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# How parasite exposure and time interact to determine *Australapatemon burti* (Trematoda: Digenea) infections in second intermediate hosts (*Erpobdella microstoma*) (Hirudinea: Erpodellidae)

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ABSTRACT

*Australapatemon* spp. are cosmopolitan trematodes that infect freshwater snails, aquatic leeches, and birds. Despite their broad geographic distribution, relatively little is known about interactions between *Australapatemon* spp. and their leech hosts, particularly under experimental conditions and in natural settings. We used experimental exposures to determine how *Australapatemon burti* cercariae dosage (number administered to leech hosts, *Erpobdella microstoma*) affected infection success (fraction to encyst as metacercariae), infection abundance, host survival, and host size over the 100 days following exposure. Interestingly, infection success was strongly density-dependent, such that there were no differences in metacercariae load even among hosts exposed to a 30-fold difference in cercariae. This relationship suggests that local processes (e.g., resource availability, interference competition, or host defenses) may play a strong role in parasite transmission. Our results also indicated that metacercariae did not become evident until ~4 weeks post exposure, with average load climbing until approximately 13 weeks. There was no evidence of metacercariae death or clearance over the census period. Parasite exposure had no detectable effects on leech size or survival, even with nearly 1,000 cercariae. Complementary surveys of leeches in California revealed that 11 of 14 ponds supported infection by *A. burti* (based on morphology and molecular sequencing), with an average prevalence of 32% and similar metacercariae intensity as in our experimental exposures. The extended development time and extreme density dependence of *A. burti* has implications for studying naturally occurring host populations, for which detected infections may represent only a fraction of cercariae to which animals have been exposed. Future investigation of these underlying mechanisms would be beneficial in understanding host-parasite relationships.

## 1. Introduction

In digenetic trematodes, infection of the second intermediate host can be affected by host immunity, intra- and interspecific interactions among parasites, and abiotic factors (Désilets et al., 2015 and references therein). Understanding whether and how such factors structure parasite populations is a daunting task that may not generalize across host taxa or the diverse range of transmission and encystment strategies employed by digeneans. Among digenean-intermediate host systems that remain poorly studied are those in which metacercariae are formed in leeches, such as members of the genus *Australapatemon*. *Australapatemon* is a genus of digenetic trematodes distributed widely throughout North and South America, Europe, Australia, and Africa. It commonly infects birds in the family Anatidae, which includes ducks, geese, and swans (Jones et al., 2005; Davies and Ostrowsk de Núñez, 2012). Sudarikov (1959) created *Australapatemon* to accommodate a species of *Apatemon* described by Johnston (1904), but Dubois and Pearson (1965) considered *Australapatemon* a subgenus of *Apatemon* (Dubois, 1953, 1968). Yamaguti (1971) and later authors recognized as a distinct genus (see also Niewiadomska, 2002) – a classification that has been subsequently supported with genomic data (summarized in Blasco-Costa et al., 2016; Gordy et al., 2017; Hernández-Mena et al., 2014). *Australapatemon* has a well-defined genital cone and ridged hermaphroditic duct as an adult (Niewiadomska, 2002) and uses leeches as

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second intermediate hosts, whereas *Apatemon* uses fishes and the smooth hermaphroditic duct of the adult lies within a less defined genital cone (Blasco-Costa et al., 2016). At present, 10 species of *Australapatemon* are recognized: *A. niewiadonmski*, *A. answeris*, *A. burti*, *A. minor*, *A. bdellocystis*, *A. canadensis*, *A. congolensis*, *A. fuhramanni*, *A. intermedius*, *A. mclaughlini*, and *A. magnacetabulum* (Gordy et al., 2017).

The life cycles of species in the genus *Australapatemon* generally involve freshwater snails, aquatic leeches, and birds (Faltýnková et al., 2007; Karvonen et al., 2017; Stunkard et al., 1941; Willey and Rabinowitz, 1938). Miller (1923, 1927) described *Cercaria burti* (now *Australapatemon burti*) from *Helisoma* (previously *Planorbis*) *trivolvis*; the same type of cercariae were detected by Cort and Brooks (1928) in the same part of northern Michigan in both *H. trivolvis* and *Lymnaea humilis* (previously *Lymnaea humilis modicella*). Subsequent studies have consistently reported trematodes in this genus to be relative generalists in their use of snail first intermediate hosts, in contrast to many trematodes (Aksenova et al., 2016; Gordy et al., 2017; Schell, 1985). For instance, *Australapatemon burti* has been detected from at least 11 different snail species in Europe, Canada, and the United States (Aksenova et al., 2016; Gordy et al., 2017). Furcocercous cercariae (forked tail) are released from infected snails and invade the tissue of freshwater leeches (Niewiadomska, 2002; Stunkard et al., 1941). Ultimately, infected leeches are ingested by definitive bird hosts (Anatidae), wherein metacercariae develop into adults and pass eggs into the host feces 14 days post-infection (Drago and Lunaschi, 2016; Faltýnková et al., 2007; Stunkard et al., 1941).

Leeches offer a useful experimental system in which to examine interactions between infectious cercariae and their second intermediate hosts (Karvonen et al., 2017; McCarthy, 1990; Stunkard et al., 1941). Beginning with detailed studies of the life cycle of *Australapatemon burti* in the 1940s, Stunkard (1941) showed that cercariae passively contact leech hosts in the water column, penetrate the host tegument, and subsequently develop into metacercariae within the vascular system. Leech species also vary considerably in susceptibility. For instance, Karvonen et al. (2017) experimentally exposed two species of European leeches (*Erpobdella octoculata* and *Helobdella stagnalis*) to 30 cercariae of *Australapatemon* sp. After 35 days, *E. octoculata* was both more likely to be infected than *H. stagnalis* (80% prevalence vs. 20%) and supported 2.5× more metacercariae per infected host. Experimental studies have further revealed that the metacercariae of *Australapatemon* spp. often have an extended, within-host development period before they are readily detectable and/or infectious to subsequent hosts. By pressing a leech between two glass microscope slides, the number of metacercariae per host can be quantified non-lethally and thus tracked in the same individual host through time (Choo, 2009; Davies and Ostrowsk de Núñez, 2012; Iles, 1959; Karvonen et al., 2017). This approach indicates that metacercariae within leeches typically require between 25 and 42 days to develop completely, frequently with little evidence of infection prior to 25 days (e.g. Choo, 2009; Karvonen et al., 2017; Stunkard et al., 1941). In the exposures conducted by Karvonen et al. (2017), 25% of metacercariae were visible after 32 days, 90% after 42 days, and 100% after 56 days (the last census performed). Although variation in leech host susceptibility to *Australapatemon* spp. has been explored, less research has been devoted to understanding how exposure dosage and time post-exposure interact to determine observed infection load, especially for parasites from western North America.

