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Development, hatching, and infectivity of *Echinostoma caproni* (Trematoda) eggs, and histologic and histochemical observations on the miracidia

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Abstract Light microscopy studies were done on the eggs and miracidia of the intestinal trematode *Echinostoma caproni*. Eggs were obtained by homogenizing adult worms derived from ICR mice in artificial spring water (ASW). To study egg development and to obtain miracidia, eggs were incubated in ASW in either a light or a dark environment for 10 days at 28° C. Egg development was characterized and staged at 2-day intervals from day 0 to day 10 postembryonation. No significant difference in development was seen in eggs maintained under conditions of light or darkness. Eggs maintained in darkness for 10 days and exposed to incandescent light produced a large synchronous hatch of miracidia within 3 h of exposure to light. As expected, miracidia used to expose *Biomphalaria glabrata* snails produced patent infections at 5–7 weeks postinfection (p.i.). Embryonated eggs aged from 0 to 10 days that had been fed to *B. glabrata* snails also produced infections within 4 weeks of infection. Miracidial longevity studies showed that 50 newly hatched miracidia maintained at either 4º, 12º, 22º, or 38° C lived for 6, 28, 14, and 5 h, respectively. Histology studies were done on whole miracidia stained in 0.01% neutral red and in Schneider's acetocarmine. Histochemistry studies of miracidia stained with alcian blue (pH 2.5) showed the presence of acid mucopolysaccharides in the epidermal plates; miracidia treated with periodic acid-Schiff plus 0.5% malt diastase showed the presence of glycogen in the body and in the epidermal/subepidermal region. Miracidia stained with Lillie's Oil Red O showed the presence of sparse neutral fat droplets in the body.

Introduction

Recent studies on the biology of echinostomes have focused on an Egyptian 37-collar-spined species, *Echinostoma caproni* (previously referred to as *E. liei*). This echinostome is easy to maintain in the laboratory and can be cycled between mice and *Biomphalaria glabrata* snails (see Huffman and Fried 1990 for review). Most of these studies have been concerned with the biology of adult parasites in rodent hosts, e.g., that of Ursone and Fried (1995), but some work is available on the biology of the larval stages, e.g., that of Krejci and Fried (1994).

Behrens and Nollen (1993) provided information on egg development and miracidial hatching of *E. caproni* derived from adult worms grown in hamsters and mice. The purpose of our study was to provide additional light microscopic observations on the eggs and miracidia of *E. caproni*. Specifically, we examined the development and infectivity of eggs derived from adult worms grown in ICR mice and synchronous hatching of the miracidia. We also made histologic and histochemical observations on the miracidia.

