Inheritance of *Schistosoma mansoni* infection incompatibility in *Biomphalaria alexandrina* snails

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In this study, we looked at the inheritance of susceptibility and resistance to Schistosoma mansoni infection *in the first generation of crossbred* Biomphalaria alexandrina *snails. Our ultimate goal is to use such information to develop a biological method of controlling schistosomiasis. We infected laboratory-bred snails with* S. mansoni *miracidia and examined cercarial shedding to determine susceptibility and resistance. Five parental groups were used: Group I contained 30 susceptible snails, Group II contained 30 resistant snails, Group III contained 15 susceptible and 15 resistant snails, Group IV contained 27 susceptible and three resistant snails and Group V contained three susceptible and 27 resistant snails. The percentage of resistant snails in the resulting progeny varied according to the ratio of susceptible and resistant parents per group; they are 7%, 100%, 68%, 45% and 97% from Groups I, II, III, IV and V, respectively. On increasing the percentage of resistant parent snails, the percentage of resistant progeny increased, while cercarial production in their susceptible progeny decreased.*

Key words: *Schistosoma mansoni - Biomphalaria alexandrina* - susceptibility - resistance - biological control

Schistosomiasis is considered the second most important parasitic disease in the world, ranking next to malaria (Combes 1990). About 200 million people in the world are affected (WHO 1985). Africa, Latin America, South West and South East Asia are foci of the disease (WHO 1993). In Egypt, it is the most important endemic parasitic disease (Nunn & Tapp 2000). Approximately 70% of the adult chronic liver diseases and 35% of child liver diseases are due to schistosomiasis (El-Khoby et al. 2000). In 2007, about 3% of rural populations showed *Schistosoma mansoni* eggs in their stools (Bakr et al. 2007).

Over the last few decades, the epidemiological distribution of schistosomiasis in Egypt has changed; *S. mansoni* has replaced *Schistosoma haematobium* in the Nile delta and has become well established in Middle Egypt (Abdel-wahab et al. 2000). This has resulted in a marked decrease in the prevalence of *S. haematobium* infection, with an increase in *S. mansoni* infection rates. The spread of *S. mansoni* infection has serious effects on public health and has increased the burden of controlling schistosomiasis.

Various methods have been used to combat schistosomiasis, including treatment of patients, health education, improvement of sanitation, provision of safe water supplies to populations and snail control. Methods for controlling schistosome transmission by reducing snail populations have included chemical (molluscicides), physical and biological methods (Sturrock 2001).

One promising component of biological control is the introduction of parasite resistant snails into endemic areas to replace the resident susceptible snails and avoid the often destructive changes to the local ecosystem that accompany other methods of snail control (Sturrock 2001).

A preliminary step towards possible field trials in replacing susceptible with resistant snails is a laboratory study of the susceptibility patterns expected from progeny of interbreeding susceptible and resistant snail populations. This study would enable workers to predict the flow of resistance genes in the population and assess the feasibility of this snail control method (Lewis et al. 2002). It has been suggested that incompatibility to *S. mansoni* infection, a refractory characteristic of *Biomphalaria alexandrina*, could be hereditary, similar to susceptibility (Abdel-Hamid et al. 2006). Thus, it may be beneficial to select actively resistant snails and mass culture them to increase the proportion of alleles for incompatibility as a potential method for controlling schistosomiasis in natural populations. To better clarify the promise in this disease control methodology, we investigated the heritability of resistance in *B. alexandrina* snails.

MATERIALS AND METHODS

We bred the 100 mature snails in four transparent plastic aquaria. Each aquaria contained 5 L of well-aerated, aged and dechlorinated tap water (DTW), changed twice a week. During winter, they were kept at 26ºC and maintained at room temperature in the summer (Haroun 1996). Fresh lettuce leaves were supplied as food every couple of days; dead snails were regularly removed (Smithers & Terry 1965, Frandsen & Christensen 1984, Lewis et al. 2002).