The current study aims to extend and deepen experimental research on interactions between *Australapatemon burti* and its leech intermediate host (*Erpobdella microstoma*). Specifically, we used multi-dose experimental exposures to (1) determine how cercarial infection success (%) and the resulting abundance of metacercariae in leech hosts varied with dosage (4 dosages), (2) evaluate the consequences of varying cercarial exposure for leech survival and body size, and to (3) understand fine-scale temporal variation in developing metacercariae (weekly observations of each host over 15 weeks), including when the observed load per host asymptotes or saturates. By taking advantage of non-lethal methods to assess infections through time on individual hosts, we quantified how the metacercarial accumulation varied both by time and by cercarial exposure dosage. Our experimental assays were complemented with field-based surveys to characterize patterns of infection (prevalence and intensity) within leeches from California ponds, and to compare these values with those documented experimentally. Understanding how exposure dosage and time interact to shape infection and pathology in intermediate hosts is an essential challenge for evaluating parasite transmission in natural systems.

## 2. Materials and methods

### 2.1. Study area and field collections

During the summer of 2017 (May 22nd through July 11th), we opportunistically sampled 14 ponds in the East Bay region of California within Contra Costa, Alameda, and Santa Clara counties. Ponds were selected from within parks or other land-owning agencies (East Bay Regional Parks District, University of California Reserve System, Santa Clara County Parks, and Easy Bay Municipal Utility District [see Table 1). At each location, a haphazard subset of *E. microstoma* was collected for dissection (range 1–50 depending on availability, mean = 17, SE = 4.31). Leeches were collected during standardized dipnet sweeps (D-frame with 900 μm mesh) through the littoral vegetation and substrate approximately every 10–20 m around the perimeter of the pond. Contents of each dipnet were examined for leeches and any collected samples were subsequently stored on ice before being examined in the laboratory. In the laboratory, leeches were nonlethally examined by gently pressing them between the top and bottom of a gridded Petri dish under an Olympus SZX10 stereomicroscope (range in magnifications from 40× to 60×). If *Australapatemon* spp. metacercariae were present, the number and position of infection in the body of the leech were noted. Leech size (length and width) was measured using digital calipers while under consistent pressure of a gridded Petri dish. Leeches were heat-killed with 100 °C boiling water. Voucher specimens were identified according to the keys of Klemm (1982) and Moser et al., (2006) and deposited in the Smithsonian Institution, National Museum of Natural History, Washington, D.C. (USNM 1618945–1618947).

To assess *Australapatemon* spp. infection in first intermediate snail hosts, we collected ~100 (mean = 146) snails from the same 14 ponds using standardized dipnet sweeps as described above. Snails were identified as *Helisoma trivolvis* based on characteristics outlined by Burch (1982): "shell spire (left side) strongly inverted, with a more or less deep conical depression, and spire side of body whorl with or without a strong keel". Additional genetic analyses from samples in this area suggest they are all consistent with *Helisoma* (*Planorbella*) *trivolvis* (Martin et al., 2020). We measured apex to rim of the outer rim of aperture and dissected each snail and identified parasitic infections using cercaria morphology (Schell, 1985). Specifically, snails were gently cracked and tissues teased apart from the shell with forceps. The internal organs and gonads were examined for rediae, sporcoysts, and mature cercariae. Heavy parasite infections were vouchered in 95% ethanol for molecular analysis (see below for details).

### 2.2. Experimental exposure studies

We conducted experimental exposure trials to determine the susceptibility of *E. microstoma* to cercariae of *Australapatemon* sp. as a function of exposure dosage and time post-exposure. We collected leeches (n = 50) from a pond at Vargas Plateau Regional Preserve in which no infection was detected among previously examined leeches and suitable snails were lacking. All collected individuals were also assessed nonlethally for metacercariae (using the method above) before use in the experiment. Leeches were individually housed in plastic containers with 325 ml of treated water (UV-sterilized, carbon-filtered,

**Table 1**
Patterns of *Australapatemon* sp. infection from ponds sampled in the Bay Area region of California. Presented for each site is the number of examined leeches (*Erpobdella microstoma*) with visible infection/number dissected, with the prevalence of infection as a percentage. The average number of metacercariae per host (including zeroes) is listed as the infection load. Similarly, for dissected snails (*Helisoma trivolvis*), we show the number of snails with putative infections (based on the presence of sporocysts bearing strigeid-type cercariae) relative to the number dissected, and the prevalence as a percentage. Two sites did not support any *H. trivolvis* snails. Molecular sequencing helped validate the observed parasites as a species of *Australapatemon* (see manuscript for details).

| Site | Latitude | Longitude | Leeches infected | Leech prevalence | Infection load | Snails infected | Snail prevalence |
|---|---|---|---|---|---|---|---|
| CA-MUD66 | 37.92147 | −122.2269 | 0/1 | 0 | 0 | 0/99 | 0 |
| CA-MUD67 | 37.92211 | −122.2181 | 0/1 | 0 | 0 | 0/100 | 0 |
| GDPND004 | 37.61184 | −122.0044 | 20/24 | 83.3 | 3.79 | 7/65 | 10.77 |
| GDPND005 | 37.62254 | −122.0002 | 1/19 | 5.26 | 0.11 | 0/97 | 0 |
| PRNTH1 | 37.67092 | −121.9563 | 2/16 | 12.5 | 0.13 | 0/323 | 0 |
| PRNTHIDK | 37.65212 | −121.9519 | 29/49 | 59.2 | 4.00 | 8/202 | 3.96 |
| PRNTHOWL | 37.65944 | −121.9600 | 2/22 | 9.09 | 0.14 | 1/250 | 0.40 |
| PRPND010 | 37.64721 | −121.9193 | 0/1 | 0 | 0 | 3/330 | 0.91 |
| CA-SF19 | 37.29215 | −121.7063 | 2/11 | 18.2 | 1.27 | 0/11 | 0 |
| SF26 | 37.27889 | −121.6981 | 10/20 | 50.0 | 1.80 | 0/100 | 0 |
| TGIF | 37.63630 | −121.9131 | 0/5 | 0 | 0 | 1/248 | 0.40 |
| VPPND005 | 37.58979 | −121.9444 | 8/9 | 88.9 | 5.67 | 0/33 | 0 |
| PRPND001 | 37.59859 | −121.8884 | 0/4 | 0 | 0 | 0 | n/a |
| VPPND004 | 37.57819 | −121.9493 | 0/50 | 0 | 0 | 0 | n/a |

dechlorinated tapwater; hereafter referred to as "treated water") at 24 °C. Exposed and control leeches were fed laboratory-raised brine shrimp and had water changes weekly. *Australapatemon* sp. cercariae were harvested from infected *Helisoma trivolvis* snails collected from three ponds in the same study area. In brief, snails were isolated into 50 ml vials filled with 50 ml of treated water and examined for cercariae every 6 h over a 24 h period. Cercariae were identified based on morphological characteristics (e.g. forked tail) (Schell, 1985), for which a subsample were verified by genetic analysis (see molecular analysis below). Exposures occurred at 22 °C temperature and 12:12 h light:dark cycle.