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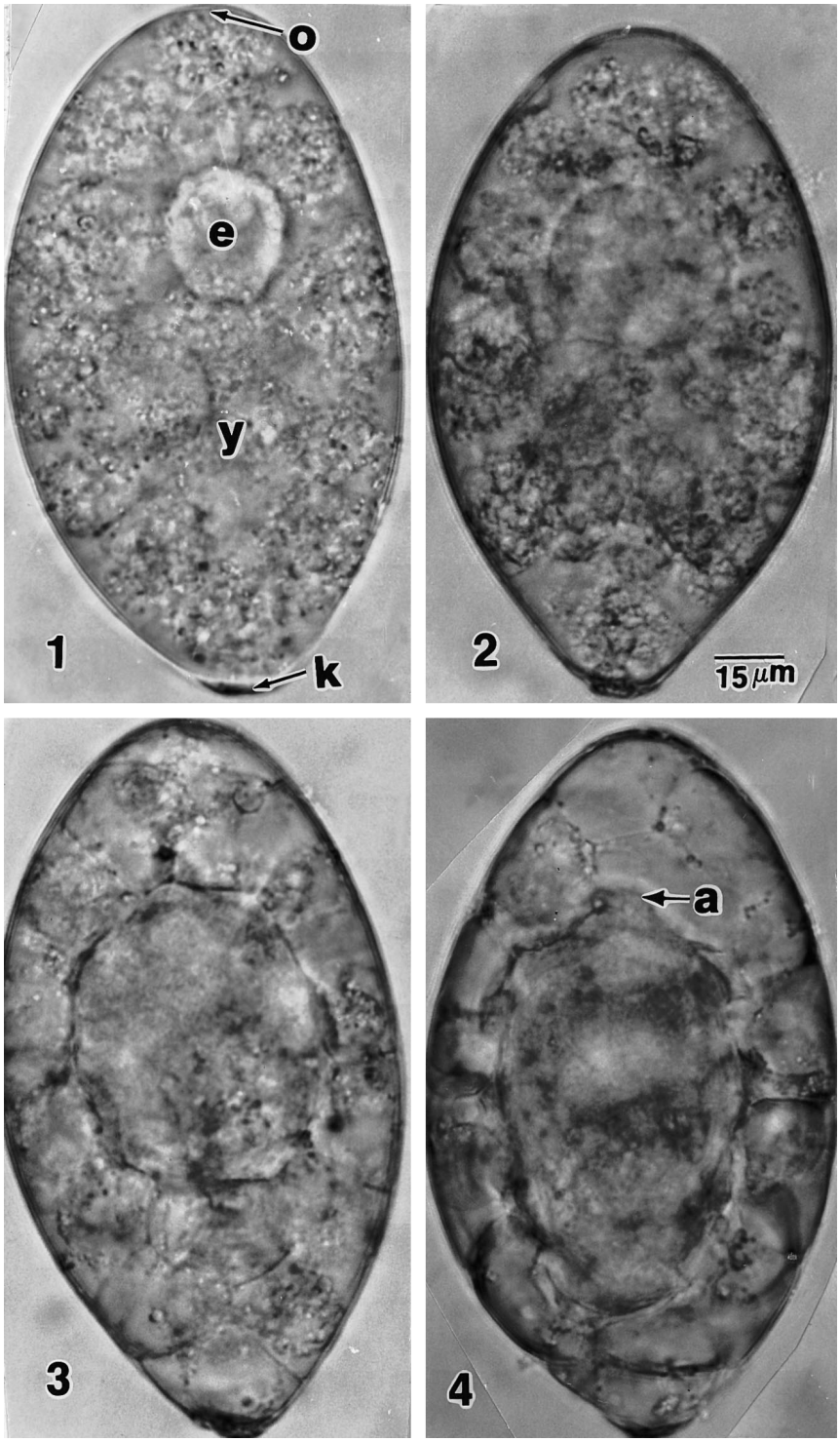
Materials and methods

Egg studies

Ovigerous worms aged 2–3 weeks were obtained from experimentally infected female ICR mice as described by Ursone and Fried (1995). Eggs were obtained by homogenizing 10 worms in 20 ml of artificial spring water (ASW; Ulmer 1970) in a Waring blender as described by Fried and Weaver (1969) for *Echinostoma trivolvis* (referred to as *E. revolutum* in that study). Approximately 200–400 eggs/worm were obtained using this procedure. During the course of this work, about 200 worms were homogenized to obtain sufficient eggs.

To obtain eggs with fully developed miracidia, about 400 eggs were placed in a disposable plastic tube containing 10 ml of ASW. The tube was covered with aluminum foil to avoid exposing the eggs to light and was incubated in a water bath at 28.2±0.2° C. Another tube was set up under the same conditions described but was left uncovered so as to expose the eggs to a constant source of incandescent light (a 50-W lamp maintained about 20 cm from the culture). Approximately 20 tubes maintained in a dark environment and another 10 tubes exposed to light were used to obtain eggs and miracidia.

