Parasite-modified behaviour in non-trophic transmission: Trematode parasitism increases the attraction between snail intermediate hosts

Eliuk, L.K.[1], S. Brown[2], R.C. Wyeth[2], and Detwiler, J.T.[1]

[1]Department of Biological Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada eliuklk@myumanitoba.ca, jillian.detwiler@umanitoba.ca

[2]Biology, St. Francis Xavier University, Antigonish, NS B2G 2W5

shelby.m.brown@hotmail.com, rwyeth@stfx.ca

Jillian Detwiler, Department of Biological Sciences, University of Manitoba, 50 Sifton Road, Winnipeg, MB R3T 2N2, Canada, 204-474-8689, fax: 204-474-7604, jillian.detwiler@umanitoba.ca

Laura K. Eliuk (L.K. Eliuk), Shelby Brown (S. Brown), Russell C. Wyeth (R.C. Wyeth), Jillian T. Detwiler (J.T. Detwiler). Parasite-modified behaviour in non-trophic transmission: Trematode parasitism increases the attraction between snail intermediate hosts

Many parasites with complex life cycles cause host behavioral changes that increase the likelihood of transmission to the next host. Parasite modification is often found in trophic transmission, but its influence on non-trophic transmission is unclear. In trematodes, transmission from the first to second intermediate host is non-trophic suggesting that free-swimming larvae (cercariae) emerging in closer proximity to the next host would have higher transmission success. We performed a series of behavioral experiments with echinostome trematodes and their snail hosts to determine if potential second hosts (*Planorbella* sp.) were more attracted to parasitized first hosts (*Lymnaea elodes* Say, 1821). In a Y-maze, a responding snail (*Planorbella* Haldeman, 1842 sp.) was placed in the base and its response to five treatments was assessed: no stimulus, duckweed (a food item, *Lemna turionifera* Landolt), non-parasitized *L. elodes*, parasitized *L. elodes*, and finally parasitized versus non-parasitized *L. elodes*. Snails showed some attraction to uninfected snails, but had a stronger response to infected first host snails. These results indicate that potential second host snails were more attracted to parasitized, heterospecific first host snails over non-parasitized heterospecific snails. This study demonstrates that echinostome trematodes alter snail behaviour by changing navigational choices in uninfected potential hosts through a chemical communication mechanism.

Keywords: *Lymnaea elodes*, Marsh Pondsnail, *Planorbella*, Ramshorn Snail, altered behaviour, parasite manipulation, trematode-snail interactions, Y-mazes

Introduction

Endoparasites with complex life cycles face the challenge of completing their development while using several different host species. To develop from larvae to adults, these parasites must encounter and successfully colonize at least 2-4 hosts in succession. Alteration of host behaviour is a common parasitic adaptation that increases the likelihood of transmission to the next host in the life cycle (Moore 1984). Parasites can alter host activity, or microhabitat choice, or both (Lafferty and Shaw 2013). This phenomenon is documented in virtually every taxa of parasitic animals and protozoans, and has been extensively documented in trematodes (Moore 2002; Lafferty and Shaw 2013).

Many trematode species cause behavioural changes in their intermediate hosts, which makes the transmission of the larval parasites to the next host more likely (Moore 1984; Cézilly et al. 2010; Lafferty and Shaw 2013). Most studies of trematode-altered behaviour have demonstrated its importance in trophic transmission whereby second intermediate hosts are consumed by definitive hosts (Lafferty and Shaw 2013). In these cases, second intermediate hosts, infected with encysted parasites (metacercariae), behave in ways that make predation and thus consumption of parasites by the next host in the life cycle more likely. For example, brain-encysting metacercariae cause ants to change their activity and microhabitat choice by freezing on the top of a blade of grass, making predation by grazing ungulate hosts more likely (Moore 1984). Likewise, fish infected with metacercariae behaved more conspicuously and were more likely to be eaten by bird hosts (Lafferty and Morris 1996).

In contrast, the role of parasite-modified behaviour in promoting non-trophic transmission is less clear. In particular, few studies have tested whether parasite-modified behaviour promotes transmission between first and second intermediate hosts. The survival of trematodes with 3-host life cycles depends on transmission from a mollusc first host to second intermediate hosts. Larval cercariae emerge from first intermediate host molluscs and must encounter and colonize a second intermediate host within 24 hours due to their short-life span (Kanev et al. 1995). The abiotic environment can present a challenge to cercarial transmission, depending upon habitat heterogeneity and flow conditions, because cercariae are not strong swimmers (Fingerut et al. 2003). Thus, altering host behaviour to increase proximity among first and second intermediates hosts increases the probability that the 3-host trematode life cycle continues.

For non-trophic transmission, the manifestation of parasite-modified behaviour may be similar to that found in trophic transmission. When parasitized by trematodes, first intermediate host gastropods alter their habitat choices to promote transmission to second intermediate crustacean hosts. Relative to uninfected snails, the estuarine snail *Nassarius obsoletus* Say, 1822 (=*Ilyanassa obsoleta* Say, 1822, sensu Curtis 1987) infected by the trematode *Gynaecotyla adunca* (Linton, 1905) Yamaguti, 1939 were more likely to aggregate further above the water level on a shoreline (Curtis 1987). This change in microhabitat use may be adaptive to the parasite because the cercariae emerging from parasitized snails would be more likely to encounter potential second intermediate host amphipods. However, many trematodes use a range of different host species as second intermediate hosts. These host species may span a wide variety of taxa from invertebrates (e.g. arthropods, platyhelminthes, molluscs) to vertebrates (e.g. fish,

amphibians). Thus, it is unclear if parasite-modified behaviour would play a significant role in transmission to all these host species, or only to particular species. Selection for parasite-modified behaviour may not be as strong if there are several potential co-occurring host species. Further, it is not clear that the type of behavioural change would be the same towards all the potential host species.

