Elucidating Factors that Influence Infection Success and the Behaviour of Gastropod Hosts of *Echinostoma trivolvis* lineage c

by

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Abstract

Wildlife trematodes are important to study due to their impact on wildlife populations, including the ability to modify the behaviour of their hosts. However, these parasites are challenging to study without a fully elucidated life cycle that can be completed in a laboratory. As such, a model for wildlife trematodes would be a great asset for learning about the ecology and behavioural manipulation found in this group. I chose *Echinostoma trivolvis* lineage c as a parasite model to study these topics in gastropod hosts. Chapter 1 determined how host species/morphotype, host size and miracidial dose affects infection success of gastropod first intermediate hosts. I confirmed reports from natural infections that *Ladislavella elodes* is a host along with it's morphotype *Stagnicola reflexa* and further determined which size and exposure dose produced the highest infection success. I used this new information to obtain infected hosts for my second chapter of my thesis. Herein, I tested whether light and species influenced the navigational behaviour of potential second intermediates host *L. elodes*. I determined that snail velocity increased when exposed to a higher light level. Using these higher light conditions, I also found that there was no attraction to infected conspecific hosts by potential second intermediate hosts. Both experiments suggest that abiotic and biotic factors can alter snail navigational behaviour and may even influence whether host behavioural modification is observed. As such, laboratory-based studies of behavioural modification may be over or underestimating the strength and frequency of the behaviours that may be occurring in nature. Examples of naturally occurring behavioural modification may be missed entirely if not tested under the right context in the laboratory.

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Thesis Introduction

Wildlife trematodes are critical to study as they affect the health of many animals including humans (Williams et. al., 2020; Metz et. al., 2023; Scholz et. al., 2024). Their effects do not impact individuals alone, but alter population, community, and ecosystem level dynamics (Ferreira et. al., 2007). For instance, wildlife trematodes influence food webs by becoming prey and facilitating predation through modifying behaviour of their hosts (Lafferty and Morris, 1996; McKee et. al., 2020). As the climate warms, wildlife trematodes are predicted to become more abundant, potentially increasing their regulatory impact on wildlife (Harvell et. al., 2002; Poulin, 2006). An increase in the abundance of parasites is a concern for wildlife that are threatened due to habitat loss and malnutrition (Hughes and Kelly, 2006; Kutz et. al., 2009; Douda et. al., 2018). Altogether, wildlife trematodes are a critical group to study, but due in part to their high species diversity and the challenges of studying them in naturally infected hosts, very little of the biology of these parasites is known. An essential next step is to elucidate the life cycles, which provides knowledge about what hosts are essential for parasite development and transmission.

Use of a mollusc host at one or more parts of their life cycle is broadly conserved across Class Trematoda (Phylum Platyhelminthes), which consists of an estimated ~45,000 species (Lockyer et. al., 2004; Carlson et al. 2020). Molluscs are always a first intermediate host, wherein the parasite develops from either an egg or a ciliated larval stage that hatches from the egg (miracidium) and then develops into one of two different larval stages (sporocyst or rediae) (Lockyer et. al., 2004). Molluscs can also serve as second intermediate hosts for some trematode species, wherein a free-swimming larval

stage penetrates the snail, migrates through the tissue, and encysts to become a metacercariae (Lockyer et. al., 2004). For some trematodes, molluscs can simultaneously be first and second intermediate hosts and harbor sporocysts or rediae as well as metacercariae that either originate from the same individual or from other nearby molluscs that are emitting the cercarial stage (Lockyer et. al., 2004). Trematodes are most host specific for first intermediate hosts, typically being found in a single family, genus or species of mollusc (Lockyer et. al., 2004). In contrast, trematodes are often less host specific to their second intermediate hosts with some being able to use a wide variety of vertebrate and invertebrate taxa (Combes et. al., 1994). Definitive hosts for trematodes are typically vertebrates as they provide an ideal environment as trematodes can accumulate in large numbers, grow to a large size and sexually reproduce at a high rate (Benesh et. al., 2014). However, some trematode species use molluscs as definitive hosts and can even complete their life cycle with only mollusc hosts. For example, *Plagioporus sinitsini* can complete a truncated, direct life cycle using only the freshwater snail *Elimia symmetrica*, with the sporocyst supporting the formation of metacercariae and later adult worms within the gastropod host (Barger and Esch, 2000). As such, freshwater molluscs specifically are associated with every stage of the trematode life cycle, highlighting the intimate association between these gastropods and the parasites that infect them. This relationship is further reflected in a coevolutionary history of trematodes and molluscs becoming specific for one another.

Trematodes, especially the redial stages, are highly influential on the fitness of their preferred host mollusc and vice versa, resulting in increasingly specific adaptions in both

host and parasite (Van Der Knapp and Loker, 1990; Lockyer et. al., 2004). Over time, these organisms have become specifically adapted for one another, which is likely reflected in the high host specificity between trematodes and their first intermediate host molluscs (Lockyer et. al., 2004). This coevolutionary history is also associated with the evolution of traits thought to improve the chances of the parasite completing its life cycle, such as the manipulation of host behaviour.

Trematodes are one of the most prolific groups of behaviour manipulators (Thomas et. al., 2005). The behaviours observed in infected hosts are highly diverse and dependent on the species of trematode (Lafferty and Shaw, 2013). Most trematode-altered behaviours are hypothesized to increase the likelihood of transmission to the next host of the life cycle, though some appear to be incidental (Combes, 2001; Lafferty and Shaw, 2013). Modified behaviour can change host activity, host microhabitat use, or both (Lafferty and Shaw, 2013). For example, the trematode *Curtuteria australis* causes significant changes in the microhabitat use of its second intermediate host, the New Zealand cockle (Mouritsen, 2002). The parasite preferentially encysts as a metacercariae on the underside of the cockle's foot, which impairs the host's ability to dig. As a result, infected cockles are often exposed from the sediment, which is hypothesized to make them more vulnerable to predation by shorebirds, the next host in the trematode's life cycle. Behavioural manipulation may also be associated with alterations to host morphology. Sporocysts of the trematode *Leuchocloridium* spp. invade the tentacles of a terrestrial gastropod, penetrating them with elongated brood sacs until they appear enlarged and display moving multicoloured banding (Welsolowska and Wesołowska, 2014; Usmanova et. al., 2023). This

morphological change is accompanied by a less obvious behavioural change in both the activity and microhabitat of the host. A study comparing the behaviour of host and nonhost gastropods in their natural environment found that snails infected with this parasite spent more time moving than uninfected conspecifics and were more likely to inhabit bright, exposed areas (Wesołowska and Wesołowska, 2014). These morphological and behavioural changes are thought to increase the likelihood of predation from an insectivorous bird (i.e., tentacles with sporocysts resemble caterpillars), the next host in this parasite's life cycle. There are more examples of mollusc host behavioural manipulation in the trematodes, most of which affects first intermediate hosts rather than second intermediate hosts (Wojdak et. al., 2013; Lafferty and Shaw, 2013). However, the effects of parasitism on potential hosts rather than current hosts have not been significantly studied in either intermediate or definitive hosts of trematodes. In addition, manipulation of hosts is often investigated in a limited context in either the field or laboratory making it difficult to understand how frequently and to what extent this phenomenon influences host-parasite interactions. Subsequently, it is difficult to determine the magnitude of the effect of parasite manipulation on host populations, communities and ecosystems. Manipulation of host behaviour may be affected by biotic and abiotic factors that could change the frequency of the phenomenon or alter the strength of its effect. Host manipulation may be affected by biotic factors such as food availability, risk of predation, presence of conspecifics and overall body condition (Selbach et. al., 2016; Gunn et. al., 2021). Abiotic conditions like light levels, temperature, humidity, time of day, and time of year also affect host behaviour, so by extension could also

influence the manipulation of host behaviour (Wehner et. al., 2019). The context could be complex as several factors likely affect an organism at any given time and could be interacting with one another. As such, careful consideration of the context and the relevant abiotic and biotic factors is important to consider in both field and lab studies of host manipulation. Otherwise, confounding variables could be introduced limiting our understanding of the context in which parasite manipulation occurs. In the laboratory, the context in which behavioural manipulation is studied is easier to control compared to semi-natural or natural studies. However, laboratory studies are limited to parasites with elucidated life cycles or life cycles that involve hosts that can be maintained in laboratory. As such, a wildlife trematode species with characteristics of a lab model is critical to better understand how context influences our understanding of host manipulation.

Model trematodes must be generally representative of a larger group of species (Toledo et al., 2007). A model can best be compared to species that share similarities in their life cycle, such as the use of phylogenetically or ecologically related host species. Model parasites should have life stages with relatively fast development so that the time between exposure and patency (i.e., development of next life cycle stage) can be completed within a few days or weeks (Russell et. al., 2017). These parasite life stages should also be able to develop in host species that can be easily maintained and manipulated in the laboratory. In this case, trematodes that use molluscs, and even the same mollusc species as both a first and second host, are ideal as it limits the number of host species that would need to be maintained in the laboratory. Having both hosts be

invertebrates is also useful in that there may be fewer required animal care regulations compared to using a vertebrate second intermediate host (CCAC, 2024).

Some wildlife trematodes in the superfamily Echinostomatidae (Looss, 1899) are broadly used in parasitological research (Toledo et. al., 2007). Some genera, such as *Echinostoma*, are a focus of research because of their impact on host populations and species, especially for some endangered anurans (Szuroczki and Richardson, 2009). Further, echinostomes are one of the wildlife trematode groups for which prevalence is expected to increase with rising global temperatures, suggesting that host populations will be even more impacted by these parasites in future years (Poulin, 2006; Koprivnikar et. al., 2009; Szuroczki and Richardson, 2009). In addition to their relevance to wildlife health, some echinostome species have traits that make them excellent models for trematodes in general. For one, some closely related species / species complexes are distributed worldwide, allowing them to be used as models within many ecosystems. Additionally, the life cycle of echinostomes typically involves easy to maintain hosts such as freshwater gastropods and chickens and can be completed in a short time span relative to other trematodes (Fried et. al., 1997; Toledo et al., 2007; Toledo et. al., 2014). For example, some echinostomes can develop from egg to adult in as little as 10 weeks (Toledo et. al., 2014). All together, these characteristics allow the life cycle of an echinostome to be completed in a laboratory setting with a lower barrier of entry than other wildlife trematodes, requiring less invested time, care and equipment. Furthermore, several echinostomes can be transmitted between two snail hosts, which allows for a wide array of different laboratory and field studies to be conducted concerning behavioural manipulation as these hosts are

easy to work with in both settings (Keeler and Huffman, 2008). Using gastropod hosts, studies have examined how echinostome infections influence temperature selection, chemical communication and resource utilization (Gray et. al., 2009; Wang et. al., 2019; Friesen et. al., 2022a). This research potential, along with their wide range and easily maintained life cycles makes echinostomes a suitable model group for studying behavioural manipulation in gastropods.

