascending to level of pharynx; posterior coils surround testes in hindbody. Metraterm distinct. Vitellarium semi-annular, long, partly post-cecal and partly pre-cecal, partly marginal, encircles ovary, cirrus-sac and caeca, reaches level of espophagus or pharynx, not enclosing testes and posterior uterine coils; vitelline ducts median, short, T-shaped. Excretory vesicle short, V- or Y- shaped. In intestine of birds (Charadriiformes, Ralliformes); eastern Palaearctic, Nearctic, and Neotropical. Type species *P. asadai* Otagaki 1958.

***Probolocoryphe uca* (Sarkisian 1957) Heard & Sikora 1969**

Synonyms: *Maritrema uca* (Sarkisian 1957)*, Mecynophallus uca* (Ching 1963)

Figs. 4.1 – 4.3

Diagnosis (for the cercariae)

Monostome, leptocercous xiphidiocercaria with distinct butterknife-like oral stylet, tegument spinose, flame cell formula of 2[(1+1) + (1+1)], and penetration glands in 4 pairs on either side of the body. Cercariae produced by daughter sporocysts forming a colony that resides in the gonadal region of the first intermediate host, with substantial infiltration of the digestive gland and the basal visceral mass. Encyst in crabs. Final host unknown but is suspected to be a bird or mammal.

Description

Colony: Colony locus in the apical visceral mass of first intermediate host; sporocysts densely packed, primarily in the former gonadal region (with no sign of functioning host gonadal tissue remaining), with substantial infiltration of the digestive gland and basal visceral mass; total sporocyst count 650 – 1320 (1013 ± 227.13, n = 7) in snails of lengths 27 – 31 mm from winter.

Daughter sporocysts: Body spheroidal to oblong (average length:width = 2:1), translucent gold or orange, thick walled, inactive but developed cercariae within are extremely active; birth pore located ~20 microns from the anterior end; developing cercariae show no obvious spatial polarization; sporocysts contain 0 – 14 (8 ± 3.3, n = 50) fully developed cercariae in summer, 0 – 23 (7 ± 4.6, n = 35) in winter; average ratio of developed cercariae to germinal balls and embryos in summer 8 : 11 (n = 50) compared to winter 7 : 23 (n = 35). Developed cercariae in sporocysts can be distinguished from developing embryos by the presence of a developed and active tail, clear oral sucker with stylet and clear penetration glands, and their frequent movement within the sporocyst.

Cercariae (shed from snails): Body semi-elliptical, translucent, colorless, widest just below oral sucker and most narrow at level of excretory bladder, approximately equal in length to tail, spinose; non-oculate; oral sucker not well developed, longer than wide; oral stylet prominent, appears straight and narrow (2.5 wide) from dorsal view, dorsal flange apparent from side profile, 5 – 7.5 (7.05 ± 0.97, n = 50) wide, with narrow handle; pharynx not apparent; penetration glands in 4 pairs; anterior penetration gland pair originating at the most anterior point of the body, more prominent, larger ducts; posterior penetration gland pair originating at the tip of the stylet, smaller, less defined ducts; ventral sucker not apparent; cystogenous glands not apparent; excretory tubes in single pair, extending anterolaterally from the excretory bladder; flame cell formula 2[(1+1) + (1+1)] = 8; excretory bladder bi-lobed, typically wider than long, U-shaped, somewhat irregular; tail simple, unarmed, ringed cuticle.

Type host: *Cerithideopsis californica*

Location in first intermediate host (daughter sporocysts): gonadal region, digestive gland region, and basal visceral mass

Second intermediate host: *Leptuca crenulata*

Type Locality: University of California Kendall-Frost Natural Reserve, San Diego, California, USA (32.8°N, 117.2°W)

Habitat: Estuaries (intertidal flats, pans, channels); type from channels

Dates of collection: April - September 2020

Deposited material & Genbank accession numbers: TBA

Etymology: This species was named by Sarkisian (1957), who used the epithet uca in reference to the second intermediate host *Leptuca crenulata* (formerly *Uca crenulata*).

Behavior / other biology: The colony undergoes slight regression from early December to late March, when sporocyst distribution throughout the snail becomes increasingly sparse and cercariae shed in lower numbers (Hechinger et al. 2009 documented the decrease in colony relative mass in winter). Sporocysts are somewhat difficult to separate from one another, but it is possible with dissecting needles. *Probolocoryphe uca* has the lowest dominance rank within the California horn snail guild (Kuris 1990; Lafferty et al. 1994). Mature colonies comprise an average of ~41% of the soft-tissue weight of an infected snail (Hechinger et al. 2009). The pigments of the sporocysts have been studied by Nadakal (1960).

Cercariae continuously swim throughout the water column by rapidly lashing their tail back and forth, occasionally extending but typically curling their body inwards to form a tight ball. There is no clear sign of cercariae being geotactic or phototactic.

The worms are moderately inactive when encysted. They primarily encyst in the thoracic musculature and connective tissue of fiddler crab second intermediate hosts. Sarkisian (1957) observed the migration of very young metacercariae through the hemocoel and pericardial sinus of fiddler crab hosts. Dunn et al. (1989) reports on excystation of the metacercariae from fiddler crab (*Uca pugilator*) hosts.