Among echinostome trematodes, cercariae have different preferences for second intermediate hosts. In laboratory choice experiments, cercariae preferred to infect heterospecific snail species relative to the first intermediate host (Detwiler and Minchella 2009; Wodjak et al. 2013). For example, cercariae emerging from *Lymnaea elodes* (also known as *Stagnicola elodes* and *Ladislavella elodes* Say, 1821) were more likely to infect *Helisoma trivolvis* (also known as *Planorbella trivolvis* Say, 1817) rather than *L. elodes* (Detwiler and Minchella 2009). A corresponding field experiment with the same snail species demonstrated a similar infection pattern whereby heterospecific snail species had higher intensities of echinostome metacercarial infection (Detwiler and Minchella 2009). Increased loads of metacercariae in heterospecific second intermediate host snails could be driven by the parasite itself. The cercariae could be using chemical cues to ensure encounters with heterospecific snail species. However, considering their short-life span, weak swimming ability, and decrease in infectivity after emergence (Lo and Cross 1975), parasites may also be altering the behaviour of hosts to promote aggregations of first and second intermediate hosts. In a Y-maze where *L. elodes* and *H. trivolvis* were simultaneously included as responder snails (n=6 each), potential second intermediate hosts (*H. trivolvis*) aggregated more quickly to an environmental stimulus (crayfish carrion) when infected first intermediate hosts (*L. elodes*) were present compared to

uninfected (Gray et al. 2009). By spending more time with carrion, they also spent more time with *L. elodes* that were parasitized by the trematode *Echinostoma revolutum* sensu lato (Fröhlich, 1802) Rudolphi, 1809. While the two snail species were in close proximity, cercariae from infected snails could more easily encounter second intermediate hosts (Gray et al. 2009). However, because the latter experiments involved several stimuli at once (crayfish carrion, groups of conspecifics and heterospecifics) more direct tests of the attractiveness of susceptible second intermediate hosts to infected and uninfected first intermediate hosts are needed.

We tested whether parasite-modified behaviour plays a role in non-trophic parasite transmission using an echinostome trematode-snail system. Echinostome trematodes use freshwater snails as first and second intermediate hosts. *Helisoma* (from now on referred to as *Planorbella,* see Johnson et al. 2013) *trivolvis* and *L. elodes* are commonly found as first intermediate hosts in wetlands throughout North America (Detwiler et al. 2010). Unlike some other trematodes, echinostomes also use snails as second intermediate hosts. The latter snail species, along with others, such as *Physa gyrina* Say, 1821 can also be second intermediate hosts (i.e. only second intermediate hosts, or simultaneous first and second intermediate hosts) (Detwiler and Minchella 2009). We used parasitized *L. elodes* as first intermediate hosts, and non-parasitized *Planorbella* sp. snails as potential second intermediate hosts to test whether parasite-modified behaviour affected heterospecific snail interactions. We hypothesized that if parasites modify host interactions, then uninfected *Planorbella* sp. snails would be more attracted to parasitized *L. elodes* first intermediate host snails compared to uninfected *L. elodes*.

Materials and methods

Obtaining first intermediate hosts

The echinostome trematode-snail system was used because the first and second intermediate hosts co-occur in wetlands throughout North America. Infected first intermediate hosts used as potential stimuli in the behavioral experiments were collected from one site in Southern Manitoba because echinostome prevalence was at least 20% in 2015. *Lymnaea elodes* were collected from two 250 m^2 ditches along a road bisecting a wetland in Netley-Libau Marsh (50°18'48"N, 96°42'29"W). A total of 60 infected individuals were used only once in the behavioural experiments (Table 1).

To determine if snails were parasitized as first intermediate hosts, field-collected snails were placed under lights in the laboratory to stimulate the emergence of cercarial parasites. This stage of the parasite is generated within another stage, either the sporocyst or redia. During the exposure to light, the latter stages remain in the gonad tissue of the snail and continue to produce additional cercariae (Schell 1985). To obtain uninfected *L. elodes*, we allowed the snails that were deemed uninfected to breed (i.e. no cercariae seen after 3 hrs under lights, Detwiler and Minchella 2009). Egg cases were removed from the containers with these adults, and raised separately to ensure a stock of uninfected stimulus snails.