For experimental exposures, 2 to 4 h old cercariae from *Australapatemon* sp. infected snails were pooled from among snails and sites (nine snails collected from three ponds) and aliquoted into one of four dosages: control (no cercariae [12 ml of treated water only], n = 20 replicate leeches), low dosage (39 cercariae; n = 10), medium dosage (170 cercariae; n = 10), and high dosage (947 cercariae, n = 10). Leeches were exposed at same time and maintained individually (i.e., no grouped housing). Dosages were determined by using a metered pipette to add 1 ml aliquots from the pooled cercariae suspension into conical tubes, for which each ml contained approximately 39 cercariae (±1 SE = 0.54). Dosages were determined by overall yield of cercariae collected and with the aim of generating broad variation in exposure. Three extra replicate aliquots were used to quantify cercarial quantities. Leeches were exposed individually in 250 ml containers with 12 ml of treated water. Eight hours post-exposure, we increased the volume to 100 ml; after 24 h, leeches were transferred into individual plastic containers with 325 ml of treated water and a rock for shelter. Collection of cercariae and exposure of leeches were performed identically across all doses. Water changes occurred weekly and leeches were fed brine shrimp daily.

Leeches were examined weekly for 15 weeks (105 days) by isolating individual leeches between the two sides of a gridded plastic Petri dish and gently applying pressure under a stereomicroscope with substage lighting to quantify metacercariae (Choo, 2009; Davies and Ostrowsk de Núñez, 2012; Karvonen et al., 2017). To validate this approach, counts of metacercariae were repeated twice per leech to assess consistency (mean = 2.3 counts per host). At 15 weeks, leeches were euthanized (using methods described above), measured to nearest mm (length and width), massed after blotting dry (wet mass), and final metacercariae counts were assessed using methods described above.

### 2.3. Genetic analysis

Genetic analysis was conducted on six cercariae samples from two snails collected at two ponds (three replicates per snail, containing 1–8 cercariae per sample), for which vouchers were deposited at the Museum of Southwestern Biology (Para:31,183 and MSB:Para:31,184). DNA extraction, amplification, and sequencing of the barcode region of the mitochondrial cytochrome *c* oxidase 1 (CO1) gene were performed using the protocols and degenerate Mplat primers of Moszczynska et al. (2009). The resulting six sequences were aligned with data from *Australapatemon* spp. from Hernández-Mena et al. (2014), Blasco-Costa et al. (2016) and Gordy et al. (2016, 2017) using Multiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar, 2004) in Geneious v.10.05 (Kearse et al., 2012). Similarity of all sequences across all nucleotides was represented in a neighbor-joining (NJ) phenogram. To assess monophyly of the NJ clusters, Bayesian (Huelsenbeck and Ronquist, 2001) and Maximum Likelihood (ML) (Stamatakis, 2014) analyses were conducted on an alignment of non-redundant CO1 sequences stripped of columns with gaps, using models of nucleotide evolution estimated with Bayesian Information Criterion in MEGA X (Kumar et al., 2018), or their nearest approximation. All codon positions in the alignment were used in ML and Bayesian analyses because of lack of evidence of nucleotide saturation (Iss < Iss.c, P = 0, Xia and Lemey, 2009; Xia et al., 2003).

### 2.4. Statistical analysis

Analytically, our goals were to assess how exposure to varying dosages of *A. burti* cercariae influenced the survival, growth, and observed number of metacercariae within leeches. We modeled the number of metacercariae per leech as an overdispersed Poisson distribution with a log-link using the lme4 package in R (Bates et al., 2015). As fixed effects, we included exposure dosage (0, 39, 170, 947) and time-post-exposure (days), with random intercept terms for leech identity (given the repeated observations per individual over 15 weeks) and an observation-level random effect to account for overdispersion. Numeric predictors were scaled and centered prior to inclusion in models. Leech hosts that died prior to the final observation date (n = 4) were omitted. Because we expected the influence of time to be nonlinear, showing saturation patterns beyond some date, we also ran a quadratic model with both time and time$^2$ as fixed effects.

To evaluate (a) whether the number of metacercariae per host differed among exposure treatments (omitting unexposed controls, all of which were uninfected), and (b) whether cercarial infection success per host differed by exposure treatments, we selected the final infection load per surviving leech on day 105 (week 15) as the response variable. We used an offset term representing the log of the number of cercariae added to convert infection load into a rate (i.e., infection success). This model included a fixed effect for exposure dosage and an observation-

level random intercept term, but without any influence of time or repeated observations per host (implemented in lme4 as described above). The effects of exposure dosage on leech survival and leech size (after $\log_{10}$-transformation) were tested using a binomial generalized linear model and a Gaussian linear model, respectively.

## 3. Results

### 3.1. Field collections

In total, 31.9% (74 of 232, 95% CI = 25.9–38.3%) of field-collected leeches (*E. microstoma*) were infected with metacerariae of *Australapatemon* sp. This included individuals from eight of the 14 sampled ponds; among ponds with at least 10 leeches dissected (n = 8), all but one exhibited evidence of infection. Four ponds supported an infection prevalence of 50% or higher (range: 50–88.9%; Table 1). For sites with at least one infected leech, the average infection load (including uninfected hosts) was 1.69 ± 0.66 (n = 8 ponds and 170 leeches), with a range of 0.11–5.7 metacercariae per leech. For individual leeches, infection intensity ranged from 1 to 22 (mean ± 1 SE = 5.34 ± 0.41; n = 74). Metacercariae were of the tetracotyle morphotype with a round or oval shape (Fig. 1). The cyst wall consisted of a thick outer wall and thin inner wall. Also distinguishable were a distinct forebody and hindbody as well as a holdfast organ, aligning well with previous morphological descriptions of the genus *Australapatemon* (Stunkard et al., 1941). Consistent with previous research, metacercariae were found in the parenchyma and musculature throughout the leech (Karvonen et al., 2017; Stunkard et al., 1941). In heavy infections, localized hemorrhaging was visible.

We also dissected 1,858 *Helisoma trivolvis* from 12 ponds (methods described above). Two examined ponds did not support any *Helisoma* spp., and did not exhibit *A. burti* infection in examined leeches. Snails infected with a strigeid trematode consistent in morphology to *Australapatemon* sp. from the description by Dubois (1968) were detected in 20 *H. trivolvis*. Forked-tail cercariae without eyespots or finfolds were associated with sporocyst stages in infected snails. Infection prevalence in *H. trivolvis* ranged from 0.4 to 10.8% (mean ± 1 SE = 1.37% ± 0.88). Among the seven ponds at which leeches with *Australapatemon* sp. were detected (with sample sizes of 10 or higher), strigeid infections were recorded in *H. trivolvis* from three ponds (i.e., putative *Australapatemon* sp. infections were detected in both first and second intermediate hosts).

