EFFECTS OF *PLAGIORCHIS ELEGANS* (DIGENEA: PLAGIORCHIIDAE) INFECTION OF *BIOMPHALARIA GLABRATA* (PULMONATA: PLANORBIDAE) ON A CHALLENGE INFECTION WITH *SCHISTOSOMA MANSONI* (DIGENEA: SCHISTOSOMATIDAE)

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ABSTRACT: Prior exposure of *Biomphalaria glabrata* to the eggs of an incompatible digenean, *Plagiorchis elegans*, rendered this snail host less suitable to a compatible species, *Schistosoma mansoni*. Although *P. elegans* failed to develop patent infections in *B. glabrata*, it reduced the production of *S. mansoni* cercariae by 88%. Concomitantly, host attributes such as reproduction, growth, and survival were compromised. The effect of *P. elegans* infection was most severe among snails that, in addition, had developed patent schistosome infections. Although few *S. mansoni* cercariae were produced, egg production by *B. glabrata* was only 4% of control values. Furthermore, no doubly infected snails survived for more than 3 wk after patency, whereas controls experienced no mortality during the same time period. The above effects were attributable to the establishment and persistence of *P. elegans* sporocysts in the tissues of the incompatible snail host. Their indirect antagonistic interaction with the larval stages of *S. mansoni* may be mediated, in part, through their long-term stimulation of the host's internal defense mechanisms. These findings are discussed with a view to use *P. elegans* and other plagiorchiid digeneans as agents in the biological control of snails and snail-borne diseases.

The concept of using digeneans as agents in the biological control of molluscan vectors of disease is not new (see Bayer, 1954). It is based on the ability of the intramolluscan developmental stages of these parasites to suppress the reproductive functions of their hosts. Reproductive damage may be direct, as in instances where rediae actively consume gonadal tissues, or indirect, most probably by way of modulating the host's neuroendocrinological system (Nassi, 1979). Parasitic castration of the snail host by digenean sporocysts is thought to be of the indirect type (reviewed by De Jong-Brink, 1995).

Digeneans also interact antagonistically within their molluscan intermediate hosts (reviewed by Combes, 1982). Rediae primarily manifest direct antagonism in that they actively prey on the larval stages of other species (Lie, 1973). Indirect antagonism, in contrast, acts at a distance, conceivably mediated through the internal defense mechanisms of the snail host. Sporocysts are thought to be capable of only indirect antagonism and are generally eliminated by concurrent, dominant rediae infections (Lim and Heyneman, 1972). Rediae belonging to the Echinostomatidae appear to be particularly effective, not only in terms of antagonistic effects on other digeneans but also in terms of suppression of host reproduction (Combes, 1982). The eggs of such dominant digeneans have been deployed, with some success, into aquatic environments to combat snail-borne diseases (Nassi et al., 1979). Until recently, research conducted with a view to use digeneans as biological control agents has focused on guilds of digenean species compatible with the target snail host. However, Zakikhani and Rau (1998a) found that eggs of *Plagiorchis elegans*, ingested by *Biomphalaria glabrata*, elicited the same rapid, severe, and permanent suppression of reproductive output in this incompatible host snail as in the compatible *Stagnicola elodes* (Zakikhani and Rau, 1999). This effect was attributed to *P. elegans* sporocysts that established and persisted in the tissues of the incompatible host. The present study determines whether such persistent, truncated *P. elegans* infections of *B. glabrata* will interfere, as well, with the

establishment and cercariae production of a compatible parasite, *Schistosoma mansoni*.

MATERIALS AND METHODS

Hamsters (*Mesocricetus auratus*) served as the experimental definitive host of *P. elegans*, whereas *S. elodes* and *Aedes aegypti* served as first and second intermediate hosts, respectively. Late-third and early-fourth instar mosquito larvae were infected with *P. elegans* cercariae to obtain metacercariae. Hamsters were each given 100–150 metacercariae by gavage, and eggs were collected 10 days later. Helminth eggs were separated from host feces by serial sieving and were trapped using a 20-μm screen. In water, at room temperature and in complete darkness, such eggs remained infective for several weeks (Zakikhani and Rau, 1998a).

