Mixed strain schistosome infections of snails and the evolution of parasite virulence

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SUMMARY

Mathematical models often propose that within-host competition between parasites can be a major factor in the evolution of increased parasite virulence. Kin selection predicts that as the coefficient of relatedness between infecting parasites decreases, the benefits of competition to individual genotypes increases. Thus where parasites can adjust their behaviour in response to current conditions, higher virulence is predicted in multiple genotype infections. There is limited experimental data, however, regarding the effects of mixed strain infections on host and parasite fitness. We investigated, for a snail–schistosome system, whether a conditional increase in replication rates occurred in mixed genotype infections and resulted in increased virulence. Four groups of *Biomphalaria glabrata* snails were exposed to 1 or 2 laboratory strains of *Schistosoma mansoni*. Mixed genotype infections were observed to be more virulent than single genotype infections, in terms of reductions in host reproductive success and survival. Parasite reproductive rate was also increased in mixed strain groups. Reduced host reproductive success was suggested to be directly due to the genetic heterogeneity of the parasitic infections resulting in increased host defence costs. Reduced host survival was consistent with an adaptive conditional parasite response.

Key words: virulence, facultative response, mixed infection, *Schistosoma mansoni*, *Biomphalaria glabrata*.

INTRODUCTION

Virulence can be defined as the reduction in host success attributable to parasitic infection (Bull, 1994). There has been considerable interest over recent years concerning the circumstances under which virulence or avirulence is expected to evolve. Much theoretical work considers parasite virulence to be a consequence of parasite adaptation in order to maximize parasite fitness (see Bull, 1994 for review) and assumes that virulence is a direct result of parasites reproducing more rapidly. Mathematical models based upon single genotype infections predict that virulence evolves to a level that maximizes the total number of infections of new hosts (e.g. Anderson & May, 1982). Where mixed genotype infections of individual hosts occur, on the other hand, it is predicted that higher levels of virulence may be favoured by natural selection (e.g. Antia, Levin & May, 1994; Bonhoeffer & Nowak, 1994; May & Nowak, 1995; Frank, 1996). This is because competition between strains may select for genotypes that exploit host resources sooner, regardless of whether they would produce fewer new infections in the single genotype situation (Bremermann & Pickering, 1983). Increased virulence may thus be viewed as an unavoidable consequence of mixed genotype infections.

Kin selection predicts that the fitness advantages of within-host competitive ability rises as the relatedness between infecting genotypes decreases. Parasites could evolve a genetically fixed virulence strategy appropriate for the frequency, and average genetic heterogeneity, of mixed infections in the population. Alternatively, if parasites were able to detect the presence of other parasite genotypes within a host, a conditional strategy might evolve, where parasites facultatively alter their reproductive rates, and hence virulence, according to the current infection (Frank, 1992; van Baalen & Sabelis, 1995). If conditional parasite responses are present, a simple prediction is therefore that mixed-strain parasite infections would be more virulent than single-strain infections, even in the first generation of passage under such conditions. However, an important point to consider is that observed virulence will be a product of both parasite and host factors (Taylor, MacKinnon & Read, 1998). Host defence against genetically heterogeneous infections may require more host resources or such defence may be less effective (Morand & Harvey, 2000; Moret & Schmid-Hempel, 2000). Thus even in the absence of facultative alterations in parasite reproductive rates, mixed-strain infections may be more virulent than single-strain infections. It is therefore necessary to compare both parasite reproductive rates and viru-

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lence in mixed and single-strain infections (Taylor *et al*. 1998). Increased virulence directly due to the genetic heterogeneity of the infection would be discernible for any given parasite reproductive rate and mixed-strain infections would be more virulent than single-strain infections, even when controlling statistically for the number of parasites present.

