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Compatibility Factors of Fascioloides magna miracidia and Four Sympatric Snail Species: Miracidial Behavior and Snail Response

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Compatibility Factors of Fascioloides magna miracidia and Four Sympatric Snail Species: Miracidial Behavior and Snail Response

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Bryan Rolfsen

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ABSTRACT

Miracidia exhibit observable host-finding behaviors, and their sympatric snails have attributes that either permit or prohibit infection. This study was designed to assess the factors involved in intermediate host finding and host-parasite compatibility in the deer liver fluke (*Fascioloides magna).* The main aim was to determine the extent to which the parasite and/or the intermediate host are involved in host-parasite compatibility. A secondary goal was to determine what factors may lead to miracidial transformation. The study used a panel of four sympatric snails (*Lymnaea caperata*, *Lymnaea palustris*, *Lymnaea exilis*, and *Physa* sp.) that display a range of susceptibility to the trematode from *L. caperata* which is the natural intermediate host, to experimentally susceptible *L. palustris,* to resistant *L. exilis* and *Physa* sp. Miracidial host finding behaviors were examined to determine the extent of the role of the miracidium in initiating infection. This was tested by observing single miracidium infections for 30 minute time periods to record number of contacts, attachment time, infection success, and whether the miracidium was harmed. Miracidia attached to susceptible *L. caperata* more often ($\chi^2 = 6.6561$, $p = 0.0359$) and for longer periods of time ($\chi^2 = 8.5290$, $p = 0.0141$) than to resistant *L. exilis* or *Physa* sp. Miracidia exposed to a physid snail were harmed more often than those exposed to the lymnaeids ($\chi^2 = 5.4000$, $p = 0.0251$). To assess the role of primary barriers of snail immunity in host-parasite compatibility, miracidia were exposed to snail mucus *in vitro* to assess snail toxicity. Following the pattern seen with intact snails, mucus from *Physa* sp. was 100% cytotoxic to miracidia at 1:3 and 1:30 dilutions. Mucus from *L. caperata, L. palustris, and L. exilis* showed no difference from control for up to 4 hours. This demonstrated an overall species effect ($F_{3,32} = 23.59$; $p<0.0001$). Mucus did not significantly stimulate transformation in any species at any dilution. To assess the role of internal snail products on compatibility and miracidial transformation, miracidia were exposed to tissue homogenate from *L. caperata, L.*

palustris, L. exilis, and *Physa* sp. in protein dilutions of 0.5, 0.05, and 0.005 mg/mL. Tissue homogenate from the natural host, *L. caperata* stimulated transformation more frequently than any other species ($F_{3,77} = 4.43$; $p = 0.0063$) particularly at the 0.5 mg/mL dilution ($F_{2,78} = 25.19$; $p<0.0001$). To examine *in vivo* snail immune response, single snails of *L. caperata*, *L. palustris*, and *L. exilis* were subjected to mass exposures of *F. magna* miracidia then fixed at 1 h, 2 h, 3 h, 4 h, and 7 h. Specimens were serial sectioned and examined for *in vivo* snail immune response. Infection progressed farthest in *L. caperata*, but no significant hemocyte accumulation was observed in any species. This study showed that miracidia play an active role in locating and attacking a preferred host. Snails may avoid infection due to toxins in mucus or tissue homogenate.

I dedicate this work to my grandmother, Virginia Rolfsen:

Without your sharp elbows, finger wagging, and intermittent nagging, I may never have finished.

I love you and I like you. Thank you for your love and support.

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INTRODUCTION

Parasitism is likely the most common life strategy on the planet. Virtually every organism is parasitized, and often by multiple species. However, many requirements must be met to facilitate host-parasite compatibility, making the phenomenon of compatibility rare.

Trematodes are a highly evolved class of flatworms that utilize parasitism as a way of life. The subclass *Digenea* has a complicated, multi-step life cycle that typically uses invertebrates as intermediate hosts and vertebrates as definitive hosts. Often larval flukes (miracidia) find and burrow into snails. Adult flukes inhabit many sites in their hosts, including the circulatory system and the digestive, respiratory, urinary, and reproductive tracts.

There are three main factors that determine host-parasite compatibility at the level of the intermediate host: host finding, penetration, and snail immunity. In the case of the snail-trematode relationship, host-finding refers to the behavior of the miracidium in its attempt to locate a suitable intermediate host. In order for infection to be initiated once parasites find a host, they must navigate the external barriers of snails; infection is contingent upon successful penetration of the epithelium (whether by penetration or ingestion). Once a trematode penetrates a snail, it then must survive any immune response elicited by its presence. Different taxa of snails will have varying levels of immunity to a given trematode larva, which impacts their resistance to infection by particular taxa of trematodes. Concurrently, specific taxa of trematodes are adept at evading the immune responses of specific taxa of snails.

The ability of the miracidium to find its host is the first step in initiating a successful infection. Many researchers have looked at environmental effects on miracidial behavior (Campbell and Todd 1955a; Chernin 1970; Wade and Nollen 1982; Nollen and Mart 1983; Markum and Nollen 1996; Ford *et al.* 1998; Munoz-Antoli and Trelis 2003). There are many

intrinsic and extrinsic factors that influence whether a host is found and penetration is achieved. Chernin (1970) called these factors miraxones, referring to any stimulus that causes a miracidium to respond. Others researched the effect of snail products on miracidial behavior (Swales 1935; Campbell 1961; Shiff and Kriel 1970; Prechel *et al.* 1976; Munoz-Antoli and Trelis 2003; McGrier and Laursen 2008). In these studies, various snail products attracted or repelled miracidia, indicating their role in influencing miracidial behavior.

The second factor is the role of the host in preventing the infection before it occurs. In cases where incompatible infections are attempted, researchers have indicated that certain snail products such as plasma (Sapp and Loker 2000) and mucus (Coyne *et al.* In-press) can harm miracidia. This indicates that certain taxa may have primary barriers to infection that prevent penetration.

The last factor is the ability of the snail to eliminate infection after it has occurred. Snails have a surprisingly advanced and efficient immune system (Bayne and Yoshino 1989; Humphries and Yoshino 2003; Zhang *et al.* 2004; Hannington *et al.* 2012). They are capable of clearing a multitude of antigens (Bayne and Yoshino 1989), and there is even evidence for immune molecule diversification analogous to adaptive immunity (Zhang *et al.* 2004; Hannington *et al.* 2012). Internal snail products also work to clear infections that have reached the sporocyst/redia stage (Sapp and Loker 2000).

This study focused on these three main factors in the *Fascioloides magna*-snail relationship using *F. magna* miracidia and four snail species: *Lymnaea caperata*, *L. palustris, L. exilis*, and *Physa* sp. Miracidial host-finding behavior was examined to determine the degree to which host specificity relies on the miracidium's ability to find its host. The effect of snail products (mucus and tissue homogenate) on miracidia *in vitro* was also analyzed. Lastly, snail

internal response was evaluated using histotechnique and microscopy to evaluate what happens *in vivo* that leads to successful or unsuccessful infection of the snail host during the first several hours of exposure.

Fascioloides magna – Life Cycle, Range, Hosts

Fascioloides magna, the large American deer liver fluke, was first described by Bassi in 1875 (Price 1953). This parasite was identified in an American elk living in an Italian zoo. However, it is native to America, and only from the export of American ruminants to Europe has it spread overseas (Price 1953). It has been found in some parts of Europe and Czechoslovakia (Chroustova 1979). In America, it has been found in Arkansas, California, Colorado, Florida, Illinois, Iowa, Kansas, Louisiana, Michigan, Minnesota, Montana, New York, Oklahoma, Oregon, Texas, and Washington (Price 1953). It has also been found in Canada, specifically in British Columbia, Alberta, and Ontario (Price 1953). Though the life cycle is consistent everywhere, there may be definitive and intermediate host differences depending on geography.

The life cycle utilizes a molluscan intermediate host, a Lymnaeid snail, and a vertebrate definitive host, usually the white-tailed deer (*Odocoileus virginianus*). Sinitsin (1933) first proposed an example of the intermediate host. Swales (1935) first documented the life cycle and Pybus (2001) reviewed it. Adult flukes live in the liver parenchyma of the definitive host and deposit eggs into the liver tissues. Eggs then join bile and are passed through bile ducts into the small intestine to be expelled in feces. Eggs embryonate in water, miracidia hatch from them, and this motile larval stage searches for an intermediate snail host. Miracidia then burrow into snails and begin larval amplification (asexual reproduction). First, they transform into mother sporocysts followed by daughter sporocysts. These produce rediae from germ balls which yield six to ten daughter rediae. Daughter rediae give rise to cercariae. This second, free-living, motile

stage spontaneously emerges from the snail and swims through the water until it encysts on a piece of vegetation and becomes a metacercaria. White-tailed deer then eat this vegetation and metacercaria excyst to become juvenile flukes. These migrate to the liver, find at least one other fluke, then encapsulate in order to attain maturity together and reproduce. As hermaphrodites, it is assumed they can mate with other flukes or with themselves while encapsulated.

The most common definitive host is the white-tailed deer, but there have been reports of *F. magna* being found in aberrant hosts such as sheep (*Ovis aries)* (Foreyt 1990), llama (*Llama glama)* (Conboy *et al.* 1988), pig (*Sus scrufa*) (Migaki *et al.* 1971), and cattle (*Bos taurus*) (Schillhorn van Veen 1987). According to Price (1953), they have also been found in hosts such as the wapiti (*Cercus canadensis*), moose (*Alces alces americanus*), coast deer (*Odocoileus columbianus*), mule deer (*O. hemionus*), goat (*Capra hircus*), bison *(Bison bison*), cattle-bison hybrids (*Bos taurus x Bison bison*), yak (*Bos grunniens*), and horse (*Equus caballus*). Different levels of pathology are associated with each aberrant host.

Of the potential hosts, the parasite is not particularly harmful to its preferred hosts, cervids. Mulvey and Aho (1992) found that *F. magna* reduced body size and total number of antler points, particularly for individuals in younger age classes, but did not cause death or a decrease in overall condition. When the parasite finds itself in an aberrant host, its life cycle is interrupted, and it is unable to pass eggs through the bile ducts and out through feces (Swales 1936). In these cases, the fluke may cause considerable tissue damage while migrating through the liver, especially in the case of sheep (Swales 1936, Foreyt 1990). In bovids, the fluke is often caught in a necrotic cyst in liver tissues before it can do an extreme amount of damage (Swales 1936).

As with the definitive host, there are natural intermediate hosts of snails that are susceptible to infection. Krull (1933) listed *Fossaria modicella, F. modicella rustica*, and *Pseudosuccinea columella* as artificially infected species in the USA. Sinitsin (1933) listed *Galba bulimoides techella* as an intermediate host in Texas. Swales (1935) named *Fossaria parva* and *Stagnicola palustris nuttalliana* (*Lymnaea palustris*) as natural intermediate hosts in Canada. Griffiths (1959) found that *S. (Lymnaea) caperata* was a natural host in Minnesota. Chroustova (1979) found that *Lymnaea palustris* was a viable intermediate host in Czechoslovakia. Within this suite of hosts, some snails are more susceptible than others.

Snail susceptibility to trematode larvae is known to be age dependent. Kendall (1950) reported that *L. stagnalis, L. palustris,* and *L. glabra* were susceptible to infection with *Fasciola hepatica* only through the first few days of their lives. Additionally, he found that *L. auricularia* was completely resistant, and *L. truncatula* could be infected at any age. This finding was supported by Smith and Crombie (1982).

The suite of Lymnaeid snails prone to infection by *F. magna* also displays differential infection capabilities based on geography. Foreyt and Todd (1978) demonstrated this by experimentally infecting a suite of lymnaeid snails from Wisconsin with *F. magna* miracidia from Texas. In this study, *L. caperata*, though competent, was a less suitable host than *L. bulimoides*, a natural host in Texas.

The current study utilizes miracidia and snails collected from Minnesota to minimize regional differences in order to compare intrinsic snail factors influencing susceptibility.

Environmental Effects on Miracidial Behavior

The first barrier to snail infection is the miracidium's ability to find its host. Host-finding behavior has been documented across a wide range of trematodes, starting with physiological properties of the eggs from which they hatch. Hatching must occur in an aquatic environment. It is important for the eggs of some species to be exposed to light. Eggs of *Echinostoma caproni* hatched more readily in a lab setting when exposed to higher light intensities than lower intensities, and eggs did not hatch to any appreciable degree in complete darkness (Markum and Nollen 1996). Once miracidia hatch from the egg, there are many intrinsic and extrinsic factors that influence whether a successful snail penetration is achieved. Chernin termed these factors miraxones (1970). Additionally, eggs that take longer to embryonate may produce miracidia that are more sensitive to these environmental cues (Campbell and Todd 1955a).