Each aquarium contained pieces of foam that served as substratum for egg deposition (Azim & Watson 1948, Shoukry et al. 1997). Newly deposited egg masses were regularly collected using a scalpel, transferred to sepa-

rate aquaria and inspected daily until hatching. After hatching, baby snails were reared for one month until they were juveniles (Shoukry et al. 1997). During this month, baby snails were fed using boiled lettuce leaves. White chalk pieces were added as a source of calcium for growth of the snail shell (Dettman et al. 1989).

Stools of infected, untreated children living in Abis village were collected, dispersed in physiological saline, strained and the filtrate centrifuged at 500 *g* for 3 min. We used the sediment containing *S. mansoni* eggs for infection of the *B. alexandrina* snails (Ragab et al. 2003).

We transferred the stool sediment to a petri dish containing about 500 mL of warm (35ºC) DTW and exposed it to direct sunlight for about 30 min to allow egg hatching. Using a fine capillary pipette, we aspirated 5-6 active, vigorously swarming miracidia and used them to infect each of the 40 juvenile snails, each placed in separate wells on tissue culture plates (Henning & Youssef 1976, El-Gindy et al. 1978, Shoukry et al. 1997). Four weeks later, we used infected *B. alexandrina* snails to harvest cercariae, by placing groups of 10 snails in 200 mL beakers, containing 50 mL DTW, under direct sunlight for about 1-2 h. After carefully shaking the cercarial suspensions, we aspirated 0.1 mL from each suspension and counted the cercariae using a dissecting microscope. We calculated the total number of harvested cercariae and then used them for animal infection (Pellegrino et al. 1962, Joy 1971).

Using the paddling technique, laboratory-bred Swiss strain albino mice were infected with 120 cercariae per mouse. Seven to eight weeks post-infection (Kogan et al. 1954), eggs obtained from the intestines and livers of infected mice (El-Gindy et al. 1985, Xu & Dresden 1989) were exposed to direct sunlight for approximately 30 min to stimulate miracidial hatching (Henning & Youssef 1976). To infect the juvenile snails, we aspirated 5-6 active, vigorously swarming miracidia (Henning & Youssef 1976, El-Gindy et al. 1978, Shoukry et al. 1997). Under the above described conditions, we transferred groups of roughly 25 snails to separate containers, each containing five litres of DTW. They were kept in darkness for about four weeks (Smithers & Terry 1965, Frandsen & Christensen 1984, Haroun 1996, Lewis et al. 2002).

Four weeks post-infection, the snails were checked individually for cercarial shedding twice per week for three weeks (McClelland 1965). Snails that shed cercariae were considered susceptible, while those that failed to shed cercariae were re-exposed to miracidia (El-Gindy et al. 1978, Shoukry et al. 1997). Four weeks later, the re-exposed snails were again tested for cercarial shedding, twice weekly for three weeks (McClelland 1965). Snails that failed to shed cercariae after the second miracidial exposure were considered resistant (Zanotti-Magalhaes & Magalhaes 1997).

To obtain the first generation (F1), 75 susceptible and 75 resistant snails were crossed in different proportions, as follows: Group I: 30 susceptible snails, Group II: 30 resistant snails, Group III: 15 susceptible, 15 resistant snails, Group IV: 27 susceptible, three resistant snails and Group V: three susceptible, 27 resistant snails.

Snails of each group were reared together in a separate aquarium containing about 4-5 L of DTW (Azim & Watson 1948, Kogan et al. 1954, Smithers & Terry 1965, Frandsen & Christensen 1984, Dettman et al. 1989, Lewis et al. 2002). To avoid fertilised eggs before the beginning of the experiment, we discarded egg batches from each of the five groups during the 1st four weeks. After the 4th week, the newly deposited batches were gently collected. Egg batches of each group were transferred to a separate container containing DTW. They were monitored daily until hatching.