Every 2 days following incubation, 20 randomly selected eggs prepared as wet mounts were measured with a calibrated ocular micrometer at 200 X. Unidentified refractile droplets were seen in empty eggs from which miracidia had emerged, and 20 such eggs

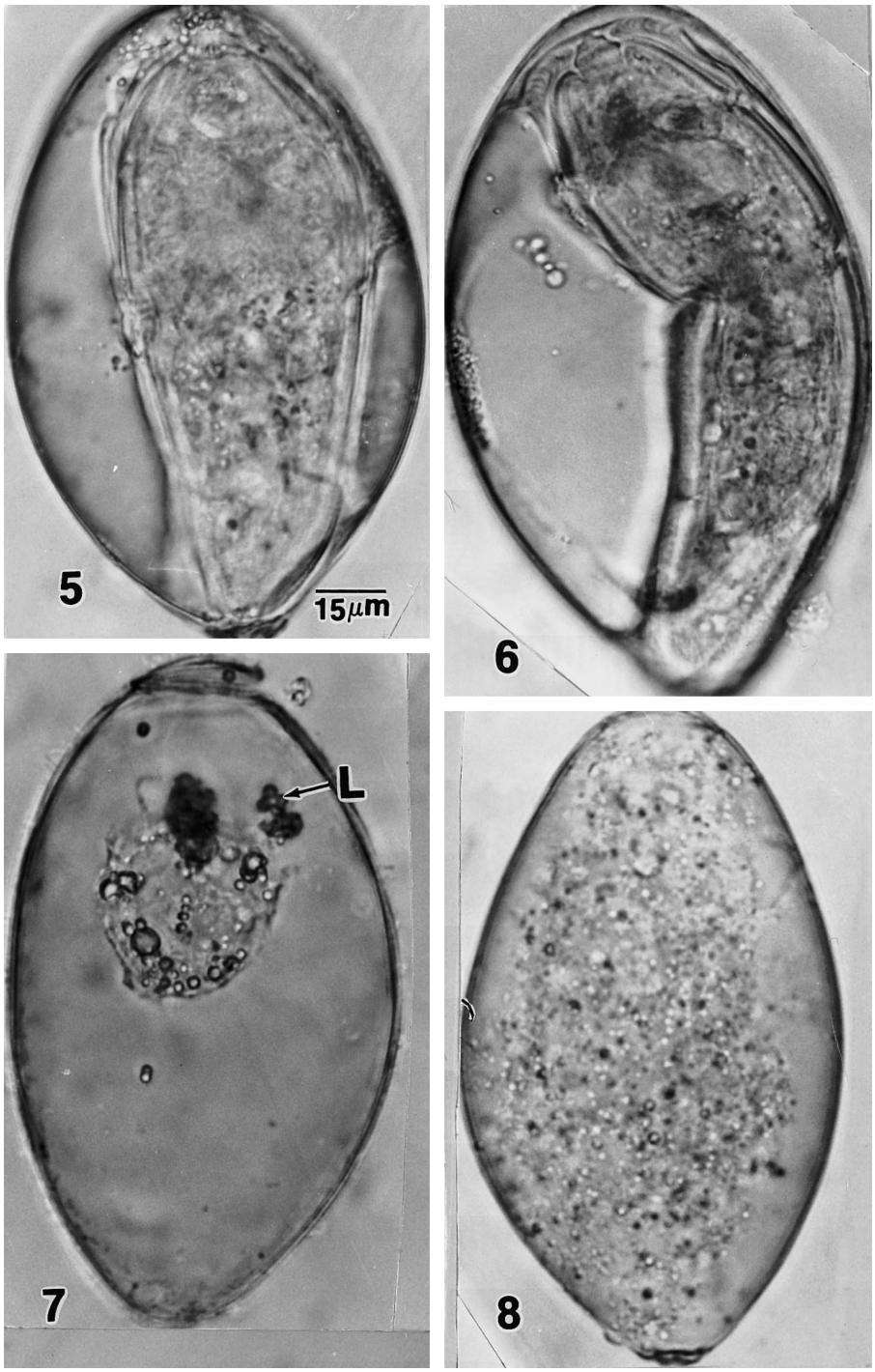


Figs. 1–4 Light micrographs of developing eggs of *Echinostoma caproni.* The bar in Fig. 2 is appropriate for all figures. **Fig. 1** A 0-day-old egg. Note the embryo (*e*), yolk (*y*), operculum (*o*), and knob (*k*). **Fig. 2** A 2-day-old egg. **Fig. 3** A 4-day-old egg. **Fig. 4** A 6-day-old egg. Note the apical papilla (*a*) in the developing miracidium

were stained in Lillie's (1944) Oil Red O (ORO) to determine if the droplets contained neutral lipids.

To compare the development of *E. caproni* eggs from mouse feces with those from homogenized worms, eggs were obtained from the feces of mice infected with 2-week-old worms. A 5-g sample of mouse feces was placed in a finger bowl with 40 ml of ASW and the feces were teased with needles to liberate the eggs. The eggs were removed with a pipet and transferred to a petri dish containing 30 ml of ASW. The procedure was repeated until the eggs were free of fecal matter. Between 150 and 200 eggs/g of feces were obtained. Approximately 400 eggs were placed in a plastic tube with 10 ml of ASW; the tube was covered with aluminum foil and incubated at 28.2±0.2° C for 10 days to obtain eggs with fully developed miracidia.

To determine if eggs could be used to infect *Biomphalaria glabrata* snails, *E. caproni* eggs at 0, 2, 4, 6, 8, and 10 days post-embryonation were fed at 20 eggs/snail (10 snails for each age group of eggs) to a total of 60 snails measuring 4–6 cm in shell diameter. The snails were maintained without food for 24 h prior to use. Each snail was placed individually in a multiwell chamber