A Puerto Rican strain of *S. mansoni* and *B. glabrata*, obtained from the Biomedical Research Institute, Rockville, Maryland, were used throughout this study. CD-1 outbred mice were infected with schistosome cercariae by skin penetration. Schistosome eggs were obtained by homogenizing infected mouse livers in 1.7% saline using a blender and passing the mixture through a series of sieves. Eggs were trapped using a 39-μm screen. Eggs were washed with saline into a beaker, allowed to sediment, and then concentrated by removing the supernatant. Eggs were hatched by diluting the egg–saline mixture with well water (1:1,000), and miracidia were collected (Smithers and Terry, 1965; Mkoji et al., 1988).

Two experimental groups of forty, 5-wk-old *B. glabrata* (maximum shell diameter 4–5 mm) were exposed individually to 8–10 fully embryonated eggs of *P. elegans* for 3 hr in a small container (diameter 4 cm) filled with enough water to cover the snail. Snails were then rinsed and transferred to individual holding containers. The remaining water and rinse were examined for *P. elegans* eggs to confirm the number of eggs ingested. Two groups of 40 snails each were sham exposed. These snails served as controls, enabling us to asses the stress associated with handling. Two weeks after exposure to *P. elegans*, snails of 1 experimental group and 1 control group were challenged individually with exposure to 7 *S. mansoni* miracidia in 1 ml of water. The remaining *P. elegans*–infected experimental group of 40 snails was sham exposed at this time, as was the remaining control group. Snails were rinsed and returned to their respective, individual holding containers (diameter 11.5 cm, height 8.0 cm) filled with 150 ml of water and maintained at 23 ± 1 C in constant light. Snails were fed fresh Romaine lettuce, supplemented with autoclaved potting soil and chalk ad libitum, in accordance with the recommendations of the supplier. Shell diameter was measured weekly to the nearest millimeter. Egg masses were detached from the surface of the container, and eggs and egg masses were counted. Water was changed weekly.

Four weeks after challenge and every week thereafter, snails were monitored for cercariae shedding. For this purpose, individual snails

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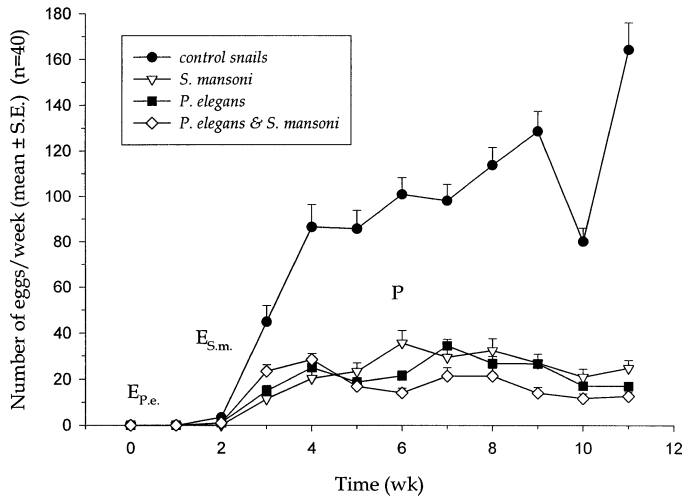
FIGURE 1. Egg production of *Biomphalaria glabrata* exposed to *Plagiorchis elegans* or *Schistosoma mansoni* (or both) (pooled patent and nonpatent snails). P = onset of patency (*S. mansoni*). $E_{P.e.}$ = exposure to *P. elegans*. $E_{S.m.}$ = exposure to *S. mansoni*.

were placed in dry, clear plastic vials for 30 min before 20 ml of distilled water was added. Snails remained in these vials for 1 hr before being returned to their respective, individual holding containers. Vials were examined for the presence of cercariae under a dissecting microscope ($\times 120$), and the mean number of cercariae per snail were estimated on the basis of ten 0.025-ml samples and the total volume. As soon as patency could be ascertained, the 2 groups exposed to *S. mansoni* miracidia were further subdivided into patent and nonpatent snails to assess the relationship between patency and egg production.

