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Patricia O'Leary

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Behavioral analysis of a digenetic trematode cercaria (*Microphallus turgidus*) in relation to the microhabitat of grass shrimp (*Palaemonetes* spp.)

by

Patricia O'Leary

(Under the Direction of Oscar J. Pung)

Abstract

The hydrobiid snail and grass shrimp hosts of the microphallid trematode *Microphallus turgidus* are found in specific microhabitats. The primary second intermediate host of this parasite is the grass shrimp *Palaemonetes pugio*. The behavior of trematode cercaria often reflects the habitat and behavior of the host species. The objective of my study was to examine the behavior of *M. turgidus* in relation to the microhabitat selection of the second intermediate host. To do so, I established a behavioral ethogram for the cercariae of *M. turgidus* and compared the behavior of these parasites to the known host behavior. I tested the phototactic, geotactic, and chemical responses of the parasite. When *M. turgidus* cercariae are placed in a half covered Petri dish I predicted that the cercariae would have negative phototaxis. For the control experiment, in the absence of light, I predicted an equal distribution of cercariae throughout the entire Petri dish. I also predicted that the cercariae would prefer the bottom of the water column, and that their behavior would change in response to chemicals in shrimp conditioned water. Both the phototaxis and geotaxis trials used lighted conditions as the experimental and unlighted conditions as the control. The phototaxis trials were performed using a half covered Petri dish and the geotaxis trials used a graduated cylinder. The chemical response trials used water from a container

which housed grass shrimp for 72 hr or unconditioned water for the control. The behavioral ethogram was based on the data of the chemical response trials. The majority of *M. turgidus* cercariae swam horizontally towards the covered side of the Petri dish for both lighted and unlighted trials, however a significantly higher percentage of cercariae swam horizontally towards the covered side of the Petri dish during the lighted trials suggesting that light affects the horizontal distribution of cercariae. *Microphallus turgidus* was also found to prefer the bottom of the water column in both lighted and unlighted trials. The behavior of the cercariae was not affected by shrimp-conditioned water. The ethogram showed that cercaria spent the largest percentage of time swimming on the bottom. The bottom dwelling behavior of *M. turgidus* cercariae corresponds to the demersal behavior of *P. pugio*. The cercariae do not appear to use chemical cues to find the host directly, rather the innate search pattern allows *M. turgidus* cercariae to find areas where the probability of encountering the primary host is highest. My findings show that the behavior of *M. turgidus* cercariae follows the demersal behavior of the grass shrimp *P. pugio* and may explain why this host is so frequently and heavily infected.

INDEX WORDS: *Microphallus turgidus*, Grass shrimp, Trematode, Cercariae, Parasite behavior

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by

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B.S., Randolph-Macon College, 2007

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Chapter I

Introduction

Microphallus turgidus (formerly *Carneophallus choanophallus*) is a digenetic trematode found in salt marshes from the coast of New Jersey to Louisiana (Heard, 1970). This parasite has a complex life cycle involving multiple hosts. The adult trematode matures and deposits eggs in the small intestine of a bird or mammal (Heard and Overstreet, 1983; Bridgman, 1969). The trematode eggs in the feces of this host are dispersed into the water, settle into the marsh sediment and are consumed by the first intermediate host, hydrobiid snails (Pung et al., 2009). The eggs hatch in the gut of the snail, releasing miracidia that develop into cercariae-producing sporocysts. Trematode cercariae emerge from the snail when induced by an increase in temperature (Fingerut et al., 2003). The cercariae leave the snail, then swim through the water column to find and infect the second intermediate host (Bridgman, 1969), primarily the grass shrimp *Palaemonetes pugio* and occasionally *P. vulgaris* (Pung et al., 2002). The life span of the free swimming cercariae of *Microphallus turgidus* can be up to 48 hr (Bridgman, 1969). In the grass shrimp the cercariae develop into metacercariae. The grass shrimp is then consumed by birds or mammals, allowing for the completion of the life cycle of the parasite (Heard and Overstreet, 1983).

Habitat Preference of Hydrobiid Snails

The most common hydrobiid snail in the marshes along the Skidaway River in Georgia is *Spurwinkia salsa* (Pung et al., 2008). *Spurwinkia salsa* represents greater than 90% of the hydrobiids in this locality and can be collected from marsh sediment and from the stems of salt marsh cord grass, *Spartina alterniflora*, but is seldom found in creek

beds (Pung et al., 2008). The distribution of trematode parasitized hydrobiids along the Skidaway River is variable and patchy. The hydrobiid snails in the marshes of the Skidaway River were found between 29-35 ppt (Pung et al., 2008). The location of trematode-infected hydrobiids and their proximity to grass shrimp second intermediate hosts is important. The greater the distance between the two hosts, the longer the ephemeral cercariae need to travel.

Habitat Preference of Grass Shrimp

The grass shrimp of the littoral zone of coastal Georgia, *P. pugio* and *P. vulgaris*, are selective in their microhabitat preferences. Both species of grass shrimp can be found within a salinity range of 10-35 ppt (Pung et al., 2002). However, lower salinities are less energetically favorable for *P. vulgaris* than *P. pugio* (Rowe, 2002). The highest prevalence of *M. turgidus* infected *P. pugio* occurred where salinity ranged from 20-29 ppt (Pung et al., 2002). Like most other shrimp species, *P. pugio* prefers resting on a substrate rather than free swimming (Gosner, 1978). Khan et al. (1995) tested substrate preferences among wood, mud, shell, and sand between grass shrimp species.

Palaemonetes vulgaris strongly preferred wood over the other substrates available in the experiment, while *P. pugio* preferred mud (Khan et al., 1995). Grass shrimp also prefer areas with macrophytic cover (Khan et al., 1997).

Host Finding Strategies: Geotaxis versus Phototaxis

Geotaxis and phototaxis need to be tested independently of each other, because a downward or upward swimming behavior could be attributed to either type of taxis. Geotaxis is identified by cercarial movement up and down in the absence of a light gradient and phototaxis can be differentiated from geotaxis by designing an experiment in

which cercariae are given a horizontal choice between light and dark. For example, the swimming behavior of *Maritrema subdolum* cercariae is attributed to positive geotaxis, because the species does not respond to light (Mouritsen, 2001). The Mouritsen (2001) experiment was performed by placing the light source above an open top light proof box.

Cercariae will stay near the bottom of the water column when they have a bottom-dwelling host (Combes et al., 1994). The cercaria-producing hydrobiid snail host of *M. turgidus* lives in marsh sediment (Pung et al., 2008) and the primary second intermediate host, the grass shrimp *P. pugio*, also prefer muddy substrates (Khan et al., 1995). Since both the first and second intermediate hosts of *M. turgidus* are benthic, the cercariae would also be expected to have benthic behavior.

