Inter-specific parasite competition: mixed infections of *Schistosoma mansoni* and *S. rodhaini* in the definitive host

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SUMMARY

Competition between parasite species has been predicted to be an important force shaping parasite and host ecology and evolution, although empirical data are often lacking. Using the *Mus musculus*-*Schistosoma mansoni* and *Schistosoma rodhaini* host-parasite systems we characterized mate choice and inter-specific competition between these two schistosome species. Simultaneous infections revealed species-specific mate preferences for both species as well as suggesting mating competition, with male *S. rodhaini* appearing dominant over male *S. mansoni*. *S. rodhaini* homologous pairs were also shown to have increased reproduction per paired female in the presence of a competitor in simultaneous infections. Overall total reproductive success was, however, similar between the two species under conditions of direct competition due to the greater initial infectivity of *S. mansoni* in comparison to *S. rodhaini*. Inter-specific competition was also implicated as increased parasite virulence to the host. The potential effects of such interactions on parasite and host ecology and evolution in nature are discussed.

Key words: *Schistosoma mansoni*, *Schistosoma rodhaini*, *Biomphalaria glabrata*, *Mus musculus*, inter-specific, competition, virulence.

INTRODUCTION

Understanding the interactions which occur when more than one species of parasite infects a host may give important insights into host-parasite systems with wide-ranging implications for our understanding of host-parasite compatibility, coevolution and perhaps even host and geographical range. Indeed, understanding of the complex population biology of multi-host and multi-pathogen systems is, in general, severely limited and has recently been declared as one of the major challenges to biomedical sciences in the 21st century (Woolhouse *et al*. 2001). Modes of interaction and inter-specific competition in the host-parasite system vary, for example, infection competition, direct interference competition, resource competition and, in certain cases, mating competition.

Schistosomes are the causative agent of schistosomiasis, a parasitic disease which is second only to malaria in terms of its socio-economic and public health importance, with approximately 600 million people exposed and 200 million infected at any time throughout the tropical world (King *et al*. 2005). Schistosomes are digenean trematodes with an indirect life-cycle involving sexual reproduction in a mammalian host and an asexual phase in a molluscan host. Schistosomes are highly unusual within the Trematoda in being dioecious, rather than hermaphroditic. Within definitive hosts, schistosomes have been shown to be polygamous (Tchuem Tchuente *et al*. 1995) with no physiological barriers to prevent inter-specific pairings (Cosgrove and Southgate, 2003*a*, *b*; Le Roux, 1954*a*; Southgate *et al*. 1995; Tchuem Tchuente *et al*. 1995; Webster *et al*. 1999). Such pairings can lead to hybridization or parthenogenesis. Generally, species that are closely related undergo sexual reproduction and hybridization while those that are more distantly related reproduce via parthenogenesis (Southgate *et al*. 1998). Specific mate recognition systems have been identified for certain schistosome combinations (e.g. *S. haematobium*/*S. intercalatum* and *S. bovis*/*S. curassoni*) but not for others (e.g. *S. mansoni*/*S. intercalatum*) (Southgate *et al*. 1998). Mating competition has also been indicated where males of one species compete for females of the other species (Cosgrove and Southgate, 2002; Southgate *et al*. 1995; Webster *et al*. 1999). In addition to mating competition, infection competition, direct interference competition and resource competition may also be predicted to occur between schistosome species within their definitive host although differentiation between these different modes of competition may be difficult.

S. mansoni and *S. rodhaini* are the most closely related species within the *S. mansoni* group (Lockyer

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et al. 2003; Morgan *et al*. 2003*b*). Both species utilize the genus *Biomphalaria* as their intermediate host and their definitive host ranges overlap in rodents (*S. rodhaini* is capable of infecting rodents, canines and very occasionally humans, whereas *S. mansoni* can infect both rodents and primates). The importance of the rodent host, common with *S. rodhaini*, in the transmission of *S. mansoni* is yet to be fully determined, although certain strains of *S. mansoni* adapted specifically for transmission through rodents have been identified in island populations (Theron, 1984*b*). Moreover, an increased role of rodents in maintaining *S. mansoni* transmission may be likely as a consequence of recent expansions of human schistosome control programme interventions, and indeed a recent study from Brazil has shown that rodents play an important role in maintaining low levels of *S. mansoni* transmission following otherwise successful control programmes in humans (Gentile *et al*. 2006). The geographical ranges of these two schistosome species overlap in several locations in Africa, including Kenya and the Democratic Republic of Congo, where mixed infections have been discovered in definitive hosts (Pitchford, 1977). The first report of a natural hybrid was from a *B. sudanica* snail from Lake Victoria, Tanzania (Morgan *et al*. 2003*a*) and a recent field collection in western Kenya has yielded more hybrids and worms with hybrid ancestors (Steinauer *et al*. 2006). Previous studies have shown that experimental hybridization crosses between the two species are viable in both directions. Hybrid miracidia have a greater snail host range than *S. rodhaini* (including *Biomphalaria alexandrina* to which only *S. mansoni* is usually infective) and hybrid cercariae show an additive chronobiological rhythm with both a diurnal peak (characteristic of *S. mansoni*) and a nocturnal peak (characteristic of *S. rodhaini*) (Le Roux, 1954*b*; Theron, 1989). The aim of this study was to use simultaneous and sequential laboratory exposures of *S. mansoni* and *S. rodhaini* to identify and characterize mate choice and potential mating competition, and identify any other form of inter-specific competition within the laboratory mouse model (*Mus musculus*). Such experimental model simulation should provide an important first step in elucidating the impact of inter-specific interactions on parasite and host ecology in natural habitats.

MATERIALS AND METHODS

Host-parasite maintenance

Parasites used were laboratory strains of *S. mansoni* and *S. rodhaini* which had been passaged routinely through *Biomphalaria glabrata* (a species well adapted for laboratory culture, strain NHM 2, originally collected in Egypt) and TO Harlan (UK) mice for >20 generations. All snails were maintained in the laboratory at 23–25 °C and subjected to a 12L (07:00–19:00) : 12D (19:00–07:00) light regime (full ultra-violet spectrum, 'Sun-glo' natural sunlight lamps). Snails were housed in groups in plastic tanks in 400 ml of Caledonian spring water (Iceland plc, UK) and changed weekly. All snails were fed *ad libitum* on fresh lettuce supplemented with fish food (Tetra Ltd) and chalk.