Most research on behavioural modification in this superfamily has focused on a particular species complex: *Echinostoma trivolvis*. Variation found in the life cycle of this parasite, followed by genetic examination, revealed that it was a species complex with three lineages in 2010 (Detwiler et. al, 2010). Prior to this discovery, the lineages a and b were primarily used for research, verifiable by the gastropod species that the parasite infected as first intermediate host (Detwiler et. al., 2012). Within these lineages, the parasite has been observed to cause first intermediate host snails to select higher temperatures than uninfected conspecifics (Wang et. al., 2019). Echinostomes also manipulate behaviour in uninfected snails that could potentially be the next host in their life cycle, causing them to be attracted to first intermediate host snails more than uninfected snails (Eliuk et. al., 2020). This example of manipulation is very interesting because in almost all cases, the first intermediate host displays the changed behaviour (Lafferty and Shaw, 2013). In these cases, manipulation is either a byproduct of the pathology or a direct change to the host's physiology (Thomas et. al., 2005; Lafferty and Shaw, 2013). The increased attraction between hosts of *E. trivolvis* is instead most likely caused by changes to the chemical signals the first intermediate host is emitting into the

water, which potential second intermediate hosts can sense and respond to (Friesen et. al., 2022a). Manipulation being seen in a potential second intermediate host rather than an infected first intermediate host creates an extra layer of complexity in the associated mechanism, with three organisms to consider. Furthermore, for the behaviour of an organism distal from the infected host to change, environmental cues must be involved, most likely chemical cues in this system, which are themselves heavily reliant on the context the organisms occur in (Friesen et. al., 2022a; Friesen et. al., 2022b). For example, pollution may cause chemical signals to degrade at a faster rate or different water flows may make tracking the signal back to its source more challenging than others (Jürgens and Bischoff, 2017; Wyeth, 2019). As such, the interactions between context and behavioural manipulation are critical, more so than they already are for other cases of manipulation. Regarding biotic context, echinostomes have a much broader host range for second intermediate hosts, making it possible for the second intermediate host snail to be either the same species as the first, a closely related species or a separate lineage of gastropod. This means that the species of gastropods within the environmental context of the host may influence the behavioural manipulation. Furthermore, unaltered behaviour is affected by factors such as light and heat (Wehner et. al., 2019), causing gastropods to select different areas and show different levels of activity. The parasitic manipulation of a host is, most simply, another factor that affects behaviour, meaning that manipulated behaviour is likely affected by these stimuli as well. In both cases, the involvement of three organisms and use of environmental cues for manipulation would be expected to increase the effect that the environmental context has on the behavioural manipulation, as the behaviour of

each organism and the chemical signals themselves are all affected by the context. The *Echinostoma trivolvis* lineage c-gastropod system is therefore an excellent model for the ways context may affect behavioural manipulation and can provide insight into how context effects other examples of behavioural manipulation too, both in wildlife trematodes and in general.

My thesis helps to develop *E. trivolvis* lineage c as a model for wildlife trematodes. We used laboratory exposures to test the suitability of several host species and morphotypes as first intermediate hosts and the effect of host size and dose on infection success. Insights into host specificity and the factors that affect infection success can be used to obtain more infected gastropods for laboratory studies of parasite manipulation. I hypothesize that if *Echinostoma trivolvis* lineage c is strictly host specific, then *Ladislavella elodes* will be the only first intermediate host and other host species / morphotypes will not be infected (*Stagnicola reflexa*, *Planorbella duryi*). Furthermore, I hypothesize that if size and miracidial dose impact infection success and survival rates, then the greatest infection success will be achieved with medium-sized snails at a high dose. In the second chapter, I used these lab-exposed hosts to investigate the context dependence of parasite manipulation of host behaviour. I determined whether infection altered host attraction between conspecific snails and then determined if changes to light levels and water temperature affect the extent of attraction between hosts. I hypothesized that if light affects the behaviour of potential second intermediate hosts, they will move at a greater rate under higher light conditions. Additionally, I hypothesized that if behavioural modification of potential second intermediate hosts is dependent on the species of

potential host, then uninfected conspecific snails will not be attracted to infected conspecifics like what has been observed between heterospecifics. My work expands the contexts in which parasite manipulation of host behaviour has been investigated. This research will inform future behavioural studies of gastropods, specifically in the ways that abiotic context can affect behaviour and shed light on the ways in which *E. trivolvis* lineage c manipulates the navigational behaviour of uninfected snails. Overall, my thesis uses *E. trivolvis* as a model wildlife trematode to fill knowledge gaps regarding life cycles and behavioural manipulation observed in this group. My research will help illuminate the effects that increasing wildlife trematode prevalence due to climate change may have on the environment and ease further efforts to investigate other aspects of this topic.

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Chapter 1: Host Species, Host Size, and Miracidial Dose influence the Infection Success of *Echinostoma trivolvis* Linage c Larvae

Studying parasites from naturally infected hosts is critical to understanding many aspects of parasite ecology. However, using naturally infected hosts can be limiting in comparison to laboratory-infected hosts. Factors such as parasite dose, host size, and exposure history are usually unknown in naturally infected hosts but are controlled in lab-infections. Likewise, the influence of parasite diversity on infection success in nature may be challenging and time consuming to determine as a host population may harbor different populations, species, genera, and families of parasites. For example, there are many cryptic complexes of helminth species that, if unaccounted for, may obscure estimates of infection success for a particular cryptic species or lineage (Detwiler et al., 2010). In comparison, using laboratory-exposed hosts allows for a much greater understanding and control over the diversity of specimens. A comprehensive understanding of the influences of these factors on infection success can be used to maximize the number of lab-infected hosts. These hosts can then be used to further explore other aspects of host-parasite interactions such as parasite-modified host behaviour.

The entire life cycle of some trematodes can be completed in the laboratory due to the use of low maintenance hosts and fast development times (Toledo et al., 2007). For instance, *Echinostoma* spp., require at most 2 host species: a single snail species can act as both the first and second intermediate host, while a small mammal or bird can serve as the definitive host (Toledo et al., 2007; Detwiler and Minchella, 2009). In addition, the life cycle can be completed within a matter of weeks (1 day to 6 weeks per life stage) (Toledo et

al., 2007). As a result, they are recognized as laboratory models for studies on host-parasite interactions (Toledo et al., 2007). Further, these echinostome species can infect wildlife and humans suggesting that insights from the laboratory can inform studies on animal and human disease (Huffman and Fried, 1990; Toledo et al., 2007). However, many nominal species within *Echinostoma* have been discovered to be cryptic species, causing some lineages to be poorly described in terms of their host specificity and life cycles, which limits their use in the laboratory (Detwiler et al., 2010).

One of these species complexes is *Echinostoma trivolvis,* which has been used to study many topics of ecological concern, including the role of parasites in amphibian decline, the interaction between pollution and parasitism on aquatic hosts, as well as the influence that climate change may have on parasitic systems (Raffel et al., 2009; Szuroczki and Richardson, 2009; Wang et al., 2019). Kanev et. al (1995) redescribed *E. trivolvis* as a nominal species to distinguish it from other species in the *Echinostoma revolutum* group (larvae and adults have 37 collar spines). This redescription used first intermediate hosts found in nature to make determinations on the species, including the elucidation of the life cycle. Lab exposures were used to explore the range of specificity towards all host stages (Kanev et al., 1995). Within this study, only one first intermediate host was observed, *Helisoma trivolvis* (synonym of *Planorbella trivolvis*), with infections being unable to be established in snail species within Lymnaeidae, Physidae, and Planorbidae. Certain larval stages (miracidia, sporocysts, rediae) are expected to occur in a single host species as a product of co-evolution (Huffman and Fried, 1990; Lockyer et al., 2004). As a result of the redescription, it was assumed that *E. trivolvis* is strictly host specific to *Planorbella*

trivolvis. However, additional genetic lineages that formed a clade with *E. trivolvis* were later discovered but used different snail species as a first intermediate host, *Lymnaea elodes* (synonym of *Stagnicola elodes* and *Ladislavella elodes*). Thus, the pattern of host use and genetic differentiation within *E. trivolvis* was greater than expected for a single parasite species, indicating that it may be a cryptic species complex.

The hypothesis that *E. trivolvis* is a cryptic species complex originated from DNA sequencing of larval parasites from naturally infected first intermediate hosts (Detwiler et al., 2010; Detwiler et al., 2012). Three distinct genetic lineages of *E. trivolvis* (a, b, and c) have been discovered through differences in mtDNA sequences (primarily ND1) and nuclear DNA (ITS) (Detwiler et al., 2010; Detwiler et al., 2012). As expected, one lineage originated from *Planorbella trivolvis*, but the other 2 lineages were found in *Ladislavella elodes*. The variation in first intermediate host use is atypical for most echinostomes, further supporting the existence of more than 1 species within this complex (Toledo et al., 2007). At present, the second intermediate host specificity for each of the lineages is unclear as few studies have sequenced metacercariae or performed experimental infections with cercariae identified as these lineages (but see Wojdak et al. 2013 for lineage a). However, multiple host species have been lab infected as second intermediate hosts of lineage c, including *Ladislavella elodes* and *Planorbella trivolvis* (Eliuk et. al., 2020). Confirmed definitive hosts through DNA sequencing show that naturally infected wildlife like muskrats can be infected with all lineages (Detwiler et al., 2010; Detwiler et al., 2012). The specificity for birds and waterfowl is less clear due to limited geographic and bird species sampling, but lineage c was found to occur only in muskrats while lineage a

occurred in mallard ducks and sand-hill cranes harvested in Manitoba, Canada (Sultana, 2018).

First intermediate host specificity can be a key characteristic in trematode species diagnoses. As such, validating the range of hosts used in an experimental context is essential. Natural infections are the product of the likelihood of encounter and compatibility, while controlled exposures ensure that encounters between the parasite and host occur, making compatibility between the parasite and the potential host paramount (Euzet and Combes, 1980). Host size may influence infection success, so determining which size classes should be used in miracidial exposures to obtain a high prevalence of infection can make *E. trivolvis* more effective models for laboratory studies of host-parasite interactions (Kuris, 1980; Jeyarasasingam et al., 1981; Huffman and Fried, 1990). In the congener *Echinostoma liei* (synonym of *Echinostoma caproni*, *Echinostoma paraensei* and *Echinostoma togoensis*), miracidial infection success was lower in smaller *Biomphalaria glabrata* snails (shell length: 1.0 – 1.9 mm) compared to larger host conspecifics (2.0 – 5.9 mm) (Kuris, 1980). However, exposures in the lab showed that miracidia of *E. caproni* had less infection success in larger *B. glabrata* snails (10 – 20 mm) compared to smaller-sized snails (2 – 5 mm) as well (Jeyarasasingam et al., 1981). This may be due to the ability for the potential host to resist infection; in small snails there is little resilience against infection, resulting in high mortality, while the larger snails have a better physiological condition, which reduces susceptibility to infection (Langeloh and Seppälä, 2018). At present, the effect of first intermediate host size on infection success is unclear for *E. trivolvis* and its genetic lineages, although Fried et al. (1987) achieved 52% (13/25) infection success after

exposing 5 miracidia of *E. trivolvis* lineage a to relatively small *Planorbella trivolvis* (3-5 mm). Given that growth rates differ between species and families of snails, it is unclear what size of lymnaeid host should be used to maximize the infection success of echinostome lineages. However, it stands to reason that the high mortality of smaller gastropods and low susceptibility to infection of large gastropods would make a medium sized potential host best for maximizing infection success.

Infection success is also influenced by parasite dose (Kuris, 1980; Fried et al., 1987). Similar to host size, the dose of miracidia used to expose potential first intermediate host may be a balance between exposure and compatibility. A higher dose may be more likely to encounter the host, but more likely to cause host mortality (Kuris, 1980). There is also the consideration of using a dose that limits the number of parasites required for exposures, as this is often a limiting factor in experimental infections. Fried et al. (1987) found that doses of *E. trivolvis* (referred to as *E. revolutum*) greater than 1 miracidia resulted in higher infection success (83-100%). At present, the dose required to maximize infection success for *E. trivolvis* lineages b and c is unknown. Further, the relationship between host size and dose is unknown making it more challenging to maximize infection success and obtain a large number of infected first intermediate hosts for laboratory studies.