Geographic distribution: Although our samples are exclusively from San Diego, the species is almost certainly wide ranging, potentially at least throughout the entire range of their first intermediate host, the California horn snail, from San Francisco to Baja California. Huspeni (2000) identified *P. uca* in *C. californica* and *C. mazatlanica* snail hosts from 7 estuaries ranging from Bolinas Lagoon (Northern California) to Bahia de Magdalena (Southern Baja California).

The original description of *P. uca* comes from metacercariae originating from sporocysts in California horn snails collected in Newport Bay, California (Sarkisian 1957). *Probolocoryphe*

uca has also been reported from Kuwait Bay, Kuwait (Al-Kandari & Al-Bustan 2010) and Florida, USA (Dunn et al. 1989) but we lack the molecular analysis necessary to confirm that these are truly the same species as *P. uca* in the California horn snail.

Table 4.2: Morphometrics for *Probolocoryphe uca* sporocysts and cercariae. All values represent the range followed by mean ± SD. Units are in microns for all except cercariae count and germ ball/embryo count. Sample size is 50 for both stages.

stage	character	*Probolocoryphe uca*
sporocyst	body L	240 - 700 (480.6 ± 97.5)
	body W	110 - 400 (247.2 ± 62.4)
	body H	110 - 370 (235.0 ± 58.4)
	cercariae count	0 - 14 (8.0 ± 3.3)
	germ ball & embryo count	3 - 27 (10.9 ± 5.2)
cercaria	body L	150 - 190 (166.0 ± 9.0)
	body W	50 - 70 (59.8 ± 3.9)
	body H	30 - 50 (40.4 ± 2.8)
	tail L	140 - 280 (212.6 ± 32.8)
	tail W	20 - 30 (20.2 ± 1.4)
	tail H	20 - 20 (20.0 ± 0.0)
	stylet L	22.5 - 27.5 (24.4 ± 1.2)
	oral sucker L	35 - 42.5 (38.7 ± 1.9)
	oral sucker W	27.5 - 37.5 (33.1 ± 2.5)
	oral sucker H	25 - 25 (25.0 ± 0.0)
	excretory bladder L	7.5 - 20 (14.5 ± 3.2)
	excretory bladder W	12.5 - 25 (14.9 ± 2.9)

Remarks

The cercariae of *P. uca* well fit within the microphallid cercaria diagnosis from Cable (1960). The molecular results (see below) confirm that this is a species of microphallid belonging in the subfamily Maritreminae. As expected, the metacercaria morphometrics collected from wild-infected fiddler crabs are comparable to the morphometrics reported in the original description (Table 4.3). Slight differences between the morphometrics are likely due to a combination of 2 factors: 1) Sarkisian measured living specimens while ours were formalin-fixed; 2) Sarkisian only provided measurements for 60-day old specimens while our

measurements are derived from wild infections that are representative of a range of ages, likely including some younger than 60 days.

Heard & Sikora (1969) proposed that *P. uca* may be a synonym of *Probolocoryphe lanceolata* (previously *P. glandulosa*) based on their similar morphology. However, Sarkisian (1957), while noting the striking similarity of the 2 species, asserting that *P. uca* precocious metacercariae are unique from *P. lanceolata* in their unlobed ovary, unlobed and regular testes, vitellaria in an inverted-U configuration, and presence of a fertilization chamber (Sarkisian 1957). These differences are sufficient to distinguish the 2 species, so they currently remain recognized as being distinct.

A provisional description of *P. uca* cercariae and sporocysts was provided in Hechinger's (2019) guide to the trematodes infecting the California horn snail. *Probolocoryphe uca* sporocyst size has also been recorded by Garcia-Vedrenne et al. (2018). The morphometrics and remarks made in both Hechinger (2019) and Garcia-Vedrenne et al. (2018) are consistent with the information presented here. The cercariae have also been reported in the literature as "xiphidiocercaria" (Maxon & Pequegnat 1949), "large xiphidiocercaria" (Martin 1955), and "Microphallid 1" (Martin 1971) from the California horn snail.