Individual size- (approximately 10 mm in diameter) and age-matched sexually mature (as measured by onset of egg laying) snails from 2 groups of 50 were exposed to either 6 miracidia of *S. rodhaini* or 6 miracidia of *S. mansoni* in 6 ml of spring water for 2 h. The 2 exposure groups were then maintained separately. At 6 weeks post-parasite exposure, snails exposed to *S. mansoni* were kept in darkness for 48 h and those snails exposed to *S. rodhaini* were kept in the light for 48 h. Cercariae were then induced to shed by placing snails exposed to *S. mansoni* under a bright (100 W) light source for 2 h and snails exposed to *S. rodhaini* in darkness for 2 h. Pooled cercariae from each group were then used to infect TO Harlan (UK) mice (of matched age and sex) in different cercarial combinations according to the experimental plan described below. The cercariae were used to infect mice, within an hour of shedding. Mice were infected by allowing the animal's feet to paddle freely for 30 min in 100 ml of spring water containing the cercarial dose.

Experimental design

Five single exposure groups (groups A–E) of 5 mice were exposed to cercariae, 1 to 110 *S. mansoni* cercariae, 1 to 220 *S. mansoni* cercariae, 1 to 110 *S. rodhaini* cercariae, 1 to 220 *S. rodhaini* cercariae and 1 to a mixed exposure of 110 cercariae of each species simultaneously (see Fig. 1). Two further multiple exposure groups of 10 mice were exposed individually to either 110 *S. mansoni* cercariae, 110 *S. rodhaini* cercariae at this time. At 2 weeks post-initial exposure, the mice from the 2 multiple species exposure experimental groups of 10 were split into 2 subgroups of 5 (groups F–I) and then re-exposed to either 110 cercariae of the same species as the first exposure (homologous sequential exposure), or 110 cercariae of the other species (heterologous sequential exposure). This experimental design, therefore, produced both simultaneous and sequential exposure to the 2 schistosome species, along with single species controls at 2 different doses. The *S. mansoni* and *S. rodhaini* strains used here have the same duration of pre-patent periods, at approximately 35 days, and therefore simultaneous infections allow the species to compete directly. In sequential infections it is assumed that the first species to infect the host (species 1) will form homospecific pairs, and the usual male-bias of schistosome infections (Liberatos, 1987), known to also be present in the

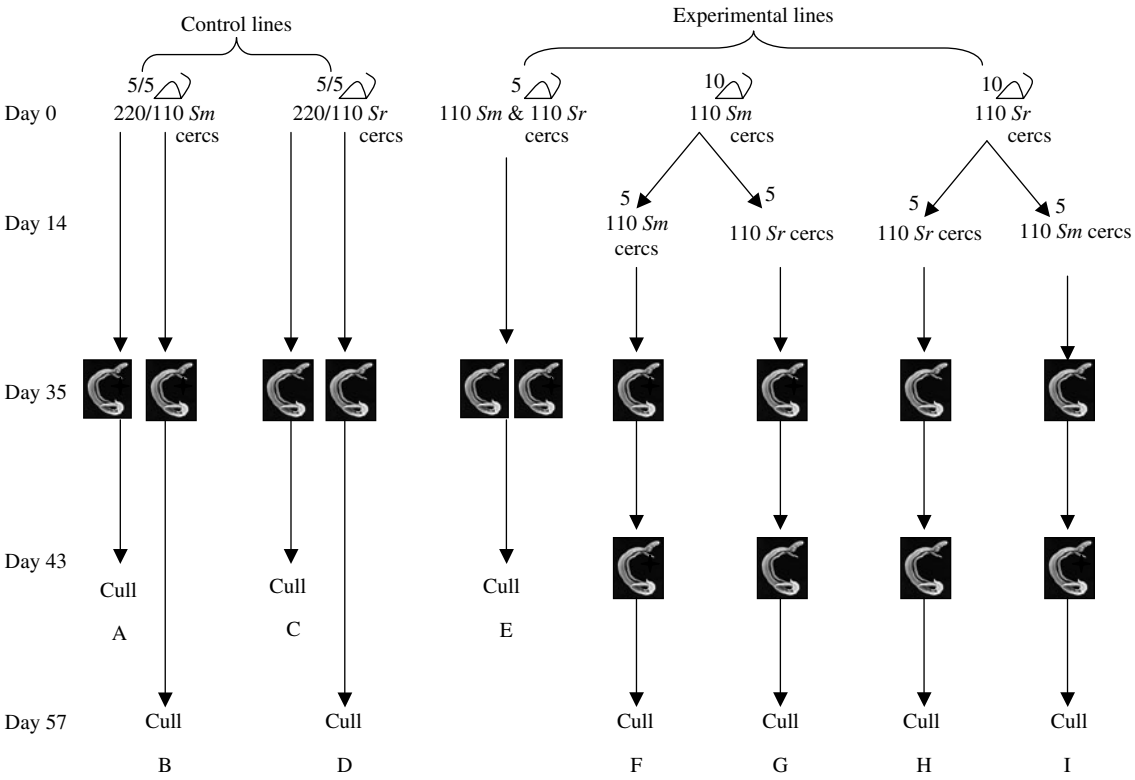

Fig. 1. Experimental design for exposure of *Schistosoma mansoni* (*Sm*) and *S. rodhaini* (*Sr*) to *Mus musculus* (▵). ■ = time of maturation of schistosome worms.

laboratory strains used here, should result in an excess of species 1 males, which may compete with species 2 males for species 2 females. If strongly dominant in the mating interaction species 2 males may also displace species 1 males from their partners.

Once all schistosome worms had matured, paired and had time to lay eggs, but before the mice showed any signs of illness, mice were euthanased with a rising concentration of carbon dioxide. Those mice exposed to 220 cercariae, in total, at the first time-point (groups A, C and E) were euthanased at 43 days post-exposure, those exposed to 110 cercariae at the first time-point (regardless of whether they were exposed or not at the second time-point – groups B, D, F–I) were euthanased at 57 days post-initial exposure (to allow maturation of any secondary infections).

