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THE INFLUENCE OF CERTAIN ENVIRONMENTAL FACTORS ON THE DEVELOPMENT OF EGGS AND SURVIVAL OF MIRACIDIA OF *ECHINOSTOMA BARBOSAI* LIE AND BASCH, 1966

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SUMMARY

The environmental factors of temperature, pH concentration, buffer solutions and pressure were studied to determine their influence on the development and hatching of the eggs of *Echinostoma barbosai*. Combinations of three temperatures (23°C, 28°C and 31°C) and five pH concentrations (8.5, 7.5, 6.5, 5.5 and 4.5) showed that maximum development and yield of miracidia occur at 28°C and pH levels of around 7.5 to 6.5. However, under certain circumstances in natural conditions, the slower hatching and release of miracidia might have some advantages over a quick emergence in a single burst of all the miracidia at the same time. Eggs developed in buffered distilled water were susceptible to the chemical composition of the buffer system used. The survival of miracidia at various pH concentrations from eggs developed in different pH solutions seemed to depend on the pH level where the miracidia were placed after hatching rather than on the level where the embryo developed. Survival in all experiments was best at pH levels of 8.5 to 7.5. Pressure, tested up to the equivalent of 34.6 ft of water, did not seem to affect embryonic development and hatching was normal.

INTRODUCTION

The life cycle of *Echinostoma barbosai* was described by LIE & BASCH [4] from the snail *Biomphalaria glabrata* SAY obtained from Recife, Brazil. The cercariae encyst in various snails; the adult worms develop in chicks, ducklings and pigeons. When *E. barbosai* and *Schistosoma mansoni* SAMBON occur together in the same snail, rediae of the former interfere with the development of sporocysts of the latter (LIE [3]). However, interaction between these two parasites is less effective than between rediae of *E. audyi* and sporocysts of certain trematodes in the snail *Lymnaea rubiginosa* MICHELIN (LIE et al. [2]).

The studies mentioned revealed that certain environmental factors, such as temperature, pH level, composition of pH buffers, and pressure, influenced the development of the eggs of *E. barbosai* and the survival of the miracidia. These influences were therefore investigated and are here described.

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MATERIALS AND METHODS

Adult *E. barbosai* worms were collected from experimentally infected 2-week-old chicks that had been fed metacercariae 19 to 24 days previously. Chicks were killed with chloroform, the rectum was dissected out between the anus and the caecum, and its contents were removed under saline solution in a large Petri dish. From there the worms were transferred in clean saline or buffer solution to 2-inch Petri dishes.

The quantity of infected material that was fed to the chicks was not standardized, so that infections with adult worms varied considerably from one host to another, ranging from 0 to 60. The number of eggs per worm also varied, usually between 50 and 150.

Eggs were teased out of the worm tissues by means of fine needles under the final buffer solution in 2-inch Petri dishes using a stereoscopic microscope with a zoom lens. The buffer solution was replaced several times to remove debris and the Petri dish closed with its top half. Eggs were incubated in the dark in all experiments.

The development of the embryo was examined daily under the compound microscope with low power objective directly from the Petri dish, from which the top half had been removed. Sixteen stages of development were recognized based on those illustrated for *E. revolutum* by JOHNSON [1].

Procedures varied for the study of the influence of different environmental factors and are described separately:

1) *Temperature and pH*

Experiments on temperature and pH were run simultaneously. The pH buffer of McIlvane was used, consisting of appropriate mixtures of 0.2 N Na_2HPO_4 with 0.1 N citric acid. Five pH mixtures (4.5, 5.5, 6.5, 7.5 and 8.5) and three temperatures (room temperature about 23°C; incubation temperatures of 28°C and 31°C) were tested.

Stages of development of the eggs, similar to those of JOHNSON [1], were determined daily and plotted against time. The resulting curves are shown in Figs. 1 to 5. The final percentages of hatching after 28 days are represented diagrammatically based on temperature (Fig. 6) and on pH concentrations (Fig. 7).

2) *Buffer solutions*

The experiments in 1 were all carried out with the citric acid-dibasic phosphate system. Two other buffer systems were tried, one based on the mixtures of mono- and dibasic phosphates, the other on Na acetate-acetic acid combinations.