Snail mortality was monitored daily. Snails that died during the course of the experiment were dissected, whenever their condition allowed, as were snails that survived to the end of the experimental period. Evidence of infections was recorded.

The experiments followed a complete randomized design with 10 replicates. Data for the number of cercariae and snail eggs produced were square root transformed before statistical analyses to satisfy the

distributional assumptions of the test. Analyses were carried out using the General Linear Model procedure of the SAS package (SAS Institute, 1999–2001). The multivariate and univariate procedure of the repeated-measures ANOVA were performed according to Crowder and Hand (1990) on weekly growth curves and cercarial shedding with week as a repetition factor and parasitic infection as the treatment factor. Overall sample means for cercariae production and snail shell diameter were calculated and compared using the Student–Neuman–Keuls test for multiple pair-wise comparisons of means. The effects of parasites and the effects of different species of parasites in the same snail host on snail reproduction were analyzed using RPM ANOVA. Mean contrasts were used for multiple comparisons of number of eggs among and between infected and uninfected snails. The effects of parasites on snail mortality over time were tested using repeated-measures ANOVA (Crowder and Hand, 1990). To determine significance among and within the treatment effects, 1-way ANOVA with time as a covariable (Zolman, 1993) was used to test the effects of single and double infections on the number of eggs produced, both before and during patency. Mean contrasts were used for multiple comparisons of survivorship among and between infected and uninfected snails.

The significance level was set at 0.05. The Greenhouse–Geisser adjustment of significance probabilities of repeated-measures ANOVA was used in a conservative approach (Crowder and Hand, 1990), and the Bonferroni correction (Miller, 1981) was applied in testing contrasts.

RESULTS

Control snails became reproductive 2 wk after the start of the experiment, and mean numbers of eggs produced per snail per week increased rapidly during the subsequent 8 wk (Fig. 1). The total number of eggs produced per snail increased by more than 50% during the 2 consecutive 5-wk periods (Table I). In contrast, in all 3 parasite-exposed groups, egg production reached a low plateau within 2 or 3 wk (Fig. 1). Exposure to either *P. elegans* or *S. mansoni* alone significantly reduced total egg production to 21 and 22% of control values, respectively; exposure to both parasites caused a decline to 16% (RPM ANOVA; infection: $F_{3, 155} = 155.78$, $P = 0.0001$; time: $F_{9, 1395} = 21.99$, $P = 0.0001$; time $\times$ infection: $F_{27, 1395} = 23.56$, $P = 0.0001$). A more detailed analysis revealed that snails with pat-

TABLE I. Univariate repeated-measures ANOVA of the effects of *Plagiorchis elegans*, or *Schistosoma mansoni* infection (or both) on *Biomphalaria glabrata* egg production over time.*

Source of variation	Weeks 2–11		Weeks 2–6†		Weeks 7–11‡	
	Observed F	$P > F$	Observed F	$P > F$	Observed F	$P > F$
Infection (I)	$F_{7, 111} = 52.10$	0.0001	$F_{7, 111} = 21.55$	0.0001	$F_{7, 111} = 74.98$	0.0001
Time (T)	$F_{10, 1110} = 11.46$	0.0001	$F_{4, 444} = 4.23$	0.0056	$F_{4, 444} = 3.13$	0.0280
I $\times$ T	$F_{70, 1110} = 10.18$	0.0001	$F_{28, 444} = 6.01$	0.0001	$F_{28, 444} = 3.71$	0.0001