As yet, few experimental studies have been conducted to test these hypotheses in animal parasite systems. Moreover, of these, most consider microparasites while there has been minimal theoretical or experimental attention paid to macroparasites. Snail–schistosome interactions are, however, a useful model system in which to investigate such topics. Schistosomes are macroparasites with an indirect life-cycle, where transmission occurs via free-swimming larval stages, miracidia (infective to the molluscan intermediate host), and cercariae (infective to the mammalian definitive host). Schistosomes can cause significant reductions in snail survival (Woolhouse, 1989; Webster & Woolhouse, 1999) and reproduction (Sturrock & Sturrock, 1970), and mixed genotype infections are common in natural foci (Woolhouse, Chandiwana & Bradley, 1990; Minchella, Sollenberger & Pereira de Souza, 1995; Davies *et al*. 1999). In this study, we exposed groups of *Biomphalaria glabrata* snails to either one or other, or a combination of two, *Schistosoma mansoni* strains. Parasite reproductive rates, transmission and virulence were compared in mixed and single genotype infections in order to investigate the existence of facultative virulence responses.

MATERIALS AND METHODS

Experimental design and set up

B. glabrata snails used were a strain (A1) originally from Brazil, that had been maintained in the laboratory without parasite pressure for in excess of 25 generations. Two strains of *S. mansoni* parasites were used, one originally from Puerto Rico (Sm-PR) and the other originally from Kenya (Sm-K). Both parasite strains had also been maintained in the laboratory for more than 25 generations, being routinely passaged through a mixed genotype pool of *B. glabrata* and CBA/CA mice.

Four size-matched groups (8–12 mm in diameter) of 30–40 sexually mature snails (as measured by the onset of egg laying) were exposed to 10 freshly hatched miracidia of one or other, or a combination of both, laboratory strains of *S. mansoni* in the following combinations: Group 1: 10 Sm-PR; Group 2: 10 Sm-K; Group 3: 6 Sm-PR, 4 Sm-K; Group 4: 4 Sm-PR, 6 Sm-K. Snails were exposed to the miracidia in 6 ml of deionized water for 2 h, a period sufficient for maximal miracidial penetration (Lewis *et al*. 1986). All exposure wells were inspected to ensure that all miracidia had apparently penetrated the snails.

Snails were maintained, for logistic reasons, in groups of 3 within experimental pots (15 × 10 × 5 cm) divided with gauze into 3 individual sections. All snails were randomly assigned to 1 of the 4 experimental groups. Each pot contained a set consisting of 1 snail of 3 of the experimental groups. Sets of possible combinations were randomly assigned to experimental pots and snails within experimental groups were randomly assigned to the appropriate set. Pot effects were controlled for in statistical analysis. All animals were maintained in a standard volume of a 50% deionized water/50% Caledonian spring water (Sainsburys Supermarkets plc., London, UK) mix. The water was changed weekly and snails were fed *ad libitum* on freshly washed lettuce. The laboratory was maintained at 23–25 °C and subject to a light regime (full UV spectrum, SAD Lightboxes Co. Ltd, High Wycombe, UK) of 12 h light and 12 h darkness.

Measurement of rate of parasite development

There is a latency or 'pre-patent' period of approximately 35–45 days from exposure of snails to miracidia until the production of cercariae. From week 5 post-infection and weekly thereafter, cercariae from each snail were 'shed' by placing snails in the dark for 24 h and subsequently exposing them, at 10.00 h to an overhead light source (100 W) for 2 h in vials containing 20 ml of deionized water. Patent infections are those that produce cercariae and thus can potentially infect definitive hosts. Individual snails were thus scored as positive or negative for cercarial production. The intensity of cercarial shedding in patently infected snails was estimated from the number of cercariae present in two 2 ml samples per snail (after ensuring a homogeneous distribution of cercariae in each vial by gentle shaking) which were stained with iodine and assayed under a dissecting microscope.