The purpose of this study was to explore how to increase infection success in the first part of the life cycle of *E. trivolvis* lineage c in a laboratory. We determined the effects of host species, host size, and parasite dose on infection success. Based on evidence from

natural infections, we hypothesized that *E. trivolvis* lineage c would only develop patent infections in a single first intermediate host species, *L. elodes* and potentially its *S. reflexa* morphotype. We also hypothesized that if dose and host size affected infection success, then medium-sized snails exposed to a higher dose would result in a higher prevalence of infection compared to the lower dose and other size combinations. Determining methods to maximize the number of lab-exposed hosts infected with *Echinostoma trivolvis* lineage c expands our ability to characterize the biology of this cryptic parasite lineage such as its species status as well as elucidating its contributions to amphibian decline, manipulation of host behaviour, and parasite responses to climate change.

To obtain snails for the exposures, uninfected snails were selected from lab-raised colonies of 2 species, *Ladislavella elodes* and *Planorbella duryi*, as well as the *S. reflexa* morphotype that is currently considered a synonym of *L. elodes* (Fig. 1.1). The lymnaeid species originated from eggs obtained from 30 adults collected in a marsh in 2022 (N 50° 11', W 97° 07') and boreal shield wetland (N 49° 41’45.2”, W 95°14’35.1”) in 2021, respectively. The planorbids originated from 30 adults that were part of a colony established in 2015 from a shipment of aquatic plants to the Biological Sciences Department greenhouse at the University of Manitoba. Voucher images of the shells from the 3 snail groups were taken according to Callomon (2019; Fig. 1). Partial genetic sequences were obtained of the cytochrome *c* oxidase I gene for the *S. reflexa* morphotype as described in Gagnon and Detwiler (2019) and deposited in GenBank (PP468355-PP468358). Using digital calipers, the length of the snail shells was measured from apex to aperture for lymnaeids, while shell diameter was measured for planorbids (Burch, 1982).

Snails were subsequently separated into 3 size categories: small (1 – 5 mm), medium (5 – 10 mm) and large (10 – 15 mm). Snails were maintained in groups of 5 in containers with 500 ml of non-chlorinated water based on species and size class over the course of the experiment. The water was replaced twice weekly, and the snails were fed green leaf lettuce *ad libitum*.

To obtain miracidia for exposures, we acquired gravid, adult specimens from muskrats (*Ondatra zibethicus*) harvested by fur trappers. The carcasses were necropsied within 24 hours of trapping and their small and large intestines as well as the cecum were searched for adult echinostomes, with specimens putatively identified as *E. trivolvis* based on their body size and number of spines found exclusively in the first third of the small intestine. Eggs from ~5 gravid adults were teased out of the uterus using forceps and pooled into sterile petri plates filled ¾ full with autoclaved non-chlorinated water. The eggs were incubated in the dark for 10 days, after which time they were placed 25 cm under incandescent lamps (100W) daily for 1-5 hours to induce hatching (Nollen, 1994). The anterior end of each adult was stored in 100% ethanol and subsequently the DNA was extracted and the partial ND1 gene amplified and sequenced to verify the identification of the parasites using methods described in Eliuk et al. (2020). A total of 47 sequences were obtained and the blastn algorithm confirmed all sequences as *E. trivolvis* lineage c that matched (> 94 query coverage and 99-100% identity) to 2 haplotypes (JQ670861.1 and GQ463052.1).

The snails were exposed to miracidia within 6 hours of hatching in 6-well plates filled ¾ full with non-chlorinated water for a period of 12 hours. To test for the effect of host

species, 2 miracidia were exposed to each of the 3 snail groups (*L. elodes*, its *S. reflexa* morphotype, and *P. duryi*, n ~ 30 per host group). To determine the interaction between size and snail species on infection success, 2 miracidia were exposed to the small, medium, and large size class of each of the 3 snail groups (N = ~30 per dose*size class combination). To determine whether miracidial dose and size influence infection success, we exposed *L. elodes* snails of all size classes to either 2 or 5 miracidia (N = ~30 per dose*size class combination). After the 24-hour exposure period, the snails were returned to their original containers and maintained as previously described for 6 weeks to allow infections to become patent (3rd generation daughter rediae with mature cercariae), at which point infection status can be tentatively determined without killing the host (Fried et al., 1997; Toledo et al., 2007). To do so, exposed snails were placed under incandescent lamps in 6-well plates for a period of 5 hours to induce cercarial emergence (Toledo et al., 2007). The wells for each snail were checked under a stereomicroscope repeatedly and any that contained cercariae were recorded as infected. Non-shedding snails were crushed to verify their infection status.

Host specificity simply constituted which of the 3 snail groups developed patent infections following exposure to miracidia. To analyze how host size and parasite dose influenced infection success, we performed 2 logistic regressions. The first analysis included all snail groups exposed to 2 miracidia that developed patent infections. We determined the effects of host species and host size class on infection success with binary infection success as the dependent variable and host group and host size class and their interaction as the independent variables. The second analysis was restricted to *L. elodes*

as this was the only snail group that was exposed to 2 dose levels. To determine the effects of host size class and parasite dose on infection success, we included both factors as well as their interaction as the independent variables. For both logistic regression models, we tested for multicollinearity between our independent variables using the variance inflation factor with values between 1-5 indicating no multicollinearity (Zuur, 2007). To assess the fit of each model, we calculated McFadden's pseudo R-squared (Louviere, 2000). Statistical analyses were performed in R version 4.2.1 using packages car (R Core Team, 2021), tidyverse (Wickham et al., 2019), emmeans (Lenth, 2022) and rcompanion (Mangiafico, 2022).

Only *L. elodes* and *S. reflexa* snails emitted cercariae at weeks 6 and 8 post-exposure (Table 1.1). Given that no *P. duryi* became infected, we excluded this species from subsequent analyses. At a dose of 2 miracidia, infection success was affected by host morphotype ($\chi^2 = 5.2$, $df = 1$, $P = 0.023$) with 5.7% of the *L. elodes* (20/353) and 10.9% (10/92) of its *S. reflexa* morphotype infected (Table 1.1). The interaction with host morphotype and host size was also significant ($\chi^2 = 14.1$, $df = 2$, $P = 0.0009$), However, host size alone ($\chi^2 = 5.2$, $df = 2$, $P =< 0.074$) was not significant with 2.6% small- (4/151), 9.2% medium- (17/185) and 6.6% large-sized (9/136) individuals of both morphotypes combined becoming infected (Table 1.1). The model had a McFadden's pseudo-R^2 of 6.1%. Tukey's post hoc test revealed that all pairwise comparisons were not significantly different.

When medium and large-sized *L. elodes* were exposed to 2 doses, neither host size ($\chi^2 = 5.22$, $df = 2$, $P = 0.07$) or dose ($\chi^2 = 0.54$, $df = 1$, $P = 0.46$) explained variation in infection success. However, the interaction between host size and parasite dose was significant ($\chi^2 =$

11.1, *df* = 2, *P* = 0.004) in a model where McFadden's pseudo-R^2 was 16.8%. Tukey's post hoc test found that more medium-sized snails became infected with a higher dose (9.5% vs 36.7%, *P* = 0.0059, Table 1.1), with a similar effect observed in small-sized snails (3.3% vs 33.3%, *P* < 0.001, Table 1.1), while there was no effect of dose on infection success in large-sized snails (3.8% vs 2.3%, *P* = 0.8774, Table 1.1).

The ability for *E. trivolvis* lineage c to infect both *L. elodes* and its *S. reflexa* morphotype supports the prediction that trematode miracidia typically infect a single species (Huffman and Fried, 1990; Lockyer et al., 2004). Although *S. reflexa* is now considered a synonym of *L. elodes*, taxonomic experts differ in opinion on the species status of *S. reflexa*. Some authorities considered them separate species (Clarke, 1981), while others consider *S. reflexa* a synonym of *L. elodes* (Johnson et al. 2013; Dillon, 2022). We have maintained separate colonies of *S. reflexa* and *L. elodes* in the lab for several years, and the difference in shell characteristics has been maintained. Further, we compared the partial cytochrome oxidase 1 gene sequences from *S. reflexa* individuals using the blastn algorithm and found the most genetically similar matches in GenBank were to *Ladislavella elodes* (query coverage 99-100 and percent identify 94.2-95.73). This preliminary analysis suggests that the synonymy of *S. reflexa* with *L. elodes* should be reinvestigated. Clarifying host taxonomy is important in determining fundamental natural history characteristics such as host specificity of parasites. If it is assumed that parasites only use one first intermediate host species, then our infection results support the current status that *S. reflexa* is a synonym of *L. elodes*. However, some echinostomes as well as other trematodes use more than one first intermediate host species (Detwiler et al. 2010;

Sultana, 2018; Laidemitt et al., 2019) suggesting co-evolutionary, ecological, or immunological similarities between gastropod species.

When the dose of miracidia of *E. trivolvis* lineage c was lower, the factor that explained the most variation in infection success was host size. Infection success between medium- and large-sized snails was similar (nearly 10%) and was 3x higher compared to small-sized snails. However, the overall prevalence in large-sized snails was driven by the *S. reflexa* morphotype. The higher infection success in large-sized *S. reflexa* compared to large-sized *L. elodes* may suggest differences in co-evolution between this parasite lineage and the two snail morphotypes. Relative to *S. elodes*, *S. reflexa* has a smaller geographic range and patchy distribution which may limit encounters with *E. trivolvis* lineage c and increase its susceptibility in the lab where the likelihood of encounter is increased (Clarke 1981, Euzet and Combes, 1980). At present, natural infections of *E. trivolvis* lineage C in *L. elodes* are limited and no natural infections of *S. reflexa* have been confirmed. However, our results also indicate that factors other than coevolution influence infection success given that morphotypes did not differ within the other size classes.

Within *L. elodes*, a higher dose of 5 miracidia increased the infection success of both medium- and small- sized snails compared to large-sized snails. At a higher dose and as host body size increases, the parasite has more chances to encounter a host leading to a higher rate of infection per miracidium (Euzet and Combes, 1980). However, increases in infection success due to encounter can also be affected by compatibility between the host and parasite (Euzet and Combes, 1980). Our study suggests that compatibility between the

host and parasite is lower for large-sized *L. elodes* and that the increase in dose was not sufficient to counteract this limit.

In our study, infection success may have been affected by host mortality. However, this factor was not monitored consistently for all snail groups and combinations and no snails were sham-exposed (i.e., negative controls), so the association between parasite exposure and mortality could not be assessed. However, the snail group with the highest infection, medium-sized *S. elodes* exposed to 5 miracidia, had no mortality. Furthermore, the increase in infection success of small-sized *L. elodes* due to increased dose suggests that mortality does not have a significant effect or is at least similar at these doses. If the goal is to obtain many infected individuals, these results suggest that at least 5 miracidia should be exposed to small and medium-sized *L. elodes*.

To our knowledge, this is the first time that this part of the *E. trivolvis* lineage c life cycle has been completed in the laboratory. Notably, we have expanded our knowledge of host specificity for this lineage as natural infections had already shown that *L. elodes* was a first intermediate host (Detwiler et al. 2010, Sultana, 2018). Our experimental exposures confirm that a genetically distinct morphotype of *L. elodes*, formerly known as *S. reflexa*, is also a viable first intermediate host. However, differences in infection success based on size class between the morphotypes of *L. elodes* suggest that the co-evolutionary, ecological, and/or physiological relationship with the parasite may differ. We have also taken steps towards understanding the relationship between species, size, and dose of parasites in relation to infection success. Our findings suggest that morphotype influences the effect of size and that size influences the effect of dose. Future studies should focus on

separating the effects of host species/morphotype, host size, and parasite dose to better understand how they affect infection success. Altogether, this study enables wider use of *E. trivolvis* lineage c in studies of host-parasite interactions by providing a description for how to obtain infected first intermediate hosts, as well as revealing key aspects of its biology.