Figs. 5–8 Light micrographs of developing eggs of *E. caproni*. The bar in Fig. 5 is appropriate for all figures. **Fig. 5** A 8-day-old egg. **Fig. 6** A 10-day-old egg. **Fig. 7** A 10-day-old egg that has released its miracidium. This egg was stained in Oil Red O. Note the lipid (*L*) droplets. **Fig. 8** A non-viable 10-day-old egg

with 2 ml of ASW and 20 eggs of the appropriate stage for 5 h. The snails were removed (10 snails for each age group of eggs), rinsed with ASW and placed in a plastic container with 40 ml of ASW, then maintained at 28° C and fed boiled lettuce ad libitum until necropsy at 3–4 weeks postexposure. The digestive gland-gonad (DGG) complex was then examined for the presence of daughter rediae.

Studies on miracidia

Approximately 200 fully developed eggs that had been maintained in a dark environment at 28° C for 10 days were transferred to a petri dish containing ASW at between 1300 and 1600 hours and were kept at 29–30° C under a constant 50-W incandescent light maintained 20 cm from the petri dish. In all, 20 replicates were thus established and, upon hatching, these miracidia were used for the various studies described herein.

Miracidial morphology was studied using organisms fixed in cold 10% neutral buffered formalin (NBF) and stained with: alcian blue, pH 2.5, for acid mucopolysaccharides; the periodic acid-Schiff reagent with and without 0.5% malt diastase (Humason 1972), for the presence of complex carbohydrates and glycogen; and Lillie's (1944) ORO for neutral fat. Live miracidia were stained in 0.01% aqueous neutral red for general morphology and in Schneider's acetocarmine (Humason 1972) for nuclear material.

The longevity of 50 miracidia each at either 4º, 12º, 22º, or 38° C was studied by placing freshly emerged miracidia in petri dishes containing 3 ml of ASW. The miracidia were examined every 2 h to determine their viability and were considered dead or moribund when they were inactive or moving slowly at the bottom of the petri dish.