After hatching, we used 150 snails in each experimental group, excluding dead snails from statistical analysis. Our analysis includes the 1st 100 snails that lived until the end of the experiment. The F1 in this study, the 150 baby snails from each group, was reared until they were juveniles. They were individually exposed to *S. mansoni* miracidia (Kogan et al. 1954, El-Gindy et al. 1978, Shoukry et al. 1997). Four weeks later, these juveniles were individually tested for cercarial shedding, returned to a separate container containing 250 mL of DTW and maintained in the same conditions described previously (Azim & Watson 1948, Kogan et al. 1954, Smithers & Terry 1965, Frandsen & Christensen 1984, Dettman et al. 1989, Lewis et al. 2002).

Cercarial shedding was done once weekly for each snail for four weeks (Pellegrino et al. 1962, Joy 1971). Snails that shed cercariae were considered susceptible, whereas any that did not shed, even after the second miracidial exposure, were considered resistant (Zanotti-Magalhaes & Magalhaes 1997).

Evaluation parameters of the F1 from different groups - (i) susceptibility rate: we determined the percentage of susceptible and resistant snails in each group (Shoukry et al. 1997, Lewis et al. 2002) and (ii) degree of susceptibility in each group: four weeks post-infection, we counted the number of cercariae shed from every susceptible snail in each group once weekly for four weeks (Pellegrino et al. 1962, Joy 1971). Total cercarial production per 100 snails (TCP/100 snails) and mean cercarial shedding per week were calculated for each group. We then used Frandsen's classes (Frandsen 1979) to assess the degree of susceptibility.

RESULTS

We recorded the susceptible and resistant traits expressed in the F1 from the various snail crosses (the 5 parent groups) of *B. alexandrina* snails, as follows.

Susceptibility and resistance rates in the F1 of the five groups - Table I and Fig. 1 show the percentages of susceptible and resistant snails in the F1 of the five parental groups. Among the F1 progeny from cross-bred Group I, containing only susceptible *B. alexandrina* parents, 93% of the snails were susceptible. Completely resistant F1 progeny were generated from Group II, in which all parents were resistant. The percentages of susceptible F1 snails resulting from mixed parental populations were 32%, 55% and 3% from parental Groups III, IV and V, respectively. The percentages of resistant snails were 7%, 100%, 68%, 45% and 97% in Groups I, II, III, IV and V, respectively.

TABLE I

Percentage of susceptible and resistant snails in the first generation (F1) obtained from the different studied groups

Groups	I (30 susceptible parents)	II (30 resistant parents)	III (15 susceptible and 15 resistant parents)	IV (27 susceptible and 3 resistant parents)	V (3 susceptible and 27 resistant parents)
Exposed F_1 snails (n)	100	100	100	100	100
SR (%)	93	0	32	55	3
RR(%)	7	100	68	45	97
X2	-	-	23.13[a]	-	-
p	-	-	0.000[a]	-	-

a: using Chi square test there was significant difference between the offspring of the five groups regarding resistance. Significant difference between groups regarding resistance. RR: resistance rate; SR: susceptibility rate.
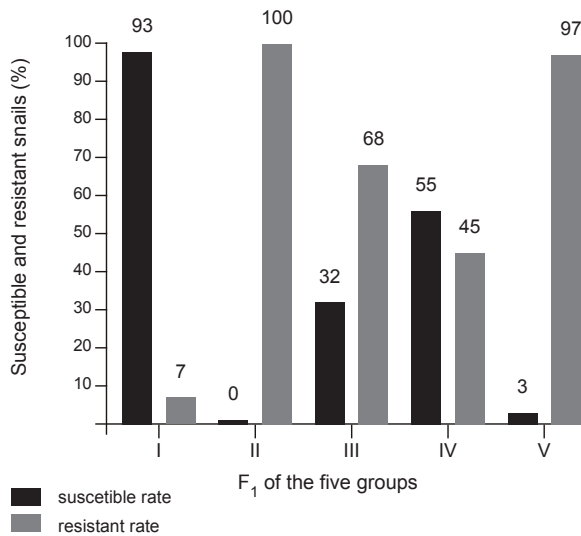