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Figure 1.1 Digital images of the snail species in 3 views (aperture, lateral, dorsal). Top row (A) = *Ladislavella elodes*, Middle row (B) = the *Stagnicola reflexa* morphotype of *L. elodes*, Bottom row (C) = *Planorbella duryi*.

Table 1.1: Infection success of *Echinostoma trivolvis* lineage c for each snail group (species or morphotype), size class and dose combination.

Snail Group	Size (mm)	Dose	Infection Success (%)
Ladislavella elodes	Small (1 – 5)	2	3.3 (4/121)
Ladislavella elodes	Small (1 – 5)	5	33.3% (10/30)
Ladislavella elodes	Medium (5 – 10)	2	9.5 (12/126)
Ladislavella elodes	Medium (5 – 10)	5	36.7 (11/30)
Ladislavella elodes	Large (10 – 15)	2	3.8 (4/106)
Ladislavella elodes	Large (10 – 15)	5	2.3 (6/256)
Stagnicola reflexa	Small (1 – 5)	2	0 (0/30)
Stagnicola reflexa	Medium (5 – 10)	2	8.5 (5/59)
Stagnicola reflexa	Large (10 – 15)	2	16.7 (5/30)
Planorbella duryi	Small (1 – 5)	2	0 (0/30)
Planorbella duryi	Medium (5 – 10)	2	0 (0/30)
Planorbella duryi	Large (10 – 15)	2	0 (0/30)

Chapter 2: Abiotic and Biotic Context Changes Behaviour of Potential Second Intermediate Gastropod Hosts of *Echinostoma trivolvis* lineage c

Introduction 2.1

Animal behaviour is influenced by the context in which it occurs (Wong and Candolin, 2015; Muller et. al., 2018; Ganguly and Candolin, 2023; Luo et. al., 2024). Context includes any abiotic environmental factor an organism can sense, such as light, salinity and heat, as well as biotic factors, such as interactions between species. Context is critical to changing behaviour as it allows an organism to respond to the current situation, typically in a way that is adaptive (i.e., increases fitness) (Sih et al., 2004). Subsequently, animals which exhibit higher levels of behavioural plasticity are usually more successful than others (Wong and Candolin, 2015). Our understanding of adaptive behaviours can be summarized into three steps: 1) animals sense a factor that is contextual, 2) the context of the factor changes that could affect the animal's fitness and 3) animals respond by altering their behaviour to the change in the contextual factor to improve their fitness. For example, a prey animal may sense high light levels, which is associated with an increased risk of predation from a predator, resulting in the prey moving less frequently to reduce the risk of being consumed (Sih et al., 2004). Animals are presumably not aware of this process, but rather act instinctively based on which adaptive behaviours have evolved, a phenomenon that is referred to as behavioral plasticity (Snell-Rood, 2013). Animals are unlikely to have developed these behaviours through learning as a failure to participate in these behaviours is typically deleterious. However, not all changes

in behaviour are adaptive, and in some cases are actively detrimental to an individual's survival to the benefit of another (Ghalambor, 2007; Zentall and Stagner, 2011; Lafferty and Shaw, 2013). No interspecific interaction shows this better than parasites manipulating host behaviour to benefit parasite fitness rather than host fitness.

Host behaviour modification is a common phenomenon among parasites, with at least 55 genera being known to manipulate behaviour (Poulin, 1994; Lafferty and Shaw, 2013). The changes to host behaviour vary significantly among parasite species, with many subtle changes. For example, the eastern mud snail *Ilyanassa obsoleta* was more likely to stay above the water line during low tides when infected with the trematode *Gynaecotyla adunca* compared to uninfected conspecifics (Curtis,1987). This change in microhabitat use due to parasitism may increases the exposure of the infected snails to predation from shorebirds, which would benefit the parasite as trophic transmission is required for the life cycle to progress. Generally, behavioural modification can be split into changes in host activity, microhabitat choice or both (Lafferty and Shaw, 2013). An example of the former may be found in parasites of the virile crayfish, *Orconectes virilis* (now known as *Faxonius virilus*). When infected with the trematode *Microphallus* sp., individuals are less likely to win competitions for sheltered areas with uninfected conspecifics (Reisinger et. al., 2015). Further, infected crayfish were more bold than healthy conspecifics, displaying an increased amount of movement in the presence of a predatory fish. Some parasite-modified behaviour is suspected to be incidental, occurring as a byproduct of infection or even to reduce the effects of infection (Combes, 2001). However, a majority of studies have

focused on behavioural modifications that may increase the fitness of the parasite, most often to the detriment of the host.

Parasites may alter the behaviour of their hosts in ways that are adaptive for the completion of their life cycle, though this has not been confirmed in most cases (Thomas et. al., 2005; Lafferty and Shaw, 2013). If the modification is adaptive for the parasite, then the altered host behaviour should be more likely to transmit the parasite to the next host in its life cycle. Context often informs interactions between organisms, meaning certain contexts are more likely to result in transmission than others (Biro et. al., 2010; Cornwell et. al., 2019). As such, behaviours caused by parasitic infection may be intrinsically tied to the context in which the host exists in the same way as any other behaviour, with an alteration occurring at the third stage of the process (i.e., a responding change in host behaviour to best match the situation for the host is replaced with a change that is best for the parasite). Using the previously mentioned *Orconectes virilis* - *Microphallus* spp. system, an uninfected crayfish could sense the chemical emissions of a predatory fish, a circumstance that is evolutionarily associated with an increased likelihood of being eaten by a fish and respond by remaining sheltered and unmoving until the chemical stimulus has reduced or disappeared (Reisinger et. al., 2015). An infected conspecific would be expected to sense the same context associated with the danger except that a modified response would be predicted, with the crayfish either altering is microhabitat use or activity to increase their likelihood of consumption by the next host in the parasite's life cycle. This change in host behaviour would most increase the fitness of the parasite when the associated context (i.e., presence of a next host predator) is present, while having a less

beneficial or even detrimental effect on the fitness of the parasite in other contexts, such as those associated with predation from a non-host species. Therefore, if behavioural modification is an adaptive trait for the parasite, it would be expected that the modified host behaviour would be dependent on context in the same way that all other behaviours are. However, this has yet to be investigated in the majority of host-parasite systems.

Parasite modified behaviour is challenging to study, even without changing contexts being considered (Thomas et. al., 2005). All the difficulties associated with studying animal behaviour, such as controlling for life history, subjectivity of behavioural categorization and variation between individuals, is amplified by having to consider not only two organisms individually, but also the interactions between them. This is the reason why most cases of manipulation have not been confirmed as adaptive for the parasite as tying a specific host behaviour to an increase in the future size of parasite populations is not feasible for most host-parasite systems (Combes, 2001; Thomas et. al., 2005). However, ignoring the effect of different contexts on behaviour could lead one to misinterpret behavioural manipulation, as cases of behavioural modification that have been described may have different degrees of change across a specific context. For example, invasive species of crayfish infected with *Microphallus* trematodes showed greatly reduced effects on their aggression and shelter finding behaviors when compared to native species under the same laboratory conditions. This example shows that host species are an aspect of the biotic context that impacts behavioural modification by parasites (Reisinger et. al., 2015). Overall, the extent of behavioural modification cannot be fully understood unless it is tested in a wide range of naturally occurring abiotic and biotic contexts. This degree of

thorough experimentation has not been conducted for nearly all host-parasite interactions currently catalogued, leading to a limited understanding of how context is expected to interact with behavioural modification (Thomas et. al., 2005). To establish this baseline, a model parasite that manipulates host behaviour should be examined in multiple contexts.

Species in the family Echinostomatidae have been studied since as early as the 18th century, with researchers increasingly adopting them as models due to their relatively easy to maintain life cycles and wide global distribution (Toledo et. al., 2014). For this study, we chose the parasite *Echinostoma trivolvis* lineage c as our model for several reasons. First, parts of the life cycle of *E. trivolvis* lineage c have been completed in the laboratory (Hodinka and Detwiler, 2024). Eggs can be acquired from adult echinostomes found in the small intestine of field-caught muskrats and incubated in water-filled petri plates until miracidia develop and emerge. This free-swimming stage can then be exposed to the freshwater snail *Ladislavella elodes* to obtain patently infected first intermediate hosts. This process takes a matter of 6-8 weeks, which is a faster period of development relative to many trematodes (Fried et. al., 1997; Toledo et. al., 2007; Toledo et. al., 2014; Hodinka and Detwiler, 2024). Second, these exposures can be conducted with lab-raised, parasite-naïve hosts, such as freshwater snails, which can be bred and maintained in large numbers (100s to 1000s) in the laboratory. Third, it is relatively easy to video record and analyze the behaviour of these freshwater snail hosts due to their relatively large size and their slow movement (Gray et al. 2009; Eliuk et al. 2020). In addition, the same snail species, *Ladislavella elodes*, can be a first and second intermediate hosts for *E. trivolvis* lineage c allowing for explorations of parasite-induced changes to both types of hosts (Detwiler,

unpublished). Fourth, the use of molluscan hosts is also a trait conserved across class Trematoda, which makes research on behavioural modification of gastropods broadly applicable for the entire group (Lockyer, 2004). Behavioural manipulation of molluscs by echinostomes has been observed in the laboratory (Eliuk et. al., 2020). In a Y-maze, potential *Planorbella duryi* second intermediate host snails showed greater attraction towards an infected *L. elodes* first intermediate host rather than an uninfected *L. elodes* (Eliuk et al., 2020). Finally, given the ability to study behavioural manipulation in the laboratory using snails and trematodes, the effects of specific abiotic and biotic factors on behavioural manipulation can be studied in isolation and in combination, to determine how context influences the extent of behavioural manipulation.

Light level is an abiotic factor that may affect the behavioural manipulation of freshwater gastropod hosts for several reasons. First, light levels influence the behaviour of freshwater gastropods in the absence of parasitism. Freshwater snails use light cues to orient themselves away from light sources within aquatic environments (Lombardo et. al., 2010). Further, snails vary their activity according to day/night light cycles, with less movement and a lower average velocity during darker hours than during the day (Lombardo et. al., 2010; Checon et. al., 2021). Second, light also has a direct influence on the development and emergence of trematode parasites (Fried et. al., 1997; Morley and Lewis, 2013). High light levels are used to increase the development of miracidia so that they hatch more quickly from eggs (Fried et. al., 1997; Hodinka and Detwiler, 2024). Further, first intermediate hosts are placed under lights to induce the emergence of cercariae from the host (Fried et al., 1997; Hodinka and Detwiler, 2024). However, the effect of light on

behavioural manipulation is less clear as typically one light level has been used. For instance, a potential second intermediate host species *Planorbella duryi* was attracted to first intermediate host, *L. elodes*, in the laboratory during the day at 1090 lux (Eliuk et al. 2020). This light level is relatively low to what freshwater snails experience in nature as light levels can be at least 5500 lux during the day at the surface of a pond (Hodinka, pers. obs). At present, it remains unclear whether attraction between hosts is affected by different light levels. However, one prediction is that by increasing light and the activity level of the parasites (i.e., emergence of cercariae), attraction between hosts would increase as this would benefit the host to bring the next host in the life cycle in close proximity to the source of the cercariae.

