Mating interactions of schistosomes: biological and epidemiological consequences

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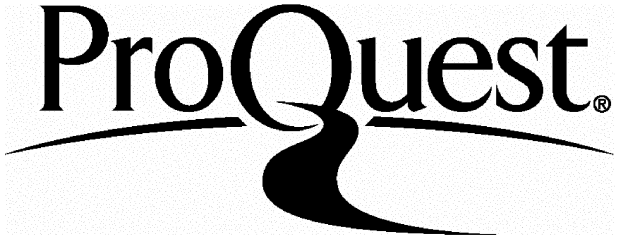

ABSTRACT

The formation of mating pairs by males and females of *Schistosoma* spp. in the liver or hepatic portal system of their definitive host is a requirement for male and female growth and sexual maturation, but is not always species-specific. Previous studies have shown that in mixed infections of two or more schistosome species, heterospecific pairs will form, leading either to hybridisation or parthenogenesis depending on the phylogenetic distance between the species. In certain species combinations, mating appears to be random: in others, particularly those involving species from different evolutionary lineages, there is a preponderance of homospecific pairs, demonstrating the existence of a specific mate preference system.

Experiments were designed to study mating interactions between different combinations of species in mixed infections in mice and hamsters. Data from infections with *S. mansoni* and *S. margrebowiei*; *S. intercalatum* (Zaire strain) and *S. mansoni*, and *S. bovis* and *S. margrebowiei*, revealed a preference for homospecific rather than heterospecific partners. A number of homospecific and heterospecific multiple-worm pairs were also obtained. Reinforcement of assortative mating by the pre-zygotic mating barrier of heterologous immunity was indicated by worm return data from these 3 mating models. The existence of mating competition between the species was demonstrated. Males of all the species were able to actively compete for paired females by pulling them away from their partners, thus effecting a change of mate. In mixed infections of *S. margrebowiei* and *S. mansoni*, neither species appeared to be competitively dominant to the other, and the data suggest that where this is the case in sequential infections, the most successful species in terms of worm return and pairing ability will be the first species to infect the host. *S. mansoni* appeared to be the more competitive species in mixed infections with *S. intercalatum* (Zaire strain), but to a lesser extent than in previously-studied mixed infections with the Lower Guinea strain of *S. intercalatum*. This suggests that the Zaire strain of *S. intercalatum* may be more competitive than the Lower Guinea strain, highlighting an important difference between these "cryptic species". There was some indication that *S. bovis* might be more competitive than *S. margrebowiei* in mixed infections of these two species.

Data from hamsters infected initially with *S. haematobium*, and subsequently with *S. intercalatum* (Lower Guinea) demonstrate the competitive dominance of *S. haematobium* over *S. intercalatum* (Lower Guinea), and suggest that *S. intercalatum* (Lower Guinea) is unlikely to become established in areas where *S. haematobium* is already present. The competitive dominance of *S. haematobium* males over *S. mansoni* males is demonstrated by data from hamsters infected initially with both sexes of *S. haematobium* and subsequently with male *S. mansoni*. A number of homosexual male pairs were obtained from mixed infections of *S. haematobium*/ *S. intercalatum* (Lower Guinea) and *S. haematobium*/ *S. mansoni*, corroborating previous findings that the clasping of objects by males is neither sex- nor species-specific.

An attempt to identify a female-specific RAPD (Randomly Amplified Polymorphic DNA) marker for *S. haematobium* resulted in the cloning and sequencing of an apparently female-specific PCR product of approximately 700 base-pairs in length. However, subsequent testing of the clone using specific primers revealed that, whilst it is present in smaller quantities in males than females, it is not specific to females, and is therefore of limited practical use as a sex-specific diagnostic tool.

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INTRODUCTION

Schistosome blood flukes are the causative organisms of schistosomiasis, a parasitic disease which is endemic in 74 countries of the tropics and subtropics (Fig 1.1), and threatens approximately one-tenth of the world's population (600 million) with infection (World Health Organization website). With more than 200 million people infected worldwide and up to 500 000 deaths from schistosomiasis each year, it is the second most prevalent tropical disease of man after malaria. It is also a disease of considerable veterinary importance, with 165 million cattle infected worldwide, in addition to other domestic livestock such as sheep, goats and buffalo (De Bont & Vercruysse, 1998). Schistosomiasis also infects a variety of wild mammals, including some species of rodents and primates.

Schistosomiasis is transmitted by freshwater snails, and mammalian infection occurs through contact with infested water. Consequently, prevalence of the disease is highest in developing countries among rural communities engaged in agriculture and freshwater fishing, although rural-urban migration is increasingly introducing it into peri-urban areas. Environmental changes linked to water resource development (dams, irrigation schemes) have also facilitated the spread of the disease to previously low- or non-endemic areas, particularly in sub-Saharan Africa. Often schistosomiasis is acquired in childhood, and despite the fact that adults acquire a degree of resistance to re-infection, the disease may persist well into adult life, with increasingly debilitating symptoms (von Lichtenberg, 1987). Depending on the intensity and duration of infection, the disease spectrum ranges from subtle morbidity (caused by anaemia, for example) to mortality resulting from bladder cancer and renal failure associated with urinary schistosomiasis, or from fibrosis and portal hypertension associated with intestinal schistosomiasis.

Currently, the main WHO objective for schistosomiasis control is to reduce the amount of disease rather than to halt transmission entirely. A co-ordinated strategy is required, encompassing a number of different disciplines. Control of morbidity with drug treatment is considered by the WHO as a feasible and effective strategy. Praziquantel is the drug of choice, as it is relatively cheap, and is effective against all forms of schistosomiasis with

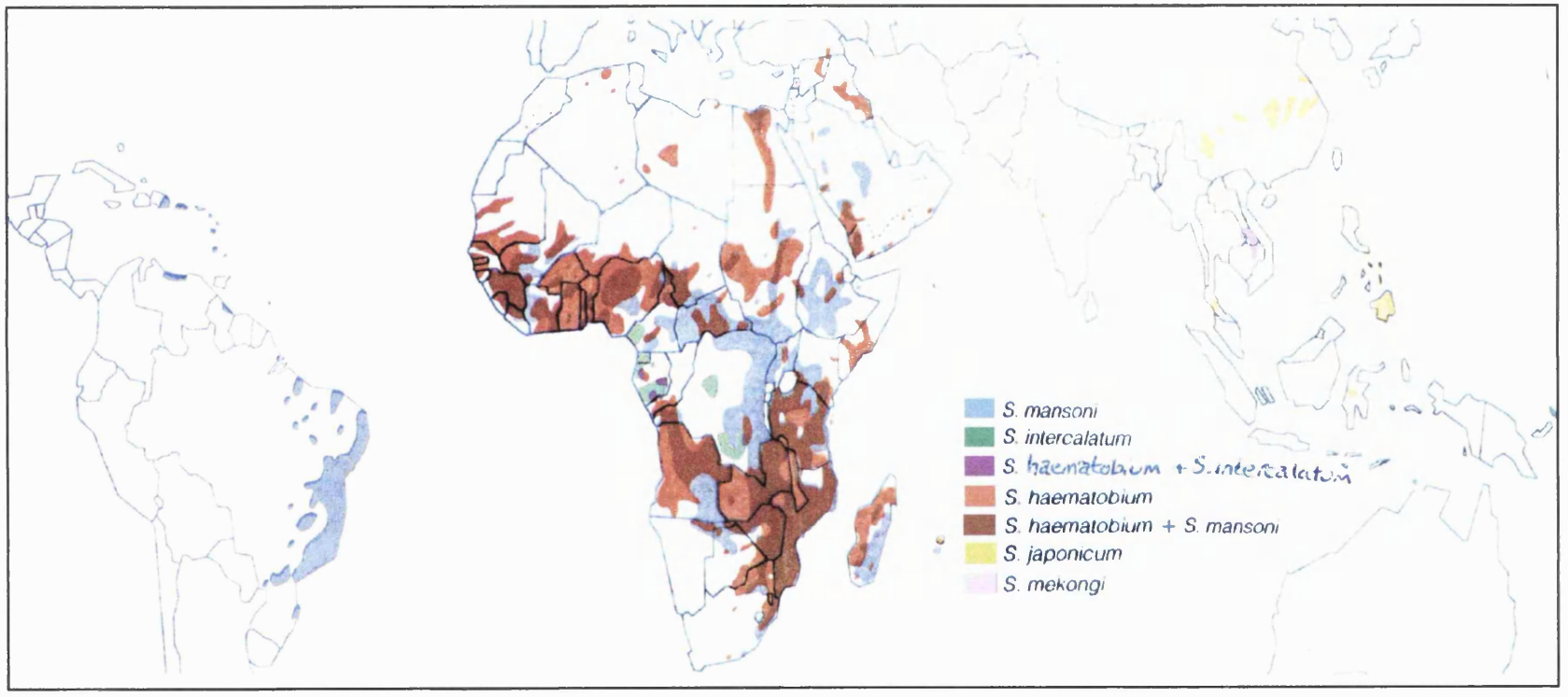


Fig 1.1. The global distribution of schistosomiasis
Source: WHO

few side effects. Oxamniquine may also be used to treat intestinal schistosomiasis caused by *Schistosoma mansoni*. The risk of developing severely diseased organs is diminished and even reversed in children which have received treatment, and a reduction in the overall number of cases of schistosomiasis can be maintained in some areas for up to 5 years without further intervention. Development of an effective vaccine against schistosomiasis is another potential method of control, progress towards which has been slow over the past few decades. However, in recent years a number of protein/peptide schistosome antigens have been identified as potential vaccine candidates, and have the advantage of being able to be produced by recombinant DNA technology (Bergquist, 1998). One of these, glutathione-S-transferase (GST), is of particular interest due to its reduction of schistosome fecundity (Capron *et al.,* 1995), and is scheduled for clinical trials. In another approach, molluscicides may be used to control the snails which transmit schistosomiasis, and are most cost-effective when applied focally to epidemiologically important water contact sites. However, effective mollusciciding requires repeated long-term application by highly skilled persons (Mott, 1987). Health education and the provision of sanitation and safe water are other important control strategies prioritised by the WHO.

Schistosoma spp. have a complex life cycle, involving both definitive (vertebrate) and intermediate (freshwater/amphibious snail) hosts (Fig 1.2).

Eggs from infected definitive hosts are voided into freshwater via the faeces or urine. The egg membrane is permeable to water, the influx of which causes the egg to swell and the membrane to rupture (Kusel, 1970), whereupon the first free-living larval stage, the miracidium, is released (Fig 1.3).

The miracidium is specialized for the sole purpose of finding and penetrating a suitable snail host (Basch, 1991) and typically varies between 150 and 180μm in length and 70 and 80μm in width. Tiers of ciliated epidermal cells cover the body to provide motility, and are interspersed with non-ciliated epidermal ridges containing sensory receptors and excretory ducts (Southgate & Knowles, 1977). The secretory ducts of the penetration glands open in an apical papilla at the anterior end, which consists of a highly folded non-ciliated epidermal membrane rich in ciliated sensory organelles (Jourdane & Théron, 1987). Embryonic germinal cells are contained within the posterior end of the body.

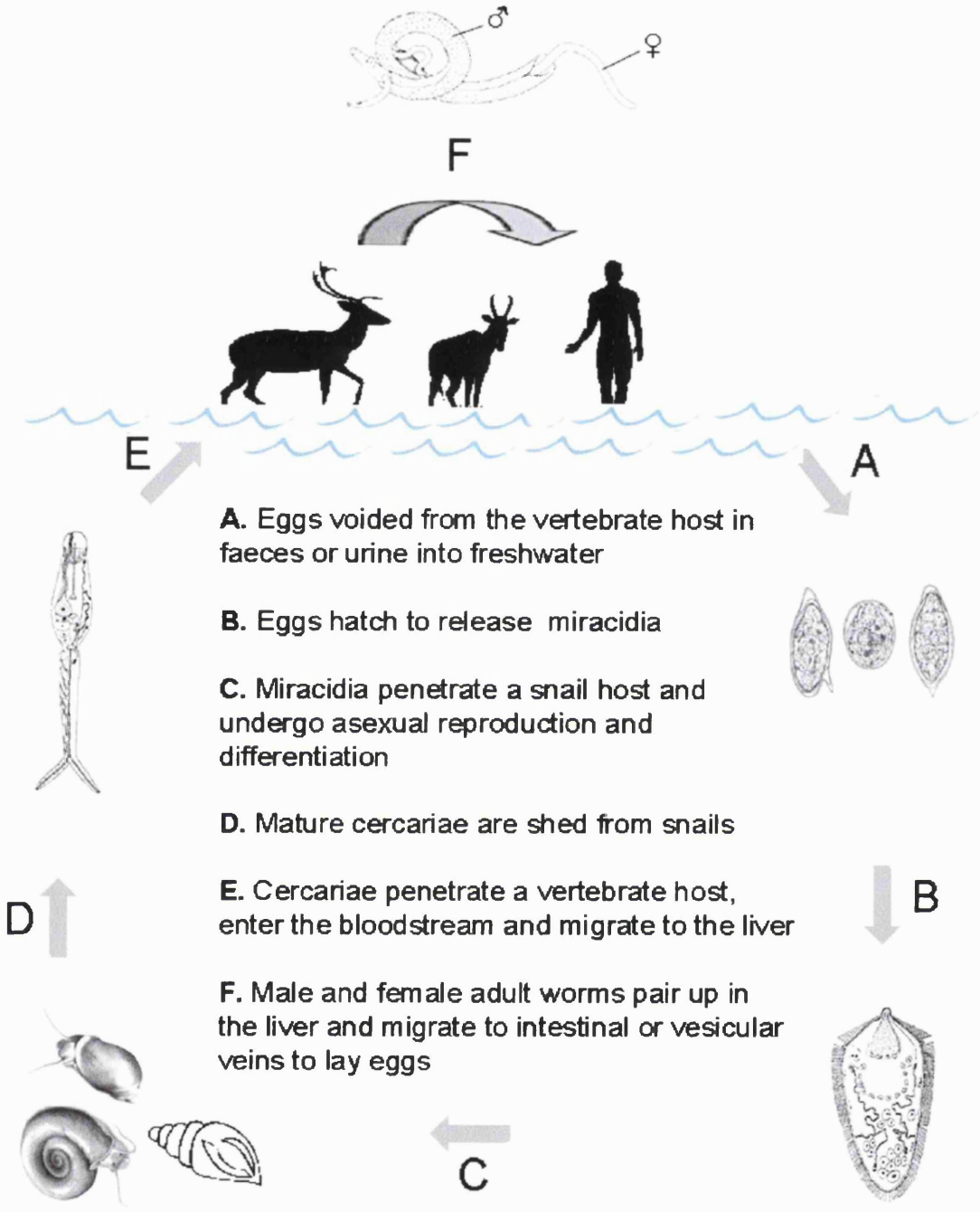


Fig 1.2. The life cycle of *Schistosoma* spp. Drawings not to scale.

