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Biological Studies on the Miracidia of Paramphistomum Microbothrioides, Price and McIntosh, and Its Intermediate Snail Host, Stagnicola (Nasonia) Cubensis, Pfeiffer.

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BIOLOGICAL STUDIES ON THE MIRACIDIA OF PARAMPHISTOMUM MICROBOTHRIOIDES PRICE AND MCINTOSH AND ITS INTERMEDIATE SNAIL HOST, STAGNICOLA (NASONIA) CUBENSIS PFEIFFER

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ABSTRACT

This study was designed as an exploratory investigation to determine experimentally some of the reactions of the miracidia of a particular species of trematode to some of the various environmental conditions that might be encountered in nature and the ability of the miracidia to penetrate and establish themselves in the intermediate snail host. Past reports on the biology of amphistome miracidia have been restricted to more or less incidental observations included in life-history accounts. A knowledge of the biology of the snail vector of any fluke is essential to a sound approach to fluke control.

Eggs of P. microbothrioides were secured by collecting the adult worms from the rumens of cows, Bos taurus, and placing them in warm water where the eggs were laid. The eggs were incubated in constant temperature ovens and stimulated by chilling to hatch at desired times.

Specimens of the intermediate snail host, S. cubensis, were exposed to miracidia in standard reference water, a laboratory-prepared medium, uniform in composition, free of organic compounds and containing the minerals usually found in surface waters.

The eggs were rapidly destroyed by desiccation when removed from water, and freezing for one to four hours caused a marked decrease in viability. Those frozen five hours or longer were completely destroyed.

Refrigeration of undeveloped eggs at 5° C. prevented development; but the viability of the eggs in one lot decreased from 69% to 30% during twelve months of refrigeration, and the minimum incubation period for

development to hatching at 30° C. increased from nine to eleven days after eleven months of refrigeration.

The time required for the miracidia to develop to the hatching stage varied with temperature. The minimum period was eleven days at 28° C., nine days at 30° C., and eight days at 35° C. Development failed to proceed to the hatching stage at 38° C. and at 40° C. There was a constant, slow hatching rate at 28° C. and at 30° C. They were never observed to hatch sporadically at constant temperatures.

Reduced oxygen tension arrested or retarded development of the eggs. They were effectively stored by sealing in boiled or unboiled water for periods of up to 60 days with little reduction in the viability of undeveloped eggs. Partially developed embryos were arrested in their development for periods of up to 74 days by sealing in boiled water.

Development and hatching were not affected by pH values between 5.5 and 8.2, but the longevity of the miracidia was considerably reduced in weakly acid waters.

Light did not serve as a stimulus for hatching, but a decrease in temperature of five or more degrees Centigrade for eggs incubated at temperatures varying between 21° C. and 35° C. stimulated immediate hatching of large numbers of miracidia. Increases in temperature failed to stimulate hatching. When mature miracidia were induced to hatch by chilling, they showed ciliary activity for about three minutes before the operculum opened. Exit from the egg shell appeared to be passive on the part of the miracidia and required about one minute.

The longevity of the miracidia was correlated with temperature. At a mean temperature of 24° C., the maximum life-span was about

fourteen hours; at a mean temperature of 33° C., it was about seven hours. A 1% solution of sucrose did not extend the longevity, nor did a 0.5% peptone solution; but 1% solutions of glucose, fructose, galactose and mannose prolonged life for several hours.

Miracidia exhibited a negative phototropism in both direct and diffuse light, and they indicated a preference for the bottoms of containers into which they were placed.

Mass exposure of the miracidia to the snail host resulted in infestation of about 87% of the snails. Exposure of single miracidia to individual snails indicated a ratio of infestibility between miracidia of ages one and four hours of about 1:1.5. At ages five, six and seven hours, the miracidia were capable of penetrating and establishing themselves in about the same ratios (or perhaps slightly less) as at age one hour. About 19% at ages one, four and five hours failed to penetrate the snail host and remained free-swimming after an hour's exposure. Single infestations by the miracidia caused no increase in the death rate of the snail host.

S. cubensis were maintained and reared in the laboratory on terraria constructed to resemble the natural habitat. Algae of the Genus Oscillatoria served as a satisfactory source of food. Algae sometimes grew in old terraria in sufficient quantities to support small colonies, but it was usually necessary to add algae for maintaining large numbers of experimental snails. They normally did not reproduce during the winter months in the terraria nor in the natural habitat, and they rarely exceeded 7 mm. in length except in May and June when they attained a shell height as great as 10 mm.

During the summer months, the snails did not normally enter the water, and they reproduced continuously, laying their eggs on mud. In terraria they entered the water at times during the summer and accumulated on clusters of algae.

In terraria during the summer months, the snails attained sexual maturity and an average length of 3.4 mm. in as few as eleven days after hatching, and they attained the usual maximum summer size of 7 mm. to 7.5 mm. within four to five weeks following hatching. Up to 50% survived aestivation for three months.

Three weeks following single exposure to the miracidia of P. microbothrioides, the infested snails averaged 0.5 mm. shorter than the non-infested ones. Varying degrees of destruction of the snail's digestive gland resulted from parasitism.

INTRODUCTION

Trematode eggs and miracidia are likely to be subjected to many varied conditions of the environment. This study was designed to determine experimentally some of the reactions of a particular species of trematode to some of the various environmental conditions that might be encountered in nature and the ability of the miracidium to penetrate and establish itself in the intermediate snail host. It was hoped also that enough of the biology of the snail host might be observed to enable a better understanding of the relationships between parasite and host.

Paramphistomum microbothrioides was chosen for this investigation because of the availability of materials and because a number of studies had already been conducted on this species in this laboratory. The intermediate host of this trematode was identified for Bennett (1936) by the late F. C. Baker of the University of Illinois as Fossaria parva (Lea); Harold W. Harry and Joseph P. E. Morrison, Associate Curator, Division of Mollusks, U. S. National Museum, have independently identified it for the present writer as Stagnicola (Nasonia) cubensis Pfeiffer.

No serious study of the biology of an amphistome miracidium has been reported; but certain authors have published life-history reports, which contain more or less incidental observations on biology. Notable among these are Krull (1932, 1934b), Bennett (1936), Sinha (1950) and Willmott (1952).

Numerous observations concerning the biology of the schistosomes

have occurred in the literature from time to time. Faust (1924) reported on the reactions of the miracidia of Schistosoma japonicum and S. mansoni. Faust and Hoffman (1934) published on the biology of the extra-mammalian phases in the life cycle of S. mansoni, and Faust and Meleney (1924) included biological observations in their monograph on S. japonicum. Gordon, et al, (1934) included notes on biology in their account of the life cycle of S. mansoni and S. haematobium. Ingalls, et al, (1949) reported their observations on S. japonicum under various conditions, and Magath and Mathieson (1946) reported on the factors affecting the hatching of ova of S. japonicum. Maldonado and his coworkers have published a number of biological studies on S. mansoni in recent years (1947, 1948, 1949, 1950a, 1950b, etc.).

The biology of the liver flukes has been reported in more or less detail in a number of instances, and several workers have attempted to correlate the biology of the intermediate host with that of the liver fluke larvae. Among the latter are Shaw and Simms (1930), Swales (1935), Olsen (1944), Roberts (1950) and Batte, Swanson and Murphy (1951).

The scarcity of information concerning the biology of free-living trematode larvae indicates a need for such investigations as the one presently reported, and Emmett W. Price (1953) of the U. S. Bureau of Animal Industry has said: "In view of the relatively large number of snail species involved in the life histories of flukes parasitic in North American ruminants, a knowledge of the bionomics of these vectors is essential to a sound approach to fluke control in the several geographic areas involved. Unfortunately, the available information on the bionomics of these intermediate hosts is rather limited and sketchy."

HISTORICAL REVIEW

According to Stiles and Goldberger (1910), the name amphistome was introduced by Rudolphi's generic name Amphistoma 1801. Fischoeder (1901) regarded all amphistomes as belonging to a single family, the Paramphistomidae, which he divided into two subfamilies, the Paramphistominae and Cladorchinae, and he erected the genus Paramphistomum, with P. cervi as the type species. Stiles and Goldberger (1910) proposed that Fischoeder's Paramphistomidae be elevated to the status of a superfamily, Paramphistomoidea, which they divided into three families, Paramphistomidae, Gastrothylacidae and Gastrodiscidae. Maplestone (1923) and Travassos (1934) accepted the views of Stiles and Goldberger, as did Dawes (1936) in his revision of the Genus Paramphistomum.

Näsmark (1937) proposed a taxonomic revision of the family Paramphistomidae, based on his study of the morphology of pharynges, acetabula and genital atria. He listed 15 subfamilies, including four new ones. In his subfamily Paramphistominae (which equals Fischoeder's Genus Paramphistomum), he listed nine genera, including seven new ones; and in the Genus Paramphistomum, he listed eleven species, including two new ones. At least three other new species have been placed in the genus since Näsmark's revision: P. microbothrioides by Price and McIntosh (1944), and P. hiberniae and P. scotiae, described by Willmott (1950).

For a complete historical survey of the Amphistomata prior to 1929, the reader is referred to Fukui (1929).

Excluding taxonomy, interest in the amphistomes has been restricted principally to life-history studies. Since Looss described in 1892 the life-history of Diplodiscus subclavatus, and in 1896 the miracidia of Gastrothylax gregarius, G. aegyptiacus and P. cervi, accounts of paramphistomide life-histories have been relatively rare. Cary (1909) published an account of the life-history of D. temperatus. Cort (1915) showed that Cary was in error, and Krull and Price (1932) repeated the work on that species. Beaver (1929) reported on the developmental stages of Allassostoma parvum, and Brumpt (1936) described the cercaria and the experimental infestation of the intermediate and final hosts of P. cervi. Willey (1941) published a report on the life-history and bionomics of Zygocotyle lunata. By far the most complete account of a paramphistomide has been given by Bennett (1936) of a species identified as Cotylophoron cotylophorum. Krull (1932, 1934b) also reported on this form. Sinha (1950) reported on the development of C. cotylophorum in India, and Willmott (1952) described the development and morphology of the miracidium of P. hiberniae. Willey and Godman (1951) described gametogenesis, fertilization and cleavage in Z. lunata.

Price and McIntosh (1944) studied the amphistome material available in the U. S. National Museum and concluded that the species referred to as C. cotylophorum by Bennett (1936) and Krull (1932, 1934b) was in reality a new species of the Genus Paramphistomum, to which they applied the name P. microbothrioides. A study of the morphology of this trematode, based on the figures and descriptions contained in Näsmark's (1937) monograph, has led the writer to conclude that it is a member of the Genus Paramphistomum and that Price and McIntosh were

probably justified in considering it a new species. The lack of Cotylophoron material for comparison, the lack of adequate figures in the literature and inadequate generic definition at the time of Krull's and Bennett's work made positive identification difficult. At that time, the only described characteristic for distinguishing between the Genera Paramphistomum and Cotylophoron was the presence in the latter of a genital sucker, set off from the surrounding tissues by a well-developed basal membrane.

MATERIALS AND METHODS

To secure the eggs of P. microbothrioides, the adult worms were collected from the rumens of cows, Bos taurus, slaughtered in various slaughter houses in the vicinity of Baton Rouge, Louisiana. These worms were placed immediately into a container of warm tap water and kept at a temperature of approximately 38° C. for several hours. During that time the worms deposited large numbers of eggs that were practically free of organic materials or other debris. At hourly intervals the eggs were removed from the container and placed in a refrigerator to prevent development until they were removed for use in experimental studies. Eggs collected by this method can be examined easily for quality and quantity, the minimum incubation period for a given temperature can be predicted with accuracy, and there is no need for changing the water in which they are incubated.

All eggs used in experiments involving free-swimming miracidia were removed from the refrigerator and incubated in a constant temperature oven which was regulated for 32° C. The water in such cultures of eggs was maintained at a very constant 30° C. in this oven. By chilling, the eggs were caused to hatch in large numbers for a period of approximately 15 minutes after being removed from the incubator. The miracidia were then poured off into another container, leaving the unhatched eggs behind. In this way, the hatching time of the larvae was known to within a very few minutes.

To determine the condition of eggs after incubation or induced

hatching, a few were taken from the culture concerned and examined with the aid of a compound microscope. Counts were made of 100 or 200 of these eggs, and each was placed in one of four categories. Those with open opercula were classed as hatched, those containing a miracidium in any stage of development, as developed, those that showed neither deterioration nor development, as unchanged, and those eggs with closed opercula and deteriorating vitelline material, or none at all, were classed as deteriorated. In each instance, the number of miracidia that had hatched was determined by the number of eggs with open or displaced opercula.