Miracidia do not actively feed; their only energy source is the glycogen reserves that they hatch with. Once these stores are depleted, the miracidia become exhausted and are less likely to be infective (Campbell and Todd 1955a; Munoz-Antoli and Trelis 2003; Ford *et al.* 1998). Ford *et al.* (1998) also found that miracidia of *Echinostoma caproni* live longer at lower temperatures, between 5 and 10 °C, than at temperatures above 15 °C and died within an hour at 45°C. They concluded that slower moving miracidia depleted less of their glycogen reserves over time. They also found some evidence that miracidia may be able to absorb nutrients through their tegument, as they noted an increase in activity with the addition of glucose to the swimming medium. Nutrient uptake was also found by Wade and Nollen (1982) when they exposed *Schistosoma mansoni* to medium (without bacteria) in which *Bacillus sphaericus* and *B. moratai* were cultured. Ford *et al.* (1998) also examined the effect of high and low pH in the swimming medium on miracidial viability and found that the longevity of *E. caproni* increased at pH 9 and pH 5. They found that saline solutions decreased the longevity and swimming

capability of these miracidia. Nollen and Mart (1983) also found that the hydrogen ion concentration elicited a strong response from *Megalodiscus temperatus* miracidia, resulting in both vertical movement in the water column and an attachment response.

Snail Products and Miracidial Behavior

Prechel *et al.* (1976) found that sialic acid from snail-conditioned water was an effective attractant for *M. temperatus* miracidia. However, the experiment did not differentiate between free and bound sialic acid. Thus, it was possible that the chemical could have been bound to a glycoprotein or glycolipid – common components in snail mucus. They concluded that the free amino acid make up of a potential host is probably not a viable tool in the host finding process. The identification of molecules, however, such as small peptides, glycopeptides, or oligosaccharides that may be unique to different species of snails may provide much more information to miracidia (Prechel *et al.* 1976).

Several researchers have found significant responses to snail conditioned water (SCW) on trematode larvae. *Schistosoma haematobium* miracidia were found to respond, and be attracted to, some sort of water-soluble compound produced by snail metabolism (Shiff and Kriel 1970). Prechel and Nollen (1979) tracked miracidial movement by taking a three second exposure picture of *M. temperatus* miracidia. There was a high level of reaction to SCW from the preferred host for up to 6 h followed by a drop off. The photographs showed the transition of miracidial paths from generally linear to very erratic with many turns following inoculation with SCW.

Munoz-Antoli and Trelis (2003) investigated the decoy effect using two species of parasites and a suite of host snails. Their experiments determined that host snails (HS) and non-host snails (NHS) do not need to be together, or even in close proximity, in order to produce a

decoy effect. In both systems (*Echinostoma friedi* and *Euparyphium albuferensis*), there was a marked decoy effect that occurred upon introduction of either non-host snail or non-host snail SCW. The most significant effect of the non-host snail was that it caused miracidia to increase host-finding behavior and attempt penetration. They found that unsuccessful penetration attempts resulted in damage and exhaustion, making it harder to infect the host snail. Additionally, unsuccessful attempts caused them to shed penetration glands, also making infection harder. They found that miracidial infection rates of host snails went down when the media was inoculated with SCW. This could be because the SCW contained a toxic substance released by the NHS that stimulated a HS regulatory response. They found that with *E. friedi,* if SCW of the NHS was added to media with a HS, host finding behaviors of miracidia increased. This caused an increase in metabolic demand, decreased selectivity, and ultimately decreased miracidial fitness due to exhaustion of glycogen reserves or aberrant attachment attempts. They proposed this decoy effect as a behavioral reason for the low prevalence of infections in host snails in areas where definitive host populations are infected with a high prevalence and there is high snail diversity.

In the specific case of *F. magna* miracidial behavior, not much work has been done. Swales (1935), Cambpell and Todd (1955a; 1955b), Campbell (1961), and McGrier and Laursen (2008) have done the majority of inquiry into the subject. Campbell and Todd (1955a) summarized the research to that point, saying that little or no chemotaxis had been found to be at play with respect to the host finding behavior of *F. magna*. The miracidia were frequently seen swimming near a suitable host without trying to attach, though at times it appeared they were attracted to a mucus trail. In their experiments, they described evidence of chemotaxis when they observed the miracidia attacking the snail *Fossaria modicella rustica* more frequently than *Stagnicola reflexa*. They asserted that perhaps eggs that took longer to embryonate were

more capable of responding to chemotaxis. Additionally, they found that the age of *F. magna* miracidia is a factor of infectivity. Very young (< 1 h) miracidia and very old (> 8 h) miracidia were less infective than those that were 1.5 to 2 h old, and younger miracidia were more effective attackers than older miracidia. Lastly, more miracidia attempted attachment during the first 15 min of exposure than during the succeeding 55 min. Campbell (1961) later realized that he could not rule out chemotaxis because the design of the previous experiment may have allowed for diffusion of snail products into the small volume of water in which the snails were located. He tried a different experiment where he placed a piece of filter paper with *Stagnicola reflexa (Lymnaea exilis)* snail tissue homogenate in a petri dish with thousands of miracidia. When he left the filter paper in the water, the miracidia became hyperactive, but did not swarm around the paper. However, when he touched the paper to the bottom of the petri dish and removed it, the miracidia swarmed around the spot where the tissue homogenate was. From this, he concluded that *F. magna* miracidia were able to recognize chemical cues (Campbell 1961). McGrier and Laursen (2008) found that *F. magna* miracidia were able to find a single host reliably in up to 1 L of water with rates dropping at 10 L. They also found that *Lymnaea* spp. decoy snails tended to decrease experimental infection rates of *L. caperata*.

Miracidial Penetration and Transformation

Once a miracidium finds its host snail, it must penetrate and transform to a mother sporocyst. In many species, it is still not understood what causes these phenomena to occur. It may be that snail components such as mucus and hemolymph provide chemical cues to stimulate penetration and transformation. At a minimum, these products must not damage the miracidium during the process.

Coil (1981) described the process of miracidial penetration and transformation in *F. magna* by following transformation at fixed time intervals. Miracidia lost their cilia early, after contact with the snail. Next, their epithelial cells either detached, or were carried into the snail body. At this point, the apical gland and vesiculated gland cells of the parasite released vesicles that lost their outer coating in the snail tissue where snail collagen fibers were concomitantly destroyed. At the end of this process, miracidia were considered transformed into mother sporocysts.

Not all trematode systems function in this way, however. In some, the eggs are eaten, hatch in the snail's intestine, and the miracidia penetrate the intestine. In others, the whole miracidium penetrates the snail. There is still much to be learned about the process of miracidial transformation. Specifically, the behavioral strategies and morphological changes associated with attachment; the role of glandular secretion in the process of penetration; the process of exciliation and shedding of epidermal plates; and the role of the apical organ in breaching the snail epithelium first proposed by Coil (1981) are still applicable today.

Studies that have produced miracidial transformation *in vitro* have helped to further understand some of these concepts. The most studied trematode is the human blood fluke (*Schistosoma mansoni*). However, *S. mansoni* can transform spontaneously into sporocysts *in vitro* in Chernin's Balanced Saline Solution without snail products (Yoshino and Laursen 1995), so this question has never been of intense study in that system.

It is difficult to force transformation in *Fascioloides magna*. However, Campbell and Todd (1955b) were able to make *F. magna* miracidia transform to sporocysts *in vitro* by simply exposing several miracidia to a snail whose shell had been peeled back. The miracidia attached to the snail, shed their cilia and epithelial plates, transformed, then detached from the snail.

Campbell and Todd (1955b) could not explain why this occurred, nor could they recreate the results reliably. Laursen and Yoshino (1998) were able to make miracidia transform into sporocysts by bathing them in a conditioned medium from a snail cell line (BGE cells) derived from embryonic cells of *Biomphalaria glabrata*. They were able to recreate the results several times, and found that applying heat and proteinase K to the system stopped transformation. They concluded that some sort of protein was responsible for the shedding of cilia and epidermal plates and ultimately, transformation.

Snail Immune Response and Host-Parasite Compatibility

After penetration and transformation occur, the trematode must be able to survive within the host. In order for a larval trematode to successfully infect a snail, the organisms must be physiologically compatible on several levels (Bayne and Yoshino 1989). The snail must be susceptible and the parasite must be suitably infective. Bayne and Yoshino (1989) felt that host-parasite compatibility is such a rare circumstance that resistance should be considered the rule, while susceptibility is considered the exception. They make this claim based on two facts. First, there is usually low prevalence of infected snails even in hyperendemic areas. Second, the snail immune system has the ability to clear many foreign substances by hemocyte attachment and encapsulation (Bayne and Yoshino 1989, Humphries and Yoshino 2003). However, hemocyte encapsulation and subsequent killing tends not to occur in compatible infections.

Snail immunity is mediated by hemocytes which attach to and encapsulate foreign molecules. They perform several specialized functions, but deal mainly in immunity and self-preservation. Hemocytes are present in hemolymph, the non-cellular, molecule-rich fluid that comprises the snail circulatory system. Though it has traditionally been thought that molluscs have a rather simplistic immune system, the work of Humphries and Yoshino (2003) suggests

that perhaps there is more homology with vertebrate immune systems than previously thought. They found that hemolymph is not only capable of microbial phagocytosis, multicellular encapsulation, and cell-mediated cytotoxicity, but is also able to bind foreign ligands to hemocyte receptors. In fact, they found various hemocyte-associated receptors that were capable of binding specific carbohydrates, extracellular matrix proteins, growth factors and cytokines.

More recently, a family of lectins called FREPs (fibrinogen related proteins) has been implicated as having a large role in snail immunity (Hannington *et al.* 2012, Zhang *et al.* 2004). FREPs have one to two immunoglobulin domains as well as a fibrinogen domain, and they have the ability to clear a large array of foreign antigens. They have also been shown to undergo diversification by means of point mutation and gene conversion (Zhang *et al.* 2004). In *B. glabrata,* the snail hosts for schistosomes, the knockdown of the FREP3 gene by small interfering RNA caused some resistant individuals to lose their resistance (Hannington *et al.* 2012). This study also verified that FREP3 is diversified in different hemocytic cells. This information provides evidence for the presence of a snail immune system that has much more specificity in its ability to clear antigens than previously realized.

It is probable that several factors work together to produce compatibility. Bayne and Yoshino (1989) proposed that one or more of the following phenomena may be occurring. Mother sporocysts may avoid being recognized by the snail immune system via molecular mimicry, acquisition of host molecules, or prevention of opsonization. Alternatively, they may be able to resist the toxic components of the host, or interfere with host hemocyte function.

In an experiment performed by Sapp and Loker (2000) where five snail species (all planorbids or lymnaeids) were exposed to four digenean species, it was found that each parasite

species produced patent infections in its known host, but not in the others. They also found that miracidia were less likely to attach to/penetrate an unsuitable host, although many specimens still tried. The researchers went on to test the effects of plasma from different species of snails on the different species of sporocysts/redia. They found that plasma was not harmful to compatible species, but that it was harmful to incompatible species – specifically, lymnaeid plasma was very harmful to the species that infect planorbids and vice versa.

In addition to these intrinsic differences in susceptibility between species, there are other factors that play a role in infectivity. For example, in lab and field experiments with avian schistosomes, infection intensity was directly correlated with snail size (Graham 2003). As mentioned above, susceptibility patterns have been found to vary with snail age; young individuals of otherwise resistant species may exhibit reduced resistance (Kendall 1950, Smith and Crombie 1982).

Most of the work on the role of snail immunity has been done on Schistostomatidae and Echinostomatidae. A novel system within Echinostomatidae, such as the one provided by *F. magna,* will help to answer fundamental questions about snail host specificity of digenean trematodes. Recently, Coyne *et al.* (In-press) found that *Helisoma trivolvis* mucus served as a barrier to infection by *F. magna*. The mucus was extremely cytotoxic to *F. magna* miracidia due to a proteinaceous component. However, mucus from compatible *Lymnaea palustris* did not display any cytotoxicity to *F. magna* miracidia. Although *Helisoma trivolvis* is ubiquitous across much of North America, it is of interest to observe the host-parasite relationship in sympatric snail species.

In this project, I tested miracidial choice behavior in regards to the ability of *F. magna* miracidia to find, attach to, and penetrate a suite of sympatric snail hosts including *Lymnaea*

caperata, L. palustris, L. exilis, and *Physa* spp. *Lymnaea caperata* has been identified as the natural intermediate host in MN (Griffiths 1962). *Lymnaea palustris* has also been identified as a natural host (Swales 1935). Meanwhile, *L. exilis* and *Physa* spp., snails that often share the same pond, are resistant to infection by *F. magna*. I also tested the effect of snail products (mucus and tissue homogenate) from this suite of species on *F. magna* miracidia. Lastly, I analyzed histological samples to determine any *in vivo* differences between susceptible and resistant species in the first four hours of infection.

This study examined multiple species of *Lymnaea*, some resistant and some susceptible, and an outside resistant genus (*Physa*). By studying snails commonly found in the same ponds in MN, I was able to compare patterns naturally affecting host finding and trematode resistance in this system.