Fig. 1: percentage of susceptible and resistant snails in the first generation (F1) obtained from the different studied groups.
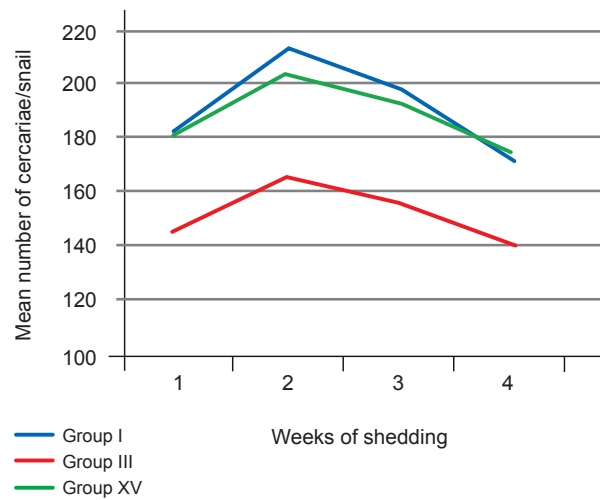

Fig. 2: mean number of cercarial shedding by susceptible snails in the progeny of Groups I, III and IV per week during the four weeks interval.

Using Frandsen's classes (Frandsen 1979), we categorised the degree of susceptibility, as assessed according to the following parameters:

Cercarial production by susceptible snails among the offspring of the different groups - Shown in Table II, we calculated the total number of cercariae produced by the susceptible snails over a period of four weeks and the mean of cercariae shed by each susceptible snail in the different studied groups. From the Group I FI, in the third Frandsen class, the total number of cercariae produced by all snails over the four weeks (TCP) was 71,205. No cercariae (TCP = 0) were shed from F1 snails of Group II, which was in Frandsen class 0. The TCP of F1 snails in Group III was 19,458, placing them in Frandsen class 2. Both Groups IV and V were categorised as class 1 because their TCP values were 41,457 and 1,344 respectively.

We observed the highest cercarial production/snail/week in the Group I progeny at 191.4 ± 30.9 cercariae, followed by Group IV progeny at 188.4 ± 22, then Group III progeny that produced an average of 152 ± 23.6. Group V progeny showed the least cercarial production, at 112 ± 14.2 cercariae/snail/week, while Group II released no cercariae at all.

Mean number of cercariae shed by susceptible offspring in each group per week over the four-week interval - Table III and Fig. 2 show that the 2nd week of shedding yielded the highest mean number of cercariae shed by *B. alexandrina* snails in the progeny of Groups I, III and IV: 211.9 ± 41.3, 164.7 ± 25.9 and 203.2 ± 26.3 cercariae/snail, respectively. Data collected from Group V were not tabulated because they were not statistically significant, as only three snails shed cercariae. Yet we observed the highest mean (117 ± 13.1) in the 3rd week

TABLE II

Total cercarial production (TCP) by offspring first generation (F1) of the five studied groups

Group	Shedding snails in F1 of each group n	TCP/100 snails over the four weeks n	Cercariae/snail/week mean ± SD
I	93	71,205	191.4 ± 30.9
II	0	0	0
III	32	19,458	152 ± 23.6
XV	55	41,457	188.4 ± 22
V	3	1,344	112 ± 14.2

TABLE III

Comparison between mean cercarial shedding per week in the progeny of Groups I, III and IV during the four weeks interval

Weeks	Mean cercarial shedding per week			F	p	Post hoc test (least significant difference)
	Group I	Group III	Group IV			
1	182.8 ± 32.5	146.2 ± 25.27	181.8 ± 22.6	21.13	0.000[a]	(I, III)[a], (III, IV)[a]
2	211.9 ± 41.3	164.7 ± 25.9	203.3 ± 26.3	22.024	0.000[a]	(I, III)[a], (III, IV)[a]
3	197.7 ± 36.8	155.5 ± 27.5	192.8 ± 25.2	21.213	0.000[a]	(I, III)[a], (III, IV)[a]
4	173.1 ± 27.3	141.5 ± 22.6	175.8 ± 22.5	22.201	0.000[a]	(I, III)[a], (III, IV)[a]

a: significant difference if $p \leq 0.05$. Group V was excluded from this analysis because only three snails shed Cercariae, which is not valid in statistical analysis. F test to compare more than two arithmetic means.

of shedding, followed by 115 ± 17.1 in the 2nd week. In the 1st and 4th weeks of shedding, the mean values were nearly the same: 108 ± 13.7 and 108 ± 13.1.