Miracidia for longevity and infestivity studies were isolated with the aid of a capillary pipette and a dissecting microscope into vials 15 millimeters in diameter and 15 millimeters long.

For maintaining snails in the laboratory, rectangular boxes, 20" x 30" x 6" deep, were constructed from 1" x 6" pine boards and filled to a depth of approximately three inches with soil from the vicinity of the natural habitat of the snail. One or two trenches were then formed in the soil of each box to allow for a continuous flow of water that kept the soil saturated. Water was fed into one end of each box through rubber tubing and led out through a hole drilled at the desired level in the opposite end. The soil in each box was seeded with algae collected from drainage ditches. These terraria were elevated to a height of two feet on wooden frames constructed of 2" x 4" timbers, and the wooden frames were roofed with plastic-filled screen wire to permit the passage of light and to prevent flooding of the terraria during rains. These terraria were

maintained in a basement window-well on the south side of a two-story, masonry building.

Individual snails were exposed to single miracidia by placing the two in a short vial with one cubic centimeter of standard reference water as developed by Hart, Doudoroff and Greenbank (1945) and modified by Abegg (1948), Williams (1948) and Freeman (1949).

Standard reference water is a laboratory-prepared medium, free of organics and containing the minerals usually found in surface waters. It was chosen as a medium for all experimental studies of the miracidium because of its uniform and constant composition and hydrogen ion concentration as opposed to the fluctuating composition of various natural surface waters.

Standard reference water is prepared from six different stock solutions, made up in advance. These stock solutions are prepared in the following manner, using chemicals of the C. P. grade:

Stock solution 1:

Magnesium sulphate-heptahydrate	71.0 grams
Potassium sulphate	6.5 grams
Manganous sulphate-tetrahydrate	0.2 grams
Redistilled water to make one liter	

Stock solution 2:

Calcium chloride-dihydrate	18.6 grams
Redistilled water to make one liter	

Stock solution 3:

Sodium bicarbonate	25.0 grams
Ammonium nitrate	3.0 grams
Potassium phosphate-dibasic trihydrate	1.1 grams
Redistilled water to make one liter	

Stock solution 4:

Calcium oxide	32.3 grams
Redistilled water to make one liter	
Bubble with carbon dioxide until a suspension is obtained	

Stock solution 5:

Sodium metasilicate-monohydrate	62.6 grams
Redistilled water to make one liter	

Stock solution 6:

Ferric chloride hexahydrate	1.2 grams
Redistilled water to make one liter	

The final dilution is prepared by adding one cubic centimeter each of solutions 1, 2 and 3 for each liter of standard reference water desired to the measured amount of redistilled water. Carbon dioxide is then dispersed into this solution through a porous gas dispenser for 15 minutes. The pH of the solution at this point is approximately 4.3. To this solution, one cubic centimeter of solution 4 is added for each liter of standard reference water, and carbon dioxide is added again until the solution becomes clear. The solution should now have a pH of approximately 5.1. Next, compressed air is bubbled through the solution for 25 minutes, raising the pH to approximately 7.9. Finally, one cubic centimeter each of solutions 5 and 6 is added for each liter of standard reference water, and the solution is aerated for at least one hour to obtain a final, constant pH value of 7.9.

Water that had been distilled in a Barnstead still and redistilled in a Pyrex glass still with an internal nichrome heating element was used in preparing the stock solutions and the subsequent dilutions of standard reference water used in this series of experiments.

OBSERVATIONS AND EXPERIMENTAL RESULTS

1. The Egg and Its Development

The adults of P. microbothrioides live in the rumen of the cow where they cling by their acetabula, buried deep among the numerous villi. Here they deposit their eggs to become mixed with the contents of the rumen and to pass into the intestine, finally to be voided with the feces.

The eggs, as described by Bennett (1936), are remarkably uniform in appearance, being nearly ovoid with a slight attenuation at the opercular end, and averaging approximately 129 by 68 microns in size.

After passing from the host, the eggs are at the mercy of the environment, which varies according to the place of deposition. They may be subjected to temperatures that range from below freezing to well above 40° C., to conditions of moisture ranging from complete desiccation to suspension in water, to conditions of zero dissolved oxygen or to high concentrations, and to pH variations on either side of neutral.

Effect of Drying. The egg shell appears to be readily permeable to water as removal from water resulted in total destruction of the ovum. As much water as possible was drained from one large sample of freshly collected eggs, and the container was left at room temperature for nine and one-half hours. Water was then added to the eggs, and they were placed at 30° C. for incubation. Examination after six days

of incubation revealed complete deterioration of the shell contents. Not one developing embryo could be found, and the vitelline cells had broken down into a homogeneous mass. Controls showed 75% developed or hatched after nine days at 30° C, Another sample of eggs was drained free of covering water and placed for one hour before an electric fan on a hot, humid day. Examination after ten days of incubation in water showed complete deterioration and no development of the ova. The majority of the shells in this case appeared nearly empty, and there was a collection of the yolk material on the outside of the shell, so located as to suggest that the operculum had loosened and allowed the yolk to flow out. Eighty-two percent of the controls developed or hatched in nine days at 30° C.

McPherson (1951) performed experiments to determine the amount of drying tolerated by these ova when suspended in cow feces. He concluded that "If the moisture content of the feces drops to as low as 7.2%, all eggs contained in the feces will be dead in a period of eight days." In feces with a moisture content of 29%, he found no decrease in viability during a period of 60 days.

Roberts (1950) found that drying for periods of one to ten hours proved fatal to the eggs of Fasciola hepatica. This was true of the undeveloped eggs as well as those that had incubated at 25° C. for 14 days.

Temperature as a Factor in Development. The extreme ranges of temperature in which the egg will survive and develop have not been determined. Some efforts at establishing these ranges were attempted, but lack of equipment for controlling temperatures made real success impossible.

At an oven temperature of 32° C., where the water containing eggs remained at a practically constant temperature of 30° C., these eggs constantly and repeatedly hatched in good numbers after nine days of incubation, but not earlier. At a constant water temperature of 28° C. they failed to hatch at the end of ten days, but hatching was obtained at the end of 11 days. At 35° C. they hatched after eight days of incubation.

Table 1 shows development at water temperatures of 28°, 30°, 35°, 38° and 40° C. Each entry represents a different collection of eggs, or eggs coming from different groups of worms. The results of these experiments indicated that fluctuations of temperature between 28° and 35° C. did not significantly influence development, except that development to the hatching stage was somewhat shortened as the temperature increased.

Samples of eggs from two different egg collections failed to develop at 38° C. They showed neither deterioration nor development after ten days at this temperature, but the vitelline material did appear slightly condensed. Eggs from a third collection showed 4% with very slight development after ten days of incubation at 38° C. A second sample from this third collection (not shown in Table 1) was constantly aerated with a Thiberg aerator during ten days of incubation at 38° C. Five percent of these produced fully developed miracidia, and hatching was induced. Another 15% of the eggs showed development of the embryo to about maximum width, but there was no elongation nor evident development of organs. The controls for these eggs, incubated at 30° C. for ten days, showed 32% developing to mature miracidia. Two other collections of eggs were incubated for ten days at 40° C. These

showed slight development in 39% and 14% of the eggs, whereas the controls at 30° C. developed mature miracidia in 71% and 32% respectively.

Bennett (1936) indicated the effect of temperature on the rate of development of this form. He found that the miracidia developed to hatching in as few as eleven days at room temperatures in June and July and that up to 29 days were required at room temperatures in January and February.

Table I. Development of P. microbothrioides Eggs Incubated at Various Temperatures.

Water Temp. °C.	Days Incubated	Percent Developed	Percent Control Developed at 30° C.
28	11	65	68
35	8	80	71
35	8	34	44
35	8	22	32
38	10	0	63
38	10	0	84
38*	10	4	31
40*	10	39	71
40*	10	14	32

* Only slight development occurred. None developed mature miracidia.

Jenkins (1951) attempted to establish the rate of development for P. microbothrioides at 21°, 27°, 32° and 38° C. She found that they began to hatch in from 8 to 19 days at temperatures of 27-38° C. Between these extremes she found a difference in the rate of development to the hatching stage, but she found no difference in the total number that developed and hatched during an incubation period of 27 days. At 21° C. she reported such a slow rate of development and hatching that she was never able to observe miracidia; however, she concluded that

the total number hatched after 43 days was as great as the number after 27 days at the higher temperatures. She reported oven temperatures, not the actual temperature of the water containing the eggs.

Sinha (1950) found that the eggs of C. cotylophorum hatched in 7-9 days in summer and 15-16 days in winter. Willmott (1952) reported that the eggs of P. hiberniae hatched between 14 and 20 days at laboratory temperatures of 20-22° C.

Numerous statements occur in the literature concerning the incubation period of trematode eggs, some of which are quoted here. Krull (1934a) found that segmentation in the eggs of F. hepatica is arrested at low temperatures, but that it is resumed at 13° C., and Ross (1930) and Thomas (1882) reported that segmentation in this form is arrested at low temperatures, but they failed to report the minimum temperature at which segmentation is resumed; nor did they report the maximum temperature tolerated. Roberts (1950) reported that the eggs of F. hepatica hatched after 14 days at 25° C. and after 5 weeks at 17° C., but Shaw and Simms (1930) observed hatching in as few as 14 days at room temperature. Beaver (1937) found that the eggs of Echinostoma revolutum hatched in from 18 to 30 days at room temperatures, and Swales (1935) reported that those of Fascioloides magna began hatching on the 29th day when incubated at a mean temperature of 24° C. (range 21-27° C.).

The Effect of Freezing. To determine the effect of freezing on the undeveloped egg, numerous eggs were suspended in a few drops of water and subjected to temperatures of approximately -5° C. for varying periods of time, after which they were incubated at 30° C. See Table 2.

Eggs that were frozen for periods of one-half hour were not

affected. Those frozen for one, two, three or four hours showed a marked decrease in the number capable of development, and those frozen five hours or longer failed to develop when thawed and incubated.

Table 2. The Effect of Freezing on the Undeveloped Eggs of P. microbothrioides.

Period Frozen	Percent Developed and Hatched after Incubation	Percent Control Developed and Hatched
½ hr.	35	35
½ hr.	34	35
1 hr.	30	35
1 hr.	11	30
2 hr.	5	30
3 hr.	4	35
3 hr.	0	30
4 hr.	3	30
5 hr.	0	30
2 days	0	35
5 days	0	35
10 days	0	35

Shaw and Simms (1930) found that newly collected eggs of F. hepatica developed and hatched subsequent to being frozen at -11° C. for 24 hours. Freezing for the same period of time destroyed those eggs with embryos. Swales (1935) reported that freezing F. magna ova in the one- to sixteen-cell stage at -5° C. in water or feces for periods up to six weeks did not affect them, but that eggs in late stages of development were completely destroyed by freezing.

Oxygen as a Factor in Development. McPherson (1951) was able to show that the eggs of P. microbothrioides failed to develop as long as they remained in cow feces, even though the moisture content was maintained at 90% or higher. Even after periods of up to 64 days at room temperature, no development was apparent, and the reduction in

the number capable of subsequent development was only very slight. He assumed that the very low oxygen tension under these conditions was responsible for the failure of the eggs to develop. Swales (1935) observed that the eggs of F. magna developed to the hatching stage in feces as long as the moisture content did not drop below 60%.

In an attempt to determine the effect of a reduced oxygen tension, several experiments were conducted during the present investigation. In one case a 15 ml. test tube was filled with water that had been boiled for 15 minutes. The tube was sealed by pouring one-half inch of melted petroleum jelly on the surface of the water. When the tube had cooled, approximately 20,000 eggs were introduced and the tube was resealed. Thirty-six days later the eggs were examined, and it was found that none had developed. About 25% appeared to have deteriorated. At this time the eggs were put into 250 ml. of water and placed in incubation at 30° C. When examined after 10 days in open water, 55% showed development and another 12% were developed to the hatching stage. Hatching began soon after removal of the culture from the controlled temperature oven. Controls from these eggs in open containers at 30° C. for 10 days showed 20% deteriorated and 75% developed or hatched.

From one collection of eggs, which showed 24% developing or hatching when incubated immediately after collection, two samples were sealed in boiled water. One sample was opened and incubated after being sealed for 35 days. Counts revealed that 29% developed or hatched. The second sample was opened and incubated after being sealed for 60 days; 18% developed or hatched.