Microphallus similis cercariae, like those of *M. turgidus*, have a demersal host; crabs that hide under rocks during the day. When the cercariae of *M. similis* encounter a light source in a Petri dish painted half black they move to the darker half. *M. similis* cercariae also prefer to swim on the bottom of the water column (McCarthy et al., 2002). Since the behavior of *M. similis* cercariae involves both geotaxis and phototaxis, *M. turgidus* should also be tested for both behaviors.

Host Finding Strategies: Photoreception

Many trematode cercariae are light sensitive. Examples include cercariae of trematodes, *Cryptocotyle lingua*, *Trichobilharzia ocellata*, and *Himasthla rhigedana* (Rees, 1975; Feiler and Haas, 1988; Fingerut et al., 2003). Phototaxis, whether towards or away from light, is dependent on the location of the next host of the cercaria (Combes et al., 1994). Cercariae of the trematode *Bunodera mediovitellata* swim horizontally toward light, but do not swim up. It was concluded that this behavior brings the parasite

into the lighted areas on the bottom of the water column, where the chance of encountering the bottom dwelling caddisfly larva host is highest (Kennedy, 1979).

While photoreceptors are used by some cercaria in finding their next host other sensory organs may play a role as well. *Cryptocotyle lingua* is an example of a cercaria that not only reacts to light but also reacts to chemical cues. In fact, fish extract cues may take precedence over phototaxis in *C. lingua* (Rees, 1975). When given both cues, *C. lingua* move away from a light source and toward the fish extract. However, cercariae of *C. lingua* are usually photopositive.

In trematode species that are phototactic, photoreceptors, often referred to as eye-spots, facilitate light perception by the cercariae (Haas, 1992). Eye-spots are identified by a dark coloration and contain pigmented granules. Both miracidia and cercariae can have photoreceptors. A miracidia is another stage of the trematode life cycle which infects mollusks (Kennedy, 1979). There is more photoreceptor variation in cercariae between species, than among miracidia (Rees, 1975). Interestingly, the miracidium of *Schistosoma mansoni* lacks eye-spots, but still has sensitivity to light. The miracidium of *S. mansoni* has large vacuoles with cilia, which might serve as photoreceptors (Roberts and Janovy, 2005). The cercariae of *M. turgidus* do not have apparent photoreceptors (Heard and Overstreet, 1983), but this does not necessarily mean they are not phototactic.

Cercariae of *Cryptocotyle lingua* have both pigmented and unpigmented photoreceptors (Rees, 1975). In cercariae with unpigmented photoreceptors the term photokinetic response is used to refer to a behavioral change due to light intensity (Combes et al., 1994). Examples of phototactic cercariae without apparent photoreceptors include the microphallids *Maritrema arenaria* and *Microphallus similis* (McCarthy et al.,

2002). Cercariae of the microphallid trematode, *Microphallus similis*, swim horizontally towards darker areas. It was concluded that this behavior would increase the probability of encountering the shore crab host (*Carcinus maenas*), that hides under rocks during the day (McCarthy et al., 2002). If phototaxis developed independently in multiple cercarial species it is logical that they would have structures analogous in function, but different in morphology.

Host Finding Strategies: Chemical Response

Another method of cercarial orientation is responding to chemical cues. Cercariae have two possible means of following a chemical gradient. For example, the cercariae of the trematode *Pseudechinoparyphium echinatum* will reverse course if the chemical components in the snail conditioned water is diluted (Haas, 1992). Swimming up a chemical gradient may enable a cercaria to find its next host efficiently.

Instead of searching for the host, some cercariae search for the microhabitat of the host and then rely on chance contact to initiate infection (Combes et al., 2002; Combes et al., 1994; Haas, 1992). A response to chemical cues is said to be lacking in cercariae that infect peripatetic (mobile) hosts (Combes et al., 2002; Haas, 1992), however, exceptions have been found. The cercariae of *Schistosoma mansoni* can orient to the chemical cue of linoleic acid mimicking human skin (Shiff and Graczyk, 1994). These parasites are attracted to human skin secretions, particularly the amino acid arginine (Granzer and Haas, 1986). Because the grass shrimp hosts of *M. turgidus* are motile but demersal (bottom dwelling), in behavior with a tendency to adhere to macrophytic vegetation and other objects (Khan et al., 1995; Khan et al., 1997), the possibility of a chemical response in *M. turgidus* is worth investigating.

Thesis Objectives

The goal of my thesis project was to examine the behavior and phototactic responses of *M. turgidus* cercariae to investigate whether or not the behavior of the cercaria reflects the habitat and behavior of its demersal grass shrimp host *P. pugio*. Once the longevity of *M. turgidus* cercariae was established my specific objectives were to (1) determine if *M. turgidus* cercariae respond to light, (2) determine the preferred elevation of *M. turgidus* in a water column in the presence and absence of light, and (3) test for a chemical response of the cercariae by establishing an ethogram for *M. turgidus* in untreated and grass shrimp-conditioned water.

Based on my own observations and those of McCarthy et al. (2002), I hypothesized that *M. turgidus* cercariae would have behavioral adaptations that put the parasite in close proximity to the primary second intermediate host, *P. pugio*. I predicted that (1) *M. turgidus* cercariae have photoreceptors. I also predicted that (2) cercariae would have a negative phototaxic response with (3) a random distribution in the absence of light. Due to the similarities between the behaviors and microhabitat preferences of the arthropod hosts of both *M. turgidus* and *M. similis*, I predicted that the cercariae would (4) prefer the bottom of the water column, because this would put them in closer proximity to their host. Finally, I predicted that (5) cercariae respond to chemical cues in grass shrimp-conditioned water.

Chapter II

Materials and Methods

Hydrobiid Snail Collection and Maintenance

Hydrobiids, primarily *Spurwinkia salsa*, were collected in high marsh sediment along the Skidaway River (31°58'31" N, 81°01'52" W) as previously described (Pung et al., 2008). The hydrobiid snail *Onobops jacksoni*, although less abundant, is also found at this collection site. A garden trowel was used to collect the upper most layer of sediment from an area approximately 1 m². Because hydrobiid snails are only a few mm in length (Pung et al., 2008), the sediment was brought to the laboratory and filtered through brass sieves of decreasing mesh size (2 mm, 710 µm, 500 µm, 150 µm; ASTM E-11 Specification, Fisher Scientific, Atlanta, Georgia) using tap water to concentrate the snails in a small volume of sediment. The remaining sediment was immediately placed in a 13.2 L rectangular plastic container filled with artificial brackish water [Instant Ocean, Aquarium Systems Inc., Mentor, Ohio, adjusted to 23 parts per thousand (ppt) salinity]. As the hydrobiid snails moved up the sides of the container over a period of 2 wk they were collected by aspiration. The snails were then screened for natural parasite infection as described below.