Exposure groups	Total number of *B. glabrata* eggs/snail (mean $\pm$ SE)§		
	Weeks 2–11	Weeks 2–6	Weeks 7–11
Control snails	1059.60 $\pm$ 58.12 a	416.13 $\pm$ 31.37 c	643.48 $\pm$ 31.99 b
S. mansoni (all snails)	229.03 $\pm$ 27.31 ef	120.35 $\pm$ 14.67 hi	107.68 $\pm$ 15.61 hij
S. mansoni (patent)	51.29 $\pm$ 13.95 k	51.29 $\pm$ 13.95 k	0.00 $\pm$ 0.00
S. mansoni (non-patent)	321.36 $\pm$ 27.89 d	157.84 $\pm$ 18.20 gh	163.52 $\pm$ 15.19 fgh
P. elegans	221.73 $\pm$ 14.29 efg	114.75 $\pm$ 8.70 hij	106.98 $\pm$ 7.23 hij
Challenge (all snails)	166.45 $\pm$ 18.36 fgh	103.00 $\pm$ 10.44 hij	63.45 $\pm$ 9.46 k
Challenge (patent)	48.43 $\pm$ 11.10 k	48.43 $\pm$ 11.10 k	0.00 $\pm$ 0.00
Challenge (non-patent)	234.16 $\pm$ 18.22 e	132.64 $\pm$ 12.29 h	101.52 $\pm$ 8.45 hij

* Circularity test for time is significant. Probability of significance was adjusted using the Greenhouse–Geisser Epsilon correction factor (G–G = 0.6423).
† Prepatent period of *S. mansoni* infection.
‡ Patent period of *S. mansoni* infection.
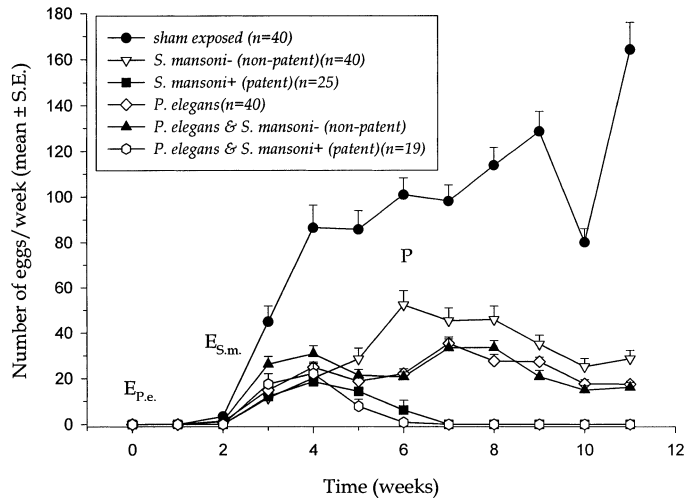§ Means with the same letter are not significantly different at the 0.05 level.

FIGURE 2.   Egg production of *Biomphalaria glabrata* exposed to *Plagiorchis elegans* or *Schistosoma mansoni* (or both) (patent and non-patent snails considered separately). P = onset of patency (*S. mansoni*). $E_{P.e.}$ = exposure to *P. elegans*. $E_{S.m.}$ = exposure to *S. mansoni*.

TABLE II.   Univariate repeated-measured ANOVA of the effects of *Plagiorchis elegans*, or *Schistosoma mansoni* infection (or both) on *Biomphalaria glabrata* growth.