3) *Survival of the miracidia*

The survival of miracidia from eggs hatched in three different pH levels was tested by their motility; the hatched miracidia were observed after being placed in homologous and various heterologous pH levels. To do this, hatching of large batches of eggs in a given pH concentration was followed under the stereoscopic microscope. Under favorable circumstances, miracidia hatched in bursts of several dozen at a time. The freshly emerged miracidia were picked up by a Pasteur pipette fitted with a rubber bulb and were transferred into a small dish filled with the pH solution to be tested. A dish about 20 mm in diameter and 18 mm deep, for use with the Beckman pH meter, was found most convenient and was small enough to allow observation of all the miracidia at one glance through the stereoscopic microscope. Between 10 and 20 miracidia were placed in each dish for observation. Tests were repeated to reduce errors and individual variation. The end of survival time was estimated to have been reached when more than 90% of the miracidia became immobile and usually sank to the bottom.

Survival was tested at three pH levels: 8.5, 7.5 and 6.5. Miracidia liberated in each pH concentration were respectively placed in five pH solutions: 8.5, 7.5, 6.5, 5.5 and 4.5. The buffer system used was the citric acid-dibasic phosphate combination.

4) *Pressure*

To evaluate development and survival of eggs under conditions of deep water, experiments were set up to observe the effect of pressure on hatching time. Eggs collected in the way described here were di-

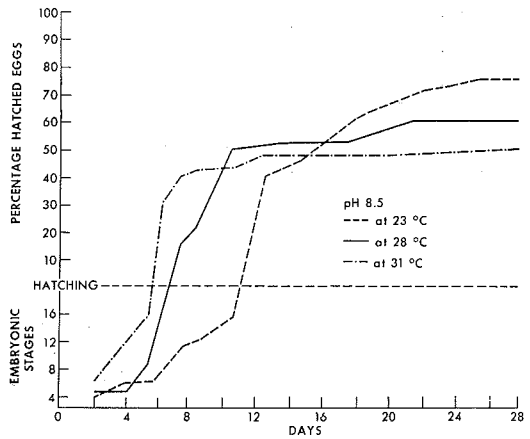
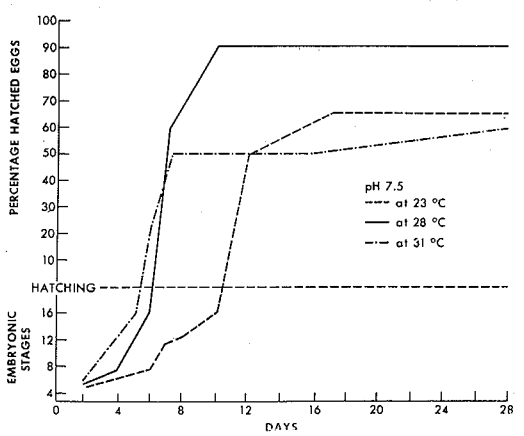


Fig. 1



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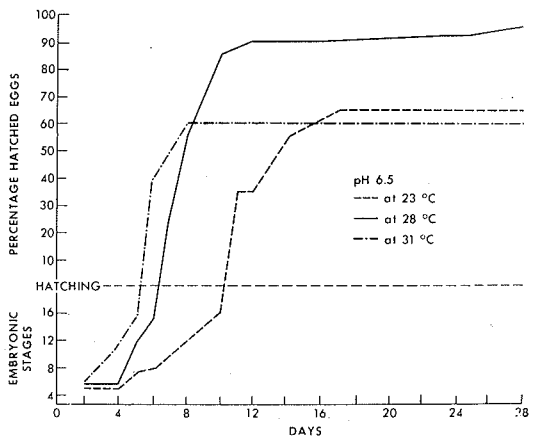

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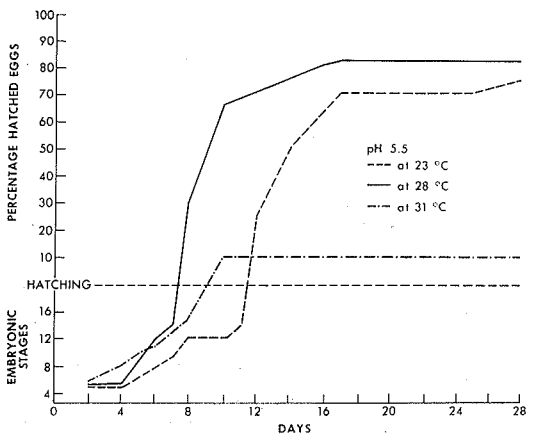

