Chapter 8

Cercariae Of Digenetic Trematodes: Use In Laboratory Investigations

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Introduction

Digenetic Trematodes - General Background

Digenetic trematodes (Phylum Platyhelminthes, Class Trematoda, Subclass Digenea) are responsible for a number of disease conditions in humans (e.g., schistosomiasis, fascioliasis, swimmer's itch, etc.). Trematodes are flatworm parasites which are found as adults in a variety of vertebrate definitive hosts. Eggs released from adult worms enter the external environment by a number of routes (e.g., feces, sputum, urine, etc.) depending on the species. The eggs are either eaten by snails or hatch and release a short-lived miracidium which penetrates a snail. Within this snail first intermediate host, the process of polyembryony occurs whereby several different intramolluscan larval stages (i.e., sporocyst, redia, and cercaria) are formed by asexual reproduction. This asexual proliferation results in the production of numerous genetic clones of the original miracidium. The cercaria is a non-feeding, short-lived transfer stage which is often an active swimmer. It breaks out of the sporocyst or redia stage in which it was formed and emerges from the snail usually in an aquatic environment. Depending on the digenean species, the cercaria either: (1) actively penetrates a second intermediate or definitive host, (2) is consumed by a second intermediate or definitive host, or (3) encysts on external matter such as vegetation. The cercaria may develop into an encysted metacercaria stage or directly into an adult worm depending on the species. If the former scenario occurs, then transmission to the

vertebrate definitive host is by the food chain. Several recent books have been published on trematodes (Fried and Graczyk 1997; Smyth and Halton 1983) which provide considerable detail on a number of topics related to this group including control, transmission, physiology, structure, behavior, and biochemistry.

Study of digenean species which are non-human pathogens provides safe models for an array of basic investigations. While commercially available from some biological supply houses, snails infected with cercariae are usually common in streams, ponds, reservoirs, rivers, and lakes. Local snail populations are ideal for field studies assessing seasonal prevalence of infection and associated recruitment/loss of trematodes. The effect of exogenous environmental triggers including light, oxygen, pH, and temperature on the timing and pattern of cercarial release from the snail host can easily be conducted within the laboratory. Post-emergence studies evaluating similar factors affecting cercarial longevity, infectivity, and swimming behavior are also possible. The results of these studies are open-ended and dependent on the digenean species in question as well as the independent variable(s) incorporated into the design. Further details regarding such studies are summarized on the Research Link 2000 website (http://www.berea.edu/BIO/CUR/mainpage.html) under LAB CORE: Cercaria Model Systems And Techniques.

Objectives

Two general experiments will be conducted with the unusually large cercaria of *Proterometra macrostoma*. Students will assess the effect of: (1) different wavelengths of light on the vertical swimming burst distance of this cercaria and (2) pH and pepsin on the emergence of the *P. macrostoma* distome (pre-adult) from its cercarial tail.

Time Considerations and Difficulty

Plan to spend one lab period with your class collecting snails and screening them for infections. This may be bypassed if the instructor elects to collect and screen without class participation. Allow 2 hours for setting up the experiments. The laboratory should require a maximum of 3 hours (i.e., approximately 2 hours for the light wavelength experiment and 1 hour for the distome emergence experiment), and is suitable for entry level biology classes.

Materials

Snail Maintenance Supplies

Reported natural sources for snails infected with *Proterometra macrostoma*:

1. Alabama - Cahaba River (Host: *Goniobasis opaca*)

2. Illinois - Des Plaines River near Evanston (Host: *Goniobasis livescens correcta*); Salt Fork Branch of the Vermilion River at Homer Park near Homer, Illinois (Host: *Goniobasis livescens*)

3. Indiana - Clear Creek approximately 10 km south of Bloomington (Host: *Elimia livescens*)

4. Kentucky - The following three sites are near Lexington - South Elkhorn Creek; North Elkhorn Creek at intersection with KY State Road 922 and Newtown Pike in Scott County; Cane Run Creek where it intersects with US HWY 460 in Scott County (Host: *Elimia* (*Goniobasis) semicarinata* at all three sites)