Some miracidia were used to test infectivity by individually placing each of 10 snails (5–8 cm in shell diameter) in a multiwell chamber with 10 newly emerged miracidia in 2 ml of ASW for 24 h. Snails were then transferred to a jar containing 700 ml of aerated ASW and were fed boiled lettuce ad libitum for 7 weeks at 22° C. These snails were isolated to determine patent infections and were then crushed and examined to confirm the presence of rediae and cercariae in the DGG at 7 weeks p.i.

Results

Egg studies

The development of eggs obtained from homogenized worms and maintained in a dark or light environment was similar. The following results were obtained from eggs kept in darkness. In all, 95% of the newly embryonated eggs (day 0) contained fine granular yolk and an embryo located near the operculum (Fig. 1). During these observations (from 0 to 10 days postembryonation at 28° C), 90% of the incubated eggs showed uniform development and 10% were nonviable. Eggs incubated for 2 days had enlarged embryos and contained defined clusters of yolk globules (Fig. 2). The 4-day-old eggs had relatively large embryos and contained large, distinct yolk globules (Fig. 3). After 6 days of incubation, the embryo had grown longer and the yolk globules were transparent; at this time the apical papilla was seen at the operculate end of the egg and the embryo began to resemble a miracidium (Fig. 4). By day 8 the miracidium showed a further increase in length and the number of yolk globules in the egg decreased markedly, leaving behind a large vacuole (the viscous cushion?) adjacent to the miracidium; at this time the miracidium was oriented along the long axis of the egg (Fig. 5). At day 10, a fully developed miracidium whose length was greater than that of the egg was seen. This miracidium was located on one side of the egg and was partially folded in the middle (Fig. 6).

As based on 20 replicates, 2–5% of the 200 eggs that had been kept in a dark environment for 10 days had hatched prior to their exposure to incandescent light. Once the eggs had been transferred to fresh ASW and maintained in the light, a synchronous hatch of 75–80% of the miracidia occurred within 3 h. Hatching data on eggs kept in constant light were not recorded. Figure 7 shows an empty 10-day-old egg that had released its miracidium and was stained in ORO. This egg contained red droplets indicative of neutral lipids. Figure 8 shows a nonviable egg that did not develop during 10 days of incubation in a dark environment at 28° C in ASW.

The mean length and width of eggs and embryos kept in darkness are shown in Fig. 9. Data on eggs kept in the light are almost identical and are not included herein.