Source of variation	Observed F	$P > F$
Infection (I)	$F_{3, 156}$ = 14.93	0.0001
Time (T)	$F_{11, 1716}$ = 83.80	0.0001
I × T	$F_{33, 1716}$ = 16.94	0.0001
Infection contrasts		
C vs. PS†	$F_{1, 156}$ = 25.94	0.0001
PS vs. P	$F_{1, 156}$ = 36.25	0.0001
P vs. S	$F_{1, 156}$ = 13.50	0.0003
S vs. PS	$F_{1, 156}$ = 0.86	0.3548

* Circularity test for time is significant. Probability of significance was adjusted using the Greenhouse–Geisser Epsilon correction factor (G–G = 0.1475).
† C, controls; P, *P. elegans* exposed; S, *S. mansoni* exposed.

ent *S. mansoni* infections, whether alone or preceded by exposure to *P. elegans*, produced significantly fewer eggs than did those that failed to shed cercariae (Fig. 2; Table I). Snails shedding *S. mansoni* cercariae produced no eggs after patency, whereas their exposed, but nonpatent counterparts continued to reproduce, albeit at significantly lower levels than sham-exposed controls (Fig. 2). Snails exposed only to *P. elegans* produced significantly more eggs than did the snails with patent *S. mansoni* infections but no more than those produced by snails that had been exposed to the schistosome but failed to shed cercariae (Table I).

Growth rates of all 3 parasite-exposed snail groups declined after 6 wk, whereas that of controls remained unchanged (Fig. 3). Exposure to *P. elegans* alone significantly reduced the growth of snails below that of controls ($F_{1, 156}$ = 5.51, P =

0.0202) but not as severely as did the exposure to *S. mansoni* alone or in combination with *P. elegans*. Growth of snails exposed to both *P. elegans* and *S. mansoni* was no slower than that of snails exposed to the schistosome alone but significantly less than that of uninfected snails (Fig. 3; Table II).

Mortality over the 11-wk experimental period was evident only among parasite-exposed snail groups (Fig. 4; Table III). Mortality was highest among doubly exposed snails and snails exposed only to *S. mansoni* (Fig. 4; Table III). In both groups, mortality occurred primarily among individuals with patent *S. mansoni* infections. Thus, snails that failed to develop patent infections after exposure to *S. mansoni* also showed no mortality during the course of the experiment. Exposure to *P. elegans* alone significantly reduced the survival of the snail host but not as severely as seen in combination with *S. mansoni*. None of the snail exposed to only *S. mansoni* died before patency. In contrast, mortality among doubly exposed snails, which later developed patent *S. mansoni* infection, was evident
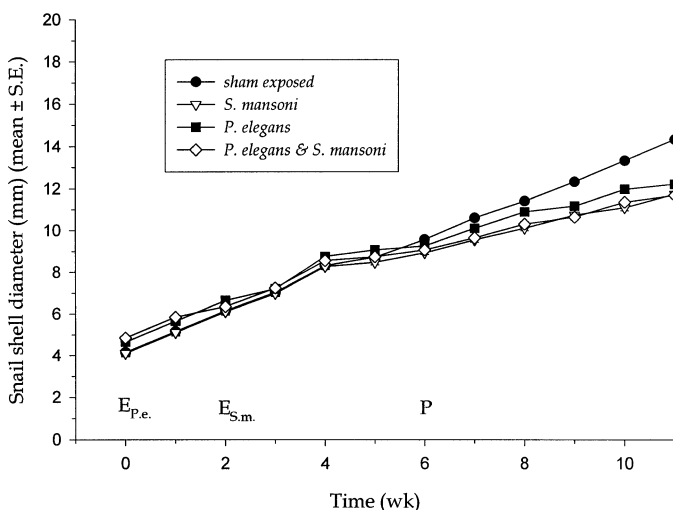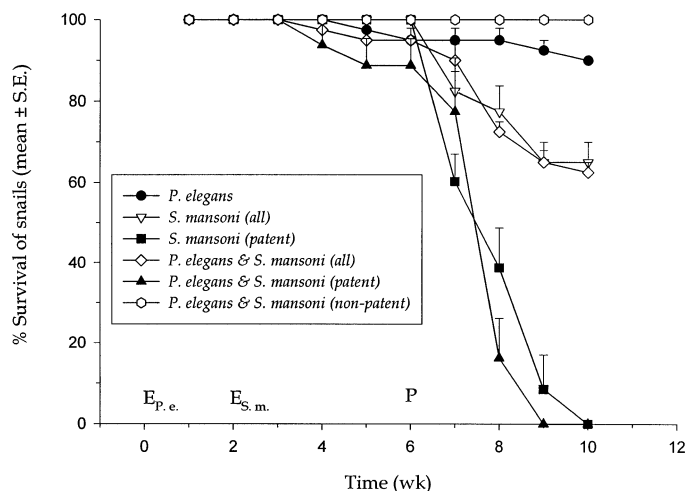