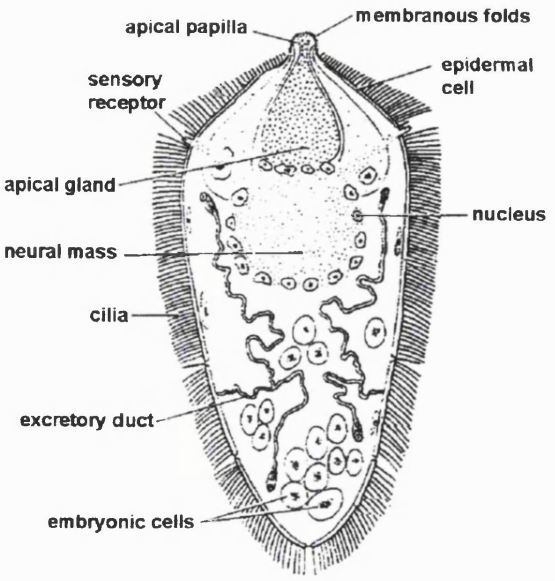


Fig 1.3. Diagrammatic representation of a miracidium. Adapted from Southgate & Knowles (1977).

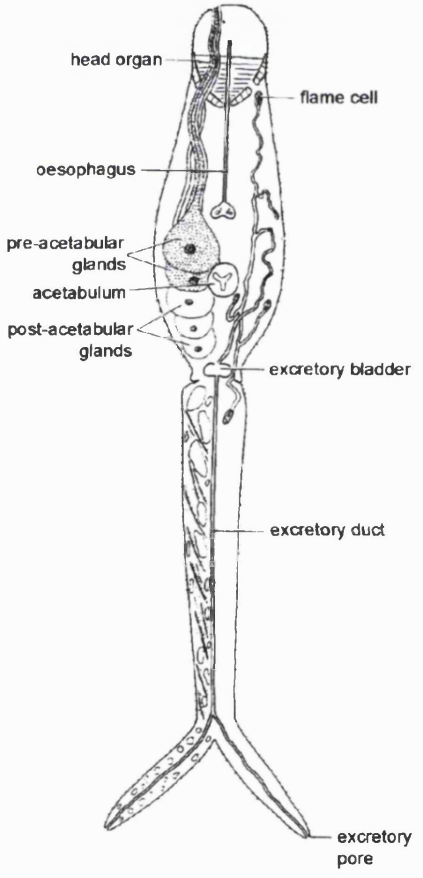


Fig 1.4. Diagrammatic representation of a cercaria. Adapted from Southgate & Knowles (1977).

Miracidia can swim at rates of up to 2mm s^{-1} but are short-lived (8-12h for *S. mansoni* (Chernin & Dunavan, 1962)) and therefore have little time in which to locate and penetrate a suitable snail host. Each schistosome species is restricted by the specific range of snail hosts in which it can successfully develop, with the degree of snail-schistosome compatibility determined to a large extent by the relative influence of parasite infectivity genes and host resistance genes (Bayne & Loker, 1987). The behavioural patterns of miracidia are closely adapted to the ecology of those snails with which they are compatible. For example, miracidia of *S. mansoni* are positively phototactic and negatively geotactic to maximise their chances of penetrating their *Biomphalaria* snail hosts which spend most of their time at the water surface attached to floating vegetation (Chernin & Dunavan, 1962).

On reaching the snail's preferred niche, the miracidia swim in long sweeping lines until they encounter various chemical attractants emitted by the snail, which Chernin (1970) termed "miraxone". Miraxone consists of a variety of compounds including certain amino and fatty acids in snail excretory products, in addition to ammonia and amines secreted by the snail foot (Mason & Fripp, 1977; Etges *et al.,* 1975). These stimuli are not species-specific (Chernin, 1970), and therefore miracidia may be diverted by non-target snails if these are present in sufficient numbers at transmission sites (Moné & Combes, 1986). Having reached the potential snail host, miracidia explore the snail surface for a suitable entry site, which is usually in the snail foot (Jourdane & Théron, 1987). The membrane folds of the apical papilla act as a sucker fixing the miracidium in place, and penetration takes place by the mechanical action of the apical papilla boring into the snail's epithelium, with the possible involvement of histolytic enzymes secreted by the penetration glands (Wadji, 1966).

Once a miracidium has entered a compatible snail, its development into a mother sporocyst commences with the loss of the ciliated plates, sensory papillae, and musculature. The ciliated epidermis is replaced by a syncytial tegument covered with microvilli, representing the change in larval function from motility to nutrient uptake (Jourdane & Théron, 1987). In the first round of asexual multiplication within the snail host, the germinal cells inside the sporocyst enlarge, divide, and eventually differentiate into secondary, daughter sporocysts, which are released when the tegument of the mother sporocyst ruptures. Daughter sporocysts migrate through the snail tissue to the hepatopancreas, where germinal

cells within them start to divide, giving rise to morulas (balls of cells bounded by an outer epithelium) in the first stage of cercariogenesis. Differentiation into the second free-living larval form, the cercaria, then occurs. In several important schistosome species such as *S. mansoni*, *S. haematobium*, *S. bovis*, *S. intercalatum* and *S. mattheei*, a second phase of sporocyst production takes place (Jourdane & Théron, 1987).

Cercarial production within snail hosts occurs daily (with the exception of *S. japonicum*, where cercarial production may cease and restart after a period) and may continue for several months, often ending with the death of the snail. The number of cercariae produced by a single snail is dependent on several factors which include the species of schistosome; the degree of compatibility between them and the health and age of the snail (Webbe, 1965).

Mature cercariae escape from the mother sporocyst but remain within the mantle cavity of the snail until an external trigger causes them to be shed. The exact daily pattern of cercarial emission is unique to each schistosome species, adapted to coincide with the period of maximum water contact of the definitive host, but it usually occurs during the photophase. Emission of cercariae follows a circadian rhythm (one emergence peak a day), with the exception of *S. margrebowiei*, which has a first emission peak early in the morning, and a second late in the afternoon (Pitchford & Dutoit, 1976). Photoperiod appears to be the most important factor influencing the emission of cercariae, with inversion of the photoperiod causing an inversion of the emission rhythm (Glaudel & Etges, 1973). In addition, the rhythm becomes desynchronized in the absence of photoperiod at constant temperature (Valle *et al.*, 1973). The quality of light is not so important: shedding will take place in infrared or ultraviolet light as well as in white light, and a low wattage bulb is sufficient to stimulate shedding in the laboratory.

Cercariae are specialised for location and penetration of a suitable definitive host, and have a body with a muscular head organ, five sets of penetration glands (two pre-acetabular and three post-acetabular – Fig 1.4) and a prominent ventral sucker (the acetabulum), together with a muscular tail which ends in two furcae, and enables the cercaria to swim. A glycocalyx coat over the surface allows the cercariae to control osmotic pressure in a freshwater environment. Glycogen stored in both the body and tail form the main energy

reserve, and, as cercariae do not feed, its quantity significantly influences the cercarial longevity (Wilson, 1987). This longevity is decreased in warmer waters where cercarial movements are rapid (Lawson & Wilson, 1980), or in fast flowing waters where cercariae must actively swim to maintain their position. Newly-emerged cercariae alternate upward swimming with passive downward sinking, which serves to maintain the cercariae at the water surface. As glycogen reserves become depleted, downward sinking prevails and cercariae progress to the bottom (Carter, 1978). Certain environmental stimuli, which might indicate the presence of a vertebrate host, alter cercarial behaviour. For example, mixing of water causes an increase in the time spent swimming, and skin-derived chemicals stimulate continuous swimming with frequent reversals of direction (Wilson, 1987). On contact with the vertebrate host, a cercaria attaches to the skin by means of the acetabulum, and penetrates the epidermis by means of histolytic enzymes secreted by the penetration glands, and muscular burrowing through the host tissue.

Upon penetration, a cercaria sheds both its tail and the glycocalyx coat, replacing the latter with a tegument composed of two closely opposed lipid bilayers. This transformed cercaria is known as a schistosomulum *in vivo*. The process of transformation takes less than one hour to complete (Cousin *et al.,* 1981). Profound biochemical changes also occur, as the schistosomulum changes from aerobic to anaerobic respiration. The schistosomula travel through the subcutaneous tissue to their route of exit from the skin into the peripheral vessels of the blood and lymphatic system. They are carried to the lungs where they remain for several days, and commence rhythmic extensions and contractions of their length which facilitate their migration through blood vessels (Wilson *et al.,* 1978; Crabtree & Wilson, 1980). Considerable loss of parasite numbers occurs in the lungs, largely through schisosomula rupturing thin-walled pulmonary capillaries and becoming trapped in alveoli (Wilson, 1987). These losses are of great significance to the final parasite load, as schistosomes do not multiply within their definitive host.

On leaving the lungs, surviving schistosomula are distributed round the body in the circulatory system, and may make several circuits of the vasculature before being trapped either in the liver (the desired location), or in a site unfavourable for development (Wheater & Wilson, 1979; Wilson & Coulson, 1986).

In the liver, where the parasite feeds on red blood cells, growth and maturation commences. Schistosomes are dioecious, and adult males and females pair up together. This stimulates growth and sexual maturation in the females, and enables mating to occur. The much larger male worm clasps the threadlike female in a groove on its ventral surface called the gynaecophoric canal (Figs 1.5 and 1.6), and paired worms migrate to the egg-laying sites: the mesenteric or rectal veins for most species of schistosome, or the veins of the vesical venous plexus for *S. haematobium*. Here oviposition commences, with the production of eggs bearing a terminal or lateral spine (or no spine at all) depending on the species. The eggs vary in shape and size according to the species, ranging from approximately 62μm x 53μm for *S. edwardiense* to approximately 456μm x 66μm for *S. nasale*. Eggs are non-embryonated when first laid, developing further in the host tissue. Numbers of eggs produced each day by a single worm pair may reach 300 for *S. mansoni*, and 3500 for *S. japonicium* (Webbe, 1982). Egg-laying continues throughout the long life-span of schistosomes in their vertebrate hosts: 3.5-12 years on average in humans, but more than 30 years in some cases (Vermund *et al.*, 1983).

Mature eggs migrate across host tissue layers to reach the lumen of the bowel or bladder by mechanisms which are not fully understood, but may involve both host factors (blood pressure, peristalsis) and egg factors (proteolytic secretions from the egg, the egg spine if present) (Jourdane & Théron, 1987). As the eggs of the schistosomes penetrate the walls of the veins and the small intestine or urinary bladder, they cause a significant amount of damage to the tissues. The tissues haemorrhage, often leading to the appearance of blood in the urine (known as "haematuria": a common sign of urinary schistosomiasis) or faeces. Once in the bowel or bladder lumen, eggs are voided with the faeces or urine to continue the schistosome life cycle. Only an estimated 35% of eggs escape their hosts in this way: the remainder die within host tissues or are swept back in the bloodstream and become lodged in the liver or spleen (Cheever, 1969).

The time taken from cercarial penetration to the first appearance of eggs in stools or urine is called the pre-patent period, and varies according to species: for *S. mansoni* this is a period of approximately 35 days, whereas for *S. haematobium* the pre-patent period is approximately 60 days (Loker, 1983).

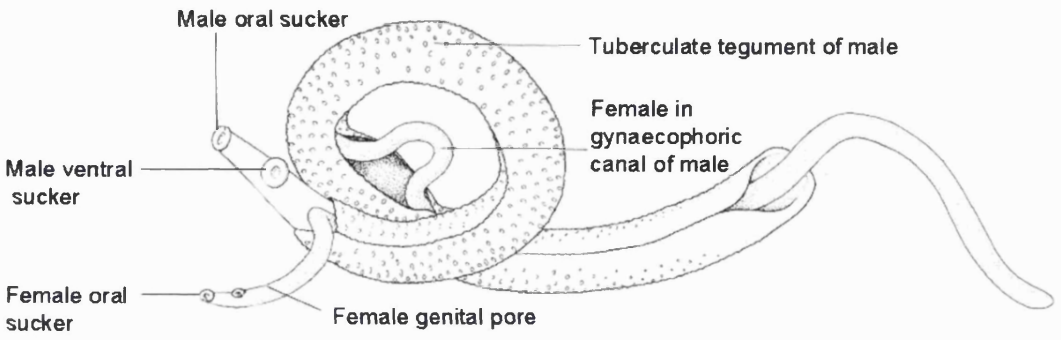


Fig 1.5. Diagrammatic representation of a worm pair. Adapted from Whitfield (1993).

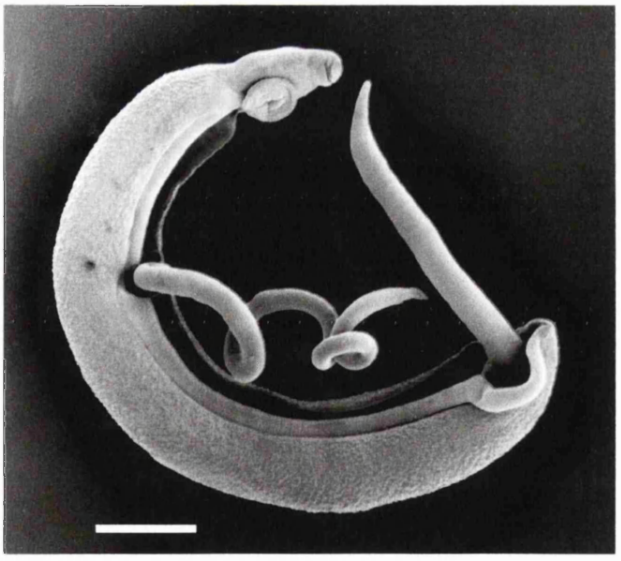


Fig 1.6. Scanning Electron Micrograph of a worm pair. Scale bar = 500µm.

The progressively debilitating pathology of chronic schistosomiasis largely results from host immune reactions to the eggs deposited in tissues, and not to the adult worms, which are largely refractory to immune attack due to their ability to acquire host antigens on their surface and masquerade as "self" (von Lichtenberg, 1987).

Dermatitis (or "swimmers itch") may result from the initial invasion of skin by cercariae, but this is usually an acquired antibody response elicited when non-compatible schistosomes have attempted penetration (Basch, 1991). In permissive hosts, there is little or no reaction to invasion, and in the early stages of the disease, individuals may have only minor symptoms or none at all. However, a few individuals may suffer "acute schistosomiasis" 1 to 2 months after initial cercarial exposure: a rare self-terminating phase lasting 2-8 weeks which is characterised by fevers and enlargement of the spleen and liver (von Lichtenberg, 1987). Whether there has been an acute phase or not, the early stages of schistosomiasis are dominated by diffuse cellular infiltrates and proliferation of cells of the immune system. This proliferation partly results from the systemic host response to antigens produced by cercariae, adult worms and eggs, and partly from local granulomatous responses to egg deposition. In the granulomatous response, soluble egg antigen (SEA) secreted by eggs causes a cellular aggregate composed of lymphocytes, macrophages and eosinophils to form around them. This leads to inflammation and damage of host tissue which gradually becomes fibrotic through collagen deposition, and may obstruct local blood flow in the liver and spleen. However, the proliferative responses of early infection are largely modulated in chronic schistosomiasis, prolonging the survival of host and parasite. Thus the pathology of severe, chronic schistosomiasis is largely due to the formation of advanced fibrovascular lesions at sites of massive egg deposition rather than to scattered egg granulomas alone. These lesions obstruct critical vessels and can lead to portal fibrosis and hypertension with congestive hepatosplenomegaly. They respond poorly to treatment in contrast to the granulomas of the early disease stages. In infections with *S. haematobium*, such lesions are capable of obstructing the urinary tract or disrupting its motor coordination, and may lead to renal failure, or a specific type of bladder cancer, the incidence of which in certain parts of Africa is 32 times higher than that of simple bladder cancer in the USA (WHO factsheet, 1996).