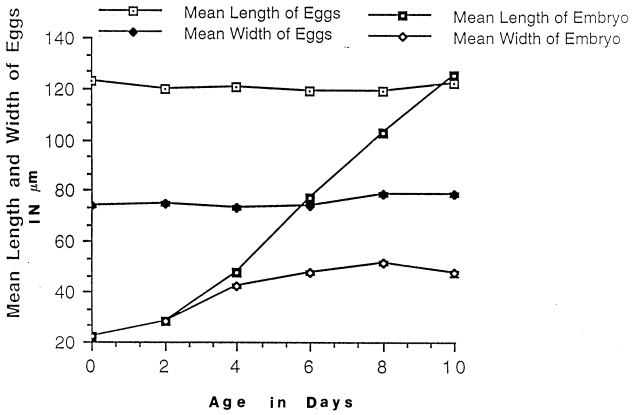
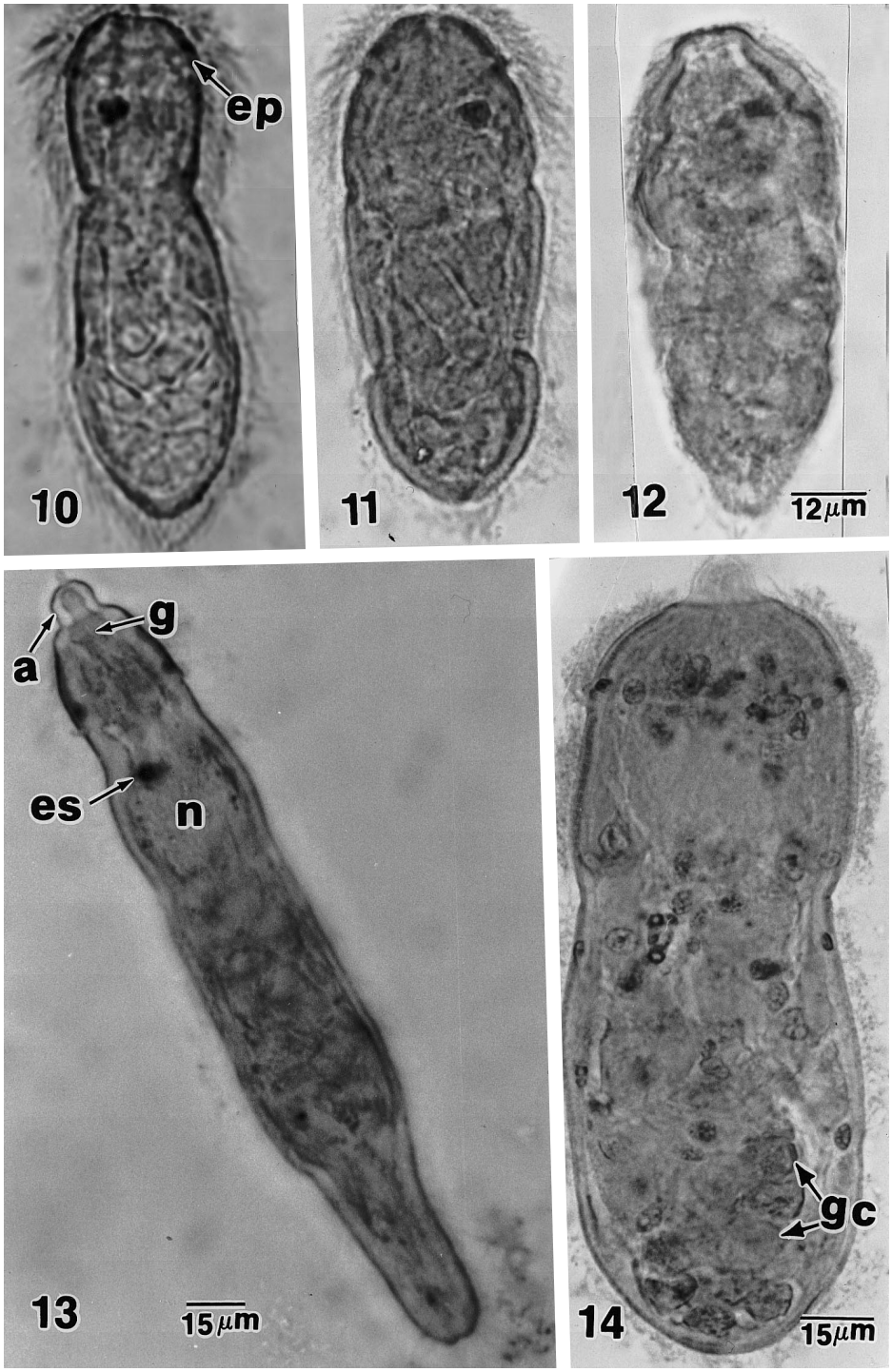


Fig. 9 Mean length and width measurements of *E. caproni* eggs maintained in a dark environment at 28° C in artificial spring water from 0 to 10 days postembryonation. In all, 20 eggs were measured at each datum point. Standard error bars are included in the graph but are too small to be visible

The mean length and width of these eggs remained almost constant throughout the incubation period. The mean length of the embryo increased slowly during the first 2 days of development and then rapidly from day 2 to day 10. The mean width of the embryo increased slowly until day 2, after which it increased rapidly until day 4 and then less rapidly until day 10.