In another experiment, eggs that had been allowed to develop

at room temperatures for six days, or until the embryos had reached maximum width without elongation, were introduced into a 250 ml. Erlenmeyer flask filled with boiled water and sealed with petroleum jelly. After 74 days, examination revealed no detectable advance in development. The eggs were then placed in an open container of fresh water and incubated at 30° C. After five days of incubation, examination revealed development of approximately 50% to mature miracidia, and hatching was induced in large numbers. The container was sealed again, and 24 hours later miracidia were still present in the contained water. After another 24 hours, no miracidia were observed. The controls from this group of eggs in open containers at room temperatures showed 76% developed or hatched in 13 days.

Another sample of these embryonated eggs that had developed for six days at room temperature, with 71% showing development, was sealed in unboiled tap water at room temperature. The seal was removed at the end of eight days, and examination revealed no further development. Seven days after the seal was removed, counts revealed that 11% of the eggs still contained embryos of about maximum width, while 60% had developed to the hatching stage. Hatching was induced at this time. The controls for these eggs required 13 days to reach the hatching stage. It is interesting to note that when the period these experimental eggs remained sealed is deducted from the incubation period, about 13 days remain. In other words, the controls hatched eight days before this group, which was sealed for eight days.

Another sample of undeveloped eggs was sealed in unboiled tap water and incubated at 30° C. for a period of 57 days. Examination

revealed no apparent development, and 20% had deteriorated. These eggs were then placed in an open vessel at 30° C., and hatching was induced nine days later. The eggs were sealed again about eight hours after hatching was induced. When next examined, 43 days later, counts revealed that 40% had hatched, and 22% of the eggs contained living, mature miracidia. The controls showed 82% developed and hatched after nine days at 30° C.

One other test was run to determine the effect of a reduced oxygen tension. Approximately 15,000 eggs were placed into 7 ml. of water in a 15 ml. test tube. The tube was closed with a cork stopper and placed on its side at 30° C. When examined after six days of incubation, only a very slight development was detectable. After 11 days of incubation, some embryos had developed into a spherical mass about 30 microns in diameter. The cork was removed at this time and the tube placed upright. After a total of 36 days of incubation, miracidia were observed swimming free in the water, and counts showed that 5% of the eggs had hatched, 6% had developed to mature miracidia and 9% had deteriorated. After 89 days of incubation, free miracidia were still being observed swimming in the water. The contents of the test tube were then lost before counts could be made of the total number of eggs that had developed or hatched. The control for these eggs showed hatching after nine days, and a total of 75% had developed or hatched after 20 days of incubation.

Although no quantitative data are presented, it has been noticed repeatedly that eggs mixed with fermenting or decaying organic materials were much slower in developing. When eggs were thus contaminated, as when they were washed from the rumen of the infested

definitive host, it became necessary to change the water at least daily in order to have large numbers of the eggs maturing and hatching together.

Sinha (1950) used distilled water for incubating the eggs of C. cotylophorum, changing the water twice daily, even after careful cleaning of the eggs to prevent bacterial infusions. He thus implied that development is retarded by a reduced oxygen tension. Shaw (1931) speaks of F. hepatica eggs lying dormant, even when under conditions apparently favorable for hatching, and he noted that they may hatch over a period of 13 months. Bennett (1936) and Jenkins (1951) indicated the need for frequent changing of the water over the eggs of P. microbothrioides, and Willey (1941) experienced much difficulty, due to bacteria and molds, in obtaining successful development of the ovum of Z. lunata. He obtained best results by keeping the eggs in Petri dishes and changing the water daily. Willmott (1952) reported an entirely different experience with the eggs of P. hiberniae. When kept in too much water, or in water containing no vegetable matter, the ova collapsed after a few days. She found that they developed better when kept in a small quantity of filtered tap water to which sterile rabbit feces or well-washed rumen content had been added. The water was not changed at all, but was replaced if it showed signs of evaporation. She failed to state whether these eggs are capable of developing in the feces of the host.

The Effect of Hydrogen Ion Concentration on Development and Hatching. To determine experimentally the effect of hydrogen ion concentration on the development of the eggs and the ability of the miracidia to escape from the egg shells, the method of Maldonado, et al,

(1950b) was used. The medium employed was standard dilution water (described in the Standard Methods for the Examination of Water and Sewage of the American Public Health Association, 9th ed., 1946). This is a buffered medium, used generally in biological oxygen-demand determinations, which possesses the average composition and general properties of natural river waters. To obtain the various pH levels desired, the buffer was correspondingly graduated with a Beckman pH meter by adding variable amounts of decinormal sodium hydroxide to the potassium acid phosphate-ammonium sulfate mixture. In this manner stocks of dilution water with the desired hydrogen ion concentrations were prepared for use in the development and hatching studies.

Four experiments were undertaken, one at room temperature, two at 30° C. and one at 35° C. Table 3 gives the various hydrogen ion concentrations tested and the experimental results as compared with the controls in standard reference water.

This particular buffer proved to be not wholly satisfactory for the ranges of pH attempted as it failed to maintain a stable condition at the high pH values. Nevertheless, this solution did serve satisfactorily to determine the effect of weakly acid conditions, such as might be encountered in nature.

It might be expected that weakly alkaline waters would be more satisfactory for development and hatching, because miracidia are known to live longer at pH values in excess of neutral. Maldonado, et al, (1950b) found that the miracidia of S. mansoni died very rapidly in acid waters, approximately 50% of them dying within the first hour at pH values between 4.97 and 6.0. They reported that hatchability was

not significantly affected by any change in hydrogen ion activity ranging from pH 5.16 to pH 8.35. Ingalls, et al, (1949) found that little hatching of S. japonicum ova occurred at pH values below 6.6, and that the hatching rate increased to a maximum at a little above pH 7.6.

No attempt was made in the present investigation to determine the actual longevity of these miracidia at the various hydrogen ion concentrations used for testing development and hatching, but it was noted in each case that the acid waters contained fewer free-swimming larvae than did the alkaline waters. Also it was noted that the alkaline waters still contained free-swimming miracidia for eight to ten hours after hatching was induced, whereas the acid waters contained none after two to three hours following induced hatching. One group of 50 miracidia isolated individually into water at pH 5.6 were all dead when examined after three hours.

From the data in Table 3 it can be seen that in each case development and hatching in the standard dilution water appeared comparable to that in the standard reference water control, with the possible exception of the test that was run at room temperature. In that instance each of the standard dilution waters showed the percentage developed and hatched to be somewhat lower than the standard reference water control. There seemed to be no significant difference in the numbers developing and hatching at the various pH levels of the standard dilution water.

Table 3. Development and Hatching of P. microbothrioides Eggs at Various Hydrogen Ion Concentrations

Medium	Initial pH	Final pH	Days Incubated	Percent Developed	Percent Hatched	Percent Unchanged	Percent Deteriorated	Temperature of Incubation
SRW-C*	8.20	8.60	12	37	0	44	19	
SDW**	6.30	6.50	12	31	1	55	13	
SDW	6.60	6.65	12	33	0	53	14	30° C.#
SDW	7.40	7.20	12	31	1	58	10	
SDW	8.00	7.40	12	34	3	48	15	
SDW	8.50	7.40	12	31	4	54	11	
SRW-C	8.20	8.05	30	34	3	44	19	
SDW	6.30	6.60	30	22	12	50	16	
SDW	6.60	6.60	30	28	4	55	13	
SDW	7.40	7.00	30	30	6	46	18	30° C.#
SDW	8.00	7.35	30	28	7	48	17	
SDW	8.50	7.30	30	34	7	44	15	
SRW-C	8.10	8.10	11	20	24	51	5	
SDW	6.40	6.80	11	28	12	51	9	
SDW	6.80	7.30	11	26	20	43	11	
SDW	7.40	7.60	11	23	19	48	10	35° C.##
SDW	7.90	7.60	11	24	20	47	9	
SDW	8.10	7.80	11	21	27	45	7	
SRW-C	8.20	8.15	18	24	26	42	8	
SDW	5.50	5.50	18	16	17	57	10	
SDW	6.50	6.40	18	21	19	50	10	Room Temp.
SDW	7.30	7.10	18	15	19	52	14	(25-32°C.)##
SDW	8.10	7.40	18	19	20	50	11	

* SRW-C, standard reference water control.
** SDW, standard dilution water.
\# Hatching not induced.
\## Hatching induced by chilling.

The Keeping Quality of Eggs under Refrigeration. The inability to secure trematode eggs in sufficient quantities for experimental studies at any given time makes it necessary that the eggs be collected in as great numbers as possible when they are available and then stored for future use.

At times during the present investigation, materials were more than ample, while at other times months passed in which no material was available. Ordinarily it was not necessary to store eggs under refrigeration for more than two to three months, and the decrease in the number viable dropped by not more than about 20% in three months. From one very large collection of worms, eggs were kept in refrigeration for a whole year. Samples were removed to incubation at 30° C. at various intervals, and the percentage viable was determined. Table 4 is a compilation of the results.

The number of eggs listed as deteriorated (because the shell was empty or nearly so, but with the operculum remaining closed) did not change significantly during the first ten months, which suggests that the egg content did not actually deteriorate, but rather that the shell was laid down with little or no vitelline material. There was a gradual decline in the number capable of developing and a corresponding gradual increase in the number of eggs that showed no change in appearance during the first ten months. During the eleventh and twelfth months, the number that remained unchanged decreased, and the number that deteriorated increased. The decrease in the number viable was more rapid during the first six-month period than during the second. Even at the lower viability rates of the later months, miracidia could still be obtained in sufficient quantities for almost any purpose that

might be desired.

Table 4. The Effect of Refrigerator Storage at 5° C. on Subsequent Development of the Ova of P. microbothrioides.

Months in Refrigeration	Days Incubated	Percent Developed and Hatched	Percent Unchanged	Percent Deteriorated
0	42	69	14	17
1	45	59	25	16
2	40	50	43	7
6	22	46	40	14
7	33	47	38	15
8	37	35	41	24
9	29	28	60	12
10	26	33	54	13
11	28	31	39	30
12	30	30	44	26

Quantitative longevity studies of the miracidia obtained from these eggs after twelve months of storage were not attempted, but the maximum length of life was observed to be comparable to that of miracidia hatched from the eggs shortly after collection, 10 to 12 hours at 29° C.

After 11 months of refrigeration, it was noted that the minimum incubation period for hatching had increased from 9 to 11 days.

Swales (1935) stored the eggs of F. magna at 2-5° C. and reported that nine months at this temperature did not retard subsequent development. Krull (1934a) investigated the hatchability and infestivity of refrigerated eggs of F. hepatica. He found that eggs refrigerated at 2-10° C. up to two years, six months and seven days hatched at room temperature in 18 days, and that the miracidia were capable of infesting the snail host. He reported no data on the percentages hatched or infestive.

2. The Miracidium

Effect of Light and Temperature on Hatching. It has been generally assumed in this laboratory that light is a stimulus to the hatching of this miracidium, and it has been the practice to remove fully-developed eggs from a constant temperature oven and to place them under bright light when miracidia were desired. It has been noted also that hatching usually occurred under these conditions during the early morning hours. Only rarely have they been thus induced to hatch in the afternoons.

Bennett and Jenkins (1950), in reporting on the longevity of this miracidium, stated: "It was observed that very few miracidia would hatch in the darkened (constant temperature) boxes but that when exposed to light they would begin to hatch within a very short time, which greatly facilitated the procuring of miracidia."

As time progressed in this study, it became more and more evident that some stimulus was effective in bringing about hatching, otherwise a slow, steady hatching rate should have been apparent instead of the sudden appearance of very large numbers of miracidia within a few minutes of time. It also became evident that light could not be the sole source of that stimulus, as it could not be depended on to give uniform results.

Controlled attempts at determining the effect of light and possibly other physical stimuli were attempted. Several samples of eggs, each in a separate container, were incubated in a dark room with a constant temperature of 31-32° C. After the eggs had incubated for twelve days, one container was placed under a bright light and a second

one was carefully covered to exclude all light. The water was poured from two other containers and aerated for ten minutes. This aerated water was then returned to the original containers, and one was placed in the light as above, the other in the dark. The constant temperature of the incubation room was maintained throughout these experimental procedures. After one hour, it was observed that no hatching had occurred in any of the containers. These containers were then placed in a vessel of cold water where they were allowed to remain until the temperatures dropped to approximately 25° C. They were then returned to their original places of darkness or bright light. Miracidia occurred in large numbers in all of the containers within 15 minutes.