From infected first intermediate hosts, live cercariae were identified as echinostomes (Family Echinostomatidae) based on the presence of collar spines (Schell 1985). Using morphology alone, it can be difficult to identify echinostomes to species (Bolek et al. 2019). Thus, we sequenced the DNA from a subset of parasites at the

NADH dehydrogenase 1 (*ND1*) gene in a Containment Level II lab (Facility Certification #BF0172-2R). We compared our sequences to others in GenBank and in our lab database for species-level identification

Infected snails were preserved in ethanol following the behavioral experiments. These snails were crushed and a redia (progenitor stage of cercariae) was removed from the gonad of each snail. The whole parasite was soaked in water to remove any traces of ethanol and placed into a 200 µl solution of 5% chelex containing 0.2 mg/ml of proteinase K, followed by incubation for 2 hours at 56°C and boiling for eight minutes at 99°C. Extracted DNA samples were stored at -20°C before running polymerase chain reaction (PCR). Genetic identification was based on amplification of partial fragments of the *ND1* mitochondrial gene because this region can delineate species (Detwiler et al. 2010). PCR was performed using a 25 µl reaction mix containing 2 µl of extracted DNA, 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer, and 0.05 unit/µl Taq polymerase to amplify partial fragment of ND1. JB11 (5′-AGA TTC GTA AGG GGC CTA ATA-3′) and JB12 (5′-ACC ACT AAC TAA TTC ACT TTC-3′) were the forward and reverse primers, respectively (Morgan and Blair 1998). The thermocycling profile began with an initial denaturation at 95°C for 3 min, followed by 35 cycles with 1 min denaturation at 94°C, 45 s for primer annealing at 52°C, and 2 min for primer extension at 72°C, with a final extension step at 72°C for 7 min. All PCR products were purified with the UltraClean PCR clean-up kit according to manufacturer instructions (MO BIO Laboratories, Inc). Sequencing was completed in the forward and reverse direction at the Hospital for Sick Children, Toronto, ON using an ABI 3730XL instrument.

Obtaining second intermediate hosts

Second intermediate hosts were the responding snails in the behavioural experiments. Lab-raised uninfected *Planorbella* snails were obtained from breeding colonies in the University of Manitoba Animal Holding Facility. Use of lab-raised snails was necessary to confirm that snails had not been previously exposed to parasites. This breeding colony was initially established from adults that were in a shipment of aquatic plants received by the Biological Sciences greenhouse at the University of Manitoba in 2015. Because their origin was unknown and there are disagreements in *Planorbella* identification and taxonomy at the species level (Eva Pip personal communication), we refer to them as *Planorbella* sp.

We performed DNA barcoding with the cytochrome oxidase 1 (*COX1*) gene to better understand the genetic identification of the lab snails as well as the snails collected from the field. DNA was extracted from two lab-raised *Planorbella* sp. and two lab-raised and field-collected *Lymnaea elodes* using the E.Z.N.A. Mollusc DNA Kit according to the manufacturer's instructions (Omega Bio-Tek, cat. No. D3373-02). A partial *COX1* fragment was amplified by PCR in 25 ul reaction volumes with 50 ng of DNA, 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer, and 0.05 unit/µl Taq polymerase. We targeted the Folmer region with LCO1490: 5'GGTCAACAAATCARAAAGATATTGG-3' and HCO2198: 5'TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). The thermocycler profile followed that of Gordy et al. (2016). All PCR products (~650 bp) were visualized in a 2% agarose gel containing ethidium bromide and cleaned with the UltraClean PCR

clean-up kit (MO BIO Laboratories, Inc). Sequencing was completed in the forward and reverse direction at the Hospital for Sick Children, Toronto, ON using an ABI 3730XL instrument.

Behavioural experiment set-up

All snails used in the experiments were kept in artificial spring water (ASW, preparation by Ulmer 1970) for at least 2-3 days before being used in the experiments. We used ASW to limit the amount of foreign biochemical content in the water and their potential affects on snail behaviour. All behavioural experiments were conducted in the Animal Holding Facility at the University of Manitoba under controlled conditions. The average temperature of the ASW was 22.9˚C, the average room humidity was 56.6%, and the average air temperature was 22.2˚C. Light levels were kept constant at 1090 lux. We also attempted to minimize differences in behavioural responses due to differences in snail size (as a proxy for age). The mean length ± SE of responding snails (lab-raised *Planorbella* sp.) was 13.79 ± 0.14 mm. Among the stimulus snails that were uninfected, the mean length of lab-raised *L. elodes* was 16.91 ± 0.18 mm. For the infected stimulus snails, field-collected *L. elodes* had mean lengths of 25.43 ± 0.44 mm.

Behavioural experiments

Five treatments were performed to assess whether parasites modify the heterospecific interactions between two freshwater snail species. Specifically, we tested whether *Planorbella* sp. second intermediate host snails moved towards odours from infected and uninfected first intermediate hosts (*L. elodes*) in a Y-maze. The Y-maze was

custom-made at the Department of Chemistry's Academic Workshop (University of Manitoba). The dimensions of the central and side arms were the same at 15.5 cm by 5 cm and the depth was 2.5 cm.

Each treatment consisted of 30 replicates. In each replicate, one *Planorbella* sp. was placed at the bottom of the central arm and the stimulus snails (uninfected or infected *L. elodes*) were placed in either of the side arms of the Y maze. Treatment 1 was a control in which no snail was placed in either arm of the tank. This control determined whether there was any bias in the direction that *Planorbella* sp. chose to travel in the absence of a stimulus. Treatment 2 used common duckweed as a stimulus to determine if the responding *Planorbella* sp. snails would respond to a stimulus in the Y-apparatus because it has been shown to be a stimulus in Y-mazes Gray et al. (2009). In Treatment 3, there was one non-parasitized heterospecific snail in one side arm, and no snail in the other side arm. This treatment determined if *Planorbella* sp. tended to aggregate with *L. elodes* in the absence of parasitism. In Treatment 4, there was one parasitized snail in one arm, and no snail in the other arm. This treatment determined if parasitism had any effect on the tendency of *Planorbella* sp. to aggregate with *L. elodes*. In Treatment 5, there was one parasitized snail in one arm of the Y, and one non-parasitized snail in the other arm. This treatment most directly addressed our hypothesis because it tested how *Planorbella* sp. would react when both parasitized and non-parasitized heterospecific snails were present in the environment.