Worm identification, measurement of parasite infectivity and virulence to the host and parasite reproductive success

Worms from each mouse were recovered by a modified hepatic perfusion technique (Smithers and Terry, 1965). They were immediately separated into pairs and single worms and the sex of the single worms was identified via visual observation through a dissecting microscope. Each worm pair was then placed in a cryopreservation tube (Nunc) and single worms of each sex were pooled and placed in separate cryopreservation tubes in which they were snap-frozen and stored in liquid nitrogen. Worm species identification was subsequently performed by phosphoglucomutase (PGM) enzyme isoelectric focussing on gels with a pH range 4–7 (Bremond *et al.* 1989). Worm pairing combinations were recorded. Parasite infectivity to the host was then calculated as the number of worms of each species recovered from an individual mouse divided by the cercarial dose of that species. Relative dominance in mating competition was tested between species by comparing the percentage of males mated at euthanasia for each species.

The liver and spleen were removed from each mouse and weighed. Parasite virulence to the host was calculated as the weight of the liver and spleen as a percentage of body weight, as the major pathological effect of both *S. mansoni* and *S. rodhaini* in definitive hosts results from granuloma formation around excreted eggs resulting in hepatosplenomegaly and it was not considered ethical to assay virulence as mortality in mice (Davies *et al.* 2001). Livers and spleens were prepared for miracidial hatching by macerating through a sieve into a sedimentation flask with 200 ml of saline. After 10 min, when the sediment had settled, the top 150 ml was removed using a suction pump. A further 200 ml of saline were added and the sedimentation process repeated reducing the sediment to 10 ml. The sediment was

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decanted into a Petri dish and the sedimentation flask was washed with 40 ml of spring water (Ulmer, 1970) to remove all remaining sediment. The sediment with spring water mixture was placed under a bright lamp for 30 min in order to stimulate miracidial hatching. The number of miracidia present in the final 50 ml was estimated from the mean number of miracidia in $10 \times 0{\cdot}5$ ml samples. Hatching of eggs from the liver and spleen rather than from the faeces is common practice in the mouse laboratory system as the mouse is not a natural host for any schistosome species and therefore eggs are not produced in the faeces in large enough numbers (if at all) for hatching before egg associated morbidity results in illness in the mice (for ethical reasons our mice are euthanased before any symptoms of illness occur). It must be acknowledged that results of parasite reproductive success from hatching eggs from the liver may not be representative of egg production in the faeces and could even be predicted to represent eggs from those worms which may have been less competitively successful in terms of resource competition (although there is no evidence for this as yet). There could also potentially be an egg hatching bias in terms of egg hatching success varying between species and hybrids; however, this is likely to reflect the actual fitness of these eggs. These issues are, however, standard limitations across this study and all similar studies, and important conclusions may still be drawn from comparisons of miracidial production as measured by egg hatching from the liver and spleen between experimental groups.

A subsample of 25 miracidia from each mouse exposed to both schistosome species was stored for later molecular identification. Miracidia were washed once in spring water and then picked up individually in 5 μl of water and pipetted on to Whatman FTA® cards. Genetic sequencing was used to identify miracidia to species and hybrids. Once molecular identification had been carried out (as described below), total parasite reproductive success was estimated by multiplying the proportion of miracidia identified to each genotype per mouse by the estimate of the total miracidial production from the liver and spleen of that mouse. Another parameter, parasite reproductive success per paired female, was also calculated by dividing total parasite reproductive success by the number of paired females present which could have produced that genotype of miracidia. One limitation of this final parameter is that worm pairings can only be recorded at the time of euthanasia and may not represent all the worm pairings that have been present for the duration of the infections.

Molecular analyses

This study takes advantage of recently developed techniques which allow the amplification of DNA from a single miracidium (Gower *et al*. 2007). For all miracidia stored, a region of the nuclear ribosomal second internal transcribed spacer (ITS2) was sequenced and compared to controls in order to identify the species of the miracidia or whether it was a hybrid. For those miracidia found to be hybrids further sequencing was carried out for a region of the mitochondrial cytochrome oxidase subunit 1 (CO1) in order to identify the hybrid's maternal genotype.

The DNA preparation was carried out on the Whatman card according to the manufacturer's protocol Whatman FTA® cards (Gower *et al*. 2007). Polymerase chain reaction (PCR) amplifications were performed on a Geneamp PCR System 2700 (Applied Biosystems). Amplification of the ITS region was performed using 'ITSSSCP2' (5′-3′ ACAAACCGTAGACCGAACC) and 'ITS2SSCP3' (5′-3′ CACGACGCACATTAAGTC) primers (Kane *et al*. 2002). Amplification of the CO1 region was performed using 'ASMIT 1' (5′-3′ TTTTTTGGGCATCTGAGGTTTAT) and 'ASMIT 2' (5′-3′ TAAAGAAAGAACATAATGAAAATG) primers (Stothard and Rollinson, 1997). Amplifications for both primer sets were performed separately in 50 μl reactions containing one plug of prepared Whatman FTA® card, 1 μl of each primer (ASMIT 1 and ASMIT 2 or ITSSSCP2 and ITS2SSCP3) at 50 pmolar concentration, 25 ml of HotStarTaq Master Mix (Qiagen) and 23 μl of pure water. Thermal cycling was performed under the following conditions for 'ITSSSCP2' and 'ITS2SSCP3': 15 min at 95 °C to activate the HotStarTaq DNA Polymerase and for denaturation, followed by 40 cycles of 1 min at each of 94 °C, 58 °C and 72 °C; followed by a final 10 min extension at 72 °C. For 'ASMIT 1' and 'ASMIT 2' thermal cycling was performed by: 15 min at 95 °C to activate the HotStarTaq DNA Polymerase and for denaturation, followed by 45 cycles of 15 sec at 94 °C, 30 sec at 40 °C and 45 sec at 72 °C; followed by a final 7 min extension at 72 °C. PCR products were purified in 96-well plates using a Millipore Montage PCR purification kit as per manufacturer's protocol.

