IN VITRO CULTURE OF REDIAE OF *ECHINOSTOMA CAPRONI*

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Summary :

Rediae of *Echinostoma caproni* (Egyptian strain) were dissected from *Biomphalaria glabrata* snails at intervals from 13-34 days post-exposure and co-cultured for up to 51 days with cells of the *B. glabrata* embryonic (Bge) cell line. Rediae readily ingested Bge cells and survived longer when co-cultured with cells than in cell-free cultures. Rediae released mostly motile cercariae throughout the observation period when in Bge medium and cells. Rediae cultured in 199 medium with Bge cells also produced progeny throughout most of the observation period. In the latter medium, progeny were much more likely to include rediae as well as cercariae. Some cercariae produced *in vitro* encysted as metacercariae. Rediae consumed cercariae released into culture but were not observed to attack one another or rediae of a different echinostome species.

KEY WORDS : Digenea, Echinostomatidae, *Echinostoma caproni*, *Biomphalaria glabrata*, rediae, *in vitro* culture.

Résumé : CULTURE *IN VITRO* DE RÉDIES D'*ECHINOSTOMA CAPRONI*

Les rédies du trématode Echinostoma caproni *(souche égyptienne) ont été isolées des tissus de* Biomphalaria glabrata *entre 13 et 34 jours après infestation, mises en culture avec la lignée cellulaire Bge (Bge cells) dérivée d'embryons de* B. glabrata, *et maintenues jusqu'à 51 jours dans ces conditions expérimentales. Les rédies ingéraient activement des cellules Bge, et présentaient une meilleure survie en présence de cellules dans le milieu de culture. Les rédies cultivées avec des cellules Bge dans du milieu Bge produisaient des cercaires mobiles tout au long de la période d'observation. Lorsqu'elles étaient cultivées avec des cellules Bge mais en présence de milieu 199, elles produisaient à la fois des rédies et des cercaires. Certaines cercaires produites* in vitro *se sont enkystées en métacercaires. Le suivi des cultures a mis en évidence l'ingestion par les rédies de cercaires produites* in vitro, *mais jamais de phases de cannibalisme entre rédies appartenant ou non à la même espèce.*

MOTS CLÉS : *Digenea, Echinostomatidae,* Echinostoma caproni, Biomphalaria glabrata, *rédies, culture* in vitro.

Several fundamental aspects of the intramolluscan biology of digenetic trematode larvae remain poorly understood. The basic developmental patterns of larval digeneans require further clarification. Intra- and interspecific interactions between digenean larvae also remain understudied. Such studies have been impeded in part by our inability to provide culture conditions that allow complete and normal *in vitro* development of digenean larval stages.

Most previous work on culturing of larval digeneans has focused on *Schistosoma mansoni*. It is possible to transform axenically miracidia into mother sporocysts (Voge & Seidel, 1972; Basch & DiConza, 1974). Miracidium-derived mother sporocysts will produce daughter sporocysts (Yoshino & Laursen, 1995) when co-cultured in the presence of cells of the *Biomphalaria glabrata* embryonic cell line (Bge cells) originally developed by Hansen (1976*a*). Daughter sporocysts derived from infected snails will release additional daughter sporocysts when cultivated with a mosquito cell line (Hansen, 1973), Bge cells (Hansen, 1976*b*), medium conditioned by Bge cells (Hansen *et al.*, 1974*a*) or even in axenic medium (Hansen *et al.*, 1974*b*). Early cercarial embryos freed from daughter sporocysts will develop into swimming but noninfective cercariae when cultured with Bge cells (Hansen, 1975; Basch & DiConza, 1977). Coustau *et al.* (1997) have also reported the development of *S. japonicum* mother sporocysts and their production of daughter sporocysts in the presence of Bge cells.