Before each trial, the Y-maze was cleaned with 70% ethanol and rinsed with deionized water to remove any snail mucus from a previous trial (as recommended by Boissier et al. 2003). The Y-maze was placed on the same spot on a table for every trial,

and light levels were kept even across the apparatus. A SONY Handicam DCR-SR68 camcorder was positioned on a tripod directly over the tank. For every trial, the Y-maze was filled with 350 mL of room temperature ASW (Ulmer 1970), and the water temperature was recorded to ensure consistency. The arm of the Y-maze that the stimulus snail was placed in was determined by a random number generator prior to each trial (odd number for left side, even number for right side). Stimulus snails were restricted to the last two centimeters of the side arms using a piece of mesh secured with silicone glue.

At the beginning of each trial, stimulus snails (parasitized or non-parasitized) were placed in their respective arms of the tank first and left for five minutes to acclimate, and then responding snails (*Planorbella* sp.) were placed in the base of the Y-tank and left for five minutes. Acclimating *Planorbella* sp. were contained in the first five centimeters of the base using a piece of Styrofoam. This acclimation period allowed all snails to become accustomed to the water and the Y-maze and allowed the stimulus snails to release mucus into the water for the responding snails to detect. At the end of the 5 min acclimation period, the Styrofoam blocker was gently removed and the behaviour of the responding snail was recorded for 30 min. If the snail attached to the Styrofoam piece, it was gently pushed off and allowed to re-emerge from its shell before starting the trial. All responding and stimulus snails were used only once.

Behavioural data analysis

Qualitative patterns of snail movement in Treatments 1-5 were compared using activity heat maps generated for each treatment, based on methods by Wyeth et al. (2011). Videos were first converted to grayscale and inverted (to ensure light snails

moving on a dark background), and then decimated to 0.3 frames/s. Motion was then enhanced in each grayscale video by subtracting an image of the Y-maze background (created from an average of a 50-fold decimation of the grayscale video). The resultant enhanced video, which highlighted the lighter snail on a black background, was then filtered with a threshold to produce a white snail on a black background for each frame. All frames in this binary video were summed to generate the heat map of activity for each trial, and heat maps of all trials in each treatment were summed to summarize activity in the treatment.

Quantitative analyses included four different measurements of the responding snails' position over time in the Y-maze, all of which were visually assessed by watching videos. First, we recorded the time it took the snail to first leave the base of the Y-maze and enter one of the side arms (s). Second, we determined the arm first chosen, which occurred when at least half of the snail's body had passed over one of the entrances to the side arms after leaving the base of the Y-maze. Third, we recorded the number of times the choosing snail entered either arm. Fourth, we determined the amount of time (s) the responder snail spent in each side arm of the Y-maze.

Two additional measures of snail locomotion behaviour were used to compare movements of animals tracked once they were inside the stimulus arm of the Y-maze. Automated tracking of the snails in the 0.3 frames/s binary videos (see above) was accomplished using the TrackMate plugin for FIJI software (Schindelin et al. 2012; Tinevez et al. 2017). Further processing of track data in the MTrackJ plugin (Meijering et al. 2012) calculated both the velocity of snails between successive points along the track, and the change in heading between successive pairs of points along the track (working

progressively along a track, each pair of points forms a vector with an angle indicating the snail heading between those two points; the change in heading is thus the difference in angle between successive vectors along the track). To acquire single measurements for each trial, we calculated the average velocity for the entire track and the standard deviation of the changes of angle (SD of dAngle) for the entire track, serving as a measure of heading variation for the track. These additional measures assessed whether finer changes in locomotion accompanied any differences in positioning in the Y-maze, helping to distinguish whether any differences arose due to changes in locomotion itself or choices in where the animals moved in the Y-maze.

Statistical analyses included testing if snails first chose either arm (Treatment 1-Negative control) or the arm with the stimulus (Treatment 2-5) compared to a 50/50 expectation using Exact tests of goodness of fit. Paired T-tests or Wilcoxon sign rank tests (if the Shapiro-Wilk test indicated a significant deviation from normality) were used to determine if there was any difference in the number of entries or the time snails spent in either arm (Treatment 1 –Negative Control) or with the stimuli (Treatments 2-5). For comparisons among multiple treatments, we excluded the duckweed treatment as comparing responses to prey versus heterospecific odour sources was not relevant. Thus, we determined if there were differences in the time to first response among and between Treatments 1, 3, 4, and 5 using a generalized linear model (GLM) with a negative binomial distribution and log link function followed by Tukey's post hoc comparisons. We report the results of this model because GLMs with Gaussian and Poisson distributions were over-dispersed (residual deviance/degrees of freedom residuals >1). Finally, comparisons of the velocity and SD of dAngle were compared among

Treatments 1 and 3- 5 using a one-way ANOVA followed by Tukey's post hoc comparisons. Analyses were performed in R version 3.5.1 or 3.5.3 (R Core Team, 2016) with the following packages: ggplot2, ggthemes, coin, ggpubr, multcompView, MASS, and multcomp (Venables and Ripley 2002; Hothorn et al. 2006; Hothorn et al. 2008; Graves et al. 2015; Wickham 2016; Arnold 2019; Kassambara 2019).