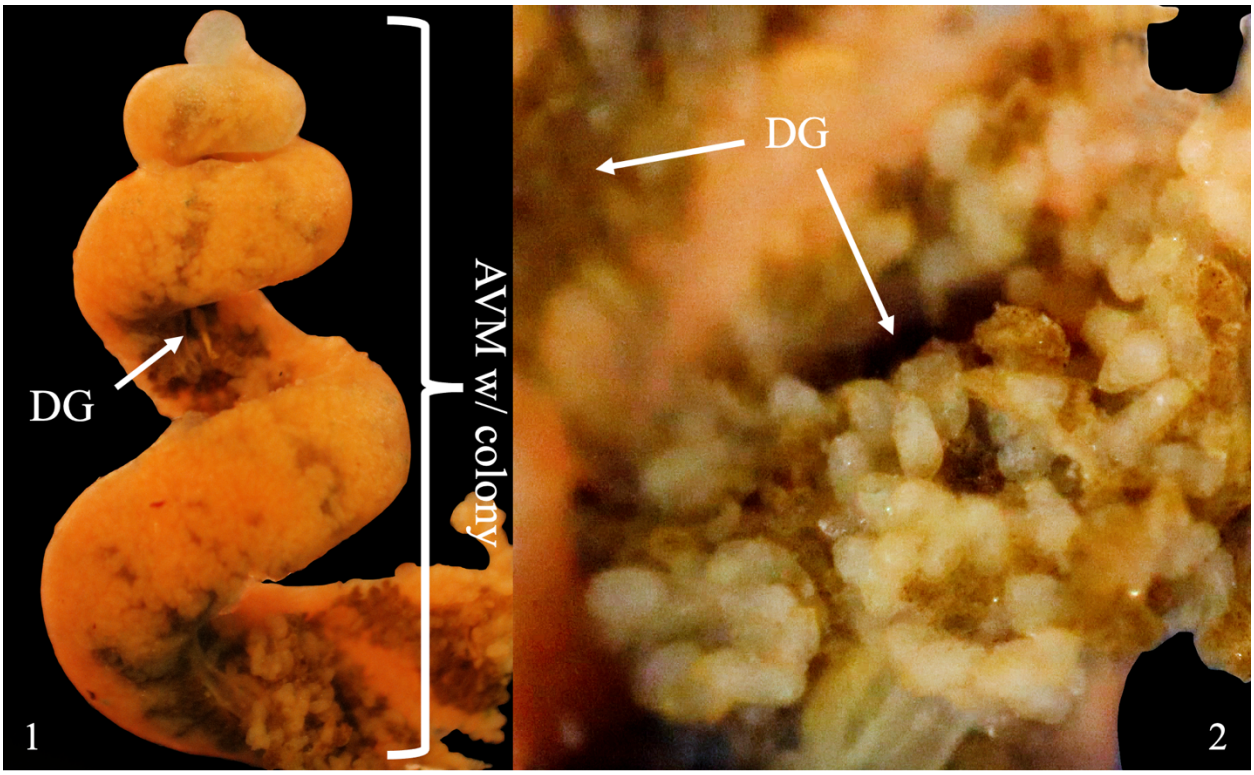

Figure 4.1: *Probolocoryphe uca* colony overview photos. (1) California horn snail removed from its shell. (2) Close-up view of *P. uca* sporocysts infiltrating the host snail digestive gland. AVM = apical visceral mass. DG = digestive gland.