METHODOLOGY

Snails – collection and lab cultures

Four snail species (*Lymnaea caperata, L. exilis, L. palustris,* and *Physa* sp.) were collected in Minnesota between 2010 and 2011 within the range of *F. magna*. The principal collection sites were Carlos Avery Wildlife Management Area in Anoka County, MN and St. Croix State Park in Hinckley (Pine County), MN. Snails were brought back to the lab and reared in 10 gal and 1 gal aquaria in artificial spring water (Ulmer 1970). They were fed frozen lettuce and fish flakes *ad libitum*. Only the offspring were used for experimentation, to avoid any confounding factors of preexisting infections. Snails used for experimentation were mature unless otherwise noted.

Miracidia – collection and lab maintenance

Trematode eggs were collected from within St. Croix State Park, during yearly deer population control hunts in the fall of 2011 and again the fall of 2012. Hunters provided deer livers at the check station upon exiting the park. Livers were dissected and eggs were collected from within liver capsules or released from adult flukes. Eggs were sedimented and cleaned in the field then brought back to the lab to embryonate at room temperature for 3-4 weeks. Activated charcoal was placed in the containers in order to minimize mold growth. Once miracidia were observed, eggs were moved to the refrigerator and stored at 1.5°C - 4°C where they remained viable for upwards of a year. Mass hatches were performed for experiments by placing miracidia in test tubes filled with water at room temperature for 1-4 h.

Miracidial Behavior

To evaluate the role of the miracidium in the infection process, I exposed three species of snails (*L. caperata, L. exilis,* and *Physa* sp.) to a single miracidium. For each 30 min trial, a snail was adhered shell down to the bottom of a well in a 24 well plate using double sided tape. The well was filled with enough DI water to completely submerge the snail. A single miracidium was added to the well and observed through a dissection microscope. The following parameters were evaluated: the number, location (shell, body, or mucus), and duration of contacts with the snail; the number and duration of attachment attempts; successful attachments (defined by presence of miracidium on snail epithelium after 2 h); and damage to the miracidum (defined by observation under a compound microscope or obvious behavioral components such as ceasing to swim or swimming abnormalities).

The number, location, duration, and frequency of attachment attempts were analyzed using a non-parametric, one-way chi-square protocol with a Kruskal-Wallis post-hoc test. The duration of attachment attempts was analyzed using an ANOVA.

Snail Response

The role of snail products in compatibility was evaluated by testing the effect of mucus and snail tissue homogenate on miracidia *in vitro*.

Snail Mucus:

Mucus was harvested from the head-foot from a suite of snails (*L. caperata, L. palustris, L. exilis,* and *Physa* sp.) using a head-foot retraction method. First the snail was wiped off with a paper towel then, using a pipette, the snail was gently pushed back into its shell and its epithelium was massaged with the pipette tip to stimulate mucus excretion. Mucus was pipetted from the snail into a 1.5 μL microtube and stored in the refrigerator (1.5°C - 4°C) until used (usually the same day, never more than 1 day). Mucus was diluted (1:3, 1:30, 1:300, and 1:3000) in Chernin's Balanced Saline Solution (CBSS) (Chernin 1963). Mucus for a single assay often came from more than one snail. Miracidia were then exposed to 40 μl of the mucus solutions or CBSS control and placed on a slide that was coverslipped using petroleum jelly around the edges. Observations were made at 1 h, 4 h, and 6 h. At each time period, miracidial condition was determined using the following categories: swimming, settled, transforming, transformed, damaged, dead, or in the petroleum jelly. For analysis, observations were grouped into three main categories: damaged, transforming, and intact. Damaged (grouping of dead and damaged) was defined as anywhere from dead to an obvious splitting of the epithelium and blebbing out of internal structures (fig. 1). Transforming (grouping of transformed and transforming) was defined as anywhere from the initial stages of losing cilia and epithelial plates to complete transformation

to mother sporocyst stage (fig. 2). Intact (combination of swimming and settled) was defined as miracidia still having all epithelial plates intact and beating cilia – these miracidia could have been swimming or settled on the bottom of the well (fig. 3).

Heat treated *Physa* sp. mucus was used in one assay to assess whether effector molecules were heat-labile, as typical of proteins. Mucus was heated (65°C in a hot water bath for 30 min) and then allowed to cool to room temperature prior to exposure to miracidia.

Snail Tissue Homogenate:

Snail shells were removed, and the head foot was excised, patted dry with a paper towel, and homogenized using a plastic mictrotube tissue homogenizer. The homogenate was centrifuged at 1,000 rpm for 1 min. The supernatant was then removed and the protein concentration was determined using a BCA protocol. A dilution series (0.5 mg/mL, 0.05 mg/mL and 0.005 mg/mL protein concentrations) was set up in 96 well plates. Between 10 and 200 miracidia were then added to tissue homogenate solution from a single snail on a slide to make a final volume of 80 μL of the test concentration. Observations were made at 1 h, 2 h, and 24 h and miracidial outcomes were measured as in the mucus assay. The observation time was 24 h in this assay to assess if miracidia transformed completely in homogenate. In two separate trials, all homogenate samples were exposed to heat treatment to evaluate the role of proteins as effector molecules. Microtubes of diluted homogenate were placed in a hot water bath at 65°C for 30 minutes. They were then centrifuged for 2 min at 10,000 RPM and allowed to cool to room temperature.

Mucus and homogenate assays were analyzed using a Repeated Measures Analysis of Variance. All proportions were arcsin transformed prior to analysis. Untransformed data were graphed for ease of visualization.

Histology

Histologic examination was used to assess snail immune response *in vivo*. *Lymnaea caperata*, *L. exilis*, *L. palustris,* and *Physa* sp. were isolated and exposed individually to many miracidia (hundreds-thousands) for predetermined time intervals of 1 h, 2 h, 3 h, and 4 h. A single snail of each species was exposed for each of the time periods. After exposure, snail shells were physically removed and the head foot was isolated and placed in AFA (9 parts 70% ethanol, 0.5 part commercial formalin, 0.5 part glacial acetic acid) for 2-14 days. Specimens were then stored in 70% ethanol until processed. They were dehydrated using a graded tert-butanol/ethanol series and embedded in paraffin (56° C melting point) (Johansen 1940). All snails were cut into 10 μm serial cross sections using a rotary microtome. Sections were attached to slides using Haupt's adhesive. They were stained using eosin (1g/L in 70% ethanol with 5 mL glacial acetic acid) and hematoxylin (0.5% aqueous) (Sanderson 1994). Sections were dehydrated in a graded ethanol series, cleared with limonene, and mounted with Permount. Pictures were taken using an OptixCam digital camera. They were uniformly adjusted in Photoshop for level, contrast, and color. Images were then examined for qualitative differences in immune response between species.

RESULTS

Role of Miracidial Behavior in Host Finding

The host finding behavior of *Physa* sp. (n = 10), *L. exilis* (n = 8), and *L. caperata* (n = 12) was examined to determine the role of the miracidium in initiating an infection. No interspecies differences were found with regard to the number, site (shell, body, mucus), or duration of contacts with the snail by the miracidium. Miracidia were found to attach to *L. caperata* significantly more often (4 did not attach, 7 attached 1 time, 1 attached 4 times) than *L. exilis* (6

did not attach, 2 attached 1 time) or P*hysa.* sp (9 did not attach, 1 attached 9 times), but no significant difference was found between *L. exilis* and *Physa* sp. ($\chi^2 = 6.6561$, p = 0.0359, df = 2) (fig. 4). Miracidia also attached for a significantly longer period of time to *L. caperata* ($\bar{x}$ = 604.1 s) than to *Physa* sp. ($\bar{x}$ = 20.2 s), but there was no significant difference between *L. caperata* and *L. exilis* ($\bar{x}$ = 167.4 s) nor *L. exilis* and *Physa* sp. (F = 4.03, p = 0.0294, df = 2,27) (fig. 5). There were no significant interspecies differences for successful attachments. When miracidia were exposed to whole snails, they were damaged more frequently by *Physa* sp. than expected ($\chi^2 = 6.6000$, df = 2, p = 0.0330). Only 25% of miracidia were damaged after exposure to *L. caperata* while 50% were damaged after exposure to *L. exilis*, and 80% were damaged after exposure to *Physa* sp. (fig. 6).

Role of Snail Mucus in Compatibility

The effect of snail mucus on miracidial condition was measured to assess the role of this primary barrier in compatibility. There was an overall species effect on the ability of mucus to cause damage to miracidia (df = 3,32; $F_{3,32} = 23.59$; p<0.0001). *Physa* sp. was the most lethal snail species, particularly at the 1:3 and 1:30 dilution (fig. 7) where it killed 100% of the exposed miracidia. There was no overall dilution effect at dilutions out to 1:300 (df = 2, 33; $F_{2,33} = 0.19$; p = 0.8315), but there was an interaction between species and dilution (df = 11, 24; $F_{11,24} = 9.62$; p<0.0001) (Appendix.1). In all three models, time had a significant effect (A.1), but there were no interactions with time. Over time, all species except *Physa* sp. trended toward higher rates of damage in the 1:3 and 1:30 dilutions (fig. 7). Any effect of mucus on miracidia disappeared at the 1:3000 dilution.

In a single trial, there was a complete negation of the toxic effect of the mucus after heat treatment. When miracidia were exposed to *Physa* sp. heat-treated mucus, all miracidia were still swimming after four hours.

There was no difference in the ability of mucus to stimulate transformation based on snail species, dilution, or the interaction between species and dilution (A.2). The only variable that was able to explain any of the variation in transformation rates was time in all three models (A.2). It should be noted here that all miracidia that started to transform, but died in the process, were moved to the damaged category. This can large Transformation was more likely to occur by 4 to 6 h (fig. 8). *Lymnaea caperata* had some stimulatory effects at the highest concentrations, but it was not statistically greater than other species. Approximately 22% of the miracidia exposed to *L. caperata* mucus at the 1:3 dilution transformed after 4 h. This effect decreased to about 5% at the 1:30 dilution and was completely gone at the 1:300 dilution. *Physa* sp. had some stimulatory effects after mucus was diluted past cytotoxicity (approximately 5% at 1:30 versus ~30% at 1:3000). *L. palustris* and *L. exilis* were variable (fig. 8).

Role of Tissue Homogenate in Compatibility

Snails were exposed to snail tissue homogenate to test the effect of internal snail products on miracidial condition. There was an overall species (df = 3, 77; $F_{3,77} = 6.76$; $p = 0.0004$) and dilution (df = 2, 78; $F_{2,78} = 18.21$; $p<0.0001$) effect on the damaging potential of tissue homogenate from the different snails (A.3). In all analyses, the amount of time that the miracidia were exposed to the homogenate had an effect (A.3). All models showed interactions between time, dilution, and species were significant (A.3). Homogenate from *Physa* sp. caused the most damage and mortality at the 0.5 mg/mL dilution after 1 h (68%) and 4 h (85%) (fig. 9). This effect decreased at 0.05 mg/mL, damaging 19% of miracidia after 4 h and was no different

from control at 0.005 mg/mL (fig. 9). At the 0.5 mg/mL dilution after 4 h, *L. caperata* (34%) and *L. exilis* (25%) were both more damaging to miracidia than the control. At 0.005 mg/mL, all were equivalent to the control. After heat treatment, damage rates were equivalent to the control (fig. 10).

Tissue homogenate was also analyzed for its ability to stimulate transformation. There was an overall species (df = 3,77; $F_{3,77} = 4.91$; $p = 0.036$) and dilution (df = 2,78; $F_{2,78} = 20.36$; $p<0.0001$) effect on transformation initiation (A.4). The amount of time that the miracidia were exposed to the homogenate also had an effect (A.4). It should be noted here that all miracidia that started to transform, but died in the process, were moved to the damaged category. All models including interactions between snail species, time, and dilution were significant. *Lymnaea caperata* stimulated the most transformation at the 0.5 mg/mL dilution (57%), but any effect was gone by 0.005 mg/mL (fig. 11). *L. exilis* also stimulated transformation significantly more than control (19%) at 0.5 mg/mL after 1 h. All effects were abrogated after heat treatment (fig. 12).

Histological Examination for* in vivo *Snail Response

Miracidia attached to all three snail species, but only successfully transformed in *L. caperata*. No hemocyte attraction was observed in early attachment of *F. magna* miracidia to *L. palustris* (fig. 13c). Miracidia were not seen attached to *L. palustris* after 2 h or 3 h of exposure. Likewise, there was no hemocyte activity in the early stages of attachment to *L. exilis* (fig. 13a). As the miracidia burrowed deeper into *L. exilis*, there was some hemocyte accumulation, but no attachment or encapsulation (fig. 14a, fig. 15a, fig. 16b). *Lymnaea caperata* did not show any significant immune response, as measured by hemocyte accumulation or encapsulation (fig. 13b, fig. 14b, fig. 15b, fig. 16b). In early attachments, hemocyte attraction was not observed (fig.