In addition to abiotic factors such as light, the biotic context of species interactions could influence behavioural manipulation of freshwater snails. Echinostome cercariae emerging from a first intermediate host can infect second intermediate hosts that can be the same individual (e.g., cercariae encyst in same host they originated from) (Kanev et. al., 1995; Fried et. al., 1997). These cercariae can also infect a conspecific or a heterospecific snail species, and even other species beyond snails such as tadpoles (Szuroczki and Richardson, 2009). Laboratory studies indicate that cercariae prefer to infect heterospecific hosts relative to conspecifics (Detwiler and Minchella, 2009; Wojdak et. al., 2013). There is evidence that cercariae are able to differentiate the secretions of heterospecific snails, enabling them to discriminate between a potential conspecific or heterospecific second intermediate host (Hertel et al., 2007; Langeloh and Seppälä, 2018). It would be adaptive for cercariae to preferentially infect heterospecific hosts over

conspecific hosts as first intermediate hosts have higher mortality than second intermediate hosts due to the consumptive development of rediae and damage caused by cercarial emergence (Kuris and Warren, 1980). If the attraction of potential second intermediate hosts to the first intermediate host is an adaptation of the parasite, the attraction may be stronger between heterospecific snails than in conspecific snails, or even completely absent in conspecific contexts. As the behavioural manipulation in this system has only been observed in a heterospecific context, testing attraction dynamics in conspecific potential hosts is needed.

We hypothesized that light level would influence snail navigational behaviour in the absence of parasites. Specifically, we predicted that under high light (5500 lux), snails would increase their velocity and wandering compared to low light (165 lux) as these levels correspond to light during the day and evening, respectively. Using those high light conditions, we then hypothesized that parasitism of conspecifics would not influence the behaviour potential second intermediate host *L. elodes*. We predicted aspects of navigational behaviour as a measure of attraction like velocity and wandering by conspecific responders will be similar when uninfected or infected conspecific snails were presented as stimuli in a Y-maze. This research will determine whether two different contexts affect both unaltered behaviours and behavioural modification of freshwater snail hosts by *E. trivolvis* lineage c. Contextual changes in modified behaviour may indicate that the scope of parasite manipulation observed in the laboratory has yet to capture the full spectrum that may be occurring in nature.

Methods 2.2

A laboratory colony of *Ladislavella elodes*, established using eggs obtained from 30 adults collected in a marsh (N 50° 11', W 97° 07') in 2021, was used to obtain both responder and stimulus snails for this study. This freshwater snail species was chosen as both field and laboratory studies indicate that *E. trivolvis* lineage c uses only this species as a first intermediate host (Detwiler et al. 2010; Hodinka and Detwiler, 2024). In 2023-2024, hatchling snails (<1mm in length) from the laboratory colony were separated into groups of five individuals and placed in 500 ml plastic containers filled with non-chlorinated water. The water was replaced twice weekly, and the snails were fed green leaf lettuce *ad libitium*. They were kept in a room with a 12-hour light/dark cycle to approximate a natural day/night cycle. Snails were raised in these containers until they reached a shell length (aperture to spire) of at least 5mm.

A total of 142 snails with shell lengths of 5-10 mm were haphazardly selected from the containers and exposed to miracidia of *Echinostoma trivolvis* lineage c following methods described in Hodinka and Detwiler (2024). Each snail was individually exposed to either 2 or 5 miracidia for 24 hours using 6-well plates and then transferred to 500 ml containers in groups of 5 and maintained with the feeding and water changing regime described above. Six weeks post-exposure, snails were placed under lamps (100 watt) in 6-well plates for 5 hours to induce cercarial emergence and confirm a patent infection. At this point, a total of 23 snails were determined to be first intermediate hosts and were used as stimuli in the behavioural assays. All snails used in behavioural assays were later crushed to confirm infection status (i.e., presence of rediae in gonadal tissue).

To test whether light or parasitism influenced snail navigational behaviour, we utilized a Y maze with arms measuring 5 cm x 15.5 cm filled to a depth of 2.5 cm with 350 ml of non-chlorinated water. Haphazardly selected, unexposed, individual *L. elodes* snails from the laboratory colony (5-10 mm in shell length) were placed at the base of the Y maze and confined to the first 2 cm of the base using a piece of Styrofoam. After a 10-minute acclimation period, the snails were allowed to move freely, and their behaviour was video recorded with either a Sony HDR-CX675 or Panasonic HC-V180 for 30 minutes. A stimulus/stimuli, when present, was placed at the end of an arm selected using an online random number generator (Haahr, 2022) at the start of the acclimation period, allowing for a chemical gradient to reach the junction between the 2 arms (the choice point).

To test the effect of light, a total of 4 treatments were performed where responding snails were exposed to either high or low levels of light. High light was provided by 4 incandescent lamps (100 watt) placed around the behavioural chamber, which created a light level of 5500 lux over every section of the chamber. For the low light treatments, the ceiling lights emitted 165 lux. For each light level, treatments consisted of a responding snail with no stimulus and another treatment with a food stimulus at the end of one randomly chosen arm (i.e., 5 grams of duckweed within a cheese cloth sack). Video analysis on each recording was performed using the StackReg (Thévenaz et. al., 1998), Trackmate (Ershov et. al., 2022) and mTrackJ (Meijering et. al., 2012) packages in ImageJ to obtain the average velocity of each responder snail (see Eliuk et. al., 2020 for more details). In addition, this software was also used to calculate the degree of wandering, which was measured as the standard deviation of the change in the heading of each responder snail.

To test the effects of species interactions (conspecifics and parasites) on *L. elodes* navigational behaviour, 5 treatments were performed including a negative (i.e., no stimulus in either arm of Y maze) and positive control (i.e., food stimulus in one randomly selected arm) using the high light of 5500 lux. Frozen cubes of brine shrimp (1.5 grams, San Francisco Bay brand, sourced from Petsmart) were used as the stimulus in the positive control as *L. elodes* has been shown to be attracted to crustacean carrion (Gray et al. 2009). The 3 experimental treatments included 1) an uninfected conspecific placed in one randomly selected arm, 2) a patently infected conspecific placed in one randomly selected arm and 3) one of each stimulus presented at the same time in randomly selected arms. All stimuli were placed at the end of each arm in a 3D printed rectangular prism (1.5 x 1.5 x 3.0 cm) lined with 70-micron nylon mesh. In contrast to the first experiment, snail behaviour was recorded for 60 minutes following 10 minutes of acclimation, as preliminary trials for this experiment found that responder snails often did not reach the decision point before 30 minutes had passed. Video analysis was performed to obtain the mean velocity of the responder in each arm and the standard deviation of the change in the heading of the responder snail in each arm. Furthermore, to measure the attraction to each stimulus in this system, the mean distance of the responder from each of the stimuli, the time the responder spent within 3 cm of the stimulus, the time the responder spent in each arm and the number of times the responder entered each arm was also obtained using the ImageJ packages StackReg (Thévenaz et. al., 1998), Trackmate (Ershov et. al., 2022) and mTrackJ (Meijering et. al., 2012). A distance of 3 cm was selected because it was within the range that cercariae can locate and penetrate a second intermediate host (Hodinka, pers. obs). A

distance of 1 cm was also analyzed but did not significantly differ from 3 cm (data not shown).

To analyze the effects of light levels on snail behaviour, we performed 4 t-tests comparing the average velocity and standard deviation of the change in the angle of heading (D angle) between the high light and ambient light conditions. Treatments were only compared with the other treatment with the same stimulus presence / absence. To test the effect of parasitism on attraction, we performed paired t-tests to compare the measures of behaviour between the two arms within each treatment. We also performed one-way ANOVAs to compare the behaviour of the responders across all treatments (excepting the positive control). For analyses, we assigned the left arm as the stimulus arm for the negative control, whereas for the double stimulus treatments, we used the measures relative to the infected stimulus snail. Analyses were performed using R version 4.2.1 with packages car (R Core Team, 2021), tidyverse (Wickham et al., 2019), emmeans (Lenth, 2022), rcompanion (Mangiafico, 2022), MASS (Venables and Ripley, 2002) and glmmTMB (Brooks et. al., 2017).

Results 2.3

Under the 2 light conditions, the average velocity of the responder snails differed when no stimulus was present ($W = 304$, $P < 0.047$). When both arms were empty, responding snails moved faster in high light (mean velocity ± standard error: 0.009 ± 0.005 cm/s) compared to low light (0.006 ± 0.006 cm/s). In the presence of the duckweed stimulus, average velocity of responder snails differed between the light levels ($W = 184$, P

< 0.001). Responders moved approximately 2x faster when exposed to high light (0.011 ± 0.005 cm/s) compared to low light (0.005 ± 0.005 cm/s). However, there was no significant difference in the standard deviation of the change in the D angle of the responder snails between the light treatments with (78.9˚ ± 1.5˚) or without (81.6˚ ± 1.3˚) a duckweed stimulus (t = 1.29, *df* = 52.5, *P* = 0.20; t = 1.97, *df* = 51.7, *P* = 0.054).

In the second experiment testing whether attraction between conspecifics is affected by parasitism, responder snails showed an attraction to the brine shrimp stimulus. Compared to an empty arm, the mean distance from the stimulus (13.9 ± 9.5 cm) differed compared to an empty arm (21.3 ± 8.7 cm; V = 121, *P* = 0.02). Further, more time was spent in the arm with the stimulus (1731.3 ± 1334.0 sec) compared to an empty arm (851.5 ± 1185.4 sec; V = 291, *P* = 0.046). Moreover, more time was spent within 3 cm of the stimulus (1350.3 ± 1214.0 sec) compared to the same area within the empty arm (506.2 ± 784.4 sec; V = 294.5, *P* = 0.01). However, there was no significant difference in the number of times the responder snail entered the arm with the stimulus (0.9 ± 0.74) compared to an empty arm (0.5 ± 0.72; V = 167, *P* = 0.058). Likewise, there was no significant difference in the average velocity traveled in the arm with and without the brine shrimp (0.023 ± 0.017 vs 0.018 ± 0.021 cm/s, V = 254, *P* = 0.25). There was also no difference in the standard deviation of the change in the D angle between the arm with the brine shrimp and without (53.1 ± 32.7 and 31.7 ± 35.8, respectively; V = 289, *P* = 0.052).

In contrast, there were no differences between the arms for any measure in the negative control (Table 2.1). Additionally, there were no differences in the six measures of navigational behaviour for the two treatments involving an empty arm and a snail stimulus

(uninfected conspecific, infected conspecific) (Table 2.1). Further, none of these measures differed when two snail stimuli (uninfected and infected) were each presented in an arm simultaneously (Table 2.1). Across the four treatments (except the positive control), the ANOVA for each measure of navigational behaviour showed no differences (Figures 2.1-2.6).