Worm burdens sustained by infected individuals vary greatly, and depend, for example, on the intensity and rate of cercarial exposure and the degree of individual resistance (Basch, 1991). One study of human post-mortem data in Brazil revealed that many asymptomatic infected subjects harboured less than 10 *S. mansoni* worm pairs, but 10% of subjects harboured between 160-320 pairs, whilst one child with a heavier fatal infection harboured more than 1600 worm pairs (Cheever, 1968). Given that it is impossible to count worms in live subjects in the field, it is common practice to measure the intensity of a schistosome infection by quantitative egg counts (Wilkins, 1987).

The taxonomic classification of *Schistosoma* spp. is given in Table 1.1. Schistosomes are trematodes belonging to the sub-class Digenea and the family Schistosomatidae. All members of sub-family Schistsomatinae are parasitic in mammals, but only schistosomes of the genus *Schistosoma* are associated with man.

Currently, 20 species of *Schistosoma* are recognized, of which 5 are considered important parasites of man: *S. mansoni*, *S. haematobium*, *S. intercalatum*, *S. japonicum* and *S. mekongi*.

Table 1.1. Classification of *Schistosoma* spp.

PHYLUM Platyhelminthes Schneider, 1873
CLASS Trematoda Rudolphi, 1808
SUB-CLASS Digenea
FAMILY Schistosomatidae Looss, 1899
SUB-FAMILY Schistosomatinae Stiles & Hassall, 1926
GENUS *Schistosoma* Weinland, 1858

The genus *Schistosoma* has been subdivided into 4 groups which mostly reflect the range of snail genera utilised by the different species in nature, and loosely reflect the egg morphology of each species (Rollinson & Southgate, 1987).

The *S. haematobium* group includes the medically important species *S. haematobium* and *S. intercalatum*, as well as *S. bovis*, *S. margrebowiei*, and *S. mattheei* which parasitize domestic livestock (and occasionally man in the case of *S. mattheei*). *S. haematobium*-group species are found in Africa and some adjacent regions, and their eggs bear a terminal spine (Fig 1.7, *a, c, e* and *f*). They are mainly transmitted by snails of the genus *Bulinus* (Fig 1.8 *b*), although *S. bovis* is also transmitted by the planorbid snail *Planorbarius metidjensis* in the Iberian peninsular.

Of the *S. mansoni* group, only *S. mansoni* itself parasitizes man. This species is widespread in Africa, the Carribbean, and South America. *S. mansoni*-group species are transmitted by snails of the genus *Biomphalaria* (Fig 1.8 *a*) and their eggs have a lateral spine (Fig 1.7 *b*).

Members of the *S. indicum* group occur mainly in parts of the Indian sub-continent and parts of south-east Asia and are parasitic in artiodactyls. They are usually transmitted by snails of the genus *Indoplanorbis*, although *S. incognitum* is transmitted by snails of the genera *Radix* and *Lymnaea*, and is also able to infect rodents and carnivores.

Species of the *S. japonicum* group are distributed throughout south-east Asia, China, Japan and the Philippines. *S. japonicum* infects humans in addition to a wide range of other mammalian definitive hosts, and is a true zoonosis. *S. sinesium* and *S. mekongi* are transmitted by snails of the genus *Neotricula*, whereas *S. japonicum* is transmitted by the polytypic, amphibious snails of the genus *Oncomelania*. Whilst eggs of *S. japonicum* (Fig 1.7 *d*) and *S. mekongi* have round, minutely spined or spineless eggs, those of *S. sinensium* bear a prominent lateral spine, similar to *S. mansoni* eggs .

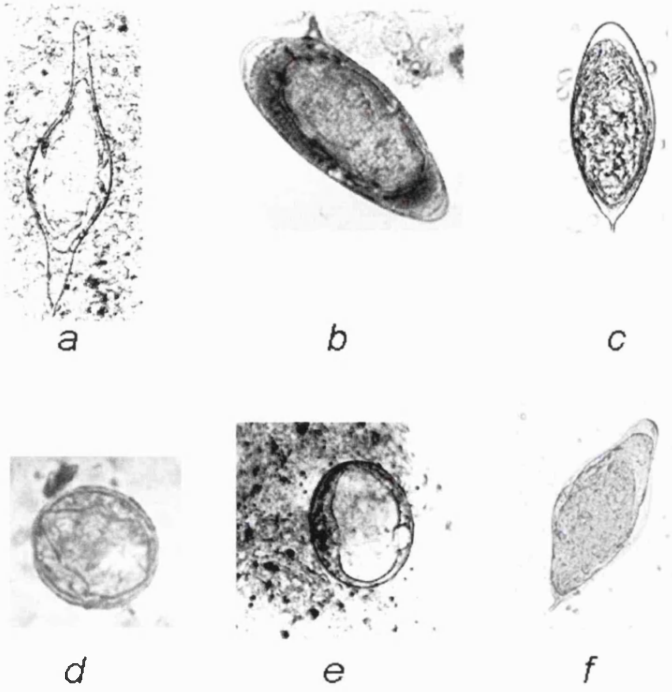


Fig 1.7. Eggs of 6 species of schistosome at various magnifications (average length of eggs in µm). a, *S. bovis* (202); b, *S. mansoni* (142); c, *S. haematobium* (144); d, *S. japonicum* (81); e, *S. margrebowiei* (87); f, *S. intercalatum* (175).

Fig 1.8. *a, Biomphalaria* spp. *b, Bulinus* spp. Taken from the Bayer Manual of Pest Control

Schistosomes are unusual amongst trematode worms in a number of ways. Firstly, they have a 2-host life cycle, whereas the majority of trematodes have a 3-host cycle in which cercariae shed from a mollusc penetrate a second intermediate host (e.g. a snail, another aquatic invertebrate or a fish) and develop into metacercariae before being ingested by a suitable definitive host. Secondly, most trematodes live in the digestive tract or related organs of their host, whereas schistosomes live in the blood-vascular system. Thirdly, apart from one recently described species which is parasitic in crocodilians (Platt *et al.,* 1991), schistosomes parasitise only homeothermic (warm-blooded) animals (birds and mammals), whereas many trematodes are parasites of cold-blooded animals (Combes, 1991). Fourthly, one of the most remarkable features of schistosomes is the fact that they are dioecious rather than hermaphrodite. This is not only in contrast to their trematode ancestors, but to the usual trend observed in many parasitic groups (Combes, 1991). Indeed, hermaphroditism has been linked with low motility, and hence with the parasitic lifestyle (Maynard Smith, 1978, 1989). Combes (1991) offers an immunological explanation for the reversion of schistosomes to dieocy in the course of evolution from hermaphrodite ancestors. The adoption of warm-blooded higher vertebrates as definitive hosts brought with it the need for the parasites to evade a far more elaborate immune system: dieocy, with its mandatory cross-fertilization would offer the genetic variability necessary for the evolution of immune evasion strategies. Basch (1990) suggests that sequential hermaphroditism, which occurs in trematodes and involves the exhibition of male sexual function prior to the onset of female sexual function (protandry) or *vice versa* (protogyny), developed in the hermaphroditic proto-schistosomes as a first step towards dioecy, and selection operated to suppress the development of the contrasex organ systems. In some circumstances, such as when clasped by a male of another distantly related species or even genus, female schistosomes may produce unfertilized parthenogenetic eggs, which are matroclonal, and yield miracidia which are slightly infective to snails (Basch & Basch, 1984; Basch, 1990, 1991; Jourdane *et al.,* 1995). This may serve as a means of allowing the female to continue her life cycle in circumstances when the host has not acquired a suitable conspecific male partner (Basch, 1990), and hints at an ancestral independence from males. Occasionally, further reminders of a hermaphroditic ancestry arise from schistosomes which display hermaphroditic characteristics. This is well documented for *S. mansoni*, particularly in guinea-pig hosts (Vogel, 1947; Short, 1948; Paraense & Santos, 1949; Saoud, 1964; Basch, 1990) and is more common in males than females, with the extent of

male feminization ranging from a few clusters of vitelline cells to reasonably developed ovaries and uteri. Thus substantial fragments of the female genome remain in males, but the loss of the contrasex genome in females is more complete. Indeed, this, and the pronounced sexual dimorphism of the schistosome sexes may result from selective pressure on the female to optimize reproductive efficiency by abandoning not only the determinants for maleness, but most locomotory and pharyngeal musculature and parenchymal tissue (Basch, 1990). By way of compensation, these stuctures have been augmented in males to serve not only their needs (i.e. fertilization of eggs), but those of their female partners. For example, it is the male worm which transports the female from the liver to the site of oviposition, and as this distance can be considerable in larger hosts such as humans and cattle, it is to the advantage of males to have as much musculature as possible. In addition, Basch (1990) suggests that the loss of pharyngeal musculature in females leaves them unable to feed efficiently on their own, whilst the muscularity of males allows them to assist females by physically pumping host blood into their intestine by muscular movements of the gynaecophoric canal. Unpaired females in unisexual infections remain stunted and sexually immature, whilst paired, sexually mature females which are separated from their partners and transferred into a naïve host undergo a physical regression that is reversible on regaining a male partner (Clough, 1981). Gupta & Basch (1987) postulated that malnutrition is responsible for the stunting and immaturity of unpaired females, and Basch (1990) speculated that feeding assistance is the key to male stimulation of female growth and development and the maintenance of her mature state.

Whilst Shaw (1977) demonstrated that reproductive stimulation of females is independent of sperm transfer, other authors (Cornford & Fitzpatrick, 1985; Skuce *et al.*, 1990; Tchuem Tchuenté *et al.*, 1996 *b*) suggest that the stimulation of females by males is chemical, perhaps hormonal, although the exact nature of such chemical transfers remains to be determined. Certainly, chemical mediation is strongly implicated in the process of attraction between males and females (Eveland *et al.*, 1982, 1983; Imperia *et al.*, 1980; Haseeb & Eveland, 1986; Tchuem Tchuenté *et al.*, 1996 *b*), with females being more significantly attracted to products released by males *in vitro*, than *vice versa*. The involvement of lipids, especially steroids has been postulated both for attraction and reproductive stimulation of females, as studies have shown that cholesterol is transferred between male and female schistosomes (Haseeb *et al.*, 1985; Haseeb & Eveland, 1991). It

is interesting to note that, to a lesser extent than with females, unpaired males may be stunted in size in comparison to paired males (Li, 1958; Lengy, 1962; Tchuem Tchuenté *et al.,* 1996 *b*), suggesting some reciprocity of chemical signalling between males and females.

Pairing itself is largely due to thigmotactic processes rather than any pheromonal signals, as demonstrated by the experiments of Basch & Nicolas (1989) in which *S. mansoni* males attempted to pair with alginate fibres *in vitro*.

Perhaps the most remarkable feature of schistosomes is that neither attraction, pairing, nor the reproductive stimulation of females appears to be species-specific: males and females of different species of *Schistosoma* can form pairs and mate, even when they are distantly related (from different evolutionary groups) (Tchuem Tchuenté *et al.,* 1993, 1995). If the species are closely related (from the same group) such interactions may potentially lead to hybridisation, whereas parthenogenesis is the likely outcome of interspecific pairing between distantly related species (Armstrong, 1965; Taylor, 1970; Jourdane & Southgate, 1992; Tchuem Tchuenté *et al.,* 1994; Jourdane *et al.,* 1995; Southgate *et al.,* 1998; Webster *et al.,* 1999).

In nature, interspecific pairing is known to occur in areas of sympatry between different species which are able to co-infect the same definitive host, and this may have some important epidemiological consequences. For example, there is a risk that one of the co-parasitizing species may be competitively excluded, either partially or totally, by the other (Tchuem Tchuenté *et al.*, 1993, 1996 *a*, 1997 *b*; Southgate *et al.,* 1995, 1998; Southgate, 1997; Webster *et al.,* 1999). Studies suggest that competitive exclusion of *S. intercalatum* by *S. mansoni* and *S. haematobium* is an important factor restricting the distribution of *S. intercalatum* in Africa (Southgate, 1978; Southgate *et al.,* 1982; Tchuem Tchuenté *et al.,* 1995, 1996 *a*). Where hybridization occurs between closely related species, backcrossing of hybrid offspring with parental species over many generations (introgressive hybridisation) may eventually lead to the emergence of a new strain of one of the parental species. This process is implicated in the complete replacement of *S. intercalatum* at Loum, Cameroon by an emergent new strain of *S. haematobium* in a period of less than 30 years (Southgate, 1978; Tchuem Tchuenté *et al.,* 1997 *b*). In addition, interspecific crosses which result in

hybridization may lead to an increase in the genetic heterogeneity of natural schistosome populations (Tchuem Tchuenté *et al.,* 1996 *b*).

The lack of specificity shown by schistosomes for their definitive hosts allows them to be studied in experimental laboratory hosts (McLaren & Smithers, 1987). Laboratory studies of schistosome mating interactions generally use mice and hamsters as definitive hosts. A possible disadvantage of using these hosts to examine schistosome mating behaviour is that the available space for mating interactions is far less than in a human, for example. Another consequence of the reduced host size in mice and hamsters is that paired *S. haematobium* worms are unable to migrate to the veins of vesical venous plexus as the vessels are too small: instead the worm pairs inhabit the mesenteric veins (Tchuem Tchuenté *et al.,* 1996 *b*). Nevertheless, mice and hamsters allow the full development of schistosomes which pair in the hepatic portal vein, migrate to the egg-laying site and lay eggs in tissues: these models are therefore of some relevance to the human situation (McLaren & Smithers, 1987).

Initial studies of schistosome mating interactions in rodents infected with two or more different species suggested that choice of mate occurred by trial and error, i.e. mating was random and independent of species identity (Armstrong, 1965; Southgate *et al.,* 1982; Rollinson *et al.,* 1990). However, more detailed, recent studies have revealed the existence of specific mate preference systems in mixed infections of *S. intercalatum* (Lower Guinea strain) and *S. mansoni* (Tchuem Tchuenté *et al.* 1993, 1994, 1995); *S. haematobium* and *S. mattheei* (Southgate *et al.,* 1995); and *S. haematobium* and *S. mansoni* (Webster *et al.,* 1999) in rodents. Each time the mate preference was for intraspecific partners, thus establishing specific "choice of mate" as an important pre-zygotic reproductive isolating mechanism, helping to maintain the genetic identity of co-infecting species (Tchuem Tchuenté *et al.,* 1993).

Studies of mixed infections of *S. intercalatum* (Lower Guinea strain) and *S. mansoni* in mice (Tchuem Tchuenté *et al.,* 1993, 1994, 1995), and *S. haematobium* and *S. mansoni* in hamsters (Webster *et al.,* 1999) have highlighted other important features of schistosome mating interactions. Mating competition and change of mate have been demonstrated, with unpaired males being able to pull paired females away from their male partners. This

challenges the long-held belief that the association between paired males and females is a permanent one. Studies of mixed infections of *S. intercalatum* (Lower Guinea strain) and *S. mansoni* demonstrated that heterospecific pairs change to homospecific pairs whenever given the opportunity (Tchuem Tchuenté *et al.,* 1995). We may therefore speculate that heterospecific pairing (which at first appears to be a particularly unsuitable mating option for distantly related species which yield low-viability, parthenogenetic, offspring) may arise from the selective advantage to pair, allowing both males and females to achieve full growth, and the female to be maintained in a reproductively mature state until a suitable homospecific partner comes along (Tchuem Tchuenté *et al.,* 1995, Jourdane *et al.,* 1995).