Immediately after collection, snails were housed in 19 L plastic buckets in artificial brackish water aerated with a sponge filter (Hydro-Sponge 1™, Aquarium Technology Inc., Decatur, Georgia) and fed twice weekly with a regular rotation between 3 marine microalgal concentrates (1 ml of Phytoplex, Kent Marine, Franklin, WI; 0.5 ml of Shellfish Diet, Reed Mariculture, Campbell, California; 0.5 ml of *Thalassiosira weissflogii*, Reed Mariculture). Fifty percent of the water was changed biweekly.

Infected snails were kept in brackish water in 650 ml plastic storage containers. Storage container lids had small slits for ventilation. While in the plastic storage containers water was changed weekly and snails were fed once a week with 0.4 ml of Shellfish Diet 1800™ diluted 1:10 in brackish water.

Screening Snails for Natural Parasite Infection and Acquisition of *Microphallus turgidus* Cercariae

Snails were screened for natural parasite infection and *Microphallus turgidus*-like cercariae (dark staining anterior penetration glands, oral stylet present, faintly staining posterior glands) as follows. Individual snails were placed in wells of culture plates (24 well, Costar, Becton Dickinson Co., Franklin Lakes, New Jersey) with 2 ml of artificial brackish water. The culture plates were placed in a 30°C incubator on a 12 hr light: dark cycle. Snails were fed (15µl Shellfish Diet etc.) and the water changed twice a week. Wells were checked for cercariae using an inverted microscope twice weekly for 3 weeks. Snails infected with nematodes or with trematodes not fitting the description of *M. turgidus* were discarded. Snails shedding microphallid cercariae were retained and the cercariae were then stained with neutral red dye (0.01% neutral red in 0.75% NaCl). If the cercariae fit the description of *M. turgidus* then the snail was used as a source of *M. turgidus*-like cercariae for preliminary studies and the cercaria longevity experiment. Snails that did not shed cercariae were considered uninfected. These snails were then infected with *M. turgidus* in the laboratory by Dr. Pung as previously described (Pung et al, 2009). All experiments, excluding the longevity experiment, used *M. turgidus* cercariae obtained from laboratory-infected snails.

Serum-coating of Glass and Plasticware

All plastic and glass containers used in the experiments were coated with bovine calf serum (GIBCO, Grand Island, New York) diluted in artificial brackish water (20 ppt) to prevent the cercariae from adhering to the plastic or glass. For example, Petri dishes (60 x 15 mm, polystyrene, Falcon, Becton-Dickinson Co., Franklin Lakes, New Jersey) were treated as follows. Forty-five μ l of the serum and approximately 10 ml of artificial brackish water were placed in the Petri dish (0.45 % serum). Five minutes later the dish was rinsed 3 times with artificial brackish water. Pipettes and pipette tips were coated with undiluted serum.

Cercariae Longevity Experiment

The longevity of the cercariae after shedding from the snail host was determined to limit the effect of age on the behavior studies. To do so, *Microphallus turgidus*-like cercariae were obtained by placing 1 or 2 naturally infected snails into a serum-coated 60 x 15 mm Petri dish containing 10 ml of 20 ppt brackish water. These Petri dishes were placed in the 30°C incubator in the evening to induce shedding of cercariae. The Petri dish was removed from the incubator after 1 hr and cercariae were transferred to a serum-coated 24-well, flat-bottom tissue culture plate (1 cercaria/well) containing 2 ml of brackish water and incubated overnight at room temperature. The following morning the cercariae were observed for mortality. Mortality was defined as 30 seconds without movement of body or tail. Cercariae were then checked hourly for mortality until they were at least 24 hr old. Scans of the culture plate were made with an inverted dissecting microscope. Approximately 15 min were needed to scan all 24 wells in the culture plate and scans were made between the 15 min to approximately the 30 min mark of each hour.

For the first replicate cercariae were chosen at random (24 total). The ratio (cercariae per snail) is unknown for the first replicate, because the 2 snails were placed together. The ratio of the number of cercariae used from an individual snail was calculated for the second replicate (23 total, 9 and 14 respectively from each snail).

Cercaria Phototaxis Experiment

For each trial, 2 laboratory-infected snails were placed in serum-coated, 60 x 15mm Petri dishes containing 10 ml of brackish water (1 snail/dish). The Petri dishes were incubated at 30°C to induce cercarial shedding. After 2 hr, the plates were removed from the incubator and checked for the presence of cercariae. Twenty cercariae from each snail were then transferred to each of 2 fresh, serum coated 60 x 15 mm Petri dishes containing 10 ml of 20 ppt brackish water. To transfer the cercariae, a serum-coated plastic micropipette tip was used. The micropipettor (P20 Pipetman, Gilson, Middleton, Wisconsin) was set to the lowest setting to prevent the liquid from being drawn fully into the tip. This would create an air bubble below the sample being transferred, increasing the likelihood the cercaria would become trapped in the pipette tip. Cercariae were placed at random locations throughout the dishes. Each Petri dish was placed on the dissecting microscope stage, in the dark and under a cardboard box, for 30 minutes. This was done to establish an initial random distribution. After this adjustment period, the cardboard box was no longer used and black construction paper was placed beneath half the Petri dish. An additional piece of construction paper was folded into the bottom piece to cover the sides of the dish without using tape or glue. Black plastic was used to cover the top half of the Petri dish on the same side as the paper. The orientation of the dark half was rotated 90° each trial. The rotation was done to ensure that the cercariae were not

predisposed to swim in one direction over the other without orienting to the covering (i.e. always swimming left or right). White LED lights were placed on each side of the microscope stage to illuminate the Petri dish from above. The 2 light sources were adjusted so that the shadow was cast in the center of the Petri dish. The room lights were then turned off so that the LEDs were the only light source and cercariae were left undisturbed for a 30 min adjustment period to allow taxis to occur. The 30 min adjustment period includes the time needed to position the lights and set the black covering in place. To limit the effect of a light source while placing the black covering, a red plastic filter was placed over a LED flashlight. [Although the effect of red light on *M. turgidus* is unknown, a similar technique with a dim red safelight was used during a darkroom experiment with *Trichobilharzia ocellata* cercariae and it was found red light did not affect cercariae behavior (Feiler and Haas, 1988).] After the initial 30 min, scans were made twice per hour for 2 hr to count the number of cercariae in the lighted half of the dish. The first scan was used for data analysis. To ensure that all cercariae were counted, scanning started at the perimeter of the dish using the edge of the black half as a guide. The Petri dish was scanned side to side at 20x. Three scans were needed to view the entire clear half. The dish was moved slowly by hand during scanning. Next, the opaque plastic cover was removed from each dish and the total number of cercariae in the dish were tallied. As each cercaria was counted, it was removed and placed in a discard container to ensure that none were counted more than once. After the last cercaria was found, the Petri dish was scanned for an additional 5 minutes to ensure that none of the cercariae were missed. The experimental control for the phototaxis study used exactly the same methods as described above except that the cercariae were exposed to light only

when the plate was set up and during the scan sampling. Also, for the experimental control the box was retained throughout the entire trial to insure total darkness except during viewing. A total of 10 experimental and 10 control trials were performed for this experiment.