13b, fig. 14b). In cases where transformation and penetration occurred, there was no hemocyte attachment or encapsulation (fig. 15b, fig. 16b). The only hemocyte accumulation observed was in *L. palustris* once partial penetration of the miracidium had occurred (fig. 16c). It was also observed that full penetration only occurred in *L. caperata* after 4 h (fig. 16b) indicating that miracidia penetrated *L. caperata* at a greater rate than *L. exilis* or *L. palustris*.

DISCUSSION

My main goal was to determine whether host-parasite compatibility is primarily controlled by the intermediate host or by the parasite in the *Fascioloides magna* system. Results indicated that both parties are active participants in the arms race. Miracidia exhibit observable host-finding behaviors, and their sympatric snails had attributes that either permitted or prohibited infection.

While *F. magna* miracidia made indiscriminate contact with all the species, they attached more often and for longer periods of time to a suitable host. The host-finding process had a species-level specificity as evidenced by the higher number of total attachments to its natural intermediate host, *L. caperata,* than to the resistant *L. exilis*. This implies that miracidial attachment and penetration is not a random process. Miracidia may make random contact with potential hosts, but when they find a suitable host they invest more energy and time into initiating infection. This evidence is contrary to the notion that the initiation of infection is the result of chance (Campbell and Todd 1955a). Instead, this supports the idea that *F. magna* miracidia must use some chemical and/or physical cues to seek out hosts and initiate infection.

Previous research has identified free amino acids, the hydrogen ion, Mg++, tissue homogenate, and other molecules in snail conditioned water (SCW) to have stimulatory or attractant effects on miracidia (Prechel *et al*. 1976, Nollen and Mart 1983, Campbell 1961). This

study supports the idea that these cues exist on a species level. It is likely that one Lymnaeid species has unique molecular markers that stimulate attachment and transformation that another species in the same genus does not possess. It could be argued that in my experimental design, the miracidia and snails were sharing such a small space that they had no choice but to make contact, but in many trials the miracidium never touched the snail. Miracidia were able to initiate contact or avoid contact with objects near them.

Of the miracidia exposed to live *Physa* sp., 80% were damaged after the 30 min trial while only 25% were damaged after exposure to *L. caperata* and 50% by *L. exilis.* This indicates that *Physa* sp. has some sort of physical/chemical barrier to infection that *Lymnaea caperata* does not. *Lymnaea exilis* may have some cytotoxic effect, but miracidia tried to attach unsuccessfully which may have decreased miracidial fitness (Munoz-Antoli and Trelis 2003). The cytotoxic effect in *Physa* sp. was strong enough that simply making contact or even being in the same water resulted in a high likelihood of damage or death.

Extended behavioral observations showed that the Lymnaeids used grooming behaviors to avoid infection. In many trials where miracidia attached and then detached, the snail was responsible for causing the detachment. Depending on where the miracidium attached, the snail was able to use its antennae or its head-foot to physically remove it from its epithelium. This behavior may be of particular importance to the Lymnaeids that do not possess the same discrete physical/chemical barrier to infection that Physids do.

In addition to miracidial behavior, I also looked at the effect of snail tissue homogenate and mucus on the viability of *F. magna* miracidia. Most significantly, mucus of the resistant *Physa* sp. was extremely cytotoxic to miracidia, and tissue homogenate of the natural intermediate host, *L. caperata,* was able to stimulate transformation. *Physa* sp. mucus, at 1:3

and 1:30 dilutions, was 100% lethal to all miracidia exposed. This, along with observational data from the behavior experiment, indicates that snail mucus can serve as an anti-parasitic barrier toward digenean flatworm miracidia. All of the snails used were collected from the same habitat and would therefore all be exposed to the same parasites in the wild. Given the strength of this miracidial toxin, it is likely that it helps keep *F. magna* from penetrating *Physa* sp. in the field. Complete negation of the effect by heating argues that the responsible molecule may be a protein(s) or glycoprotein(s), which are common in snail mucus. Many types of molecules from mucus that have been shown to have positive, or stimulatory, effects on miracidia are generally unaffected by heat (Ford *et al.*1998; Nollen and Mart 1983), so the attractant and cytotoxic molecules in this study are likely different. However, it is possible that resistant snails share some of the same attractant or stimulatory molecules as susceptible snails. Campbell (1961) illustrated this when he found *F. magna* miracidia to be stimulated by tissue homogenate from *L. exilis*. I also observed hyperactivity when miracidia were exposed to heat treated snail products from *Physa* sp. This decoy effect may have far-reaching implications in the wild.

The mucus from *L. caperata, L. exilis*, and *L. palustris* was not significantly more cytotoxic to miracidia than CBSS control and there were no differences between these species. Apparently the effector molecule(s) causing the cytotoxic effect seen in *Physa* sp. are not found in these three lymnaeids. It is likely that there is an internal barrier that is different between susceptible *L. caperata* and *L. palustris,* and non-susceptible *L. exilis*. Furthermore, resistance mechanisms seem to differ between nonsusceptible *L. exilis* and resistant non-lymnaeids.

The toxic effect of mucus has been observed by other researchers. Coyne *et al.* (In-press) demonstrated that *Helisoma* sp. mucus was toxic to *F. magna* as well. Perhaps there is a protein or complex of proteins that can be identified as creating this primary barrier to parasitic

infection. Not only would this be of great interest to parasitology, as it would help to better understand the mechanisms underlying host specificity, but it may have ecological implications as well.

There was no significant overall species-specific effect on the ability of mucus to stimulate transformation. However, I did identify a trend. As the cytotoxicity of *Physa* sp. mucus decreased, the transforming stimulus increased. By the 1:3000 dilution, nearly 30% of miracidia were transforming. This implies that there may be a common molecule or set of molecules that has different effects at different concentrations. Perhaps the responsible molecule(s) creates a general stimulation that at high levels is fatal, but at low levels is stimulatory. It is possible that even higher dilutions could result in greater transformative stimulation. Coyne *et al.* (In-press) found some evidence for highly diluted mucus of *Helisoma* sp. stimulating *F. magna* transformation as well. This study supports those results, but there was no time frame or dilution in which mucus was significantly more effective at stimulating transformation than the CBSS control. Time was a significant model for explaining the variation. This was probably due to the fact that individuals that started to transform, but died in the process, were moved from the transforming category to the damaged category.

Tissue homogenate from *Physa* sp. was also harmful to *F. magna* miracidia. In the protocol, I tried to remove as much of the mucus from the head-foot as possible, but I cannot definitively say if this effect was the result of the homogenate itself or mucus in the homogenate preparation. Some mucus from the surface was likely incorporated when the homogenate was prepared. It is possible that mucus-secreting glands in the snail tissue were stimulated to produce mucus during the preparation. If the effect was from the homogenate itself, then there is support for *Physa* sp. having some internal defense system against *F. magna*

in addition to the external mucus barrier. This coincides with research done by Sapp and Loker (2000) which found that plasma from planorbid snails was extremely harmful to lymnaeid-infective sporocysts. *Physa* sp. could not be infected with *F. magna* miracidia due to mucus cytotoxicity, but that does not preclude additional internal immunity. In nature, *F. magna* miracidia would not see the inside of a snail. Therefore, transformed sporocysts from *F. magna* need to be exposed to mucus-free snail tissue homogenate, hemolymph, or plasma from these snails to better understand the mechanisms underlying internal snail host immunity of *Physa* sp. to *F. magna.* This would help determine if *F. magna* is capable of infecting *Physa* sp. once past the mucus barrier. Unfortunately, at this time, there is no reliable method to produce newly transformed mother sporocysts of *F. magna in vitro* in the numbers required to complete such a study.

Tissue homogenates from *L. caperata* and *L. exilis* were able to stimulate transformation at high concentration. *Lymnaea caperata* was more effective than *L. exilis*, but *L. exilis* stimulated more transformation than *L. palustris* or *Physa* sp. at the highest concentration. These effects were most clearly seen 1 h after exposure. This makes sense as penetration and transformation take at least 2 h (Coil 1981). By 4 h *in vivo,* miracidia should be completely transformed and penetrated. However, in this study, many miracidia died during the transformation process *in vitro* and were counted as dead and not transformed at the 4 h time frame. The homogenates from *L. palustris* and *Physa* sp. did not have this same transformative effect. Any effect was almost completely negated after heat treatment of the homogenate. Once again, this suggests that the responsible molecule(s) is a protein.

It was not surprising that tissue homogenate from *L. caperata* would stimulate transformation, as it is known to be the natural intermediate host in Minnesota. Mucus from *L.*

caperata did not stimulate transformation, so apparently there must be effector molecule(s) within the snail epithelium or underlying tissues that stimulate transformation when the apical papillae makes contact. Even though transformation was initiated, very few specimens transformed completely. Almost all of those that did transform completely died within 24 h, and many died before the 4 h mark. With the loss of epithelial plates and the extensive membrane rearrangement taking place during transformation, it is possible that *F. magna* requires the internal environment of the host to complete the process. Even the lowest dilution of 1:3 for protein concentration represented a very different osmotic environment than what exists in the mantle or head-foot of an intact snail. It is also possible that necessary nutrients or molecules are concentrated in miracidial entry points, but when homogenized become too dilute to support transformation.

While it was expected that *L. caperata* would stimulate transformation, it was surprising that *L. exilis* did so as well, supporting nearly 20% transformation at 0.5mg/mL. *Lymnaea exilis* is a non-susceptible snail that miracidia only infrequently attached to, but did not exhibit cytotoxicity in its mucus or tissue homogenate. Therefore, either an internal barrier likely prevents sporocyst development in live *L. exilis* if miracidia do attach.

To date, it has not been possible to complete the life cycle of *F. magna*, or any other digenetic trematode *in vitro,* which limits its use as a model in research. Laursen and Yoshino (1999) were able to get the miracidia to transform into mother sporocysts using BGE cell supernates and into daughter sporocysts in co-culture with BGE cells. Unfortunately, it has been difficult and labor intensive to reproduce these results. While the homogenate assay in my study was not designed to stimulate miracidia to transform into sporocysts, it does provide an interesting pathway toward a reproducible method for doing so. *Fascioloides magna* miracidia

shed their epithelial plates as they burrow into the snail's epithelium. It would be useful, from a research standpoint, to reliably and easily transform miracidia into sporocysts *in vitro*. The reaction of the hemolymph and plasma with sporocysts could then be analyzed. If a protein, glycoprotein, or complex of either could be isolated, it could help us better understand the mechanisms that trigger transformation in *F. magna* and other digeneans.

Bayne and Yoshino (1989) proposed three factors that could lead to successful infection by a miracidium: 1) mother sporocyst is capable of resisting cytotoxic effects of snail immune system; 2) mother sporocyst is capable of evading an immune response by molecular mimicry, acquisition of host molecules, or prevention of opsonization; 3) mother sporocyst disrupts hemocyte function. To see if any of these factors were at play, snails that were experimentally infected with *F. magna* miracidia were sectioned to observe the early effects of snail immunity *in vivo*. *Lymnaea caperata*, the natural host in Minnesota, showed little to no immune response from early attachment through complete penetration. There was very little hemocyte activity surrounding any penetrating miracidia, and encapsulation was not observed. Thus, after transformation, the mother sporocyst likely relied on the second or third factor, as evidenced by the minimal hemocytic attraction to the parasite (fig. 13b, fig. 14b, fig. 15b, fig. 16b). It is possible that the mother sporocyst had similar molecular markers to snail cells, or acquired host molecules during the penetration phase of infection. Since hemocytes were not observed to accumulate near the mother sporocyst, their ability to locate or attach to the parasite may have been disrupted.

There was some evidence of hemocyte accumulation around the site of penetration In *L. palustris* (fig. 13c, fig. 16c). However, I did not observe any appreciable hemocyte attachment, and there was no encapsulation. It appears that the presence of hemocytes was dependent on

the penetration site, since surrounding tissues had similar concentrations of hemocytes. *Lymnaea palustris* has been identified as a natural host in Czechoslovakia (Chroustova 1979) and in the lab in the USA. As a competent host, it is unsurprising that the immune response is low. *Fascioloides magna* miracidia did not completely penetrate *L. palustris* in the given time frame. In contrast, they were able to penetrate and transform in *L. caperata* within 3 h. This may simply be a result of no variation in the sample since only one snail was sectioned for each infection time.

In the resistant snail, *L. exilis*, there was also a very weak immune response. There was some hemocyte accumulation at the penetration sites (fig. 14a, fig. 16a), but the concentration was similar in surrounding tissues. Once again, there was no definite hemocyte attachment or encapsulation. Complete penetration and transformation to mother sporocysts was not observed. While my sample size was small, it is possible that although *L. exilis* is a suitable host for miracidia, to the point of permitting attachment, there is some internal barrier that causes them to fall off of the snail. As was seen with *L. palustris*, miracidia were not able to progress through the infection process in the same time frame as in *L. caperata*. Though there was not an obvious barrier in mucus, tissue homogenate, or early snail immunity, *L. exilis* does not readily harbor *F. magna* infections. The barrier to infection in *L. exilis* is unclear at this point.