Little work has been undertaken with the *in vitro* culture of other digeneans, particularly those species that feature redial stages in their larval development. Rediae of *Fascioloides magna* were maintained for up to ten days in simple media containing amino acids and sugars (Friedl, 1961*a,b*), and Basch & DiConza (1975) reported that *Echinostoma paraensei* rediae can be maintained for more than a month in axenic culture. They noted that *E. paraensei* rediae dissected from snails at 18 days post-exposure subsequently released small rediae within the first few weeks of culture but

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did not produce cercariae. Rediae were also observed to eat *S. mansoni* mother and daughter sporocysts in vitro (Basch & DiConza, 1975). Augot *et al.* (1997) cultured rediae of *Fasciola hepatica* axenically for intervals up to 16 days to quantify the number of daughter rediae and cercariae produced *in vitro.*

As so little previous work has been done to study rediae in culture, the present study was undertaken to observe rediae of *Echinostoma caproni.* Recently, Ataev *et al.* (1998) reported that sporocysts of this species lived for up to 17 weeks in the presence of Bge cells as compared to only two weeks when in cell-free medium. For the present study, *E. caproni* rediae were placed into culture wells in which Bge cells were being propagated in two types of media. We sought to determine if 1) survival of rediae was prolonged by the presence of Bge cells; 2) rediae would consume Bge cells; 3) progeny rediae or cercariae would be released and over what interval of time; and 4) if *E. caproni* rediae would attack or prey upon other rediae or cercariae of the same or different echinostome species.

MATERIALS AND METHODS

Echinostoma caproni (Egypt) was maintained in hamsters and albino *Biomphalaria glabrata* (Brazil). An isolate of *Echinostoma sp.* from Niger was maintained in hamsters and *Bulinus globosus. Biomphalaria glabrata* of 5-10 mm shell diameter were individually exposed to 20-30 miracidia of *E. caproni.* At intervals ranging from 13-34 days post-exposure (dpe), rediae were harvested from these snails. Snails were soaked for one hour in water containing 1 % penicillin, streptomycin and fungizone, swabbed in 70 % ethanol, and then crushed in a pool of serum-free medium 199 diluted to half normal strength for use with snails (Loker *et al.*, 1992). Rediae were transferred to fresh medium and rinsed to remove snail debris. Concurrent dissection of infected snails and examination of the redial populations present indicated that rediae harvested before or on 20 dpe were mother rediae, whereas those collected at later dates represented mixed generations. From 1 to 22 rediae were placed in individual flat- or round-bottomed wells in 96-well culture plates (Becton Dickinson Co., Franklin Lakes, NJ).

Most wells were seeded with Bge cells in Bge medium (Hansen, 1976*a*) supplemented with 10 % fetal calf serum (FCS) and gentamycin, the day before rediae were to be added. The source and maintenance of Bge cells were as described by Yoshino & Laursen (1995). At the time rediae were added, the medium in each well was changed, either to Bge or 199 medium. Depending on the well, the medium contained 0, 5 or 10 % heat-inactivated FCS. All media contained gentamycin (43 µg/ml). Cultures were incubated at 24-27 °C under normal atmospheric conditions and medium in 0.75 ml volumes was changed on a weekly basis. No additional Bge cells were added once the cultures were initiated. Six cultures lacking Bge cells were also set up, each receiving a different combination of medium (Bge or 199) and FCS (0, 5, or 10 %). All cultures were examined immediately after initial set up and at weekly intervals thereafter. An additional 11 cultures were established, each containing Bge cells and a single redia each of *E. caproni* and of *Echinostoma sp.* The number and condition of larvae in each culture was monitored as described above.