It is the aim of this thesis to characterise the mating interactions of a number of new combinations of African schistosome species which have not previously been studied, using mice as the definitive host. It is proposed to establish the existence or otherwise of a specific mate preference system, mating competition, and change of mate for these mixed mating models. One application of this information, for example, may be to indicate the likely outcome of interactions between these species in the field (if they occur sympatrically and utilise the same definitive hosts), and/or to increase the accuracy with which the outcome of mating interactions between other combinations of species may be predicted. To this end, experiments were conducted which examine the mating interactions between *S. mansoni* and *S. margrebowiei*; *S. intercalatum* (Zaire strain) and *S. mansoni*; and between *S. margrebowiei* and *S. bovis,* in mice. In addition, the interactions between *S. haematobium* and *S. intercalatum* (Lower Guinea strain) and between *S. haematobium* and *S. mansoni* in hamster hosts were studied, with a view to furthering existing knowledge of the mating behaviour of these species in mixed infections (Southgate *et al.,* 1982; Webster *et al.,* 1999).

CHAPTER 2

Materials and Methods

CHAPTER 2
MATERIALS AND METHODS

2.1 MAINTENANCE OF LIVE MATERIAL

2.1.1 Overview

Schistosomes were maintained in the laboratory by repeated cycles ("passages") of snail (intermediate host) and mouse/hamster (definitive host) infection. The length of time for which they can be maintained in laboratory passage is highly species, strain and even isolate dependent: *S. mansoni*, for example, can be maintained for many years whereas *S. haematobium* rarely survives beyond one or two laboratory passage cycles. Schistosomes were introduced into the laboratory by infected snails: either naturally infected, wild-caught snails, or wild-caught or laboratory-bred snails deliberately exposed to miracidia hatched from eggs originating from the livers, urine or faeces of naturally infected definitive hosts.

2.1.2 Snail infection procedure

In the laboratory, snails were infected by exposing them to miracidia hatched from eggs from the livers of infected laboratory rodents: albino TO outbred mice (*Mus musculus)* and golden hamsters (*Mesocricetus auratus*). Infected livers were removed from freshly sacrificed rodents (culled in a $C0_2$ chamber) and forced through a 1mm metal mesh into a beaker containing 0.85% saline. The liver suspension was allowed to sediment under gravity for 30 mins and the supernatant was then discarded. The sediment was re-suspended in saline and washed several times, and the supernatant discarded after each wash. After washing, the sediment containing schistosome eggs was resuspended in tank water (which had been biologically filtered by guppies to remove residual heavy metals) to initiate hatching, which is stimulated by a drop in osmolarity. The suspension was transferred to petri dishes and placed under a lamp for 30 mins to provide heat and light: both are pre-requisites for miracidial hatching (Erasmus, 1972). After this incubation, the petri-dishes were examined using a binocular microscope: miracidia were clearly visible and could easily be removed using a pasteur pipette. Snails were infected by placing them individually in small pots containing filtered tank water and 5-7 miracidia. The snails were left overnight at 26°C, and then pooled and transferred to a plastic tray containing fresh filtered tank water. Snails were maintained at ambient temperatures of 26°C with a 12 hour light/dark cycle; high intensity illumination; and on a diet of dried lettuce and tropical fish

food flakes. Cercariae were shed from snails from approximately 28 days post infection onwards (depending on the schistosome species).

2.1.3 Obtaining cercariae from infected snails

Infected snails were transferred into small beakers of fresh filtered tank water and placed under strong illumination. All species of schistosome used in the mating experiments in this work have cercariae which emerge in response to the sunlight (as opposed to some species, such as those parasitic in rodents, which emerge during the night): strong illumination in the laboratory mimics the effect of the sun to stimulate cercarial emergence from the snails.

2.1.4 Procedure for infecting rodents

Water containing newly shed cercariae was transferred into a petri dish, and the cercariae observed under a binocular microscope where they were easily visible. The number of cercariae required to infect each rodent was removed using a pasteur pipette and transferred to a 500ml opaque plastic beaker containing filtered tank water (200ml for mouse infections, 300ml for hamster infections). The mouse or hamster was allowed to paddle in the infected water for approximately 30 minutes (the 'partial immersion" technique of infection (Watson & Azim, 1949)), sufficient time for the cercariae to penetrate the skin.

2.1.5 Recovery of worms from infected animals by perfusion

After waiting for the full development of the adult worms, mice were culled using $C0_2$, and the worms recovered by the following perfusion technique:

1. Using a sharp pair of scissors, the area of skin covering the abdomen was cut away to leave the gut and liver clearly exposed
2. The ribcage was cut open to expose the heart
3. The rodent was pinned onto a vertical perfusion rack, and a 100μm sieve positioned immediately beneath, to retain the perfused worms
4. The hepatic portal vein leading from the gut to the liver was cut
5. A 60ml syringe was filled with citrated saline (0.75% sodium citrate, 0.85% sodium chloride) and a large bore needle attached
6. The needle was inserted into the base of the heart the fluid injected with some force to push the worms out of the circulatory system onto the sieve

7. The abdominal cavity was washed with 0.85% saline and the washings allowed to drain through the sieve
8. The worms retained by the sieve were washed into a separate pot of saline
9. Any worms which had retained their attachment in the mesenteric veins were dissected out.

Worm pairs were quickly segregated after recovery as the constituent partners often separate after approximately 20 minutes, and may be confused with unpaired worms.
Care was taken to number each pair and each individual worm, prior to taxonomic identification.

2.2 ORIGIN OF SPECIES, EXPERIMENTAL INFECTIONS AND SPECIES IDENTIFICATION FOR THE MATING MODELS IN CHAPTERS 3-7

2.2.1 CHAPTER 3 : *S. margrebowiei/ S. mansoni* interactions

Origin of species

The isolate of *S. mansoni* used in these experiments originated from Egypt and was introduced into the laboratory by Prof Mike Doenhoff (University of Bangor) where it was maintained for several years in *Biomphalaria glabrata* and albino TO mice.

The isolate of *S. margrebowiei* used in these experiments originated from the Lochinvar National Park, Zambia and was isolated in 1991 by exposing laboratory-bred *Bulinus wrighti* to miracidia recovered from the freshly deposited faeces of *Kobus Leche* (lechwe). It was maintained in the laboratory in *Bulinus natalensis* and albino TO mice.

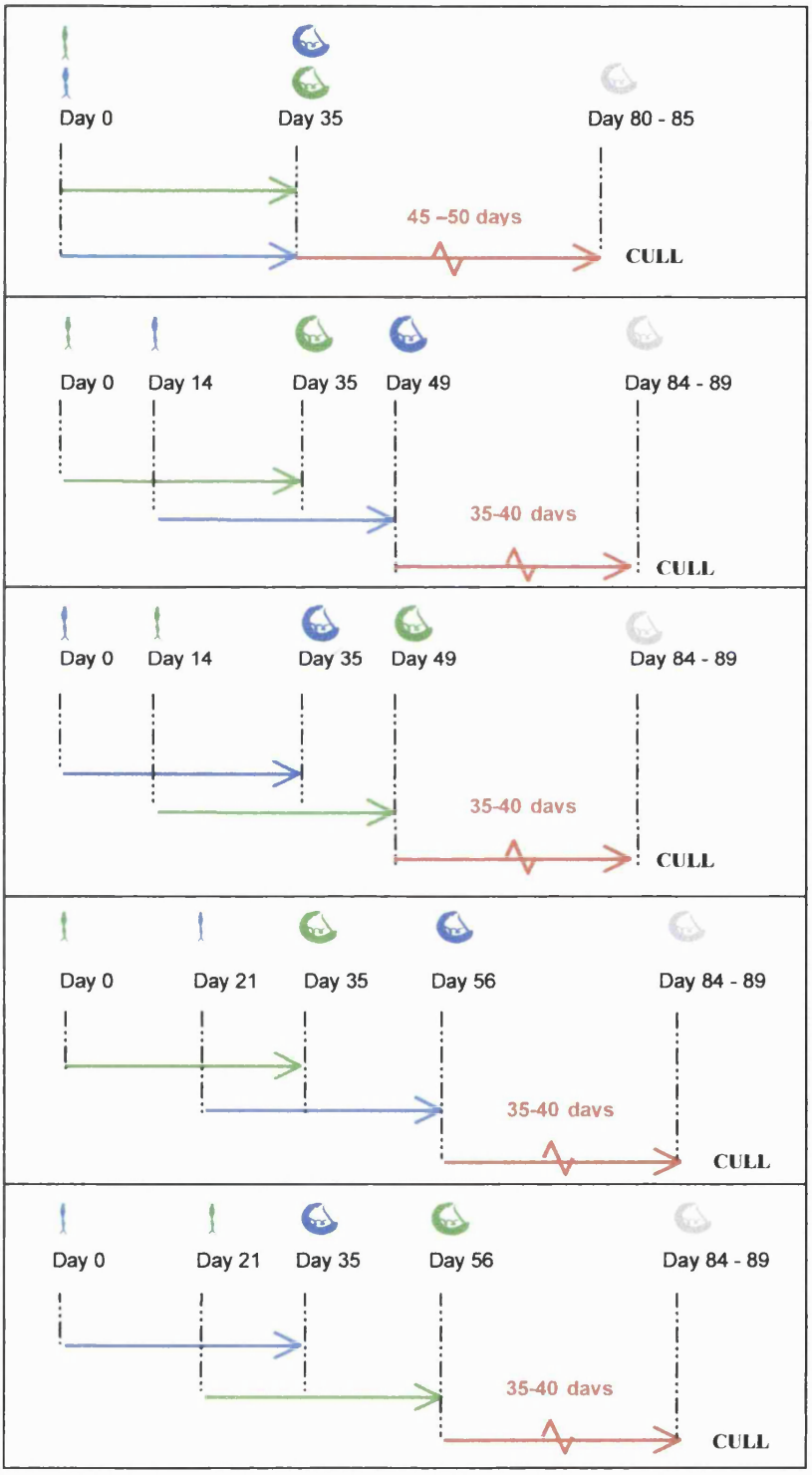
Experimental Infections

The experimental design was aimed at determining how male and female worms of both species interact in mixed infections in the vertebrate host, and whether a specific mate recognition system and mating competition exists between the two species. Mice were exposed individually by the paddling technique (section 2.1.4) to a fixed number of male and female cercariae of both species in a series of 5 experiments (Fig 2.1).

The experiments were of two types, the first of which (experiment #1) involved the simultaneous infection of mice with 150 mixed-sex cercariae of *S. margrebowiei* and 150 mixed-sex cercariae of *S. mansoni*, thus allowing the schistosomes total choice of mate from either species. The second type (experiments #2-5) involved the sequential infection of mice: firstly with 150 mixed-sex cercariae of *S. mansoni*, and two weeks later with 150 mixed-sex cercariae of *S. margrebowiei* (experiment #1) and *vice versa* (experiment #2), and repeated with a three-week interval between infections (experiments #3 and #4).

.Fig 2.1 Schematic representation of the time-course of infections in chapter 3.

= infection with *S. mansoni* cercariae = infection with *S. margrebowiei* cercariae = onset of patency (and pairing) for *S. mansoni* = onset of patency (and pairing) for *S. margrebowiei* = recovery of heterospecific and/or homospecific worm pairs, plus unpaired worms



Experiment 1

Experiment 2

Experiment 3

Experiment 4

Experiment 5

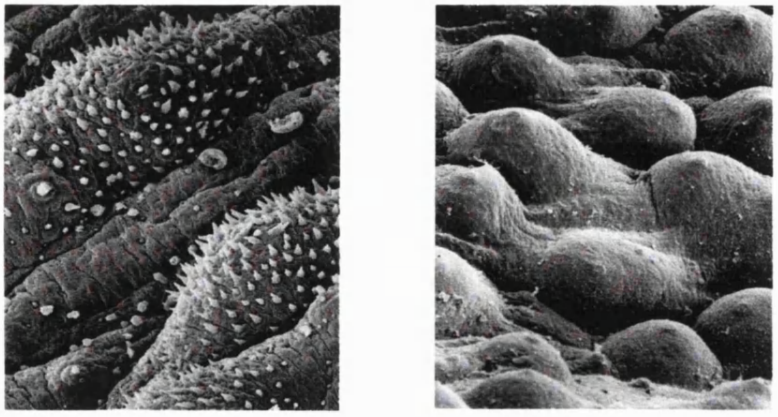
In sequential infections it is assumed that the first species to infect the host ('species 1') will form homospecific pairs, and the usual male-bias of schistosome infections (Liberatos, 1987; Mitchell *et al.*, 1990) should result in an excess of 'species 1 males' which may compete with 'species 2 males' for 'species 2 females'. Comparison of sequential experiments with 2-week and 3-week time lags between infections should reveal any highly time-dependent features of mating interactions.

After infections had reached patency, mice were killed and worms collected by perfusion and dissection of the hepatic portal vein and mesenteric venous systems of each infected mouse (section 2.1.5): 45-50 days post-infection for experiment #1 and 35-40 days post-reinfection for experiments #2-5. Each pair of worms recovered, and any unpaired worms recovered from the infected mice were segregated into individual containers and the male and female worms from each pair were separated. Females were taxonomically identified whilst still alive: male worms were stored in Anal R 100% v/v ethanol in separate wells of a 96-well microtitre plate (with resealable lids) prior to identification.

Species identification

Females were taxonomically identified by microscopic examination of the morphology and number of the intrauterine eggs: *S. mansoni* females produce single, lateral spined eggs (Fig 1.7 *b*) whereas *S. margrebowiei* females produce several smaller, rounder eggs with a barely discernible terminal spine (Fig 1.7 *e*).

Males were identified by microscopic examination of the morphology of the dorsal tubercles: those of *S. mansoni* possess many spines (Fig 2.2 *a*) whereas tubercles of *S. margrebowiei* are spineless (Fig 2.2 *b*).



a b

Fig. 2.2. Male dorsal tubercles. *a. S. mansoni*, x3500, with spines *b. S. margrebowiei*, x1000, without spines

Immature (unpaired) males with poorly developed tubercles were identified by extracting and purifying DNA from them individually (section 2.3.1) and subjecting a sample of the DNA to PCR amplification of the first internal transcribed spacer (ITS1) region of rRNA. This region contains tandem repeats, the number of which is diagnostic for some species (Kane *et al.*, 1996). There are 4 repeats in the ITS1 region of *S. margrebowiei* compared with 2 in *S. mansoni*, and therefore specific PCR amplification with *S. margrebowiei* DNA results in a larger band which runs at a higher (slower) position on an agarose gel than the amplification product from *S. mansoni* (Fig 2.3), allowing the 2 species to be distinguished from one another.

For the amplification of the ITS1 sequence, 25µl PCR reactions were performed as below for each DNA extraction and for 2 control DNA samples (from a known *S. mansoni* and a *S. margrebowiei* male), using the primers ETTS1 (5'-TGCTTAAGTTCAGCGGGT) and ETTS2 (5'-TAACAAGGTTTCCGTAGGTGAA) (Kane & Rollinson, 1994)

Reagent	**Volume**
DNA	1µl of 20µl extraction
dNTP	4µl (at 1.25mM)
10X PCR Buffer	2.5µl
Taq DNA polymerase (Cambio)	0.25µl (at 5U/µl)
Primer	1µl of each at 30pmol/µl
ddH_20	15.25µl
TOTAL	**25µl**

Samples were overlaid with mineral oil as the PCR machine used (a Perkin-Elmer 480 Thermocycler) did not have a heated lid.