Ethogram of Cercariae Behavior in Normal and in Shrimp-Conditioned Water

For this experiment, control water was defined as 20 ppt artificial brackish water. Shrimp-conditioned water was obtained by maintaining grass shrimp, *P. pugio*, in 10 L of 20 ppt artificial brackish water for 72 hr. Cercariae were obtained from lab-infected snails. Next, one cercaria was transferred to a serum-coated 60 x 15 mm Petri dish containing 10 ml of normal or shrimp conditioned water. The behavior and the location of the cercaria was recorded using a voice tape recorder. The cercaria was viewed with a mirrored dissecting microscope and the following behaviors were recorded: bottom of water column at rest, top of water column at rest, bottom of water column swimming, top of water column swimming, bottom crawl, stuck to bottom, mid-water column swim, mid-water column rest, swimming up, swimming down, and sinking. Each of the cercaria used was viewed for 9 min 30 sec after a 30 second acclimation period. The horizontal direction and distance of travel was determined by using 1 cm² boxes that were drawn with indelible marker on the outside bottom of the Petri dish. The number of boxes entered by the cercariae during the observation period was recorded. A total of 36 trials were performed for cercariae in control and conditioned water (18 trials each). All trials were performed blind (i.e., the observer did not know whether control or conditioned water was being used). The containers were code labeled A or B, and then filled by another individual.

Cercaria Geotaxis Experiment

Microphallus turgidus cercariae were obtained from laboratory-infected snails for the phototaxis experiment. A serum-coated 10 ml glass graduated cylinder (diameter = 17 mm, height = 140 mm, Pyrex No. 3025, Lowell, MA) was filled with 9 ml of 20 ppt artificial brackish water. Thirty cercariae were transferred to the graduated cylinder which was then fitted with a black tube and black square top with a 1 cm² opening to ensure that light could enter only from above. After a 60 min adjustment period cercariae were transferred in 3 ml increments to 3 separate serum-treated Petri dishes. 3 ml of water was equivalent to 27 mm in height. The Petri dishes were designated top, middle, and bottom. Finally, the cercariae were counted by removal. Cercariae were removed one at a time using a P20 micropipet and this final count was used in data analysis. To make sure that insertion of the 10 ml pipette did not disturb the water and potentially influence the position of the cercariae, methylene blue crystals were placed in the graduated cylinder prior to the first trial. The methylene blue did not move during pipetting, suggesting that cercariae distribution would not be influenced. The geotaxis experiment was repeated 6 times for both the lighted and unlighted trials.

Statistical Analysis

For all experiments a power analysis was performed based on initial data to determine the number of replicates necessary (JMP Software, Cary, NC). The longevity study used scan sampling, while the chemical response study used focal continuous sampling methods with a t-test (bottom swim) and Wilcoxon rank-sum tests (all other behaviors) for statistical analysis. The non-parametric t-test equivalent, Wilcoxon rank-sum test was chosen for the other behaviors, because the data was not normally

distributed. A roaming index, based on the number of boxes travelled in the ethogram studies, was analyzed with a Wilcoxon rank-sum test. For the phototaxis experiment, a Chi-square analysis was used and the significance was evaluated using a method for combining probabilities from independent tests (Sokal and Rohlf, 1998). Combining probabilities from independent tests was used, because the total number of cercariae varied slightly between trials. The statistical significance is based on the $-2\sum \ln P$ and a statistical table was used to determine the critical values (Rohlf and Sokal, 1981). A Wilcoxon rank-sum test was also used in the phototaxis experiment to determine if there was a statistical difference between light and dark trials. The swimming depth experiment was analyzed with replicated goodness-of-fit G-tests (Sokal and Rohlf, 1998). The df and G-values were used to calculate P for each depth trial. After the sums were calculated the number of cercariae in the bottom, middle, and top portions of the graduated cylinder were compared.

Chapter III

Results

Longevity of *Microphallus turgidus*-like Cercariae

Microphallid cercariae from naturally infected snails were monitored to determine their life span in the laboratory. In both trials there was no cercariae mortality (ie., no movement for 30 sec) within the first 15 hr post shedding (Fig. 1). A mortality of greater than 50% was observed between 24-25 hr. One hundred percent mortality was not observed during the duration of either replicate, but by hour 24 of trial #1 and hour 25 of trial #2 most of the remaining live cercariae, though active, were no longer free swimming.

Ethogram Results

The behavior of *M. turgidus* cercariae was recorded and used to construct an ethogram. *Microphallus turgidus* cercariae were observed to have 11 distinct behaviors. They were bottom swim, swimming up, swimming down, bottom rest, top swim, top rest, sinking, stuck, mid-water column swim, mid-water column rest, and bottom crawl. Definitions of behaviors and pictorial examples are found in Figures 2 and 3. Cercariae spent most of their time swimming on the bottom or resting on the bottom (Fig. 4).

Chemical Response Trials

Shrimp-conditioned water had no effect on the behavior of *M. turgidus* cercariae (Table I, Fig.4). A roaming index (rudimentary distance travelled) was calculated. The cercariae in control water did not travel significantly farther than the cercariae in the shrimp-conditioned water (Fig. 5, Wilcoxon rank-sum, $P = 0.157$).

Phototaxis Trials

When cercariae in the uncovered half of the Petri dish were exposed to white light the percent of cercariae differed from the expected 50:50 ratio ($-2\sum \ln P$ of 122.4 > critical value 37.6). The same statistical analysis was performed on the 10 control replicates that were kept in the dark. Similarly, when not exposed to light, the null hypothesis of a 50:50 ratio was rejected ($-2\sum \ln P$ of 55.0 > critical value 37.6). In both cases more cercariae were found in the covered half of the dish. However, the mean percentage of cercariae in the uncovered side of the Petri dish was significantly lower in the dishes exposed to light than in those kept in the dark (Fig. 6, Wilcoxon rank-sum, $P = 0.007$)

Geotaxis Trials

When placed either in the light or the dark, cercariae in a graduated cylinder were not evenly distributed after 60 min [(Lighted trials, replicated goodness-of-fit G-test, summed $G=317.9$, 12 df, $P < 0.001$, Table II)(Dark trials, replicated goodness-of-fit G-test, summed $G= 278.6$, 12 df, $P < 0.001$, Table III)]. Whether exposed to light or kept in the dark, nearly all of the cercariae were found in the bottom third of the water column (Fig. 7).

Chapter IV

Discussion

Longevity of *Microphallus turgidus*-like Cercariae

Since no mortality was observed from 0 hr to 15 hr in my longevity trials with *M. turgidus*-like cercariae, I decided to use cercaria no more than 12 hr old in my experiments. Trematode cercariae are generally short lived (McCarthy, 1999; Mouritsen et al., 1997). For example, *M. turgidus* cercariae were previously reported to live up to 48 hr (Bridgman, 1969). Because the *M. turgidus*-like cercariae are short lived, it is possible that, in the wild, they are released in close proximity to the grass shrimp host and that they have behaviors that permit them to find a potential host or host microhabitat quickly.