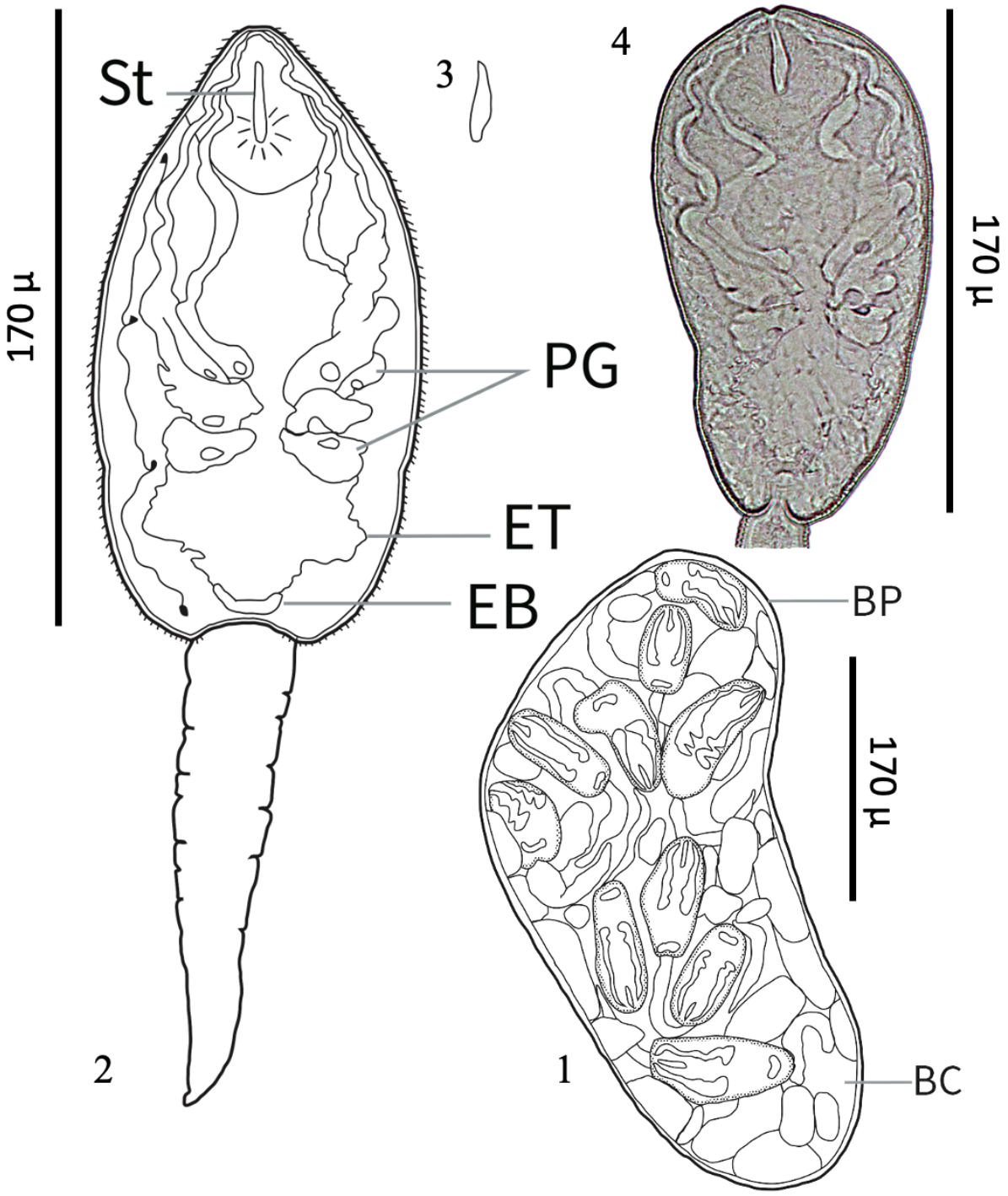

Figure 4.2: *Probolocoryphe uca* daughter sporocyst (1), ventral view of cercaria (2), sinistral view of oral stylet (3), and ventral view of cercaria squashed to permit sinistral view of oral stylet (4). The following abbreviations are used to label morphology: BC = body cavity, BP = birth pore, EB = excretory bladder, ET = excretory tubules, PG = penetration glands, St = stylet.

Remarks on *Probolocoryphe* sp. from *Pachygrapsus crassipes*

The excysted precocious metacercariae fit well the diagnosis for *Probolocoryphe* adults (DeBlock 2008). The encysted metacercariae bear a striking resemblance to those of *P. uca*, which may be why they were not distinguished from *P. uca* in previous studies (e.g. Lafferty & Dunham 2005; Hechinger et al. 2011). The encysted metacercariae of both species are 180 – 230 (207 ± 17.50, n = 10 for each species, total n = 20) in diameter, in agreement with the morphometrics reported by Sarkisian (1957). Morphological differences become more apparent in the excysted metacercariae. These differences are as follows: 1) *Probolocoryphe* sp. is 21% larger than *P. uca* in body length, 2) *Probolocoryphe* sp. individuals have vitelline fields forming a partially enclosed square-shaped ring around the cirrus, as opposed to inverse U-shaped vitelline fields 3) in *Probolocoryphe* sp. the ventral sucker is circular in shape, while in *P. uca* it is wider than long, 4) *Probolocoryphe* sp. individuals have less prominent tegumental spines, 5) *Probolocoryphe* sp. individuals have a more narrow rostellum relative to their body size, and 6) *Probolocoryphe* sp. individuals have irregular testes, while the testes of *P. uca* are smooth and rounded.

Based on the morphology it is very likely that *Probolocoryphe* sp. is *P. lanceolata*, which was originally described from a little blue heron, *Florida caerulea caerulea*, in Oaxaca, Mexico (Coil 1955). *Probolocoryphe lanceolata* has also been found in clapper rails, *Rallus longirostris*, in Alabama and Florida (Heard 1970) and racoons, *Procyon lotor*, in Florida (Heard 1970), both of which are common in California estuaries. Additional molecular work is needed to confirm that *P. lanceolata* occurs in California and is, in fact, *Probolocoryphe* sp. from shore crab second intermediate hosts.

Table 4.3: Morphometrics for *Probolocoryphe uca* excysted metacercariae from wild-infected fiddler crabs, *Probolocoryphe* sp. excysted metacercariae from shore crabs, and *P. uca* metacercariae from Sarkisian's (1957) experimentally infected fiddler crabs. Values represent the range followed by mean ± SD. Sarkisian (1957) only provided single measurements from a 60 day-old specimen. Units are in microns. Sample size is 15 for fiddler crab metacercariae and 20 for shore crab metacercariae.