Discussion 2.4

Our results show that one type of abiotic context, differing light levels, affected the navigational behaviour of *L. elodes* snails. Compared to low light, high light led the snails to travel more quickly (cm/s) without a stimulus suggesting that light levels alone influence the pace at which snails navigate. No matter the light conditions, snails moved at a quicker pace when the food stimulus was present, but the velocity was ~2x greater with high light conditions. Thus, light and food resources may be two stimuli that have an additive effect on snail velocity. This is congruent with other results found for this system, as snails show a low time to first response for food sources, a comparable metric to velocity (Grey et. al., 2009; Eliuk et. al., 2020). Our light levels in the laboratory correspond to light levels that snails can experience in nature. We measured 5500 lux at the surface of a pond at 12pm on a cloudy day and 165 lux was recorded at the same pond at 9pm during the evening (Hodinka, unpublished data). In nature, gastropods tend to be less active during daylight hours and have been observed feeding during late evening and night when light levels are low (Brown, 1985; Orvain and Sauriau, 2002; Checon et. al., 2021). In these studies, a number of factors changed over the course of the study, such as water temperature, light levels, and time of day, so it is challenging to isolate the effect of any of one of them on

snail behaviour. Furthermore, research on how aquatic gastropods respond to lights has focused primarily on marine species, which may not accurately represent the behaviour of freshwater species (Orvain and Sauriau, 2002; Checon et. al., 2021). In contrast, our laboratory-based experiments were performed between 9 am and 5 pm with a constant light level and similar water temperatures between the light levels. Snail species could also vary in their responses to light depending upon their microhabitat preferences. *Ladislavella elodes* tends to occur in more shallow, light areas compared to other species like *Planorbella trivolvis* that occur in deeper, darker areas of aquatic wetlands (Brown, 1982). Further, the environment in which snails are raised could affect their responses to light, with snails that had only experienced artificial lights less likely to react to natural light levels in the same way that wild individuals would.

If our results are reflective of what is occurring naturally with the species, there are significant implications when the relation between higher light and emergence of *E. trivolvis* infective life stages is considered. Emergence patterns in trematode larvae have been observed to correlate with the activity patterns of their next host (Combes, 2001). The second host specificity of *Echinostoma trivolvis* lineage c has not been investigated in laboratory or in naturally infected hosts, but presumably it can infect a variety of different gastropod species, similar to other echinostome species (Detwiler and Minchella, 2009). For example, *Echinostoma trivolvis* (i.e., likely lineage a based on first intermediate host use, Detwiler et al. 2010), cercariae can successfully develop, migrate, and become viable metacercariae in conspecific and heterospecific snails that co-occur with first intermediate host *L. elodes* as well as other non-gastropod taxa such as tadpoles (Kanev

et. al., 1995). Thus, in this case, higher snail activity level under these two conditions may improve the likelihood that free-swimming larvae emerging from the first host would encounter a second host. Although we did not test the response of *L. elodes* to cercarial parasites directly, we predict that behavioural modification is most adaptive for the parasite at high light because higher light levels increase the activity of the host as well as the parasite as increased light induces emergence of cercariae to allow for transmission to other second intermediate hosts as well as the emission of molecules that may drive attraction between the snails (Friesen et al. 2022). Host navigation is one type of behaviour that could affect the transmission rate between first and second intermediate hosts because it affects the proximity of parasites to the next host in the life cycle. Thus, we suggest that exploring whether parasites modify the movements of snails is best done in high light.

The lack of attraction to infected snails supports our prediction that behavioural modification of potential second intermediate hosts is not beneficial for the parasite. In this set of treatments, responder snails were attracted to another food source, brine shrimp, indicating that the lack of attraction is likely not due to the experimental design or inclusion of higher light levels. Rather, the lack of attraction is most likely due to the preference of cercariae for heterospecific second intermediate hosts (Detwiler and Minchella, 2009; Wojdak et al. 2013). If it is adaptive for the parasite to infect heterospecific second intermediate hosts, then it would also be adaptive for the behavioural manipulation to be greatest when heterospecific potential hosts are present. However, the complete lack of attraction was unexpected, as attraction of conspecifics

would be adaptive for the parasite as it would decrease the chance of reinfection of the current host. The lack of attraction could be related to the mechanism by which the behavioural manipulation occurs. A less sophisticated mechanism may only be able to target heterospecific snails, with no ability to invoke a more moderate response in conspecifics (Lafferty and Shaw, 2013). Alternatively, the mechanism may not change the behaviour of conspecifics due to an adaptive mechanism on the part of the gastropod instead of the parasite. To date, behavioural manipulation of potential second intermediate hosts has occurred in both heterospecific gastropods which do and do not occur with *L. elodes* or *E. trivolvis* lineage c in Manitoba. Naivety in the non-native species may leave them more vulnerable to the behavioural manipulation while snail species that naturally co-occur and have evolved with the parasite are better adapted to resist changes to their behaviour as being a second intermediate host of this parasite is suspected to have significant fitness costs (Combes, 2001). However, considering that behavioural manipulation has been observed in some co-occurring species, the effect of naivety on the susceptibility to behavioural modification in this system is likely low. Regardless of the lack of attraction to conspecifics, our results show an effect of biotic context on behavioural manipulation.

Overall, these results indicate that both abiotic and biotic context may have an impact on parasitic manipulation of behaviour and, as such, should be considered when behavioural assays are performed. This is especially true in laboratory experiments which both have a higher level of control over the context and are less likely to match naturally occurring contexts in which the behaviour occurs. These results have implications for

previous studies of parasitic manipulation of behaviour where behavioural manipulation was evaluated with a set of specific conditions, which limits our knowledge of the spectrum of behavioural manipulations that may occur in nature. Future research should focus on changing single contextual factors of behavioural assays in which behavioural manipulation has been observed, as this will provide a more thorough understanding of factors that influence manipulation. This approach could also aid in understanding the mechanisms by which behavioural manipulation occurs. For example, the difference in attraction found in conspecific and heterospecific potential second intermediate hosts in this system indicates that chemical signaling molecules may differ depending upon species. Indeed, Friesen et al. (2022) found that infected and uninfected *Planorbella pilsburyi* snails had significant differences in their oxylipin profiles, suggesting that chemical emissions are involved in the modification of behaviour in this system. Testing attraction towards specific molecules emitted in higher concentrations by infected gastropods could help determine which specific chemicals are responsible for the changed behaviour, as they will also be reacted to differently between conspecific and heterospecific snails. Furthermore, knowing which contexts cause manipulated behaviour to strengthen or weaken can indicate when it is occurring in nature and point to how the behaviour may be adaptive for the parasite.

Research on parasitic manipulation of behaviour would greatly benefit from an increased perception of the importance of context to behaviour. Both abiotic and biotic factors are able to affect changed behaviour in the *E. trivolvis* lineage c-snail interaction, which indicates that it is likely true for a much broader range of parasitic systems.

Understanding the relationship that context has with parasitic manipulated behaviour will further our understanding of manipulated behaviour as a whole and allow us to more confidently assert that behavioural changes are occurring in the natural world.

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Tables and Figures

Table 2.1: Responder gastropod activity in the arms of the behavioural chamber in treatments with a brine shrimp stimulus (BS), no stimulus (NS), and infected conspecific stimulus (IS), an uninfected conspecific stimulus (US) and both an infected and uninfected conspecific stimulus (DS). The left arm was considered the stimulus arm for the negative controls, while the arm containing the infected conspecific was considered the stimulus arm for the double stimulus treatment.

	Treatment	Stimulus Arm Average	Non-Stimulus Arm Average	Results of Statistical Analysis
Average Distance to Stimulus Cube (cm)	BS	13.9 ± 9.5	21.3 ± 8.7	V = 121, $P = 0.02$
	NS	19.4 ± 6.0	17.9 ± 5.6	t = 0.81, df = 29, $P = 0.42$
	IS	17.0 ± 6.2	18.3 ± 6.5	(t = -0.14, df = 29, $P = 0.89$)
	US	21.3 ± 5.6	18.7 ± 6.3	(t = -1.41, df = 29, $P = 0.17$)
	DS	18.7 ± 5.6	19.7 ± 4.9	(t = -0.66, df = 29, $P = 0.51$)
Time Spent within 3cm of the Stimulus Cube (sec)	BS	1350.3 ± 1214.0	506.2 ± 784.4	(V = 294.5, $P = 0.01$)
	NS	426.4 ± 658.7	567.1 ± 660.0	(t = 0.71, df = 29, $P = 0.48$)
	IS	509.6 ± 569.4	531.8 ± 659.3	(t = -0.11, df = 29, $P = 0.91$)
	US	259.0 ± 405.3	509.6 ± 717.3	(V = 62.5, $P = 0.20$)
	DS	341.9 ± 426.5	316.7 ± 406.7	(t = 0.21, df = 29, $P = 0.84$)
Time Spent in Arm (sec)	BS	1731.3 ± 1334.0	851.5 ± 1185.4	(V = 291, $P = 0.046$)
	NS	974.5 ± 991.5	1161.1 ± 886.2	(t = -0.63, df = 29, $P = 0.53$)
	IS	1269.0 ± 993.5	1082.5 ± 1132.3	(t = 0.51, df = 29, $P = 0.13$)
	US	600.3 ± 907.3	1024.5 ± 1122.5	(t = -1.41, df = 29, $P = 0.17$)
	DS	1051.5 ± 1010.3	799.8 ± 908.4	(t = 0.86, df = 29, $P = 0.40$)
Times Arm was Entered	BS	0.9 ± 0.74	0.5 ± 0.72	(V = 167, $P = 0.058$)
	NS	1.1± 0.83	1.1 ± 0.93	(V = 79.5, $P = 0.90$)

	IS	1.0 ± 0.80	0.7 ± 0.69	(t = 1.54, df = 29, *P* = 0.89)
	US	0.63 ± 1.2	0.7 ± 0.64	(V = 61, *P* = 0.45)
	DS	1.0 ± 0.91	0.7 ± 0.68	(V = 95.5, *P* = 0.15)
Average Velocity in Arm (cm/s)	BS	0.023 ± 0.017	0.018 ± 0.021	(V = 254, *P* = 0.25)
	NS	0.034 ± 0.024	0.031 ± 0.025	(t = 1.80, df = 29, *P* = 0.08)
	IS	0.030 ± 0.022	0.023 ± 0.022	(t = 1.52, df = 29, *P* = 0.14)
	US	0.015 ± 0.021	0.020 ± 0.020	(V = 77, *P* = 0.19)
	DS	0.031 ± 0.026	0.026 ± 0.026	(t = 0.90, df = 29, *P* = 0.38)
Standard Dev. of ΔD Angle in Arm	BS	53.1 ± 32.7	31.7 ± 35.8	(V = 289, *P* = 0.052)
	NS	60.4 ± 32.2	47.0 ± 35.0	(t = 0.13, df = 29, *P* = 0.90)
	IS	51.0 ± 34.3	40.9 ± 36.9	(t = 0.90, df = 29, *P* = 0.38)
	US	21.0 ± 31.1	40.8 ± 37.2	(t = -2.3, df = 29, *P* = 0.02)
	DS	40.9 ± 32.2	38.8 ± 34.5	(V = 171, *P* = 0.56)

Figure 2.1: Average distance in centimeters from the responder snail to the stimulus box for all replicates across the no stimulus (NS), uninfected stimulus (US), infected stimulus (IS) and double stimulus (DS) treatments, displayed with group mean and standard deviation. The stimulus arm refers to the left arm for the NS treatment and the infected gastropod for the DS treatment.