Results

Control treatments

Comparison of the two control treatments demonstrated the Y-maze was effective at testing *Planorbella* sp. responses to attractive odours sources. In the negative control (both arms empty), there were no significant differences in the number of times either arm was entered (Table 1), nor the time spent in either arm (Table 2). In contrast, in the positive control with duckweed (a food item) used as a stimulus, the snails entered the stimulus arm significantly more often than the non-stimulus arm (Table 1), and spent significantly more time with the duckweed than the empty arm of the tank (Table 2). Further, they responded more quickly to the duckweed, reaching the choice point of the maze in significantly less time than in an empty Y-maze (Mann Whitney U test: $W = 687$, $P = 0.0005$). The only measurement that showed no significant differences in both the positive and negative controls was the frequency at which the stimulus versus non-stimulus arms were first chosen (Table 3). Overall, the clear differences in behaviour in the presence of duckweed as an attractive odour source (shown qualitatively in Fig. 1A versus Fig 1B) verified that our Y-maze could be used to test for responses to heterospecific snail odours.

Heterospecific snail treatments

Responder snails made different choices in the Y-maze, depending on the presence of uninfected and infected stimulus snails. Qualitatively, heat maps of activity did not show noticeable differences in the stimulus arm with an uninfected *L. elodes* compared to the empty arm (Fig. 1C). In contrast, infected snails appeared to be more attractive, resulting in greater activity at the head of the stimulus arm, both when paired with an empty arm (Fig. 1D) and an uninfected *L. elodes* (Fig. 1E). Quantitative analysis suggests the differences we observed arise from differences in the choices made by the snails. Comparing the time to the first response (i.e. the time to traverse the stem of the Y-maze to the choice point), there was a significant difference among the negative control and three heterospecific snail treatments (negative binomial log linked GLM: $F_{3, 9.67} = 128.99$, $P = 0.022$). Tukey's posthoc tests show that this difference is driven solely by differences between the treatment with uninfected snails (Treatment 3) and treatment without snails (Treatment 1) ($Z = 0.49$, $P = 0.029$).

Once the responding snails reached the choice point of the maze, a stimulus arm with an uninfected heterospecific snail was entered significantly more often than an empty arm (Table 1), but there was no significant effect on total time spent in or first choice of that stimulus arm (Tables 2 and 3). Infected snails in the stimulus arm had more substantial effects. The stimulus arm was chosen first significantly more often (Table 3), and as trials progressed, the stimulus arm was entered significantly more often (Table 1). This presumably accounts for the significantly greater time responder snails spent in the stimulus arm compared to an empty arm (Table 2, Fig. 2).

When uninfected and infected heterospecific snails were simultaneously presented as stimuli in opposite arms (Fig. 1E), the quantitative analyses showed the snails to have much less of a preference. There was no significant difference in whether the stimulus or non-stimulus arm was chosen first (Table 3). There were also diminished, but still significant differences between the average number of entries and total duration in the stimulus versus non-stimulus arms (Tables 1 and 2).

Measurements of locomotion parameters solely in the stimulus arms showed little evidence of any differences that depended on the stimulus snails (Fig. 3). There were no significant differences in instantaneous crawling velocities (Table 4). The variation in crawling heading was significantly different among treatments, but the only significant pairwise difference among the treatments was between crawling without any stimulus snail and just one of the two treatments with an infected stimulus snail (Treatment 5, with infected versus uninfected snails). Thus, the significant differences among the treatments in where the snails move in the Y-maze (Tables 2 and 3) do not appear to be a consequence of any changes in basic locomotion, and are therefore attributable to changes in where the animals choose to locomote.

Parasite and snail species diversity in behavioural trials

We confirmed with DNA sequencing that all field-collected snails were infected with echinostomes. The field-collected *L. elodes* were infected by three species. In Treatment 4, 488 bp of the *ND1* gene were successfully sequenced from rediae from six total snails. BLAST searches of genetic sequences from GenBank showed that one snail was infected with a new haplotype (MN996927) that was 99% identical over 476 bp to *Echinoparyphium* sp. C (MH369083). Three snails were each infected with a different

haplotype of *Echinoparyphium* lineage 2 (MN996930, MN996931, MN996932). MN996931 was 100% identical to MH369025 and MH368953 except for our sequence was longer. MN996932 was a 100% match to 10 sequences in GenBank except for our sequence was longer (MH369119, MH369134, MH369068, MH368983, MH369074, MH369143, MH369099, MY369036, MH368995, MH369033). The fifth and sixth snails were infected with a haplotype of *E. revolutum* sensu lato (MN996925) that was 100% similar at 474 bp to GQ463083). In Treatment 5, 488 bp of the *ND1* gene was successfully sequenced from rediae that originated from six of 30 snails. Five snails were infected with a total of four haplotypes of *Echinoparyphium* lineage 2. Two of these haplotypes were also used in Treatment 5 (MN996931, MN996932 x two snails) and two were only found in Treatment 5 (MN996929, MN996928). The sixth snail was infected with a haplotype (MN996925) that was 100% similar to *Echinostoma revolutum* sensu lato at 474 bp (GQ463089).