In two other separate tests, eggs failed to hatch in either light or darkness inside the constant temperature room where they had incubated. After chilling, miracidia occurred and counts revealed that equal numbers had hatched in light and in darkness.

Cultures of eggs that were incubated and maintained at a water temperature of 30° C. never swarmed with miracidia, even when incubation exceeded the minimum incubation period for hatching (nine days) by two or three weeks, thus giving the impression that little or no hatching occurred. Four containers with eggs that had so incubated for three weeks were removed to a room of approximately the same temperature. After 30 minutes, when no miracidia had occurred in any of the containers, one container was cooled by 5° C., and miracidia began to appear almost immediately. A second container was warmed by 5° C. by placing it in warm water. No hatching occurred within 30

minutes; but miracidia did occur in very large numbers soon after this second container had been chilled to approximately 25° C.

To avoid any increase in oxygen tension that might result from cooling the water, the remaining two containers were sealed by pouring petroleum jelly on the surface of the water to a depth of one-half inch. One was chilled; the other was not. Miracidia occurred in very large numbers during the next 15 minutes in the chilled container, while none occurred in the other. But when the second sealed container was chilled, equally large numbers of miracidia occurred.

Even eggs that had been incubated at 21° C. were induced to hatch by chilling. When the temperature was lowered to 15° C., miracidia occurred in large numbers. No hatching occurred when such eggs were warmed to 29° C. Nor did hatching occur when the temperature dropped back to that of incubation; but when the eggs that had been so warmed were chilled to 15° C., larvae hatched in large numbers. Counts were made on eggs that had incubated for 37 days at 21° C. Before chilling, it was found that 2% had open opercula and 26% contained mature miracidia. After chilling an additional 20% hatched.

Ordinarily when hatching was induced to obtain miracidia for experimental purposes, no care was taken as to the exact amount the temperature of the eggs was lowered. The eggs were flooded with water at a temperature of about 15° C., or the container in which they had incubated was placed in a bath of about 15° C. This stimulated immediate hatching.

Willey (1941), in his study of Z. lunata, found that the majority of the eggs hatched between 10 p.m. and 2 a.m. He concluded that darkness was not the controlling factor, for just as many larvae

hatched in a culture dish placed under a strong beam of light as in dishes covered with black paper. His attempts to induce the hatching of miracidia by the stimuli of light and darkness were entirely unsuccessful.

Willmott (1952) found that the eggs of P. hiberniae can be induced to hatch by warming them up to 26° C. from laboratory temperatures of 20-22° C., or by bringing them from darkness into light, but that hatching may be delayed from 30 minutes to several hours after stimulation.

Swales (1935) found that F. magna ova hatched sporadically, with most of the swarms emerging at night. He reported that hatching was not affected by light, but that sudden changes in temperature often stimulated hatching, e.g., eggs taken from the refrigerator at 3° C. frequently hatched in less than an hour at laboratory temperatures of 22-24° C. This method he found far from infallible. Conversely, he found that the eggs occasionally hatched on being removed from the laboratory to the refrigerator.

Jepps (1933) maintained that chilling the eggs of F. hepatica will provide the necessary stimulus for hatching, but Roberts (1950) was unable to confirm this. He found that those eggs incubated in the dark hatched only on exposure to light and that violet and blue wavelengths of the spectrum were the essential part of the light stimulus. Shaw and Simms (1930) found exposure to sunlight an effective stimulus for the hatching of F. hepatica ova.

Maldonado (1949) and Maldonado, et al, (1950a) reported that light had a decided effect on the hatching of S. mansoni, with a much higher percentage hatching under brilliant illumination than in the dark.

Ingalls, et al, (1949) stated that the hatching of S. japonicum eggs was almost completely inhibited by refrigeration and by temperature of about 37° C. They also found that variations in light intensity had no apparent effect on hatching, and they observed no diurnal or nocturnal cycle in hatching frequency. Magath and Mathieson (1946) reported good hatching for this form at 29° C., and no hatching at 37° C.

It had been noted that the summer temperature of the water in a drainage ditch where snails infested with P. microbothrioides had been collected was about 28° C. Thus the failure of miracidia to occur in appreciable quantities in cultures at 30° C. suggested that perhaps a somewhat lower temperature might be conducive to a faster rate of hatching. To check the actual hatching rate within a constant temperature oven, two collections of eggs were placed in incubation, one at 30° C. and the other at 28° C. In that way, each served as a control for the other and a comparison could be made between the two temperatures. No large collection of eggs was obtainable; therefore it became necessary to use two different collections. One collection had been refrigerated for 38 days; it was incubated at 28° C. The other had been refrigerated for five days; it was incubated at 30° C. About 75% of the eggs set at 28° C. were viable, but, unfortunately, only about 30% of the second lot was viable. The difference in viability is not explained. An occasional collection of low viability was noted, but no attempt was made to determine the cause.

Samples from the culture at 28° C. could not be induced to hatch at the end of ten days; but, at the end of eleven days, hatching was induced by cooling. At the end of twelve days, and every five days thereafter, a sample of the collection was removed from the culture, and

counts were made to determine the fraction that had hatched in the oven. Another sample was removed and chilled two hours before counting. The results of these counts were tabulated in Table 5. The sample of eggs for determining the degree of hatching in the oven was killed in each case with a drop of formaldehyde as soon as the sample was taken from the culture.

Table 5. The Hatching Rate of P. microbothrioides Ova at 28° C. and as Obtained by Chilling at Five-Day Intervals.

	Condition of Ova in 28° C. Oven				Percent	
Days Incubated	Percent Hatched	Percent Developed	Percent Unchanged	Percent Deteriorated	Hatched by Chilling	Total Hatched
10	1	65	3	31	0	0
12	7	68	2	23	6	13
17	20	44	4	32	17	37
22	26	46	1	27	16	42
27	46	24	4	26	14	60
32	52	19	5	24	9	61
37	46	24	3	27	9	55
42	51	16	5	28	14	65
47	50	16	4	30	9	59
52	55	10	3	32	7	62
62	58	8	2	32	4	62

The egg-counts obtained indicated that the number hatched in the oven gradually increased throughout the period of incubation. The number induced to hatch by chilling remained more or less constant throughout, with a possible slight increase at 17, 22 and 27 days. The combined total of those hatched in the oven and by chilling remained uniform after the 27th day of incubation.

Samples from the culture at 30° C. could not be induced to hatch on the eighth day of incubation. At the end of eleven days, hatching was induced by chilling, and counts were made then and at five-day

intervals thereafter as described above. The counts obtained are recorded in Table 6.

With such a small percentage of the eggs developing in this case, it was difficult to compare the rate of hatching to that which occurred at 28° C. It seemed, however, that there was a constant, slow hatching rate at both temperatures, and that at 30° C. hatching was completed in a shorter period of time.

Table 6. The Hatching Rate of P. microbothrioides Ova at 30° C. and as Obtained by Chilling at Five-Day Intervals.

	Condition of Ova in 30° C. Oven				Percent	
Days Incubated	Percent Hatched	Percent Developed	Percent Unchanged	Percent Deteriorated	Hatched by Chilling	Total Hatched
8	2	26	10	62	0	2
11	5	22	8	65	11	16
16	7	18	19	56	20	27
21	9	20	8	63	14	23
26	8	17	10	65	20	28
31	10	17	14	59	14	24
36	17	11	5	67	2	19
41	14	11	12	63	6	20

Cooling after 36 days at 30° C. produced only an occasional miracidium, and subsequent counts revealed that 9% of the eggs contained dead miracidia, or miracidia in which the only evidence of life was a feeble flickering of the flame cells.

The Hatching Phenomenon. Bennett (1936) has given a detailed description of the hatching phenomenon for P. microbothrioides. His observations led him to think that the mucoid plug had to be destroyed by glandular activity before the miracidium could exert the necessary pressure for opening the operculum of the shell. Sinha (1950) recorded similar observations concerning the hatching of C. cotylophorum eggs,

stating that the miracidium was observed "...to strike the plug with its anterior end. The plug was gradually destroyed and the miracidium became free to exert its pressure against the operculum. It forces the operculum in a very short time and squeezes out its body through the aperture, which is slightly smaller than the diameter of the body."

Willmott (1952), working with P. hiberniae, and Willey (1941), working with Z. lunata, found that no mucoid plug develops in those forms, and they found evidence in each case that the actual opening of the operculum is not the result of physical pressure exerted by the miracidium.

Swales (1935) stated that the mucoid plug of F. magna is not destroyed prior to hatching, and he indicated that the opening of the operculum is the direct result of physical pressure by the miracidium.

Bennett (1936) noted that the mucoid plug of P. microbothrioides begins to form at the opercular end of the egg after the flame cells start to function and that it continues to increase in size until the miracidium begins to destroy it. He stated also that it is easy to find all stages of destruction of the plug in an egg culture in which miracidia are hatching. It is true that plugs of various sizes do occur in a given sample of eggs, but the impression gained during this study was that these plugs became progressively larger as incubation proceeded up to the hatching stage. Then, if the eggs were not stimulated to hatch, these plugs continued to increase slowly in size, or at least they did not decrease in size. It seemed that the size of the plug was dependent on the stage of development or growth, rather than on the stage of destruction. An attempt was made at measuring these plugs in cultures just at the hatching stage for comparison with the plugs

from older cultures, but the rapid hatching that occurred outside the oven made this impracticable.

Assuming that the glands of the miracidium did play a part in the hatching phenomenon, and that the mucoid plug was destroyed prior to hatching, an attempt was made to observe the accelerated destruction of the plug following stimulation by chilling. Eggs that had developed past the minimum hatching period were taken with the least amount of water possible, suspended in a few drops of cold water and examined under the compound microscope. Every egg observed by this method contained large, well-developed mucoid plugs with no evidence of partial destruction. None of the miracidia appeared especially active. They exhibited infrequent, arhythmical muscular twitchings and occasional, slow muscular contractions, for the most part. A few were seen to demonstrate rather rapid and severe muscular contractions. To observe the actual hatching of the miracidium, an egg was chosen at random and observed until the miracidium emerged. In every case observed, the only indication that the miracidium was soon to emerge was the beginning of ciliary activity. The epithelial cilia normally were not observed to function, even during the periods of muscular activity, until just before the actual emergence of the larva. The onset of the beating of the cilia was sudden, and activity was confined usually to about the middle third of the body. This activity was sufficient to set up a current toward the posterior end of the body, as evidenced by the movement of what appeared to be bits of vitelline material. This ciliary activity continued for periods of from two and one-half to four and one-third minutes before the operculum opened. The average time for those observed was three and one-fourth minutes. It was with a sudden

explosiveness that the operculum finally opened. This occurred with such force as to displace the egg in the water where it was suspended. The mucoid plug began immediately to flow from the egg shell and to diffuse into the surrounding water. As the plug flowed from the egg a configuration similar to that of the astral rays in the mitotic figure developed, due supposedly to the different indices of refraction as the substance of the plug diffused into the water. The miracidium began to emerge immediately behind the mucoid plug, and this emergence from chilled eggs appeared to be more of a passive flowing through the opercular opening, rather than the result of any active behavior on the part of the miracidium. Almost no ciliary activity was observed until the larva was all but free of the shell, at which time a violent beating of the cilia began and the miracidium was hastened in its exit. Two eggs with miracidia so oriented as to place the posterior end toward the operculum were observed to hatch with no difficulty. They made their exit, posterior end first, as rapidly as did those of normal orientation. Exit normally required not more than one minute after the operculum opened.

In cultures of eggs that had incubated long past the minimum hatching period, miracidia were frequently seen showing violent ciliary activity over the entire surface of the body. A number of these were observed for long periods, but they were never able to open the egg and emerge. Such miracidia were occasionally seen in cultures of shorter incubation periods.

Longevity of the Miracidium. Bennett and Jenkins (1950) have reported the maximum life-span for this miracidium as being 16 hours and the average longevity as being 9.9 hours in standard reference

water at temperatures varying between 23° and 26° C. They reported a somewhat longer, but perhaps not significant, average life-span in filtered stream and lake waters. Weber (1950) and McPherson (1951) each listed several maximum and average life-spans at various temperature ranges, but they made no correlation between temperature and longevity. McPherson did conclude, however, that longevity is reduced at higher temperatures.

Some effort was made in this study to determine the mean life-span for the miracidium at various prevailing room temperatures, which ranged from 23° C. to 34° C., and at correlating temperature and longevity. The data obtained are presented in Table 7.

