

AN ABSTRACT OF THE THESIS OF

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AND INFECTION OF THE FIRST INTERMEDIATE HOST

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Development of the unembryonated eggs of Nanophyetus salmincola was found to occur only in eggs washed from feces exposed to 5°C for from one to ninety-two days and incubated at temperatures from 15 - 30°C. Development was described and illustrated up to and including the miracidium.

Hatching of fully-embryonated eggs occurred at low rates after 58 days of incubation. The process of hatching was described and illustrated. Spontaneous hatching and violent aeration induced hatching in approximately one percent of the eggs, while shaking with sand caused dehiscence of opercula of approximately 50 percent of fully-embryonated eggs. Hatching was induced in eggs incubated 37 days or longer when they were exposed to the snail, Oxytrema silicula, concurrently with the addition of fresh water.

Miracidia penetrated O. silicula by chance. Sporocysts, measuring 0.50 - 0.61 mm in diameter, were taken from the foot and adjacent tissues of the snail after 33 days and immature rediae were present after 44 days of development. Rediae leave the sporocyst by a birth pore. Sporocyst development was described and illustrated.

Development of the Egg of Nanophyetus salmincola (Chapin)
and Infection of the First Intermediate Host

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TABLE OF CONTENTS

	<u>Page</u>
INTRODUCTION	1
METHODS AND MATERIALS	8
RESULTS	13
Egg Development	
Experiment I	14
Experiment II	14
Experiment III	15
Experiment IV	16
Experiment V	16
Experiment VI	17
Experiment VII	18
Experiment VIII	19
Egg Hatching	
Experiment IX	22
Experiment X	24
Experiment XI	26
Experiment XII	26
Experiment XIII	27
The Miracidium	28
<u>Oxytrema silicula</u> Infection	
Experiment XIV	31
Experiment XV	32
Experiment XVI	33
Experiment XVII	33
The Sporocyst	35
DISCUSSION	38
BIBLIOGRAPHY	59

LIST OF TABLES AND FIGURES

<u>Table</u>	<u>Page</u>
1 Effects of fecal material on embryo development in <u>Nanophyetus salmincola</u> .	49
2 Incubation of 50-egg samples of <u>Nanophyetus salmincola</u> under stable experimental conditions.	50
3 Incubation of 50-egg samples of <u>Nanophyetus salmincola</u> under experimental conditions with routine water change.	51
4 Incubation of 200-egg samples of <u>Nanophyetus salmincola</u> under experimental conditions using large water volumes.	52
5 Effect of exposure to 5°C before incubation on development and hatching percentage on 500-egg samples of <u>Nanophyetus salmincola</u> .	53

<u>Figure</u>		
1 Detailed drawings of embryo development of <u>Nanophyetus salmincola</u> incubated through ten days at 20 - 22°C following exposure to 5°C.		54
2 Detailed drawings of embryo development of <u>Nanophyetus salmincola</u> when incubated from 11 through 30 days at 20 - 22°C following exposure to 5°C.		55
3 Detailed drawings illustrating the hatching process of the miracidium of <u>Nanophyetus salmincola</u> .		56
4 Detailed drawings of the miracidium of <u>Nanophyetus salmincola</u> .		57
5 Detailed drawings of the structure of the sporocyst of <u>Nanophyetus salmincola</u> , freshly dissected from <u>Oxytrema silicula</u> .		58

DEVELOPMENT OF THE EGG OF NANOPHYETUS SALMINCOLA (CHAPIN)
AND INFECTION OF THE FIRST INTERMEDIATE HOST

INTRODUCTION

Numerous workers have studied the biology of Nanophyetus salmincola (Chapin). This trematode is of economic importance in the Pacific Northwest of the United States of America in that it is a vector for Neorickettsia helminthoeca, a rickettsial organism which causes severe and often fatal infections in canids. The disease is commonly called "salmon poisoning". A compilation of the biology of N. salmincola and "salmon poisoning" disease was made by Millemann and Knapp (1970). Since 1970, N. salmincola studies have been concerned with the effects of the cercarial stage on the swimming ability of fish hosts (Butler and Millemann, 1971) and with aspects of the rickettsial organism (Noonan, 1973).

The first life cycle of a digenetic trematode was reported by Thomas (1883) on Fasciola hepatica. Since that time hundreds of trematode life cycles have been reported. The pattern used for publication is nearly always the same. The cycle is demonstrated in the laboratory and includes a review of the literature; the location and destructiveness of the parasite in the definitive host; followed by descriptions of the adult, or adults in dioecious species; eggs produced by the fluke and their pathway out of the definitive host; the miracidium and observations of its hatching; sporocyst and/or redial stages in the molluscan host; the cercaria and its encystment, and the metacercarial stage, when present, is described. Workers commonly omit certain early stages of the trematode life cycle. These

are the development of the fertilized egg culminating in the mechanism of miracidial hatching and the direct infection of the molluscan host by the miracidium.

It is understandable that trematode embryology is commonly neglected, as the eggs are extremely difficult to study since the zygote is typically surrounded by 20 to 30 vitelline cells, which are, in turn, surrounded by a tanned, protein shell often opaque and extremely impermeable (Smyth and Clegg, 1959). The mechanism and initiation of egg hatching is another difficult topic to study. Kusel (1970) studied hatching of Schistosoma mansoni eggs and stated that the hatching mechanism of nonoperculate eggs is still unknown. The possible mechanisms and triggering of hatching of operculate eggs for numerous trematodes have been reported (Thomas, 1883; Barlow, 1925; Mattes, 1926; Onorato and Stunkard, 1931; Rowan, 1956, 1957; Friedl 1960; Lengy, 1962; Wilson, 1968, and Harris, Harkema and Miller, 1970). None of these workers has shown conclusively the mechanism of miracidial hatching, although hatching was induced by various methods.

Each parasite appears to behave differently to different environmental stimuli. Factors which appear most influential in bringing about hatching are specific humidity, temperature and oxygen tension exposure (Smyth, 1966). Environmental stimuli appear to be vital cycles of life, including those of parasites. Berntzen (1966) compared a parasite with a computer governed by a set of physiological clocks and keys which are "triggered" by physiochemical environmental factors. Otto (1958) said in his retirement address to the American Society of Parasitologists:

"No one parasite is completely known, but in many cases the many facets of information available give us a clue at least to its ecology. It is human nature to respond favorably to finite data, but to be wary of vague subtleties. Thus, when two sets of data appear to be in direct conflict, there is almost automatic reaction directed toward learning which is the truth and which is in error. While these extremes do occur and must be resolved, more often than not this appearance of conflicting information signified that there are large gaps in our knowledge. These gaps in our knowledge of the ecology of parasitology are wider and more frequent than any other aspect of parasites and parasitism."

Various facts have been reported concerning egg development and miracidial hatching of Nanophyetus salmincola. Donham (1925) first reported large numbers of small trematodes and their eggs in the intestinal tract of dogs which had been fed salmon from several streams of Western Oregon and Washington. The trematode, N. salmincola, was described by Chapin (1926, 1928) after studying material collected by Donham. Chapin described the eggs as yellowish-brown and measuring 0.075 - 0.085 mm. long by 0.055 mm. wide. Donham, Simms and Miller (1926) simply reported the eggs as oval with a greatest diameter of 0.075 mm. Other workers reported that the eggs of N. salmincola measured 0.064 - 0.097 mm. long by 0.034 - 0.055 mm. wide when taken from feces of a variety of definitive hosts (Price, 1929; Simms, et al., 1931; Witenberg, 1932; Bennington, 1951, and Bennington and Pratt, 1960). Filimonova (1965, 1968) reported that the eggs of the Siberian subspecies, Nanophyetus salmincola schikhobalowi, are slightly smaller than N. salmincola, measuring 0.052 - 0.082 mm. long by 0.032 - 0.056 mm. wide.

Eggs were found in fecal matter five to eight days after the definitive host had ingested fish infected with metacercariae (Simms,

Donham and Shaw, 1931 and Filimonova, 1965). Unembryonated eggs were present in the uterus of the adult worm by the sixth day after the host ingested metacercariae (Bennington, 1951 and Bennington and Pratt, 1960). The eggs were unembryonated when released with feces (Millemann and Knapp, 1970), but were illustrated in an embryonated state by these authors.

Price (1929) first reported that the eggs of N. salmincola were operculated. Bennington (1951) and Bennington and Pratt (1960) reported that there was a small, blunt point at the end opposite the operculum and that the light-brown eggs sank quite rapidly in water.

None of the workers studying N. salmincola eggs reported precise methods used in handling of egg-bearing feces or how eggs retrieved from feces were handled before incubation. Nyberg (1971), in a personal communication with the author, stated that he believed eggs used in preliminary hatching studies performed for Millemann and Knapp in 1965-66 were refrigerated for about ten days before incubation. In a study of "salmon poisoning" disease transmission, Nyberg, Knapp and Millemann (1967) stated that eggs were collected and washed at 20°C and suspended in Hank's balanced salt solution. The eggs were held in ice baths between washings and until they were injected into dogs. It is not known whether this method was used by Millemann and Knapp (1970).

There is no published record of the disposition of yolk cells or the zygote in the egg of N. salmincola, nor the developmental stages which produce the miracidium.

The record of hatching of N. salmincola eggs is highly

variable. Donham et al. (1926) and Donham (1928) were unable to get embryonation or hatching of eggs retrieved from feces of infected dogs. Eggs were incubated outside and at room temperature for periods of up to 90 days. Price (1929) reported hatching of eggs from infected dog feces in about three months. Simms et al. (1931) reported a personal communication with Price indicating that he actually observed hatching on the 66th through the 139th day. Simms, Donham and Shaw (1931) wrote that eggs from infected dog feces required 75 days to hatch when held in running water with a temperature range of 52 - 65°F, but that eggs held at room temperature in standing water were largely embryonated on the 58th day of incubation when a single miracidium hatched. This miracidium, as compared to that of F. hepatica, was small, possessed long cilia and swam very rapidly. Bennington (1951) and Bennington and Pratt (1960) reported that N. salmincola eggs collected from feces of hamsters and held in tall bottles of water at room temperature hatched earliest at 87 days, with others hatching through 200 days. Running tap water or aeration produced no change in the rate of development. Miracidia within egg shells were active, with back and forth contractions and elongations which appeared to exert force against the operculum. Ciliary motion was not noted until the operculum was released.

Filimonova (1965) noted hatching of the eggs of Nanophyetus salmincola schikhobalowi after 160 days when held in standing water at 16 - 22°C. When a layering of ice was present, hatching would occur in 35 to 45 days. Eggs did not develop at 3°C or at temperatures of 33 - 37°C and light did not influence development.

Millemann and Knapp (1970), in a preliminary, inconclusive

study, reported that eggs of N. salmincola held in test tubes with 60 ml. of water at pH 7 and constant light began hatching at about 31 days at 21°C, 26°C and 31°C. By the 156th day of incubation, 75 percent of the eggs had hatched. Hatching increased with decrease in temperature and egg mortality increased with increase in temperature. Aeration and sand substrate did not influence development.

A complete description of the miracidium of N. salmincola or its hatching process has not been reported. Bennington (1951) and Bennington and Pratt (1960) reported that the newly-hatched miracidium measured 0.087 - 0.105 mm. long by 0.037 mm. wide and swam in characteristic, long, graceful curves. Only one flame cell was observed by Bennington and Pratt (1960). They were unable to demonstrate miracidial attraction to the snail host, Oxytrema silicula (Gould), although abundant infections of redia were reported in wild snail populations. The early developmental stages of N. salmincola in its snail host have not been described.