The number of parasite stages produced early in the infection is a measure of the parasite reproductive rate (Davies, Webster & Woolhouse, 2001*a*; Taylor *et al*. 1998). We used the number of cercariae produced at day 47 as a composite measure of parasite reproductive rate determined by the frequency of patent infections, the length of the pre-patent period and the intensity of cercarial shedding. Day 47 was chosen since this was the median length of pre-patent period exhibited in these infections and thus was considered the most sensible measure to reflect the contribution of all three of these factors, each of which are important components of parasite reproductive rate. Though there is wide variation in cercarial production over time, even for individual snails, such variation would be expected to contribute to within-group variation, and thus will tend

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to underestimate the differences between groups. Collection of cercarial data weekly until 12 weeks post-infection further allowed estimation of an index of overall parasite success, measured as the total production of cercarial stages, and confirmation of the rate of parasite development in early patency.

Transmission success of cercariae produced by each group was assayed at week 9. Eight snails were randomly selected from each of the 4 experimental groups; their cercariae were pooled and used to infect four 6-week-old inbred CBA/CA mice at a dose of 220 cercariae/mouse by paddling for 1 h in 30 ml of infected water.

At 7 weeks post-infection, before signs of pathology had occurred, mice were killed using a rising concentration of carbon dioxide. Adult schistosomes were recovered by a modified hepatic perfusion technique (Smithers & Terry, 1965). Worms were sexed and counted. Miracidia from each of the livers were stimulated to hatch by macerating through a sieve and exposure to a bright light (100 W) source for 30 min in 100 ml of deionized water. Miracidial numbers were estimated from the mean of five 1 ml samples assayed under a dissecting microscope.

Measurement of virulence

Virulence was defined as the reduction in host fitness attributable to parasite infection. Thus an infection was considered to be more virulent than another infection if the host in the former infection had a lower survival or reproductive success. Intermediate host survival was measured as the number of days until death. All snails were followed until death, where the maximum survival was 51 weeks. There were 2 measures of host reproductive success, (i) the number of egg masses laid, a measure of potential reproductive capacity, and (ii) the number of viable offspring produced, that is the actual reproductive success. Host reproduction was measured thrice weekly; hatchlings were removed following counting but new egg masses were marked and left, to allow further hatching of offspring.

Statistical analysis

Chi-square analysis (χ^2) was used to compare the frequency of patent infections between groups. Evidence of differences in fitness traits between experimental groups was investigated using analysis of variance (in a generalized linear modelling procedure). Parasite reproductive rate (number of cercariae produced at day 47), host survival, host reproductive success, and the total number of cercariae produced were used as dependent variables. Dependent variables were transformed as necessary to meet the model assumptions of normality of error and homogeneity of variance. Experimental group, pot and initial snail size were used as independent variables. Minimal models are presented, with non-significant terms excluded. Means and standard errors were back-transformed as necessary for presentation.

A second set of analyses was conducted using host survival and host reproductive success as dependent variables. Here experimental group, pot, initial snail size and the parasite reproductive rate (number of cercariae produced at day 47) were used as independent variables. Thus this measured evidence of a difference in virulence between mixed and single-strain infections, when controlling for the number of parasites present.

Differences between mixed and single-strain infections in the transmission success to, and reproduction in, definitive hosts were investigated using Mann-Whitney U tests.

RESULTS

There was no evidence of a difference between the Sm-PR and Sm-K strains in any of the measures studied, nor between the 2 groups of mixed-strain infections, (see Figs 1 and 2), and thus we pooled the 2 single-strain and 2 mixed-strain groups to increase statistical power.

Parasite reproduction and transmission

There was no evidence of a difference between groups in the frequency of patent infection (Group 1 = 41·7%, Group 2 = 46·2%, Group 3 = 52·0%, Group 4 = 44·4%; $\chi^2 = 0{\cdot}56$, D.F. = 3, $P = 0{\cdot}91$) and thus all snails were included in the analysis. Parasite reproductive rate (the number of cercariae produced at day 47) was significantly higher in mixed strain than single-strain infections (Fig. 1A) ($F_{1,133} = 4{\cdot}10$, $P = 0{\cdot}04$). However, despite the increase in early reproductive rate in mixed-strain infections, overall parasite success, as measured by the total number of cercariae produced during the experiment, showed no difference between groups ($F_{1,133} = 0{\cdot}21$, $P = 0{\cdot}65$) (Fig. 1B). Figure 1C demonstrates that the difference between single and mixed infections was apparent throughout the first 3 weeks of patency.