### 3.2. Genetic analysis

Partial CO1 sequences (597–623 bp in length) from six samples of cercariae of *Australapatemon* sp. obtained from two *H. trivolvis* snails (GenBank accession numbers MT827011-MT827016; MSB:Para) varied by 0–2.5% (mean 1.46%), with no variation in translated amino acids. The most similar sequences in GenBank were those of *Australapatemon burti* LIN1 deposited for Gordy et al. (2017), which differed by 0–6.9% (mean 2.66%) from data we obtained (Fig. 2). As in Gordy et al. (2017), the large LIN1 clade contains clusters and unresolved subclades that drive high overall divergence (up to 6.5% divergence in *A. burti* LIN1 in Gordy et al., 2017). In an NJ phenogram, these clusters corresponded to host and geographic provenance, with data in the present study grouping with cercariae from planorbid snails in California, and the next most similar clade a group of sequences from planorbids in Alberta and eastern Canada. These two subclades are generally well supported, but nested within the large cluster of CO1 from cercariae collected from *Stagnicola elodes* in Alberta (Supplementary Figs. S1 and S2 and data S1 and S2)

### 3.3. Experimental exposures

*Australapatemon burti* infections in leech intermediate hosts varied over time and as a function of experimental dosage. While no unexposed (control) leeches exhibited any infection over the 15-week experiment (n = 20), 93% of exposed leeches supported one or more metacercariae by the end of the trial. There was no significant effect of cercarial exposure dosage on leech survival (binomial generalized linear model [GLM], P = 0.47) or on final leech body length and mass (LM on $\log_{10}$-transformed values; all P > 0.4 [see Supplementary data 3]). Four leeches died prior to the end of the trial, including two from the medium dose (170 cercariae) and one each from the high (947 cercariae) and low dosages (39 cercariae). These hosts were excluded from all further analyses.

Days post-exposure had a strong, positive influence on the number of metacercariae detected, regardless of exposure dosage (omitting the unexposed leeches). No metacercariae were observed prior to four weeks post-exposure (Fig. 3). This number increased monotonically over time, eventually saturating after 13 weeks across all doses except controls (Fig. 3). Thus, the best-supported statistical model included a quadratic term for time (overdispersed Poisson generalized linear mixed models [GLMM]; scale (days) = 5.24 ± 0.33, P < 0.00001; scale (days$^2$) = −3.24 ± 0.25, P < 0.00001), with no additional influence of exposure dosage (P = 0.12).

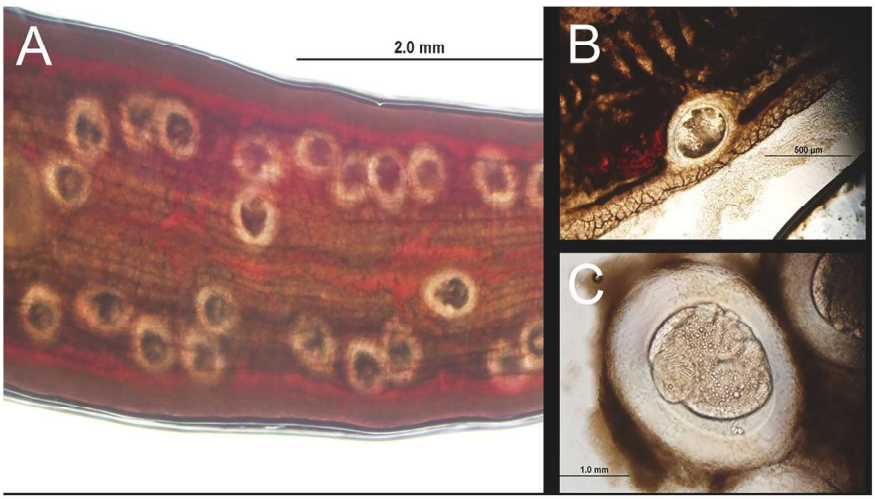


**Fig 1.** (A) A leech (*Erpobdella microstoma*) infected with metacercariae of *Australapatemon burti* post-experimental exposures; (B) metacercarial cysts within the tegument of an infected leech; and (C) a single encysted metacercaria.

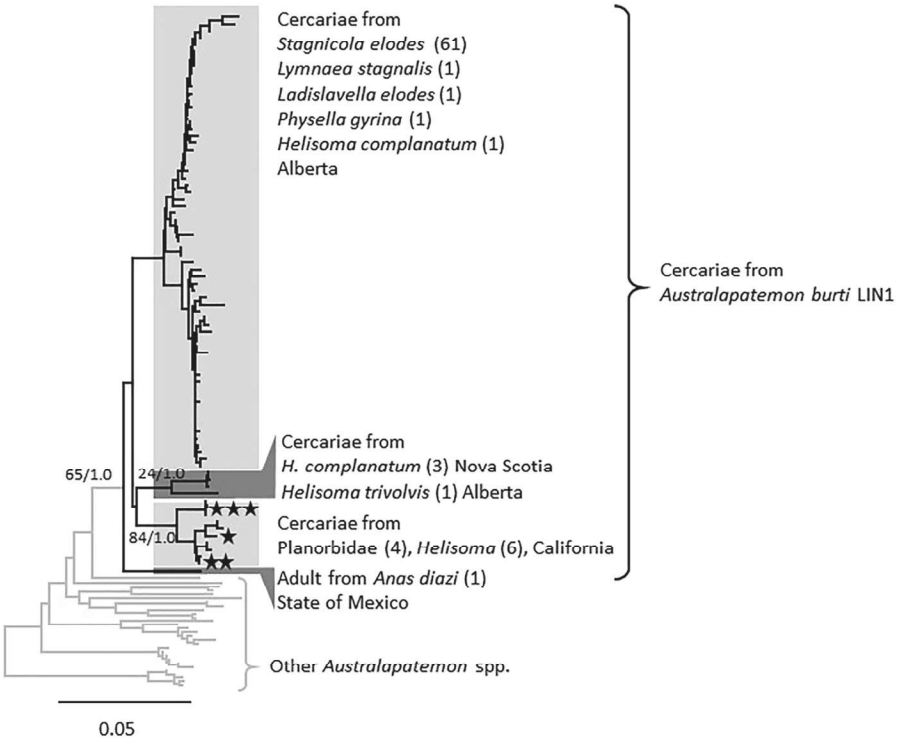


**Fig 2.** Neighbor-joining analysis of partial sequences of cytochrome *c* oxidase I from a species of *Australapatemon*, using all alignment sites. Stars indicate six sequences obtained in the present experimental study in comparison with cercariae shed by *Helisoma* spp. snails collected from California ponds. Shaded clusters within *A. burti* LIN1 are labeled by geographic provenance and hosts of material sequenced with number of replicates in parenthesis. Statistical support in separate maximum-likelihood and Bayesian analyses shown only for *A. burti* LIN1; lack of annotation indicates no support (proportion of 1000 ML bootstrap replicates/ posterior probability). *Australapatemon burti* LIN1 was distinguished from other putative and established species of *Australapatemon* by Gordy et al. (2017). Sequences from the present study are MT827011-MT827016; sequences from other studies are HM385485-6, HM385534-58, JX977725-8, KT334176-81, KT831346, KT831351, KY207548-624, KY587394, KY587396, KY587397-401.