The responder snails, *Planorbella* sp., were genetically similar (1.1% mean group distance for 452 bp) to snails identified as *Planorbella trivolvis* (AY651208, MH087568, EU038397 and MH087626), *Planorbella tenuis* Dunker, 1850 (ER012174) and *Planorbella duryi* Wetherby, 1879 (KY514384). The shell morphology was similar to *Planorbella pilsburyi* F.C. Baker, 1926 (Eva Pip personal communication). Thus, we refer to our responder snails as *Planorbella* sp. until the snail taxonomy is resolved. The stimulus snails identified by shell morphology as *L. elodes* closely matched *Ladislavella elodes* (KT831386) collected from Alberta, Canada. These two sequences differed by 0.60% at 672 bp. The change in nomenclature from *Lymnaea* (*Stagnicola*) to *Ladislavella* was recommended by Vinarski et al. (2012).

Discussion

The behavioural experiments demonstrated that choosing *Planorbella* sp. snails responded to uninfected and infected heterospecific *L. elodes* snails. However, there was a stronger attraction to trematode-infected snails compared to uninfected snails. When the other arm was empty, there was a significant response to infected snails in three of the four choice behaviours assessed (first choice of stimulus arm frequency, number of entries, and duration in the stimulus arm), while only one of the four was significant for uninfected snails (number of entries into the stimulus arm). Further, when given the choice between the parasitized and non-parasitized stimuli, they spent more time with infected snails compared to uninfected snails. Thus, we find evidence that parasites modify the odours of *L. elodes* and those changes make the parasitized snails more attractive to *Planorbella* sp. As measured in our experiments, this change in response is manifested as differences in positioning in the Y-maze, rather than changes in locomotion parameters (namely, velocity and variation in headings during crawling). This suggests the changed odours from parasitized snails do not simply trigger a modification to normal crawling, but instead do indeed influence the navigational choices of the responding snails. The increased attraction of potential second intermediate host snails to infected first intermediate hosts may be advantageous for the echinostome parasites. Closer proximity to the next host in the life cycle will give the short-lived and poor-swimming cercarial larvae a greater probability of successful transmission from first to second intermediate host.

Although echinostome parasites had been implicated as a factor that affected snail interactions, a more direct test involving fewer stimuli was needed (Gray et al. 2009). By testing the responses to only one stimulus at a time, we found similar results to the previous study that heterospecific attraction was modified by echinostome trematodes (Gray et al. 2009). In addition, by presenting two stimuli simultaneously (uninfected and infected snails), our study shows that there was an attraction to infected snails even when an uninfected snail was present. Worldwide, there are at least 34 species of echinostome trematodes that use freshwater snails as second intermediate hosts (Keeler and Huffman 2009). If they all have similar effects on snails, then echinostome parasitism may be a common, but overlooked, influence on snail aggregations.

By colonizing a heterospecific snail, the parasite may be increasing its dispersal and survival. Echinostomes can use their first intermediate host as a second intermediate host, where cercariae will re-enter the host they have emerged from and encyst as metacercariae (Detwiler and Minchella 2009). However, the progenitor stage of cercariae, the redia, feeds on the gonad of the snail, which negatively affects survival of the first intermediate hosts and subsequently the parasite's survival as well (Sorensen and Minchella 1998). Echinostome cercariae can also colonize uninfected snails of the same species as their 1st intermediate host. Yet, laboratory exposures showed that when echinostomes were given a choice between homospecific and heterospecific snails, they preferred to infect heterospecific hosts (Detwiler and Minchella 2009; Wojdak et al. 2013). However, to better understand the context dependent nature of snail-snail transmission, experiments are needed to test the attraction of echinostomes to both uninfected and infected homospecific and heterospecific hosts.

We also demonstrated heterospecific snail attraction in the absence of parasitism, which could be adaptive in reducing individual risk of predation (Parrish and Edelstein-Keshet 1999) or finding food resources (Gray et al. 2009). Aggregation among snails is most often studied by assessing trail-following behaviour. Most trail-following studies have involved snails that prey on other gastropods, such as the species *Euglandina rosa*, which is capable of following the trails of its prey species as well as the trails of conspecifics (Cook 1985; Clifford et al. 2003). In studies of freshwater snails, individuals usually follow the trail of conspecifics, but not heterospecifics. For example, *B. glabrata* could follow conspecifics, but not the mucus trails of a heterospecific species, *Lymnaea stagnali*s (Townsend 1974). It is important to note that *B. glabrata* and *L. stagnalis* do not co-occur in nature and would likely not have evolved to recognize each other. In another study, the freshwater snail *Physa parkeri* followed the trails of conspecifics, but avoided the trail of a naturally co-occurring heterospecific snail *Campeloma decisum* (Karowe et al. 1993). In contrast, we demonstrated that *Planorbella* sp. was attracted to *L. elodes* rather than not attracted or actively avoiding as shown in previous studies. Further, our study demonstrates that heterospecific attraction between snails can occur without trail following and suggests that just as pheromones are known to be important for a number of aquatic gastropods, kairomones or other signalling molecules that induce heterospecific snail aggregations may also be a common aspect of chemical communication in gastropods (Wyeth 2019).

Documenting more systems in which parasite-modified behaviour affects transmission will contribute to a better understanding of the underlying mechanisms. Behavioural experiments often hypothesize that trematodes alter the chemical

composition of snail exudates to make parasitized snails more attractive to other snails (Boissier et al. 2003; Curtis 1987; Gray et al. 2009). Several studies have compared the organic compounds such as amino acids and neutral lipids in snail-conditioned water between uninfected snails and echinostome-infected snails (reviewed in Sherma and Fried 2011). Concentrations of free fatty acids in snail-conditioned water did not differ between uninfected and infected snails (Schneck et al. 2004). However, employing High Performance Liquid Chromatography (HPLC) analysis on snail exudates will help identify the role of more specific signalling molecules like eicosanoids (oxidized fatty acids) which are produced by both parasites and snails and have been shown to mediate host-trematode interactions (Stanley-Samuelson 1994; Belley and Chadee 1995). We can combine analyses of the chemical signalling molecules with behavioural experiments to test alternative hypotheses raised in this experiment. For example, the behaviour of responding snails could have been affected by the size and activity of the stimulus snail if these factors affect the emission of signalling molecules. At present, these hypotheses remain untested, but future experiments could test whether these factors affect the amount and type of signalling molecules emitted from the snail host.