Amplification was performed in a Perkin-Elmer 480 Thermocycler programmed for: 95°C for 5 mins; 29 cycles of [95°C for 30 secs, 60°C for 30 secs and 72°C for 1.5 mins]; 60°C for 30 secs, 72°C for 10 mins.

10µl of each sample was mixed with 2µl of loading dye and run out on a 1% agarose gel containing ethidium bromide.

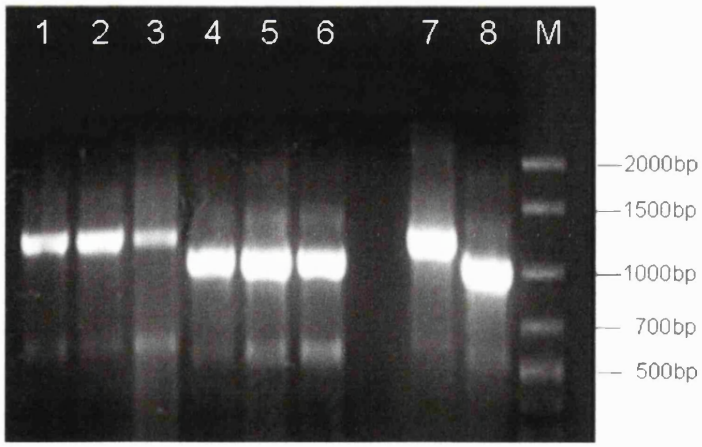


Fig. 2.3. PCR amplification of ITS1 markers to distinguish between males of *S. margrebowiei* (lanes 1, 2, 3, and 7) and *S. mansoni.* (lanes 4, 5, 6 and 8). Lane M, size marker (Cambio "Biomarker® EXT").

Some smaller males did not yield any ITS1 products following PCR amplification. For these males, identification was achieved by testing a sample of the DNA extracted from them for PCR amplification of the *S. mansoni*-specific marker Sm107. This marker, first identified by Hamburger *et al.* (1998) is a highly repetitive 121-base pair sequence specific to *S. mansoni*, specific amplification of which results in a characteristic "ladder" pattern on an agarose gel, enabling *S. mansoni* worms to be distinguished from other species (Fig 2.4). Moreover, since Sm107 is reported to form approximately one-tenth of the *S. mansoni* genome, this allows it to be amplified from very small quantities of DNA such as that yielded by single immature worms. However, whilst a positive result identifies *S. mansoni*, a negative result does not conclusively demonstrate that the sample is of non-*S. mansoni* origin, as it could mean that the PCR reaction failed for some other reason.

For the amplification of the Sm107 sequence, 25µl PCR reactions were performed as below for each DNA extraction and for two control DNA samples (from a known *S. mansoni* and a *S. margrebowiei* adult worm), using the primers 5'-GATCTGAATCCGACCAACCG and 5'-ATATTAACGCCCACGCTCTC (Hamburger *et al.*, 1998).

Reagent	**Volume**
DNA	1µl of 20µl extraction
dNTP	4µl (at 1.25mM)
Buffer F (Invitrogen)	2.5µl
Taq DNA polymerase (Cambio)	0.25µl (at 5U/µl)
Primer	1µl of each at 20pmol/µl
ddH_20	12.75µl
TOTAL	**25µl**

NB. Buffer F from the Invitrogen PCR Salt Optimization Kit was used in the reactions, as previous optimization of Sm107 amplification showed this buffer, containing 10mM $MgCl_2$ to yield the best results (Wamachi, 2000).

Samples were overlaid with mineral oil as the PCR machine used (a Perkin-Elmer 480 Thermocycler) did not have a heated lid.

Amplification was performed in a Perkin-Elmer 480 Thermocycler programmed for: 95°C for 2.5 mins; 35 cycles of [95°C for 30 secs, 55°C for 30 secs and 72°C for 1.5 mins]; 72°C for 10 min.

10µl of each sample was mixed with 2µl of loading dye and run out on a 1% agarose gel containing ethidium bromide.

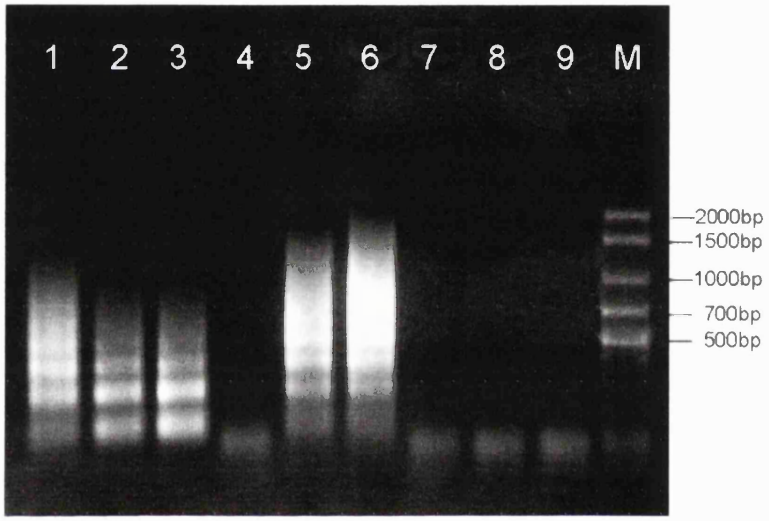


Fig. 2.4. PCR amplification of marker Sm107 to distinguish between males of *S. mansoni* (lanes 1, 2, 3, 5 and 6) and *S. margrebowiei* (lanes 4, 7, 8 and 9). Lane M, size marker (Cambio "Biomarker®" EXT").

2.2.2 CHAPTER 4: *S. intercalatum* (Zaire strain)/ *S. mansoni* interactions

Origin of species

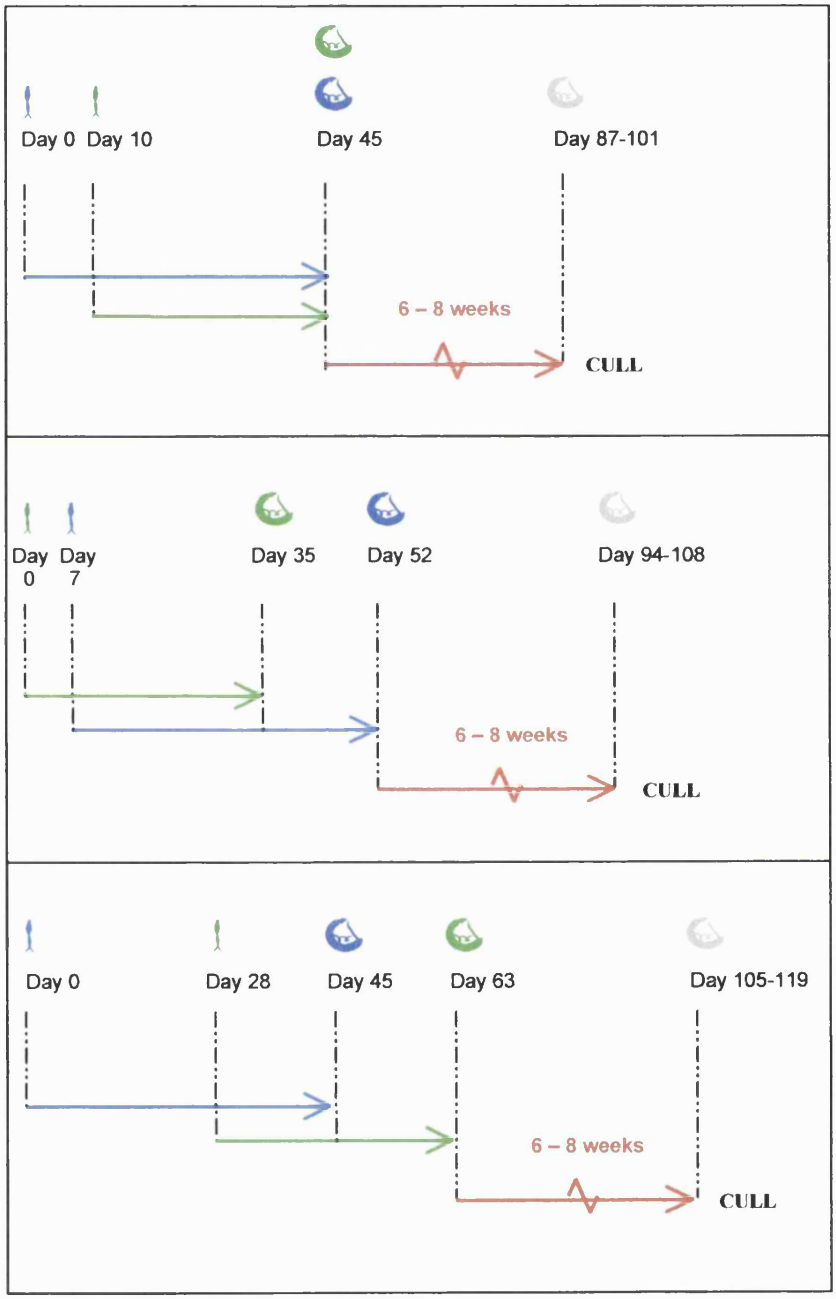
The isolate of *S. intercalatum* (Zaire strain) used in these experiments originates from Kinshasa, Democratic Republic of Congo, where an authochthonous focus of *S. intercalatum* was described in 1987 by De Clercq. Tchuem Tchuenté *et al.* (1997 *a*) noted a possible phosphoglucomutase (PGM) isoenzyme difference between this isolate and the original one from Kisangani described by Fisher (1934), but it utilizes the same snail host (*Bulinus globosus*) and remains distinct from the Lower Guinea strain. It was isolated in 1994 from faeces of infected primary school children in Kinshasa by L. A. Tchuem Tchuenté and maintained in the laboratory at the University of Perpignan in *B. globosus* from Zambia and Swiss OF1 mice for several years. It was introduced into the laboratories of The Natural History Museum in 2001 courtesy of L. A. Tchuem Tchuenté where it was maintained in laboratory-bred *Bulinus wrighti* from Oman, and albino TO mice for two passages prior to its use in this study.

The isolate of *S. mansoni* used in these experiments originated from Richard Toll, Senegal and was isolated from wild-caught *Biomphalaria pfeifferi* in 1993. Since then, it has been maintained in laboratory-bred *B. pfeifferi, B. glabrata* and albino TO mice.

Experimental infections

As for the *S. margrebowiei/ S. mansoni* mating model, the experimental design was aimed at determining how male and female worms of both species interact in mixed infections in the vertebrate host, and whether a specific mate recognition system and mating competition exists between the two species. Mice were exposed individually by the paddling technique (section 2.1.4) to a fixed number of male and female cercariae of both species in three experiments (Fig 2.5). These were of 2 types, the first of which (experiment #1) involved the infection of mice with 150 mixed-sex cercariae of *S. intercalatum* (Zaire) and 10 days later with 150 mixed-sex cercariae of *S. mansoni*, to ensure that both species reached patency simultaneously and therefore had total choice of mate from either species.

Fig 2.5. Schematic representation of the time-course of infections in chapter 4.
= infection with *S. mansoni* cercariae = infection with *S. intercalatum* (Zaire) cercariae = onset of patency (and pairing) for *S. mansoni* = onset of patency (and pairing) for *S. intercalatum* = recovery of heterospecific and/or homospecific worm pairs, plus unpaired worms



Experiment 1

Experiment 2

Experiment 3

The second type of experiment (experiments #2 and #3) involved the sequential infection of mice, with the time interval between infections adjusted according to the differences in patency of the two species in mice (approximately 45 days for *S. intercalatum* and approximately 35 days for *S. mansoni* (Loker, 1983)), so that the first species would reach patency 2-3 weeks before the second. Mice in experiment #2 were infected firstly with 150 mixed-sex cercariae of *S. mansoni* and then with 150 mixed-sex cercariae of *S. intercalatum* (Zaire) one week later, whilst mice from experiment #3 were infected firstly with 150 cercariae of *S. intercalatum* (Zaire), and 4 weeks later with 150 cercariae of *S. mansoni.*

Mice were killed and worms collected by perfusion and dissection of the hepatic portal vein and mesenteric venous systems of each infected mouse 6-8 weeks post reinfection (section 2.1.5). Each pair of worms, and any unpaired worms recovered from the infected mice were segregated into individual containers and the male and female worms of each pair were separated. Females were taxonomically identified whilst still alive: male worms were stored in separate, labelled cryotubes in liquid nitrogen prior to identification.

Species identification

Females were identified by microscopic examination of the morphology and number of the intrauterine eggs: *S. mansoni* females produce single, lateral spined eggs (Fig 1.7 *b*), whereas *S. intercalatum* females produce several eggs which have a conical posterior end tapering to a terminal posterior spine (Fig 1.7 *f*).

Since the taxonomic identity of the male worms could not be determined by morphology, individual male worms were examined for glucose-6-phosphate-dehydrogenase (G6PDH) activity using the isoelectric focusing technique (section 2.3.3). Male worms of the isolates of both species used in this study are monomorphic for G6PDH but differ in the pI value of the enzyme, producing an identifiable profile which enables the two species to be distinguished from one another (Fig 2.6).

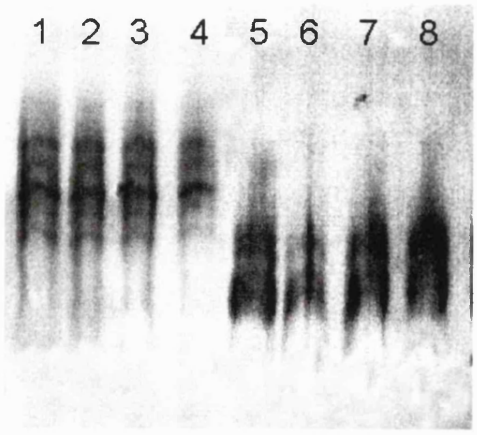


Fig. 2.6 G6PDH enzymes from extracts of adult male worms. Lanes 1-4, *S. intercalatum* (Zaire); Lanes 5-8, *S. mansoni*.