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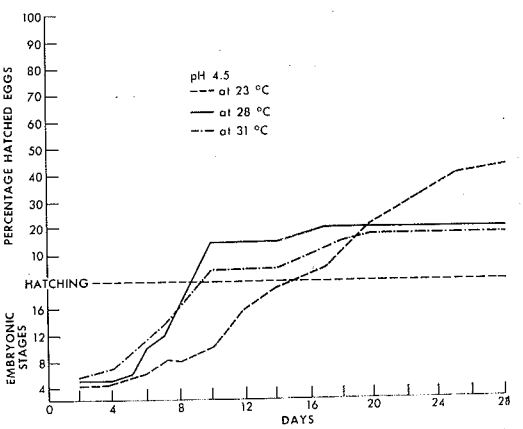

Fig. 5

Figs. 1-5 — Curves showing development of embryo in egg (lower section) and percentage of hatched eggs (shown above "hatching" line). Embryonic stages based on JOHNSON, 1920.

vided into two batches in separate Petri dishes in pH 7.5 buffered water. Experiments were carried out in the incubator at 28°C with one batch used as a control. The second Petri dish was placed inside a pressure jar, known as an anaerobic bacteriological incubation jar, closed by means of a metal, screw-top lid and provided with an air outlet and a pressure gauge. Once closed, the pressure inside the jar was raised by connecting the outlet with an air-pressure system to obtain the desired pressure; then the valve was closed and the whole jar placed in an incubator.

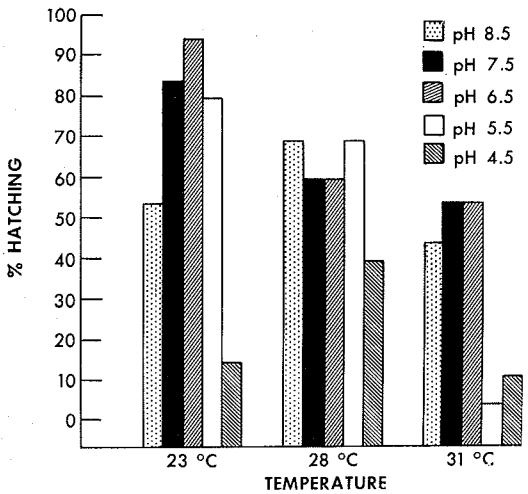

Fig. 6 — Percentage of eggs hatching 28 days after their removal from the worms for each pH level, at temperatures of 23°, 28° and 31°C.

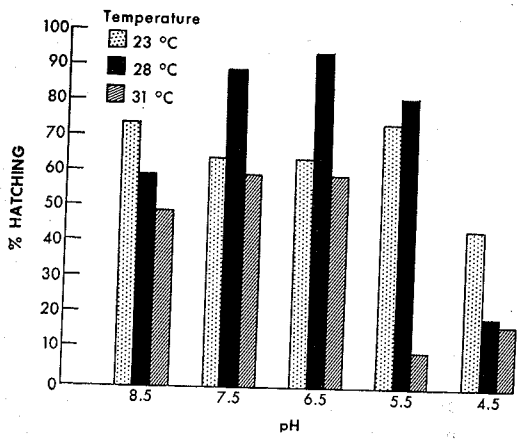

Fig. 7 — Percentage of eggs hatching 28 days after their removal from the worms, at pH levels of 8.5, 7.5, 6.5, 5.5 and 4.5 for each of the three temperatures.

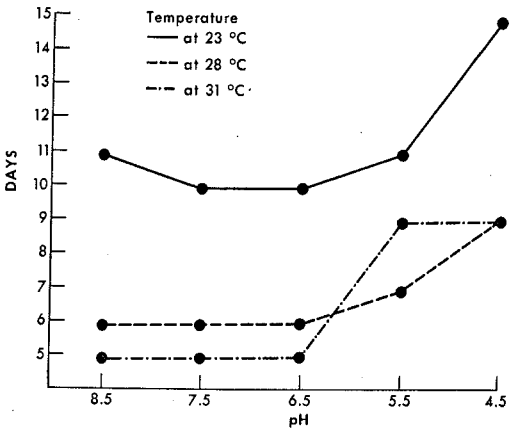

Fig. 8 — Number of days for eggs to start hatching at the three temperatures and the five pH levels tested.