Cercariae from wild-caught snails were used for the longevity study because parasites from laboratory-infected snails were not available at the time. Unfortunately, *M. turgidus* cercariae are morphologically indistinguishable from the cercariae of certain other microphallids found in the wild. For example, *Microphallus basodactylophallus*, a parasite of blue crabs (Heard and Overstreet, 1983), is common in the southeast and easily confused with *M. turgidus*. Consequently, I cannot be certain that the cercariae used in the longevity study were actually *M. turgidus* and not another closely related microphallid trematode. However, the results of my longevity study are in agreement with those of other investigators (Bridgeman, 1969). Also, no cercaria mortality was observed in any of my subsequent studies, all which were performed using *M. turgidus* cercariae from laboratory-infected snails.

Effect of Light on the Behavior of *Microphallus turgidus* Cercariae

Cercariae with sensitivity to light find their next host by using either pigmented

eye-spots (Haas, 1992), unpigmented eye-spots (Roberts and Janovy, 2005) or both (Rees, 1975). Cercariae swim towards or away from light depending on the location of the next host (Combes et al., 1994). In my study, the majority of *M. turgidus* cercariae swam horizontally towards the covered side of the Petri plate in both the lighted and the unlighted trials. However, a significantly higher percentage of cercariae swam horizontally towards the covered side of the plate in the lighted trials. One of the second intermediate hosts of *M. turgidus* is the grass shrimp *P. pugio* (Bridgman, 1969). Like the crab host of *M. similis*, *P. pugio* naturally seeks areas that provide cover (Khan et al., 1997). My results indicate that *M. turgidus* cercariae, like those of *M. similis* (McCarthy et al., 2002), might swim horizontally towards the dark to increase the probability of finding the demersal host of the parasite.

I expected that the *M. turgidus* cercariae not exposed to light would be randomly distributed after the dark trials and cannot explain why many of them swam under the covered side of the Petri dish. It was impossible to set up this experiment without initially exposing the parasite to some light. In a study on host finding behavior in cercariae of the trematode *Trichobilharzia ocellata*, the intensity and duration of the stimulus was found to be an important factor in the percentage of cercariae that responded to it (Feiler and Haas, 1988). Perhaps *M. turgidus* cercariae have residual memory for light or dark and, even after a brief exposure to light, were able to orient to cover.

Ethogram Analysis

I observed that *M. turgidus* cercariae spend most of their time swimming or resting on the bottom of the water column. This may be accounted for by the fact that *P. pugio*, the second intermediate host, is benthic. In addition I observed 2 distinct

behavioral patterns during the ethogram study. They were horizontal movement (bottom swim) and vertical movement (swimming down, swimming up, and sinking). Individual cercaria seemed to favor one over the other. For example a cercaria might swim up, top rest, sink to the bottom, bottom swim, and swim up again in a cyclic pattern. Other cercariae would bottom swim without the cyclic vertical pattern. The use of multiple behavioral strategies by *M. turgidus* cercariae is logical because, despite a predictable host environment, preferred microhabitats might be patchy in distribution.

Effect of Shrimp Conditioned water on Cercariae Behavior

To test whether or not *M. turgidus* cercariae respond to grass shrimp secretions, I placed cercariae in shrimp conditioned and control water. I predicted that, if the parasite responded to grass shrimp secretions its behavior would change when placed in shrimp-conditioned water. However, shrimp conditioned water did not significantly change the behavior of the cercariae. Most attempts to find a cercarial response to a nearby host are unsuccessful (Combes et al., 2004). Cercariae are believed to generally respond to chemicals only at a short distance (Combes et al., 2002). My results support the findings of other studies that cercariae often do not use chemical cues to find mobile hosts (Combes et al., 2002; Haas, 1992).

The chemical content of the shrimp-conditioned water was not determined. Chemicals known to be produced by grass shrimp include sex pheromones. Grass shrimp sex pheromones are not dispersed into the water. They are insoluble and are known as contact sex pheromones (Caskey and Bauer, 2005). Urine is an example of a dispersed, water soluble chemical secreted by crustaceans. Some crustaceans, Caribbean spiny lobsters (*Panulirus argus*) for example, use urine as a form of communication (Shabani et

al., 2009). If the cercariae of *M. turgidus* changed behaviors when exposed to shrimp conditioned water this would indicate a response to a chemical cue. Future experiments should test the response of *M. turgidus* to directly contacted chemicals.

Preferred Depth of *Microphallus turgidus* Cercariae

I found that *Microphallus turgidus* cercariae were more likely to be located at the bottom 3 ml of the graduated cylinder than in the middle or top of the water column. This was observed when the cercariae were exposed to overhead light and when kept in the dark. This observation is supported by the fact that, in the ethogram study, the most frequently observed behaviors were bottom swim and bottom rest. Similarly, the cercariae of the trematode *Bunodera mediovitellata*, which also has a benthic second intermediate host (caddisfly larva), were found to not move more than 3 to 5 mm from the bottom, even in the presence of overhead illumination (Kennedy, 1979). Cercariae of *M. similis*, infect shore crabs, and are found at the bottom of the water column during lighted trials, but maintain an even distribution throughout the water column during unlighted trials (McCarthy et al., 2002). In contrast, *M. turgidus* cercariae stay at the bottom of the water column when in the dark.

The precise depth of *M. turgidus* cercaria could be better determined by using a dissecting microscope placed horizontally (Mouritsen, 2001; Feiler and Haas, 1988). This method would allow the cercariae to be viewed in situ, rather than employing the pipetting technique I used to assess the distribution of the parasites. The pipetting method indicates only that the parasites were in the bottom third of the water column, not their exact height.

Future Studies

Future studies could include in situ cage trials where *P. vulgaris* is moved to areas in the field where *P. pugio* is located. If *P. vulgaris* became more heavily infected this would indicate *M. turgidus* is less prevalent or absent in the preferred microhabitat of *P. vulgaris*. This could be further supported by a lab study where *P. vulgaris* is kept in small cages in a larger tank at varying depths. Cercariae could be released into the tank and the grass shrimp could be evaluated for prevalence of cysts.

The interaction of *M. turgidus* with light should be further investigated to verify that they are reacting to the light itself, not the heat produced by the light source. Placing the Petri dish in a water bath could further limit the possible effect of temperature by increasing water volume to absorb heat without increasing the swimming area of the cercariae. The behavioral reaction of grass shrimp to light and dark should also be worthy of further testing. Additional experiments might also include tactile chemical responses in *M. turgidus*. The cercariae did not respond to soluble chemicals in shrimp conditioned water, but that does not mean the parasites are incapable of reacting when they contact the intended host species.