2.2.3 CHAPTER 5: *S. margrebowiei/S. bovis* interactions

Origin of species

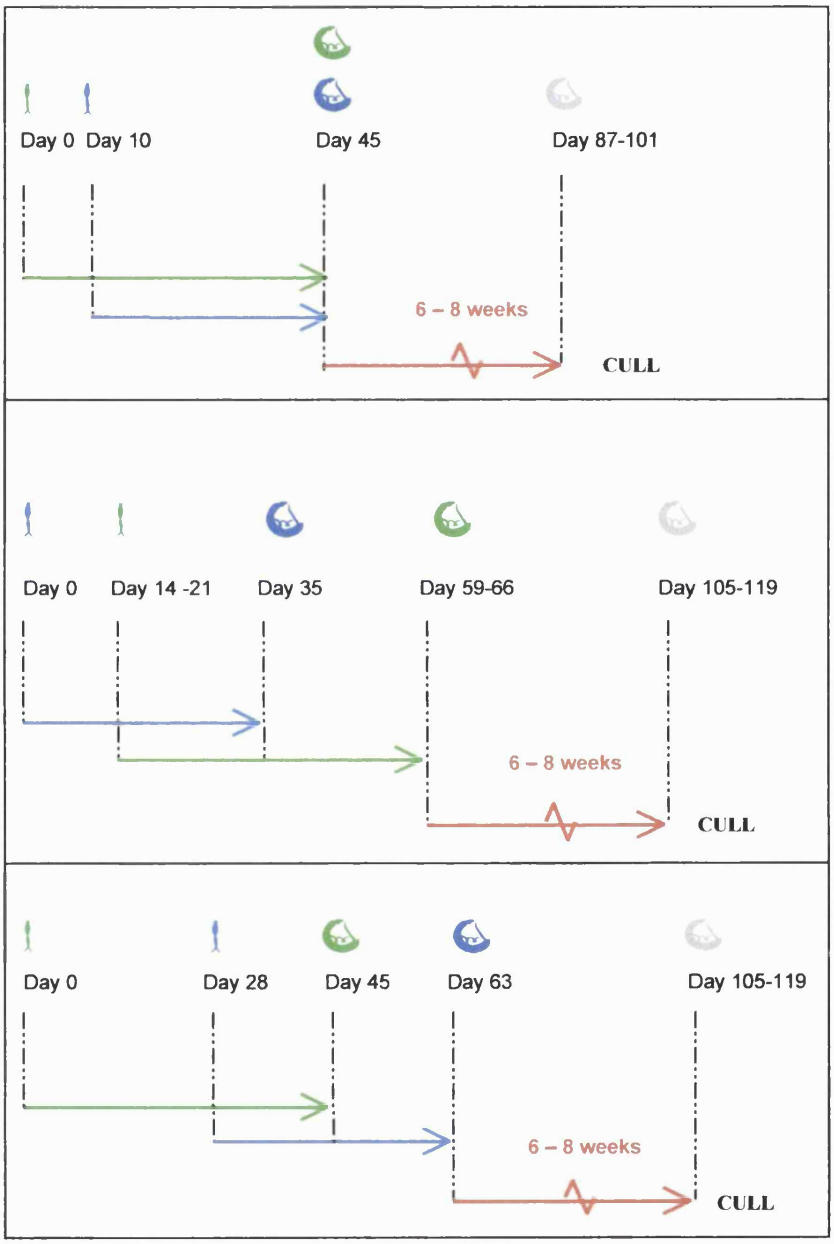
The isolate of *S. bovis* originated from St Louis, Senegal where it was isolated in 2000 by exposing laboratory-bred *B. wrighti* to miracidia from the fresh faeces of an infected animal.The isolate was passaged once in the laboratory through *Bulinus truncatus* and albino TO mice.

The isolate of *S. margrebowiei* originated from the Lochinvar National Park, Zambia and was isolated in 1991 by exposing laboratory-bred *B. wrighti* to miracidia from the freshly deposited faeces of *Kobus leche* (lechwe). It was maintained in the laboratory in *Bulinus natalensis* and albino TO mice.

Experimental design

As with the *S. margrebowiei/S. mansoni* and *S. intercalatum* (Zaire)/*S. mansoni* mating models, the experimental design was aimed at determining how male and female worms of both species interact in mixed infections in the vertebrate host, and whether a specific mate recognition system and mating competition exists between the two species. Mice were exposed individually by the paddling technique (section 2.1.4) to a fixed number of male and female cercariae of both species in 3 experiments (Fig 2.7). These were of 2 types, the first of which (experiment #1) involved the simultaneous infection of mice with 150 mixed-sex cercariae of *S. bovis*, and 10 days later with 150 mixed-sex cercariae of *S. margrebowiei*, to ensure that both species reached patency simultaneously and therefore had total choice of mate from either species. The second type of experiment (experiments #2 and #3) involved the sequential infection of mice, with the time interval between infections adjusted according to the differences in patency of the two species in mice (approximately 45 days for *S. bovis* and approximately 35 days for *S. margrebowiei* (Loker, 1983)), so that the first species would reach patency 3-4 and 2-3 weeks before the second species for experiments #2 and #3, respectively. Mice in experiment #2 were infected firstly with 150 cercariae of *S. margrebowiei*, and 2 weeks later with 150 cercariae of *S. bovis*, whilst mice from experiment #3 were infected first with 150 cercariae of *S. bovis* and 4 weeks later with 150 cercariae of *S. margrebowiei*.

Fig 2.7. Schematic representation of the time-course of infections in chapter 5.
= infection with *S. bovis* cercariae = infection with *S. margrebowiei* cercariae
= onset of patency (and pairing) for *S. bovis* = onset of patency (and pairing) for *S. margrebowiei* = recovery of heterospecific and/or homospecific worm pairs, plus unpaired worms



Experiment 1

Experiment 2

Experiment 3

Mice were killed and worms collected by perfusion and dissection of the hepatic portal vein and mesenteric venous systems of each infected mouse (section 2.1.5) 6-8 weeks post reinfection. Each pair of worms recovered, and any unpaired worms from the infected mice were segregated into individual containers and the male and female worms from each pair were separated. Females were taxonomically identified whilst still alive: male worms were stored in separate, labelled cryotubes in liquid nitrogen prior to identification.

Species identification

Females were identified by microscopic examination of the morphology of the intrauterine eggs: *S. margrebowiei* females produce round eggs with a minute terminal spine (Fig 1.7 *e*), whereas *S. bovis* females produce spindle-shaped eggs with a terminal spine (Fig 1.7 *a*).

Since the taxonomic identity of the male worms could not be determined by morphology, individual male worms were examined for acid phosphatase (AcP) activity using the isoelectric focusing technique (section 2.3.3). Male worms of the isolates of both species used in this study are monomorphic for AcP but differ in the pI value of the enzyme, producing an identifiable profile which enables the two species to be distinguished from one another (Fig 2.8).

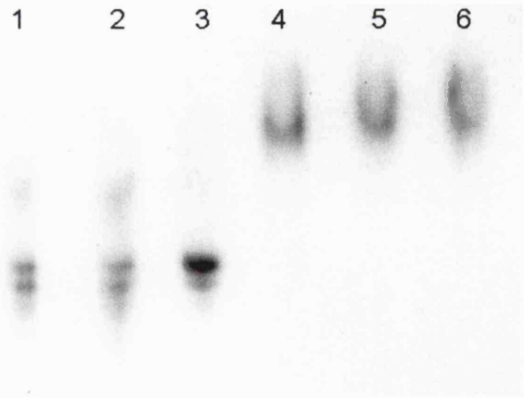


Fig. 2.8. AcP enzymes from extracts of adult male worms. Lanes 1-3, *S. margrebowiei*; Lanes 4-6, *S. bovis*.

2.2.4 CHAPTER 6: *S. haematobium/ S. intercalatum* (Lower Guinea) interactions

Origin of species

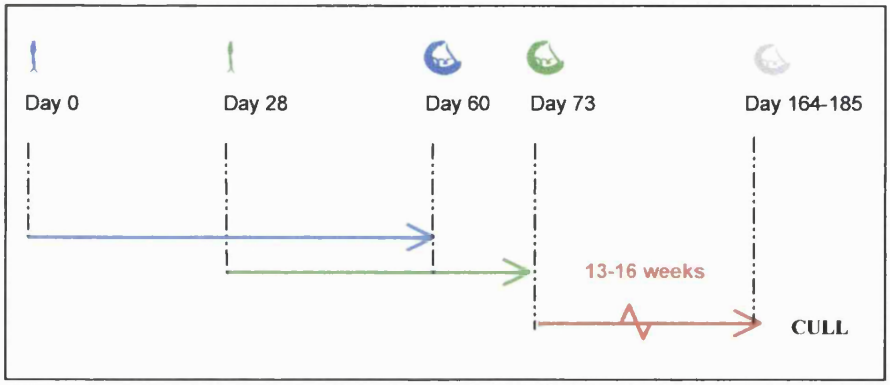
The Lower Guinea strain of *S. intercalatum* used in this study was isolated from 26 naturally infected *Bulinus forskalii* snails collected in Edea, Cameroon in 1998. It was maintained for 2 years in laboratory-bred *B. wrighti* and albino TO mice prior to use.

Eggs of *S. haematobium* were isolated from urine collected from the population of the village of Barombi Mbo, Cameroon in 2000. Laboratory-bred *B. wrighti* were exposed to the miracidia hatched from the eggs, and the isolate was passaged once in the laboratory through albino TO mice and *B. wrighti* prior to use in this study.

Experimental infections

The experimental design was aimed at determining the interactions between males and females of *S. haematobium* and *S. intercalatum* in sequential infections in hamsters with *S. haematobium* as the first infection and *S. intercalatum* as the second (Fig 2.9). The experiment extends the work carried out by Southgate *et al.* (1982) on the interactions between these two species in both simultaneous infections, and infections with *S. intercalatum* as the first infection, and *S. haematobium* as the second. Taking into account the differences in patency of the two species in hamsters (approximately 60 days for *S. haematobium* and approximately 45 days for *S. intercalatum* (Loker, 1983)) hamsters were exposed individually by the paddling technique (section 2.1.4) to 150 mixed-sex cercariae *of S. haematobium*, and 4 weeks later to 150 mixed-sex cercariae of *S. intercalatum* (Zaire strain) so that *S. haematobium* would reach patency approximately 2 weeks earlier than *S. intercalatum*. It is assumed that *S. haematobium* will form homospecific pairs before *S. intercalatum,* and the usual male-bias of schistosome infections should result in an excess of *S. haematobium* males which may compete with *S. intercalatum* males for *S. intercalatum* females.

Fig 2.9 Schematic representation of the time-course of infections in chapter 6.
= infection with *S. haematobium* cercariae = infection with *S. intercalatum* (Lower Guinea) cercariae = onset of patency (and pairing) for *S. haematobium* = onset of patency (and pairing) for *S. intercalatum* = recovery of heterospecific and/or homospecific worm pairs, plus unpaired worms



Hamsters were killed and worms collected by perfusion and dissection of the hepatic portal vein and mesenteric venous systems of each infected hamster (section 2.1.5), 13-16 weeks post reinfection. Each pair of worms recovered, and any unpaired worms from the infected mice were segregated into individual containers and the male and female worms from each pair were separated. Females were taxonomically identified whilst still alive: male worms were stored in separate, labelled cryotubes in liquid nitrogen prior to identification.

Species identification

Females were identified by microscopic examination of the morphology of the intrauterine eggs: eggs of *S. haematobium* are oval at their anterior end and conical at the posterior end, tapering to a terminal posterior spine (Fig 1.7 *c*), whereas eggs of *S. intercalatum* are larger, with a more conical anterior end than eggs of *S. haematobium* (Fig 1.7 *f*).

Since the taxonomic identity of the male worms could not be determined by morphology, individual males were examined for glucose-6-phosphate-dehydrogenase (G6PDH) activity using the isoelectric focusing technique (section 2.3.3.). Worms of the isolates of both species used in this study are monomorphic for G6PDH but differ in the pI value of the enzyme, producing an identifiable profile which enables the two species to be distinguished from one another (Fig 2.10)

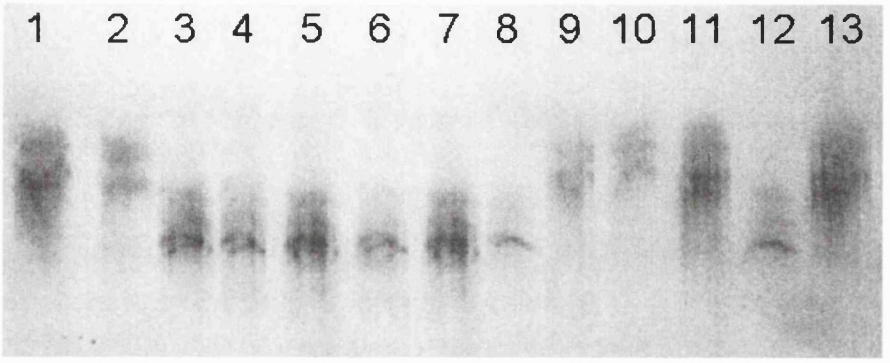


Fig. 2.10 G6PDH enzymes from extracts of adult male worms. Lanes 1, 2, 9-11 and 13, *S. haematobium* ; Lanes 3-8 and 12, *S. intercalatum* (Lower Guinea)

2.2.5 CHAPTER 7: *S. haematobium/ S. mansoni* interactions

Origin of species

The isolate of *S. haematobium* used in this study is as described in section 2.2.4.

The isolate of *S. mansoni* used in this study is as described in section 2.2.2.

Experimental infections

The experimental design was aimed at determining the mating interactions which take place when a single sex of *S. mansoni* is introduced into a pre-established infection of mixed male and female *S. haematobium*. *S. mansoni* male and female cercariae were obtained from *B. glabrata* snails infected by a single miracidium. As male and female cercariae cannot be morphologically distinguished from one another, the sex of the *S. mansoni* infections from the snails was determined in two separate ways. Firstly, DNA was extracted and purified from cercariae shed from each unmiracidially infected snail (section 2.3.2), and tested for PCR amplification of the *S. mansoni* female-specific W1 marker. The W1 sequence yields a PCR amplification product of ~ 450bp with female *S. mansoni* DNA, together with a series of higher molecular weight products resulting from degenerate regions in the repeats. Under the PCR conditions below, male DNA produces a complex pattern of non-specific amplification products which contrasts significantly with that produced by female DNA (Fig 2.11).

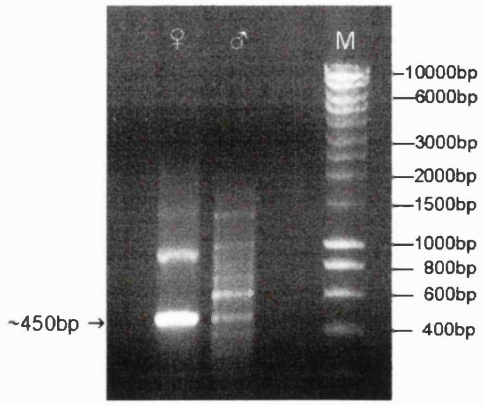


Fig 2.11. PCR amplification of female-specific marker W1 from cercariae shed from unimiracidially infected snails. Note the strong band of approximately ~450bp in the female lane. Lane M, size marker (Bioline "Hyperladder I").

For the amplification of the W1 sequence, 25µl PCR reactions were performed as below for each extraction and for two control samples (from a male and a female adult worm), using the primers 5'-GTGAAATTCTTCCTTCACAC and 5'-GACATTCAACTCAATGTTCG (Dias Neto *et al.*, 1993).

Reagent	**Volume**
DNA	0.5µl of each extraction
dNTP	4µl (at 1.25mM)
10X PCR buffer	2.5µl
Taq DNA polymerase (Cambio)	0.25µl (at 5U/µl)
Primer	0.5µl of each at 100pmol/µl
ddH_20	16.75µl
TOTAL	**25µl**

Samples were overlaid with mineral oil, as the PCR machine used (a Perkin-Elmer 480 Thermocycler) did not have a heated lid.

Amplification was performed in a Perkin-Elmer 480 Thermocycler programmed for: 94°C for 5 mins; 30 cycles of [94°C for 1 min, 50°C for 1 min and 72°C for 2 mins]; 72°C for 5 mins.