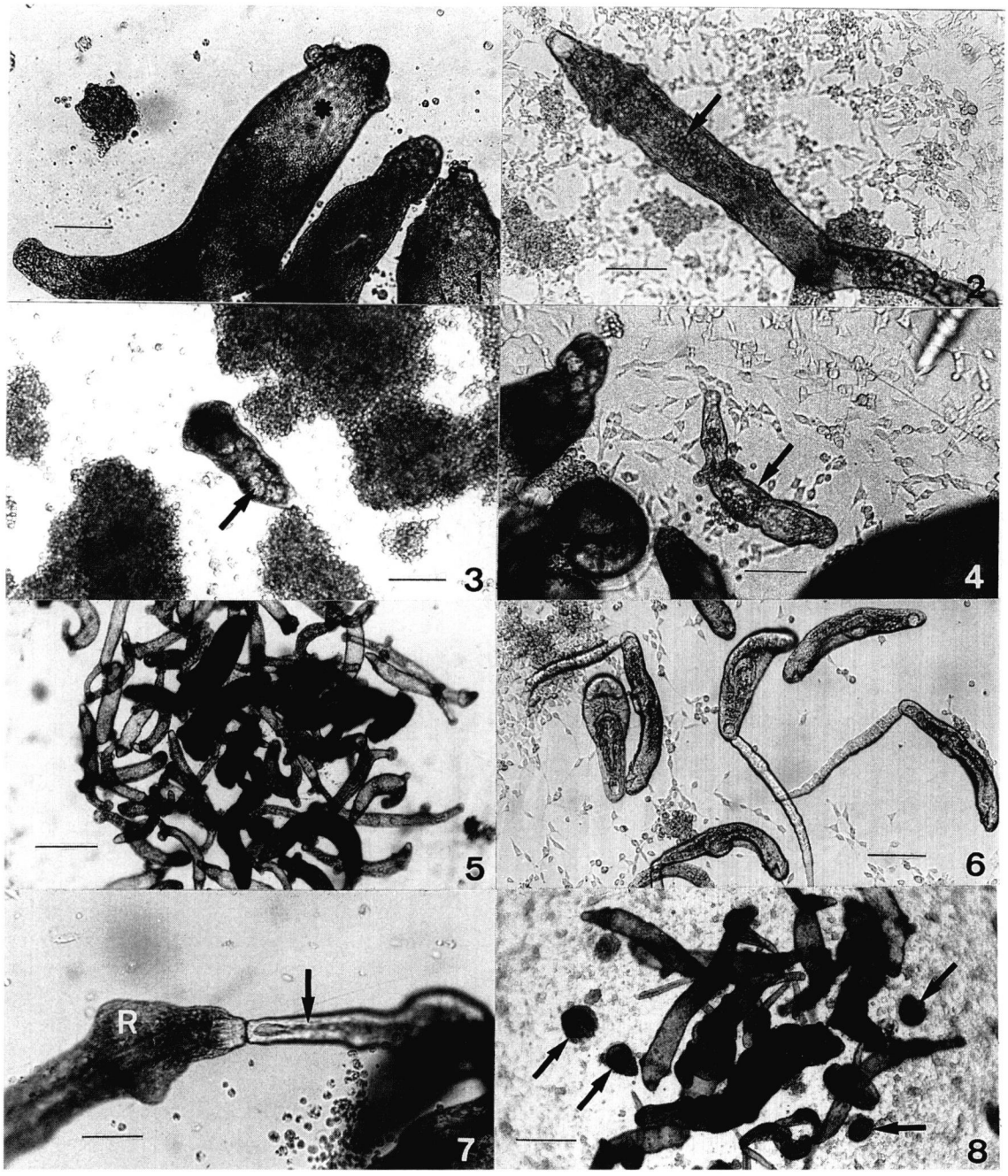
RESULTS

Rediae in cultures lacking Bge cells, regardless of the medium or amount of FCS present, were initially motile and released cercariae and rediae within the first four days of culture. By one week, they possessed a gut that was abnormally inflated, their body wall was brown and rough instead of clear and smooth, and they were only weakly motile. By 20 days they were dead.

All remaining efforts focused on cultures containing Bge cells. Bge cells grew well in both Bge and 199 media. Rediae in Bge medium initially developed a bloated gut and opaque body wall (Fig. 1), but seemed to recover and when the cultures were terminated, many contained developing progeny. Rediae in cultures containing medium 199 did not become bloated and released more progeny, particularly progeny rediae, than did rediae in cultures with Bge medium (Table I). No obvious effect on Bge cell production or progeny production by rediae was noted in cultures weaned of FCS.

Rediae cultured with Bge cells readily ingested the cells (Fig. 2) and in general, did much better than rediae from cultures lacking cells. Some produced germinal balls (Fig. 3) that increased in size and some released progeny even at 51 days, the longest interval that cultures were followed. As long as Bge cells were present, rediae routinely survived for longer than 30 days in culture.