The various mean temperatures, based on hourly readings during each observation, ranged from 23.88° C. to 33.10° C. The maximum life-span ranged from 6.0 to 14.0 hours, and the average life-span from 4.9 to 9.9 hours.

When the formula for determining Pearson's produce-moment coefficient was applied to the data in Table 7, it was found that the coefficient of correlation between temperature and longevity was 0.856. A coefficient of 0.77 for any two variables tested by this formula represents a fairly high correlation and indicates a marked relationship between the two variables. The samples of miracidia involved represented several different egg collections, and the temperatures were far from constant. If miracidia from a single adult parasite population were tested at various uniform temperatures, perhaps the correlation between temperature and longevity would be even more striking.

Table 7. Maximum and Average Life-Spans of the Miracidia of P. microbothrioides at Various Temperatures.

Temp. Range ° C.	Mean Temp. ° C.	Maximum Life Span (Hrs.)	Average Life Span (Hrs.)	Sample Size
23.0-26.0	23.88	14	9.90	50
25.0-26.0	25.55	11	8.23	100
26.0-29.0	27.20	12	7.49	100
29.0-31.0	29.88	9	6.68	50
29.0-31.0	30.00	9	7.00	50
28.0-31.0	30.55	11	7.30	50
30.0-31.0	31.00	9	4.90	50
29.5-32.0	31.10	8	7.00	50
29.5-32.0	31.33	11	6.82	50
30.5-32.0	31.43	7	5.70	50
31.0-33.0	31.50	9	6.10	50
31.0-33.0	32.00	6	5.20	50
32.0-34.0	32.64	8	5.90	50
32.0-34.0	33.10	7	4.98	50

Bennett and Jenkins (1950) reported also on the effect that glucose in standard reference water had on the life-span of this miracidium. They found that the maximum life-span increased from 16 to 29 hours and that the average longevity increased from 9.9 to 16.1 hours over plain standard reference water at comparable temperatures. Prince (1950) had shown by a study of the miracidia and cercariae of S. mansoni in 1948 and 1949 that the presence of at least certain organic materials in water was conducive to a significant increase in the maximum and average life-spans of that form.

During the course of this study an effort was made to compare the effect of different sugars on the life-span of the miracidium; but the inability to control laboratory temperatures caused the experiments to be discontinued after a single test of 50 miracidia for each of 5 sugars (sucrose, glucose, fructose, galactose, mannose) against 50

control miracidia in standard reference water. The sugar solution in each case was one percent in standard reference water. The temperature for the various experiments ranged between 30° C. and 34° C.

Sucrose produced no apparent effect on longevity, as the average life-span in sucrose was 6.06 hours as compared to 5.7 in the control.

Glucose extended the maximum life-span from 11 to 14 hours and the average span from 7.3 to 8.68 hours. The cessation of swimming was the criterion used for determining death.

No average longevity was calculated for fructose. The controls were all dead at eight hours and the average life was 5.9 hours. Those in the fructose solution showed only very feeble swimming after eight hours. For the most part, those surviving eight hours showed only muscular twitchings or feeble, worm-like, creeping movements along the bottom of the isolation vial. Observations were discontinued after 14 hours when only two miracidia were showing feeble swimming movements. The remaining 21 were lying on the bottoms of the containers, and close observation revealed slight muscular contraction in each. Observations were resumed at the end of 25 hours in the fructose solution. Thirteen of the larvae had not deteriorated, and examination in a hanging drop under the compound microscope revealed muscular contractions, active flame cells and lashing cilia, but none were able to swim. At the end of 27 hours in the fructose solution, all the miracidia were dead.

The controls for the miracidia in galactose were all dead at the end of seven hours with an average life-span of 4.98 hours. At the end of six hours in galactose, only nine miracidia were able to

swim. Another 27 were living, but not swimming. At the end of nine hours none were swimming. Observations were not continued to determine how long life continued after swimming ceased.

In a solution of mannose, no miracidia were capable of swimming after 18 hours, but 12 lived beyond that time. None survived longer than 30 hours. The calculated average longevity was 11.44 hours; that for the controls with a maximum life-span of 9.0 hours was 4.9 hours.

A solution of 0.5% peptone was tested as a medium for miracidia. In one test of 50 miracidia, the maximum life-span was 8.0 hours and the average was 6.0 hours. The controls had a maximum span of 9.0 hours and an average of 7.0 hours. In a second test, miracidia did not live beyond 7.0 hours in a 0.5% peptone solution, as compared to 9.0 for the controls. The average longevity of these experimental miracidia was 5.84 hours. For the controls it was 6.68 hours.

Response to Light. The present investigation failed to confirm Bennett's (1936) observation that these miracidia possess a positive phototropism. Their response to light first became evident during the isolation of individual miracidia. It was noted that when a glass container with numerous individuals was placed under the dissecting microscope there was always a concentration of the miracidia on the side of the container that had been turned away from the north, lighted end of the laboratory. If this container was so oriented under the microscope as to place the concentration of miracidia toward the microscope lamp, it was noted that great swarms of the larvae always moved across the field of the microscope away from the source of light. These observations suggested that they were repelled by

weak, diffuse light, as well as by strong, direct light.

When large beakers (400-500 ml.) with thousands of miracidia were allowed to stand undisturbed on the laboratory table for some time and were placed in the strong beam of the microscope light in such a way as to orient the accumulated miracidia toward the light source, the miracidia were seen to advance almost in unison across the diameter of the beaker. So concentrated was the advancing front of miracidia that they were visible to the unaided eye. When care was taken to gently rotate the beaker 180 degrees, just as the mass of miracidia was reaching the side opposite the light source, the advancing front reversed its direction and again traveled away from the light. With care this procedure was repeated several times without undue dispersal of the miracidia.

To check the above observations quantitatively, two methods were employed. By the first method a horizontal staining dish with inside dimensions of 80 mm. x 55 mm. x 32 mm. deep was filled to a depth of approximately 15 mm. with water containing miracidia and placed on a laboratory table about 15 feet from the window with the long side parallel to the lighted end of the room. After one hour a glass microscope slide was placed across the middle length of the dish and formaldehyde was added to the water in each side of the dish to kill the miracidia. Those from either side were then counted with ease. By the second method, two fifteen-inch lengths of glass tubing with an inside diameter of eight millimeters were connected with a two-inch length of black rubber tubing. One length of glass tubing was wrapped with black paper to exclude light, while the other was left uncovered.

This assemblage was stoppered with corks at each end. To determine the effect of light on miracidia, this apparatus was filled with water containing miracidia and left in the light of a 60-watt bulb for one hour. By clamping the connecting length of rubber tubing, each length of glass tubing was emptied into an individual container where the miracidia were killed for counting.

Table 8 records the counts obtained by the above methods. Tests No. 1 and 2 were done on clear, sunny days, Test No. 3 on an overcast, rainy day. Possibly this negative phototropism would have appeared quantitatively much stronger had it been tested in larger containers.

Table 8. The Response of the Miracidia of P. microbothrioides to Light.

Test No.	Percent Miracidia (in lighted side of staining dish)	Percent Miracidia (in opposite side of staining dish)
1	19	81
2	27	73
3	35	65
	(in lighted end of glass tube)	(in darkened end of glass tube)
4	25	75
5	28	72
6	18	82

Willey (1941) observed no phototropism in the miracidia of Z. lunata and stated that they do not show any tendency to concentrate in any one place or in any way to influence each other. Swales (1935) stated that the miracidia of F. magna "do not appear to be attracted to either direct or diffused light. A strong microscope light does not

affect them in any way." Barlow (1925) made no mention of a phototropism concerning the miracidia of Fasciolopsis buski, but he observed that "There is a swarming behaviour in which the miracidia gather in a wide, roughly oval-shaped band, swimming mostly in the same direction and preferring one side of the dish to the other..."

Response to Gravity. The miracidia of P. microbothrioides did not always appear vertically equally distributed in the water. When the water in which they were swimming was as much as two to three inches deep, they always appeared to be concentrated toward the bottom part of the vessel. To determine the water level preferred, numerous miracidia were placed in a 100 ml. graduated cylinder, which was filled with water to a level of nine inches. After a lapse of one hour, to allow the miracidia to seek the level desired, the water was siphoned from the cylinder inch by inch into nine different containers. Formaldehyde was added to each of the nine containers, and the miracidia in each sample were counted. Three separate tests were conducted. The results are tabulated in Table 9.

Table 9. The Response of the Miracidia of P. microbothrioides to Gravity.

	No. of Miracidia in Various One-Inch Strata from Top to Bottom									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	Total
Test No. 1	7	10	9	11	36	46	22	60	1950	2151
Approx. %	.5	.5	.5	.5	2	2	1	3	90	
Test No. 2	416	351	273	273	152	195	182	481	1560	3883
Approx. %	11	9	7	7	4	5	4.5	12.5	40	
Test No. 3	169	130	117	78	65	47	143	228	3900	4877
Approx. %	3.5	2.5	2.5	1.5	1.5	1	3	4.5	80	

Uniform lighting of the cylinder, to avoid the influence of light, was attempted by conducting each test near the windows of the laboratory. The ages of the miracidia when siphoned from the cylinder were five hours, two and one-half hours and one hour in the order listed in Table 9.

In each of the three tests a concentration of miracidia occurred in the bottom inch of water. Only small variations occurred at the various depths above. No explanation is offered for the relatively small percentage of miracidia in the bottom inch of Test No. 2 as compared to the other two tests.

Willey's (1941) statement that the miracidia of Z. lunata show no tendency to concentrate in any one place suggests that they have no preference as to water level, but he does not say so specifically. Concerning the miracidium of F. buski, Barlow (1925) has said, "It swims more below the surface of the water than at the surface, because the snail, for which it has a predilection, is thoroughly aquatic in habit." Faust and Hoffman (1934) found the miracidia of S. mansoni in the top inch or the bottom half inch of water five inches deep. Faust (1924) reported that the miracidia of S. japonicum swim around in the bottom of the container for a brief period and then rise to within a few millimeters of the surface and that those of S. hematobium distribute themselves throughout the container a little while after hatching.

Infestation of the Intermediate Host by Mass Exposure. Bennett (1936) found that in most cases he was able to get 100% infestation of the intermediate host by this miracidium when he placed a large number of snails in water with hundreds of free-swimming miracidia, and Weber

(1950) reported an average mass infestation of 67%. In the present investigation every effort was made to use snails of a uniform size for all penetration experiments. The size selected for use was a snail with an overall length of 3 mm. from the apex to the most anterior tip of the body whorl. The snails were never measured to determine their exact size. To have done so would have required subjecting them to much handling and the possibility of injury. A sample of snail shells with a known length of 3 mm. was taken to the terraria at the time of collecting snails for experimental purposes and used as a comparison for selecting the desired size.

For determining the rate of infestation by mass exposure of the miracidia, snails were collected and placed in finger bowls with a diameter of 10 cm. and a depth of 4 cm. Large numbers of miracidia in standard reference water were then added to the bowls. In each case the miracidia were one hour old when placed with the snails. After a one-hour exposure, all the water was poured from the snails and they were rinsed with water before being placed on terraria to await development of the penetrated larvae. Because of the snails' inclination to leave the water, it was necessary to fill completely the finger bowl in each case and to place a piece of glass over the top, making it impossible for them to get out of the water.

After a period of 18 to 21 days, random samples of the snails thus exposed to miracidia were dissected and examined for the presence of redia and immature cercariae. Up to three weeks, the progeny of the experimental snails did not grow enough to be confused with the parent generation, but in many cases during the hot summer months a greater lapse of time made the selection of the original snails

extremely difficult because of the rapid growth-rate of the young. Two weeks after exposure, no cercariae occurred free in the host tissues, and the rediae were often so small and so few in number as to make positive diagnosis of infested individuals very difficult or, perhaps, even impossible.

Table 10 compiles the results of seven such mass exposures of the miracidia to the snail host. The percentage found positive for infestation by dissection ranged from 55% to 100%, with an average of 87%. The water temperature at the time of exposure was comparable in each case, varying from 29° to 31° C. The ratio of miracidia to snails was not controlled in these experiments. It was estimated that not less than 1000 miracidia were present in each case.

Willey (1941), working with the paramphistome Z. lunata, obtained infestations of from 10% to 55% by exposing masses of miracidia to the intermediate snail host, Helisoma antrosum.