Sequencing was carried out using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) in 10 ml reactions containing 3 ng of DNA for every 100 bps of PCR product, 1 pMol primer, 3 μl Big Dye dilution buffer ($2{\cdot}5\times$), 1 μl Big Dye reaction mix and xμl dH_20. Thermal cycling was performed under the following conditions: 5 min pre-denaturation at 96 °C without Big Dye reaction mix, pause of cycler whilst samples transferred to ice and Big Dye added. Then followed 25 cycles of 10 sec at 96 °C, 5 sec at 50 °C, 4 min at 60 °C. Samples were then run on a 3730 DNA Analyser sequencer (Applied Biosystems) for 2 h as per the manufacturer's run module. Sequences

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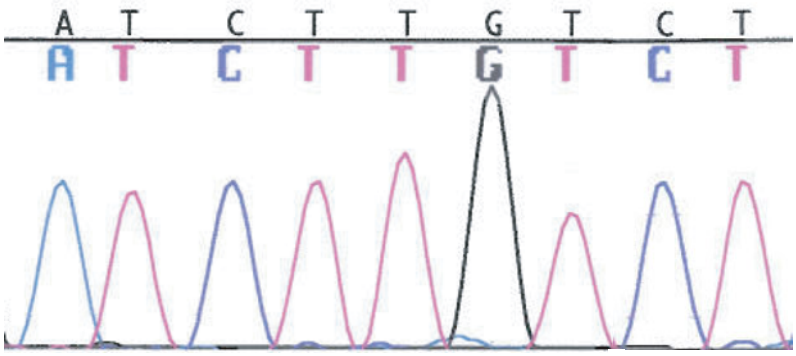
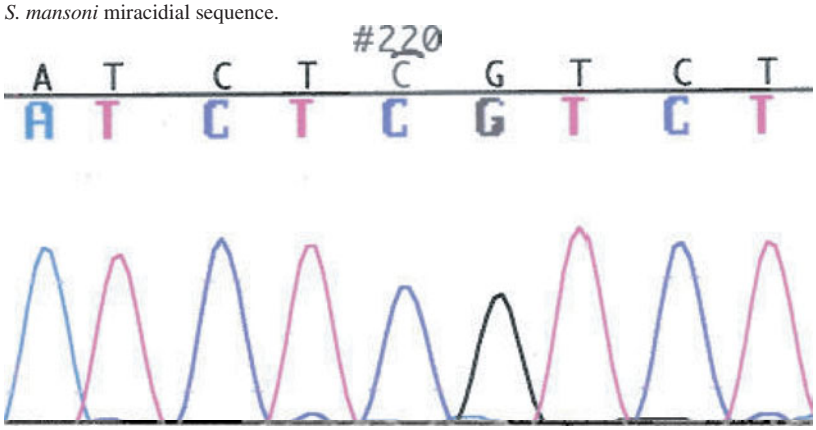
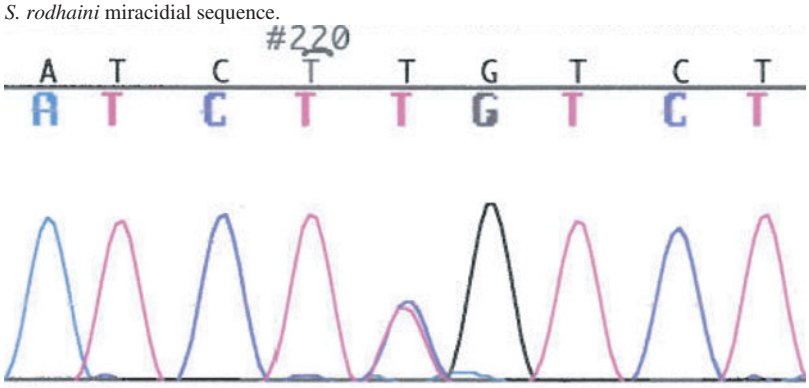


S. mansoni miracidial sequence.



S. rodhaini miracidial sequence.



Hybrid miracidial sequence. Both C and T peaks are clearly present at the base of difference between the 2 species, even though only T is called.

Fig. 2. Sequence data for a region of the nuclear ribosomal second internal transcribed spacer (ITS2) used to identify hybrid miracidia by the presence of a double peak (C and T peaks) at a locus of difference between *Schistosoma mansoni* and *S. rodhaini*.

were then imported into Sequencher vs. 3.1.1. (GeneCodes corp.) and compared to control sample sequences in order to identify the species of schistosome miracidium present in each sample. Hybrids were identified by the presence of a double peak (Webster *et al*. 2007) at the loci that differed between *S. mansoni* and *S. rodhaini* in the ITS sequences (one peak representing the base pair for *S. mansoni* and a different one for *S. rodhaini*) (see Fig. 2). True hybrids could be distinguished from mistaken amplification of 2 miracidia (1 of each species) by the presence of a double peak in their ITS sequence and only a single peak in their CO1 sequence (in comparison to a double peak in both sequences).

Statistical analyses

Statistical analyses were carried out separately on the 4 different dependent variables measured in this study; parasite infectivity, parasite virulence, quantity of worm pairings and parasite reproduction, as the complexity of the study and inherent differences in measuring these variables meant that different combinations of experimental groups needed to be analysed in order to investigate these different

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variables. In addition to this, since infectivity is highly sensitive to environmental conditions and because egg production and parasite virulence will depend on the length of the infection, all comparisons were made between experimental groups that were infected at the same time-point and for the same duration.