Mean differences in the length and width of 20 zero-day-old eggs obtained by homogenization versus the same number obtained by fecal sedimentation were compared using Student's *t*-test, and values of $P<0.05$ were considered significant. There was no significant difference ($P=0.26$) between the mean length of fresh eggs from homogenized worms (122.8±0.7) and that of eggs from the feces (121.8±0.6). There was no significant difference ($P=0.15$) between the mean width of eggs from homogenized worms (74.0±0.5) and that of eggs from the feces (74.0±0.6). There was no significant difference ($P<0.081$) between the mean length of embryos of eggs from homogenized worms (22.0±0.6) and that of embryos of eggs from fecal smears (20.8±0.4). Similarly, there was no significant difference ($P<0.34$) between the mean width of embryos of eggs obtained from homogenized worms (22.3±0.6) and that of embryos of eggs removed from the feces (21.5±0.5). Eggs from the feces maintained in a dark environment in ASW at 28° C for 10 days contained fully developed miracidia, but detailed studies on the development or hatching of these eggs were not done.

Microscopic observations of the cultures containing the 60 snails that had been fed 0- to 10-day-old eggs showed that the snails had ingested most of these eggs within 5 h. Dissection of additional snails fed eggs showed the presence of eggs in the stomach and intestines within 24 h of feeding. Of the 60 snails, 59 survived until necropsy at 3–4 weeks postexposure. Of the survivors fed 0-, 2-, 4-, 6-, 8-, and 10-day-old eggs, 4, 3, 9, 7, 8, and 10 were infected with daughter rediae, respectively.



Figs. 10–14 Light micrographs of miracidia of *E. caproni*. **Fig. 10** A miracidium fixed in 10% neutral buffered formalin (NBF) and stained with alcian blue (pH 2.5). Note the positive reaction in the epidermal plates (*ep*). The bar in Fig. 12 appropriate for Figs. 10 and 11 **Fig. 11** A miracidium fixed in NBF and treated with periodic acid-schiff (PAS). The body and epidermal/subepidermal areas are positive. **Fig. 12** A miracidium fixed in NBF and treated with PAS plus diastase. The body and epidermal/subepidermal areas are much lighter, suggesting the presence of glycogen (see Results). **Fig. 13** A live miracidium stained with neutral red. Note the presence of the apical papilla (*a*), gut (*g*), eyespot (*es*), and neural mass (*n*). **Fig. 14** A miracidium fixed and stained in acetocarmine to show the presence of germ cells (*gc*)

Studies on miracidia

Miracidia stained with alcian blue showed the presence of acid mucopolysaccharides in the epidermal plates (Fig. 10). Miracidia stained with PAS confirmed the presence of complex carbohydrates in the body and epidermal plates (Fig. 11). In miracidia that had been treated with diastase, glycogen was depleted from the body and the epidermal/subepidermal region of the larvae (Fig. 12). Neutral red staining showed the appearance of live miracidia, including the apical papilla, gut, neural mass, eyespots, and two flame cells (Fig. 13; not all of these structures are seen in the figure). Staining with acetocarmine showed the location of germinal cells in the posterior half of the miracidial body (Fig. 14). ORO staining showed the presence of sparse lipid droplets measuring less than 1 μm in diameter in the body of miracidia (photograph not shown).

The longevity of 50 newly hatched miracidia was 6 h at 4° C, 28 h at 12° C, 14 h at 22° C, and 5 h at 38° C. Of 10 snails exposed to newly hatched miracidia, 6 of 7 survivors were infected with *Echinostoma caproni* rediae and cercariae at 7 weeks p.i.