Our study contributes to understanding how trematodes alter the behaviour and ecology of their host species to increase success in reaching the next host. We demonstrated that a freshwater snail (*Planorbella* sp.) had an increased attraction to a heterospecific snail (*L. elodes*) when it was parasitized as a first intermediate host with echinostome trematodes. This suggests that echinostome parasites alter the behaviour and aggregation of second intermediate hosts by modifying the odours of the first intermediate hosts. Our study is one of few to demonstrate that trematode parasites affect

the behaviours of uninfected, potential hosts (Gray et al. 2009). Instead of avoiding infected hosts, the odour change attracted susceptible hosts, which could increase parasite transmission. Given that freshwater snails and echinostomes are ubiquitous in freshwater systems, these parasites may play an important role in mediating interspecific interactions among gastropods.

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Table 1. The mean number of entries by responder snails (*Planorbella* Haldeman, 1842 sp.) for each arm in the Y-maze, with Wilcoxon sign-rank tests of significant differences between the two arms for each treatment (*Z* statistic; *n* = 30 each). Significant *P*-values (<0.05) are bolded. Heterospecific stimulus snails were *Lymnaea elodes* Say, 1821.

	Mean # arm entries ± SE			
Treatment	Stimulus	Non-stimulus	*Z*	*P*-value
1 - negative control (no stimuli)	1.5±0.13	1.4±0.16	0.396	0.692
2 - positive control (duckweed)	2.0±0.17	1.4±0.18	2.443	**0.015**
3 – uninfected snail	2.1±0.20	1.2±0.12	3.705	**0.0002**
4 – infected snail	2.0±0.16	1.2±0.11	3.618	**0.0003**
5 – infected and uninfected snail*	1.9±0.14	1.5±0.14	2.216	**0.027**

*Snails were simultaneously presented, one in each arm, the arm with the uninfected snail is considered the non-stimulus.

Table 2. Comparing the amount of time a responder snail (*Planorbella* Haldeman, 1842 sp.) spent with the stimulus and non-stimulus in the Y-maze. One side arm was empty in Treatments 2-4. In Treatment 5, each side arm had one snail (infected or uninfected). Heterospecific stimulus snails were *Lymnaea elodes* Say, 1821. Significant *P*-values (<0.05) are bolded.

	Mean time spent in arm (s)				
Treatment	Stimulus	Non-stimulus	*t*	df	*P*-value
1 - negative control (no stimuli)	418.0	433.1	-0.170	29	0.87
2 - positive control (duckweed)	875.4	331.5	-5.469	29	**<0.0001**
3 – uninfected snail†	520.3	411.2	-0.984	29	0.33
4 – infected snail	585.0	338.7	-3.144	29	**0.0038**
5 – infected and uninfected snail*	607.8	454.4	2.0946	29	**0.045**

† Shapiro-Wilk test indicated data was non-normal ($W = 0.89$, $P = 0.0057$), Wilcoxon signed rank test was also not significant ($V = 174$, $P = 0.24$).

*Snail were simultaneously presented, one in each arm, the arm with the uninfected snail is considered the non-stimulus.

Table 3. The frequency of arm first chosen by the responder snail (*Planorbella* Haldeman, 1842 sp.) in the Y-maze. Heterospecific stimulus snails were *Lymnaea elodes* Say, 1821. For all treatments, the expected frequency of choice was 15:15 between the arms. Significant *P*-values (<0.05) are bolded.

	# times chosen first		
Treatment	Stimulus	Non-stimulus*	*P*-value
1 - negative control (no stimuli)	15	15	0.52
2 - positive control (duckweed)	20	10	0.10
3 – uninfected snail	20	10	0.10
4 – infected snail	22	8	**0.016**
5 – infected and uninfected snail*	16	14	0.86

*Snails were simultaneously presented, one in each arm, the arm with the uninfected snail is considered the non-stimulus.

Table 4. Comparing activity in the stimulus arm among four of the five treatments with ANOVA and Tukey posthoc tests. Treatment 2 (duckweed as stimulus) was not included).

					Treatments			
Measure	df1	df2	*F*	*P*-value	1	3	4	5
Velocity (cm/s)	3	112	1.59	0.196	a	a	a	a
SD of dAngle (deg)	3	112	3.03	0.032	b	ab	ab	a

Figure Captions

Figure 1. Activity heat maps for each of the five Y-maze treatments comparing *Planorbella* Haldeman, 1842 sp. responses to different odour sources: empty Y-maze arms, duckweed (prey), uninfected *Lymnaea elodes* Say, 1821, and *L. elodes* infected with echinostomes. Arrowheads indicate regions of qualitatively higher activity in response to duckweed and infected *L. elodes*.