Mother rediae that were relatively young when taken from snails (13 dpe) did not release progeny in culture, but after 27 days of observation, one contained an elongate developing redia that continued to grow for the remaining 20 days of observation. Individual mother rediae collected from snails at 20 dpe released as many as 37 daughters over a 40-day



Figs 1-8. – *In vitro* cultivation of *Echinostoma caproni* rediae.

Fig. 1. Rediae taken from snail with 24 day-old infection, in Bge medium with Bge cells for three days. Note enlarged, empty gut (*) and opaque body wall. Scale bar = 145 µm.

Fig. 2. Mother redia taken from snail with 13 day-old infection, in culture with 199 medium and Bge cells for six days, with gut containing ingested Bge cells (arrow). Scale bar = 143 µm.

Fig. 3. Redia taken from snail with 19 day-old infection, in culture with 199 medium and Bge cells for 29 days, containing germinal balls (arrow). Scale bar = 145 µm.

Fig. 4. Progeny redia (arrow) released from a redia taken from snail with 30 day-old infection and held in culture with 199 medium and Bge cells for 10 days. Scale bar = 109 µm.

Fig. 5. Numerous (approximately 40) progeny rediae released over a 39 day period of culture, derived from 12 original rediae (see dark, opaque rediae) taken from snail with 30 day-old infection, in 199 medium and Bge cells. Scale bar = 439 µm.

Fig. 6. Recently released cercariae, some tail-less, in a culture established for 41 days. The original rediae were taken from a snail with a 19 day-old infection, and were cultured in 199 medium and Bge cells. Scale bar = 139 µm.

Fig. 7. Ingestion of tail (arrow) of recently-produced progeny cercaria by a redia (R), 33 days after initiation of culture. The original rediae were taken from a snail with a 30 day-old infection and cultured in Bge medium with Bge cells. Scale bar = 107 µm.

Fig. 8. Encysted metacercariae (arrows) derived from cercariae released *in vitro*, from a four day-old culture containing 199 medium and Bge cells. The original rediae were taken from a snail with a 30 day-old infection. Scale bar = 417 µm.

	199	Bge
Age of infection in snails (days) when rediae to be cultured were collected	13-34	13-34
Number of of cultures set up	25	14
Average # of rediae/culture (± SE)	4.68 ± 1.04	4.0 ± 0.95
# of cultures in which no progeny were released	10	7
# of cultures in which only rediae were released	4	1
# of cultures in which only cercariae were released	5	6
# of cultures in which both rediae and cercariae were released	6	0
Average # of progeny released per cultured redia (± SE)	2.89 ± 0.78	0.94 ± 0.41
Total # of rediae released	127	1
Average # of rediae released per cultured redia (± SE)	1.48 ± 0.69	0.01 ± 0.01
Total # of cercariae released	160	54
Average # of cercariae released per cultured redia (± SE)	1.40 ± 0.48	0.93 ± 0.41
Longest observed culture interval (days) after which new progeny were noted		
progeny rediae	45	51
progeny cercariae	41	45
Longest observed interval (days) during which progeny were produced within a single culture	> 44	> 44

Table 1. – Summary of results for *Echinostoma caproni* rediae cultures containing Bge cells and either 199 or Bge medium.

interval. No cercariae were released by these mother rediae.

Cultures with rediae derived from snails > 20 dpe likely contained mixtures of mother and daughter rediae which released progeny rediae (Figs. 4 and 5) and cercariae (Fig. 6), or both (Table I). In no case was a single redia observed to release both cercariae and rediae. In most cases, progeny were produced 10 days or longer after culture establishment and in several, progeny were released 40 days or longer.

Progeny rediae ingested Bge cells and in some cases contained developing germinal balls and several were living when the cultures were terminated. Motile cercariae were released as late as 45 days after initiation of cultures. Cercariae were observed to be ingested by rediae (Fig. 7). Cercariae also encysted in culture (Fig. 8), and the resultant cysts were covered by Bge cells. Moribund cercarial bodies separated from their tails were also noted.

In no case were rediae observed to attack one another. Bge cells were not observed sticking to living rediae or cercariae but occasionally were observed to encapsulate rediae believed to be moribund or dead. After several weeks of culture, some rediae were surrounded by what appeared to be remnants of Bge cells. It is not known if these were cells that died as a normal part of Bge cell culture, if they were killed by the presence of rediae, or if they had been regurgitated from redial guts.