Attraction to snail hosts of numerous different trematode miracidia has been demonstrated (Faust, 1924; Campbell and Todd, 1955; Wright, 1959; Kawashima, Tada and Miyazaki, 1961; Chernin and Dunavan, 1962; Plempel, 1964; MacInnis, 1965; Kinoti, 1968; Schwabe and Kilejian, 1968; Shiff, 1968; Shiff and Kriel, 1970 and Chernin, 1970). On the contrary, Stunkard (1943), working with Zoogonoides laevis, reported that the miracidia of this species invaded the snail host by chance.

Filimonova (1965) reported that living miracidia of N. s. schikhobalowi measured 0.063 - 0.105 mm. long by 0.021 - 0.042 mm. wide with four rows of ciliated epithelium arranged in tiers, with six,

seven, three and one cells, respectively, anterior to posterior. The first row of epithelial cells had four cells with one pore and two cells with two pores. In the third row of cells, two cells had one pore each. There were four pores between the second and third rows of cells and two apical pores in the miracidium. Neutral red staining showed one apical gland surrounded by two multicellular glands with posteriorly directed ducts. At the level of the second and third epithelial cells, there were two glands with anteriorly directed duct openings. Germ cells were located between these anterior and posterior paired glands. One pair of flame cells was present with pores opening between the second and third rows of epithelial cells. Filimonova (1965) was unable to determine attraction of the newly-hatched miracidia of N. s. schikhobalowi to their known snail hosts, Semisulcospira laevigata and S. cancellata.

The snail hosts of N. salmincola and N. s. schikhobalowi have not been infected experimentally.

The purposes of this research were (1) to gain a more complete understanding of the life cycle of N. salmincola by experimentally showing the environmental requirements which initiate the development of the egg through miracidial hatching; (2) to describe this development; (3) to accurately describe the miracidium of N. salmincola; (4) to demonstrate experimental infection of the snail host by the miracidium, and (5) to describe the early developmental stages of N. salmincola within the snail host, Oxytrema silicula.

MATERIALS AND METHODS

Metacercariae of N. salmincola were taken from kidneys of Oncorhynchus tshawytscha, the Chinook salmon, and Salmo clarki clarki, the cutthroat trout, provided by the Alsea Fish Hatchery, Alsea, Oregon. Fresh kidney tissue infected with metacercariae was also used from Salmo gairdneri, the rainbow trout, and cutthroat trout taken from the Siletz River, Lincoln County, Oregon. Metacercarial infection of these fish was easily confirmed by visual examination for small, white specks in the kidneys, as described by Shaw (1947), and the use of a dissecting microscope, following the description of Bennington (1951) and Bennington and Pratt (1960). Metacercariae were force-fed to the definitive hosts in intact kidney tissue and as isolated cysts from kidney tissue, following the technique of Nyberg (Gebhardt et al., 1966).

Experimental definitive hosts used in this study were Mesocricetus auratus, the golden hamster, and Canis familiaris, the common dog. Both mammals were housed in cages with slatted or mesh bottoms so that the feces would drop from the cage. The dogs and hamsters had unrestricted, balanced diets of dry food and water. Body temperatures and the general conditions of infected hosts were recorded daily. From the sixth to tenth day after ingestion of metacercariae, the dogs were given 50 milligrams of oral tetracycline hydrochloride per pound of body weight, as effective treatment for "salmon poisoning" disease (Noonan, 1973).

Fecal samples were caught in water bath trays placed under

hamster cages or collected fresh from clean pans three or four times a day for 15 days after host ingestion of metacercariae. Fecal smears were made of all collections to note the presence and abundance of parasite eggs. Identification of N. salmincola eggs was determined following the description of Bennington (1951) and Bennington and Pratt (1960).

Most of the N. salmincola eggs used in this study were separated from fresh dog and hamster feces, never more than three hours old, collected on the sixth through tenth days following host ingestion of metacercariae. These feces were dispersed in dechlorinated water at 20°C and the homogenate was passed through a series of Tyler standard screen sieves of decreasing mesh opening diameters of 0.246, 0.147, 0.104 and 0.074 mm. to remove fecal matter. The eggs were washed five times by allowing them to settle eight to ten minutes in 1500 ml. of dechlorinated water at 20°C and decanting the supernatant.

A second method was used to obtain clean eggs and to estimate degrees of hamster infection. Hamsters were killed and their intestinal contents examined on the seventh day after they had ingested metacercarial cysts. Adult flukes were recovered from the small intestine, counted and identified as N. salmincola following the description of Chapin (1926) and Bennington and Pratt (1960). Washed flukes were held overnight in covered dishes of dechlorinated water at 10°C. Eggs shed by the adults were collected from the bottom of the dish by pipette.

Collections of large numbers of washed eggs to be used in experiments, numbered V, VI, VII, VIII and IX, were maintained for six to twelve months in covered, eight-inch culture dishes with 1500 ml. of

unaerated, dechlorinated water. These collections were maintained at 5°C and 20 - 22°C and approximately pH 7. Light exposure was a day/night cycle.

Ten fresh eggs from each collection were measured. Measurements were made with the use of a calibrated, ocular micrometer and/or photomicrographs of a stage micrometer and then recorded in millimeters.

Snails, Oxytrema silicula, used for this study were collected from Dixon Creek, within the city limits of Corvallis, Oregon. No infections of N. salmincola were observed in 76 snails, with shell lengths of from 7 - 25 mm., collected during April, May and June, 1972. Flumenicola virens (Lea), small, stream snails commonly found living in the same habitat as O. silicula were collected from Greasy Creek, near Philomath, Oregon. Snails were maintained in aerated, dechlorinated water at 20 - 22°C in covered, eight-inch culture dishes. All snails were fed fresh lettuce and their water was changed every other day.

Illustrations of egg development were made from living material using light and phase microscopy. Detail was added from photomicrographs at various stages of development. Outlines of some structures were made by use of a Wild drawing arm.

Photomicrographs were taken using a Zeiss RA microscope with a Nikon Microflex automatic photomicrograph attachment. Films used were Kodak High Contrast Copy 5069 developed with Kodak D-19 and Kodak high-speed Ektachrome color film developed commercially. Prints were made on single-weight Kodak Polycontrast F paper.

A total of 17 experiments were conducted in this research.

Experiments I through VIII were designed to determine the effects of varied environmental conditions on the development of N. salmincola eggs. Experiments IX through XIII were designed to discover if any environmental stimuli were responsible for hatching of the miracidium of N. salmincola. The last four experiments, numbers XIV through XVII, were performed to determine if there was attraction of N. salmincola miracidia to the snail, O. silicula, and if infection of this first intermediate host could be induced.

In all embryogenesis experiments described, N. salmincola eggs from fresh, egg-laden fecal samples or reserve egg collections were maintained or incubated in covered Syracuse stack dishes, 23 x 55 mm., containing 40 ml. of dechlorinated water at a pH of six to seven. Eggs were examined after one day and thereafter at least every other day to observe and record any developmental change.

All experiments requiring eggs in feces were begun using egg-laden feces passed by the host animal within the hour. Fresh eggs used in this study were shed by the adult fluke no longer than 12 hours or separated from feces no longer than four hours before their use in experiments.

In embryogenesis and hatching experiments, large numbers of eggs (100 or more) were approximated by counting the number of eggs in a pipette drop from a thoroughly-stirred egg collection.

Miracidial structure (Figure 4B) was determined by observation of both hatched miracidia and fully-embryonated eggs. Anatomical terminology followed Bennett (1936). Hatched miracidia were obtained by induction of their hatching by the introduction of one O. silicula into

a culture dish containing embryonated eggs and the addition of 10 - 20 ml. of dechlorinated water fresh from the tap.

Most of the anatomy of the miracidium and sporocyst stages of N. salmincola were worked out from living specimens either intravitaly stained with neutral red or methylene blue or slowed with Protoslo (Carolina Biological Supply Company). Photomicrographs were used to add detail to drawings. Epithelial cell boundaries of the miracidium and ciliary patterns (Figure 4A) were demonstrated using the dry technique, silver-line system of Klein (Guyer, 1939).

RESULTS

In preparation for recovery of eggs of Nanophyetus salmincola to be used in this research, examinations of dog and hamster feces showed that eggs first appeared in low numbers late on the fifth day after the host had ingested metacercariae. From the sixth through the ninth day the greatest numbers of eggs were recovered. After the tenth day of infection, the numbers of eggs found in host feces began to decrease. One hamster killed ten days after ingestion of metacercariae was infected with nearly 1,500 adult N. salmincola, mostly in the duodenal area. An estimated 16,000 eggs had been collected from its feces during the seventh day of infection.

None of the symptoms of "salmon poisoning" disease was noted in infected hamsters, but when high numbers of metacercariae were fed to these animals, their feces became loose and mucus-laden ten to eleven days after infection. Some of these hamsters died 18 to 24 days after infection, apparently from severe enteritis induced by the heavy infection of trematodes. Dog hosts showed typical symptoms of "salmon poisoning" beginning with elevated body temperatures the fifth or sixth day following ingestion of metacercariae. Tetracycline hydrochloride was found to be effective treatment to combat "salmon poisoning" disease without inhibiting the trematode infection. Infected dogs rarely ate, drank or defecated during the period when their body temperature was elevated, but after treatment their appetite returned and egg-bearing fecal material was passed regularly.

Egg Development

Experiment I

The purpose of this experiment was to demonstrate the influence of the presence of feces on embryogenesis under various experimental conditions. On July 26, 1971, hamster fecal pellets were macerated and one placed in each of 13 dishes. These dishes of egg-bearing feces were incubated under varied experimental conditions for two weeks (Table 1).

All eggs observed in this experiment became filled with clear vacuoles after two to six days of incubation. The yolk cells appeared degenerate as they changed color, from light brown to light yellow, and lost their cellular uniformity. No development of the zygote was noted under any experimental condition. It was noted that yolk cell degeneration seemed to be slowed at lower temperatures of incubation and by dilution of fecal material in association with eggs.

Experiment II

The purpose of this experiment was to demonstrate the effect of a period of desiccation on fresh eggs. On July 27, 1971, hamster fecal pellets were incubated at temperatures of 9 - 10°C and 20 - 22°C and allowed to desiccate. One pellet was macerated after 12 hours and added to each of two dishes of water and incubated at their respective desiccation temperatures. Continuous lighting was used. The experiment was run for two weeks.

After desiccation, all egg contents collapsed to one side of the shell. When the eggs were incubated in water subsequent to desiccation, no appreciable swelling of egg shell contents was noted and all contents had degenerated by the ninth day. The zygote was not visible at any time after initiation of the experiment.

Experiment III

The purpose of this experiment was to determine conditions which might induce egg development under stable, experimental situations. On July 30, 1971, 50 washed eggs from hamster feces or shed from adult flukes were placed in each of 13 dishes. The eggs were incubated under varied experimental conditions for 70 days (Table 2). On August 25, 1971, this experiment was rerun in part using eight dishes containing freshly-washed eggs (Table 3). The conditions were modified in that the water was changed after every examination by decanting 30 ml. of supernatant and adding fresh water maintained at incubation temperatures.

In these experiments it was noted that all egg content degeneration was inhibited at an incubation temperature of 5°C. These eggs retained an appearance of freshly-collected, unembryonated eggs (Figure 1B) through the 70 days of the experiment.

All other eggs, incubated at higher temperatures and varied lighting, typically showed a central clearing between yolk cells, appearing the fifth to sixth day. The zygote was not discernible after the sixth day of incubation and no cleavage or embryonic stages were observed under any of the experimental conditions.

It was noted that total degeneration of all enclosed egg contents occurred only after a period of 30 to 36 days at incubation temperatures ranging from 20 - 39°C. After this time it was frequently noted that the operculum would drop off the egg shell.