5. Michigan - Carp Lake River where it intersects Michigan State Road 31 approximately 3 miles north of Levering, Emmet County, Michigan (Host: *Elimia (Goniobasis) livescens*)

6. New York - Oneida River near Lake Oneida (Host - *Goniobasis livescens*)

7. Ohio - Olentangy River just north of the intersection with Lane Avenue within the boundaries of Ohio State University at Columbus, Franklin County (Host: *Elimia (Goniobasis) livescens*)

8. Wisconsin - Oconomowoc River (Host - *Pleurocera acuta*)

NOTE: The Indiana, Kentucky , Ohio, and Michigan sites have been recently verified. The author is willing to provide snails when he makes his fall and spring collections as long as the recipient is willing to cover shipping charges; please contact him in the early fall of each academic year for dates.

8 white plastic tubs (approximate length = 36 cm; width = 31 cm; depth = 13 cm)
4 aquarium pumps with airstones
1 environmental chamber with dark/light cycling *or* fluorescent grow lights and access to "dark" cabinets
-- source of dechlorinated water
-- lettuce

Light Wavelength Experiment (Supplies for eight groups of four students)

8	plastic pipettes (for handling cercariae; cut off tips to enlarge opening)
8	gooseneck lamps *or* ring stands with clamps & portable hand lamps
8	25 W green party light bulbs
8	25 W blue party light bulbs
8	25 W red party light bulbs
8	25 W white party light bulbs
8	1,000 *or* 2,000 mL scaled glass graduated cylinders (same make)
8	stopwatches
8	cm rulers
1	light meter (if available)
1	lab without windows (i.e., lab can be made completely dark); not mandatory, but provides better results

Distome Emergence Experiment (Supplies for eight groups of four students)

8	dissecting microscopes (transmitted light) Note: Students may wish to view this process in more detail with the low power objective of a compound microscope after the initial experiment is completed.
24--48	30 mL glass beakers
8	rolls lab tape
8	permanent markers
12	125 mL Ehrlenmeyer Flasks; each will contain 100 mL of the various "emergence" fluids; the instructor should prepare these prior to lab with the chemicals and materials listed below.
2,000 mL	Ringer's saline for cold-blooded vertebrates (6.5 g/liter NaCl; 0.05 g/liter KCl; 0.16 g/liter $CaCl_2$ x $2H_20$; 0.39 g/liter $MgSO_4$ x $7H_2O$; 0.2 g/liter $NaHCO_3$)
1	dropper bottle with concentrated HCl
10 g	Pepsin (Catalog # - Sigma P 7125)
1	pH meter and standards
1	magnetic stirrer with 1/2 " magnetic stir bar
1	2,000 mL beaker
1	balance

Student Outline

Introduction

Proterometra macrostoma is a digenetic trematode which is widely distributed in the eastern United States. The life cycle of *P. macrostoma* is indirect and incorporates a snail intermediate host and a fish definitive host. The adult worm is found in the esophagus and stomach of sunfish (Family Centrarchidae). Eggs containing fully-developed miracidia are

released into water with fecal material and are subsequently ingested by snails in the Genus *Elimia* (formerly *Goniobasis).* Intramolluscan stages include both sporocyst and redia generations which increase in number by asexual polyembryony. Within the redia, macroscopic furcocysticercous cercariae develop. Just prior to emergence of the cercaria from the snail, the distome body of the cercaria retracts and enters a vesicle/cavity within the cercarial tail. Following release into the water, the subsequent swimming behavior and size of the *P. macrostoma* cercaria make it attractive to potential definitive hosts which rapidly ingest the worm. There is no intervening metacercarial stage, and the digenean body, once liberated from the cercarial tail in the fish stomach, matures directly into the adult worm. Pictures of various stages of this life cycle are available on the Research Link 2000 website (http://www.berea.edu/BIO/CUR/mainpage.html) under LAB CORE: Examples of Research-Based Labs with Trematode Cercariae: *Proterometra macrostoma* Life Cycle Images.