Table 10. Infestive Rates of Miracidia of P. microbothrioides at Age One Hour When Mass Exposed to the Snail Host, S. cubensis.

No. Snails Exposed	No. Snails Dissected	Percent Infested	Percent Not Infested
250	60	85	15
200	39	97	3
90	20	100	0
100	20	90	10
100	22	55	45
100	18	89	11
100	24	83	17
940	202	87	13 (Totals)

Infestation of the Intermediate Host by Single Exposure. In order to determine the efficiency of the individual miracidium for

penetrating and establishing itself in the intermediate host, groups of 100 newly-hatched miracidia were individually isolated into 100 separate vials, each containing one milliliter of standard reference water. When the miracidia were one hour old, one snail, 3 mm. in length and selected as described above, was added to each vial where it remained for one hour. The snails usually do not remain voluntarily in the water for long periods during the summer months. To insure uniform exposure for each snail, it became necessary to keep the snail forcibly submerged in the water with the miracidium. The only way to do this, and still avoid the loss of any free-swimming miracidia, which might be present at the end of the exposure period, was to sit by and push each emerging snail back below the surface of the water with an applicator stick. At the end of the exposure hour, each vial was carefully examined for the presence of a free-swimming miracidium. The snails from those vials containing such miracidia were then discarded as being negative for infestation. The remainder of the snails were placed on laboratory terraria to await a developmental period of approximately three weeks.

Seven experiments of this type were attempted during the early part of this study. The results are tabulated in Table 11. The total infested ranged from 5% to 25%. The mean total was 12%. (All fractions in Tables 11, 12, 13 and 14 have been recorded as the nearest whole percentage). These exposures were made at laboratory temperatures, which varied from 23-29° C. Eggs from which the miracidia were hatched were taken from three different collections of eggs, as indicated in the column headed "Collection Number." Each of these collections represented a different stock or population of the adult parasite, and it is noted that the miracidia from Collection No. 2 consistently gave a much

lower percentage of infestation than did miracidia from either Collection No. 1 or Collection No. 3. It is noted also that the temperature (23-26° C.) for each of the series of Collection No. 2 was somewhat lower and that the refrigeration period (36-96 days) for these eggs was much longer than for either of the other two collections. Another variable was the incubation period, which ranged from 9 to 12 days. If any trend is indicated by the incubation period, it is that the longer period is conducive to a higher degree of infestation. It is possible that the range in the total infested is only that of normal sample variation. This is suggested by the fact that the largest sample, 200 miracidia in Experiment No. 1, gave a total infested of 12%, which is exactly the mean for the series of samples. These variables and their possible significance will be discussed in more detail below in connection with the other tabulated data.

Table 11. Infestive Rates of Miracidia of P. microbothrioides at Age One Hour When Individually Exposed to the Snail Host, S. cubensis.

Experiment No.	Age of Miracidia in Hrs.	Eggs			Temperature at Exposure (° C.)	Snails							
		Collection No.	Days Refrigerated	Days Incubated		Sample Size	Discarded (Miracidia Present)	Placed in Terraria	Surviving				Calculated Total Percent Infested
									Number	Percent	No. Infested	Percent Infested	
1	1	1	14	9	29	200	87	113	45	40	8	18	12
2	1	2	36	9	24	100	54	46	36	78	7	19	9
3	1	2	65	9	23	100	7	93	39	42	2	5	5
4	1	2	96	11	26	100	25	85	16	19	1	6	5
5	1	3	0	10	27	100	31	69	17	25	4	24	16
6	1	3	7	12	28	100	56	44	7	16	4	57	25
7	1	3	16	9	28½	100	26	74	56	76	13	23	17
					Totals	810	286	524	216	41	39	18	12

It seemed desirable to determine the effect of the age of the miracidium on its ability to establish itself in the snail host; therefore, representative eggs were chosen for comparison of miracidia that had been hatched together from a single egg collection. The ages of one, four and seven hours were chosen for working convenience, so that an experiment could be completed within a single day with as few variables as possible. Only one experiment was run at that time. The results are presented in Table 12, and they indicate a considerable increase in the infestibility of age four hours over age one hour, with the total infested increasing from 24% to 37%. The miracidia of age seven hours, with a total infested of 32%, appear less infestive than the four-hour olds, but still somewhat more infestive than the one-hour olds.

Table 12. Infestive Rates of Miracidia of P. microbothrioides at Ages One, Four and Seven Hours When Individually Exposed to the Snail Host, S. cubensis.

Experiment No.	Age of Miracidia in Hrs.	Eggs			Temperature at Exposure (° C.)	Snails							
		Collection No.	Days Refrigerated	Days Incubated		Sample Size	Discarded (Miracidia Present)	Placed in Terraria	Surviving				Calculated Total Percent Infested
									Number	Percent	No. Infested	Percent Infested	
8	1	4	17	14	25	100	17	83	21	25	6	29	24
8	4	4	17	14	25	100	14	86	44	51	19	43	37
8	7	4	17	14	25	100	9	91	48	53	17	35	32

All subsequent experiments relating to the infestation of the intermediate host were conducted with miracidia from a single, large

collection of eggs, which had been refrigerated for a considerable period of time.

Three experiments like the one described in Table 12 were run in close succession, using miracidia one, four and seven hours old. The data thus obtained are tabulated in Table 13. The variables for these experiments were reduced to a minimum. The temperature of the water at the time of exposure varied by not more than 1° C. The miracidia came from a single collection of eggs. The periods of refrigeration and incubation varied but little. With the exception of the first series of each age group (Experiment No. 9), the results were quite uniform within the age groups. The snails of that experiment (No. 9) were dissected 14 days following exposure to the miracidia, and it is believed that not all cases of infestation were detected. As stated earlier, no cercariae occur free in the snail tissues in so short a period, and the rediae may be so small or few in number as to make detection of infestation very difficult. The fourteen-day developmental period was attempted as a method of reducing the loss of snails to a minimum. All other snails were allowed to remain on the terraria for 21 days so that infestation might be detected more easily. In spite of the decline in the total infested in Experiment No. 9, it is believed that the ratio of infestation for the various age groups should not have been affected.

The results of this series of experiments indicated quite conclusively that there was an overall increase in the infestibility of the miracidium between the ages of one and four hours. It also appears from the data in Table 13 that there was a decrease in infestibility between the ages of four and seven hours to a point lower than at one

hour; but when the death rate of the miracidia during the eighth hour of life is considered, a different concept is gained.

Table 13. Infestive Rates of Miracidia of P. microbothrioides at Ages One, Four and Seven Hours When Individually Exposed to the Snail Host, S. cubensis.

Experiment No.	Age of Miracidia in Hrs.	Eggs			Temperature at Exposure (° C.)	Snails							
									Surviving				
		Collection No.	Days Refrigerated	Days Incubated		Sample Size	Discarded (Miracidia Present)	Placed in Terraria	Number	Percent	No. Infested	Percent Infested	Calculated Total Percent Infested
9	1	5	48	12	30½	100	20	80	68	85	6	9	7
10	1	5	50	11	30	100	12	88	69	78	11	16	14
11	1	5	53	11	30	100	23	77	72	94	14	19	15
					Totals	300	55	245	209	85	31	15	12
9	4	5	48	12	29½	100	31	69	66	96	15	23	16
10	4	5	50	11	30	100	12	88	68	77	15	22	19
11	4	5	53	11	30	100	23	77	56	73	17	30	23
					Totals	300	66	234	190	81	47	25	19
9	7	5	48	12	30½	100	1	99	88	89	5	6	6
10	7	5	50	11	30	100	2	98	89	91	11	12	12
11	7	5	53	11	30½	100	7	93	78	84	8	10	10
					Totals	300	10	290	255	88	24	9	9

To determine the death rate during various hours of the miracidia's life, three groups of 100 miracidia from Collection No. 5 were isolated exactly as for the infestibility determinations, except that no snails were added to the isolation vials. The temperatures were comparable also, ranging from 29.5° C. to 30° C. It was found that an average of 2% died during the second, fifth and sixth hours, each, and that 13% and 39% died during the seventh and eighth hours,

respectively.

The effect of the death of 2% of the miracidia during the second and fifth hours on the total infestation for those hours is negligible. If it is assumed that the miracidia that died during the eighth hour of life were unable to penetrate the snail host, and if adjusting calculations are made for that assumption, it is found that the mean calculated total infested by the seven-hour age group is raised to 15%, somewhat higher than the mean calculated total for the one-hour age group, but still lower than that of the four-hour age group.

After observing the trend of the results in the foregoing experiments, it became desirable to test the infestibility of ages five and six hours. It was physically impossible for a single investigator to test these two age groups simultaneously; therefore, three series of age six hours were run in close succession, followed by three of age five hours in close succession. See Table 14. Since these two ages could not be tested simultaneously, it seemed desirable that each be tested against one-hour old miracidia as a base of comparison. Had this not been done, the impression might have been gained that the efficiency of the miracidium increases considerably between ages four and five hours; but Table 14 reveals that the calculated total infested by the one-hour old miracidia also increased over those of like age in Table 13. Adjusting calculations for the miracidia dying during the seventh hour of life raises the mean calculated total infested by the six-hour old miracidia from 19% to 22%. The effect of the death of 2% of the miracidia during the sixth hour is negligible.

In comparing the data of Tables 11, 12, 13 and 14, it is noted

that the mean calculated total infested by the one-hour old miracidia is 12% in both Tables 11 and 13, even though several egg collections are involved, and in spite of the fairly wide range in temperatures and the varied refrigeration periods involved. In no case in these two sets of data does the incubation period for the eggs exceed 12 days. The two series of one-hour old miracidia from Collection No. 5 (Table 14) show a comparable mean calculated total infested of 29% and 31%, which is much higher than the same age groups in Tables 11 and 13, although the temperatures are comparable with those of Table 13. The one-hour age group from Collection No. 4 in Table 12 shows a calculated total infested of 24%, which is somewhat lower than that for the same age in Table 14, but still double that of the one-hour olds in Table 13, even though the temperature in Table 12 is considerably lower (approximately 5° C.).

In every case where the total infested ran higher than 12% for the one-hour old miracidia it is noted that the incubation period of the eggs also ran higher than 12 days. This is the only variable that consistently suggests a trend toward a lower or a higher infestibility on the part of the miracidia. This indication that a forced aging of the miracidium within the egg shell increases its ability to penetrate and establish itself in the snail host seems not unreasonable, especially in view of the fact that aging after hatching indicates a definite increase in that ability.

The suggestion gained from the data in Table 11 (Collection No. 2) that the refrigeration period might be a factor in reducing infestibility is opposed by the data in Table 14 where the refrigeration period is double that of Table 13 but still showing a much higher rate

of infestation.

The lower temperature (23-26° C.) in Table 11, as a possible factor in the lowering of infestation, is opposed by the data in Table 12. The temperature of exposure in Table 12 is 25° C., comparable to that in Table 11; yet the total infestation is much higher in Table 12.

Table 14. Infestive Rates of Miracidia of P. microbothrioides at Ages One, Five and Six Hours When Individually Exposed to the Snail Host, S. cubensis.

Experiment No.	Age of Miracidia in Hrs.	Eggs			Temperature at Exposure (° C.)	Snails							
									Surviving				
		Collection No.	Days Refrigerated	Days Incubated		Sample Size	Discarded (Miracidia Present)	Placed in Terraria	Number	Percent	No. Infested	Percent Infested	Calculated Total Percent Infested
12	1	5	96	14	30½	100	23	77	60	78	22	36	28
13	1	5	97	15	30	100	23	77	44	57	12	27	21
14	1	5	100	13	29½	100	17	83	46	55	24	52	43
					Totals	300	63	237	150	63	58	39	31
12	5	5	96	14	31	100	20	80	63	79	16	25	20
13	5	5	97	15	30	100	19	81	36	44	14	39	31
14	5	5	100	13	30	100	11	89	32	36	13	41	36
					Totals	300	50	250	131	52	43	33	27
15	1	5	83	13	30	100	16	84	80	95	31	39	32
16	1	5	85	13	31	100	17	83	72	87	24	33	28
17	1	5	86	15	30	100	18	82	74	90	25	34	28
					Totals	300	51	249	226	91	80	35	29
15	6	5	83	13	30	100	7	93	86	92	22	25	23
16	6	5	85	13	31	100	7	93	90	97	12	13	12
17	6	5	86	15	31	100	15	85	76	89	20	26	22
					Totals	300	29	271	252	93	54	21	19

None of the above considerations of known variables rule out the possibility of varying infestibility among different stocks, strains

or populations of the adult parasite.