Experiment IV

The purpose of this experiment was to demonstrate the effect of a period of freezing on fresh eggs. On August 16, 1971, a fecal pellet and freshly-washed eggs from the feces of a hamster were placed in separate dishes of water and exposed to a temperature from -1° to 0°C for 12 hours and then thawed at 16 - 17°C. The fecal pellet was then macerated and incubation of both the egg-bearing feces and the washed eggs was at 16 - 17°C for two weeks.

The washed eggs were vacuole-filled by the third day of incubation. The yolk cells appeared degenerate by the fourth day of incubation. Those eggs incubated with fecal material were vacuole-laden and the yolk cells disrupted after 24 hours of incubation. No development was observed in either of these test situations.

Experiment V

The purpose of this experiment was to demonstrate the effect of large volumes of water on egg development under varied experimental conditions. On October 4, 1971, eggs washed from hamster feces were placed in six dishes. Approximately 200 eggs were placed in each dish. These dishes were then covered with a single-layer thickness of regenerated cellulose dialysis tubing with an average pore radius of

24 Å⁰ (Carolina Biological Supply Company). These coverings were secured tightly with rubber bands. The dishes were incubated for 70 days while submerged in aquaria containing large volumes of water (four to ten liters) under various experimental conditions (Table 4). Water temperatures were maintained by use of aquarium heaters or enclosure of aquaria in controlled temperature chambers. Continuous lighting was used.

Eggs incubated under these conditions did not develop. Results were very similar to those of Experiment III where comparable incubation temperatures were used, even when aeration was applied (Table 4). When eggs were incubated at 9 - 10°C, it was noted that degeneration of egg yolk cells was retarded.

Experiment VI

The purpose of this experiment was to demonstrate the influence of an extended period of low temperature exposure prior to elevated incubation temperature on egg development. Beginning on September 27, 1971, two covered dishes containing 100 freshly-washed eggs each from hamster feces were continually maintained at a temperature of 5°C for 92 days. On December 27, 1971, both dishes were transferred to incubation at 20 - 22°C and maintained 45 days.

Eggs used in this experiment remained in a normal, unembryonated state (Figure 1B) as long as they were maintained at a temperature of 5°C. No degeneration of yolk cells or embryonic development was observed during this cold exposure. When these dishes of eggs were transferred to incubation at 20 - 22°C, development of a two-cell stage

(Figure 1C) was noted on the fifth day. A spherical embryo (Figure 2D) capable of some movement was developed by the nineteenth day and after 36 days of incubation at 20 - 22°C, nearly 100 percent of the eggs were fully-embryonated (Figure 3A). No hatching of miracidia was observed through the duration of this experiment, nor were empty egg shells seen in either of the experimental groups.

Experiment VII

The purpose of this experiment was to demonstrate the influence on embryo development of Nanophyetus salmincola after exposure at 5°C for various periods of time prior to incubation at varied, elevated temperatures.

The eggs used in this experiment were taken from two reserve collections obtained from dog feces on November 11, 1971, continually maintained at 5°C and 20 - 22°C, respectively. Five hundred washed eggs were incubated in each of 14 dishes. At intervals, November 11, 12 and 22, 1971; December 6, 14 and 20, 1971; January 18 and 25, 1972, and March 5, 1972, 500 washed eggs were removed from reserve collections and incubated under varied temperatures and constant lighting (Table 5). This experiment was run for six months.

In this experiment those eggs incubated at 5°C remained in an unembryonated state (Figure 1B). Eggs which were incubated at a temperature of 8 - 10°C did not develop, but the contents degenerated within the egg shell by 45 days. The contents of eggs incubated at a temperature of 20 - 22°C and 30°C with no exposure to 5°C were degenerated by 34 and 30 days, respectively.

All eggs exposed to a temperature of 5°C and then incubated at higher temperatures developed to at least a four-cell stage (Figure 1D) by the sixth day of incubation (Table 5).

Eggs which had been maintained at 5°C for 113 days developed to an ovoid-embryo stage (Figure 2B) by 15 days of incubation at 20 - 22°C, but had stopped development and degenerated by the 35th day of incubation at that temperature. Many eggs exposed to 5°C for from one to seventy days contained fully-mature miracidia (Figure 3A) by 37 days at all elevated incubation temperatures employed (Table 5). It was noted that beginning on day 58, less than one percent of these miracidia had hatched. After day 58, a small number of miracidia hatched weekly through the duration of the experiment, but nearly 90 percent of the fully-embryonated eggs had still not hatched after six months.

Experiment VIII

The purpose of this experiment was to describe the developmental stages of the eggs of N. salmincola. A reserve collection of unembryonated eggs (Figure 1B) was washed from dog feces on June 12, 1972, and continuously maintained at 5°C. From June 13, 1972, through July 2, 1972, 1,000 eggs were removed from the reserve collection and placed in a separate dish each day and incubated at 20 - 22°C. A seasonal day/night lighting period was used. The maximum period allowed for development was 48 days. Samples of eggs from all dishes were examined at appropriate intervals to observe and record daily developmental changes (Figure 1B-F, Figure 2A-E and Figure 3A).

Development of the embryo of Nanophyetus salmincola was found

to be more uniform within a given egg sample the longer the eggs were exposed to a 5°C temperature prior to incubation. By the fifth day of incubation, two unequal cells were formed (Figure 1C). By day six, a four-cell embryo (Figure 1D) had developed and on the eighth day an eight to nine-cell stage (Figure 1E) was apparent.

It was noted that the developing embryos were enclosed by a vitelline membrane with a perivitelline space which enlarged as development progressed. This enlargement of the perivitelline space was concurrent with the disappearance of yolk cells and granules and with embryonic growth.

A 22 - 24 cell stage (Figure 1F) measuring 0.018 - 0.020 mm. by 0.020 - 0.023 mm. was developed by the tenth day of incubation.

By the 12th day of incubation, the embryo was ovoid (Figure 2A) measuring 0.020 - 0.022 mm. wide by 0.038 - 0.042 mm. long. Primitive epithelial cells covered the surface in an arrangement consisting of a covering of five rows of epithelial cells arranged in anterior to posterior tiers made up of one, six, seven, three and one cell, respectively.

The 14-day embryo (Figure 2B) measured 0.023 - 0.025 mm. wide by 0.043 - 0.047 mm. long. The primordium of the apical papilla was apparent. At this stage there was often close apposition of the vitelline membrane to the surface of the epithelial cells, especially at junctions between adjacent epithelial cells. Frequently yolk granules were seen appressed to the outer surface of the vitelline membrane at these points. During later stages, described below, this occurrence of yolk granules at the outer surface of the vitelline

membrane was seen in all cases, so much so, that often the outlines of many of the epithelial cells could be determined by the arrangement of the yolk granules.

The embryo had grown by 16 days of incubation (Figure 2C) to measure 0.030 - 0.035 mm. wide by 0.045 - 0.047 mm. long. The apical papilla was quite distinct and ciliary primordia could be distinguished on the surface of some primitive epithelial cells.

By the 20th day of development (Figure 2D) there was no appreciable change in the size of the embryo from day 16, but more ciliary primordia were visible and yolk material had diminished. At the 20-day stage, the first embryonic movements were observed. These involved a longitudinal contraction of the posterior part of the body from the tip anteriorly through the level of the fourth tier of epithelial cells.

In the 30-day embryo (Figure 2E), the gut sac was first apparent. In this stage germ cell primordia were also first observed in the subepithelial mass. The embryo measured 0.037 - 0.042 mm. wide by 0.058 - 0.065 mm. long at this stage and nearly filled the perivitelline space. This stage was capable of both circumferential and longitudinal contraction over its entire body. Nearly all yolk material had been used. Ciliary primordia were evident on most epithelial cells, but no ciliary processes were observed by day 30.

Fully-embryonated eggs (Figure 3A) containing mature miracidia, measuring 0.025 - 0.030 mm. wide by 0.070 - 0.075 mm. long at rest, were present at day 37 of incubation. These miracidia were capable of considerable contraction within the egg shell, often shortening

their length by as much as one-third. No beating of cilia was observed in these fully-embryonated eggs. Two vacuoles (Figure 3A) became apparent lying outside the vitelline membrane at the level of the third and fourth epithelial tiers, beginning on day 33 of incubation. These vacuoles contained small granules and fluid. As the embryonated eggs aged, the quantity of granules increased in these vacuoles, but the vacuole size remained the same until hatching was imminent. (See Experiment X, p. 24-25.)

Egg Hatching

Experiment IX

The purpose of this experiment was to attempt to determine possible experimental stimuli which would induce hatching of the miracidia of Nanophyetus salmincola. From January through April, 1972, fully-embryonated eggs were observed while exposed to various stimuli.

Physical pressure was applied to embryonated eggs by pushing down on a cover slip covering them while in a temporary wet mount. It was noted that in incompletely embryonated eggs (Figure 2D and E) the egg shell would typically split equatorially and that the operculum was never released. The incompletely developed miracidia were always destroyed by this procedure. When fully-embryonated eggs were used, the opercula would usually burst open and the active miracidia were released in an instant. Epithelial ciliary beating and flame cell activity were initiated only after the miracidium was released and came into contact with water. These hatched miracidia swam abnormally and always had some materials from the egg adhering to their caudal

cilia. Miracidia of Nanophyetus salmincola normally showed rhythmic, longitudinal body contractions every three to eight minutes while in the egg shell.

When miracidia in fully-embryonated eggs were exposed to a range of subdued to intense lighting, using an illuminator with a rheostat or open sunlight, they showed no noticeable change in activity within the egg case. It was noted that younger miracidia in embryonated eggs, 37 to 50 days old, usually showed slightly more frequent body contractions than did older miracidia from 60 to 180 days old.

When fresh water was added to temporary wet mounts, no change in behavior was noted and the movements of the miracidium continued at a normal rate. If a drop of water from an aquarium containing Oxytrema silicula was added to the edge of a cover slip of a wet mount of fully-embryonated eggs, 140 days old, these miracidia responded after about 30 seconds by accelerated body contractions lasting for about two minutes, at which time the contraction rate slowed again to the normal rate. No activity of epithelial ciliature was observed. This procedure was repeated over a period of two hours and the results were consistent. No hatching was induced by this method. The activity of flame cells during contractions could not be observed, but when contractions slowed, the flame cells did not appear to be active.

When fully-embryonated eggs were exposed to violent bubbling of water by pipette, miracidial release through the operculum of the egg shell was induced in an estimated one percent of the eggs. The released miracidia swam abnormally and were very similar to those

forced out of the egg shell by physical pressure described above.

When a small quantity of sand grains was introduced into a vial containing fully-embryonated eggs and shaken, miracidia were released from about 50 percent of the eggs, but these miracidia again appeared abnormal.

Experiment X

The purpose of this experiment was to demonstrate the experimental conditions under which extensive hatching of embryonated eggs of N. salmincola occurred. On May 15, 1972, one medium-sized Oxytrema silicula, 20 mm. in shell length, was placed in each of three dishes, A, B and C, with 30 ml. of water and 50 fully-embryonated eggs, 120 days old. Temperature was maintained at 20 - 22°C and a seasonal day/night light cycle was used. Dish A held eggs and stale water from a dish in which embryonated eggs had been maintained. Dish B contained eggs and aerated stale water taken from a dish in which embryonated eggs had been maintained and dish C held eggs and 20°C dechlorinated water fresh from the tap. This experiment was run for three days with observations three times a day.