There was no evidence of any differences in the infectivity to, or miracidial production in, the definitive hosts from mixed and single-strain infections for a fixed cercarial dose (U = 44·5, $P = 0{\cdot}78$ and U = 46·5, $P = 0{\cdot}57$ respectively).

Virulence

Figure 2 shows the virulence of mixed and single-strain schistosome infections. Host survival was lower (i.e. virulence was higher) in mixed-strain

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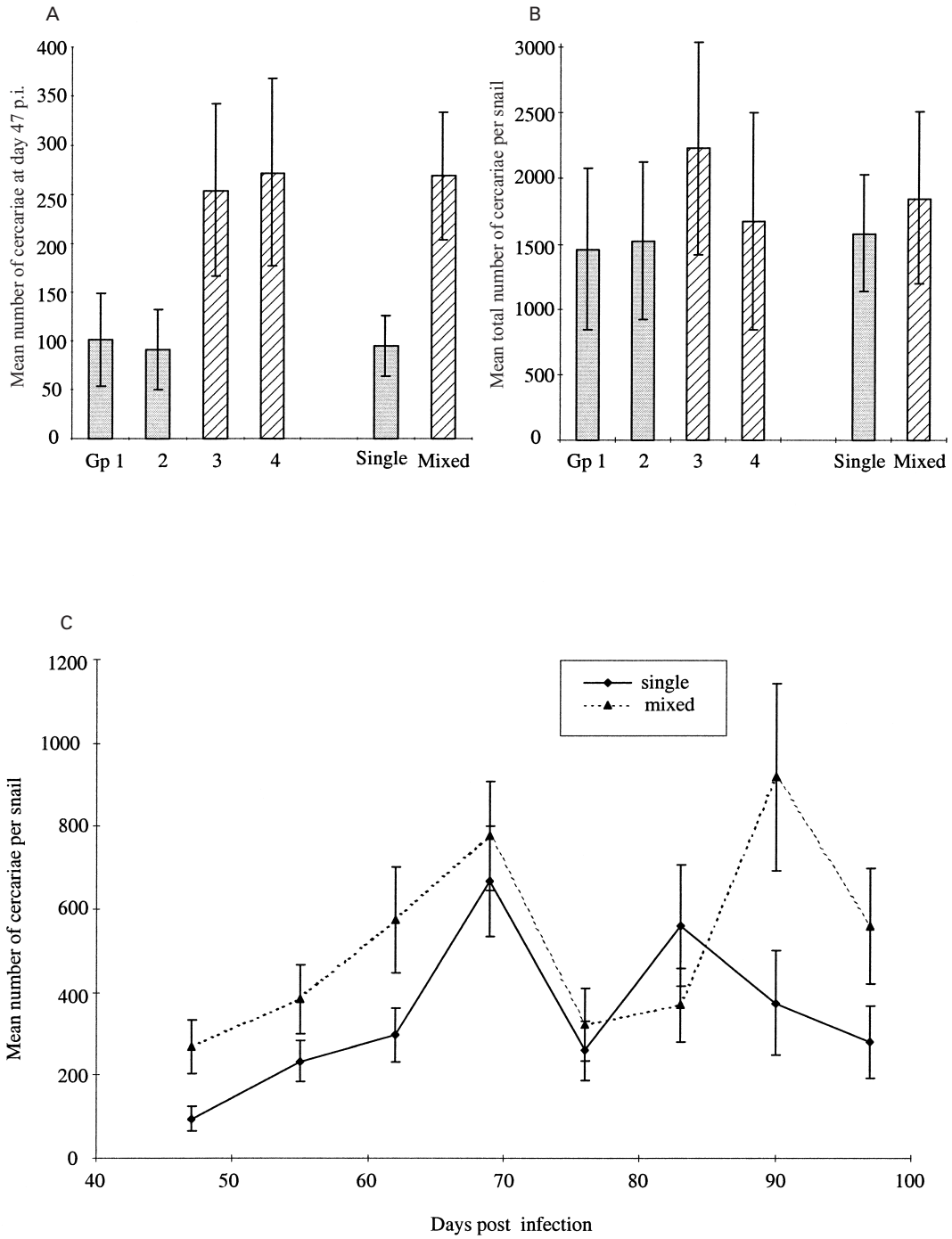