Table III shows the mean cercarial shedding for the different groups' progeny during the four weeks interval. In the 1st week, the mean values for the progeny in Groups I, III and IV were 182.8 ± 32.5, 146.2 ± 25.3 and 181.8 ± 22.6, respectively, where F = 21.13 and p = 0.000. During the 2nd week, 211.9 ± 41.3, 164.7 ± 25.9 and 203.3 ± 26.3 were the mean values of Group I, III and IV, respectively, where F = 22.024 and p = 0.000. For the 3rd week, the mean values of Group I, Group III and Group IV were 197.7 ± 36.8, 155.5 ± 27.5 and 192.8 ± 25.2, respectively, where F = 21.213 and p = 0.000. As for the 4th week, mean values of Group I, Group III and Group IV were 173.1 ± 27.3, 141.5 ± 22.6 and 175.8 ± 22.5, respectively, where F = 22.201 and p = 0.000. A post hoc test was calculated for the four-week interval and revealed a significant difference between Groups I and III, as well as between Groups III and IV.

DISCUSSION

Schistosomiasis presents significant economic and public health consequences in many developing countries and is considered a serious problem in Egypt (WHO 1985). Genetics is one form of biological control, first discussed by Newton (1952), who found that susceptibility of *Biomphalaria glabrata* to *S. mansoni* in-

fection is hereditary. In 1958, Hubendick demonstrated that susceptible snail populations could be reduced by applying populations that are genetically resistant to the parasite. The flow of genetic resistance to *S. mansoni* in *B. glabrata* and *Biomphalaria tenagophila* was studied in the Western Hemisphere (Rosa et al. 2005, Coelho et al. 2008). However, little is known about the resistance genes in *B. alexandrina*.

Susceptibility of *B. glabrata* to *S. mansoni* infection is age dependant; in juvenile snails, susceptibility is controlled by at least four genes, each with multiple alleles, while in adulthood there is only one dominant gene that controls susceptibility (Richards & Shade 1987). Rosa et al. (2005) found that two dominant genes determine resistance in *B. tenagophila*. In the same year, El-Khayat et al. reported that genetics is one of the most important approaches for biological control of susceptibility of *B. alexandrina* to *S. mansoni* infection. In 2006, Abdel-Hamid et al. found that, similar to susceptibility, resistance is hereditary and is controlled by a genetic factor in *B. alexandrina*. In 2008, Coelho et al. introduced large numbers of resistant *B. tenagophila* into the field to cross-breed with the local snails. In their study, Coelho et al. applied molluscicides, which obliged the remaining local population to cross-breed with the resistant snails and generate *S. mansoni* resistant offspring. In light of such studies, we assessed the inheritance of *S. mansoni* resistance in *B. alexandrina* snails, using the progeny of interbreeding resistant and susceptible populations.

Analysis of these results revealed the appearance of resistant snails in the F1 progeny population in various snail crosses, which we attribute to a dominant resistance characteristic in *B. alexandrina* snails. Our result concurs with the Lewis et al. (2002) study using *B. glabrata* snails. Further, previous investigations documented dominant resistance heritability in *B. glabrata* snails (Richards & Merritt 1972).