For these 4 dependent variables the 2 main hypotheses being tested were firstly whether the variables differed for infections of *S. mansoni* and *S. rodhaini* and secondly whether the variables differed as a result of mixed species infections in comparison to single species infections. Differences in parasite infectivity were tested for between species (between experimental groups B and D and within group E) and between individual species infections and mixed species infections (between group B and E for *S. mansoni* and between group D and E for *S. rodhaini*). Differences in parasite virulence were tested for between species (between groups A and C and between groups B and D). Differences in parasite virulence were also tested for between individual and mixed species infections for simultaneous infections (between groups E and A for *S. mansoni* and groups E and C for *S. rodhaini*) and for sequential infections (between groups I and H for *S. rodhaini* and G and F for *S. mansoni*). Differences in numbers of worm pairings and percentages of males paired were tested for within groups. Simultaneous mating choice and competition was investigated within group E and sequential mating choice and competition were investigated within groups G and I. Differences in parasite reproductive success per paired female were tested between species (between groups A and C and groups B and D) and between simultaneous mixed species infections and single species infections (at both doses) (between groups A and E and B and E for *S. mansoni* and between groups C and E and D and E for *S. rodhaini*). Differences in reproductive success per paired female of worm pairs within the sequential heterologous exposure groups were also tested for (groups G and I). Total reproductive success gave similar trends for analyses between species as reproductive success per paired female (these results are therefore not included in the Results section). For group E, however, results for total reproductive success differed from those for reproductive success per paired female and are therefore included in the Results section.

All tests carried out between groups used 2 sample *t*-tests or analysis of variance (in a generalised linear modelling procedure) in Minitab Release 14 (Minitab Inc., State College, PA). Dependent variables were transformed as necessary to meet the generalised model's assumptions of homogeneity of variance and normality of error. Where variables could not be sufficiently transformed non-parametric Wilcoxon and Mann Whitney tests were used. A Bonferroni-corrected *P*-value of 0·013 (adjusted for the number of tests performed on each biologically distinct subset of the data) was considered significant to control for potential type-1 errors.

RESULTS

Parasite infectivity

S. mansoni was shown (Table 1) to be significantly more infective than *S. rodhaini* in both the simultaneous exposure experimental group (group E) and control groups exposed to 110 cercariae of each species (groups B and D) ($F_{1,19} = 24{\cdot}78$, $P < 0{\cdot}01$). There was no difference in the infectivity for the 2 parasite species under conditions of simultaneous parasite exposure (group E) in comparison to control groups exposed at the same individual dose (110 cercariae) of each species individually (groups B and D) ($F_{1,19} = 0{\cdot}22$, $P = 0{\cdot}645$).

Parasite virulence

There was no significant difference (Table 1) between the virulence of each parasite species at either cercarial infection dose of control group ($F_{1,19} = 0{\cdot}07$, $P = 0{\cdot}793$) or between the virulence of the mixed infection experimental group (group E) (220 cercariae exposed in total) and the control lines of either species exposed to 220 cercariae (groups A and C) ($F_{1,14} = 0{\cdot}23$, $P = 0{\cdot}642$). Heterologous sequential exposures resulted in higher virulence than that produced by homologous exposures. Significantly higher virulence was observed for *S. rodhaini* then *S. mansoni* heterologous sequential exposure (group I) in comparison to *S. rodhaini* homologous sequential exposure (group H) ($t = 3{\cdot}64$, D.F. $= 9$ $P = 0{\cdot}008$) and there was a non-significant higher virulence of *S. mansoni* then *S. rodhaini* heterologous sequential exposure (group G) in comparison to *S. mansoni* homologous sequential exposure (group F) ($t = 0{\cdot}88$, D.F. $= 9$, $P = 0{\cdot}41$).

Worm pairings

For control groups A and C all but a very small number of worms were paired if a mate was available (see Table 2).

For experimental group E homospecific pairings of both species and heterospecific pairings in both directions were all present, as predicted. These results from worm pairings for those mice exposed to both *S. mansoni* and *S. rodhaini* simultaneously (group E) indicated the presence of a species-specific mate preference system, with males of both species making a greater number of homospecific pairings than heterospecific; however, the presence of several heterospecific pairings in both directions indicate that this preference may not be particularly strong,

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Table 1. Parasite infectivity, sex-ratios, virulence and reproductive success in the definitive host for all control and experimental lines of *S. mansoni* (*Sm*) and *S. rodhaini* (*Sr*)

Control/ experimental line (cercarial dose)	Parasite	Parasite infectivity (no. of worms recovered/ cercarial dose) (±S.E.)	Parasite sex ratios (mean no. of ♂ and ♀ worms recovered per mouse) (±S.E.)	Parasite virulence (mouse liver and spleen weight as % of body weight) (±S.E.)	Parasite reproductive success (estimate of the no. of miracidia produced per paired female by each worm pairing combination) (±S.E.)	Total parasite reproductive success (estimate of the no. of miracidia produced by each worm pairing combination) (±S.E.)
220 *Sm* (group A)	*Sm*	0·194±0·02	27♂±5·3 15·4♀±1·2	4·902±0·2	37·0±10·2	508·86±137·8
110 *Sm* (group B)	*Sm*	0·190±0·01	16·4♂±2·4 8♀±2·2	8·436±1·2	163·1±32·9	1382±468·4
220 *Sr* (group C)	*Sr*	0·202±0·03	26·4♂±5·9 17·8♀±2·4	5·440±0·6	34·4±12·9	542·52±173·2
110 *Sr* (group D)	*Sr*	0·094±0·02	5♂±1 5·2♀±0·97	7·504±0·6	10·0±3·8	45·6±18·7
110 *Sm* & 110 *Sr* (group E)	*Sm* *Sr* *Sm* ♂/*Sr* ♀ *Sr* ♂/*Sm* ♀	0·190±0·03 0·072±0·02	16·6♂±2·6 10·8♀±2·7	4·950±0·3	23·6±9·91 125·4±45·1 52·0±25·4 13·7±10·9	134·9±47·4 228·1±92·3 154·4±43 26·9±34·8
110 *Sm* then 110 *Sm* (group F)	*Sm*	0·204±0·03	23·8♂±2·6 21♀±5·8	7·012±0·4	89·1±14·8	1780·8±679·7
110 *Sm* then 110 *Sr* (group G)	*Sm* *Sr* *Sm* ♂/*Sr* ♀ *Sr* ♂/*Sm* ♀	0·064±0·02	9·8♂±2·3 13♀±2·9	7·514±0·4	146·8±49·4 35·4±28·7 21·6±19·7 74·8±21·3	720·6±197·4 51·3±41·4 21·6±46·2 81·6±196·4
110 *Sr* then 110 *Sr* (group H)	*Sr*	0·078±0·01	13♂±2·9 4·2♀±1	7·804±0·4	29·7±12·6	112·8±38·2
110 *Sr* then 110 *Sm* (group I)	*Sr* *Sm* *Sm* ♂/*Sr* ♀ *Sr* ♂/*Sm* ♀	0·176±0·02	26·8♂±1·5 15·4♀±2·2	9·744±0·3	11·58±3·1 12·34±5·2 0 46·3±26·3	69·6±31·9 84·9±21·3 0 644·2±82