Conclusion

My study provides new information about host-parasite relationships and reveals how *M. turgidus* find its hosts. My work also provides clues that suggest why some hosts are more likely to be infected than others. The grass shrimp *P. pugio* and *P. vulgaris* are both second intermediate hosts of *M. turgidus*. However, *P. pugio* is often more heavily infected than *P. vulgaris* (Pung et al., 2002). It is not known why these two species differ in prevalence and intensity of *M. turgidus* metacercariae. However these two sympatric

species of grass shrimp differ in their preferred microhabitat. The cumulative data of this thesis indicates that *M. turgidus* would most likely be present and actively swimming in the muddy bottom areas where *P. pugio*, but not *P. vulgaris*, typically lives.

When the results of these experiments are looked at as a whole, the interaction between *M. turgidus* and its host becomes apparent. I found that the cercariae did not respond to shrimp conditioned water, which suggests that the cercariae may not use chemical cues to find the next host. The findings of the geotaxis study suggest that the cercariae are, instead, staying within the demersal microhabitat where the grass shrimp *P. pugio* has the highest probability of being found. *Palaemonetes vulgaris*, on the other hand, is less likely to be exposed to *M. turgidus* cercariae at their preferred microhabitat. If *M. turgidus* cercariae had positive phototaxis or responded to shrimp-secreted chemical cues in the water, *P. vulgaris* might have a higher prevalence and intensity of infection. The parasite was negatively phototactic. This suggests that the parasite is searching for a particular microhabitat consistent with that of its host.

Some *M. turgidus* cercaria chose to spend more time swimming up vertically or swimming horizontally compared to other cercariae, which suggests that behavior varies between individual cercariae. The parasite swims horizontally in response to light, but not vertically, suggesting that they swim along the bottom to shaded areas where grass shrimp are likely to be found. This behavior was confirmed by the results of my behavior ethogram of the parasite in which the cercariae spent most of their time swimming or resting on the bottom. The results of the phototaxis study are fascinating, because the cercariae appear to respond to even a very low level cue of some kind. The behavioral

mechanism might involve the light itself or the small amount of heat produced by the LED light source.

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Table I. Ethogram of the behavior of *Microphallus turgidus* cercariae in both control and shrimp-conditioned brackish water. Behaviors were compared using Student's t-test or Wilcoxon rank-sum tests. Percents (%) equal percentage of time spent in each activity. (n= 1 cercaria/ trial, 18 trials per treatment group)

Behavior	Time spent in activity in sec \pm SD (%)		
	Control water	Shrimp-conditioned water	P value
Bottom swim	363.1 \pm 143.9 (63.7%)	380.0 \pm 136.7 (66.7%)	0.7
Bottom Rest	61.9 \pm 53.1 (10.9%)	70.9 \pm 64.8 (12.5%)	0.8
Swimming up	37.0 \pm 49.7 (6.5%)	38.8 \pm 49.6 (6.8%)	0.7
Swimming down	20.4 \pm 26.8 (3.6%)	17.8 \pm 27.5 (3.1%)	0.9
Top rest	29.6 \pm 40.2 (5.2%)	22.2 \pm 34.4 (3.9%)	0.9
Sinking	33.2 \pm 56.8 (5.8%)	20.8 \pm 41.0 (3.7%)	0.6
Bottom crawl	1.1 \pm 4.5 (0.2%)	0.0 \pm 0.0 (0%)	0.0
Mid water column rest	0.8 \pm 2.8 (0.2%)	0.0 \pm 0.0 (0%)	0.0
Mid water column swim	1.8 \pm 7.8 (0.3%)	0.5 \pm 2.1 (0.1%)	1.0
Top swim	8.3 \pm 18.6 (1.5%)	16.6 \pm 33.3 (2.7%)	0.4
Stuck	12.7 \pm 28.6 (2.2%)	2.3 \pm 5.8 (0.4%)	0.3

Table II. Geotaxis replicates for *Microphallus turgidus* cercariae in a graduated cylinder exposed to light. Replicated goodness-of-fit tests found that all replicates and the sum of replicates were statistically significant from the expected 1:1:1 ratio. (target n= 30, actual n ranged from 28-30, 6 trials)

Replicate	df	G	P
1	2	55.02	<0.001
2	2	57.15	<0.001
3	2	42.36	<0.001
4	2	42.45	<0.001
5	2	55.02	<0.001
6	2	65.92	<0.001
Total	12	317.92	<0.001

Table III. Geotaxis replicates for *Microphallus turgidus* cercariae in a graduated cylinder not exposed to light. Replicated goodness-of-fit tests found that all replicates and the sum of replicates were statistically significant from the expected 1:1:1 ratio. (target n= 30, actual n ranged from 24-29, 6 trials)

Replicate	df	G	P
1	2	40.25	<0.001
2	2	63.72	<0.001
3	2	11.56	<0.005
4	2	44.42	<0.001
5	2	57.13	<0.001
6	2	61.52	<0.001
Total	12	278.61	<0.001

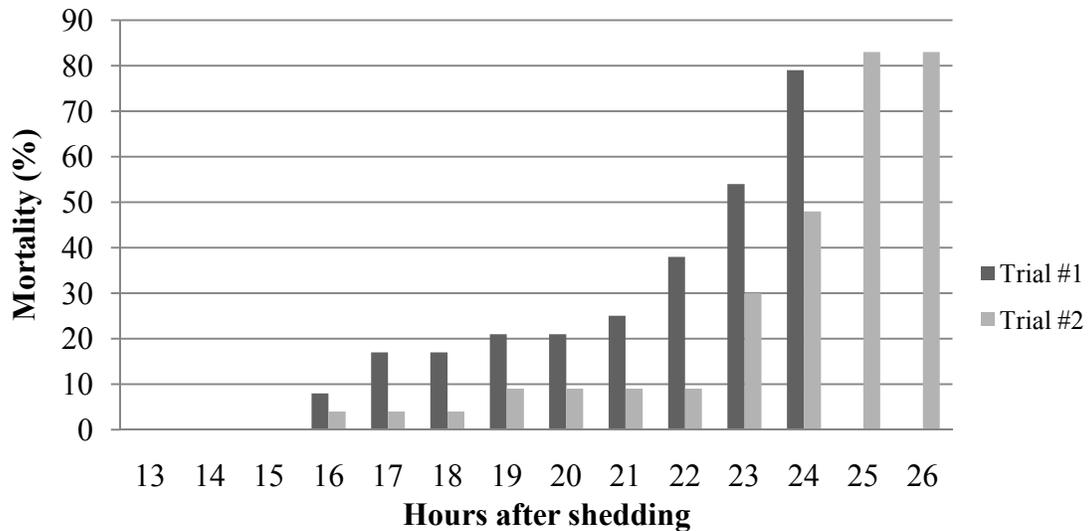


Figure 1. Longevity of *Microphallus turgidus*-like cercariae in the laboratory. Cercariae were obtained from parasitized snails (*Spurwinkia salsa* and *Onobops jacksoni*) screened for natural microphallid infection using light microscopy and neutral red staining of cercariae. (n=23-24 cercariae/trial, 2 trials)