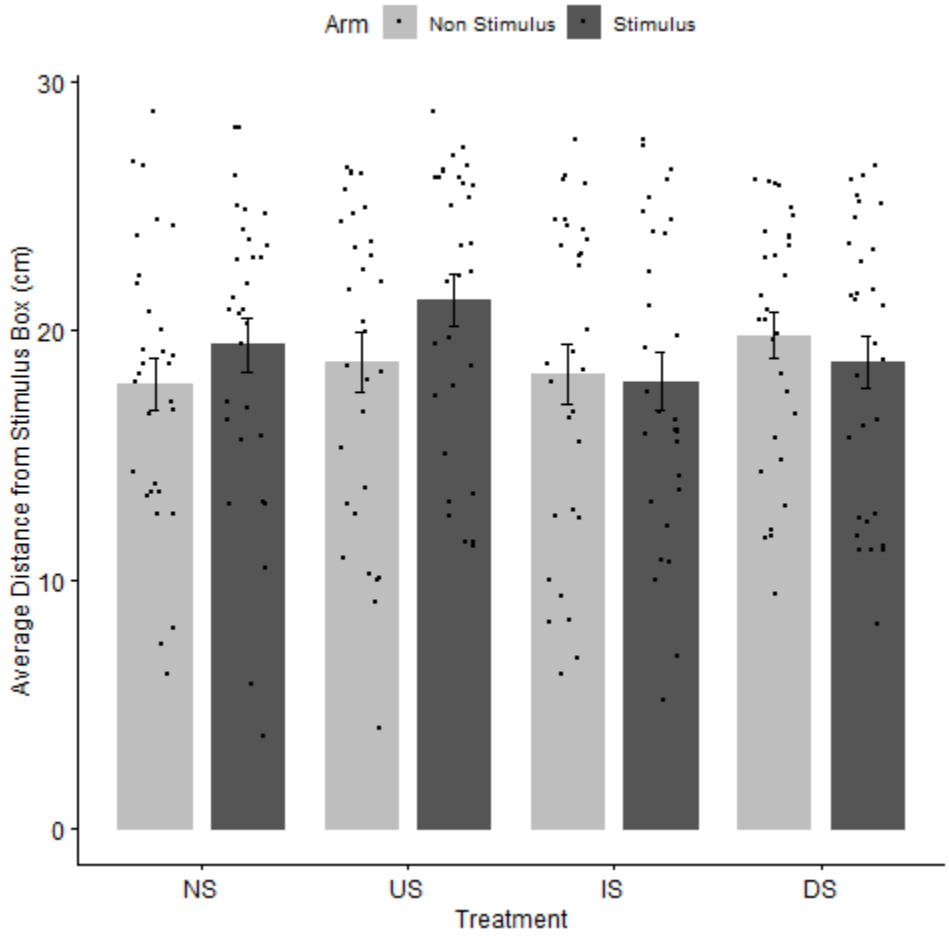


Figure 2.2: Time in seconds that the responder snail spent within 3cm the stimulus box for all replicates across the no stimulus (NS), uninfected stimulus (US), infected stimulus (IS) and double stimulus (DS) treatments, displayed with group mean and standard deviation. The stimulus arm refers to the left arm for the NS treatment and the infected gastropod for the DS treatment.

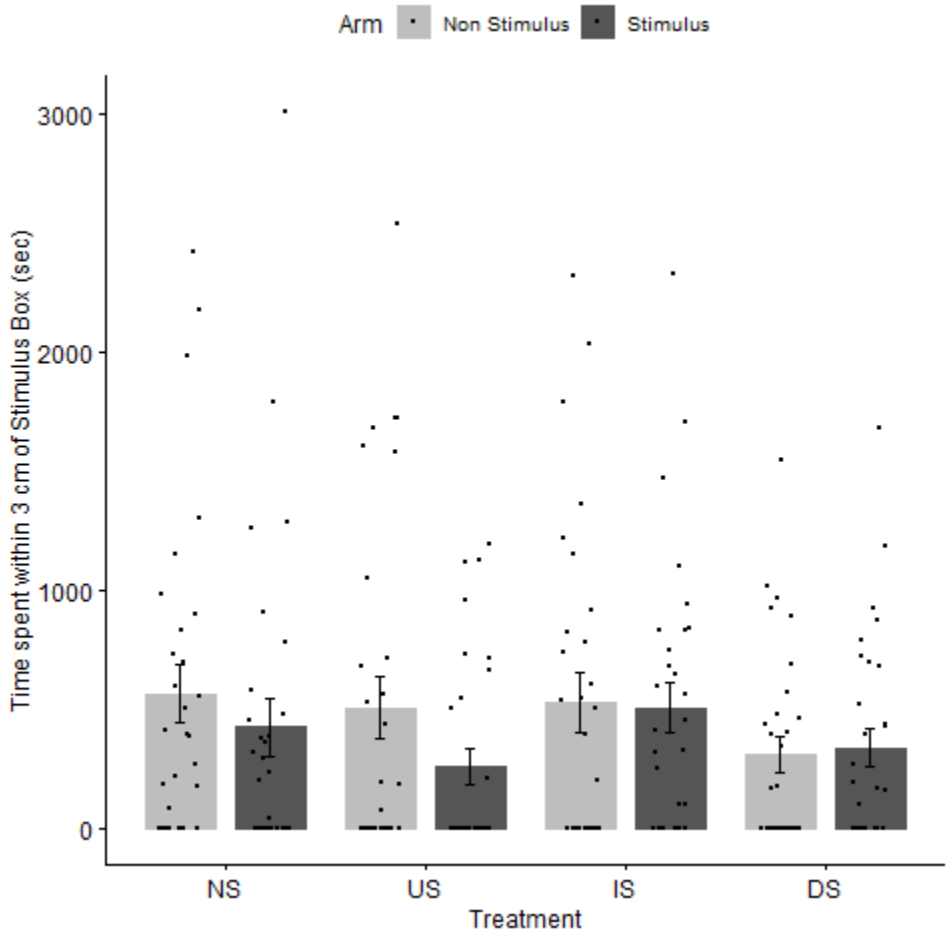


Figure 2.3: Time in seconds that the responder snail spent inside the indicated arm of the behavioural chamber for all replicates across the no stimulus (NS), uninfected stimulus (US), infected stimulus (IS) and double stimulus (DS) treatments, displayed with group mean and standard deviation. The stimulus arm refers to the left arm for the NS treatment and the infected gastropod for the DS treatment.

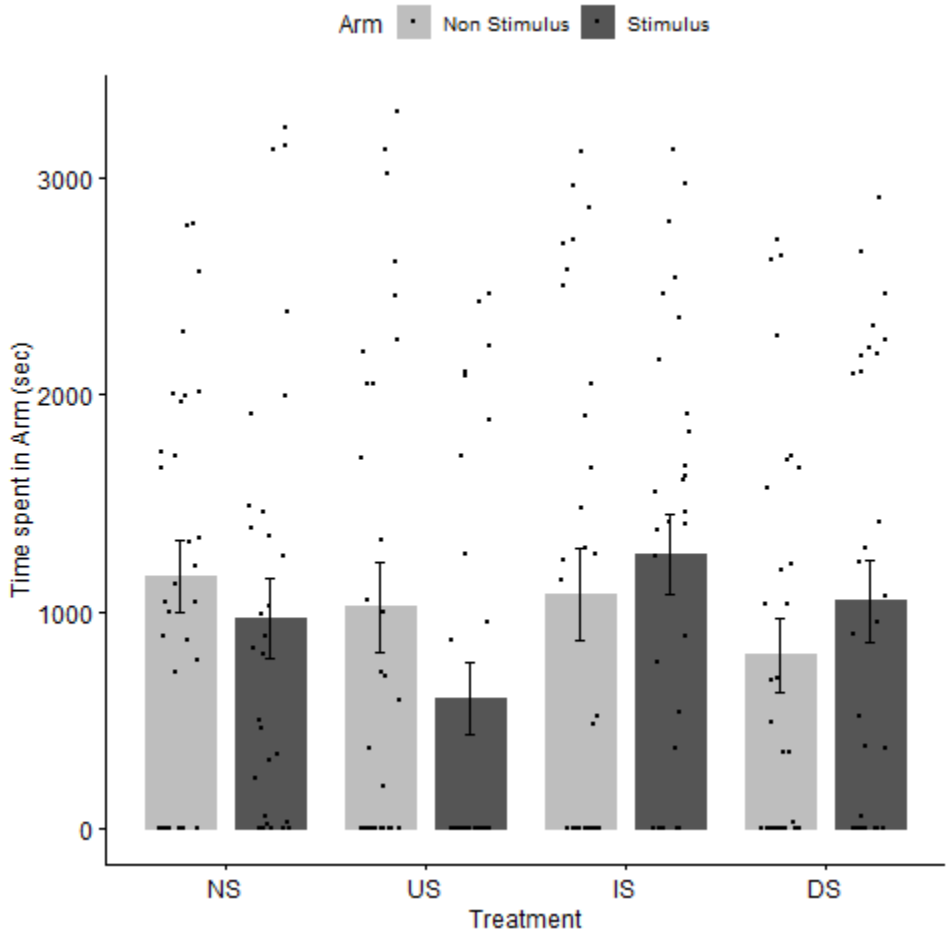


Figure 2.4: Counts of the number of times the responder snail entered the indicated arm of the behavioural chamber for all replicates across the no stimulus (NS), uninfected stimulus (US), infected stimulus (IS) and double stimulus (DS) treatments, displayed with group mean and standard deviation. The stimulus arm refers to the left arm for the NS treatment and the infected gastropod for the DS treatment.

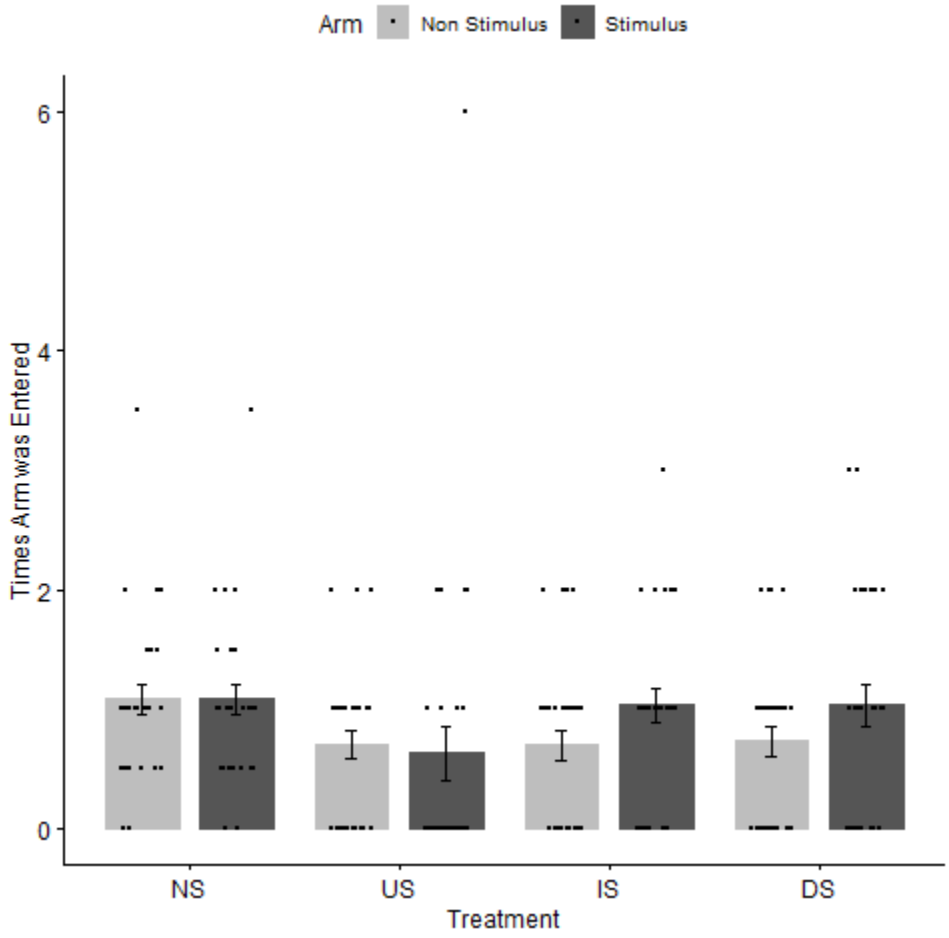


Figure 2.5: Average velocity in centimeters per second of the responder snail while in the indicated arm of the behavioural chamber for all replicates across the no stimulus (NS), uninfected stimulus (US), infected stimulus (IS) and double stimulus (DS) treatments, displayed with group mean and standard deviation. The stimulus arm refers to the left arm for the NS treatment and the infected gastropod for the DS treatment.