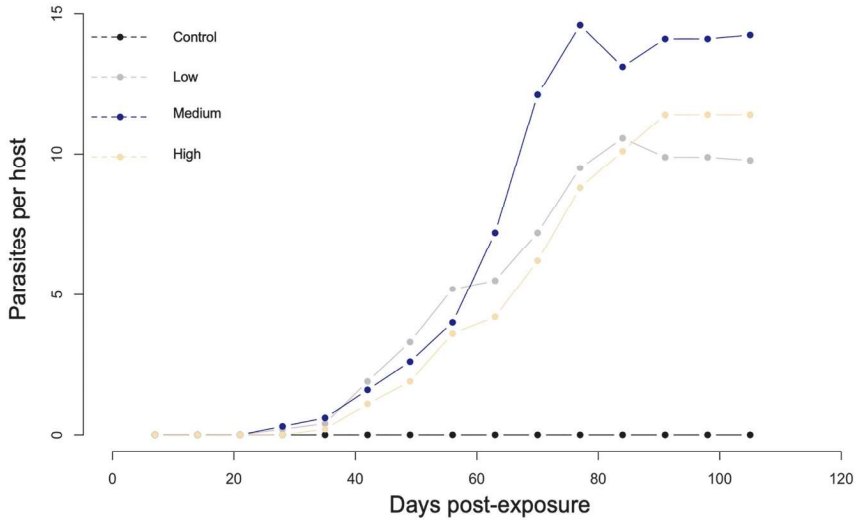


**Fig. 3.** The mean number of encysted *Australapatemon burti* metacercariae observed post-exposure as a function of exposure treatment (control [n = 0 cercariae], low [n = 39], medium [n = 170], high [n = 947]) in *Erpobdella microstoma* leeches. The total census period was 14 weeks with leech hosts examined individually every 1 week.

Overall infection success was low, such that only a small fraction (~10%) of administered cercariae formed metacercariae by the end of the experiment. Thus, after 15 weeks, there were no significant differences among exposed treatments in mean infection load (overdispersed Poisson GLMM: scale (dose) = −0.06 ± 0.14, P = 0.68), despite a nearly 25-fold variation in the initial dosage (Fig. 4). Across doses (excluding controls, all of which were uninfected), most leeches supported infection (25/27), with an average (±1 SE) of 11.70 ± 1.40 metacercariae after 15 weeks (range: 0–32). Converting these values to infection success with an offset term (i.e., the proportion of administered cercariae successful in forming metacercariae), parasite dosage had a sharply negative influence on infection success (overdispersed binomial GLMM: scale (dose) = −1.48 ± 0.18, P < 0.0001). More specifically, infection success decreased from 25% in the low dosage to 8.4% in the medium and 1.2% in the highest dosage). These observations indicate that either cercariae penetration or establishment is strongly density-dependent, leading to a parasite carrying capacity within the hosts.

## 4. Discussion

We detected *Australapatemon burti* infections in 74 *Erpobdella microstoma* leeches (second intermediate hosts) and 20 *Helisoma trivolvis* snails (first intermediate hosts) over a three-month sampling period of ponds in the Bay Area of California, representing one of the first field surveys for this parasite in the western USA. Of the 14 ponds sampled, 11 supported *A. burti* in snail or leech host species, with an average

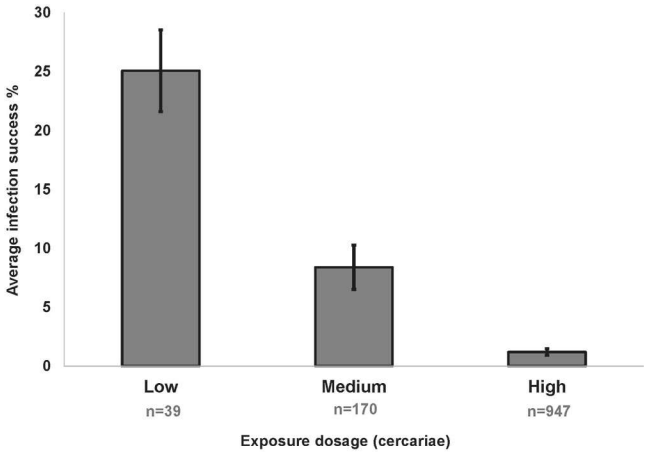


**Fig. 4.** Average infection success (%) ± 1 SE (as indicated with error bars) of *Australapatemon burti* cercariae in *Erpobdella microstoma* leeches at day 105 in relation to exposure dosage (low [n = 39], medium [n = 170], high [n = 947]), excluding controls (as none were infected) and any leeches that died prior to 105 days.

infection prevalence of 32% in leeches. Phylogenetic analysis of CO1 sequences from cercariae identified them as part of a lineage of *Australapatemon* spp. previously recorded from multiple snail genera in Canada (Alberta and Nova Scotia) and the USA (California, based on samples collected by our group) (Gordy et al., 2017). By comparison, researchers in Finland have reported that >35% of leech species in multiple lakes support *A. burti* infection, with a mean intensity of ~40 metacercariae per infected leech, compared with 2.1 metacercariae per host in the current study (Choo, 2009; Karvonen et al., 2017). We also detected intra-molluscan stages of infection within *H. trivolvis* that were consistent with published descriptions and genetic sequences of *A. burti*. Overall prevalence of infection in snails was low (~1%), and observed within only 3 of the 12 ponds where infected leeches were noted. *Australapatemon burti* has been reported from a wide variety of snail hosts (Gordy et al., 2017 and references therein), which suggests the other unexamined snail species (e.g., physid or lymnaeid snails) at our field sites may have concurrently hosted infection, thereby explaining the discrepancy with infected leeches. Alternatively, in light of the low prevalence observed in snails, we may not have sampled enough *H. trivolvis* in most ponds.