Fig. 1. Mean (A) parasite reproductive rate and (B) parasite success for groups of snails exposed to 10 Sm-PR miracidia (Gp 1), 10 Sm-K (Gp 2), 6 Sm-PR, 4 Sm-K (Gp 3) and 4 Sm-PR, 6 Sm-K miracidia (Gp 4). The mean of the 2 single genotype (Gps 1 and 2) and 2 mixed-strain infections (Gps 3 and 4) are also shown. In (C) the mean cercarial shedding for the mixed and single genotype infections is shown over a period of 8 weeks.

infections than single-strain infections ($F_{1,133} = 5{\cdot}31$, $P = 0{\cdot}02$). Similarly, the rate of offspring production was reduced in mixed-strain infections ($F_{1,133} = 4{\cdot}61$, $P = 0{\cdot}03$). In contrast, there was no difference in the rate of egg mass production between the 2 groups $F_{1,133} = 0{\cdot}43$, $P = 0{\cdot}51$).

Within experimental groups, there was a significant relationship between increased parasite reproductive rate and reduced host survival ($F_{1,132} = 4{\cdot}47$, $P = 0{\cdot}04$), but not between parasite reproductive rate and host reproductive success ($F_{1,132} = 0{\cdot}001$, $P = 0{\cdot}97$). When parasite reproductive rate (the number of parasites present early in the infection) was controlled for, the difference between mixed and single-strain infections in host survival was lost ($F_{1,132} = 2{\cdot}75$, $P = 0{\cdot}10$). In contrast, mixed-strain infections were more virulent than single-strain infections in terms of reductions in host reproductive success for any given parasite reproductive rate ($F_{1,132} = 4{\cdot}87$, $P = 0{\cdot}03$).