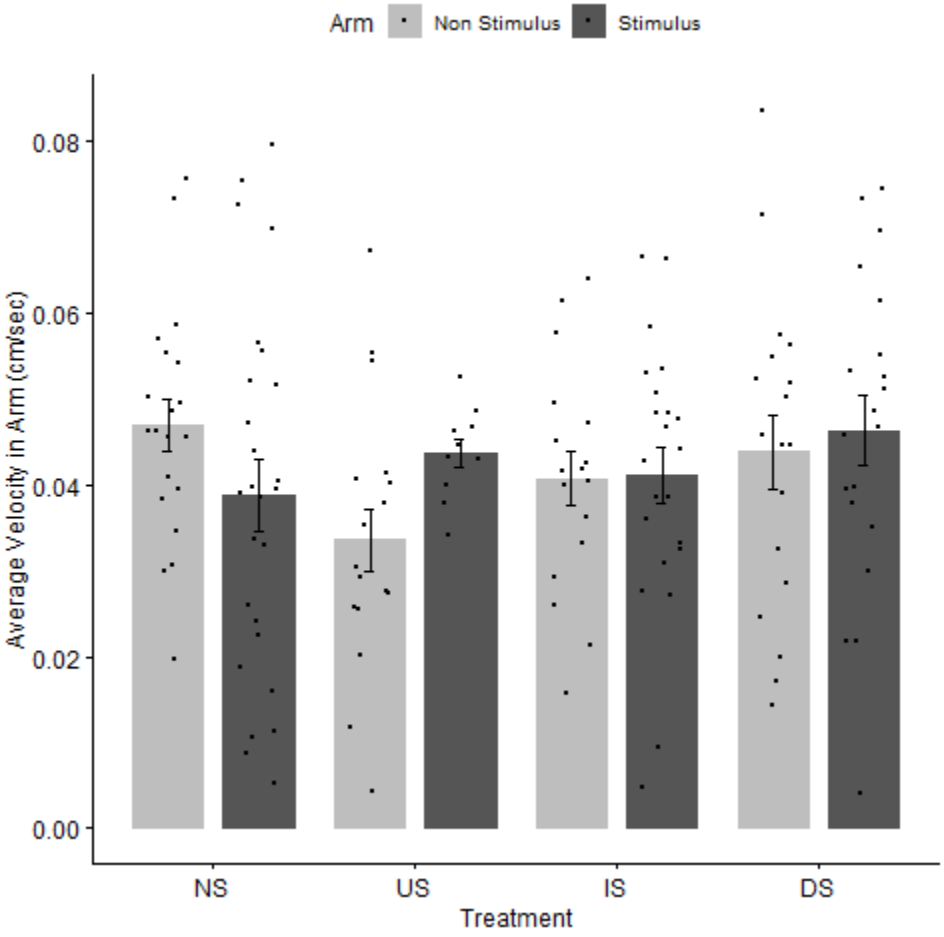
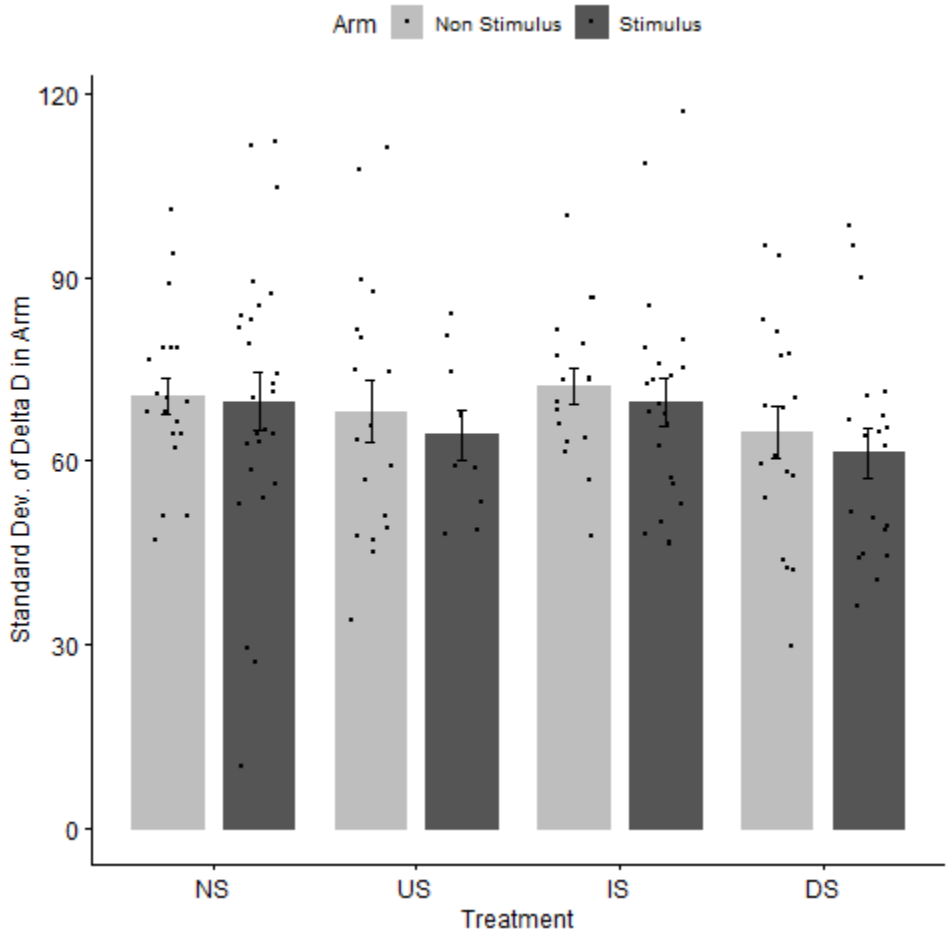


Figure 2.6: Standard deviation of the change in the responder snail's heading while in the indicated arm of the behavioural chamber for all replicates across the no stimulus (NS), uninfected stimulus (US), infected stimulus (IS) and double stimulus (DS) treatments, displayed with group mean and standard deviation. The stimulus arm refers to the left arm for the NS treatment and the infected gastropod for the DS treatment.



Thesis Conclusion

My research broadened our knowledge of the life cycle of *Echinostoma trivolvis* lineage c to increase its ability to be considered a model for studies on behavioural modification by wildlife trematodes. We can use this knowledge to obtain higher numbers of infected hosts for further experimental work. Further, we can better understand the importance of behavioural modification in transmission of trematodes between first and second intermediate hosts.

My first chapter showed the effects of three key factors, host species, size, and dose, on infection success for first intermediate hosts. Regarding host specificity, our results implied that either this parasite lineage is able to infect a wider range of first intermediate snail hosts than other lineages, or that the morphological differences between *Ladislavella elodes* and *Stagnicola reflexa* are not significant enough to greatly impair the parasite's ability to find, penetrate or establish in the snail. This has important connotations for determining whether *S. reflexa* should be considered a separate species from *L. elodes* rather than a morphotype, with my results suggesting that it is not a different species, if only from a parasitological perspective. The fact that *Planorbella duryi* was not found as a viable first intermediate host indicates that the second option is more likely the case. However, due to *P. duryi* being non-native to the area in which the parasites were obtained, it is possible that the actual usable host range of this parasite is wider than this result would imply, with similar naturally occurring species like *P. trivolvis* and *P. pilsburyi* being more likely to become first intermediate hosts. Future research should investigate this naturally occurring range of potential first intermediate hosts. However, for the

purposes of obtaining first intermediate hosts for lab studies, either *L. elodes* or *S. reflexa* can become infected at similar rates.

In the first chapter, the metric of size was used as a proxy for both host age and host condition, with my results indicating that medium-sized snails become infected at the highest rate. This study could be refined to further separate these metrics and generate more specific data on which value is optimal for both. For example, the snails could have been tracked from hatching to exposure date to have specific comparable age groups. Furthermore, certain measures of body condition such as growth rate, food consumption or respiration rate could have been used to more specifically understand how body condition interacts with infection success. Regardless, my approximation of a medium sized snails is useful for optimizing infection success in viable gastropods.

Finally, the effects of exposure dose on infection success seem to indicate that an exposure of five miracidiae, while producing a high rate of infection, could be increased to further optimize the rate of successful infections. This broadly implies a low rate of establishment per miracidium, which is especially interesting considering that the chance of encounter was raised significantly compared to what would be expected in natural conditions. Further investigation of this to more accurately determine both the rate at which successful encounters become successful infections and the optimal exposure dose for medium sized snails should be preformed. Despite this and other expandable areas for the other factors, this research will allow future exposures to be performed at a much lower cost, regarding both effort and number of potential hosts for downstream applications such as investigations of behavioural manipulation.

My second chapter confirmed that behavioural modification is influenced by the context in which the modification occurs. Using video analysis, I determined the effects that light as an abiotic factor and conspecific interactions as a biotic factor had on the ways in which snails navigate. First, I found that light levels which closely matched naturally occurring light levels increased the rate at which uninfected snails moved when compared to lower levels. This implies that behaviour may be affected by any abiotic stimulus the subject may be able to sense, and that careful consideration must taken to not only ensure that untested variables remain constant between treatments, but also that these variables are consistent with what may occur in nature. Otherwise, it is likely that any change in behaviour seen in an experimental trial may not occur in nature, thus creating false assumptions about natural processes both in the context of behavioural manipulation and outside of it.

Second, I discovered that conspecific snails were not attracted to *E. trivolvis* lineage c infected first intermediate host snails. Not only does this provide no evidence for parasite manipulation, but this result is in contrast to what heterospecific snails have been observed to do when parasitized with echinostome species. Similarly to the results of host specificity in Chapter 1, this could potentially be influenced by the subject snail being invasive to the parasite's range and therefore having a much reduced coevolutionary history with the parasite. It is possible that other, closely related naturally occurring species would also not show a change in behaviour. Future research should use species such as *Planorbella pilsbryi* or *Planorbella trivolvis* as responders in behavioural assays to confirm this result. Regardless, the differences in behavioural modification observed in the

two species contexts suggest that parasitic modification of behaviour is dependent on biotic context in a similar way as abiotic context.

Overall, my thesis has eased the process of using this parasite species for experimental studies and confirmed that behaviour modification is context dependent. These studies have wide implications for *E. trivolvis* lineage c as well as general parasitic behavioural manipulation. Future studies should focus on optimizing lab infections of first intermediate hosts by testing more specific measures of gastropod physiological condition, as well as a greater array of both naturally occurring species and exposure doses. More research should also be focused on the ways in which other abiotic contexts such as water temperature and chemical properties like salinity and oxygenation, as well as biotic contexts like the use of naturally occurring potential second intermediate host species as responders affect behavioural manipulation in this system. Finally, the elucidation of the first part of this parasite's life cycle should be used to test whether the previously discovered behavioural manipulation applies to *E. trivolvis* lineage c specifically in a heterospecific context.