Upon crossing susceptible and resistant snails in parental Groups III, IV and V, the resulting offspring contained resistant individuals in a higher percentage than in the parent groups. In *B. glabrata*, Lewis et al. (2002) generated almost comparable susceptibility phenotypes to those for *B. alexandrina* herein. The main difference between both studies was the 7% resistant progeny for *B. alexandrina* from completely susceptible parents, absent in the progeny of the analogous parental group for *B. glabrata*. A probable explanation for the 7% resistant *B. alexandrina* progeny is that the parent group includes snails that carry resistance genes and the resistance alleles increase among successive generations. Reinforcing this assumption, in 1997, Shoukry et al. reported the appearance of resistant *B. alexandrina* snails in the progeny of susceptible parents. Studying three successive *B. alexandrina* generations, they reported a decrease in susceptibility from one generation to the next among snail progeny originating from susceptible snails.

Containing completely resistant *B. Alexandrina*, all of the Group II offspring were resistant. Similarly, in 2002, Lewis et al. did not find any susceptible snails in the progeny of *B. glabrata*-resistant parents. However, whether or not susceptible progeny will appear as a result of crossing successive resistant generations requires further investigation. In 2006, Abdel-Hamid et al. validated this prediction using *B. alexandrina*, where they found that parent snails of susceptible progenies could be infection resistant.

In the current paper, susceptibility assessment by the TCP in each group revealed that the five groups fell within the lower degrees of Frandsen's categorisation (Frandsen 1979). According to Frandsen, Group I is compatible (class 3), as it produced the highest cercarial yield; this compatibility could be attributed to the snail crosses of this parental group that contained totally susceptible parents. Both Groups III and IV fell into class 2 of Frandsen (poorly compatible). Progeny of Group V were not very compatible (class 1). Group II lay within class 0, being completely resistant or incompatible; they shed no cercariae because their parents were completely resistant snails. Therefore, increasing the number of resistant parent snails decreases the degree of *B. alexandrina* susceptibility to *S. mansoni* infection. Notably, in 1996, Haroun collected *B. alexandrina* snails from two Egyptian Governorates and infected each group of snails with its homologous strain of *S. mansoni* miracidia. Haroun found that both groups lay within Frandsen's second, poorly compatible class.

Haroun's (1996) and our results show that *B. alexandrina* snails lie within the lower categories of compatibility compared to other *Biomphalaria* species studied by Frandsen in 1979. In Lewis et al. (2002), the *B. glabrata* parent group that contained totally susceptible snails did not give rise to any resistant progeny, while the analogous group in our *B. alexandrina* study yielded seven resistant snails. We reason that *B. alexandrina* may contain more resistance alleles in the susceptible population than those in other *Biomphalaria* species, thus accounting for its lower susceptibility and the appearance of resistant progeny from completely susceptible parents.

We used the mean cercarial shed/snail/week as our second parameter in assessing degree of *B. alexandrina* susceptibility to *S. mansoni* miracidia infection. This varied from one group to the other according to the percentage of resistant parents: the highest mean was from Group I progeny, having purely susceptible parents, followed by Group IV, containing only three resistant parents, then Group III, with 15 resistant parents. We observed the lowest cercarial production in Group V, consisting of 27 resistant parents. Comparing the mean cercarial shedding of Groups I, III and IV each week during the four weeks of shedding, there was a significant difference between both Groups I and III and Groups III and IV. In 2000, Knight et al. reported that even among susceptible snail stocks, certain individuals were more resistant than others.

Excluding Group II, which contained only resistant snails, we observed the highest cercarial count in each of the shedding groups in the 2nd week of shedding. Group V exhibited its highest cercarial count between the 2nd-3rd weeks of shedding. These results concur with Haroun's (1996) experiments that showed the highest cercarial production in shedding snails occurred mainly in the 2nd week of shedding.

Importantly, our results show that upon increasing the proportion of resistant parents, the percentage of their resistant progeny increased, while cercarial production in their susceptible progeny decreased. This study supports the introduction of parasite resistant *B. alexandrina* into endemic areas to replace the resident susceptible ones as a promising method of biological control for *S. mansoni* in Egypt. Future studies in the resistance and susceptibility of *B. alexandrina* to *S. mansoni* necessarily include the related genetic markers.

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