It is well-documented that *P. macrostoma* cercariae emerge from their snail intermediate host at night (Lewis et al. 1989). This non-feeding transfer stage may live for more than 24 hours in the water, although its swimming activity declines rapidly after 12 hours (Lewis et al. 1989). This behavior would likely expose swimming cercariae to periods of both light and dark in the field, although active, newly emerged larvae undoubtedly spend more time under nocturnal conditions. While previous studies of digeneans have evaluated the effect of light wavelength on cercarial emergence (Asch 1972), little is known about the effect of different wavelengths of light on cercarial swimming behavior. Since fish are attracted by the swimming bursts of *P. macrostoma* cercariae, any exogenous factor such as light which accentuates or diminishes this behavior would be of importance with regard to completion of this species' life cycle.

As previously noted, the distome body of *P. macrostoma* emerges from the cercarial tail chamber following ingestion by the sunfish definitive host. The role of host stomach HCl in this emergence has been considered qualitatively (Horsfall 1934), but the time frame for this phenomenon and the precise conditions promoting this "emergence" have only recently been quantified (Rosen et al. 2000).

The objectives of this laboratory are to: (1) determine the effect of light wavelength (i.e., red, green, white, and blue) on the vertical swimming burst distance of *P. macrostoma* cercariae and (2) evaluate the effect of pH and pepsin on the emergence of the distome body from the cercarial tail.

General Methods

Place collected snails in white plastic containers filled with filtered creek water. Maintain the snails at 20--25^{o} C under continuous light, and feed lettuce ad libitum. The water and lettuce in the containers should be changed every 2 days. When cercariae are required for experiments, any previously emerged cercariae are to be removed from these pans. The snail cultures should

then immediately be placed in an environmental chamber in the dark at 20^{o} C. This should promote a copious release of new cercariae within 2--4 hours.

Light Wavelength Experiment

Construct four light sources with 25 watt colored light bulbs (green, blue, red and white). Remove cercariae from cultures within 8 hours following their emergence from snails. Only one cercaria will be used at a time by each group. Pipette a single cercaria into a 2.0 liter scaled, graduated cylinder (previously filled to 2.0 liters with filtered creek water). Position the lamp several inches above the cylinder and turn off all room lights.

For the next hour, expose the cercaria to 5 minute periods of red, white, red, green, red, blue, and red light (following a 2 minute acclimation period to each of these treatments). During the 5 minute trial periods, make one "burst" observation at the beginning of each 1 minute interval. Record the height at which the cercaria initiates and terminates its initial swimming burst in the cylinder (Appendix A). Calculate the total distance traveled by the cercaria in mL from these two measurements (Appendix A). (Note: You will later convert the mL recordings into mm by measuring the distance between the volumetric units on the cylinder with a cm ruler). Change the light sources at the end of each treatment, and then initiate the next 5 minute period of light treatment following a 2 minute acclimation period to the new light. Use a red light to rearrange light sources during the brief intervals between treatments. A complete experiment will require approximately 1 hour (i.e., seven 2 minute acclimation periods, seven 5 minute light treatments, and time for switching light sources), and thus no cercaria will be more than 9 hours post-emergence from the snail at the end of an experiment.

Determine the average distance traversed by a cercaria during a swimming burst from the five consecutive 1 minute trials for each light treatment. These averages will then be used to obtain a class mean $\pm$ SD (standard deviation) for all *P. macrostoma* cercariae under each light condition. Construct a bar graph (x axis = type of light treatment; y axis = swimming burst distance in mm) with the pooled class data and interpret your results. Several questions are provided below for your consideration.

1. Do your results really represent a wavelength effect or is this simply a dark/light response?
2. Do the tested light wavelengths penetrate the water column to the same extent?
3. How would the behavior you have identified here benefit the cercaria of *P. macrostoma* during daylight hours?

Distome Emergence Experiment

Prior to the experiment, make a Ringer's solution for cold-blooded vertebrates and acidify with concentrated HCl to obtain the following levels of pH: 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0. For the pH + pepsin experiments, make 0.5% pepsin solutions with each of the acidified salines just prior to use.