Snail age may explain some of the differences seen in my study, as snails of different ages were used for analysis. Mature individuals were used for *L. caperata* and younger individuals were used for *L. palustris* and *L. exilis*, but their ages were inconsistent. Given that snail age corresponds to differences in susceptibility (Foreyt and Todd 1978, Swales 1935, Soulsby 1982), some of the observed variation is likely explained by age differences.

Digenean-snail compatibility depends on the interplay of behavioral and physiological properties of parasite and host. When a miracidium finds a suitable host, infections may be successful or not depending on its ability to discriminate the snail as such and the immune response of the snail. My research elucidated some general patterns in the *F. magna* system. In all parasite-host relationships, the first step is host-finding. In the lab, *F. magna* demonstrated the capability to differentiate between host genera and even host species in order to initiate a potentially successful infection. In the field there are decoy effects, predation, and variable environmental conditions that may decrease the utility of this ability. Even with many miracidia in an aquatic system, these factors can make it unlikely that a miracidium will find a suitable host, contributing to the over dispersed nature of parasitic infection in snails and parasites in general.

In an outbred population, it is unlikely that all individuals in a species will be equally susceptible, or that some species will be definitely and irreversibly resistant. In the *F. magna* system, I found that the sympatric genus, *Physa*, was able to avoid infection due to the cytotoxicity of its mucus. This primary barrier to infection not only prohibited miracidial penetration, but it significantly reduced miracidial survival. In the field, these factors may play a role in down-regulating *F. magna* infection in suitable hosts, also contributing to overdispersion.

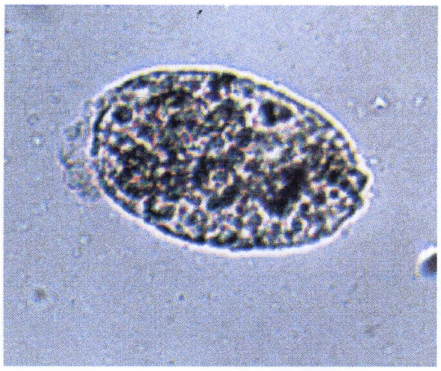
In the event that a miracidium does find a snail to which it can attach without being harmed, it must be physically capable of penetrating the host and surviving its immune response. The three lymnaeid species tested did not exhibit any cytotoxic properties in their mucus, but only two of them regularly support infection, *L. caperata* and *L. palustris*. Still, *F. magna* miracidia attached and attempted to initiate infection in all lymnaeid species. There are many examples of parasites with extremely high fecundity that rely heavily on opportunism as a

life strategy. It may be the case with *F. magna* that unsuccessful attempts at penetration are superior to being too discriminating and entirely missing an attempt at infection.

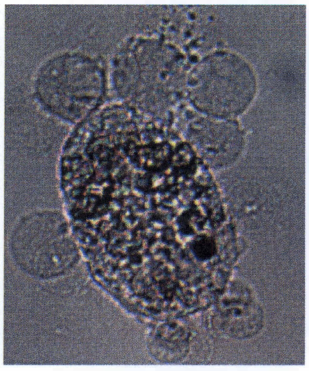
The effect of decoy snails and miracidial toxicity may have far-reaching ecological impacts. When a miracidium attempts to attach to an incompatible host, its likelihood of then finding and attaching to a compatible host is reduced. This may be due to a decrease in fitness because of cytotoxicity or an unnecessary expenditure of energy reserves during an attempted attachment (Campbell and Todd 1955a; Ford *et al.* 1998; Munoz-Antoli and Trelis 2003). Either way, these cytotoxic or incompatible snails may serve to regulate parasite populations by decreasing their likelihood of initiating a successful infection. Without decoy snails in an ecosystem, it is likely that parasitic infection would be more prevalent.

Host-parasite compatibility in digeneans is clearly, as Bayne and Yoshino (1989) found, an exception to the rule of resistance. It is also a dynamic process. Host-parasite relationships can change over time. Though they have some discriminating capabilities, miracidia still opportunistically attempt to penetrate non-susceptible hosts. Eventually, an evolutionary pioneer will have a mutation that allows infection in a non-susceptible host and non-susceptibility will immediately be compromised. For animals that utilize parasitism as a way of life, it is beneficial for a population to overcome strict host-parasite specificity, even if it is a dead end for many individuals that try.

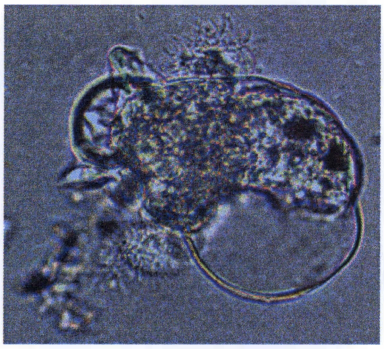
FIGURES



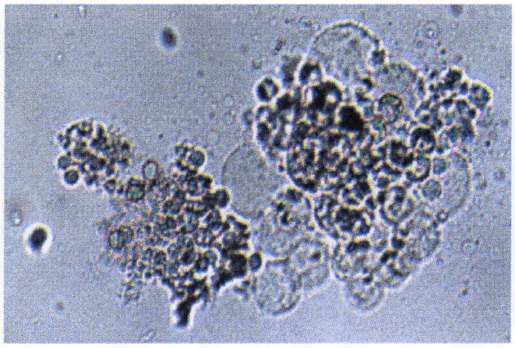
(a)



(b)

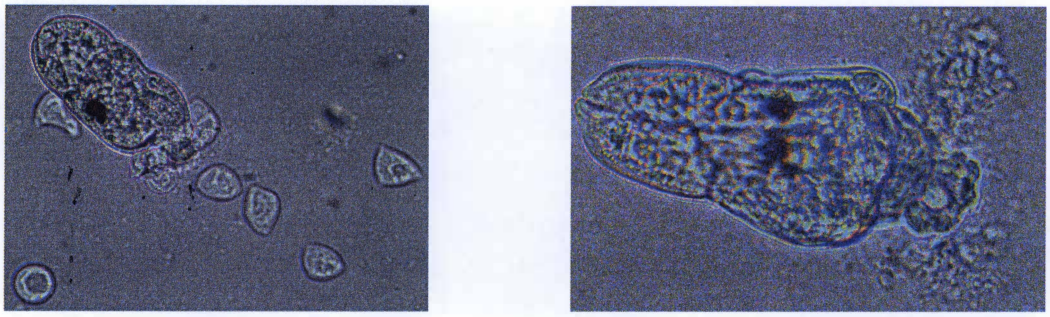


(c)



(d)

Figure 1: A series of pictures displaying miracidia included in the category of "damaged". This category ranges from damaged (a, b, c), where the miracidium displays a ruptured epithelium and blebbing out of internal structures to completely dead (d).



(a) (b)

Figure 2: A series of pictures displaying miracidia included in the category of "transforming". It is made up of transforming and transformed. Transforming is characterized by the shedding of epithelial plates (a). Transformed is characterized by a loss of epidermal plates and a lack of cilia (b).

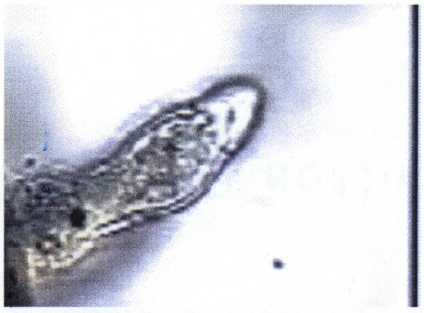


Figure 3: A picture displaying miracidia included in the category of "intact". These miracidia have intact epidermal plates and actively beating cilia. They may be swimming throughout the water column or settled on the bottom of the well.

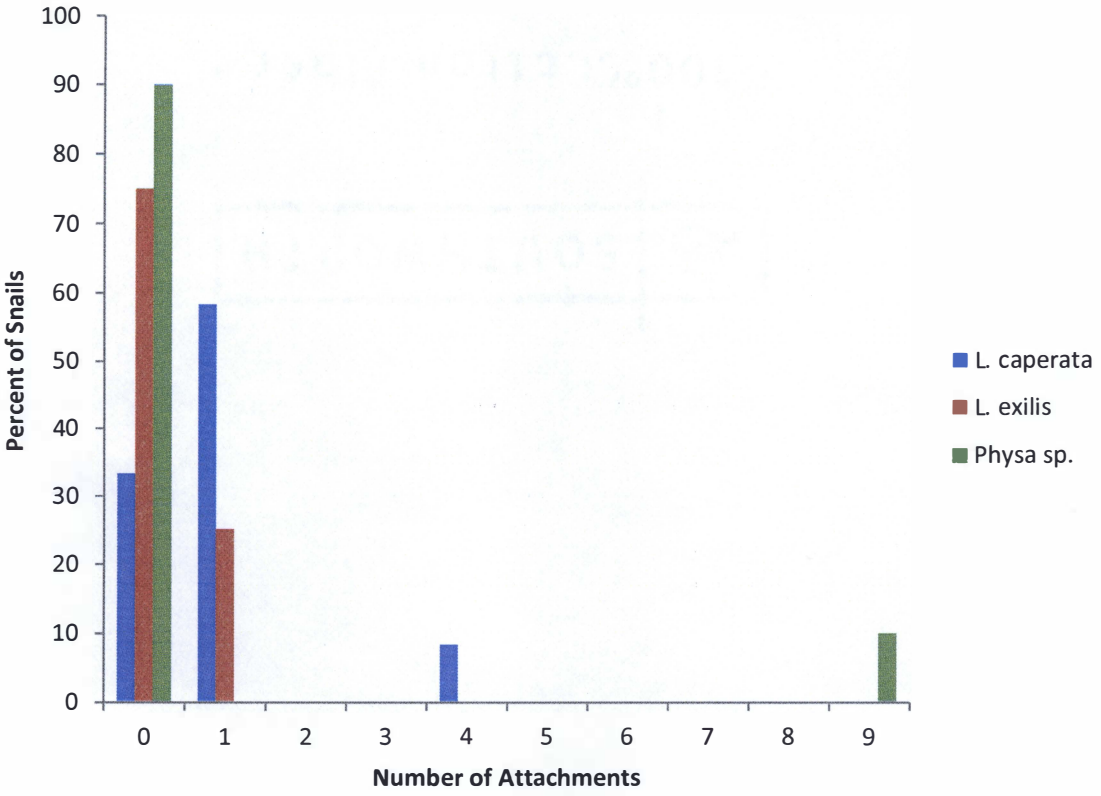


Figure 4: Influence of snail species on miracidial choice in initiating attachment attempts. Histogram comparing the number of attachments to *L. caperata* (n = 12), *L. exilis* (n = 8), and *Physa* sp. (n = 10). Non-parametric analysis revealed that miracidia attached to *L. caperata* significantly more often than to *L. exilis* or *Physa* sp, but there was no significant difference found between *L. exilis* and *Physa* sp. (x^2 = 6.6561, p = .0359, df = 2). Only 10% of miracidia attempted to attach to *Physa* sp. while 66.7% of miracidia attempted to attach to *L. caperata.*

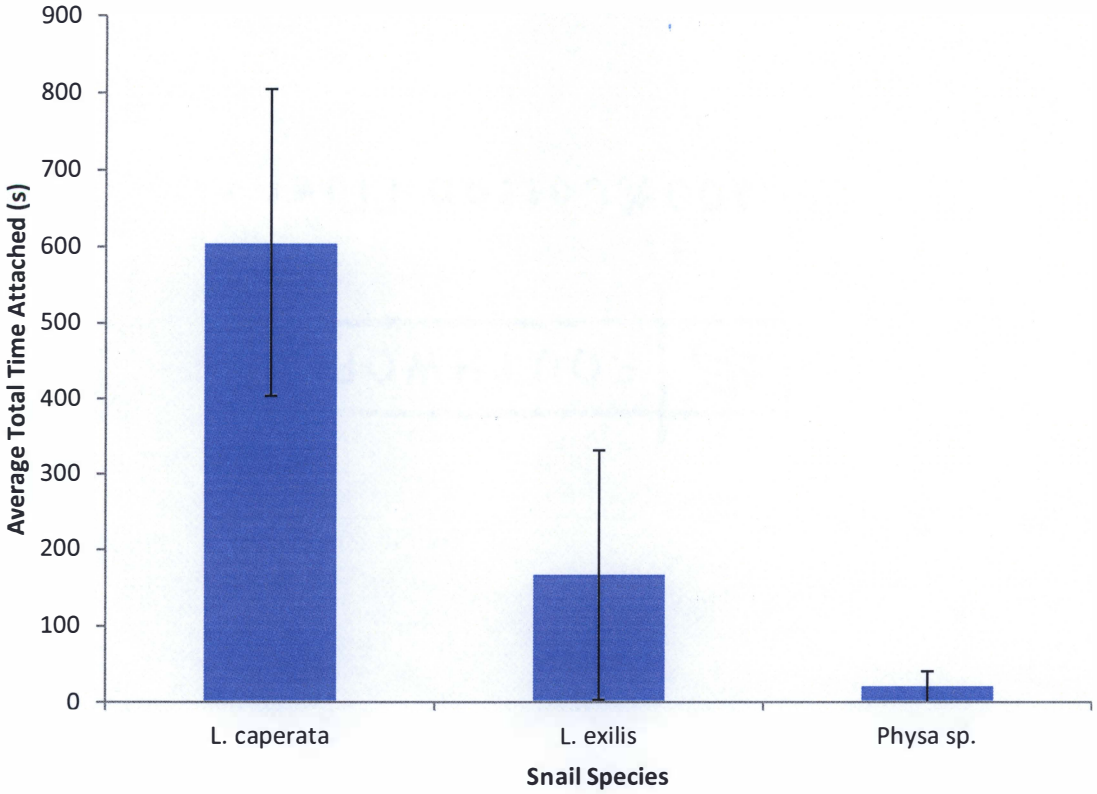


Figure 5: Influence of snail species on length of attachment attempt. Mean attachment time (+/- SE) Miracidia attached for a significantly longer period of time to *L. caperata* ($\bar{x}$ = 604.1 s) than to *Physa* sp. ($\bar{x}$ = 20.2 s), though there was no significant difference between *L. caperata* and *L. exilis* ($\bar{x}$ = 167.4 s) nor *L. exilis* and *Physa* sp. (F_{crit} = 3.51, F = 4.03, p = 0.0294, df = 2).