Phylogenetic analysis of CO1 gene sequences from our leech and snail infections identified them as a lineage of *A. burti,* one of nine sympatric species that (Gordy et al., 2017) recovered mainly in Alberta. Not all the species of *Australapatemon* that Gordy and colleagues (2017) distinguished genetically separated morphologically or through host affiliation, which is also true for related digeneans (Blasco-Costa and Locke, 2017). In about half of the studies reviewed by Blasco-Costa and Locke (2017), the number of species initially expected based on morphology or ecology differed from the detected with molecular data, with genetically delineated species richness usually exceeding morphospecies richness. The matching CO1 gene sequences from our experimental materials with that of other field-collected materials in a single, well-supported clade supports an important premise of our work, namely that all of cercariae studied herein belong to a single species belonging to *A. burti* LIN1 clade, which we view as species complex. Gordy et al. (2017) also considered that the large *A. burti* LIN1 clade may consist of multiple species. Longer CO1 gene sequences or data from other, more variable markers could resolve the status of species or lineages within *A. burti* LIN1.

Results from the experimental exposures of *Erpobdella microstoma* leeches provided detailed information on the temporal patterns and outcomes of infection within second intermediate hosts. While no adverse effects on growth or survival were observed, regardless of exposure dosage, we observed distinctive patterns in infection load and success as a function of dosage and time post-exposure. Using data gathered over 100 days post-exposure, our results confirmed the exceptionally long development time for metacercariae in leeches (Choo, 2009; Davies and Ostrowsk de Núñez, 2012; Stunkard et al., 1941; Willey and Rabinowitz, 1938). Based on repeated, non-lethal examinations of leeches, no metacercariae were evident until approximately four weeks post-exposure, and infection loads per host climbed thereafter until ~13 weeks post-exposure, despite no new cercariae being administered. These results are consistent with a previous study; Stunkard et al. (1941) reported 90% encystment by 40–42 days post-exposure. This delayed development of infection in second intermediate hosts has also been reported for amphibian larvae exposed to cercariae of *Clinostomum marginatum*, which exhibit metacercariae only after three weeks post-exposure (Calhoun et al., 2019). Whether early stage metacercariae are too small or too indistinct for earlier detection, or whether they reside in a different location inside the host, remains to be determined. These long developmental periods have important implications for field-examined animals: early examination of hosts could result in reporting of a false negative, for instance. This suggests that field surveys involving *Australapatemon* spp. require multiple sampling events or an extended post-collection holding period to provide an accurate assessment of infection prevalence and quantify burden. Even in our own research, we found that leeches with no evidence of infection subsequently exhibited metacercariae after a period of captivity in the laboratory (i.e., 1–2 weeks). The use of fluorescently labeled cercariae could provide further insights into this question, including where the parasites reside prior to detection (Keeney et al., 2008; LaFonte and Johnson, 2013; Leung and Poulin, 2011). Clearance of infection, in which the number of metacercariae decrease over time, was not detected during our experiment, in contrast to some other trematodes (Calhoun et al., 2015; LaFonte and Johnson, 2013; Stutz et al., 2019).

One of the most striking results from the varied dosage level exposures was the strong evidence for density-dependence in metacercarial establishment. Infection success, or the proportion of establishing parasites relative to the number of administered cercariae, decreased sharply with exposure dosage (Fig. 4). As a result, there were no actual differences in final infection load after 15 weeks, despite nearly 25-fold differences in the administered numbers of cercariae (range 39–947; Fig. 4). While other studies of trematode-host interactions have also reported a decrease in infection success with higher cercariae exposure (Koprivnikar et al., 2017; Orlofske et al., 2018), the extreme magnitude of the current result is noteworthy. By calculating the number of established metacercariae relative to the number of administered cercariae (i.e., infection success), we estimate that infection success decreased from ~25% in the lowest dose to ~1.2% in the highest treatment dosage. Using dosages of <30 cercariae per leech (albeit with different exposure methods), Choo (2009) reported 75% infection success in leeches (*Erpodella*) from Finland. These patterns raise intriguing questions about the dosages to which field-caught hosts are actually exposed. For instance, while we found similar infection loads in field-collected leeches (0–22) relative to the experimentally-exposed leeches (0–32), the strong density-dependence makes it difficult to determine the actual number of cercariae to which naturally occurring hosts were exposed; with such strong density dependence, it is possible that field-observed leeches were exposed to much larger numbers of cercariae. However, the rate of infection also warrents consideration as pulse infections (repeated exposures over time) have higher infection success than a single exposure (Lindell, et al., 2017).

We hypothesize that the observed density-dependent reductions in infection success could stem from at least two possible mechanisms: (1) a reduction in the initial penetration of cercariae into the host caused by competitions (i.e., 'cercariae interference') or shifts in host behavior (e.g. Stunkard et al., 1941) and/or, (2) a decrease in the post-penetration establishment of metacercariae through alterations in the innate immune response by the host (e.g., Fredensborg and Poulin, 2005; Loker,

2012, Pulze, et al., 2017). Previous researchers have noted that species of *Australapatemon* cercariae depend on passive encounters with their leech hosts (i.e., rather than active searching behaviors) (Stunkard et al., 1941), which could lead to a change in success rate if hosts reduce activity or movement following heavier infections. For instance, fathead minnow encounters with *Ornithodiplostomum* sp. cercariae decreased directly in response to host activity (James et al., 2008). Others have suggested intraspecific competition between encysting parasites. Hoverman et al. (2013) found that early encysting parasites reduced the encystment success of later arriving parasites by as much as 41%, which they reasoned was mediated by host immune responses and/or competition for space (Cox, 2001; Klemme et al., 2016; Poulin, 1999). Strong interspecific negative associations among metacercariae within and between spatially limited sites in fish eyes have also been attributed to competition for space and concomitant host immunity (Désilets et al., 2015). Similarly, crowding of trematodes can decrease overall infection load within hosts, delay parasite development time, or limit parasite body size (Fried and Nelson, 1978; Valero et al., 2006; Yao et al., 1991). For example, *Fasciola hepatica* in sheep exposed to 200 metacercariae has a pre-patent period (days to be infective) of 63 days, whereas in those exposed to 2000 metacercariae parasite development was delayed by 13–15 weeks (Valero et al., 2006).

The strong density-dependency results uncovered here indicate that fine-scale local process may play a strong role in this parasite's transmission to its second intermediate host (Esch et al., 2002, Fredensborg and Poulin, 2005). As a result, observed infection loads of metacercariae among field-collected leeches may yield relatively little insight about the dosage to which hosts have been exposed. For example, for 'snapshot' surveys in which hosts have either not had enough time to manifest infections (i.e., prior to 4 weeks post-exposure) or for which density-dependent infection success has set in, the observed number of metacercariae may be strongly decoupled from the actual number of infectious parasites in the environment. Importantly, however, in natural systems parasite exposure likely involves repeated encounters with small numbers of cercariae, rather than single massive doses. For example, in fish studied by Rauch et al. (2005), metacercariae of *Diplostomum pseudospathaceum* were genetically distinct rather than clonal, and genetic diversity in these metacercariae far exceeded that found in cercariae from sympatric snails, which indicates that fish acquired metacercariae incrementally, rather than in large packets. This type of serial exposure gives hosts opportunities to behaviorally avoid (Daly and Johnson, 2011) or immunologically respond to infection, such that linking laboratory-based findings to field infections will require further investigations.