Discussion

Most *Echinostoma caproni* eggs (0 days old) from homogenized worms contained fertilized embryos that were not well developed. Our study provides detailed information on the development of these eggs to the fully developed miracidial stage. *E. caproni* eggs derived from worms grown in ICR mice took 10 days to become fully developed miracidia in ASW at 28.2±0.2° C. At day 10 a synchronous hatch of 75–80% of the miracidia was observed. Studies by Behrens and Nollen (1993) described the incubation of *E. caproni* eggs from worms grown in mice (unspecified strain) at 27° C under conditions of light and darkness. Their study showed that regardless of whether the eggs were kept in constant light or darkness, they became fully developed in 10 days and hatched as early as within 13 days. In contrast, their mouse-source eggs maintained in a dark environment for 11 days required 6 additional days to reach maximal hatching after they had been exposed to light (Behrens and Nollen 1993). A large synchronous hatch of miracidia on a specific day postembryonation was not reported by Behrens and Nollen (1993).

Behrens and Nollen (1993) showed that light was not a prerequisite for the development of *E. caproni* eggs, and our study confirmed that observation. Although light is not a prerequisite for hatching of miracidia of this species, it is necessary for a large synchronous miracidial hatch. The value of obtaining such a hatch on a particular day is important when larvae are needed for teaching and research purposes.

The homogenization method of obtaining eggs was less tedious than that of teasing eggs from the uteri of gravid worms (Behrens and Nollen 1993) or the fecal sedimentation procedure (see Pritchard and Kruse 1992 for details). Eggs obtained from the homogenization procedure developed at about the same rate as those obtained by fecal sedimentation. We did not test hatching of fecal eggs. Interestingly, McKindsey and McLaughlin (1994) found that the hatching success of *Cyclocoelum mutabile* was dependent on the source of eggs, with significantly greater numbers of eggs hatching from eggs teased from worms than from eggs obtained from host feces.

The results of our study show that *Biomphalaria glabrata* snails can become infected by ingesting eggs of *E. caproni* at as early as day 0 postembryonation. These eggs probably develop within the lumen of the snail gut, eventually releasing miracidia, which penetrate the gut and migrate to target sites in the snail host. The possibility also exists that some eggs may have entered the snail via sites other than the gut, e.g., between the shell and the snail body, with infection occurring via a nonenteric route. To verify egg infectivity in snails, histology studies on snails exposed to eggs should be performed. We have not seen other studies in which embryonated echinostome eggs were used to infect snails, although Etges (1992) used such a procedure to infect *Helisoma anceps* snails with embryonated eggs of *Zygocotyle lunata.*

The results of *E. caproni* miracidial infection of *B. glabrata* snails were similar to those reported by Jeyarasasingam et al. (1972), who found 90% infection at 5 weeks p.i. after infecting 10 snails of 5±1-mm shell diameter with 10 miracidia/snail.

The duration of survival of *E. caproni* miracidia was dependent on the environmental temperature. The results of the present study showed that miracidia lived the longest at 12° C and the shortest at 38° C, findings similar to those reported by Smyth and Halton (1983) on the miracidia of *Fasciola hepatica.* Future studies are needed to investigate the infectivity of *E. caproni* miracidia to *B. glabrata* snails at different temperatures.

The presence of neutral lipid droplets in empty eggs raises questions as to the origin of these fats. Perhaps these droplets are residues of fat from yolk reserves in the egg or, possibly, excretory/secretory products released by miracidia.

Other than silver nitrate staining to show the epidermal plate pattern of the miracidium of *E. caproni* (see Jeyarasasingam et al. 1972; Richard and Brygoo 1978), histology studies on this larval stage are not available. Our studies provide additional information on the structure of this stage.

The histochemistry studies were done to examine neutral lipids and complex carbohydrates in the miracidia. Other histochemistry studies for proteins and nucleic acids were not done, although such work would provide informative data on miracidial development. The histochemistry studies using carbohydrate staining procedures confirmed the presence of glycogen in miracidia of this species (see the review by Ginetsenkaya 1988 of this topic). Further study will be required to elucidate the functional significance of the complex carbohydrates and neutral lipids identified in the present study.

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