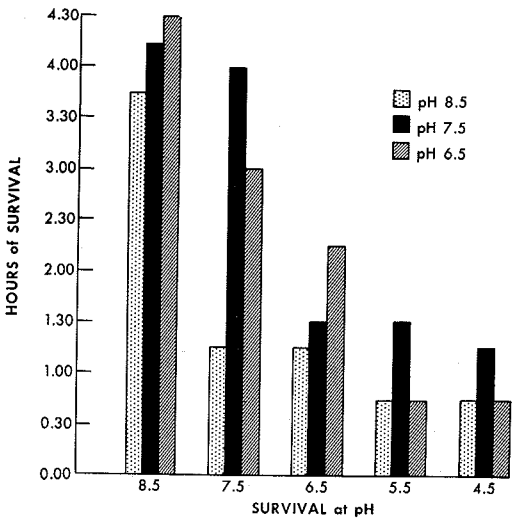

Fig. 9 — Time of survival in hours of freshly hatched miracidia at room temperature at combinations of pH levels of the incubated egg and of the buffer tested.

RESULTS

1) *Temperature and pH*

The experiments, which were repeated with very similar results as part of other experiments, showed that: a) The highest percentage of hatching eggs occurred at 28°C at pH levels of 7.5, 6.5 and 5.5; the highest yields were 95% at pH 6.5. b) More constant results for all pH levels occurred at 23°C, although the peak at this

temperature was only 75% (at pH 8.5 and 5.5). c) Results at 31°C were definitely less satisfactory than at 23 and 28°C. Averages of hatching for all pH levels were calculated for each temperature, with the following results: at 28°C — 71%; at 23°C — 65%; at 31°C — 40%. d) Optimum hatching under the conditions of these experiments occurred at 28°C at the 6.5 to 7.5 pH levels, when 90 to 95% of eggs hatched. This is clearly shown in Fig. 7.

Averages for all temperatures at each pH level were: at pH 8.5 — 61%; at pH 7.5 — 71%; at pH 6.5 — 73%; at pH 5.5 — 56%; at pH 4.5 — 31%.

The speed of embryonic development, that is, the time lapse between teasing out the eggs and the start of hatching, is shown in Table I.

TABLE I

pH level	Temperature in Centigrade	Day hatching starts
8.5	23	11
	28	6
	31	5
7.5	23	10
	28	6
	31	5
6.5	23	10
	28	6
	31	5
5.5	23	11
	28	7
	31	9
4.5	23	15
	28	9
	31	9

The more alkaline solutions, i.e. those between pH 6.5 and 8.5, seemed the most favorable, and the temperatures of 28 and 31°C shortened hatching time. As previously mentioned, poorer results percentage-wise were obtained at 31°C. Figure 8 shows development curves obtained when time was plotted against pH levels.

The rate of hatching in some experiments was gradual, any time within the 28 days of observation; in others maximum hatching took place shortly after the first eggs started to emerge, usually when hatching conditions were favorable. Examples of "drawn-out" curves were seen at pH 4.5, 5.5 and 8.5 at 23°C and at pH 7.5 at 31°C (Figs. 1, 2, 4 and 5). In all these experiments the number of eggs that were in process of hatching was still increasing, although slowly, at the end of the 28 days of observation. This phenomenon has certain survival value for the species: if, under highly favorable conditions, the sudden liberation of large numbers of hatching miracidia has a relatively high potential of infective value through sheer numbers at a given time, this also means that within the life span of this stage (not more than about 4 hours, as subsequent experiments showed) all suitable snail hosts must be found and invaded to insure survival of any particular batch of eggs. On the other hand, with slow emergence, the lower number of available miracidia is compensated for by their presence over a longer period of time, i.e., abundance is replaced by longer time of exposure.