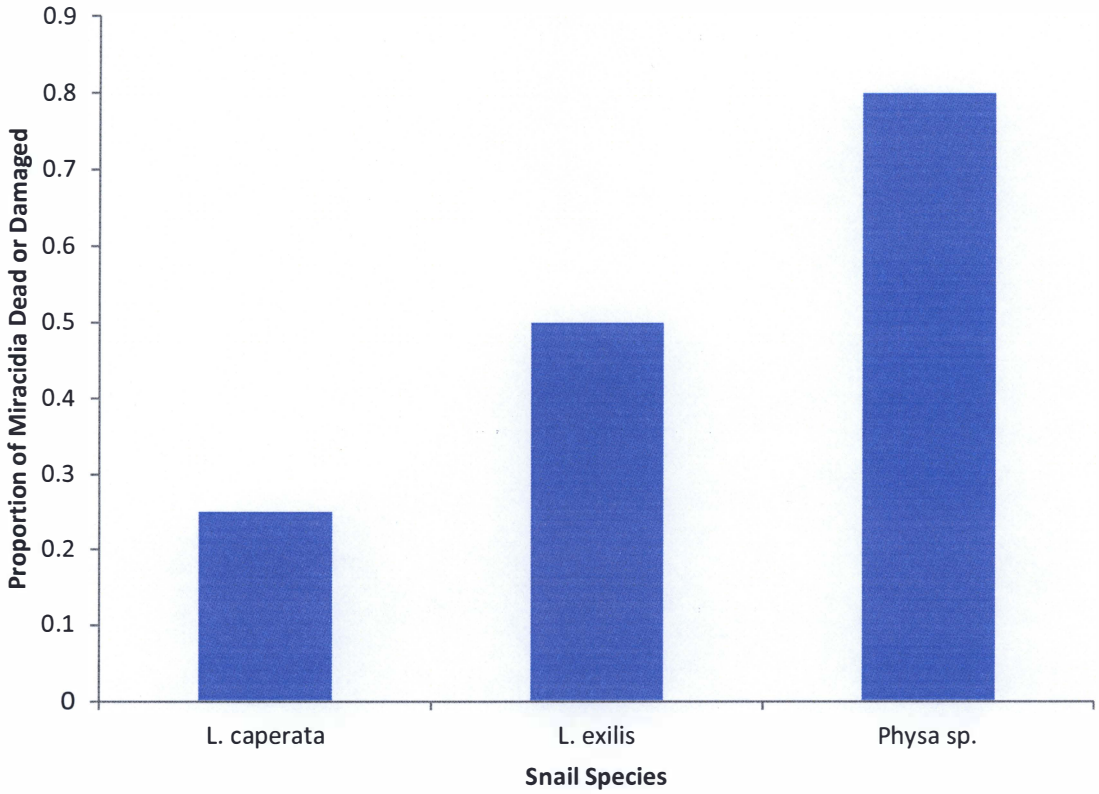


Figure 6: Whole snail effects on miracidia. After 30 min exposures, 25% of miracidia were damaged in presence of *L. caperata*, 50% in presence of *L. exilis*, and 80% in presence of *Physa* sp. More miracidia were damaged as a result of exposure to *Physa* sp. ($x^2 = 6.6000$, $df = 2$, $p = 0.0330$)

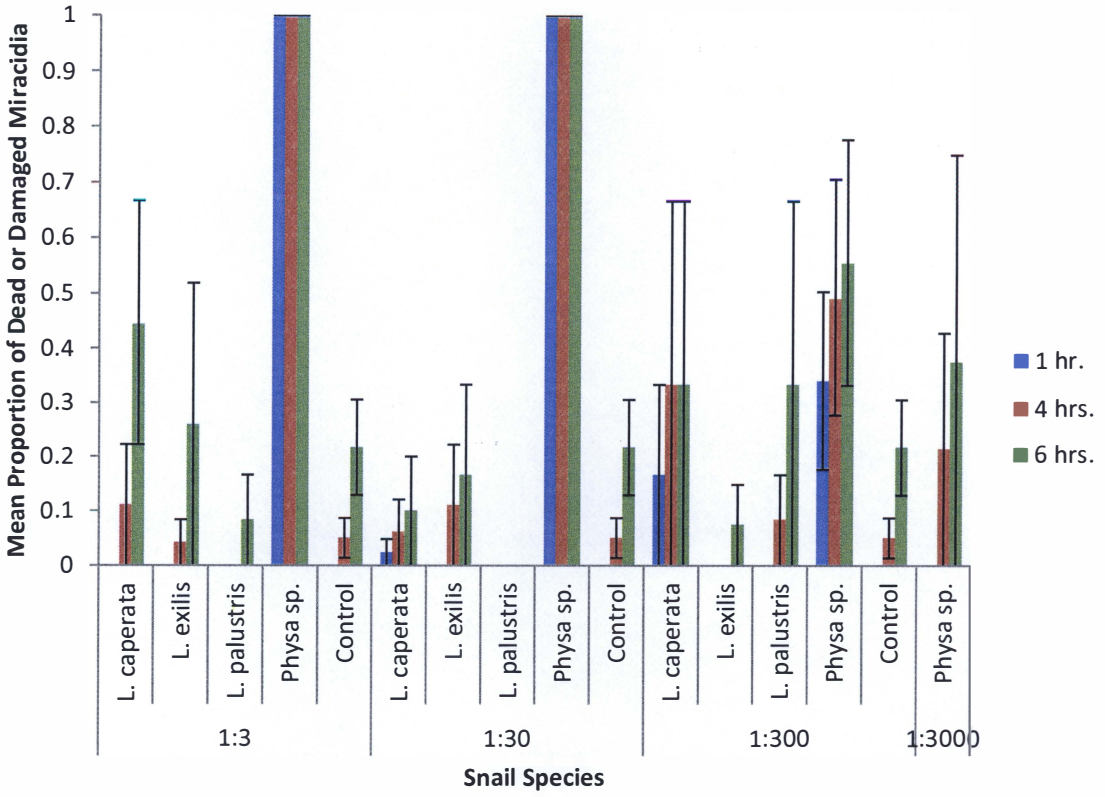


Figure 7: Role of snail mucus in miracidial toxicity. The mean proportion of dead or damaged miracidia (+/- SE) over time (1 h, 4 h, 6 h) after exposure to dilutions of snail mucus (control, n = 12; *Physa* sp., 1:3000, n = 2; all others, n=3). The control treatment is the same throughout the experiment (n=12), but graphically repeated to ease visual comparison within each dilution. There was an overall species effect on the ability of mucus to cause damage to miracidia (df = 3,32; F = 23.59; p<0.0001). *Physa* sp. was significantly more cytotoxic (100%) at both the 1:3 and 1:30 dilutions, but the effect was gone by 1:3000 dilution. There was no overall dilution effect (df = 2, 33; F = 0.19; p = 0.8315), but there was an interaction between species and dilution (df = 11, 24; F = 9.62; p<0.0001) (A.1). In all three models, time had a significant effect (A.1), but there were no interactions with time.

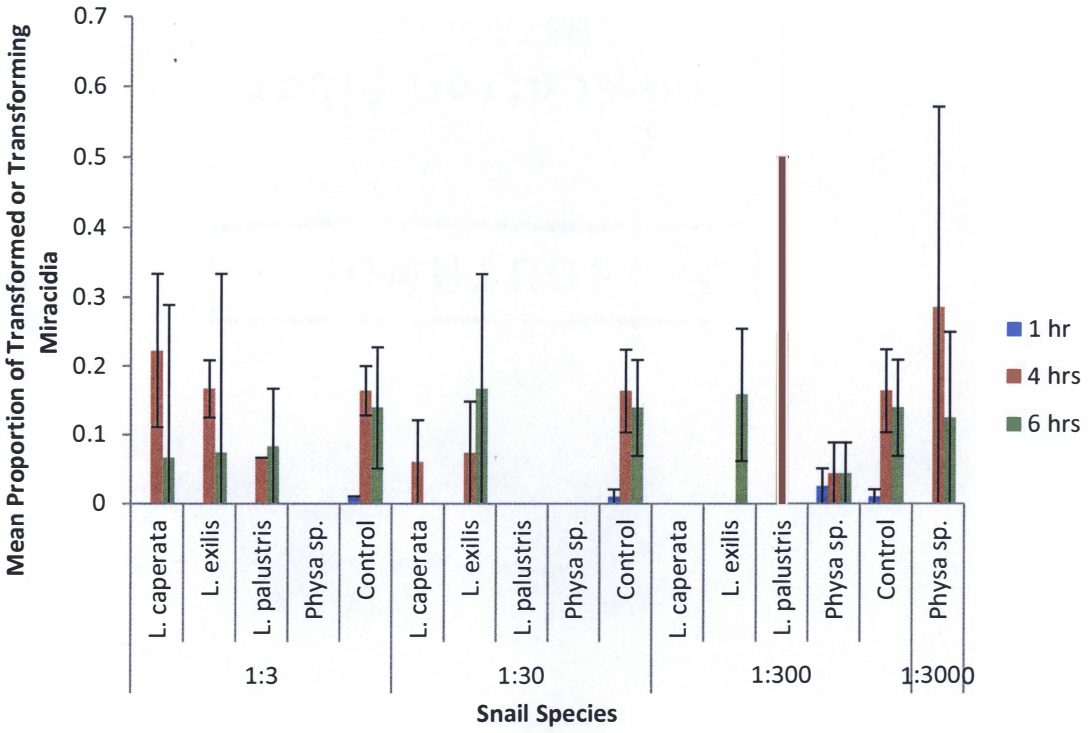


Figure 8: Role of mucus in stimulating miracidial transformation. The mean proportion of transformed or transforming miracidia (+/- SE) over time after exposure to mucus from 4 snail species (control, n = 12; *Physa* sp., 1:3000, n = 2; all others, n=3). The control treatment is the same throughout the experiment (n=12), but graphically repeated to ease visual comparison within each dilution. There was no significant difference in the ability of mucus to stimulate transformation based on snail species, dilution, or the interaction between species and dilution (A.2). Time was the only variable that was able to explain any of the variation in transformation rates in all three models (A.2). Transformation was more likely to be occurring in the 4 h or 6 h time frame in all species. Mucus from *L. caperata* and *L. exilis* displayed some stimulatory effects, but was only significantly different from *Physa* sp. Mucus from *Physa* sp. killed miracidia at the highest concentrations, but had some stimulatory effects after dilution. Effects of mucus from *L. palustris* and *L. exilis* were variable.