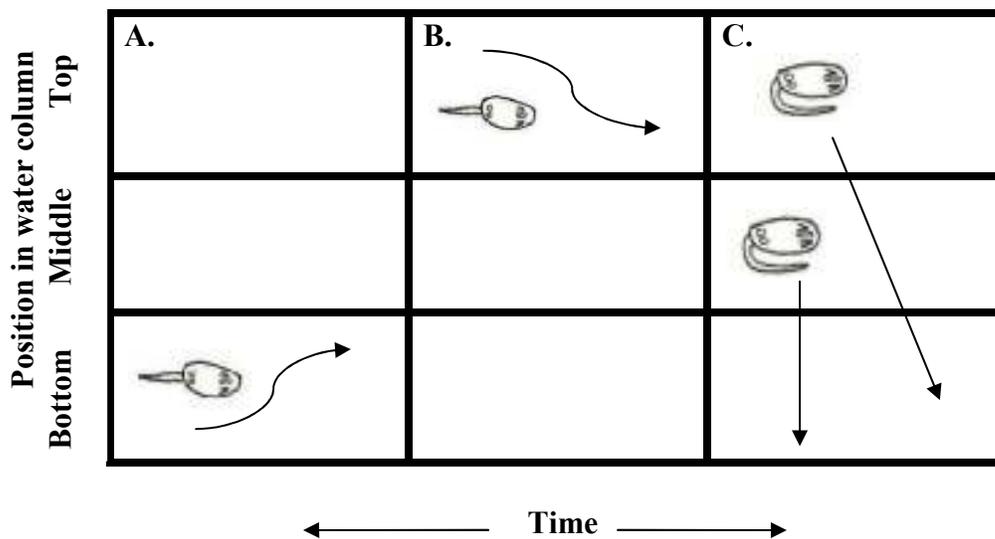


Figure 2. Cercariae behaviors. (A) Bottom Swim. Cercaria swims along the bottom, moving slightly up or down. Once the cercaria swims up and becomes out of focus of the bottom the cercaria is in the middle of the water column. (B) Top Swim. Cercaria swims in the top of the water column, moving slightly up or down. Once the cercaria swims down and becomes out of focus of the surface the cercaria is in the middle of the water column. (C) Sinking. The cercaria rests by tucking the tail under the body. Both the body and tail remain stationary while the cercaria sinks towards the bottom. The cercaria can sink straight down or sink at an angle. This behavior can occur at any level of the water column.

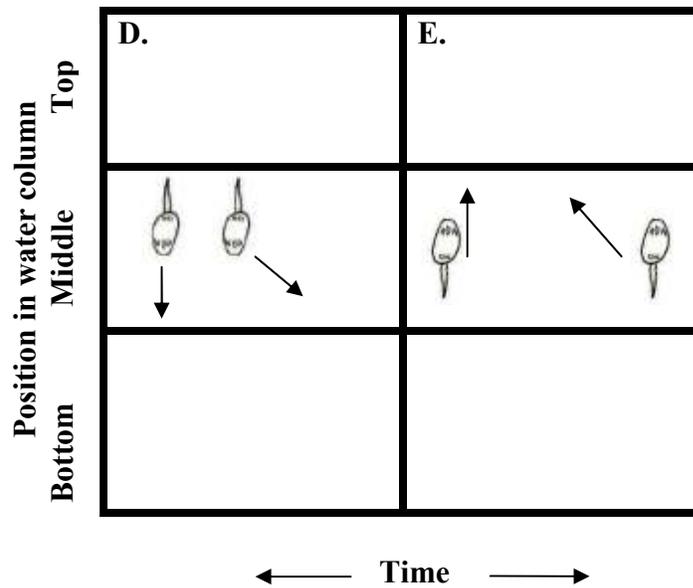


Figure 3. Cercariae behaviors continued. (D) Swimming Down. The cercaria swims directly down or swims down at an angle. The cercaria is followed by adjusting the focus. The “swimming down” behavior can extend until the cercaria reaches the plastic bottom of the Petri dish. (E) Swimming Up. The cercaria swims directly up or swims up at an angle. The cercaria is followed up by adjusting the focus. The “swimming up” behavior can extend into the top of the water column until the cercaria reaches the surface.

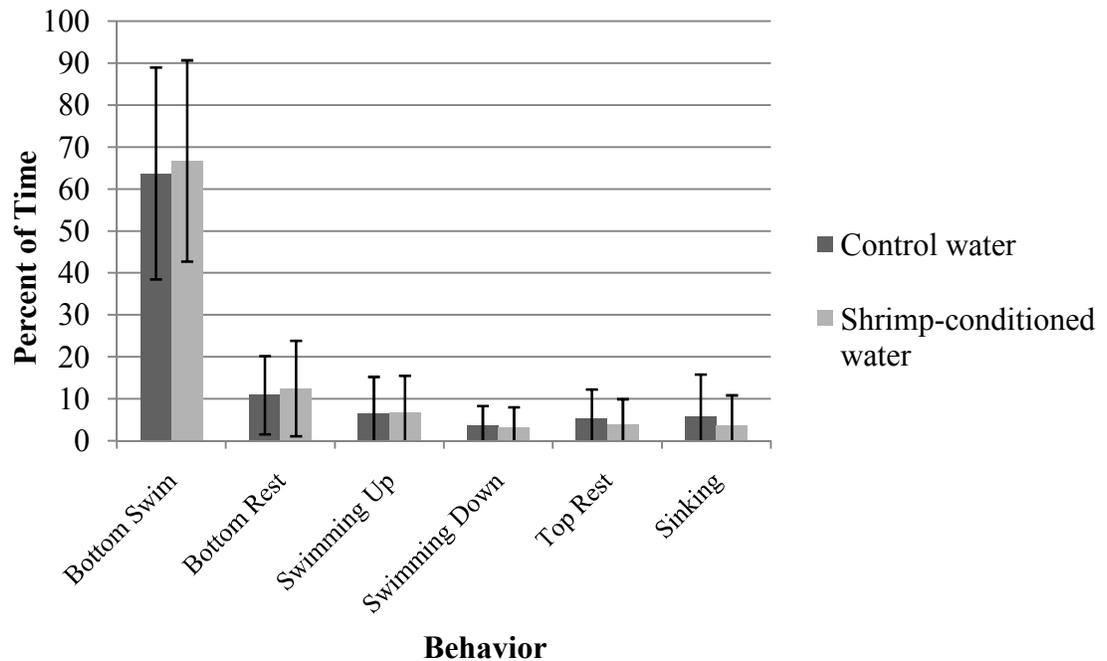


Figure 4. Average percent of time *Microphallus turgidus* cercariae spent in each behavioral state higher than 3%. No significant difference between the unconditioned and shrimp conditioned trials for all 6 behaviors shown.