The miracidia in eggs in dish A and B did not respond or hatch. Those miracidia in dish C began to respond by showing an accelerated body contraction rate within five minutes after the snail was added to the dish containing eggs and fresh water. The first hatching of miracidia was observed after 20 minutes in dish C. The hatching process was illustrated sequentially (Figure 3A-F). Following an increased contraction rate by an unhatched miracidium,

flame cell activity began after about three minutes. By five minutes after the onset of the hatching process, the two vacuoles adjacent to the vitelline membrane (Figure 3B) had increased in size. By eight to ten minutes the miracidium had moved toward the opercular end of the egg shell and the vacuoles had enlarged (Figure 3C). After 15 minutes from the onset of this process the vacuoles enlarged and appeared to coalesce to fill nearly 50 percent of the egg shell (Figure 3D). The miracidium was now contracted into a tight ball at the opercular end of the egg. The apical papilla was closely appressed and continually moved over the inner opercular surface at this time. Body contractions were nearly constant. The miracidium appeared to push against the operculum, apparently using the expanded vacuole to push against.

Miracidial hatching (Figure 3E) occurred 20 to 30 minutes after the onset of the hatching process and miracidial escape required about three seconds following operculum release. Surface cilia of the miracidium began beating the instant of opercular release. The operculum was always observed to remain attached to the egg shell at one point. It was noted that the vacuole remained in the egg shell or slowly escaped (Figure 3F) after some time.

Not all of the eggs in dish C hatched at one time. The duration of the hatching process appeared quite constant, but its initiation in the dish occurred over a period of 30 hours, by which time all eggs had hatched.

Experiment XI

The purpose of this experiment was to demonstrate possible snail species specificity for egg hatching induction in N. salmincola. On May 23, 1972, the same procedure was followed as in Experiment X, except that the snail, Flumenicola virens was used instead of Oxytrema silicula. This experiment was run for six days.

It appeared that the unhatched miracidia of N. salmincola showed no response to the presence of F. virens as compared to O. silicula.

No response or hatching was induced under any of the three test situations. Miracidial movements within the egg shell were never more than a normal contraction every three to ten minutes.

Experiment XII

The purpose of this experiment was to demonstrate the presence of possible soluble substances produced by O. silicula, which might induce egg hatching. On May 23, 1972, three dishes, D, E and F, containing 50 fully-embryonated eggs were set up. Dish D held eggs and 40 ml. of aerated water from an eight-inch culture dish holding 12 O. silicula, dish E held eggs in 40 ml. of fresh water and approximately one ml. of washed snail mucus, and dish F held eggs in 40 ml. of fresh water. This experiment was run for three days. Observations were made three times daily.

In dish D it was noted that many miracidia within the embryonated eggs, following addition of snail water, showed accelerated

contraction rates similar to those observed in eggs which were induced to hatch. (See Experiment X, p. 24.) These accelerated contractions slowed to a normal rate after nearly ten minutes and remained stable for the duration of the experiment. In dishes E and F no changes were observed in miracidial activity. No hatching was observed in any of these three experimental dishes.

Experiment XIII

The purpose of this experiment was to demonstrate permeability of a hatching inducer produced by O. silicula across a dialysis membrane and the action of the inducer on the fully-embryonated eggs of N. salmincola. On June 7, 1972, two covered, eight-inch culture dishes containing 1500 ml. of fresh, aerated, dechlorinated water were set up. Dish one contained nine, large O. silicula, 23 - 25 mm. in shell length. Dish two contained only 1500 ml. of water. One hundred embryonated eggs were placed in each of two, 23 x 55 mm., Syracuse dishes, G and H, with 40 ml. of water. Both dish G and H were separately placed inside ten-inch pieces of regenerated cellulose dialysis tubing (Carolina Biological Supply Company) so that the only contact between eggs and snails would occur across this membrane. Dish G was suspended in dish one and dish H in dish two and both maintained for 48 hours. The eggs were examined at four-hour intervals during the first 12 hours and thereafter every 12 hours. Aeration was stopped after 24 hours. A normal day/night lighting period was used and the temperature was controlled at 20 - 22°C. The egg-containing dishes, G and H, were removed from dishes one and two after 48 hours and one O. silicula was placed

in each dish. Eggs were examined at 20-minute intervals after exposure to snails.

It was noted that three miracidia had hatched by 12 hours in dish G, but that no other hatching occurred in dish G or H throughout the 48-hour experimental period.

When a snail was placed in dish G after the 48-hour period, hatching of all the remaining eggs occurred within three hours. The introduction of a snail into dish H initiated first hatching after 25 minutes and the remaining eggs hatched through the next 36 hours.

The Miracidium

The hatched miracidium of Nanophyetus salmincola (Figure 4B) measured from 0.025 - 0.040 mm. wide by 0.060 - 0.100 mm. long.

During its short life span of some 36 hours, the miracidium swam constantly, moving in apparent random directions. Its body measurements changed as it moved. When observed at low magnifications in open water, the swimming behavior of the miracidium appeared as a slow, counterclockwise spiral, parallel to the bottom of the dish for a distance. When the miracidium was changing direction in mid-water its anterior progress stopped and its posterior end sank. The body became vertically oriented while still spinning in a tight, counterclockwise spiral. The miracidium broke this tight spiral after a few seconds and resumed the parallel swimming motion again. When the apical papilla of the miracidium struck an object while swimming, it backed up and its body twisted to direct it away from the object. As the miracidium neared death, it slowed, contracted into a spherical body and sank to

the bottom.

The body of the miracidium was made up of a covering of ciliated epithelial cells over a central subepithelial mass (Figure 4A and B). The epithelial cells covered this mass, except at the apical papilla, at epithelial cell junctions and where 23 pores opened to the surface. The epithelial cells were singly nucleated and were arranged in five tiers. The first tier consisted of one cell which surrounded the apical papilla and the opening of the two pores of the penetration glands which lie just lateral and at the base of the apical papilla. The second tier of epithelial cells was made up of six cells. There were six pores opening from the subepithelial mass at the cell junctions between the first and second epithelial tiers. There were seven epithelial cells in the third tier. There were 13 pores opening from the subepithelial mass between the second and third epithelial tiers of cells, one at each cell angle (Figure 4A). The fourth tier contained three cells. The two excretory pores opened between the third and fourth tiers opposite each other across the diameter of the miracidium. The fifth epithelial cell tier consisted of one cell ensheathing the posterior end of the miracidium.

The body of the miracidium was considered to be anatomically divided into two parts -- the cephalic region, extending posteriorly through the level of the second tier of epithelial cells, and the caudal region.

Ciliation on the miracidium had a characteristic pattern of rows (Figure 4A). Epithelial tier one had one row of anterior cephalic cilia and two rows of lateral cephalic cilia. Tier two had nine rows

of lateral cephalic cilia. Epithelial tier three and four had 14 and 10 rows of lateral caudal cilia, respectively, and tier five had five caudal ciliary rows.

The large gut sac was centrally located in the cephalic region with its posterior end extending into the anterior caudal region. The anterior gut sac tapered gradually and opened at the apex of the apical papilla by a minute mouth. The contents of the gut sac were agranular and opaque.

Two flask-shaped glands, presumably penetration glands, one at each side, were lateral to the gut sac with their rounded posterior ends at the mid-cephalic level.

The minute ducts of these penetration glands passed anteriorly and opened at pores on either side at the base of the apical papilla.

Both penetration glands and the gut sac were closely surrounded by a dense arrangement which appeared to be yolk granules.

A pair of flame cells occurred mid-laterally just posterior to the gut sac. They showed rhythmic beating only after exposure to the hatching stimulus. (See Experiment X, p. 24-25.) A single excretory duct left each flame cell and curved medially to about mid-body and then turned laterally to open at an excretory pore.

From 35 to 50 germ cells, measuring 0.008 - 0.009 mm. in diameter, were centrally located in the subepithelial mass with the majority occurring in the posterior caudal region. Occasionally a few germ cells were seen as far anteriorly as the posterior part of the gut sac.

The musculature of the miracidium was not determined except that there was a circular arrangement of apical retractor muscles surrounding the base of the apical papilla. All epithelial cells appeared capable of lateral and longitudinal contractions to nearly one-half their diameter. The subepithelial mass was also capable of longitudinal contractions independent of the epithelial surface cells.

Oxytrema silicula Infection

Experiment XIV

The purpose of this experiment was to attempt to observe the penetration of Oxytrema silicula by active miracidia of Nanophyetus salmincola and obtain early developmental stages in the snail host. On June 12, 1972, 20 ml. of fresh, dechlorinated tap water and two medium-sized O. silicula were added to a dish containing approximately 500 embryonated eggs to induce hatching of miracidia. The snail and miracidial activity was observed six times daily for a period of 48 hours. The snails were then moved to an eight-inch culture dish and maintained at 20 - 22°C for three weeks with a normal day/night light cycle. One snail was examined after two weeks and the other at the end of three weeks.

The first hatching of miracidia occurred by about 20 minutes after the experiment was initiated. Numerous miracidia were often seen swimming in and around snails during the 48-hour experiment. No penetration of snail tissue by miracidia was observed, although the miracidia repeatedly collided with the snail and turned away. It was noted that miracidia were repeatedly carried in and out of the mantle

cavity with water currents produced by the snail.

Both snails moved freely in the dish and ingested large numbers of yet unhatched eggs, as well as empty egg cases. Numerous eggs, which were caught and dragged in strands of snail mucus, were seen to hatch. The mucus-encased fecal pellets passed by the snails contained great numbers of these embryonated eggs and egg cases. Examination of the defecated embryonated eggs showed that they contained active miracidia. These defecated eggs were not observed to hatch.

Thorough examinations of the snails for early developmental stages of N. salmincola, after two and three week intervals, were negative.

Experiment XV

The purpose of this experiment was to demonstrate possible attraction of the miracidia of N. salmincola for snail tissue of O. silicula. On June 13, 1972, miracidial hatching was induced as described in Experiment XIV. About 100 active miracidia were transferred into a 15 x 100 mm. petri-dish containing approximately 12 ml. of 20 - 22°C dechlorinated water. After one hour, a piece of freshly-dissected digestive gland of O. silicula, approximately one millimeter in diameter, was placed in the dish near one side. The activities of the miracidia were observed for two hours after exposure to the snail tissue.

It was observed that the active miracidia were almost evenly distributed throughout the thin water layer of the petri-dish after one hour. Within ten minutes following the insertion of digestive gland tissue of the snail to the dish, an estimated 60 percent of the

miracidia were seen swimming within a centimeter radius of this tissue, while others were evenly distributed throughout the dish. No penetration of this tissue by miracidia was observed.

Experiment XVI

The purpose of this experiment was to attempt to observe miracidial penetration into a tissue of O. silicula. On June 14, 1972, active miracidia were added to separate water drops, each containing a small portion of either shell, mucus, digestive gland, stomach wall, gills, foot, mantle or gonad of O. silicula. The activity of miracidia in association with these tissues was observed at 100 diameters magnification.

Miracidial swimming activity observed in this experiment was very similar to that described for the miracidium (see p. 28), but was apparently accelerated due to the fact that observations were made at a higher magnification. The rapidly swimming miracidia were repeatedly seen to contact snail tissue, but they never penetrated. It was observed in two out of a dozen or more contacts by miracidia with gill epithelium that they would hesitate for a few seconds before swimming away. When contact was made with other tissues the miracidia always changed course at once and sped away.

Experiment XVII

The purpose of this experiment was to attempt to demonstrate the early developmental stages of Nanophyetus salmincola in the snail, Oxytrema silicula. On June 7 and 14, 1972, 18 snails, 17 to 25 mm. in

shell length, were exposed to estimated concentrations of 1,000 hatched miracidia for a period of 24 hours. No penetration by miracidia was observed during the 24-hour exposure period. Six snails died during the first three weeks in the laboratory, but were not infected. Beginning the fourth week following exposure to miracidia, at least two snails were examined each week.