## Author role

D.M.C, E.E., and P.T.J.J designed the study. All authors collected data. D.M.C. collected data with the University of Colorado. P.T.J.J. and E.E. analyzed data. D.M.C wrote the initial draft, and all authors edited and provided feedback on the manuscript.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2020.108002.

## References

Aksenova, O.V., Bespalaya, Y.V., Bolotov, I.N., Kondakov, A.V., Sokolova, S.E., 2016. First molecular identification of *Australapatemon burti* (Miller, 1923)(Trematoda: Digenea: Strigeidae) from an intermediate host *Radix labiata* (Rossmaessler) (Gastropoda: Lymnaeidae) in Europe. Zootaxa 4132, 588–590.

Bates, D., Kliegl, R., Vasishth, S., Baayen, H., 2015. Parsimonious mixed models. arXiv preprint arXiv:1506.04967.

Blasco-Costa, I., Locke, S.A., 2017. Life history, systematics and evolution of the Diplostomoidea Poirier, 1886: progress, promises and challenges emerging from molecular studies. Adv. Parasitol. 98, 167–225.

Blasco-Costa, I., Poulin, R., Presswell, B., 2016. Species of *Apatemon* Szidat, 1928 and *Australapatemon* Sudarikov, 1959 (Trematoda: Strigeidae) from New Zealand: linking and characterising life cycle stages with morphology and molecules. Parasitol. Res. 115, 271–289.

Burch, J.B., 1982. North American freshwater snails. V. Keys to the freshwater gastropods of North America. Walkerana 4, 217–222.

Calhoun, D.M., Leslie, K., Riepe, T., Achatz, T., McDevitt-Galles, T., Tkach, V., Johnson, P.T.J., 2019. Patterns of *Clinostomum marginatum* infection in fishes and amphibians: integration of field, genetic, and experimental approaches. J. Helminthol. 1, 1–12.

Calhoun, D.M., Schaffer, P.A., Gregory, J.R., Hardy, K.M., Johnson, P.T.J., 2015. Experimental infections of bluegill with the trematode *Ribeiroia ondatrae* (Digenea: cathaemasiidae): histopathology and hematological response. J. Aquat. Anim. Health 27, 185–191.

Choo, J.M., 2009. *Australapatemon* sp. (Trematoda) Infection in *Valvata macrostoma* and in two leech species, *Helobdella stagnalis* and *Erpobdella octoculata*. University of Jyväskylä.

Cort, W., Brooks, S., 1928. Studies on the holostome cercariae from douglas lake, Michigan. Trans. Am. Microsc. Soc. 47, 179–221.

Cox, F., 2001. Concomitant infections, parasites and immune responses. Parasitology 122, S23–S38.

Daly, E.W., Johnson, P.T.J., 2011. Beyond immunity: quantifying the effects of host anti-parasite behavior on parasite transmission. Oecologia 165, 1043–1050.

Davies, D., Ostrowsk de Núñez, M.O., 2012. The life cycle of *Australapatemon magnacetabulum* (Digenea: Strigeidae) from northwestern Argentina. J. Parasitol. 98, 778–783.

Désilets, H.D., Locke, S.A., McLaughlin, J.D., Marcogliese, D.J., 2015. Community structure of *Diplostomum* spp. (Digenea: Diplostomidae) in eyes of fish: main determinants and potential interspecific interactions. Int. J. Parasitol. 43, 929–939.

Drago, F.B., Lunaschi, L.I., 2016. Digenea, Strigeidae, *Australapatemon canadensis* Dubois and rausch, 1950: first record in South America and a new host record. Check List. 6, 382–385.

Dubois, G., 1953. Systématique des Strigeida. Complèment de la Mongraphie. Mèmoires de la Sociètè des Sciences Naturelles de Neuchatel 8, 1–141.

Dubois, G., 1968. Synopsis of the Strigeidae and of the Diplostomatidae (Trematoda). Complèment de la Mongraphie. Mèmoires de la Sociètè des Sciences Naturelles de Neuchatel 10, 1–258.

Dubois, G., Pearson, J.C., 1965. Quelques Strigeidae (Trematoda) d'Australie. Bull. Soc. Neuchatel. Sci. Nat. 88, 77–99.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Esch, G.W., Barger, M.A., Fellis, K.J., 2002. The transmission of digenetic trematodes: style, elegance, complexity. Integr. Comp. Biol. 42, 304–312.

Faltýnková, A., Našincová, V., Kablásková, L., 2007. Larval trematodes (Digenea) of the great pond snail, *Lymnaea stagnalis* (L.),(Gastropoda, Pulmonata) in Central Europe: a survey of species and key to their identification. Parasite 14, 39–51.

Fredensborg, B.L., Poulin, R., 2005. Larval helminths in intermediate hosts: does competition early in life determine the fitness of adult parasites? Int. J. Parasitol. 35, 1061–1070.

Fried, B., Nelson, P., 1978. Host–parasite relationships of *Zygocotyle lunata* (Trematoda) in the domestic chick. Parasitology 77, 49–55.

Gordy, M.A., Kish, L., Tarrabain, M., Hanington, P.C., 2016. A comprehensive survey of larval digenean trematodes and their snail hosts in central Alberta, Canada. Parasitol. Res. 115, 3867–3880.

Gordy, M.A., Locke, S.A., Rawlings, T.A., Lapierre, A.R., Hanington, P.C., 2017. Molecular and morphological evidence for nine species in North American *Australapatemon* (Sudarikov, 1959): a phylogeny expansion with description of the zygocercous *Australapatemon mclaughlini* n. sp. Parasitol. Res. 116, 2181–2198.

Hernández-Mena, D.I., García-Prieto, L., García-Varela, M., 2014. Morphological and molecular differentiation of Parastrigea (Trematoda: Strigeidae) from Mexico, with the description of a new species. Parasitol. Int. 63, 315–323.