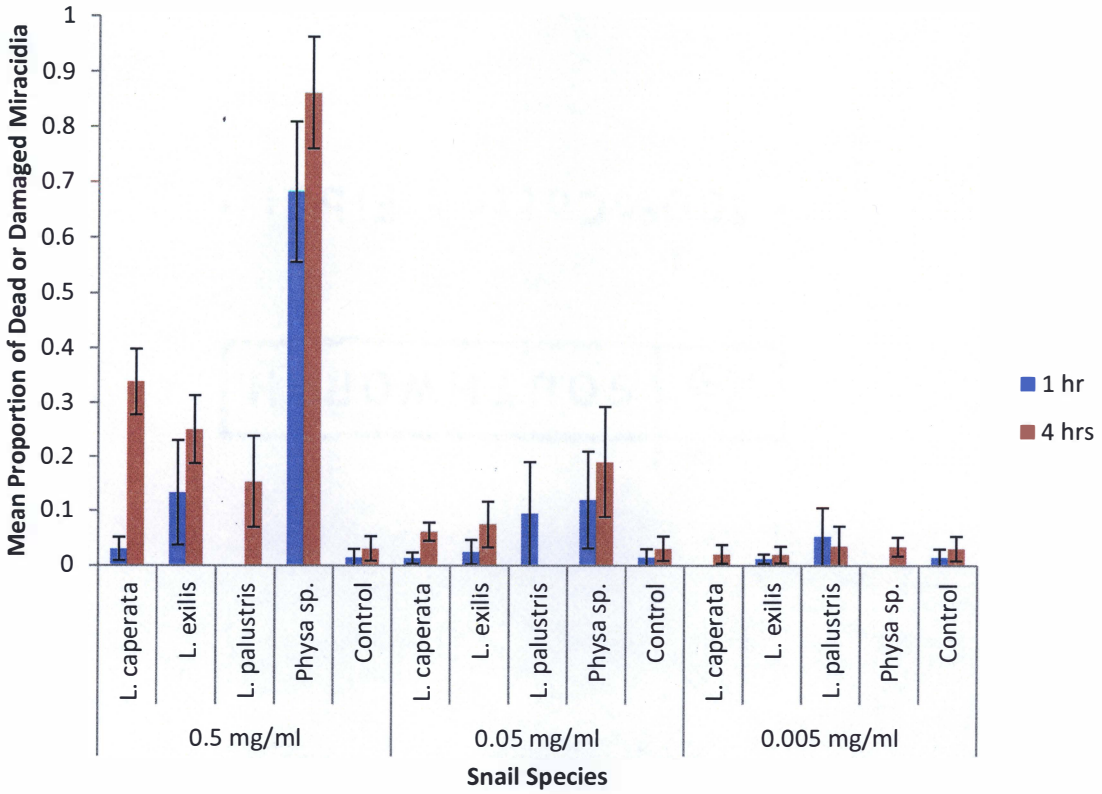


Figure 9: Role of snail homogenate on miracidia condition. Mean proportion of dead or damaged miracidia (+/- SE) over time after exposure to dilutions of snail tissue homogenate (*L. palustris,* n = 3; all others, n = 8). The control treatment is the same throughout the experiment (n=12), but graphically repeated to ease visual comparison within each dilution. There was a significant effect of species (df = 3, 77; F = 6.76; p = 0.0004), dilution (df = 2, 78; F = 18.21; p<0.0001), and time (A.3). All models showing interactions between time, dilution, and species were significant (A.3). Homogenate from *Physa* sp. caused significantly more damage and mortality at the 0.5 mg/mL dilution after 1 h and 4 h (fig. 9). This effect decreased at 0.05 mg/mL, and after 4 h there was no difference from control at 0.005 mg/mL (fig. 9). *Lymnaea caperata* and *L. exilis* homogenates were both more damaging to miracidia than control, but only at the 0.5 mg/mL dilution after 4 h. No species was any more damaging than control at 0.005 mg/mL.

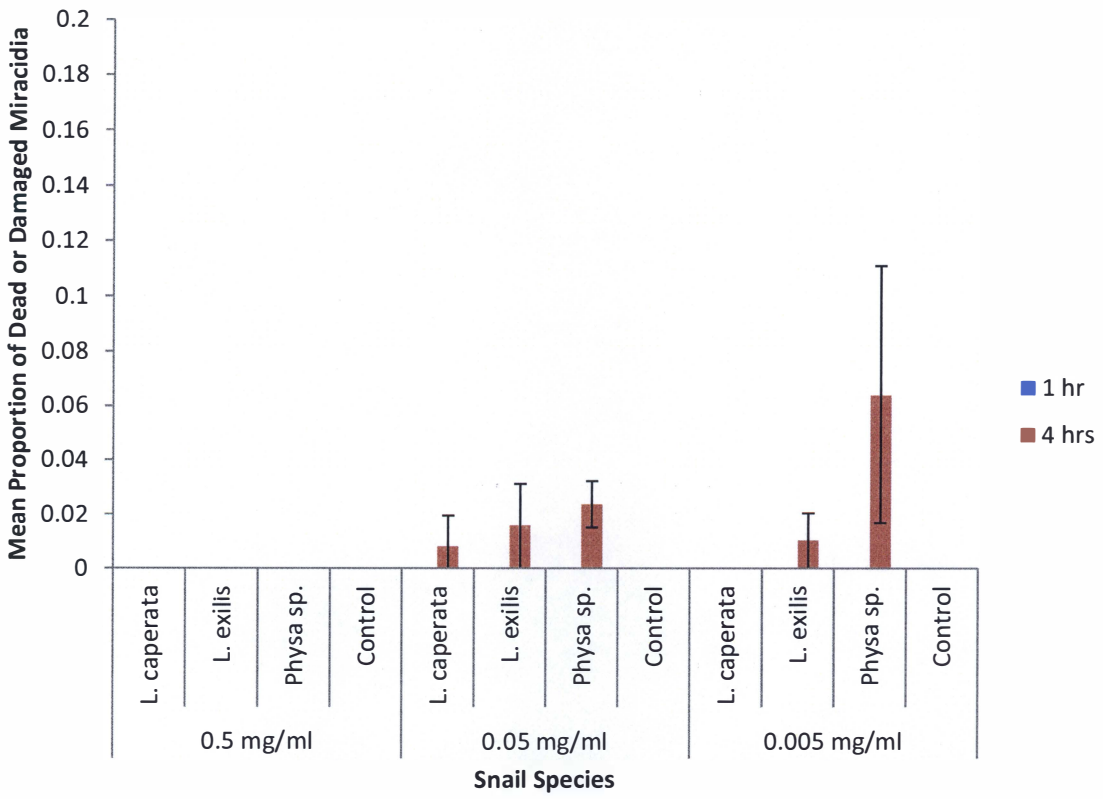


Figure 10: Effect of heat treatment on snail homogenate induced miracidial toxicity. The proportion of dead or damaged miracidia (+/- SE) across all dilutions at 1 h and 4 h after heat treatment of tissue homogenate (n = 2). The damaging effects that were seen in *Physa* sp., *L. caperata*, and *L. exilis* were almost completely negated by heat treatment. Minimal damage was observed by *Physa* sp. at the 0.05 mg/mL and 0.005 mg/mL dilutions.

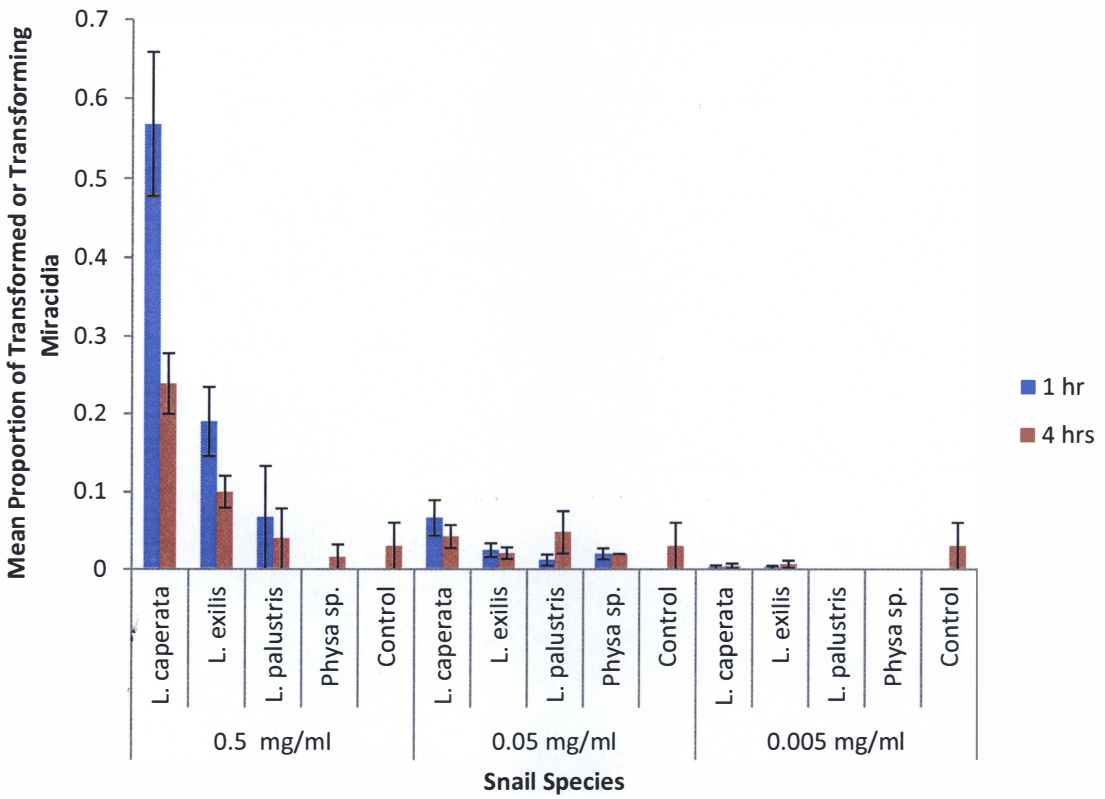


Figure 11: Effect of snail homogenates on miracidial transformation. Proportion of transformed or transforming miracidia (+/- SE) across all dilutions 1 h and 4 h after exposure to snail tissue homogenate (*L. palustris,* n = 3; all others, n = 8). The control treatment is the same throughout the experiment (n=12), but graphically repeated to ease visual comparison within each dilution. There was an overall effect of species (df = 3,77; F = 4.91; p = 0.036), dilution (df = 2,78; F = 20.36; p<0.0001), and time (A.4). All models including interactions between snail species, time, and dilution were significant (A.4). *L. caperata* stimulated significantly more transformation at the 0.5 mg/mL dilution (57%) and any effect was gone by 0.005 mg/mL. *L. exilis* also stimulated transformation significantly more than control at 0.5 mg/mL after 1 h.

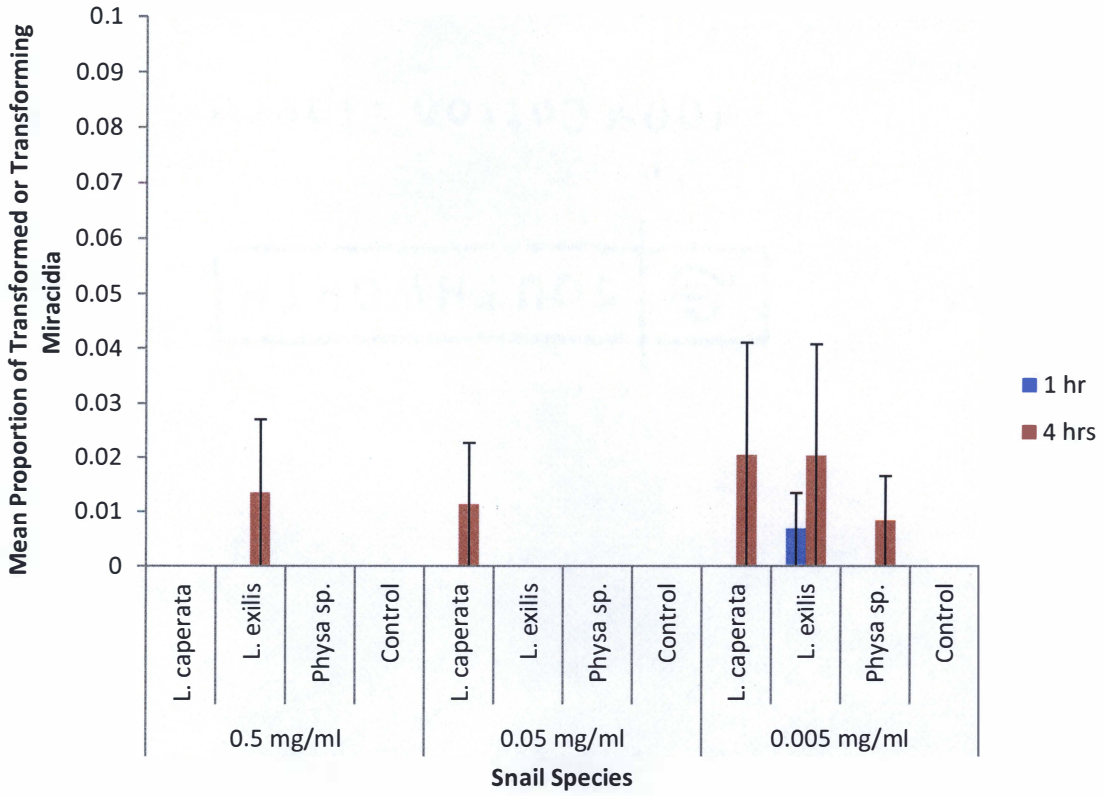


Figure 12: Effect of heat treatment on snail homogenate-induced miracidial transformation. Proportion of transformed or transforming miracidia (+/- SE) across all dilutions 1 h and 4 h after heat treatment of homogenate (n = 2). Minimal amounts of transformation were observed, but they did not differ significantly from control.