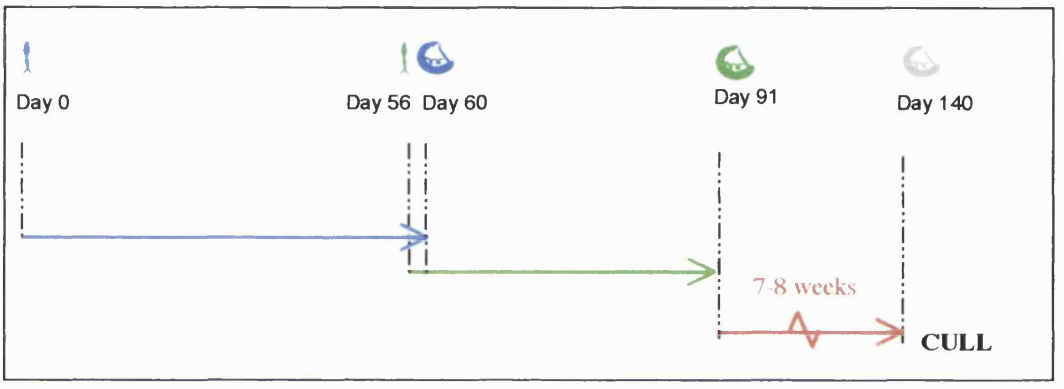
20µl of each sample was mixed with 3µl of loading dye and run out on a 1% agarose gel containing ethidium bromide.

In addition to the W1 method of sex determination, controls for each snail were carried out by infecting one control mouse for each shedding snail and waiting approximately 35 days for the development of the adult schistosomes (which have morphologically distinct sexes) before culling the mice to reveal the schistosome sex.

Hamsters were exposed individually via the paddling technique (section 2.1.4) to 200 mixed male and female cercariae of *S. haematobium*, and then either to 100 male cercariae of *S. mansoni* (experiment #1) or to 100 female *S. mansoni* cercariae (experiment #2) 8

weeks later, so that worms of the initial *S. haematobium* infection would be ready to form pairs approximately 5 weeks before the *S. mansoni* worms were ready to do so (Fig 2.12)

Fig 2.12 Schematic representation of the time-course of infections in chapter 7.
= infection with both sexes of *S. haematobium* cercariae = infection with either female or male *S. mansoni* cercariae = onset of patency (and pairing) for *S. haematobium* = onset of patency (and pairing) for *S. mansoni* = recovery of heterospecific and/or homospecific worm pairs, plus unpaired worms



The hamsters were culled, and worms collected by perfusion and dissection of the hepatic portal vein and mesenteric venous systems of each infected mouse 7-8 weeks post- reinfection (section 2.1.5). Each pair of worms recovered, and any unpaired worms from the infected mice were segregated into individual containers and the male and female worms from each pair were separated. Mature females were taxonomically identified whilst still alive: immature females and male worms were stored in separate, labelled cryotubes in liquid nitrogen prior to identification.

Species identification

Gravid females were taxonomically identified by the microscopic examination of the morphology of intrauterine eggs: those of *S. haematobium* have a terminal spine (Fig 1.7 *c*), whilst *S. mansoni* eggs have a pronounced lateral spine (Fig 1.7 *b*).

Non-gravid females with a clearly discernible ovary were identified by the ovary postion which tends to be at/in the posterior third of the body in *S. haematobium*, but in the anterior half of *S. mansoni* females (Fig 2.13) (Cowper, 1971).

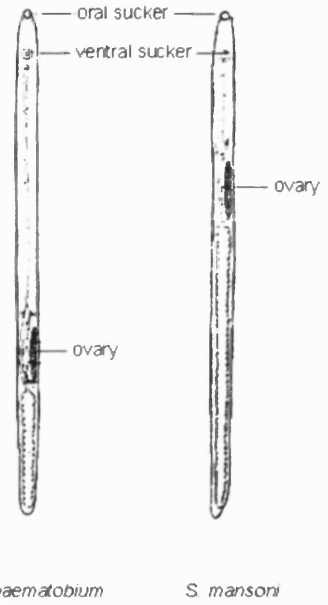


Fig. 2.13. Adult females of *S. haematobium* and *S. mansoni* (diagrammatic), showing the differing position of the ovary. Adapted from Cowper (1971).

Immature females without a discernible ovary were identified by extracting and purifying DNA from them (section 2.3.1) and testing a sample of the DNA for PCR amplification of the *S. mansoni*-specific Sm107 marker as described in section 2.2.1. This marker produces a characteristic "ladder" pattern for *S. mansoni* , allowing *S. mansoni* to be distinguished from other species such as *S. haematobium* (Fig 2.14).

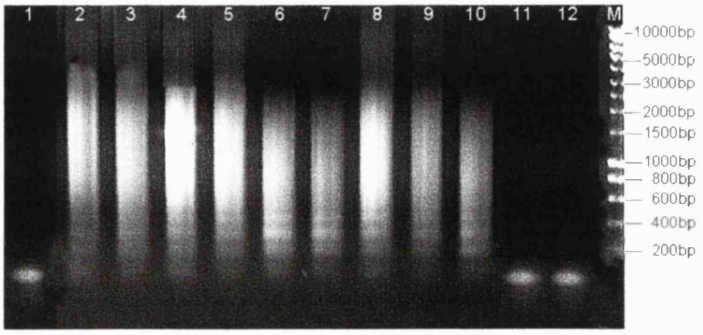


Fig. 2.14. PCR amplification of marker Sm107 to distinguish between immature females of *S. mansoni* (lanes 2-10) and *S. haematobium* (lanes 1, 11 and 12). Lane M, size marker (Bioline "Hyperladder I").

Male worms were identified by examining them for glucose-6-phosphate-dehydrogenase (G6PDH) activity using the isoelectric focusing technique (section 2.3.3). *S. haematobium* and *S. mansoni* are both monomorphic for G6PDH but differ in the pI value of this enzyme, producing an identifiable profile which enables the two species to be distinguished from one another (Fig 2.15)

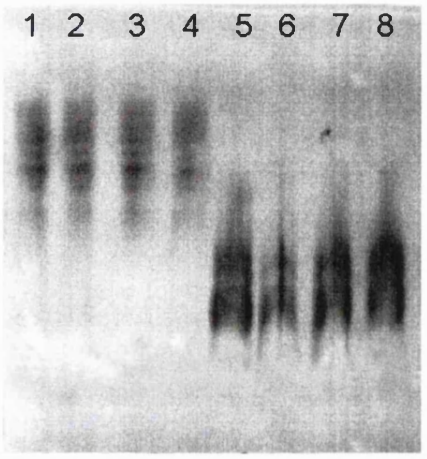


Fig. 2.15 G6PDH enzymes from extracts of adult male worms. Lanes 1-4, *S. haematobium*; lanes 5-8, *S. mansoni*.

2.3 PROTOCOLS FOR CHAPTERS 3-7

2.3.1 EXTRACTION OF DNA FROM SINGLE WORMS USING PHENOL /CHLOROFORM

NB. If worms had been fixed and stored in ethanol, they were soaked in a large volume of ddH_20 for at least 2 hours prior to commencing the extraction.
Buffer and reagent recipes are given in section 2.5

1. The worm was placed in a 1.5ml microfuge tube
2. 10µl of Extraction Buffer 1 (without SDS) was added and the worm was homogenised using a pestle.
3. 10µl of Extraction Buffer 2 (containing 1% SDS) was added to the homogenate (**NB.** Addition of SDS before homogenising the worms results in foaming and reduced sample recovery, hence it is added afterwards)
4. 5µl of Proteinase K stock solution was added
5. The tubes were incubated at 37°C overnight
6. 40µl TE Buffer pH 7.6-8.0 was added to increase the sample volume
7. An equal volume (i.e. 65µl) of a 50:50 mixture of phenol and "chloroform-IAA" was added
8. The tubes were centrifuged at 13 000 rpm for 2 mins
9. The upper aqueous layer was transferred to a clean microfuge tube (NB. Wide bore pipette tips were used to avoid damaging the DNA by excess shearing)
10. Steps 7 and 8 were repeated
11. The upper aqueous layer was transferred to a clean microfuge tube
12. An equal volume (65µl) of chloroform-IAA" was added, and the tubes gently inverted approximately 10 times to mix the contents.
13. The tubes were centrifuged at 13 000 rpm for 2 mins
14. The upper aqueous layer was transferred to a clean microfuge tube
15. A 10% volume of 3M sodium acetate solution was added
16. 2 volumes of chilled 100% Ethanol was added
17. The tubes were placed at -20°C for 15 mins
18. The precipitated DNA was recovered by centrifugation at 13 000 rpm, 4°C for 5 mins

19. The supernatant was removed and 1ml of 70% ethanol added to wash the DNA
20. The tubes were centrifuged at 4°C, 13 000 rpm for 2 mins
21. The supernatant was removed and the pellet "air-dried" at 37°C until the last traces of ethanol had evaporated
22. The pellet was dissolved in 10µl of TE Buffer pH 7.6-8.0, at 37°C for 1 hr
23. The extractions were stored at -20°C until use.

2.3.2 EXTRACTION OF DNA FROM BULK CERCARIAE

1. Water containing freshly-shed cercariae was drawn into a 60ml syringe
2. A 0.2µm membrane filter was attached to the syringe and all except 1-2ml of the water was filtered
3. With the membrane filter still attached, the remaining water was drawn back into the syringe barrel to resuspend the cercariae concentrated on the membrane
4. Thbe cercarial concentrate was transferred to a 2ml microfuge tube
5. The tube was placed on ice for 5 mins to slow cercarial activity
6. The tube was centrifuged at 4°C at 13 000 rpm for 5 mins
7. The supernatant was quickly decanted into 80% ethanol and discarded: the tube containing the cercarial pellet was retained
8. The DNA was extracted and purified from the cercarial pellet as described for single worms in section 2.3.1.

2.3.3 ISOELECTRIC FOCUSING

Overview

Isoenzymes are enzymes with the same catalytic function but which are structurally different, and those belonging to certain enzyme systems are often characteristic of different species and strains of schistosome (Wright *et al.,* 1979 *b*; Fletcher *et al.,* 1981; Wright & Ross, 1983). Every protein possesses an isoelectric point (pI): the pH at which its net charge is zero and consequently migration in an electric field ceases. Polyampholines, amphoteric molecules (acidic and basic character) are contained within the gel, and align to form a stable pH gradient when a charge is applied. Isoelectric focusing is an electrophoretic technique in which proteins are separated on a polyacrylamide gel with a pH gradient running between the electrodes – proteins migrate through the gel until they reach the pH corresponding to their pI. The technique usually results in a very good separation of isoenzyme bands, and the pH gradient of the gel can be substantially modified by mixing carrier ampholytes. Isoenzyme separation is visualized by overlaying the gel with agar containing the appropriate enzyme substrate, which produces a strong colour in the presence of the enzyme.

Sample preparation

1. Worms were recovered from liquid nitrogen and thawed
2. Each worm was transferred into a separate 1.5ml tube
3. 12µl of cold ddH_2O was added and the worms homogenised thoroughly using a pestle.
4. The tubes were centrifuged at 4°C, 13 000 rpm for 10 mins
5. The supernatants were transferred to fresh tubes and kept on ice until ready for use (NB. The technique depends upon the enzyme remaining active, and therefore samples were kept on ice as much as possible).

Preparation of acrylamide gels (for the LKB Multiphor Horizontal IEF System)

1. A small quantity of ddH_2O was applied to a plain glass plate and a sheet of GelBond® Pag film (from Amersham Pharmacia Biotech) rollered onto it
2. Another glass plate with spacers was clipped onto the GelBond® and the cassette stood upright

3. The acrylamide gel solution was made up as below:

Reagent	**Volume**
ddH_20	12.0 ml
Acrylagel (National Diagnostics)	3.15 ml
Bis-acrylagel (National Diagnostics)	1.50 ml
Ampholine (pH 4-7.5 for G6PD; pH 3.5-9.5 for AcP) (Amersham)	1.45ml
10% ammonium persulphate	94µl

4. The solution was drawn into a syringe and a wide bore needle attached
5. The needle was inserted between the gel plates, and the solution poured into the cassette
6. The gel was left for 30 mins to polymerise
7. Once set, a scalpel was inserted between the gel bond and the plain glass plate to remove the latter, and the GelBond® peeled away from the remaining plate
8. Gels were used immediately or stored overnight at 4°C in a sealed polythene bag.

Pre-focusing of gels to establish the pH gradient

1. Two wicks were soaked in anode electrolyte (1M H_3P0_4) and cathode electrolyte (1M NaOH), respectively; blotted, and the ends trimmed to the length of the gel
2. 2ml of 0.1% Triton-X was applied to the gel cooling plate of the electrophoresis tank, and the gel lowered on top, ensuring no air bubbles were trapped.
3. Any excess liquid was blotted from around the gel, and the anodic wick placed along the top edge of the gel, and the cathodic wick along the bottom edge of the gel
4. The electrodes were positioned over the wicks
5. The gel was pre-focused for 20 mins at the following settings:

	1 gel	2 gels
Power (W)	15	30
Voltage (V)	2000	2000
Current (mA)	15	30

Focusing

1. When pre-focusing was complete, 5µl of each sample was applied to the gel, at the cathodic end for G6PDH and at the anodic end for AcP
2. At the far right-hand end of the gel 2µl of haemaglobin was applied at the anode as a focusing control
3. The gel was focused for 1 hr at the following settings:

	For G6PD	**For AcP**
Power (W)	25	25
Voltage (V)	2000	2000
Current (mA)	30	50

4. While the gel was running, the enzyme development mixture was prepared as below
5. When the gel was focused and the enzyme development mixture was ready, the gel was removed from the electrophoresis tank, placed in a developing tray, and the wicks discarded.

Enzyme development

1. 100ml of buffer (0.2M Tris-HCl, pH 8.0 for G6PDH; 0.1M sodium acetate, pH 5 for AcP) was boiled, and, while mixing fast, 1.5g of agar added
2. Heating was continued until the agar had dissolved, being careful not to let it boil over.
3. The agar was kept at approx 60°C until required.
4. 50ml of the enzyme development mixture was prepared (section 2.5.5) and combined with the hot agar
5. The development/agar solution was poured over the gel in the developing tray
6. For G6PDH, the tray was covered with a light-proof lid so that the gel was in darkness (the enzyme reaction is light sensitive)
7. When the agar had set, the tray was placed at 37°C to facilitate the enzyme reaction and speed up colour development.
8. When the enzyme reactions were sufficiently developed, they were stopped with a solution of 5% glacial acetic acid and 4% glycerol
9. The gel was left overnight in stop solution (keep G6PDH gel in darkness)

10. The stop solution was then poured off, and the gel washed with a solution of 5% glacial acetic acid and 3% glycerol.
11. The wash solution was changed 2-3 times daily until the gel background had completely de-stained.
12. The agar was then carefully peeled away and discarded, and the gel (on its gel-bond mount) placed on a plate warmer at 40°C for approximately 24 hours until dried down.

STATISTICAL ANALYSIS OF DATA

The data from the experiments in chapters 1-5 were analysed using the Mantel-Haenszel test (Mantel & Haenszel, 1959) to evaluate the significance of the observed proportions. This test examines the data from each infected animal as a separate "2 by 2" table, as below. The test calculates the ratio of data values in the top row of the table to those in the bottom row; or the ratio of data values in the left-hand column to those in the right-hand column, or the ratio of data values in one diagonal to those in the other diagonal, and translates these into "odds ratios", and "log odds ratios". For example, to compare the proportion of males of both types (Sh and Sm) paired heterospecifically in the example table below, the Mantel-Haenszel test would calculate odds ratios based on the ratio of one of the diagonals to the other. These values are then assessed to see if they differ significantly from a nil log odds ratio.