No larval stages were observed from two snails examined 28 days following exposure to miracidia.

Two of the three snails examined 33 days after exposure to miracidia were found to be infected with sporocysts (Figure 5A). Two sporocysts were found in a 20 mm. snail and three sporocysts were found in a 23 mm. snail. The sporocysts, measuring 0.050 - 0.061 mm. in diameter, were found in both snails loosely associated with connective tissue adjacent to the stomach just above the foot. The central cavities of these sporocysts contained from 20 to 35 germ balls. Each sporocyst was encapsulated by a fibrous cyst wall 0.013 - 0.016 mm. thick at this stage. The sporocyst is described below. (See p. 35.)

Two 20 mm. snails examined 36 days following exposure to miracidia were found to be infected with sporocysts. Three sporocysts were observed, all just under the epidermis at the margin of the foot of the snail. When observed under the dissecting microscope, the deeply-pigmented upper surface of the foot appeared lighter in color over the slightly swollen areas containing sporocysts. When the sporocysts were dissected out, they adhered slightly to surrounding tissues. These sporocysts appeared very similar to those described above, except that they were larger, their central cavities were

completely filled with larger, slightly more developed germ balls and the fibrous cyst encapsulating them was much thinner. (See p. 36.)

After 44 days following exposure to miracidia, two sporocysts were observed from the foot of a 22 mm. snail. These sporocysts (Figure 5B) were considerably larger, (see p. 36), than earlier sporocyst stages observed and were completely filled with immobile, immature rediae. No vestige of the fibrous cyst wall observed in earlier sporocysts was present. The sporocysts were easily popped out once the host tissue was torn.

One redia (Figure 5C) was observed to pass out through a narrowed area, presumably a birth pore (Figure 5B), at the blunt end of the sporocyst while coverslip pressure increased during examination. This redia showed no motion.

The immature rediae were identified as those described by Bennington (1951) and Bennington and Pratt (1960) as immature rediae of Nanophyetus salmincola.

The Sporocyst

The sporocyst of Nanophyetus salmincola (Figure 5A) observed after 33 days of development in Oxytrema silicula was nearly spherical and measured from 0.50 - 0.61 mm. in diameter. There was an apparent fibrous capsule surrounding the sporocyst that was 0.013 - 0.016 mm. thick. This capsule was presumably host-derived. Spaces were usually present between the capsule and the sporocyst in specimens not compacted with a cover slip.

The sporocyst was relatively simple in structure and consisted

of a body wall composed of a dense outer region 0.016 - 0.018 mm. thick and a less dense inner one 0.031 - 0.047 mm. thick. The body wall surrounded a central cavity which contained 16 to 20 germ balls loosely attached to the inner body wall. Agranular germ cells, 0.012 - 0.018 mm. in diameter were seen scattered in the inner body wall region.

Germ balls of the sporocyst were composed of from 20 to 38 agranular cells measuring 0.004 - 0.007 mm. in diameter. Sizes of germ balls varied from 0.047 - 0.078 mm. in diameter.

Sporocysts taken from the snail after 36 days measured 0.59 - 0.66 mm. in diameter. The fibrous cyst wall had thinned and measured only 0.004 - 0.006 mm. thick. The epithelial wall of the sporocyst measured 0.022 - 0.054 mm. in thickness and did not appear differentiated as observed in earlier sporocysts described above. Germ balls at this stage varied in size from 0.094 - 0.117 mm. in diameter and contained a central cell mass surrounded by a single layer of cells, presumably primitive epithelium.

Sporocysts removed from the snail after 44 days of development appeared somewhat flask-shaped (Figure 5B) and measured 0.70 - 0.76 mm. wide by 0.98 - 0.105 mm. long. The sac-like body wall of germinal epithelium varied in thickness from 0.023 - 0.057 mm. At one end of the sporocyst, the epithelial walls tapered to a blunt point. There was a funnel-like opening from the central cavity of the sporocyst leading into this blunt point. When pressure was exerted by the coverslip, a redia moved up this funnel and passed out on an otherwise unseen birth pore. The cavity of the sporocyst was filled with 23 - 25 immobile, immature rediae.

The immature redia (Figure 5C) measured from 0.14 - 0.16 mm. wide by 0.36 - 0.47 mm. long. The body was vermiform, narrowing slightly to a blunt anterior end and to a rounded, or slightly pointed, posterior end. The body wall of the redia consisted of a thin, non-cellular covering, 0.003 - 0.004 mm. in thickness, over a germinal epithelial layer 0.022 - 0.028 mm. thick. The anterior end of the redia was centrally depressed at the mouth which opened into a large, muscular pharynx, measuring 0.055 - 0.067 mm. wide by 0.56 - 0.70 mm. long. A 0.26 - 0.30 mm. long, sac-like gut extended free, posteriorly from the pharynx, into the cavity of the redia. The body cavities of the rediae were partially or nearly completely filled with spherical germ cells measuring 0.014 - 0.026 mm. in diameter. No birth pore was evident in rediae taken from the sporocyst and no movement of the sporocyst or immature rediae contained in the sporocyst was observed.

DISCUSSION

Dispersal of the eggs of Nanophyetus salmincola was reported to be accomplished in nature by defecation of infected, warm-blooded, definitive hosts (Farrell and Lloyd, 1962). Millemann and Knapp (1970) reported that numerous carnivores indigenous to the Pacific Northwest of the United State have been found to be infected with this parasite.

In preparation for this research, the author attempted to hypothesize environmental situations into which the eggs of Nanophyetus salmincola might fall. It was assumed egg-bearing feces might fall onto land or into water at all times during the year, but most commonly during late fall and winter when spawned-out, dead salmon carrying metacercariae are commonly on the river banks and easily eaten by carnivores. These eggs might then be exposed to temperature extremes, desiccation, fecal concentration, or be washed from feces into streams or pools.

When unembryonated eggs were first collected for this research I assumed that the washed eggs of N. salmincola would develop when incubated as reported by most earlier workers. (See p. 46.) This assumption required nearly five months of experimentation to be proved false. Research was begun in mid-July, 1971. First development of eggs was observed in November, 1971, following an extended period of egg exposure to 5°C prior to incubation at 20 - 22°C.

My first experiment to determine the effect of fecal material on the eggs of N. salmincola indicated that the eggs deteriorated more rapidly at higher temperatures of incubation and when exposed to more concentrated fecal material. Smyth (1966) reported that the presence

of feces generally inhibits hatching or embryonation in trematode eggs, although Fasciola hepatica eggs remain viable in feces. The precise influence by fecal material upon the eggs of N. salmincola has not been determined, but the vacuolization of the eggs suggested a possible osmotic disruption. This experiment suggested that the eggs of N. salmincola must be washed away from feces in nature if they are to remain viable.

Desiccation of N. salmincola eggs indicated the water permeable nature of these eggs. Water loss was quite evident and the eggs were not able to recover when rehydrated. Clegg and Smyth (1968) reported that most trematode eggs studied were permeable to water and solutes up to a molecular weight of 150. This experiment indicated that drying of eggs of this trematode in nature was probably lethal.

Experiment III showed quite clearly that the eggs of N. salmincola degenerated after they were washed from feces and incubated at various temperatures ranging from 17 - 39°C. These results agreed with those of Donham et al. (1926) and Donham (1928), but differed from results of numerous workers who reported development at temperatures within this range (Price, 1929; Simms, Donham and Shaw, 1931; Bennington, 1951; Bennington and Pratt, 1960; Filimonova, 1965, and Millemann and Knapp, 1970). The fact that eggs remained in the unembryonated state (Figure 1B) when maintained at 5°C, indicated that the unembryonated eggs may remain viable in nature for up to 70 days in water at low temperatures.

It appeared that freezing temperatures destroyed the unembryonated eggs of N. salmincola. The results were somewhat similar to

egg exposure to fecal material. The appearance of vacuoles within the egg shell suggested that during the freezing or thawing process, osmotic imbalance occurred. Bečejac and Lui (1959), studying the development of Fasciola hepatica eggs, reported that the predevelopmental and final stages were least resistant to freezing. In nature, eggs of N. salmincola that chance to freeze probably do not develop, either as a result of water loss during freezing or due to destruction by ice crystals within the egg shell. It was not determined in this study whether or not later embryonic stages of N. salmincola were resistant to freezing.

Bennington (1951) suggested that development and hatching were enhanced when eggs were in larger volumes of water. The results of Experiment V did not substantiate his findings, but rather agreed with the results of Experiment III in that degeneration of all eggs occurred.

The results of both Experiments III and V indicated that development was dependent on a factor or factors not yet attempted by this worker.

Harris, Harkema and Miller (1970), working with the eggs of Procyotrema marsupiformis, reported that chilling at 4°C was instrumental in the development process and hatching of these eggs. The author attempted cold exposure on the eggs of N. salmincola prior to incubation at an elevated temperature. It was already known from Experiment III that eggs remained in a normal, unembryonated state at 5°C. The results of Experiments III, V and VI were interpreted to indicate that the unembryonated eggs of N. salmincola had an absolute requirement for exposure to 5°C in this study before they would develop

at an elevated temperature.

Earlier workers, studying egg development of N. salmincola did not report whether or not cold exposure prior to incubation was used. In the opinion of the author, Donham et al. (1926) and Donham (1928) must have incubated freshly-washed eggs from feces since no development was observed. In order to have obtained development, other workers, cited in the introduction, for convenience must have refrigerated or somehow cold-treated egg or fecal samples for a period of time prior to incubation and neglected to report this fact.

The results of Experiment VII again showed that 5°C exposure acted somehow to "trigger" development in subsequent warmer incubation. This experiment also indicated that the number of days of this cold exposure influenced the percentage of eggs that developed within a sample. The optimum exposure time at 5°C was found to be 40 to 64 days. Following these periods of cold exposure, 20 - 22°C, incubation produced 100 percent development of eggs to a fully-embryonated stage (Figure 3A). This data, correlated with earlier degeneration rates noted in eggs not exposed to 5°C, suggested that those N. salmincola eggs passed in host feces during the colder winter months should have the best chance of developing when stream temperatures elevated in the spring. Short and extremely long, cold exposure probably decreases the chances that eggs would develop in nature.

Clegg and Smyth (1968) reported that "overwintering" of turbellarian eggs occurred commonly, but that little was known of trematode cold resistance. Fasciola hepatica eggs have been reported to

remain viable for two and one-half years in a refrigerator (Clegg and Smyth, 1968). Friedl (1960) reported that only 50 percent of the eggs of Fascioloides magna were viable when held one year at 5°C and Kusel (1970) stated that eggs of Schistosoma mansoni remained viable only one week at 8°C. Eggs of N. salmincola exposed for 113 days to 5°C apparently were "triggered" to develop when incubation was begun, but this extended cold exposure somehow reduced their viability and having reached the stage illustrated in Figure 2B, they degenerated.

The low percentage of egg-hatching noted in Experiment VII suggested that there were some environmental factors yet untried by this worker that would influence hatching. These results seemed to correlate with an earlier report by Simms, Donham and Shaw (1931) where only one miracidium of N. salmincola hatched. Bennington and Pratt (1960) reported hatching of N. salmincola miracidia, but the percentage was not reported. Pratt (1972), in a personal conference with this worker, stated that the degree of hatching of N. salmincola eggs observed by him and Bennington had been very low.