Each group will choose at least three different acidified salines $\pm$ pepsin to assess. Pipette a cercaria into a 30 mL beaker containing 25 mL of a particular pre-cooled (20^{o} C) acidified saline or acidified saline + pepsin. Place the beaker into an environmental chamber or water bath set at 20^{o} C, and check for complete distome emergence from the cercarial tail every 5 minutes for 1 hour (Appendix B). Calculate a mean $\pm$ SD for the % distome emergence for each time period based on the combined class data. Construct line graphs (x axis = time in minutes; y axis = mean % distome emergence) with the pooled class data and interpret your results. Following the completion of your experiment, please address the following question:

1. What condition promoted the quickest emergence of distomes from the cercarial tails? Explain your answer with regard to host digestive physiology.

Notes For The Instructor

Light Wavelength Experiment

P. macrostoma cercariae used in this experiment may be between 1 minute--9 hours old provided that infected snails and released parasites have been kept at temperatures between 15--20^{o} C. There is little reduction in swimming burst distance during this time at these temperatures.

If a light meter is available, it would be advantageous (but not mandatory) to regulate light intensity at the same level at the top of the cylinder for the various wavelengths used by adjusting the light sources above the cylinders accordingly. Measurements may also be taken at the bottom of the cylinder for future reference. Calibrating should be done prior to the initiation of the experiment so that students will be able to adjust these distances quickly when the experiment is in progress. The light gradient produced in the cylinders seems to be important and likely simulates field conditions. In this regard, pilot experiments have shown that the effect achieved in this experiment is abolished when light gradients are eliminated (i.e., light intensity is kept constant throughout the cylinder using a bank of fluorescent lights to surround the cylinder). Students will find that *P. macrostoma* cercariae undergo longer swimming bursts in red light when compared to white, blue, and green light. They should consider whether this is truly a wavelength effect or simply a result of "dark vs. light" given that red light is absorbed much more rapidly in the water column than other wavelengths in the visible spectrum. What would be the significance of this phenomenon in natural systems? Even though *P. macrostoma* cercariae are released at night, their active swimming period extends well into daylight hours. It has been proposed that a "shadow effect" produced by passing fish may stimulate an immediate increase in the vertical swimming of some cercaria thus increasing the probability of contact with their fish host (Donges 1964). In the case of *P. macrostoma*, such a shadow phenomenon would increase the likelihood that this cercaria might be detected by a fish host and subsequently ingested.

Distome Emergence Experiment

Twelve acidified solutions will be made up and student groups will be allowed to select from three to six of these to test. Previous background on fish digestive physiology (Norris et al. 1973) should assist them in their chose of solutions (pH's between 2.0-2.5 + 0.5% pepsin work best). The emergence of the distome from the cercarial tail is visible with the naked eye, but a dissecting microscope is recommended. Following the formal experiment, students may wish to observe this process in more detail. Place the cercaria on a slide, cover it with a selected acidified saline $\pm$ pepsin under a coverslip, and view the events under low power magnification with a compound microscope. The distome, which shows limited movement prior to treatment, can be seen to initiate a series of contractions and expansions with this method, and the subsequent rupturing of the tail and emergence of the distome will be visible.

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Appendicies

Light Wavelength Data Sheet

Treatment	1 minute	2 minutes	3 minutes	4 minutes	5 minutes
Red 1					
Initiation					
Termination					
Distance					
White					
Initiation					
Termination					
Distance					
Red 2					
Initiation					
Termination					
Distance					
Green					
Initiation					
Termination					
Distance					
Red 3					
Initiation					
Termination					
Distance					
Blue					
Initiation					
Termination					
Distance					
Red 4					
Initiation					
Termination					
Distance					

Distome Emergence Data Sheet

Treatment	% Distomes Emerged Over 60 Minutes											
	5	10	15	20	25	30	35	40	45	50	55	60
Saline Only												
pH 1.5												
pH 2.0												
pH 2.5												
pH 3.0												
pH 3.5												
pH 4.0												
Saline + Pepsin												
pH 1.5												
pH 2.0												
pH 2.5												
pH 3.0												
pH 3.5												
pH 4.0												