character	*Probolocoryphe uca* in *Leptuca crenulata*	*Probolocoryphe* sp. in *Pachygrapsus crassipes*	*P. uca* from *Leptuca crenulata* in Sarkisian (1957)
body L	250 - 320 (282.7 ± 22.8)	270 - 410 (342.5 ± 38.8)	500
body W	110 - 150 (129.3 ± 12.8)	120 - 170 (145.5 ± 16.1)	N/A
body H	50 - 70 (58.3 ± 7.7)	55 - 90 (74.3 ± 10.0)	N/A
oral sucker L	35 - 47.5 (41.3 ± 3.4)	40 - 55 (46.3 ± 4.8)	N/A
oral sucker W	35 - 50 (39.2 ± 3.6)	37.5 - 47.5 (41.9 ± 3.3)	62
oral sucker H	35 - 40 (37.5 ± 2.5)	35 - 40 (37.8 ± 2.6)	N/A
ventral sucker L	30 - 42.5 (35.7 ± 3.9)	35 - 45 (39.8 ± 3.3)	N/A
ventral sucker W	32.5 - 47.5 (36.8 ± 3.9)	35 - 47.5 (40.3 ± 3.3)	46
ventral sucker H	30 - 35 (31.3 ± 2.3)	30 - 35 (34.0 ± 2.1)	N/A
pre-pharynx	12.5 - 30 (22.2 ± 6.5)	12.5 - 35 (23.4 ± 6.0)	25
pharynx L	25 - 30 (27.5 ± 2.1)	22.5 - 30 (26.9 ± 2.5)	30
pharynx W	20 - 25 (21.2 ± 1.6)	20 - 25 (22.9 ± 2.0)	23
pharynx H	20 - 25 (20.3 ± 1.3)	20 - 25 (22.0 ± 2.5)	N/A
esophagus L	5 - 25 (15.5 ± 5.4)	10 - 22.5 (15.8 ± 3.5)	25
ceca L	45 - 60 (51.3 ± 4.6)	45 - 60 (51.9 ± 3.9)	60 - 68
excretory bladder L	50 - 90 (69.3 ± 12.0)	60 - 87.5 (75.4 ± 7.0)	70
excretory bladder W	7.5 - 20 (13.8 ± 3.8)	10 - 22.5 (16.8 ± 3.5)	25
right testis L	35 - 55 (45.7 ± 6.2)	35 - 55 (47.0 ± 5.8)	94
right testis W	35 - 50 (42.8 ± 4.5)	40 - 52.5 (46.0 ± 4.1)	76
left testis L	32.5 - 52.5 (42.7 ± 5.1)	32.5 - 47.5 (39.4 ± 3.8)	85
left testis W	32.5 - 50 (42.8 ± 4.7)	40 - 52.5 (46.5 ± 3.5)	68
ovary L	22.5 - 27.5 (24.8 ± 2.0)	22.5 - 30 (26.6 ± 2.6)	N/A
ovary W	22.5 - 30 (24.7 ± 2.5)	22.5 - 30 (26.6 ± 3.0)	52
vitellaria arms L	52.5 - 87.5 (74.5 ± 10.6)	80 - 115 (94.9 ± 10.9)	150
collar W	47.5 - 60 (54.7 ± 4.1)	50 - 62.5 (55.9 ± 3.7)	N/A