Millemann and Knapp (1970) reported first hatching after 31 days of incubation, but did not indicate the hatching percentage until 156 days of incubation when 75 percent hatching had occurred. None of the above workers stated that they actually observed hatching so that a description of hatching was not available. The hatching success reported by Millemann and Knapp (1970) was in direct conflict with this worker's results. Using comparable incubation methods, except that periods of 5°C exposure were used to induce development, only ten percent hatching of eggs had occurred after 180 days. After considerable

consternation about these discrepancies in results, this worker concluded that since Millemann and Knapp had not described the miracidium of N. salmincola, after reporting 75 percent hatching, their observations might not have been hatching, but might be attributed in part to results observed in Experiment III (see p. 16) where up to 25 percent opercular release was observed in degenerating eggs after only 70 days of incubation.

With the results obtained in earlier experiments as a guideline, Experiment VIII clearly illustrated the distinctive developmental stages of the miracidial development of N. salmincola. These results concurred with those of numerous workers studying N. salmincola eggs, as well as other trematode eggs, indicating that trematode egg development was not significantly influenced by light exposure and that temperature elevation tended to accelerate development (Jepps, 1933; Bečejac and Lui, 1959; Bennington and Pratt, 1960; Rowcliffe and Ollerenshaw, 1960, and Filimonova, 1965).

Rees (1940) reported that there was similarity in all miracidial development involving a modified spiral determinate cleavage when compared to the acoels. There have been few reports on trematode egg development. The most complete reports were those of Johnson (1920); Price (1931); Bennett (1936); Pin-Dji Chen (1937), and Rees (1940). This work with N. salmincola embryonic development is in agreement with these workers.

The low hatching rates observed by other workers studying N. salmincola, cited in the introduction, as well as the results observed in Experiment VII, prompted an investigation into methods to

obtain extensive hatching of the miracidia of N. salmincola.

The accelerated activity of miracidia in fully-embryonated eggs, observed in Experiment IX, following exposure to snail aquarium water, suggested that some substance or substances were produced by Oxytrema silicula to induce hatching. ✓

Experiment X showed that hatching of extensive numbers of miracidia occurred when (1) one Oxytrema silicula was placed in a dish containing fully-embryonated eggs and (2) fresh water was added to the container. All fully-embryonated eggs in the dish would hatch by 36 hours, with first hatching occurring after 20 minutes, following the combination of eggs, snail and fresh water.

The results of Experiment XI suggested that the hatching inducer produced by O. silicula may be species specific, because hatching was not induced by the snail, Flumenicola virens, which is the other common stream snail coexisting with Oxytrema populations.

The hatching inducer produced by O. silicula, observed in Experiment XII, did not appear to remain long in water taken from aquaria containing these snails nor was it apparent in snail mucus. These results, correlated with Experiment X, suggested to the author that the inducer was continually produced by O. silicula, but must somehow change chemically or was no longer present in the water after a few minutes. This inducer was not only short-lived, but was of relatively small molecular weight and soluble to a degree, since it passed across a dialysis membrane to condition somehow fully-embryonated eggs. ✓ ✓

Only two other instances of host-produced hatching inducers affecting helminth eggs have been reported. Clegg and Smyth (1968)

reported there was evidence that hatching of eggs of the monogenean, Squalonchocotyle torpedinis, was stimulated by a chemical factor released by the fish host, Torpedo sp. The other report (Rogers and Sommerville, 1963) was analogous in that the hatching of eggs of the potato-root nematode, Heterodera rostochiensis, was stimulated by "eclectic" acid diffusion from roots of the host plant. Byrd and Maples (1964) suggested that eggs of the fluke, Dasymetra conferta, hatched in response to a stimulus supplied by the host snail.

I concluded that the low percentage of "spontaneous" hatching that occurred from noninduced, fully-embryonated eggs of N. salmincola was due solely to muscular movements of the miracidium within the egg shell. The hatching process induced in nature probably involves the presence of fully-embryonated eggs of N. salmincola immediately adjacent to the snail, Oxytrema silicula, in the flowing stream or in pools along the streams following a rain shower. ✓

Much more research is required to determine the precise hatching mechanism for N. salmincola eggs. Observations made during Experiment X following hatching induction suggested that at least two and possibly three factors may act in miracidial hatching. The hatching inducer noted in this study not only stimulated an increased rate of muscular contractions in the miracidium, but also acted somehow to initiate flame cell activity. Reisinger (1923) reported that flame cell activity was associated with osmotic adjustment in miracidia in egg capsules of Schistosoma haematobium. It was probable that activated flame cells of unhatched miracidia of N. salmincola regulated water balance and caused enlargement of egg shell vacuoles during the

hatching process. This water uptake phenomenon was also suggested by Mattes (1926), Rowan (1956) and Wilson (1968) in that the uptake of environmental water by the eggs of Fasciola hepatica was essential to egg hatching.

Kusel (1970) reported that vacuoles found in embryonated eggs of Schistosoma mansoni were connected to the miracidium in some manner and acted in environmental water uptake. Vacuoles within the egg of N. salmincola appeared to be associated with the vitelline membrane in fully-embryonated eggs.

Hatching enzymes produced by miracidial penetration glands were reported to act in opercular release by Barlow (1925), Rowan (1956, 1957) and Clegg and Smyth (1968). The constant movement of the apical papilla over the inner opercular surface by the miracidium of N. salmincola following the hatching stimulus suggested that a hatching enzyme may be secreted in this species.

Nearly every reported instance of trematode egg hatching has indicated muscular movement aided hatching. Muscular movements were suggested to aid hatching in N. salmincola by Bennington (1951) and Bennington and Pratt (1960). Strong muscular movements produced by miracidium in this study definitely act in opercular release.

The hatched miracidium of Nanophyetus salmincola was found to be dissimilar in many respects to the miracidium of the Siberian subspecies, N. s. schikhobalowi, as described by Filimonova (1965). (See Introduction, p. 6-7 and The Miracidium, p. 28-31.) The incomplete miracidial description of Bennington (1951) and Bennington and Pratt (1960) more closely resembled miracidia of N. salmincola. The

anatomical structures of the miracidium described in this study were named using the classic terminology after Bennett (1936). The so-called gut sac opening at the mouth probably has no digestive function, but rather acts in hatching or as a gland in conjunction with host penetration (Reisinger, 1923; Price, 1931; Bennett, 1936, and Rees, 1940).

I was able to obtain and observe great numbers of Nanophyetus salmincola miracidia and produced the first reported experimental infections of Oxytrema silicula. Results obtained in Experiments XIV and XVI were inconclusive. Although repeated contacts by miracidia with snails or snail tissue were observed, no penetration by miracidia was seen. The only suggestion of miracidial attraction to their snail host, so widely reported for other trematode species, was observed in Experiment XV when most miracidia tended to aggregate near snail digestive gland tissue. ✓

Sporocyst stages taken from Oxytrema typically occurred in the foot region of the snail. Infections were light, even though massive miracidial exposure was used. It is postulated that N. salmincola miracidial penetration of O. silicula is a chance occurrence, similar to the infection of the snail host by Zoogonoides laevis (Stunkard, 1943). Development of the sporocyst of N. salmincola was quite slow. The earliest stages were not discovered until the fourth week following exposure to miracidia. The fibrous encapsulation of the sporocyst was probably of host origin, since as development progressed and the sporocyst grew, this capsule was reduced and disappeared, possibly due to a digestive activity of the sporocyst wall. Sporocyst wall digestive

function has been suggested by Clegg and Smyth (1968).

The immature rediae, developed from germ balls in the sporocyst, were nonmotile. Characteristics of these immature rediae fitted the description of motile, immature rediae of N. salmincola found in gonad and digestive gland of O. silicula by Bennington and Pratt (1960).

Philip (1955) reported that it was possible that one or a few miracidia may be responsible for large numbers of asexually-produced generations occurring in snail hosts. Bennington and Pratt (1960) reported numerous, heavy redial infections in Oxytrema. If light N. salmincola miracidial infections occurred in nature, as produced by this worker in the laboratory, two or three miracidia could produce from 50 to 75 rediae, a considerable infection.

Table 1. Effects of fecal material on embryogenesis in Nanophyetus salmincola. Twenty-five eggs were observed and their appearance recorded at each examination.

Incubation Temperature (C°)	Lighting	Water	Total Yolk Cell Degeneration (Day of Incubation)	Egg Content Vacuolization (Day of Incubation)
9 - 10	D	WU	10	1
15 - 16	D	WU	7	1
17 - 18	N	WU	5	1
17 - 18	D	WU	7	1
20 - 22	D	WU	3	1
20 - 22	N	WU	5	1
23 - 24	D	WU	2	1
9 - 10	D	FMD	15	4
17 - 18	D	FMD	10	3
17 - 18	N	FMD	12	3
20 - 22	D	FMD	10	6
23 - 24	D	FMD	9	5

WU = water unchanged; FMD = fecal material diluted; D = continuous lighting exposure;
N = continued dark exposure.

Table 2. Incubation of 50-egg samples of Nanophyetus salmincola under stable experimental conditions.

Incubation Temperature (C°)	Egg Collection	Lighting	Total Yolk Cell Degeneration (Day of Incubation)	Opercular Release (%)	
				Day 31	Day 70
5	W	D	-	0	0
5	W	N	-	0	0
5	S	D	-	0	0
15 - 16	W	D	45	0	0
17 - 18	W	D	43	0	0
17 - 18	S	D	40	0	0
20 - 22	W	D	36	3	10
20 - 22	S	D	35	2	10
20 - 22	S	N	36	2	9
20 - 39	S	D/N	32	3	15
20 - 39	S	D	30	3	20

W = egg collection washed from feces; S = collection from eggs shed by isolated trematodes;
D = continuous lighting; D/N = day/night light cycle; N = continuous dark exposure.

Table 3. Incubation of 50-egg samples of Nanophyetus salmincola under experimental conditions with routine water change.

Incubation Temperature (C°)	Lighting	Water Source	Total Yolk Cell Degeneration (Day of Incubation)	Opercular Release (%)	
				Day 31	Day 70
5	D/N	DcW	-	0	0
5	N	DcW	-	0	0
17 - 18	D/N	DcW	45	0	0
17 - 18	D/N	SnW	43	0	0
20 - 22	D/N	DcW	36	4	12
20 - 22	N	DcW	36	3	10
25 - 27	D/N	DcW	34	5	15
20 - 39	D/N	SnW	30	5	25

D/N = day/night light cycle; N = continuous dark exposure; DcW = dechlorinated water;
SnW = water from snail aquarium.

Table 4. Incubation of 200-egg samples of Nanophyetus salmincola under experimental conditions using large water volumes.

Incubation Temperature (C°)	Aeration	Total Yolk Cell Degeneration (Day of Incubation)	Opercular Release (%)	
			Day 31	Day 70
9 - 10	Continuous	55	0	0
9 - 10	None Used	50	0	0
20 - 22	Continuous	37	2	8
20 - 22	None Used	35	3	10
27 - 30	Continuous	20	5	15
27 - 30	None Used	18	5	18

Table 5. Effect of exposure to 5°C before incubation on development and hatching percentage on 500-egg samples of Nanophyetus salmincola.

Incubation		Exposure 5°C (Days)	Day 6 (Stage)	Day 15 (Stage)	Day 37 % Fully-Embryonated	Day 180 % Hatched Eggs
Date Begun	C°					
11-11-71	5	0	1B	1B	0	0
11-11-71	20 - 22	0	-	-	0	0
11-11-71	30	0	-	-	0	0
11-12-71	8 - 10	1	1B	1B	0	0
11-12-71	20 - 22	1	1D	2B	10	1
11-22-71	20 - 22	11	1D	2B	60	3
11-22-71	15 - 30	11	1E	2C	40	2
12- 6-71	20 - 22	25	1D	2B	85	6
12-14-71	20 - 22	33	1D	2B	95	8
12-20-71	20 - 22	40	1D	2B	100	10
1-18-72	20 - 22	64	1D	2B	100	10
1-18-72	15 - 30	64	1E	2C	70	5
1-25-72	15 - 30	71	1E	2C	65	5
3- 5-72	20 - 22	113	1D	2B	0	0

1B = unembryonated egg; 1D = four-cell stage; 1E = eight to nine-cell stage; 2B = 0.023 - 0.025 mm. by 0.043 - 0.047 mm. embryo; 2C = 0.030 - 0.035 mm. by 0.015 - 0.47 mm. embryo.