FIGURE 3.   Growth of *Biomphalaria glabrata* exposed to *Plagiorchis elegans* or *Schistosoma mansoni* (or both). P = onset of patency (*S. mansoni*). $E_{P.e.}$ = exposure to *P. elegans*. $E_{S.m.}$ = exposure to *S. mansoni*.

FIGURE 4.   Survivorship of *Biomphalaria glabrata* exposed to *Plagiorchis elegans* or *Schistosoma mansoni* (or both) (patent and nonpatent snails considered separately). P = onset of patency (*S. mansoni*). $E_{P.e.}$ = exposure to *P. elegans*. $E_{S.m.}$ = exposure to *S. mansoni*. (control and *S. mansoni* nonpatent snails not shown).

TABLE III. Univariate repeated-measures ANOVA of the effects of *Plagiorchis elegans*, or *Schistosoma mansoni* infection (or both) on the survival of *Biomphalaria glabrata*.

Source of variation	Weeks 2–11		Weeks 2–6†		Weeks 7–11‡	
	Observed F	$P > F$	Observed F	$P > F$	Observed F	$P > F$
Infection (I)	$F_{3, 156}$ = 7.05	0.0002	$F_{3, 156}$ = 2.09	0.0104	$F_{3, 156}$ = 8.07	0.0001
Time (T)	$F_{10, 1560}$ = 26.36	0.0001	$F_{4, 624}$ = 4.05	0.0292	$F_{4, 624}$ = 7.85	0.0010
I × T	$F_{30, 1560}$ = 5.93	0.0001	$F_{12, 624}$ = 1.70	0.1422	$F_{12, 624}$ = 2.22	0.0500
Contrasts§						
C vs. PS	$F_{1, 156}$ = 13.61	0.0003	$F_{1, 156}$ = 4.65	0.0326	$F_{1, 156}$ = 12.99	0.0004
PS vs. P	$F_{1, 156}$ = 14.59	0.0002	$F_{1, 156}$ = 0.01	1.000	$F_{1, 156}$ = 18.20	0.0001
P vs. S	$F_{1, 156}$ = 6.60	0.0111	$F_{1, 156}$ = 0.85	0.3568	$F_{1, 156}$ = 9.54	0.0024

* Circularity test for time is significant. Probability of significance was adjusted using the Greenhouse–Geisser Epsilon correction factor (G–G = 0.2155).
† Prepatent period of *S. mansoni* infection.
‡ Patent period of *S. mansoni* infection.
§ C, controls; P, *P. elegans*-exposed; S, *S. mansoni*-exposed.

2 wk before cercariae were shed and increased significantly during patency (Fig. 4).

Necropsy of snails confirmed the presence of *P. elegans* mother sporocysts in almost half the exposed snails. Nevertheless, all snails exposed to eggs of this parasite manifested retarded development of their reproductive systems. Similar developmental effects were observed in snails exposed to *S. mansoni* miracidia, whether or not patent infections had developed.