When rediae of *E. caproni* and *Echinostoma sp.* were cultured together, they showed no tendency to attack each other. Consumption of released cercariae, including cercariae of the other species, was observed.

DISCUSSION

As noted by Yoshino & Laursen (1995) for *S. mansoni* sporocysts, the presence of Bge cells clearly had a salutary effect on cultured rediae of *E. caproni*. Many of the rediae cultured with Bge cells were alive and appeared to contain developing progeny when observations were terminated at 51 days. In some cultures, progeny were released on as many as seven separate occasions over intervals as long as 44 days. Consumption of Bge cells by cultured rediae was prominent but the beneficial effects of Bge cells were not derived from eating cells alone. Some rediae consistently had guts packed with Bge cells but did not undergo germinal cell development and others were observed to eat few cells yet produced progeny. Laursen & Yoshino (1999) noted that cultured rediae of *F. magna* contained particulate matter but did not observe rediae to feed on Bge cells.

Yoshino & Laursen (1995) observed that Bge cells attached to and then encapsulated *S. mansoni* mother sporocysts and suggested that the intimate contact with cells was a requirement for development of daughter sporocysts. Ataev *et al.* (1998) observed no contact between Bge cells and *E. caproni* sporocysts in their culturing experiments. Bge cells also did not attach to or encapsulate *F. magna* rediae (Laursen & Yoshino, 1999). Similarly, in the present study, Bge cells did not attach to or encapsulate viable *E. caproni* rediae although moribund rediae and metacercariae were encapsulated. This pattern is similar to that noted with echinostome sporocysts, rediae and metacercariae

and host hemocytes *in vivo* (Loker *et al.*, 1987), supporting the concept of Yoshino & Laursen (1995) that Bge cells have a least some properties reminiscent of hemocytes. The tendency of Bge cells to adhere to schistosome mother sporocysts but not to echinostome sporocysts and rediae is similar to the pattern noted by Loker & Adema (1995) with respect to *in vitro* interactions between schistosome and echinostome larvae and hemocytes.

As the rediae placed in culture were all obtained from infected snails, it is difficult to know with certainty which, if any, of the progeny they released had developed exclusively under i*n vitro* conditions. However, it is likely that many of the progeny released in cultures containing Bge cells had developed largely in vitro. As compared to rediae cultured without Bge cells, rediae co-cultured with cells released more progeny over much longer intervals. Also, the appearance in vitro of germinal balls that developed into elongate redial embryos indicates that larval development occurred *in vitro.*

Medium 199 supported production of Bge cells for over 50 days in culture. This medium was more favorable to rediae during the early stages of culture and supported greater overall production of progeny, especially of progeny rediae. In contrast, after initially looking quite abnormal in the presence of Bge medium, rediae seemed to recover and survived and remained productive for longer time intervals in this medium than in medium 199.

Basch & DiConza (1975) noted that *E. paraensei* rediae dissected from snails at 18 dpe released progeny rediae over two to three weeks in cultures lacking Bge cells. They did not observe cercariae or advanced cercarial embryos to be produced. In the present study, motile cercariae were released as late as 45 days after initiation of culture, and some encysted. Stein & Basch (1977) observed that cercariae of *E. paraensei* released from infected *B. glabrata* were capable of normal encystment in cultures containing Bge cells or in medium conditioned by Bge cells, but not in unconditioned medium. They also observed metacercariae to be encapsulated by Bge cells *in vitro.*

In agreement with Basch & DiConza (1975), we noted no tendency for cultured rediae to attack other rediae. We noted that rediae consumed cercariae, both of the same or of a different species, but their tendency to do so under the conditions of culture employed can only be considered as moderate.

Our results provide evidence that the presence of Bge cells has a distinctly beneficial effect on both survivorship and progeny production for cultured redial stages. Additional studies with cultured rediae should be undertaken because the prospects for considerably improving culture conditions are high, and because it will open up interesting possibilities for study with respect to interactions between larval digeneans of the same or different species.

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