Figure 1. Detailed drawings of embryogenesis of Nanophyetus salmincola incubated through ten days at 20 - 22°C following exposure to 5°C.

- A. External surface of egg shell as seen with reflected light.
- B. Unembryonated egg prior to incubation.
- C. Two-cell stage as seen at five days of incubation.
- D. Four-cell stage as seen at six days of incubation.
- E. Eight to nine-cell stage as seen at eight days of incubation.
- F. Twenty-two to twenty-four cell stage as seen at ten days of incubation.

Symbols:

ak = abopercular knob	vm = vitelline membrane
e = embryo	yc = yolk cell
op = operculum	yg = yolk granule
ps = perivitelline space	z = zygote

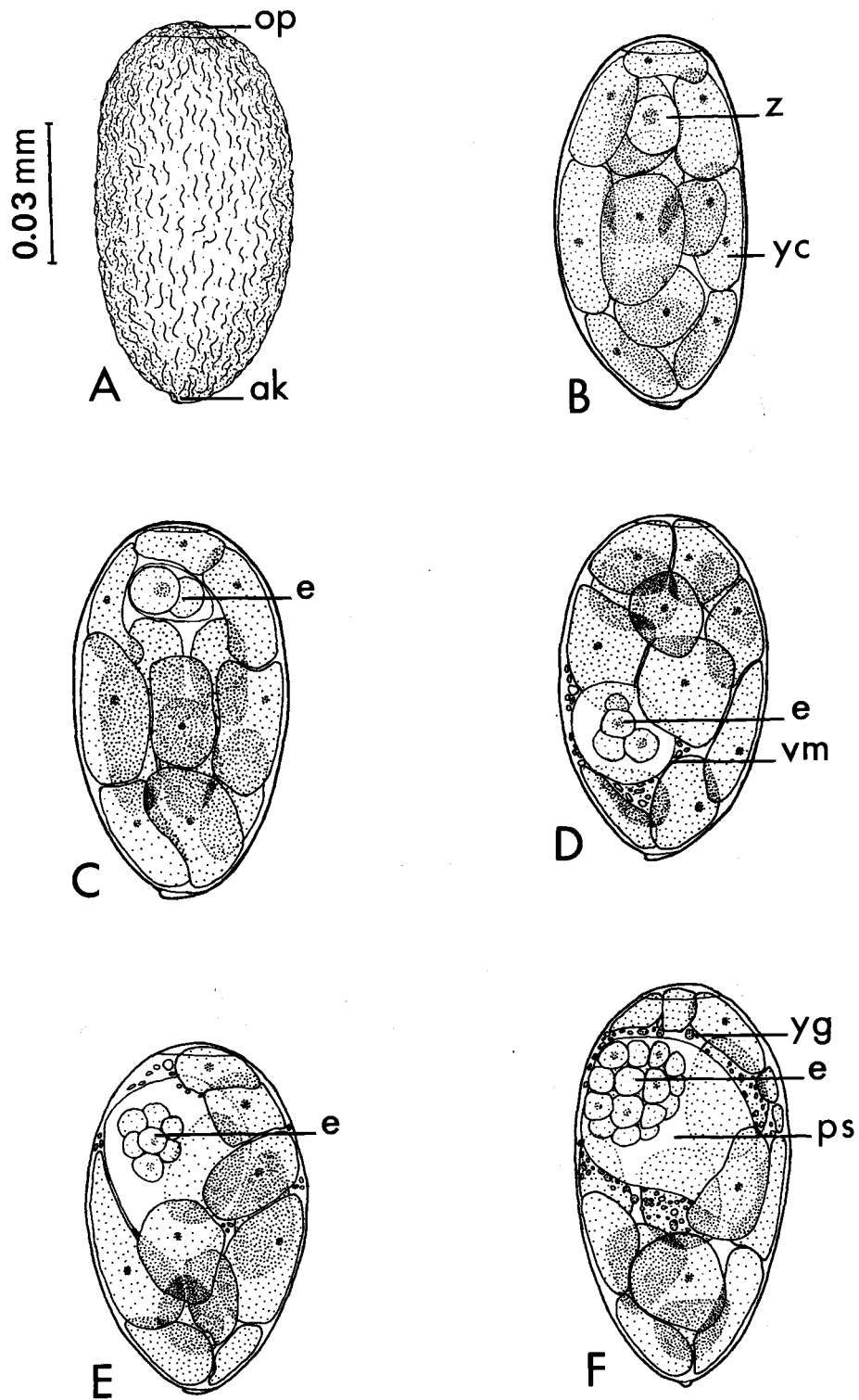


Figure 1

Figure 2. Detailed drawings of embryo development of Nanophyetus salmincola when incubated from 11 through 30 days at 20 - 22°C following exposure to 5°C.

- A. Ovoid embryo after 12 days of incubation
- B. Embryo after 14 days of incubation.
- C. Embryo after 16 days of incubation.
- D. Embryo after 20 days of incubation.
- E. Embryo after 30 days of incubation.

Symbols:

ap = apical papilla
cp = ciliary primordium
ep.n₄ = nucleus of epithelial cell of tier four
gcp = germ cell primordium
gs = gut sac
pep₁₋₅ = primitive epithelial cells of tiers 1-5
se = subepithelium

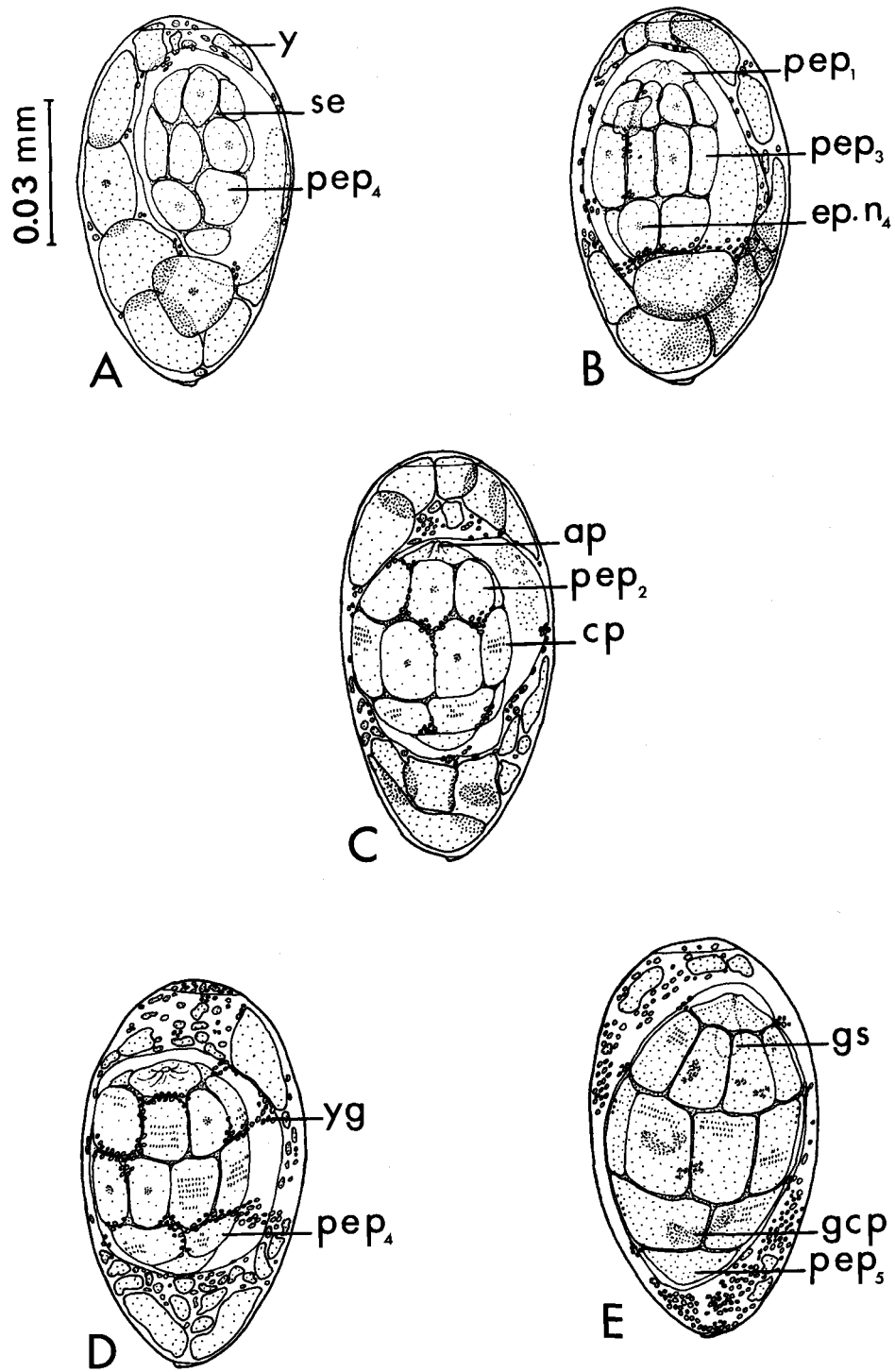


Figure 2

Figure 3. Detailed drawings illustrating the hatching process of the miracidium of Nanophyetus salmincola.

- A. Appearance of fully-embryonated egg containing a miracidium when incubated from 37 to 180 days.
- B. Appearance of contracting miracidium and enlarging vacuoles five minutes after exposure to the snail, Oxytrema silicula, and fresh water to initiate the hatching process.
- C. Contracted miracidium and enlarged vacuoles eight to ten minutes following initiation of the hatching process.
- D. Contracted miracidium and enlarged vacuole 15 minutes following initiation of the hatching process.
- E. Escaping miracidium after 20 to 30 minutes following initiation of hatching.
- F. Empty egg case of Nanophyetus salmincola showing escaping vacuole three hours after hatching.