Hoverman, J.T., Hoye, B.J., Johnson, P.T.J., 2013. Does timing matter? How priority effects influence the outcome of parasite interactions within hosts. Oecologia 173, 1471–1480.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.
Iles, C., 1959. The larval trematodes of certain fresh-water molluscs: I. The furcocercariae. Parasitology 49, 478–504.
James, C., Noyes, K., Stumbo, A., Wisenden, B., Goater, C., 2008. Cost of exposure to trematode cercariae and learned recognition and avoidance of parasitism risk by fathead minnows *Pimephales promelas*. J. Fish. Biol. 73, 2238–2248.
Johnston, S.J., 1904. Contributions to a knowledge of Australian Entozoa. N° III. On some species of Holostomidae from Australian Birds. Proceedings of the Linnean Society of New South Wales 29, 108–116.
Jones, A., Bray, R.A., Gibson, D.I., 2005. Keys to the Trematoda. CAB International and Natural History Museum, London.
Karvonen, A., FaltÝnkovÁ, A., Choo, J.M., Valtonen, E.T., 2017. Infection, specificity and host manipulation of *Australapatemon* sp. (Trematoda: Strigeidae) in two sympatric species of leeches (Hirudinea). Parasitology 144, 1346–1355.
Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649.
Keeney, D.B., Lagrue, C., Bryan-Walker, K., Khan, N., Leung, T.L., Poulin, R., 2008. The use of fluorescent fatty acid analogs as labels in trematode experimental infections. Exp. Parasitol. 120, 15–20.
Klemm, D.J., 1982. The leeches (Annelida: Hirudinea) of North America. Kendall/Hunt pub, Dubuque, Iowa.
Klemme, I., Louhi, K.R., Karvonen, A., 2016. Host infection history modifies co-infection success of multiple parasite genotypes. J. Anim. Ecol. 85, 591–597.
Koprivnikar, J., Riepe, T.B., Calhoun, D.M., Johnson, P.T.J., 2017. Does spatial heterogeneity in host distribution drive parasite aggregation among hosts? Oikos 127, 99–110.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. Mega X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549.
LaFonte, B.E., Johnson, P.T.J., 2013. Experimental infection dynamics: using immunosuppression and in vivo parasite tracking to understand host resistance in an amphibian–trematode system. J. Exp. Biol. 216, 3700–3708.
Leung, T.L., Poulin, R., 2011. Intra-host competition between co-infecting digeneans within a bivalve second intermediate host: dominance by priority-effect or taking advantage of others? Int. J. Parasitol. 41, 449–454.
Lindell, C., Welsh, J.E., van der Meer, J., Thieltges, D.W., 2017. Effect of dose and frequency of exposure to infectious stages on trematode infection intensity and success in mussels. Dis. Aquat. Org. 125 (2), 85–92.
Loker, E.S., 2012. Macroevolutionary immunology: a role for immunity in the diversification of animal life. Front. Immunol. 3, 25–29.
Martin, K.R., Johnson, P.T.J., Bowerman, J., Li, J., 2020. Biogeography of the freshwater gastropod, *Planorbella trivolvis*, in the western United States. PloS One 15, e0235989.
McCarthy, A.M., 1990. Experimental observations on the specificity of *Apatemon (Australapatemon) minor* (Yamaguti 1933) (Digenea: Strigeidae) toward leech (Hirudinea) second intermediate hosts. J. Helminthol. 64, 161–167.
Miller, H.M., 1923. Notes on some furcocercous larval trematodes. J. Parasitol. 10, 35–46.
Miller, H.M., 1927. Furcocercous larval trematodes from San Juan island, Washington. Parasitology 19, 61–83.
Moser, W.E., Klemm, D.J., Richardson, D.J., Wheeler, B.A., Trauth, S.E., Daniels, B.A., 2006. Leeches (Annelida: Hirudinida) of northern Arkansas. J. Ark. Acad. Sci. 60, 84–95.
Moszczynska, A., Locke, S.A., McLaughlin, J.D., Marcogliese, D.J., Crease, T.J., 2009. Development of primers for the mitochondrial cytochrome c oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminths. Mol. Ecol. Resour. 9, 75–82.
Niewiadomska, K., 2002. Family Strigeidae Railliet Keys to the Trematoda. CABI Publishing and the Natural History Museum, Wallingford, Oxon, U. K. In: Gibson, D. I., Jones, A., Bray, R.A. (Eds.), pp. 231–241, 1919.
Orlofske, S.A., Joseph, M.B., Fenton, A., Melbourne, B.A., Johnson, P.T.J., 2018. Experimental investigation of alternative transmission functions: quantitative evidence for the importance of non-linear transmission dynamics in host-parasite systems. J. Anim. Ecol. 87.3, 703–715.
Poulin, R., 1999. The functional importance of parasites in animal communities: many roles at many levels? Int. J. Parasitol. 29, 903–914.
Pulze, L., Baranzini, N., Girardello, R., Grimaldi, A., Ibba-Manneschi, L., Ottaviani, E., et al., 2017. A new cellular type in invertebrates: first evidence of telocytes in leech *Hirudo medicinalis*. Sci. Rep. 7 (1), 1–12.
Rauch, G., Kalbe, M., Reusch, T., 2005. How a complex life cycle can improve a parasite's sex life. J. Evol. Biol. 18, 1069e1075.
Sudarikov, V.E., 1959. Die biologischen besonderheiten der Trematoden der Gattung alaria. Trudy gel´mintol Labor Akad Nauk SSSR 9, 326–332.
Schell, S.C., 1985. Handbook of Trematodes of North America North of Mexico. University Press of Idaho, Moscow, ID.
Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313.
Stunkard, H.W., Willey, C.H., Rabinowitz, Y., 1941. *Cercaria burti* (Miller, 1923), a larval stage of *Apatemon gracilis* (Rudolphi, 1819) Szidat, 1928. Trans. Am. Microsc. Soc. 60, 485–497.
Stutz, W.E., Calhoun, D.M., Johnson, P.T.J., 2019. Resistance and tolerance: a hierarchical framework to compare individual versus family-level host contributions in an experimental amphibian-trematode system. Exp. Parasitol. 199, 80–91.
Valero, M., De Renzi, M., Panova, M., Garcia-Bodelon, M., Periago, M., Ordoñez, D., Mas-Coma, S., 2006. Crowding effect on adult growth, pre-patent period and egg shedding of *Fasciola hepatica*. Parasitology 133, 453–463.
Willey, C., Rabinowitz, Y., 1938. The development of *Cercaria burti* Miller, 1923 in leeches and ducks. J. Parasitol. 24, 1–8.
Xia, X., Lemey, P., 2009. Assessing Substitution Saturation with DAMBE. Cambridge University Pess, Cambridge, MA.
Xia, X., Xie, Z., Salemi, M., Chen, L., Wang, Y., 2003. An index of substitution saturation and its application. Mol. Phylogenet. Evol. 26, 1–7.
Yamaguti, S., 1971. Synopsis of Digenetic Trematodes of Vertebrates, vol. I and vol. II. Keigaku Publishing Company, Tokyo.
Yao, G., Huffman, J., Fried, B., 1991. The effects of crowding on adults of *Echinostoma caproni* in experimentally infected golden hamsters. J. Helminthol. 65, 248–254.