allowing the possibility of mating competition. The median difference between *S. mansoni* male homospecific and heterospecific pairings was estimated to be 2·5 (95% CI: 1–5) using the Wilcoxon test and between *S. rodhaini* male homospecific and heterospecific pairings was estimated to be 1 (95% CI: −1–2). Due to variability in sex ratios and species ratios of worms established between experimental mice mating competition was investigated by comparing the percentage of males of each species paired (whether homospecifically or heterospecifically). Mating competition was indicated with 74% of *S. rodhaini* worms paired in comparison to only 52% of *S. mansoni* worms. This indicates that in terms of direct mating competition *S. rodhaini* males might be more successful at pairing than *S. mansoni* males. It must, however, also be noted that despite *S. rodhaini's* apparent mating dominance a greater mean number of worm pairings per mouse was achieved by *S. mansoni* males than *S. rodhaini* males due to the greater infectivity of *S. mansoni* ($t=3·30$, $P=0·03$).

In the heterologous sequential infection group (group G) where mice were exposed to *S. mansoni* at the first time-point and *S. rodhaini* at the second time-point the initial *S. mansoni* infection produced more female worms than male worms. This was contrary to predictions that the usual male-bias of schistosome infections would result in an excess of *S. mansoni* males, which could compete with *S. rodhaini* males for *S. rodhaini* females and instead resulted in all *S. mansoni* male worms being paired to *S. mansoni* female worms by the time the *S. rodhaini* worms matured. This is evident in Table 2 as the significantly higher number of unpaired female *S. mansoni* worms in comparison to unpaired male worms of the same species for this group (w=15, D.F.=9, $P=0·01$). No *S. mansoni* males swapped partners in order to pair with *S. rodhaini* females once they had matured. *S. rodhaini* males were therefore able to freely pair with either their own *S. rodhaini* females or the excess of single *S. mansoni* females.

In the heterologous sequential infection group where mice were exposed to *S. rodhaini* at the

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Table 2. Worm pairing combination frequencies (worm or w) and estimated numbers of miracidia produced per paired female worm (mir.) for all mice from groups A, C, E, G and I for *Schistosoma mansoni* (*Sm*) and *S. rodhaini* (*Sr*)

Experimental group (cercarial dose)	Mouse	*Sm* ♂/*Sm* ♀		*Sr* ♂/*Sr* ♀		*Sm* ♂/*Sr* ♀		*Sr* ♂/*Sm* ♀		*Sm* ♂	*Sr* ♂	*Sm* ♀	*Sr* ♀
		worm	mir.	worm	mir.	worm	mir.	worm	mir.	w	w	w	w
220 *Sm* (group A)	1	15	62·4	n/a		n/a		n/a		0	n/a	3	n/a
	2	13	36·9	n/a		n/a		n/a		0	n/a	2	n/a
	3	18	10·7	n/a		n/a		n/a		16	n/a	0	n/a
	4	14	18	n/a		n/a		n/a		22	n/a	0	n/a
	5	12	57	n/a		n/a		n/a		25	n/a	0	n/a
220 *Sr* (group C)	1	n/a		25	37·0	n/a		n/a		n/a	9	n/a	0
	2	n/a		12	83	n/a		n/a		n/a	3	n/a	1
	3	n/a		10	24	n/a		n/a		n/a	1	n/a	2
	4	n/a		20	18	n/a		n/a		n/a	1	n/a	0
	5	n/a		19	10·1	n/a		n/a		n/a	2	n/a	0
110 *Sm* & 110 *Sr* (group E)	1	8	33·9	2	83·2	3	111	2	56·9	6	1	0	1
	2	7	13·3	3	70·5	6	35·3	1	0	7	0	0	0
	3	4	56·8	2	289	2	113·5	0	0	9	2	0	0
	4	6	13·9	1	151·2	2	0	2	8·82	7	1	0	0
	5	1	0	1	33·1	0	0	0	2·88	6	1	0	0
110 *Sm* then 110 *Sr* (group G)	1	5	126	1	149	0	0	2	0	0	0	2	0
	2	4	214	3	0	0	0	1	0	1	2	6	3
	3	11	83·5	2	0	0	0	2	0	0	0	4	0
	4	4	296·1	5	20	0	100·1	4	100·1	0	0	3	0
	5	0	14·4	0	7·7	0	7·68	0	7·68	1	1	2	1
110 *Sr* then 110 *Sm* (group I)	1	4	29·6	6	13·5	1	0	3	143·5	7	4	0	0
	2	0	0	9	2·5	0	0	1	1·4	13	5	0	0
	3	9	17·1	8	19·2	3	0	3	59·2	5	4	0	0
	4	2	3·6	7	14·1	2	0	2	7·2	6	5	0	0
	5	6	11·4	8	8·6	2	0	1	20·3	6	2	0	0

first time-point and *S. mansoni* at the second time-point (group I), both infections showed a strong male bias. This is evident in Table 2 by the presence of a large number of unpaired *S. mansoni* and *S. rodhaini* male worms and the absence of any unpaired female worms. *S. rodhaini* unpaired male worms were able to compete successfully with maturing *S. mansoni* males in order to pair with a proportion of their females (32% of *S. mansoni* females were paired by *S. rodhaini* males). However, *S. mansoni* males were also able to take a small proportion of paired females away from *S. rodhaini* to pair with them (17·4% of *S. rodhaini* females were paired by *S. mansoni* males). This was, however, less than the proportion of *S. mansoni* females paired by *S. rodhaini* males. Indeed *S. rodhaini* appeared dominant overall with a greater percentage (71%) of its males paired than *S. mansoni* (44%) and a greater mean number of males worms per mouse paired than *S. rodhaini* ($t=2·02$, $P=0·114$).