Symbols:

ee = empty egg shell	op = operculum
ep ₂ = epithelial tier two	se = subepithelium
fc = flame cell	v = vacuole
gc = germ cell	wg = waste granule
gs = gut sac	yg = yolk granule
m = miracidium	

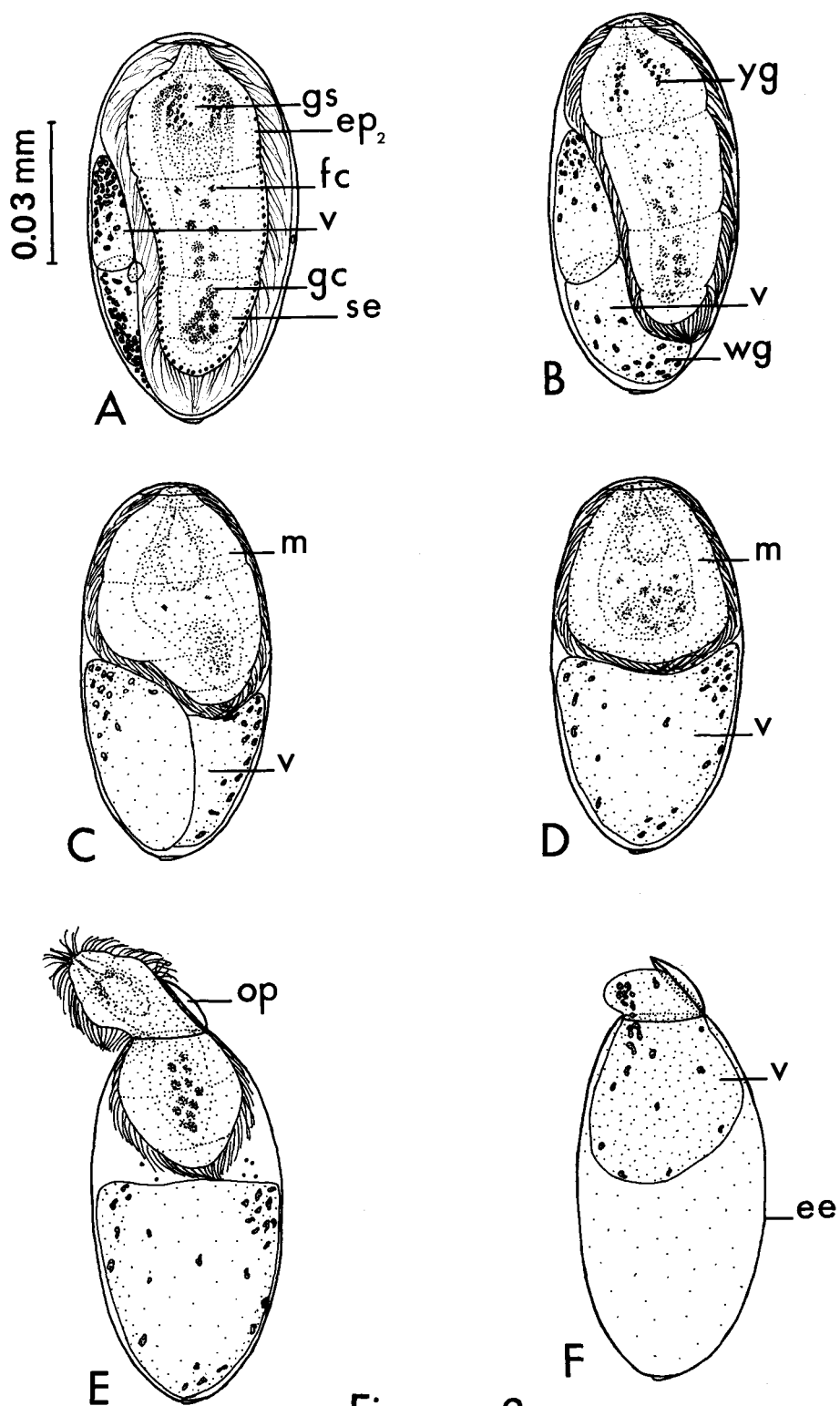


Figure 3

Figure 4. Detailed drawings of the miracidium of Nanophyetus salmincola.

A. Surface diagram showing cilia arrangement, openings of subepithelial pores and the outline of epithelial cells.

B. Hatched miracidium.

Symbols:

acc	= anterior cephalic cilium
ap	= apical papilla
cc	= caudal cilium
cr	= ciliary row
ep	= excretory pore
ep ₁₋₅	= epithelial cells of tiers 1-5
et	= excretory tubule
ep.n ₃₋₄	= nuclei of epithelial cells of tiers 3-4
fc	= flame cell
gc	= germ cell
gs	= gut sac
lcc	= lateral cephalic cilium
mo	= mouth
pg	= penetration gland
pgd	= penetration gland duct
rm	= apical retractor muscle
se	= subepithelium
sp	= subepithelial pore
yg	= yolk granule

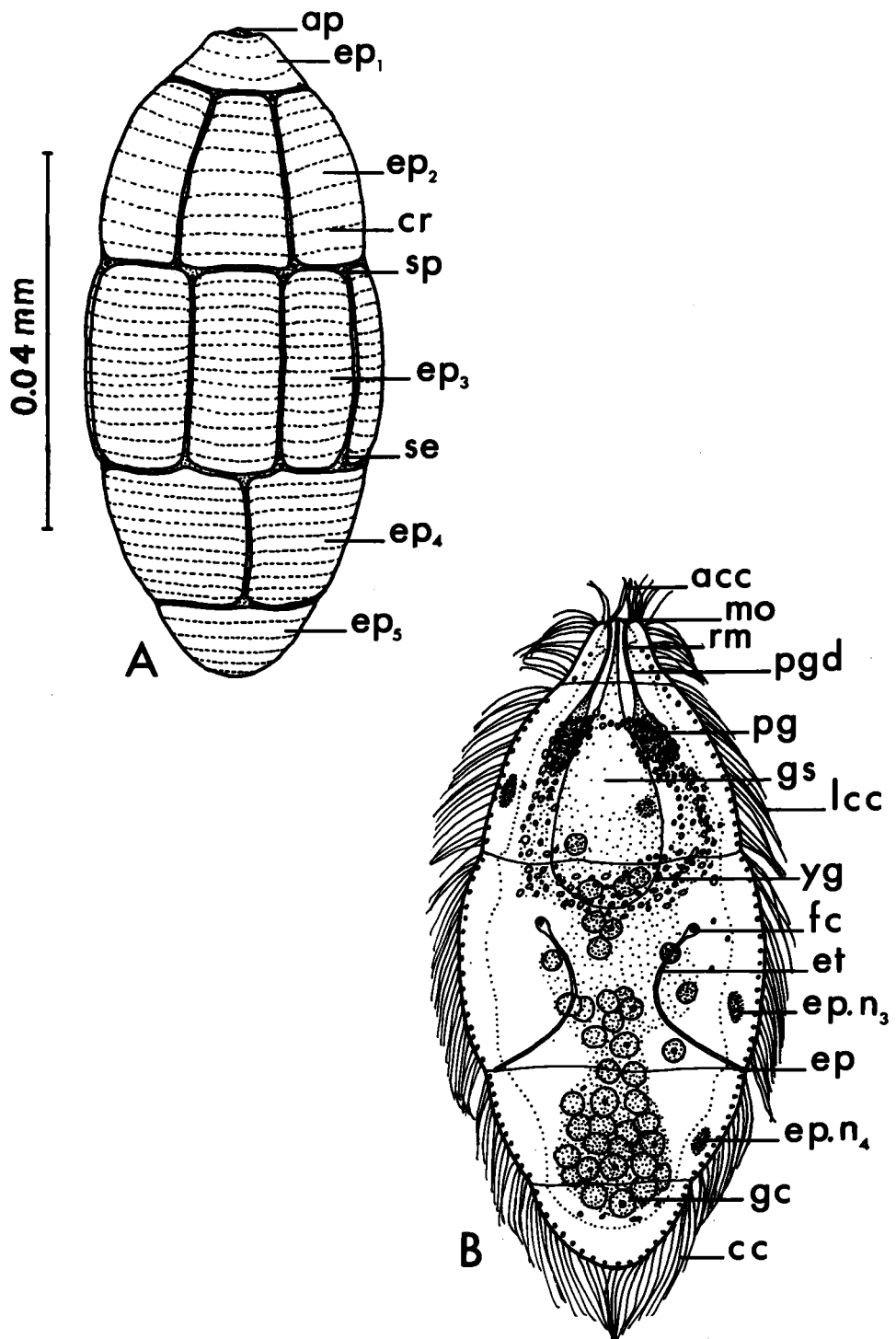


Figure 4

Figure 5. Detailed drawings of the structure of the sporocyst of Nanophyetus salmincola, freshly dissected from Oxytrema silicula.

- A. An encapsulated, immature sporocyst after 33 days of development.
- B. The mature sporocyst containing immature rediae after 44 days of development.
- C. Immature redia taken from the mature sporocyst.

Symbols:

bp = birth pore	iw = inner region of sporocyst wall
cv = cuticle	mo = mouth
g = gut	ow = outer region of sporocyst wall
gb = germ ball	ph = pharynx
gc = germ cell	r = redia
ge = germinal epithelium	
hc = host-derived capsule	

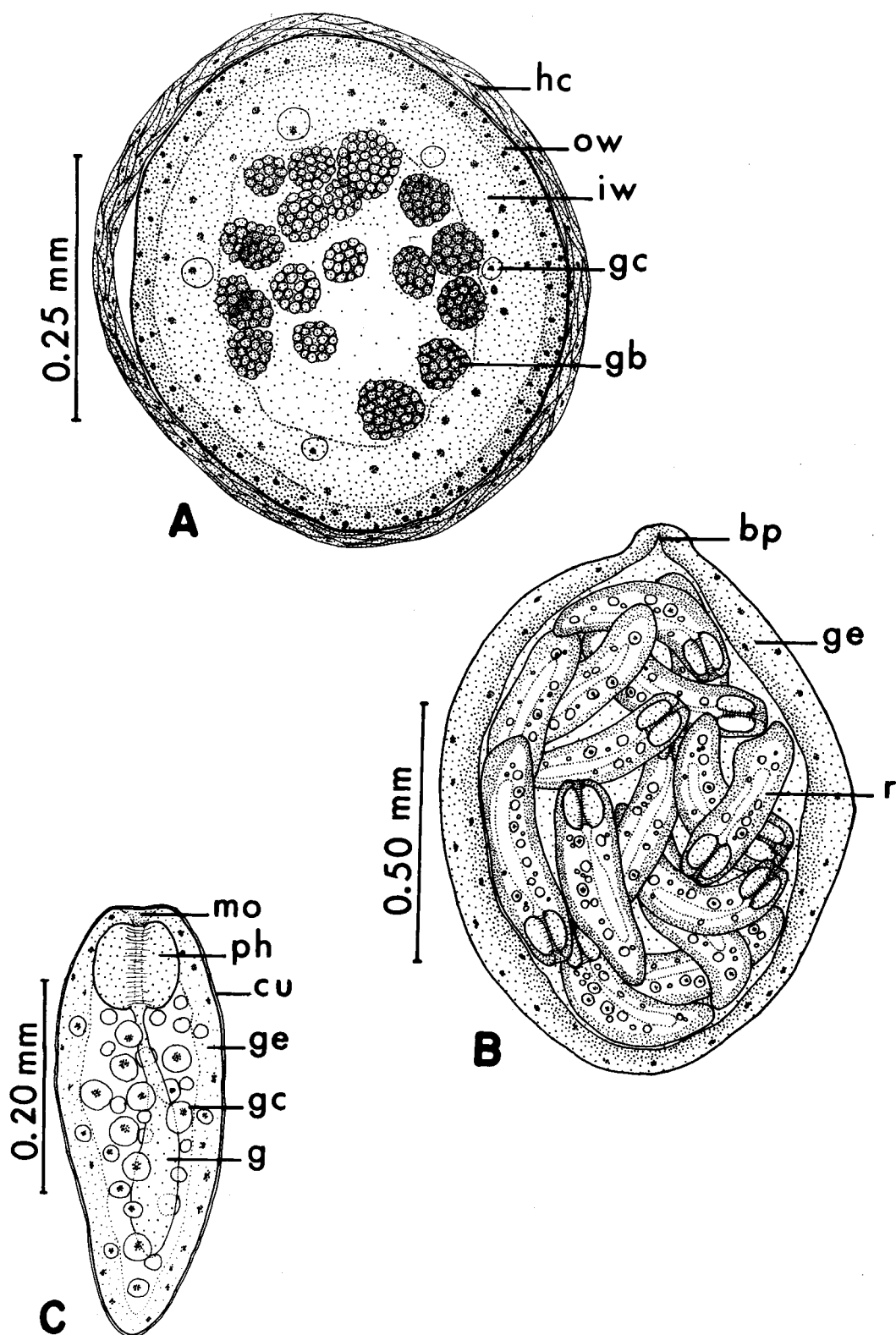


Figure 5

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