Because of the variations in the rate of infestation shown by the one-hour old miracidia, it becomes necessary to use this age group as a base for comparing the rates of infestation among the other age groups. The ratios, both unadjusted and adjusted, for the infestation rates of the various age groups are as follows:

Table 12, Unadjusted	Ratio:	1	1.56			1.35
	Age in Hrs:	1	4	5	6	7
Tables 13 and 14, Unadjusted	Ratio:	1	1.58	.896	.658	.757
	Age in Hrs:	1	4	5	6	7
Tables 13 and 14, Adjusted	Ratio:	1	1.58	.896	.758	1.21
	Age in Hrs:	1	4	5	6	7

The data of Experiment No. 8, Table 12, cannot be adjusted to allow for the death rate of the miracidia during the eighth hour of life, because the death rate has not been determined for the temperature of that experiment (25° C.). On the basis of the data in Table 7 (Page 36), it can be assumed that this death rate was considerably lower than at 30° C. Likewise it can be expected that the decline in infestation beyond the fifth hour of life would be slower than at higher temperatures.

With the exception of the miracidia from Collection No. 1, 2 and 3 (Table 11), the number of miracidia that remained free and swimming at the end of each exposure period was quite constant for each age group. When the totals of these miracidia from Tables 12, 13 and 14 are combined by ages, the mean percentages are as follows:

Mean Percentages:	19	20	17	10	5
Age in Hrs.:	1	4	5	6	7

It is noted that there is little variation in the number of free-swimming miracidia remaining in the one-, four-, and five-hour old

groups. The death rates for these ages, as already shown, is low and uniform.

It is a matter of speculation as to whether all the miracidia unaccounted for at the end of the exposure hour succeeded in penetrating the host. If they did, many of them failed to establish themselves and produce rediae and cercariae. No doubt some of them were clinging to the snail and could not be seen. Others probably entered the mantle cavity and were lost to view. There is no possibility that any appreciable number of them died during the exposure period, at least not those of ages one, four and five hours. The presence of much debris from the snail made it impracticable to determine the number of dead in each case.

The decrease in the number of free-swimming miracidia remaining from age groups six and seven hours is probably explained by the known high death rates for these ages. It is hardly conceivable that there would be a marked increase in penetration ability after age four hours without a corresponding increase in the total number infested. But if the decrease in the number of free-swimming miracidia for the older stages is attributed to death, then it must follow that a considerable number of miracidia ordinarily did penetrate the snail host but failed to develop there. This possibility is strenghtened by the fact that the number of miracidia remaining free from the first three age groups (where the death rate is low and uniform) was essentially constant, whereas the number infested showed considerable variation between age groups.

No explanation is offered for the large numbers of miracidia that remained free-swimming in Experiments No. 1-7 (Table 11).

With the exceptions of Experiments No. 7 and 8, all the snails used in the foregoing studies were reared in laboratory terraria. Controls of 100 snails each were run on each series of experiments as a check on the possibility of natural or accidental infestation of the experimental snails. These controls also served to determine the effect of infestation, if any, on the death rates of the snails experimentally exposed to miracidia. The control snails were treated in every instance exactly like the experimental snails, except that no miracidia were placed into the isolation vials with them. In every case the control samples were placed on one side of a terrarium that had been divided longitudinally into approximate halves with plastic screening. Experimental snails were placed on the opposite side of the terrarium. Table 15 shows a comparison between the survival of experimental and control snails for twelve such terraria. From these data it can be concluded that single infestations did not contribute to a higher mortality in a snail population and that experimental snails exposed to individual miracidia died at random so far as infestation was concerned.

Maldonado and Acosta-Matienzo (1948) exposed individual miracidia of S. mansoni to the snail host, Australorbis glabratus, and they concluded that as long as the miracidium maintains its vigor, irrespective of age, the power of penetration is retained. Using penetration as the criterion of infestivity, they found that about three-fourths of the larvae were infestive. Schreiber and Schubert (1949) exposed miracidia of S. mansoni to A. glabratus one to two hours after hatching and left them exposed until they had penetrated or died. Fourteen percent of the snails that were exposed to single miracidia shed cercariae, and

snails exposed to one, three, seven and twelve miracidia each produced an increasing percentage of cercariae-producing individuals as a result of increasing numbers of miracidia used for exposures.

Table 15. Comparative Death Rates Between Control Snails, S. cubensis, and Those Exposed to a Single Miracidium of P. microbothrioides.

Percent Surviving at 3 Weeks	
Experimental Snails	Control Snails
40	44
49	36
89	78
91	91
82	68
84	56
96	99
89	80
57	61
44	48
73	85
92	96
Mean 74	70

3. The Snail Host, Stagnicola (Nasonia) cubensis Pfeiffer

For a number of years the workers in this laboratory thought that this snail was Fossaria parva (Lea), but a study of Hubendick's (1951) recent monograph on the Lymnaeidae has led to the impression that this species more nearly fits his description and figures of S. cubensis (Lymnaea cubensis in Hubendick's revision of the family). This impression has now been confirmed by Dr. Joseph P. E. Morrison's identification.

The Natural Habitat. Baker (1928) stated that the members of

the Subgenus Nasonia "exhibit terrestrial habits to a much larger degree than any other group of Lymnaeas, being found usually on wet banks or bars of mud, quite out of the water."

All collections of S. cubensis used in this study were made from a continuous stream or drainage ditch located in a meadow just south of the Louisiana State University Campus in Baton Rouge. Water fills the stream the year around, and it has a maximum width of about ten feet and steep banks that may be as high as six feet. At places silt deposits have partially filled the stream bed and formed mud bars of varying width along the water's edge. It is along these mud bars that the snails are found in greatest abundance; however, they may be found along the steep banks near the water at almost any place along the stream. This species may be found also on the mud around the edges of small ponds or lakes and along recurrent, roadside drainage ditches that are dry for long periods during the summer months. If vegetation occurs along these muddy edges, the snails are always found beneath the plants, never on them.

Laboratory Rearing. For rearing and maintaining the snails in the laboratory, terraria were constructed as described earlier under Materials and Methods. The best arrangement was that of dividing the soil in the wooden trays into three approximately equal parts by forming two longitudinal trenches and by maintaining a water flow in the trenches so as to just fill them without flooding the surface of the soil. Tap water was trickled through the trenches at a rate sufficient only to maintain the desired water level.

Snails were transferred from their natural habitat to the

terraria without apparent damage. Egg masses were always apparent in large numbers in the terraria within 24 hours after putting in a collection of the snails.

Algae were supplied as a source of food for the snails. Floating masses of algae that consisted principally of the Genus Oscillatoria were collected from local drainage ditches and placed in the water-filled trenches and on the mud surfaces. The algae grew well in the terraria, and it was often possible to maintain snails for several weeks without renewing the food supply; however, it was necessary to collect and add algae from time to time when large numbers of snails were involved. Various types of diatomes were present in the collections of algae, and these were found in the digestive tracts of dissected snails along with fragments of algae. At times good growths of Spriogyra developed in the terraria, but the snails apparently never made use of that alga as a source of food.

When algae were placed in the water of a terrarium and not on the mud surface, the snails left the mud and accumulated on the algae in the water. When food became scarce, it was noted that many of the snails left the wet surfaces, climbed onto the dry sides of the trays and went into aestivation.

Winter Habits. S. cubensis is rarely found in its natural habitat between the months of December and April. The winter of 1950-51 was a rather severe one for the Baton Rouge area, and it was not until May 5, 1951, that the snails were first observed. These were all very large (ranging from 6 mm. to slightly more than 9 mm. in height), suggesting that they had lived through the winter without reproducing.

It was not until June 9, 1951, that smaller snails (ranging from 2 to 5 mm. in height) were observed in numbers.

The winter of 1951-52 was extremely mild, and field observations in September, October and November revealed that the snails were continuously present; however, no winter collection was made until December 8, 1951. Approximately 1000 snails were taken on that date, and, although no measurements were made, it was observed that a wide range of sizes was represented. The size most desired in that collection was about 3 mm., and these were plentiful.

The snails were never found in quantities and reproducing during the winter months except on one particular mud bar that was exposed to the sunlight throughout the whole of each day. They were observed on this bar on mornings when the temperature was at or near freezing, but there was never any indication that they had gone into the water. Only an occasional snail could be located at other places along the stream. On May 17, 1952, it was noted that young snails had begun to occur on the continuously shaded mud bars.

On January 8, 1952, and during the second weeks of February, March, April and May, quantitative collections of snails were made. Because it appeared that these snails were reproducing more or less continuously, it was felt that accurate samples would require the collection of all the snails from a given area. To accomplish this, areas of one square foot were marked off and all the snails that could be seen were hand picked from at least two of these square-foot areas for each sample. To secure very small individuals, it was necessary to immerse the top layer of soil in water and remove the snails when they climbed

from the water onto the sides of the containers. No one sample contained less than 220 snails, and the largest sample was composed of 870 snails. Eggs were observed at the time of each of these collections, but they were particularly abundant in January and March.

Measurements of the shell heights of these population samples gave distributions that suggested cyclic reproduction during the first four months in which the collections were made. Each sample, when plotted graphically, gave a population curve that was trimodal, with the principal mode occurring at about 1.5 mm. or at about 5.5 mm. The January and March population curves very nearly coincided, with principal modes occurring between 5 mm. and 6 mm. and with minor modes at 2 mm. and between 7 mm. and 9 mm. The February and April population curves were likewise very similar, with principal modes occurring at 1.5 mm. and with minor modes at about 6 mm. and 8 mm. Observations of the snails in laboratory terraria and in their natural habitat indicated that reproduction was not cyclic during the summer months, and the population curve for May, 1953, was unimodal, being almost flat between 2 mm. and 4 mm. and dropping off rapidly on either end.

At the time of each of the above quantitative collections, samples were taken from an estimated ten square feet of the bottom of the stream. In January three snails were thus secured and in February only one. Such samples yielded none in March, April and May.

During the late summer of 1952, the drainage ditch was dredged, and the mud bar on which snails had reproduced during the previous winter was destroyed. Field observations throughout the winter of 1952-53 failed to reveal the presence of snails as during the preceding

winter, but dredged samples from the bottom of the water produced snails in small quantities during December, January and February.

Little attention was paid to the care of laboratory terraria during the winter of 1952-53, but several of them maintained snails throughout the whole winter. The snails were observed frequently, and they were always found to be active to some extent. They seemed to prefer the water during the winter months, but they did not restrict themselves to it. No reproduction occurred after early December. The first egg masses were observed on March 15, and the first young snails were seen on March 28, 1953.

Hoff (1936) concluded that when the air temperature reached 40° F. (4.4° C.) or lower Fossaria parva goes into the water and remains there until the temperature rises above 40° F. As a check on his field observations he performed experiments to demonstrate the reaction of F. parva to changes in temperature and found that snails placed in jars at room temperature crawled out of the water and up the sides of the jars; however, when these containers were placed in a refrigerator, the snails returned to the water. When Hoff's experiments were performed with S. cubensis, results identical to his were obtained.

To check the survival of the snails in water, ten of them were placed in filtered pond water and maintained in a refrigerator at a temperature of approximately 3° C. without food. They never crawled out of the water, and at the end of one month nine were surviving. At the end of two months none was surviving. This was repeated, using filtered water without food and water to which mud and algae had been added. No snails survived more than 45 days.

Summer Habits. S. cubensis, when collected from the natural habitat, rarely exceed 7 mm. in height; but the entire population of spring snails is composed of relatively large individuals, and May and June collections may contain a few as large as 10 mm. Soon after their appearance on the mud along the streams, egg masses become apparent and young snails begin to show up. Observations in the field and in the laboratory terraria indicated that reproduction is continuous throughout the summer months.

Quantitative samples were collected and measured in June, July and August of 1952. When plotted graphically, each sample gave a bimodal population curve, with the principal mode occurring at 2 to 2.5 mm. and the lesser one at 5 to 5.5 mm. Frequent summer rains caused the stream to flood and wash away many of the snails from the mud bars, and it was felt that little could be learned from such population curves.

Snails have not been observed in the water of the natural habitat during the summer months. They may occur from the water's edge to a distance as far from the water as the soil is thoroughly saturated. Occasionally one occurs in the very edge of the water where the depth is not sufficient to cover the snail. They deposit their eggs on the wet soil, rather than in the water, and in the terraria they often climbed onto the sides of the tray and deposited eggs just above the water line where the water happened to cover a part of the mud. In the confined spaces of a terrarium, they were often seen in the water where they appeared to be feeding.