Figure 2. Responder snails (*Planorbella* Haldeman, 1842 sp.) spend more time with infected *Lymnaea elodes* Say, 1821 snail compared to non-stimulus (empty arm) in Treatment 4. Points above the one-to-one line denote snails that spent more time with the stimulus than the non-stimulus (e.g. mean difference was positive).

Figure 3. Locomotion measurements from tracking snails in stimulus arms for each of the five Y-maze treatments comparing *Planorbella* Haldeman, 1842 sp. responses to different odour sources: empty Y-maze arms, duckweed (prey, *Lemna turionifera* Landolt), uninfected *Lymnaea elodes* Say, 1821, and *L. elodes* infected with echinostomes. Measures include instantaneous velocity and heading variation (standard deviation of the change of angles between successive vectors between track point; SD of dAngle).

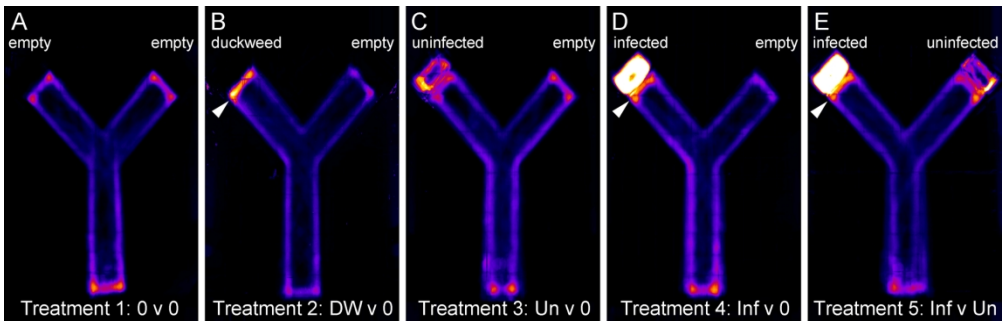

Figure 1. Activity heat maps for each of the five Y-maze treatments comparing *Planorbella* Haldeman, 1842 sp. responses to different odour sources: empty Y-maze arms, duckweed (prey), uninfected *Lymnaea elodes* Say, 1821, and *L. elodes* infected with echinostomes. Arrowheads indicate regions of qualitatively higher activity in response to duckweed and infected *L. elodes*.

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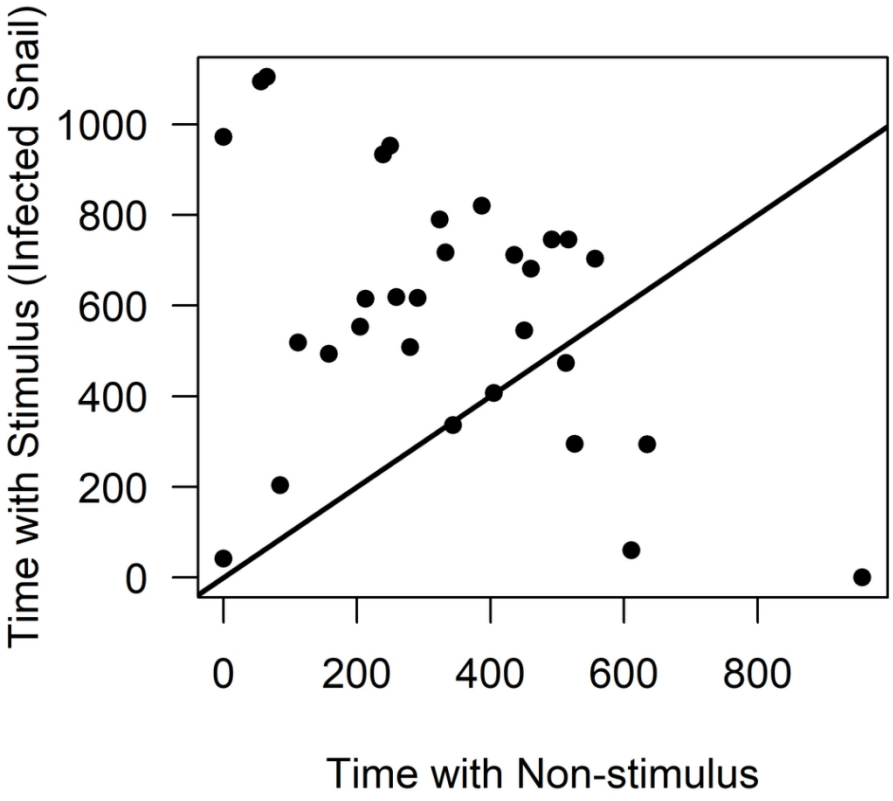

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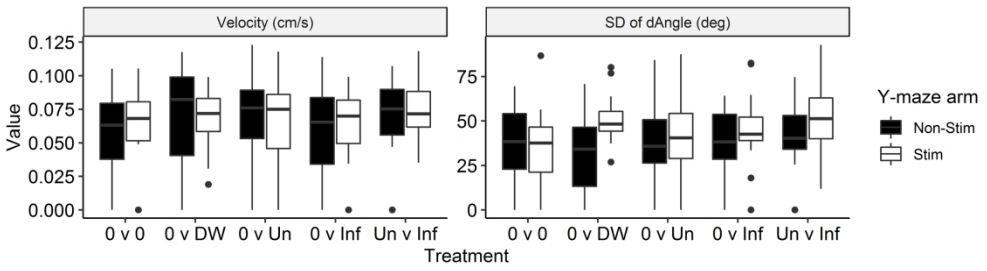

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228x63mm (300 x 300 DPI)