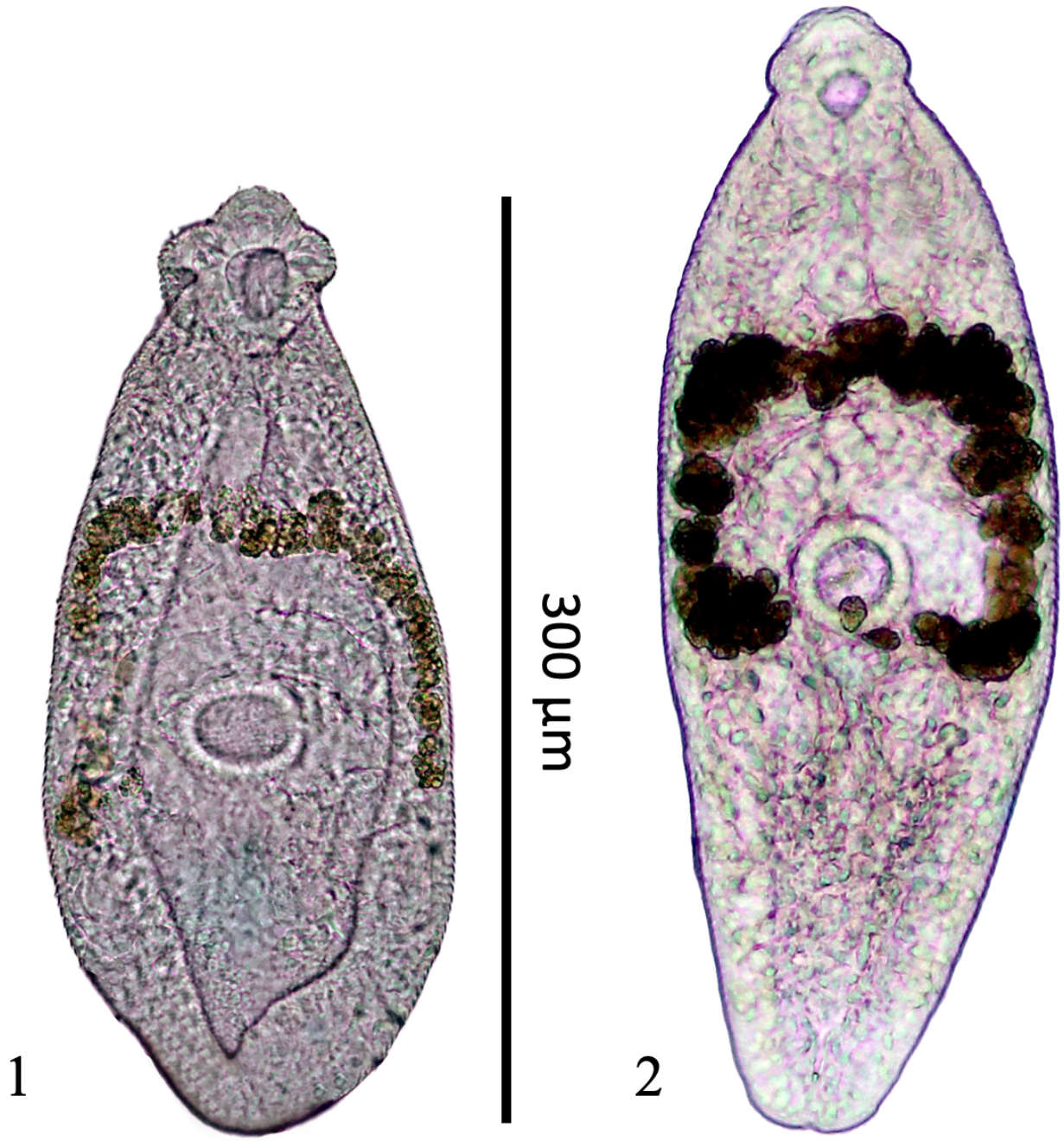

Figure 4.3: Ventral view of *Probolocoryphe uca* excysted metacercariae from *Leptuca crenulata* (1) and *Probolocoryphe* sp. excysted metacercariae from *Pachygrapsus crassipes* (2). Specimens are live and not stained.

Molecular results

The CO1 alignment was 461 base pairs in length. In the CO1 phylogeny (Figure 4.4), the *P. uca* sequences from 17 unique colonies in first intermediate host snails and those from 2 metacercariae from fiddler crabs form a distinct monophyletic clade. Some *P. uca* colonies had between 1 and 3 single base pair mutations (maximum divergence of 1.10%). The BI analysis produced a tree congruent to the maximum likelihood analysis. Unfortunately, we were not able to successfully sequence the cryptic *Probolocoryphe* species at the CO1 locus.

The 28S alignment was 890 base pairs in length. In the 28S phylogeny (Figure 4.5) the *P. uca* sequences from 5 unique colonies in first intermediate host snails and the *P. uca* sequences from 5 metacercariae from fiddler crabs form a distinct clade with high bootstrap support. The sequences of 3 metacercariae from shore crabs, however, form a distinct sister clade to *P. uca* with 1.83% divergence. This divergence value is greater than the divergence observed between cryptic species of *Cloacitrema* (see Chapter 1) and *Parorchis* (see Chapter 2). Both *P. uca* and the cryptic species group sister to members of the genus *Maritrema*, with minimum divergence of 7.7% from the closest related species, *M. subdolum*. In our 28S analysis, the same relationships between taxa were recovered as in Tkach et al. (2003). The BI analysis produced a tree congruent to the maximum likelihood analysis. Unfortunately, none of the sequences for our unidentified microphallid adults were identical to the 28S sequences of *P. uca* or *Probolocoryphe* sp.

In the 28S phylogeny, "small microphallid" clearly groups within the genus *Maritrema*. "Small microphallid" groups closely with *Maritrema heardi* (previously *Floridatrema herardi*) with 0.4% divergence. "Small microphallid" also groups closely with an unidentified microphallid adult from a marbled godwit, *Limosa fedoa*, in our wild bird data set with 0.7%

divergence. Additional morphological analysis is needed in order to confirm the identity of "small microphallid" as *Maritrema heardi*, but we can now confidently place it within the genus *Maritrema*.