Growth Rate and Sexual Maturity. Thirty-five egg masses from snails 5 mm. to 7 mm. in length were found to contain from three to

twenty-six eggs each, with an average of eight eggs per mass. During July and August of 1951 the eggs of snails in terraria usually required eight days for hatching. On one occasion some eggs hatched in seven days. The daily air temperatures in the basement window-well where the terraria were maintained ranged from a low of 21° C. to a high of 38° C. One group of eggs laid July 16 produced young snails that measured 3.7 mm. to 5.3 mm. and averaged 4.4 mm. in height 18 days after hatching. Eggs laid August 1 produced young snails that ranged from 4.5 mm. to 5.5 mm. and averaged 5.0 mm. in height 16 days after hatching. Eggs laid August 18 produced young snails that were sexually mature and laying eggs just 11 days after hatching. These ranged in height from 3.0 mm. to 4.25 mm. and averaged 3.4 mm.

The length of life of this snail has not been determined but it appears to be extremely short during the summer months. The above growth rates indicate that a height of 3 mm. may be attained in about one week. It was not unusual for experimental snails of that size to reach a length of 7 mm. during the following three weeks. Thus it appeared that the maximum summer size of 7 mm. to 7.5 mm. was reached within four to five weeks following hatching.

Response to Drying. Certain snails of the family Lymnaeidae are known to be capable of withstanding long periods of drought. Hoff (1937) collected F. parva from a dried stream bed during the driest part of a summer by digging beneath the hardened upper layer and removing soil to moist containers where they responded to a gradual addition of water. Olsen (1944) found that S. bulimoides techella burrowed into drying soil and were capable of withstanding at least five months of drought and summer temperatures of at least 40° C.

The dried bed of roadside ditches where S. cubensis is known to occur during wet seasons were not examined, but drying experiments in the laboratory failed to indicate that they actively burrowed into the soil. Snails that had climbed onto the sides of a flooded terrarium were examined three months after the water was drained away, and it was found that 50% of them responded and showed life when placed in water.

Effect of Parasitism. While dissecting snails that had been exposed to the miracidia of P. microbothrioides, the impression was gained that there was a tendency on the part of the infested individuals to be smaller than the non-infested ones. The snails from 20 experimental groups that had been individually exposed to the miracidia were measured to the nearest quarter of a millimeter just before each snail was dissected. The length of each snail was recorded along with a notation as to whether that snail had been found parasitized. The data thus obtained are recorded in Table 16. The ratio of infested to non-infested snails in each group was relatively low, but in every case, with one exception (Group No. 17), the infested individuals were found to have a shorter average length than did the non-infested ones. For the 20 groups combined, the average length of the infested snails was 0.5 mm. less than that of the non-infested snails.

Histological observations of the pathology produced by the parasite were not attempted, but gross observation of infested snails at dissection revealed varying stages of destruction of the digestive gland. Many snails were observed in which the entire visceral hump was filled with rediae and cercariae in various stages of development; and in some no trace of a digestive gland could be seen, but such snails had been able to move about in apparent good health.

Table 16. Length of Snails (*S. cubensis*) in Millimeters Three Weeks Following Exposure to a Single Miracidium of *P. microbothrioides*.

Group No.	Snails Infested		Snails Not Infested	
	No. Measured	Ave. Length	No. Measured	Ave. Length
1	6	7.166	62	7.210
2	15	6.983	51	7.147
3	5	6.140	82	6.442
4	11	7.000	58	7.215
5	15	6.966	53	7.200
6	14	5.821	58	6.069
7	17	5.691	39	6.077
8	8	5.594	70	5.943
9	31	5.943	49	6.260
10	22	5.727	64	6.172
11	23	6.087	48	6.687
12	12	5.625	78	6.195
13	25	5.990	49	6.449
14	19	5.644	56	6.298
15	27	5.314	54	5.388
16	11	5.091	31	5.331
17	12	5.437	32	5.422
18	14	5.303	22	5.534
19	24	5.469	22	5.661
20	13	5.346	19	5.592
Total	324	5.856	997	6.361

DISCUSSION

Although the foregoing observations and experimental data have been discussed in more or less detail and the pertinent literature has been cited in each section, it might be well to attempt an overall correlation of the various observations and experimental results with the total life-history of the trematode concerned. The application of experimental data to the organism in its natural habitat is difficult, and any such attempt results in speculation; therefore the interpretations offered in the following discussion may or may not be wholly correct.

In working with a trematode such as *P. microbothrioides*, it sometimes seems difficult to imagine that the life-cycle could ever by completed in sufficient numbers for the survival of the species, in spite of the very large number of eggs produced by each adult parasite. In the first place, the eggs must be deposited so that they will reach water where they can develop and hatch. Secondly, the miracidia must contact a suitable snail host during its relatively short life-span. The results of experimental infestations indicate that a very small percentage of those miracidia individually exposed to the snail are capable of penetrating and establishing themselves, and with an amphibious snail as the intermediate host, such as *S. cubensis*, which appears seldom to enter the water, the possibility of contact between host and parasite seems remote.

In considering the reactions of the eggs and miracidia to the environmental conditions to which they were subjected during this

investigation, it appears that many of them actually have survival value for the organism. Although the eggs were rapidly destroyed when removed from water, McPherson (1951) found that they survived long periods in cow feces without undue loss of viability, so long as the moisture content did not drop below 29%. This implies that they can survive for long periods after passing from the definitive host and still develop normally, provided that they finally reach a body of water. It appears then that the eggs do not have to be deposited directly into water, but that they may survive to reach water indirectly, as when washed from the feces by rain and carried into a stream or lake.

Once the eggs are enveloped in water where the dissolved oxygen content is sufficient, they may proceed in their development until mature miracidia are hatched. From the data obtained during this investigation, it would appear that the eggs could survive an oxygen tension below the minimum required for development, as undeveloped eggs were effectively stored for periods of up to 60 days in sealed water and eggs with partially developed embryos were stored in the same manner for periods of up to 74 days. If such low oxygen tensions were encountered in a stream, either before or after development had begun, they might be washed out by the running water and deposited in more suitable conditions, where development could proceed normally.

The findings of this study also indicate that the eggs would not be adversely affected in their development and hatching by various hydrogen ion concentrations between pH values of 5.5 and 8.2.

It appears that the snail host never enters the water during the summer months, but they are unable to escape the rising waters of

drainage ditches following summer rains. Surface water drains into the ditches and floods the mud bars on which the snails live and reproduce. Thus the snails are forced to remain in water for some time, or until the water level in the ditches returns to normal. Under such conditions, most of the snails remain on the mud bottom. They have not been observed to rise and cling to the surface as often happens in the case of aquatic snails.

The temperature of the water in the drainage ditches of this area remains at approximately 28° C. throughout the summer. Sudden summer showers cause a drop of several degrees in the temperature of the water, and, in the laboratory, such decreases in the temperature have consistently served as a stimulus for the hatching of large numbers of miracidia. Thus the presence of the miracidia in large numbers would increase the possibility of a relatively high percentage of infestation of the snails, as indicated by mass exposure experiments. The negative response of the miracidia to light and their apparent preference for the bottom layers of water would tend to concentrate them on the bottom of the stream where the snails are available.

Experimental data gave some indication that the miracidia gain in their ability to penetrate and establish themselves in the snail host by aging in the egg shell beyond the minimum incubation period required for hatching. If this is the case, chilling would result not only in producing greater numbers of miracidia at a given time, but these miracidia would be more efficient by having delayed their hatching until stimulated by a decrease in temperature.

SUMMARY AND CONCLUSIONS

The eggs of P. microbothrioides were rapidly destroyed by desiccation when removed from water.

Freezing of the eggs for periods of one to four hours caused a marked decrease in viability. Eggs frozen five hours or longer were completely destroyed.

Refrigeration of undeveloped eggs at 5° C. prevented development. The viability of one lot of eggs decreased from 69% to 30% during 12 months of refrigeration, and the minimum incubation period for development to the hatching stage at 30° C. increased from nine to eleven days after eleven months of refrigeration.

The time required for the miracidium to develop varied with temperature. The minimum incubation period for development to the hatching stage was eleven days at 28° C., nine days at 30° C., and eight days at 35° C. Development failed to proceed to the hatching stage at 38° C. and at 40° C.

Eggs incubated and maintained at 28° C. and at 30° C. did not hatch sporadically. At 28° C. hatching was practically complete after 62 days; at 30° C., it was practically complete after 41 days.

Reduced oxygen tension arrested or retarded development. Eggs were effectively stored by sealing in boiled or unboiled water for periods of up to 60 days. Little reduction in the viability of undeveloped eggs occurred during such storage. Partially developed embryos were arrested in their development for periods of up to 74 days by

sealing in boiled water.

Development and hatching were not affected by pH values between 5.5 and 8.2. The longevity of the miracidium was considerably r educed in weakly acid waters.

Light did not serve as a stimulus for hatching. A decrease in temperature of five or more degrees Centigrade for eggs incubated at temperatures varying between 21° C. and 35° C. stimulated immediate hatching of large numbers of miracidia. An elevation of the temperature failed to stimulate hatching.

When mature miracidia were induced to hatch by chilling, they showed ciliary activity for about three minutes before the operculum opened. Exit from the egg shell appeared to be passive on the part of the miracidium and required about one minute.

The longevity of the miracidium was correlated with temperature. At a mean temperature of 24° C., the maximum life-span was about 14 hours; at a mean temperature of 33° C., it was about seven hours.

Sucrose did not extend the longevity of the miracidium, but glucose, fructose, galactose and mannose prolonged life for several hours. Longevity was not increased by a 0.5% peptone solution.

Miracidia exhibited a negative phototropism in both direct and diffuse light.

Experimental results indicated that the miracidia had a preference for the bottoms of containers into which they were placed.

Mass exposure of the miracidia to the snail host resulted in infestation of about 87% of the snails.

Exposure of single miracidia to individual snails indicated a ratio of infestibility between miracidia of ages one and four hours of

about 1:1.5. At ages five, six and seven hours, the miracidia were capable of penetrating and establishing themselves in about the same ratios (or perhaps slightly less) as at age one hour. About 19% at ages one, four and five hours failed to penetrate the snail host and remained free-swimming after an hour's exposure.

Single infestations by the miracidium caused no increase in the death rate of the snail host.

S. cubensis were maintained and reared in the laboratory on terraria constructed to resemble the natural habitat. Algae of the Genus Oscillatoria served as a satisfactory source of food. Algae sometimes grew in old terraria in sufficient quantities to support small colonies, but it was usually necessary to add algae for maintaining experimental snails in large quantities.

The snails usually did not reproduce during the winter, which they normally spend in water.

The snails rarely exceeded 7 mm. in length except in May and June when they attained a shell height as great as 10 mm.

During the summer months, the snails did not normally enter the water, and they reproduced continuously, laying their eggs on mud. In terraria they entered the water at times during the summer and accumulated on clusters of algae.

In terraria during the summer months, the snails attained sexual maturity and an average length of 3.4 mm. in as few as 11 days after hatching, and they attained the usual maximum summer size of 7 to 7.5 mm. within four to five weeks following hatching.

Up to 50% of the snails survived aestivation for three months.

Three weeks following single exposure to the miracidia of P.

microbothrioides, the infested individuals averaged 0.5 mm. shorter than the non-infested ones. Varying degrees of destruction of the snail's digestive gland resulted from parasitism.

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BIOGRAPHY

B. Earl Prince was born at Sikes, Louisiana, on September 25, 1912. After graduating from the Sikes High School, he attended the Meadows-Draughon College in Shreveport, where he received training in bookkeeping and stenography. In 1937 he registered in Louisiana State University and was awarded the Bachelor of Science degree in 1940. From September of 1940 until February of 1942, when he entered upon active duty in the United States Navy, he attended the Louisiana State University as a graduate student in Zoology. He returned to that university in June of 1949 and was awarded the Degree of Master of Science in Zoology in June of 1950. He is now a candidate for the degree of Doctor of Philosophy in the Department of Zoology, Physiology and Entomology.

EXAMINATION AND THESIS REPORT

Candidate: Buford Earl Prince

Major Field: Zoology

Title of Thesis: Biological Studies on the Miracidia of *Paramphistomum microbothrioides* Price and McIntosh and Its Intermediate Snail Host, *Stagnicola* (*Nasonia*) *cubensis* Pfeiffer.

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

May 11, 1953