Parasite reproductive success per paired female

Table 1 and Table 2 also show the parasite reproductive success per paired female for worm pairs produced from all control and experimental groups. *S. mansoni* and *S. rodhaini* have a very similar level of parasite reproductive success per paired female from cercariae exposed at a high dose (groups A and C) and *S. mansoni* has significantly higher parasite reproductive success per paired female than *S. rodhaini* from cercariae exposed at a low dose (groups B and D) ($t=6·49$, D.F. $=9$, $P<0·001$). In simultaneous infections of the 2 species (group E) *S. rodhaini* worms achieved significantly greater reproductive success per paired female in comparison to either of their single exposure control lines (groups C and D) ($F_{2,14}=12·8$, $P=0·001$). In contrast, *S. mansoni* worms achieved a similar level of reproductive success per paired female to their high dose exposure control line. This resulted in *S. rodhaini* worms in homologous pairings exhibiting higher reproductive success per paired female than *S. mansoni* worms in simultaneous infections (group E) ($t=2·71$, D.F. $=9$, $P=0·04$). All worm pairings involving male *S. rodhaini* worms (homologous *S. rodhaini* pairings and heterologous pairings with an *S. rodhaini* male) also exhibited a non-significant higher reproductive success per paired female than for all male *S. mansoni* worm pairings (homologous *S. mansoni* pairings and heterologous pairings with a *S. mansoni* male) ($t=1·0$, D.F. $=12$, $P=0·338$). Heterologous worm pairs with an *S. rodhaini* female

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produce a non-significant trend for a larger number of miracidia than those with an *S. mansoni* female (group E) ($t=2{\cdot}01$, D.F. $=5$, $P=0{\cdot}18$).

In sequential infections the first parasite exposure had 2 weeks longer to produce eggs in comparison to the second parasite exposure. In accordance with this, homologous sequential infections of both species at a dose of 110 cercariae resulted in an intermediate level of reproductive success in comparison to that of their 110 cercarial and 220 cercarial dose control lines. In the heterologous sequential infection group where mice were exposed to *S. mansoni* at the first time-point and *S. rodhaini* at the second time-point (group G), *S. mansoni* had greater reproductive success than *S. rodhaini* ($t=2{\cdot}27$, D.F. $=9$, $P=0{\cdot}06$). This is in accordance with the fact that *S. mansoni* is the first species to infect the group. The heterologous worm pairs with *S. mansoni* females also had a greater reproductive success than those with *S. rodhaini* females ($t=2{\cdot}53$, D.F. $=9$, $P=0{\cdot}05$). In the heterologous sequential infection line where mice were exposed to *S. rodhaini* at the first time-point and *S. mansoni* at the second time-point (group I) there was no significant difference between the parasite reproductive success for *S. rodhaini* and *S. mansoni* ($t=0{\cdot}4$, D.F. $=9$, $P=0{\cdot}70$). Heterologous pairs with an *S. mansoni* female showed a non-significant higher reproductive success than that of either species' homologous pairs ($F_{1,13}=2{\cdot}93$, $P=0{\cdot}113$). No heterologous pairs were formed with *S. rodhaini* females.

Total parasite reproductive success

Table 1 shows the total parasite reproductive success for all groups. Results presented here are only those for group E where they differ from parasite reproductive success per paired female. In simultaneous infections of the 2 species (group E) *S. rodhaini* achieved intermediate total reproductive success in comparison to its 2 single exposure control lines (groups C and D) and was not significantly different to either of them (group C $t=1{\cdot}6$, D.F. $=6$, $P=0{\cdot}16$, group D $t=1{\cdot}94$, D.F. $=4$, $P=0{\cdot}125$). *S. mansoni* exhibited lower total parasite reproductive success in group E in comparison to both of its single exposure control groups (groups A and B) ($F_{2,14}=5{\cdot}1$, $P=0{\cdot}025$). In contrast to reproductive success per paired female the total parasite reproductive success of *S. rodhaini* homologous pairings for group E was not significantly greater than the total reproductive success for *S. mansoni* homologous pairings ($t=0{\cdot}89$, D.F. $=6$, $P=0{\cdot}409$) (although there was a non-significant trend). In addition to this there was no difference between total reproductive success for all male *S. mansoni* worm pairings (homologous *S. mansoni* pairings and heterologous pairings with a *S. mansoni* male) in comparison to total reproductive success for all male *S. rodhaini* worm pairings (homologous *S. rodhaini* pairings and heterologous pairings with a *S. rodhaini* male) for group E ($t=0{\cdot}25$, D.F. $=16$, $P=0{\cdot}804$).

DISCUSSION

Inter-specific interactions and competition have been identified between these 2 parasite species in both simultaneous and sequential infections, as well as differences in the interactions of the 2 species *S. mansoni* and *S. rodhaini* with their mouse definitive host. The molecular identification of single miracidium enabled us to elucidate the effects of such interactions on parasite reproductive success directly for the first time. The use of simultaneous and sequential exposures in this study allowed us to investigate several potential scenarios for interactions which may occur between *S. mansoni* and *S. rodhaini* within their definitive host in nature. For interpretation of these results, however, the potential limitations of the experimental model in terms of the necessary assumption made that miracidial production from the liver is representative of parasite reproductive success (as discussed in the methodology), must be acknowledged. All conclusions made about parasite reproductive success are based on this assumption.

Simultaneous infections of the 2 parasite species indicated that both species have species-specific mate preference systems with preponderance for homospecific mate pairings over heterospecific. These will decrease the level of genetic interaction between the different parasite species co-infecting the same host in nature. The presence of several heterospecific pairings does, however, indicate that either such species-specific mate preference systems may not be particularly strong or, in the case of limited numbers of unpaired worms, that worms of each species would rather mate with a non-preferred mate rather than not mate at all, therefore providing the opportunity for mating competition. In simultaneous infections of the 2 parasite species, mating competition was indicated, with *S. rodhaini* appearing dominant over *S. mansoni* (although due to the greater infectivity of *S. mansoni* it achieved a greater overall mean number of mate pairings per mouse). Due to the unusual female bias of *S. mansoni* worms in the heterologous sequential exposure, where *S. mansoni* was the first species to be exposed, there were no unpaired *S. mansoni* males available to compete with the secondary *S. rodhaini* infection males for their females. Unfortunately, this meant that we were unable to determine any effects of mating competition for this group. In heterologous sequential infections where *S. rodhaini* was the first species to infect, however, *S. rodhaini* again appeared to be dominant in the mating interaction.