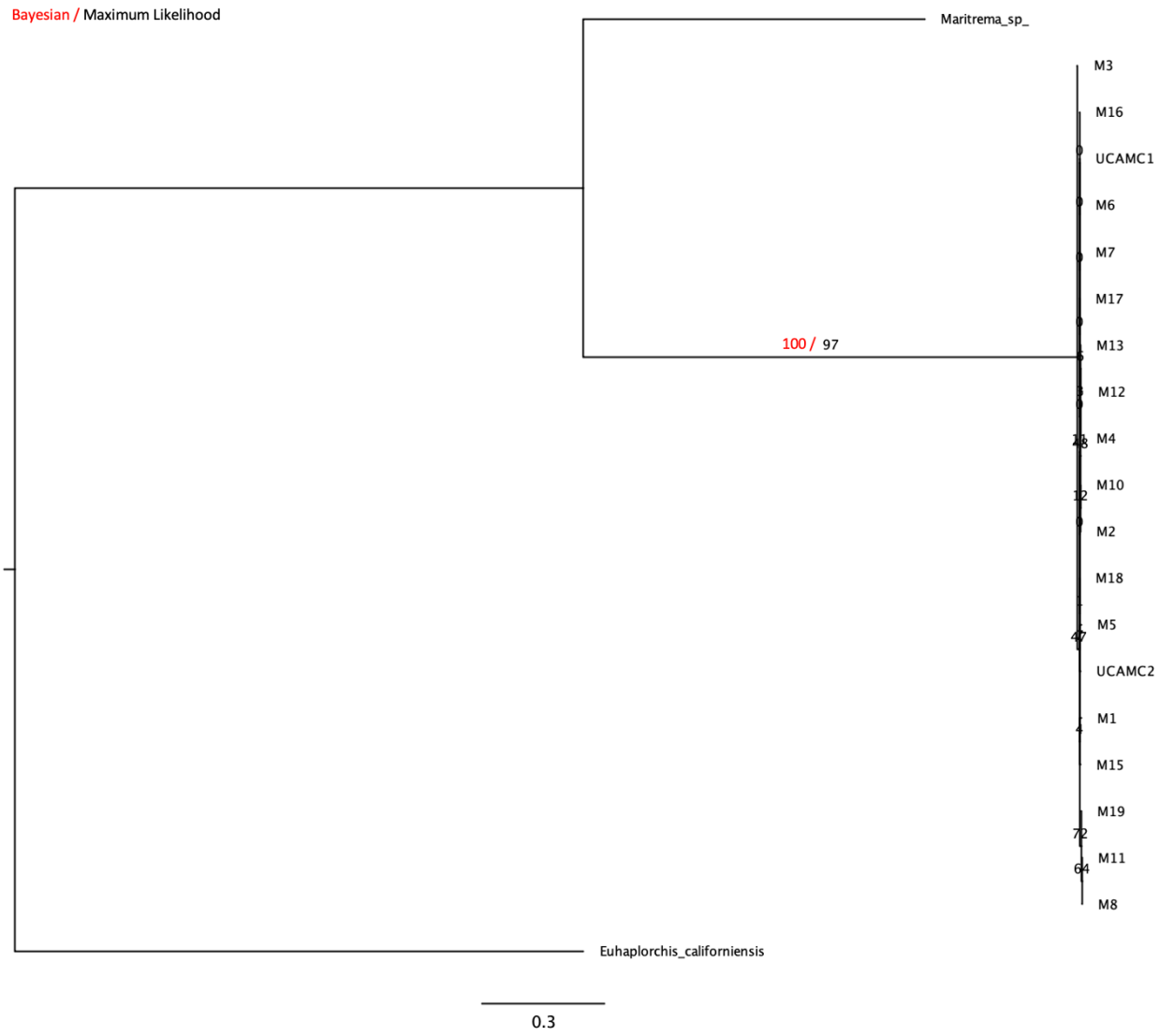

Figure 4.4: CO1 phylogenetic tree for *Probolocoryphe uca* individual colonies (PRUC in the tree) and *Probolocoryphe uca* metacercariae from fiddler crab second intermediate hosts (UCAMC in the tree). Red values represent Bayesian Inference branch lengths and black values represent Maximum Likelihood branch lengths. Numbers after species names designate unique colonies from which the sequences derive or unique second intermediate hosts (for the metacercariae) from which the sequences derive.