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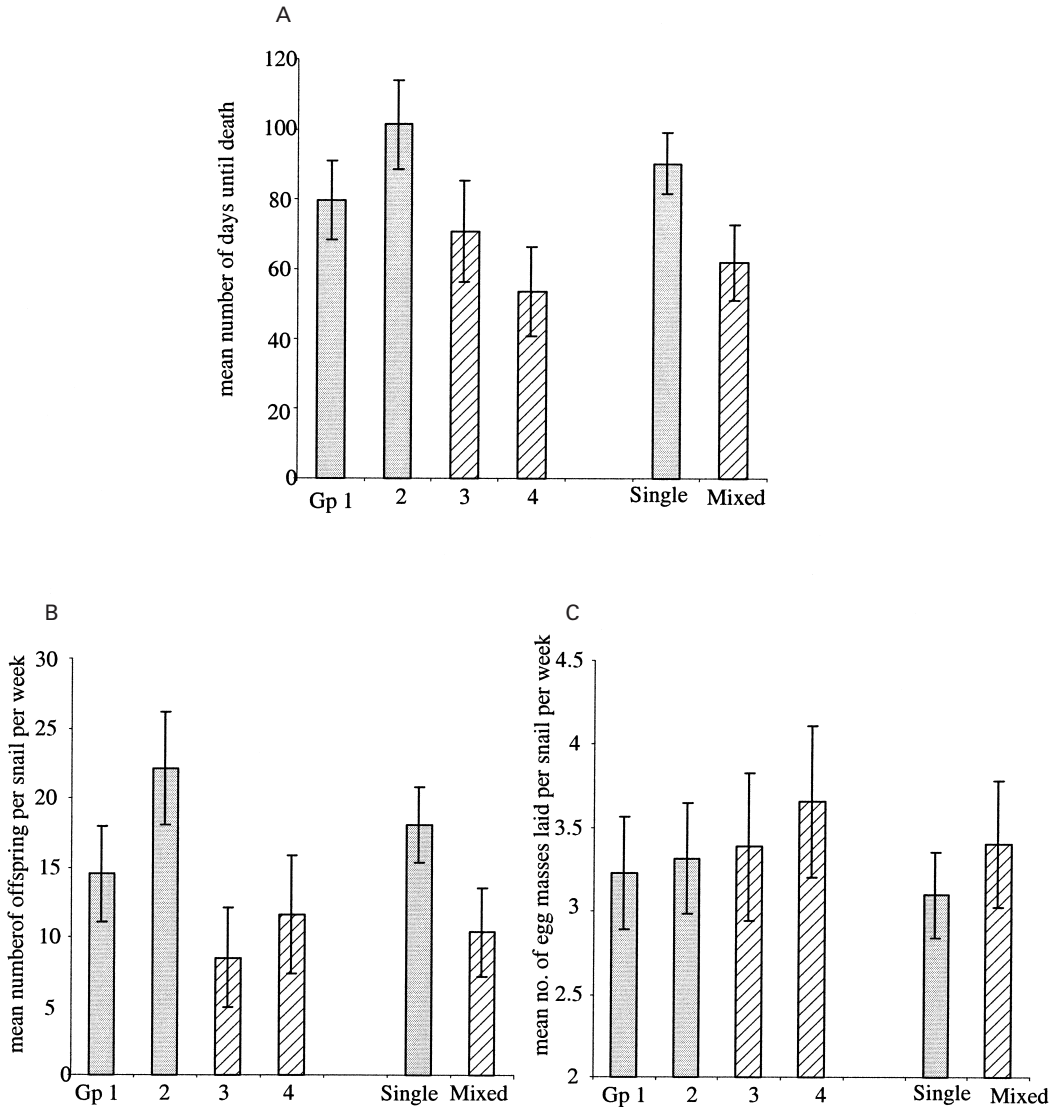

Fig. 2. Mean (A) number of days survived (B) rate of offspring production and (C) rate of egg production for groups of snails exposed to 10 Sm-PR miracidia (Gp 1), 10 Sm-K (Gp 2), 6 Sm-PR, 4 Sm-K (Gp 3) and 4 Sm-PR, 6 Sm-K miracidia (Gp 4). The mean of the 2 single genotype (Gps 1 and 2) and 2 mixed-strain infections (Gps 3 and 4) are also shown.

DISCUSSION

This study aimed to investigate the existence of conditional virulence levels in parasites in response to varying levels of parasite relatedness within their hosts. Consistent with the predictions of such a model, virulence was significantly higher in mixed strain than single-strain infections, both in terms of reductions in host survival and in host reproductive success. Moreover, the parasite reproductive rate was also increased in mixed-strain infections. There was no evidence of a difference between parasites in the infectivity to, or fecundity in, definitive hosts and thus we may assume that increased production of cercarial stages would result in increased transmission. However, the key test for the existence of a facultative virulence response is whether the higher virulence observed is associated with the increased parasite reproductive rate or whether virulence is higher in mixed-strain infections for any particular number of parasites, as would be expected if higher virulence results directly because of the genetic heterogeneity of the infection.

There was no evidence that snail reproductive success was correlated with parasite reproductive rate, and moreover, for any given reproductive rate, mixed-strain infections were still more virulent than single-strain infections. In contrast, we found that the high rate of parasite development and reproduction was associated with an increase in virulence as regards snail survival within experimental groups, and that controlling for parasite reproductive rate meant a loss of the difference between single and mixed-strain infections. Thus we infer that the genetic heterogeneity of the parasitic infections *per se* is costly to the host, and that this cost is manifested as a reduction in reproduction. Costs of defence in terms of reduced reproductive success in *B. glabrata*