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In nature, *S. rodhaini* is primarily a parasite of rodents, whereas *S. mansoni* is primarily a parasite of primates. It may therefore have been predicted that *S. rodhaini* should have been the better adapted of these 2 species here, in terms of infectivity to and reproduction within, this experimental mouse definitive host. However, in our low exposure-dose control groups (B and D) and simultaneous exposure group (group E) *S. mansoni* was found to have higher infectivity and (for groups B and D) reproductive success per paired female than *S. rodhaini*. The *S. mansoni* strain used in these experiments had been passaged through laboratory mice for several generations, and hence was also under, albeit more recent, potential selection towards adaptation through a murine definitive host. In simultaneous infections of the 2 parasite species, however, in addition to showing competitive mating dominance, *S. rodhaini* homologous pairs had increased reproduction per paired female in the presence of the competitor, with a significant increase in comparison to control groups. One possibility is that this was a result of a facultative increase of *S. rodhaini* reproduction in the presence of a competitor, indeed such conditional alteration of parasite behaviour in the presence of a mixed infection has previously been shown for different strains of *S. mansoni* within the intermediate snail host and may indicate that in both the intermediate and definitive host there is a trade-off for the parasite between reproduction and life-span (Gower and Webster, 2005). Alternatively, increased *S. rodhaini* reproduction in the presence of a competitor may be due to a reduced host response to *S. rodhaini* in mixed species infections in comparison to single species infections, rather than a parasitic facultative change. The increase observed in the current study resulted in *S. rodhaini* miracidial production being higher than that of *S. mansoni* per paired female, indicating that *S. rodhaini* worm pairs are competitively dominant to *S. mansoni* in some way in addition to mating competition. Specifically, determining the mechanisms of this additional method of competition was beyond the scope of this study, although we would suggest that resource competition may be a likely explanation. Heterologous pairings from simultaneous infections with an *S. rodhaini* female worm were also shown to produce more miracidia per paired female than those with an *S. mansoni* female. This may indicate that it is the female worm which controls the level of reproduction of a pair. Indeed, in heterologous sequential infections where mice were exposed to *S. mansoni* at the first time-point and *S. rodhaini* at the second time-point the heterologous worm pairings with the more mature *S. mansoni* female worms have greater reproductive success per paired female than those with less mature *S. rodhaini* female worms. These results therefore show that although male schistosomes have traditionally been seen as the dominant sex in mating competition, facultative changes in the females' reproductive output could have a large effect on the relative proportions of genotypes that pass into the next generation.

Total parasite reproductive success is a composite variable which measures the overall reproductive success of each parasite species. It is affected by the infectivity of the parasites, their mating competitive ability and reproduction per paired female. It therefore enables the overall reproductive success based on a matched cercarial infective dose to be determined for each species under conditions of competition. It is very interesting therefore that as a result of the fact that *S. mansoni* is significantly more infective than *S. rodhaini* in simultaneous competition (group E) and *S. rodhaini* is putatively dominant in terms of mating competitive ability and significantly dominant in reproductive success per paired female (group E) that these different factors combine to result in there being no significant difference in total parasite reproductive success between these 2 species under conditions of direct competition, as a result of a matched cercarial infective dose.

Competition also had implications for parasite virulence in this system, indicating that inter-specific parasite competition may also affect the host species. Heterologous sequential infections produced significantly increased parasite virulence to the host in comparison to homologous sequential exposure of the parasites to the mouse host. These differences in virulence could putatively be due to resource or mating competition between the parasites within the mouse host (with inter-specific competition being potentially stronger and more detrimental to the host than intra-specific competition), which may therefore reduce the length of time over which inter-specific competition may occur.

These results may have implications for our understanding of interactions between parasite species in nature. The only factor in our investigation implicated in reducing hybridization between these 2 species in nature is the increased virulence to the host caused by sequential mixed species infections. It has been suggested that despite the high level of genetic compatibility between these 2 schistosome species, differences in their definitive hosts and shedding times have helped to maintain their separation in nature (Wright and Southgate, 1976; Theron and Combes, 1995). In certain areas, however, *S. mansoni* has been shown to be specifically adapted to transmission through its rodent host in terms of its chronobiology (Theron, 1984*a*) and a recent study has also shown the temporal difference in shedding peak between *S. mansoni* and *S. rodhaini* in Western Kenya to be only 3 h (Steinauer *et al*. 2006). Indeed, also, potential changes in human lifestyles associated with increased sanitization and disease awareness in African countries may result in an increased passage

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of *S. mansoni* through its rodent definitive host, common with *S. rodhaini*, and in areas of geographical overlap greater inter-specific interaction would be predicted. From our experimental results we would predict that in areas where these 2 schistosome species regularly interact that their species distinction may be reduced or even lost, with unpredictable results for the ecology of these species with infectivity and virulence traits to both intermediate and definitive hosts from both species likely to be expressed. An important next step therefore is the use of the molecular methods such as those from this study to determine the degree of mixing that naturally occurs in populations. Indeed, if hybridization is common as predicted, further investigation into the definitive host range and virulence of such hybrids would be necessary in order to determine their potential impact on definitive host populations.

In terms of parasite inter-specific interactions in general this experimental study has revealed the complexity of such interactions. The intricacies of inter-specific competition have been highlighted with one species dominant in infectivity to the host but the other species putatively dominant in both 'worm pairing ability' in simultaneous infections and also in terms of its females' reproductive output from worm pairings (which may be another facet of resource competition). Inter-specific competition (of unknown mechanism) has also been implicated in increasing virulence of sequential mixed species infections to the host. Indeed, as the total parasite reproduction results show, neither parasite appears to have overall dominance in these complex interactions, rather one species is dominant in certain aspects of competition and the second in others. These complex results therefore highlight the need for such detailed experiments in order to determine the interactions occurring and potential impacts of these on parasite and host ecology and evolution where parasite species are found within the same hosts in nature.

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