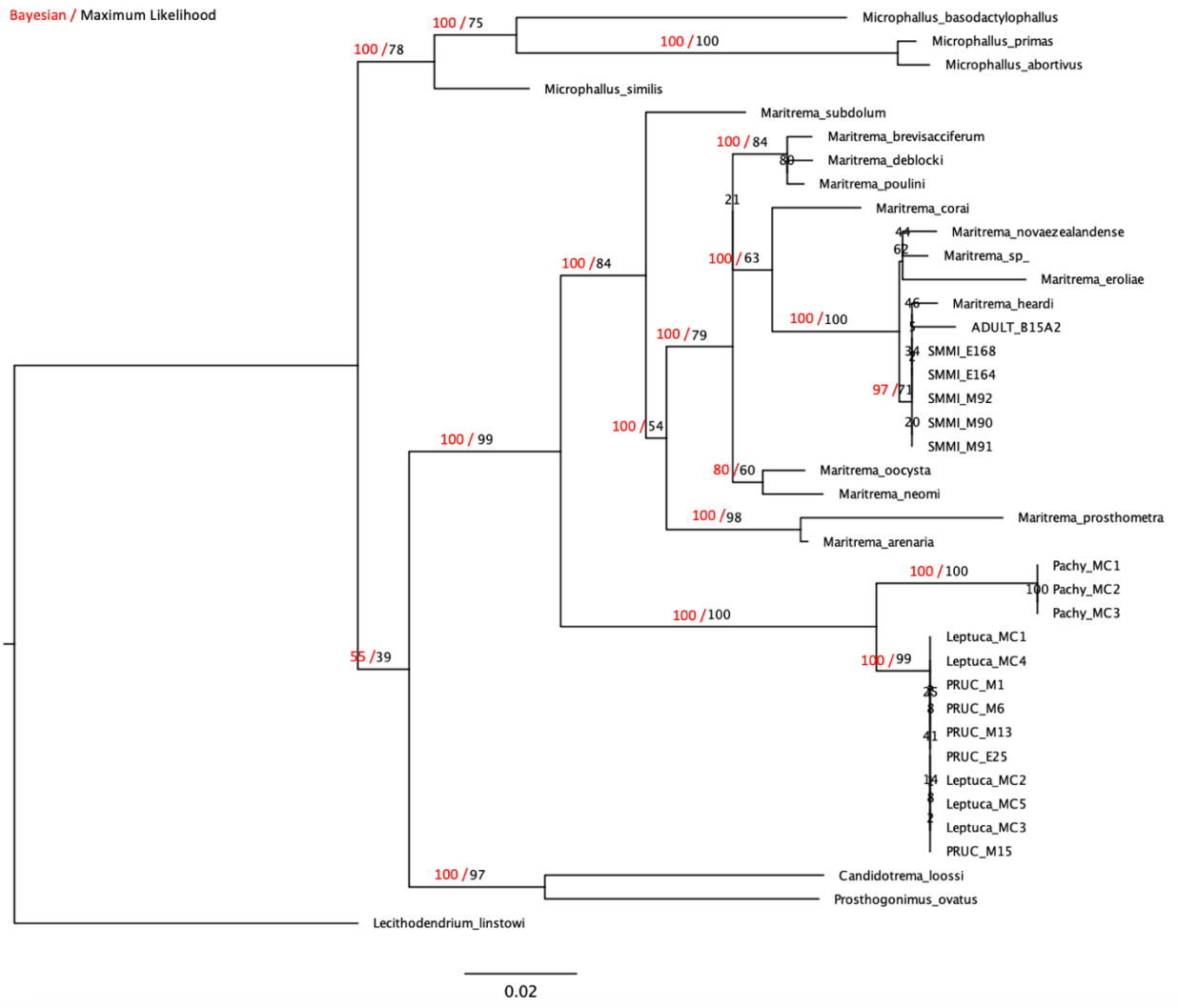

Figure 4.5: 28S phylogenetic tree for *Probolocoryphe uca* individual colonies (PRUC in the tree), *Probolocoryphe uca* metacercariae from fiddler crab second intermediate hosts (Leptuca_MC in the tree), *Probolocoryphe* sp. metacercariae from shore crab second intermediate hosts (Pachy_MC in the tree), and "small microphallid" individual colonies (SMMI in the tree). Red values represent Bayesian Inference branch lengths and black values represent Maximum Likelihood branch lengths. Numbers after species names designate unique colonies from which the sequences derive or unique second intermediate hosts (for the metacercariae) from which the sequences derive.

DISCUSSION

Here, we thoroughly describe *P. uca* daughter sporocysts and cercariae from wild-infected California horn snails. We obtained and analyzed genetic data from 17 unique *P. uca* colonies to produce CO1 and 28S phylogenetic trees. For those same trees, we obtained and

analyzed genetic data from 7 wild-infected second intermediate host fiddler crabs and 3 wild-infected second intermediate host shore crabs, which unveiled the presence of an undocumented cryptic *Probolocoryphe* species. Based on the morphology, this species is likely *P. lanceolata*, which is known from the Gulf of Mexico. We provide the first CO1 and 28S sequences for any *Probolocoryphe* species and recommend additional molecular work be done on the genus to confirm the presence of *P. lanceolata* in California. Our final host experimental infections were unsuccessful, but we were able obtain morphometrics of precocious excysted wild *P. uca* and *Probolocoryphe* sp. metacercariae to compare our specimens to those originally described by Sarkisian (1957).

All 17 of the *Probolocoryphe* colonies collected from Kendall-Frost California horn snails were *Probolocoryphe uca*, which presents the question: where are *Probolocoryphe* sp. metacercariae coming from? Most likely, greater sampling effort would reveal that *Probolocoryphe* sp. also uses horn snails as first intermediate host. However, we cannot discount the possibility that *Probolocoryphe* sp. uses a different first intermediate host, as there is an undescribed microphallid that uses *Assiminea californica* in California estuaries (Fowler 1977; RFH unpub. obs).

The inability of *P. uca* to infect final host chickens and ducks is surprising given the fact that members of the family Microphallidae typically parasitize bird final hosts (Cable 1960; DeBlock 2008), but not improbable since chickens and ducks are not their natural hosts. Further, observations of large numbers of crab exoskeletons near raccoon latrines (Lafferty & Dunham 2005), the unsuccessful bird experimental infections in the present study and by Sarkisian (1957), and the absence of *Probolocoryphe uca* and *Probolocoryphe* sp. from Dr. Sheehan's wild bird data set (see molecular results) are all evidence that raccoons or other mammals may serve

as the final host for both *Probolocoryphe* species in California. Future studies should investigate the identity of *Probolocoryphe* sp. in relation to *P. lanceolata* and explore the potential of mammals as a final host for both *Probolocoryphe* sp. and *P. uca*.

With our 28S phylogenetic analysis, we can confidently place “small microphallid” from the California horn snail (see Hechinger 2019) into the genus *Maritrema*. Additional morphological and life cycle work is needed to formally describe the species and differentiate it from the closely related *Maritrema heardi*, but this newfound taxonomic clarity will facilitate these types of future studies.

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Chapter 4, in part, is currently being prepared for submission for publication of the material. Nelson, Alexandria; Hechinger, Ryan. The thesis author was the primary investigator and author of this material.

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