Snails exposed only to *S. mansoni* first shed cercariae 4 wk later. Peak production was reached 6 wk after exposure (Fig. 5). The prevalence of infection among such snails was 62.5 ± 6.2%. Previous exposure to *P. elegans* reduced this to 47.5 ± 8.5% but not significantly ($F_{1, 78}$ = 1.81, P = 0.1819). The total number of schistosome cercariae produced by snails previously exposed to *P. elegans* (639.0 ± 181.9) was only 12% of the number shed by snails exposed to *S. mansoni* alone (5,214.6 ± 898.3). Concomitantly, the mean daily release of cercariae per snail, measured at weekly intervals during a period of 5 wk, was reduced by 91% from 2,300.00 ± 461.3 to 219.7 ± 62.5. The effect of exposure to
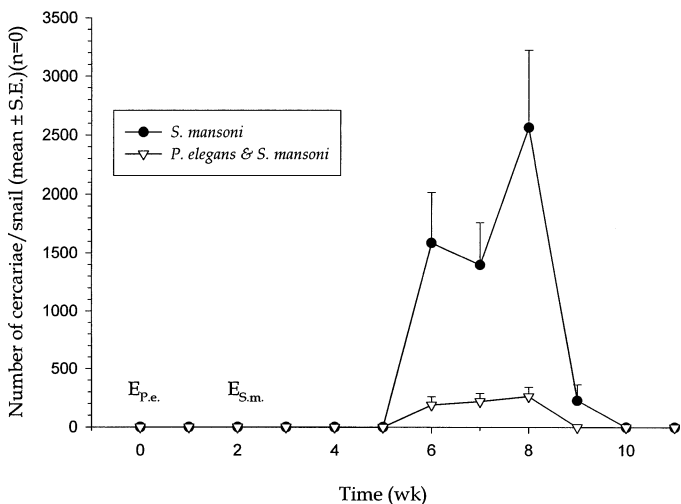

FIGURE 5. Cercariae production of *Biomphalaria glabrata* exposed to *Plagiorchis elegans* or *Schistosoma mansoni* (or both) (pooled patent and nonpatent snails). P = onset of patency (*S. mansoni*). $E_{P.e.}$ = exposure to *P. elegans*. $E_{S.m.}$ = exposure to *S. mansoni*.

P. elegans on *S. mansoni* cercariae production was highly significant (RPM ANOVA; infection: $F_{1, 78}$ = 22.06, P = 0.0001; time: $F_{6, 468}$ = 10.83, P = 0.0001; time × infection: $F_{6, 468}$ = 7.52, P = 0.0003).

DISCUSSION

As judged by their greatly reduced fecundity and by the presence of sporocysts in their tissues, all snails exposed to *P. elegans* eggs, *S. mansoni* miracidia, or both developed infections. Because *P. elegans* and *B. glabrata* are considered to be incompatible, the absence of cercariae production was not unexpected. The effects of the above 2 parasites on *B. glabrata* egg production were immediate, confirming the findings of Crews and Yoshino (1989) for *S. mansoni* and of Zakikhani and Rau (1998a) for *P. elegans* infections. Snails exposed to *S. mansoni* that failed to develop patent infections, snails exposed only to *P. elegans*, and snails with nonpatent infections after challenge exposure produced significantly lower numbers of eggs than did the controls. However, the duration of egg production did not differ and extended to the end of the 11-wk experimental period. In contrast, egg production by snails with patent *S. mansoni* infections, either alone or in conjunction with *P. elegans* infections, began to decline 2 wk after challenge exposure and ceased entirely at 6 wk, 1 wk after cercariae were first shed. This suggests that the damage caused by the migration of schistosome cercariae through the tissues of the snail host may require a greater compensatory reduction in reproduction to maintain homeostasis (reviewed by De Jong-Brink, 1995) than the mere presence of sporocysts of either species.

The role of cercariae as stressors was also reflected in host growth and mortality. Among snails exposed only to *S. mansoni* miracidia, the onset of patency was followed closely by a dramatic increase in host mortality. This supports the findings of Pan (1965) that mortality tends to be maximal during periods of peak cercariae shedding. The data also suggest that prior infection with *P. elegans* may greatly enhance the deleterious effects of patent *S. mansoni* infection. The data revealed that although snails with prior exposure to *P. elegans* shed few cercariae on challenge with the schistosome, their levels of mortality were comparable with those of snails

with 8 times their level of cercariae shedding but without prior exposure to *P. elegans* (Figs. 4, 5).