2) *Buffer solutions*

The two buffer systems tried out gave far less satisfactory results than the citric acid-dibasic phosphate system used in previous experiments. In fact, no development resulted when the Na acetate — acetic acid combination was used, and results were very poor for the mono- and dibasic phosphate mixtures at pH 4.5, 5.5 and 6.5. The mono- and dibasic phosphate buffer gave somewhat better results at pH 7.5 and 8.5, but still yielded fewer miracidia than the citric acid-phosphate combination. Comparison between these two buffer systems at 28°C showed:

Citric acid — phosphate: 80% hatching after 8 days.

Phosphate — phosphate: 63% hatching after 8 days.

Citric acid — phosphate: 92% hatching after 11 days.

Phosphate — phosphate: 74% hatching after 11 days.

Embryos developed normally in tapwater at a pH of 7.2; 82% hatched after 11 days

at 28°C. Development in distilled water at pH 5.7 was also normal, 75% of the eggs hatching after 11 days. These results were very similar to those obtained with buffered solutions at the same pH levels (Figs. 2 and 4). Both ion-concentration and chemical composition seemed important to the development of the embryos.

3) *Survival of miracidia*

Hatchings from pH 5.5 and 4.5 were also attempted, but they yielded so few miracidia at a given time that the regular tests described were practically impossible. However, in one experiment the hatched miracidia from a pH 5.5 solution could be followed at pH 8.5, 7.5 and 6.5 levels, and all survived about 30 minutes, which is considerably less time than in more alkaline solutions.

Results, diagrammatically presented in Fig. 9, show greater survival at the higher alkaline levels of 8.5 and 7.5, decreasing rapidly at lower pH levels. It is noteworthy that survival time depended on the pH of the environment in which the miracidia were placed after hatching rather than at time the eggs (embryo) developed. In only two cases did the homologous pH systems (of the eggs and of the miracidia) seem to provide optimum survival conditions, namely those of pH 7.5 and 6.5. In the lower ranges, pH 7.5-hatched miracidia did somewhat better than those hatched from the two other pH levels.

4) *Pressure*

The first experiment on pressure was carried out at 5 lb/sq in, the next at 15 lb/sq in (equal to about 11.5 ft and 34.6 ft of water respectively). In both experiments the time of development and hatching of the eggs were the same as for the control and the percentage of hatched eggs after a given time was about the same.

CONCLUSIONS

This series of simple tests indicates that the development of the eggs of *Echinostoma barbosai* and the survival of the miracidia are best at pH 6.5 to pH 7.5, at temperatures around 28°C. Moreover, development was not impaired when pressure was applied to simulate a depth of about 35 ft of water. It is assumed that the pH levels and temperatures found favorable in these tests are those representing conditions in the current natural environment of this parasite. While repeated experiments largely confirmed the results noted here, some batches of eggs deviated in hatching and survival time. The reasons for these discrepancies have not been investigated. It would be interesting to obtain measurements of pH, temperature and chemical composition from various natural habitats of this parasite in the field and compare them with laboratory results.

RESUMO

Influência de certos fatôres ambientais sôbre o desenvolvimento de ovos e a sobrevivência de miracídios de Echinostoma barbosai LIE & BASCH, 1966.

Fatôres ambientais como a temperatura, concentração hidrogeniônica e pressão, foram estudados quanto à sua influência sôbre o desenvolvimento e a eclosão de ovos do *Echinostoma barbosai*. Combinações de três temperaturas (23°C, 28°C e 31°C) e cinco concentrações de pH (8,5 - 7,5 - 6,5 - 5,5 e 4,5) demonstraram que o maior desenvolvimento e a produção máxima de miracídios ocorrem a 28°C e níveis de pH em tôrno de 7,5 e 6,5. No entanto, em certas circunstâncias, a eclosão lenta e uma produção reduzida de miracídios podem em condições naturais, apresentar vantagens em relação a uma emergência múltipla, numa só eclosão sincrônica, de todos os miracídios. Ovos desenvolvidos em água destilada tamponada mostraram-se sensíveis à composição química do tampão utilizado. A sobrevivência de miracídios em diferentes soluções de pH parecia depender mais do nível de pH em que se colocavam os miracídios após a eclosão do ôvo, do que da concentração hidrogeniônica em que se desenvolveu o embrião. Em todos os experimentos, a sobrevivência foi maior em níveis de pH de 8,5 a 7,5. Pressão, testada até o equivalente de 34,6, não pareceu afetar o desenvolvimento embrionário e a eclosão foi normal.

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