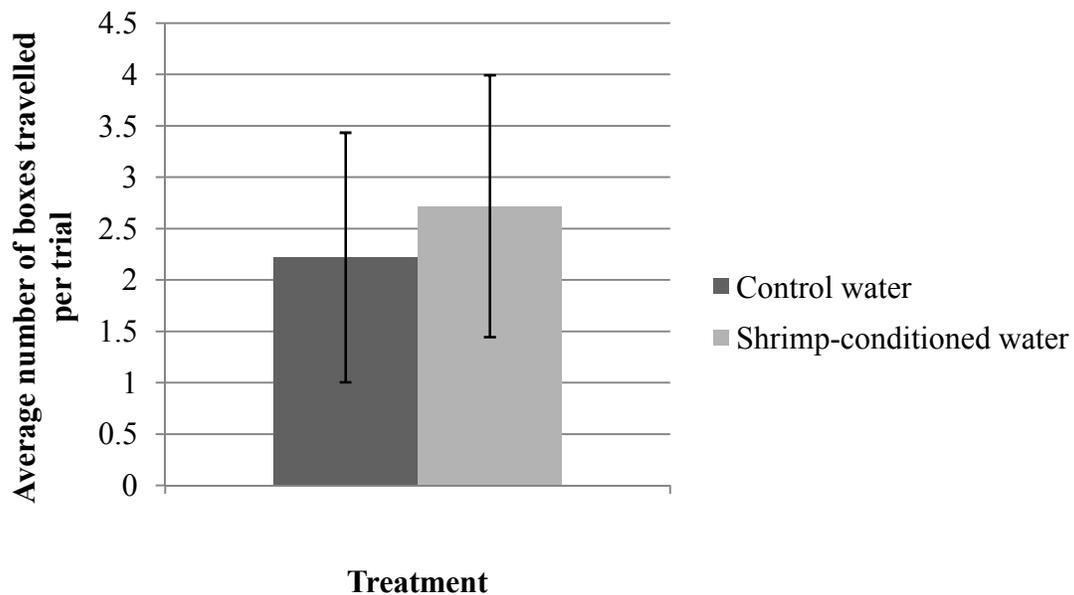


Figure 5. Average number of boxes travelled by *Microphallus turgidus* cercariae per trial for shrimp-conditioned and control water. No significant difference was found for either roaming index. Control water: mean (2.2±1.2 SD). Shrimp-conditioned water: mean (2.7±1.3 SD).

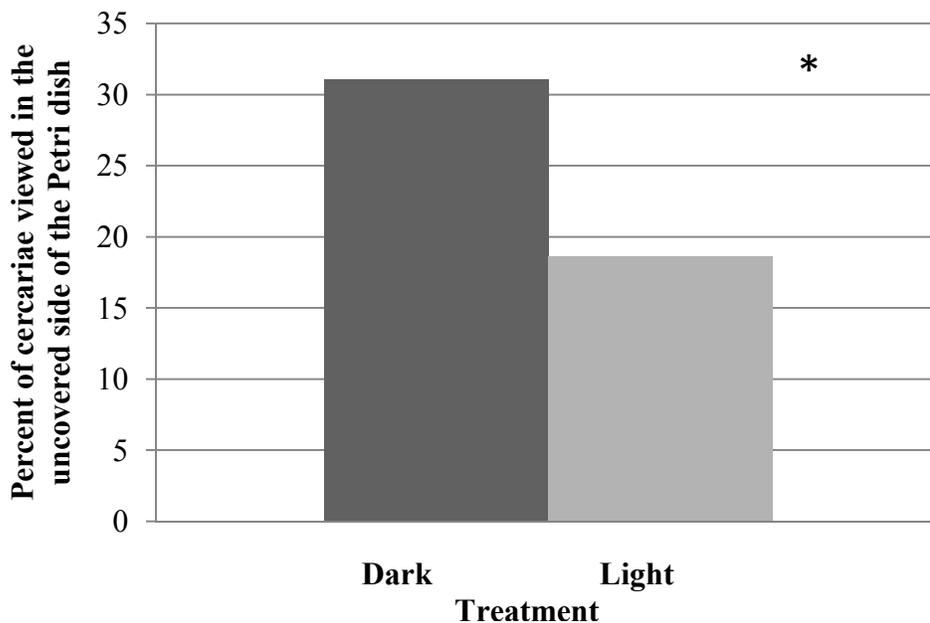


Figure 6. When exposed to white LED light, less *Microphallus turgidus* cercariae are viewed in the uncovered half than during unlighted conditions. Less cercariae viewed in the uncovered half shows that more cercariae are seeking cover. Asterisk indicates that lighted trials significantly differed from unlighted trials ($P = 0.007$). Dark: mean (31.09 ± 10.5 SD), (target $n = 20$, actual n ranged from 18-22, 10 trials). Light: mean (18.62 ± 13.5 SD), (target $n = 20$, actual n ranged from 19-25, 10 trials).

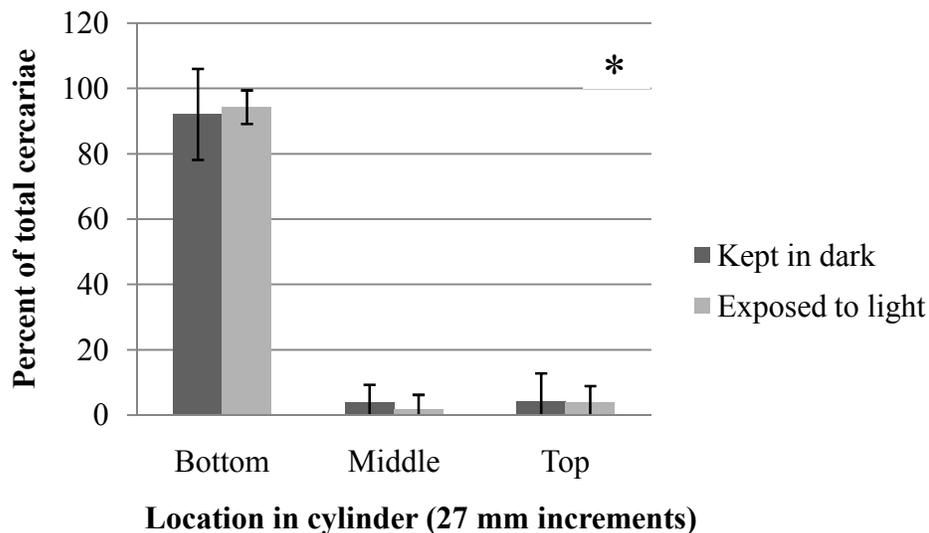


Figure 7. When allowed to swim in a graduated cylinder most *Microphallus turgidus* cercariae remain at or near the bottom. Percent of total cercariae tallied based on location in cylinder. Asterisk indicates significant difference between groups (Dark and light trials $P < 0.001$ between bottom, middle, and top).