The stress associated with the onset of patency of the schistosome infection in *B. glabrata* is reflected in a decline in snail growth, as previously documented by Minchella (1985). The present study suggests a similar effect of *P. elegans* on the incompatible host. It is noteworthy that in the compatible host, *S. elodes*, *P. elegans* induces a modest increase in host growth (Zakikhani and Rau, 1999).

Prior infection of *B. glabrata* with *P. elegans* dramatically reduced the number of cercariae shed by a superimposed infection with *S. mansoni*. Infections with only the schistosome yielded almost 8 times as many cercariae. This decline was not attributable to differential mortality between the 2 groups nor was the decline reflected in the prevalence of the schistosome infections. The latter may be due to the relatively large number of *S. mansoni* miracidia administered. Although there may have been a significant reduction in the number of schistosome sporocysts that became established in the tissues of the snail, a decline in their subsequent asexual proliferation, or both, the data do not allow us to distinguish between these effects.

The successful establishment of *S. mansoni* sporocysts in the tissues of their compatible host requires the action of excretory–secretory factors that suppress the activation of hemocytes, which are the primary cellular component of the molluscan internal defense system (Núñez et al., 1994). Hemocytes of *B. glabrata*, infected with *S. mansoni*, manifested a reduction in mobility (Lodes and Yoshino, 1990), phagocytic activity, and superoxide production (Conners and Yoshino, 1990). Interference with the internal defense system is common and is particularly strong in echinostome infections. Thus, *Echinostoma paraensei* may diminish the capacity of *B. glabrata* hemocytes to encapsulate and destroy its sporocysts to such an extent that it may put the host at risk from opportunistic infections, among them other species of digeneans (Loker et al., 1992; reviewed by Loker, 1994).

There is evidence to suggest that the interactions between the snail host's internal defense system and the sporocysts of at least some plagiorchiid digeneans may take another form. Monteil and Matricon-Gondran (1991) report that hemocytes of *Lymnaea truncatula* may recognize the sporocysts of the parasite *Haplometra cylindracea* as foreign and are attracted to them. Hemocytes adhere to the surface of the parasite where they are rendered inactive and become part of the paletot, a peculiar structure enveloping the sporocysts of various plagiorchiid digeneans, which serves to protect and nourish them. Monteil and Matricon-Gondran (1991) also found that the tegument of the growing sporocysts appears to capture most hemocytes, so that they become rare in the hemal spaces of the compatible host. It is not clear whether *P. elegans* elicits similar responses in the incompatible host, *B. glabrata*. Conceivably, sporocysts of *P. elegans* may stimulate the internal defense system, but because the sporocysts remain small, the number of free hemocytes may remain high, diminishing the success of a subsequent infection with *S. mansoni*. The dynamics of the relationship between the number of hemocytes and susceptibility to infection has been described for *E. paraensei* in *B. glabrata* by DeGaffé and Loker (1998).

In summary, infection of the incompatible host, *B. glabra-ta*, with *P. elegans* results in parasitic castration, premature host death, and a severe reduction in the cercariae output of challenge infections with *S. mansoni*. This may render *P. elegans* and other plagiorchiid digeneans of value in the management of human schistosomiasis. Because the life cycle of *P. elegans* is readily maintained in the laboratory and adult worms are highly prolific, producing approximately 1 million eggs per hamster per week, large numbers of eggs can be deployed into the habitat of target snail species. Such eggs may remain a significant source of infection for more than 2 wk (Zakikhani and Rau, 1998a). In the field, snails may acquire the infection by accidentally ingesting such eggs as they forage. Combes (1982) suggests that a ratio 600:1 (eggs–molluscs) is realistic and may be appropriate as a basis for future field trials.

ACKNOWLEDGMENTS

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