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snails selected to be genetically resistant to *S. mansoni* infection have been previously reported, suggesting that host reproduction and defence may be coupled (Webster & Woolhouse, 1999). This cost of resistance was manifested as a reduction in the number of viable offspring successfully produced and not in the number of eggs masses or embryos laid and thus is consistent with the present study where the rate of offspring, though not egg, production was reduced in the mixed-strain group. Such an effect is unlikely to be due to an alteration in the hosts energy budget since egg production was not reduced, but possibly due to the presence of a limiting factor or resource, used in both defence and reproduction, that prevents eggs laid from developing into embryos or hatching into viable offspring (Cooper *et al*. 1994; Cousin *et al*. 1995; Webster & Woolhouse, 1999). Furthermore, host defence has been shown to be specific to the infecting parasite strain in this system (Webster & Woolhouse, 1998; Webster, 2001) and thus it is plausible that the increased virulence of mixed-strain infections results from a need to allocate more of the limiting factor or resource to defence rather than reproduction, in order to deal with multiple parasite genotypes. Host effects have also been reported to be determinants of virulence in mixed-strain infections of 2 other host–parasite systems (Taylor *et al*. 1998; Imhoof & Schmid-Hempel, 1998).

The genetically heterogeneous, or mixed-strain, infections also had a higher reproductive rate and this was associated with a reduction in host survival. This is consistent with, but not proof of, a conditional increase in parasite reproductive rate following detection of the presence of other parasite genotypes. Parasite reproductive rate is thought to be strongly influenced by the number of miracidia successfully establishing in the host snail (Theron, 1992). Thus we could envisage, perhaps, direct interaction by way of excretory–secretory (E–S) products between the establishing larvae (Sire, Rogon & Theron, 1998), consistent with theory for the evolution of virulence based on maximizing parasite fitness (Bull, 1994). However, the results are also consistent with a less effective host defence in mixed-strain infections allowing an increase in the parasite reproductive rate and resulting in decreased host survival. Similarly, the results could be explained by separate effects of genetic heterogeneity on parasite reproductive rate and virulence resulting in the statistical association seen. Conclusive evidence of a direct interaction between infecting genotypes could be investigated by exposing infecting parasites to E–S products of other parasite strains in the recently developed *B. glabrata in vitro* culture system (Coustau & Yoshino, 2000).

Though we observed an increase in early reproduction in mixed-strain infections in this study, overall parasite success was not increased, due to the reduced survival of this group. This would be consistent with selection for early reproduction because of competition between the two parasite strains and could, perhaps, reflect a limit to the parasite output from a snail before snail death occurs. A conditional virulence response is adaptive, if the level of competition experienced by parasites is variable (Taylor *et al*. 1998). Snail and schistosome populations in natural foci have an extremely patchy distribution. *Biomphalaria* populations are susceptible to extreme fluctuations in population size due to high levels of extinction through drought or molluscicide treatments and rapid population growth following heavy rainfall (Woolhouse & Chandiwana, 1989; Woolhouse, 1992). Populations also exhibit wide spatial and temporal genetic variation (Hoffman *et al*. 1998; Webster *et al*. 2001; Davies *et al*. 2001*b*) and are characterized by a dynamic variability in schistosome susceptibility (Michelson & DuBois, 1978; Manning, Woolhouse & Ndamba, 1995; Morand, Manning & Woolhouse, 1996). Infection of hosts by schistosome miracidia is strongly influenced by climatic factors, water flow and habitat type (e.g. Sturrock & Upatham, 1973; Prah & James, 1977). Thus it seems likely that competition between schistosome genotypes within hosts will indeed be highly variable. It is noted that host survival, but not reproduction will directly influence parasite success, since parasites are obligatory horizontally transmitted from snail hosts to long-lived definitive hosts.

In summary, mixed-strain infections are likely to be important in the evolution and maintenance of virulence in schistosome infections of snails. Both host mortality and parasite reproductive rate were increased in mixed-strain infections and this is the first report that is consistent with facultative virulence levels due to increasing parasite reproductive rate. Reductions in host reproductive success were also observed in mixed-strain infections but were thought to result directly from the genetic heterogeneity of the infection.

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