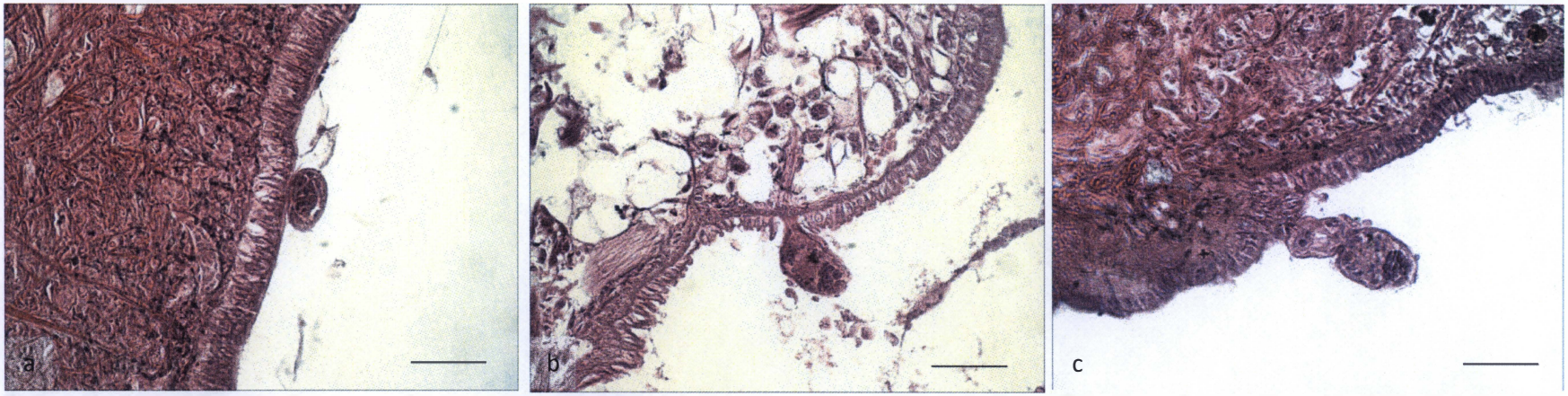


Figure 13: Miracidial attachment to three snail species after 1 h exposure: (a) *L. exilis,* mid ventral head-foot. (b) *L. caperata,* left dorso lateral mantle. (c) *L. palustris,* right head-foot, ventro lateral. Scale bars = 50 µm.

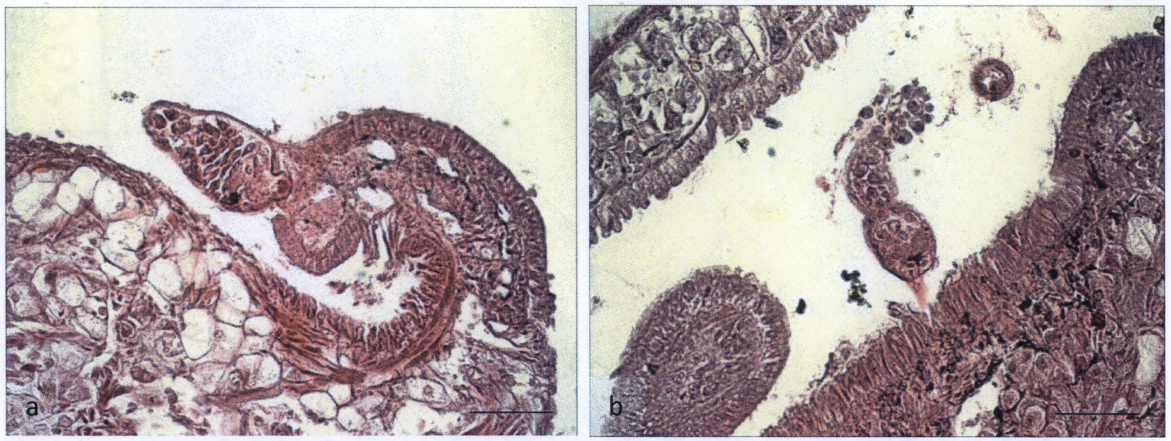


Figure 14: Miracidial attachment to two snail species after 2 h exposure: (a) *L. exilis,* left lateral mantle. (b) *L. caperata,* left, dorsal head-foot, between mantle and head-foot. Scale bars = 50µm.

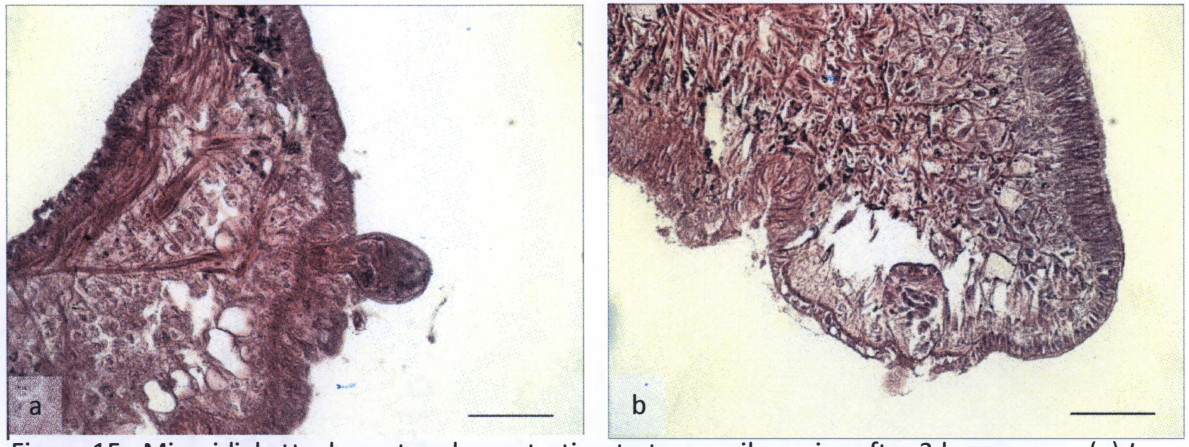


Figure 15: Miracidial attachment and penetration to two snail species after 3 h exposure: (a) *L. exilis,* left, dorsal lateral head-foot. (b) *L. caperata,* late stage miracidium, early mother sporocysts, completely penetrated epithelium, loss of cilia and epithelial plates, right, ventro-lateral head-foot. Scale bars = 50µm.

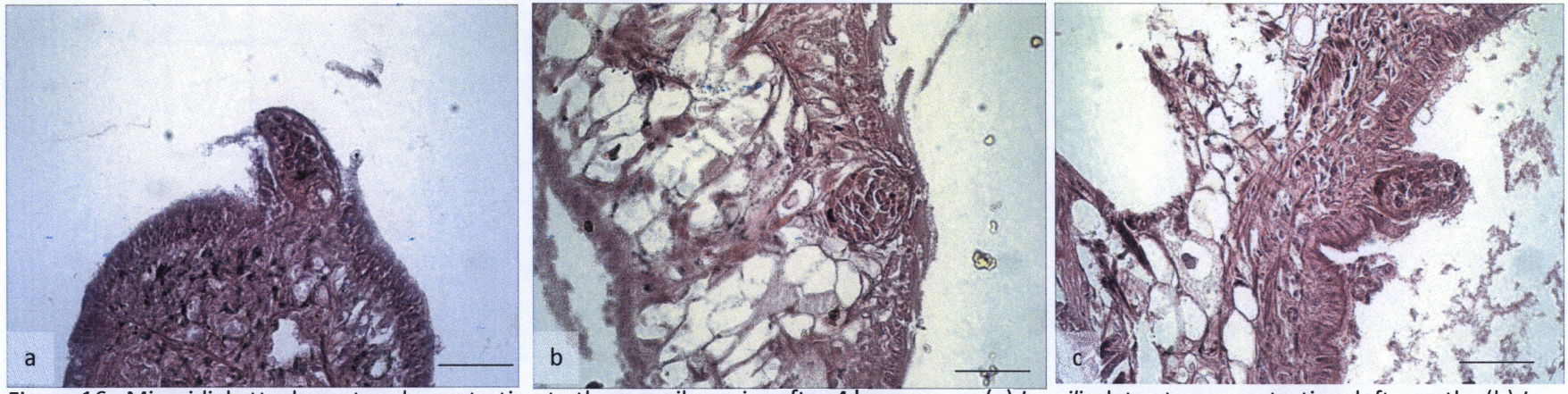


Figure 16: Miracidial attachment and penetration to three snail species after 4 h exposure: (a) *L. exilis,* late-stage penetration, left mantle. (b) *L. caperata,* fully penetrated epithelium, right dorso-lateral mantle. (c) *L. palustris,* late stage penetration, mid dorso lateral mantle. Scale bars = 50µm.

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APPENDIX

A.1

Table 1: Results of repeated measures analysis of the ability of mucus collected from different species and administered in 1:3, 1:30, and 1:300 dilutions to cause damage or death. Sources containing time (1, 4, or 6 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Species*dilution	11, 24	9.62	<0.0001
Hour	2, 23	4.87	0.0172
Hour x species*dilution	22, 48	0.70	0.8150

Table 2: Results of repeated measures analysis of the ability of mucus collected from different species to cause damage or death. Sources containing time (1, 4, or 6 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Species	3, 32	23.59	<0.0001
Hour	2, 31	5.33	0.0102
Hour*species	6, 64	0.56	0.7619

Table 3: Results of repeated measures analysis of the ability of mucus administered in 1:3, 1:30, and 1:300 dilutions to cause damage or death. Sources containing time (1, 4, or 6 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Dilution	2, 33	0.19	0.8315
Hour	2, 32	5.50	0.0088
Hour *dilution	4, 66	1.00	0.4116

A.2

Table 4: Results of repeated measures analysis of the ability of mucus collected from different species and administered in 1:3, 1:30, and 1:300 dilutions to stimulate transformation. Sources containing time (1, 4, or 6 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Dilution*species	11, 24	0.64	0.7738
Hour	2, 23	5.12	0.0145
Hour*dilution*species	22, 48	0.95	0.5397

Table 5: Results of repeated measures analysis of the ability of mucus collected from different species to stimulate miracidial transformation. Sources containing time (1, 4, or 6 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Species	3, 32	0.69	0.5668
Hour	2, 31	5.85	0.0070
Hour*species	6, 64	1.68	0.1402

Table 6: Results of repeated measures analysis of the ability of mucus administered in 1:3, 1:30, and 1:300 dilutions to stimulate miracidial transformation. Sources containing time (1, 4, or 6 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Species	2, 33	0.61	0.5489
Hour	2, 32	5.47	0.0091
Hour*species	4, 66	0.42	0.7938

A.3

Table 7: Results of repeated measures analysis of the ability of tissue homogenate collected from different species and administered in 0.5, 0.05, 0.005 mg/mL protein dilutions to cause damage or death. Sources containing time (1 hr or 4 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Species*dilution	11, 69	18.61	<0.0001
Hour	1, 69	6.40	0.0137
Hour*species*dilution	11, 69	4.98	<0.0001

Table 8: Results of repeated measures analysis of the ability of tissue homogenate collected from different species to cause damage or death. Sources containing time (1 hr or 4 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Species	3, 77	6.76	0.0004
Hour	1, 77	4.37	0.0398
Hour*species	3, 77	2.49	0.0662

Table 9: Results of repeated measures analysis of the ability of tissue homogenate administered 0.5, 0.05, 0.005 mg/mL protein dilutions to cause damage or death. Sources containing time (1 hr or 4 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Dilution	2, 78	18.21	<0.0001
Hour	1, 78	8.31	0.0051
Hour*dilution	2, 78	5.47	0.0060

A.4

Table 10: Results of repeated measures analysis of the ability of tissue homogenate collected from different species and administered in 0.5, 0.05, 0.005 mg/mL protein dilutions to stimulate transformation. Sources containing time (1hr or 4h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Species*dilution	11, 69	14.99	<0.0001
Hour	1, 69	40.68	<0.0001
Hour*species*dilution	11, 69	4.62	<0.0001

Table 11: Results of repeated measures analysis of the ability of tissue homogenate collected from different species to stimulate transformation. Sources containing time (1 hr or 4 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Species	3, 77	4.91	0.0036
Hour	1, 77	28.19	<0.0001
Hour*species	3, 77	2.02	0.1186

Table 12: Results of repeated measures analysis of the ability of tissue homogenate administered in 0.5, 0.05, 0.005 mg/mL protein dilutions to stimulate transformation. Sources containing time (1 hr or 4 h) or interactions with time are within-subject tests and report an F-test using Pillai's trace.

Source of variation	DF	F	P
Dilution	2, 78	20.36	<0.0001
Hour	1, 78	56.55	<0.0001
Hour* dilution	2, 78	14.98	<0.0001