Example "2 x 2" table:

	Sh ♀	Sm ♀
Sh ♂	Sh ♂ x Sh ♀	Sh ♂ x Sm ♀
Sm ♂	Sm ♂ x Sh ♀	Sm ♂ x Sm ♀

2.4 PROTOCOLS FOR CHAPTER 8

2.4.1 DNA EXTRACTION FROM MULTIPLE WORMS

Separate stocks of male and female *S. haematobium* DNA were prepared from a pool of worms originating from Nigeria; Senegal (Mbodienne); Cameroon (Barombi Kotto) and South Africa: 15 worms of each sex from each country were used (with the exception of Nigeria where only 5 worms of each sex were available), giving 50 males and 50 females in total.

DNA was purified using the the phenol/chloroform extraction protocol (with ethanol precipitation) in section 2.3.1, but with volumes of reagents scaled up to 250µl of each extraction buffer, and 500µl of each organic phase. In addition, after incubation with Proteinase K at 37°C (step 4 in section 2.3.1), 5µl of DNAse-free RNAse stock solution (10mg/ml) was added to each extraction and incubated at 37°C for approximately 3 hours prior to carrying out the organic extraction steps. The DNA pellet was dissolved in 100µl TE Buffer pH 7.6-8.0.

An estimation of the quantity of DNA in each sample was obtained by taking spectrophotometric readings at a wavelength of 260nm using a GeneQuant™ machine (Pharmacia). To check the quality of the DNA (i.e. to check that it was not sheared) a sample of each DNA preparation was run out on an agarose gel (result not shown) and both DNA samples observed to be intact.
An aliquot of the female DNA preparation (at a concentration of 125ng/µl) and the male DNA preparation (at a concentration of 390ng/µl) was diluted to give a concentration of 10ng/µl for each sample, ready for use as template DNA in PCR reactions.

2.4.2 PCR AMPLIFICATION OF RAPD MARKERS

A total of 50 decamer oligonucleotides (RAPD Primer Set from Operon Corp) were used for the amplification of random DNA markers to reveal any differences between *S. haematobium* males and females (Table 2.1).

Table 2.1. 5' to 3' sequences for all 50 RAPD primers used in screening for female-specific markers

PRIMER	5' to 3' sequence
AB 9-1	5'-ACTCCACGTC-3'
AB 9-2	5'-CACCGCAGTT-3'
AB 9-3	5'-AGCCAGGCTG-3'
AB 9-4	5'-GGCGTAAGTC-3'
AB 9-5	5'-GGGTGCAGTT-3'
AB 9-6	5'-GGGAACCCGT-3'
AB 9-7	5'-TCGCTGCGGA-3'
AB 9-8	5'-AAGGCTGCTG-3'
AB 9-9	5'-GGGGGAGATG-3'
AB 9-10	5'-CTGTGTGCTC-3'
AB 9-11	5'-GTCCATGCAG-3'
AB 9-12	5'-AACGGCGGTC-3'
AB 9-13	5'-CTTCCAGGAC-3'
AB 9-14	5'-AGCCGGGTAA-3'
AB 9-15	5'-TGATGCCGCT-3'
AB 9-17	5'-TCAGCACAGG-3'
AB 9-18	5'-TGTCCTGCGT-3'
AB 9-19	5'-ACCACGCCTT-3'
AB 9-20	5'-GAGTCCTCAC-3'
AB 10-2	5'-GTCCTCGTGT-3'
AB 10-4	5'-GTCTTGGGCA-3'
AB 10-5	5'-GTCACCTGCT-3'
AB 10-7	5'-GACGAGCAGG-3'
AB 10-8	5'-GGCTGCCAGT-3'
AB 10-11	5'-ACCGTGCCGT-3'
AB 10-12	5'-TGACCAGGCA-3'
AB 10-13	5'-CACGGACCGA-3'
AB 10-14	5'-TCGCAGCGTT-3'
AB 10-16	5'-AACCCTTCCC-3'
AB 10-17	5'-AGTTCCGCGA-3'
AB 10-18	5'-GTTGCGCAGT-3'
AB 10-19	5'-TGACAGCCCC-3'
AB 11-1	5'-GGCATGACCT-3'
AB 11-2	5'-TGGGCGTCAA-3'
AB 11-4	5'-GACTGCACAC-3'
AB 11-5	5'-ACGCAGGCAC-3'
AB 11-6	5'-GAGGGAAGAG-3'
AB 11-8	5'-AGCAGGTGGA-3'
AB 11-9	5'-TGCGAGAGTC-3'
AB 11-10	5'-TGGGAGATGG-3'
AB 11-11	5'-ACGATGAGCC-3'
AB 11-12	5'-GGGCGGTACT-3'
AB 11-13	5'-ACCGCCTGCT-3'
AB 11-14	5'-GTGACAGGCT-3'
AB 11-15	5'-AAGAGAGGGG-3'
AB 11-16	5'-AGGTTGCAGG-3'
AB 11-17	5'-AGCCTGAGCC-3'
AB 11-18	5'-ACCACCCACC-3'
AB 11-19	5'-GAGTGGTGAC-3'
AB 11-20	5'-TGGTGGACCA-3'

For amplification of the RAPD markers, 25µl PCR reactions were set up as below:

Reagent	**Volume**
DNA	1µl at 10ng/µl
dNTP	4µl (at 1.25mM)
Promega buffer	2.5µl
Promega *Taq* DNA polymerase	0.125µl (at 5U/µl)
Oligonucleotide primer	1µl (at 16 pmol/µl)
dH_20	16.375µl
TOTAL	25µl

Amplification was performed in a Biometra UNO PCR machine programmed for 2 cycles at low stringency, the first with an initial long denaturation; and 33 cycles at higher stringency, once the initial fragments have formed, and then 1 cycle with a long final extension as follows:

1 cycle at 95 °C for 5 mins, 30°C for 2 mins and 72°C for 1 min
1 cycle at 95 °C for 30 secs, 30°C for 2 mins and 72°C for 1 min
33 cycles at 95°C for 30 secs, 40°C for 2 mins and 72°C for 1 min
1 cycle at 95°C for 30 secs, 40°C for 2 mins and 72 °C for 5 min

After amplification, the 25µl samples were mixed with 5µl of loading dye and run out on 2% agarose gels containing ethidium bromide. The gel was examined for any female-specific markers.

From the above reactions, only oligonucleotide AB10-18 amplified an apparently female-specific PCR product, approximately 700bp in length (called Sh700). This result was verified by repetition of the PCR amplification reaction using primer AB10-18. To be certain that the ~700bp PCR product represented a discrete marker, rather than several, co-migrating RAPD-generated fragments of very similar size, it was necessary to clone the PCR product (see below), and sequence multiple clones: if all sequences are identical, then the 700bp product represents a discrete fragment.

2.4.3 EXTRACTION AND PURIFICATION OF A PUTATIVE FEMALE-SPECIFIC RAPD-PCR PRODUCT

To obtain the larger quantities of this putative marker neceesary for gel purification and cloning, RAPD amplification was repeated using AB10-18 as primer and female *S. haematobium* template DNA (as above). The RAPD products were run out on 2% agarose gels containing ethidium bromide. Bands of the correct size were then cut from the gel with a scalpel blade and purified, using QIAquick® Gel Extraction columns (Qiagen) and the following protocol:

1. Gel slices containing the DNA bands were placed into 2 separate, pre-weighed 2ml tubes and weighed.
2. 480µl of QG buffer was added to each sample (equivalent to 6 gel volumes, assuming 100mg of gel = 100µl. Note: for < 2% agarose gels use 3 gel volumes)
3. Samples were incubated at 50°C for 10 min to solubilise the gel
4. After incubation, the solution pH was checked using its internal reference dye: a yellow colouration indicates that the pH is $\leq$ 7.5, the only pH conditions under which DNA will efficiently bind to the membrane in the QIAquick® spin columns
5. 80µl (1 gel volume) of isopropranol was added to each sample
6. QIAquick® spin columns were placed in 2ml microfuge tubes
7. To bind DNA, the samples were applied to the spin columns and centrifuged for 1 min at 13 000 rpm
8. The flow-through was discarded and 0.5ml of Buffer QG placed in the column to wash the membrane and remove all traces of agarose
9. The column was centrifuged for 1 min at 13 000 rpm
10. The flow-through was discarded and 0.75ml of Buffer PE added to the column to wash the DNA
11. Samples were left to stand for 5 mins and centrifuged for 1 min at 13 000 rpm (this incubation is recommended if DNA is destined for salt-sensitive applications)
12. The flow-through was discarded and the QIAquick® columns centrifuged for an additional 1 min to completely dry the membrane
13. The QIAquick® columns were placed in clean 1.5ml tubes and 50µl of Buffer EB placed on the centre of the membrane in each column to elute the DNA

14. Columns were centrifuged for 1 min at 13 000 rpm
15. The QIAquick® column was discarded and the flow-through, containing the eluted DNA, retained.

The samples containing the purified DNA were pooled, and the DNA concentrated by ethanol precipitation (steps 15-21, section 2.3.1) and resuspended in 20µl TE Buffer, pH 7.6-8.0.

To check the sample for recovery, purity and yield of the ~700bp Sh700 fragment, 5 µl of sample was mixed with 3µl loading dye and run out on a 2% agarose gel containing ethidium bromide. By comparison with the intensity of the Hyperladder I size marker bands, the quantity of DNA in the 5µl of sample loaded was estimated to be 5ng, giving a concentration of 1ng/µl.

2.4.4 CLONING

The ~700bp Sh700 fragment was cloned using the pGEM®-T Easy Vector System cloning kit (Promega). Cloning PCR products into a T-vector (such as pGEM®-T Easy Vector) exploits the fact that *Taq* polymerase adds single 3' adenosyl overhangs to PCR products, which can pair with unpaired 3' thymidyl overhangs on the supplied, unlinearised vector. This is a highly efficient cloning method because of the improved ligation between PCR product and vector and the prevention of vector recircularization due to non-complementarity of vector ends (Sambrook & Russell, 2001; Promega pGEM®-T Easy Vector System manual) (Figs 2.16-2.18).

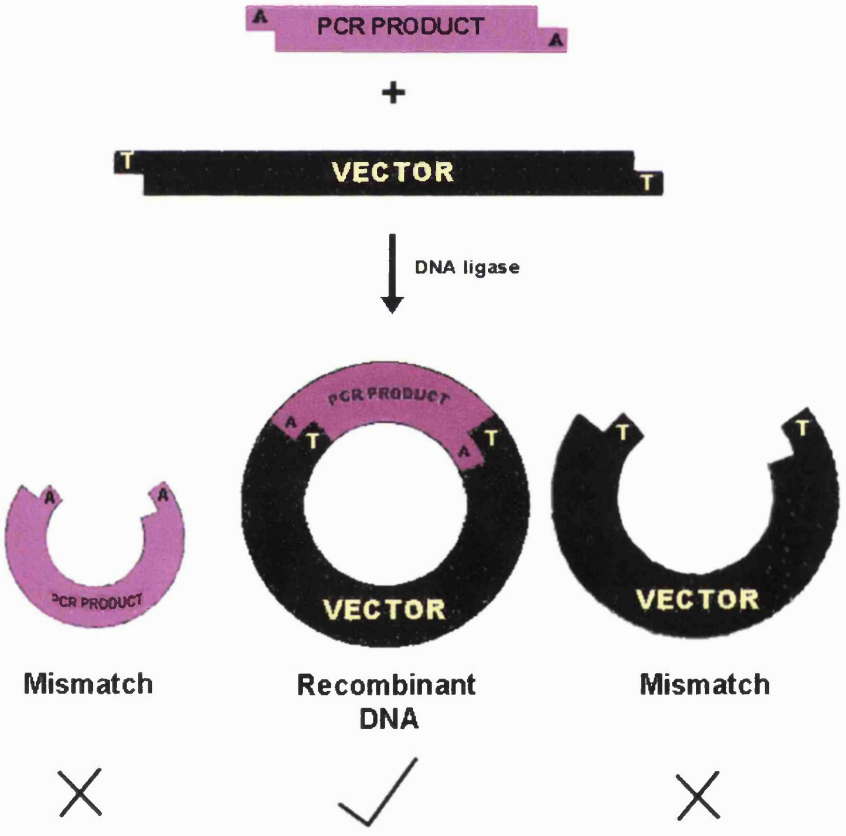


Fig 2.16 Schematic representation of the constructs for cloning. In the presence of DNA ligase, the complementary single "A" and "T" overhangs of the PCR product and the T-Vector ensure that only recombinant DNA consisting of vector ligated to insert can recircularize.

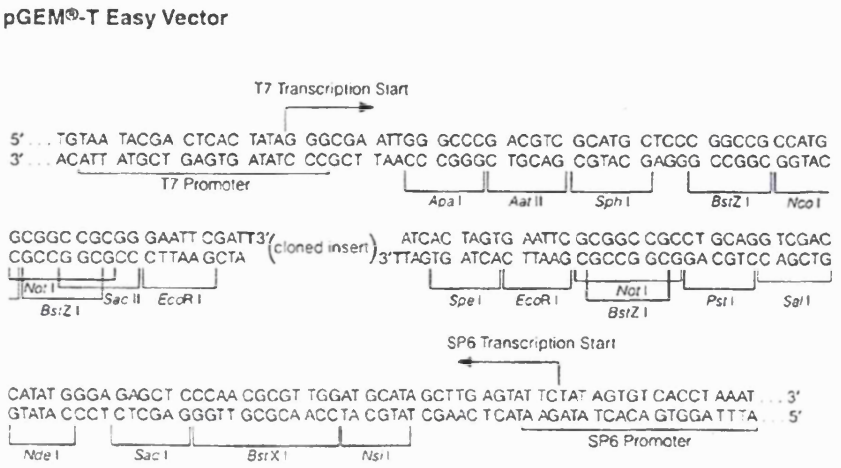


Fig 2.17 The promoter and cloning site sequence of the pGEM®-T Easy Vector (taken from the Promega pGEM®-T Easy Vector System manual)

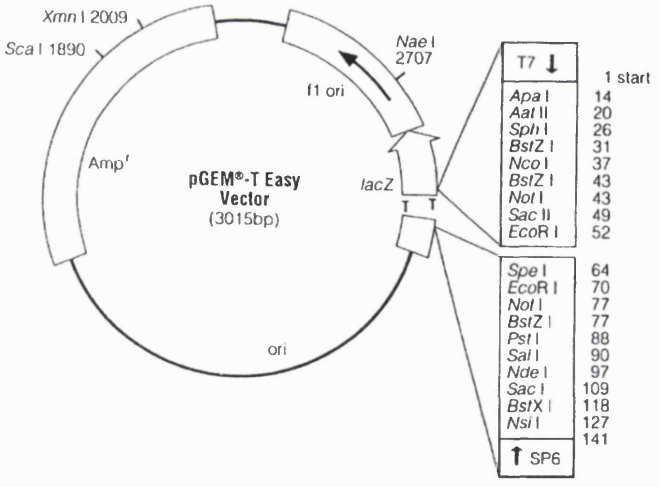


Fig 2.18 pGEM®-T Easy Vector circle map and sequence reference points (taken from the Promega pGEM®-T Easy Vector System manual)

2.4.4.1 Ligation protocol

The ligation reaction was set up as below, using an approximately 1:2 molar ratio of PCR product: vector, together with a positive control (with known and supplied insert) and a background control (no insert). The pGEM®-T Easy Vector and Control Insert tubes were briefly centrifuged prior to use to collect the contents at the bottom of the tube. The 2X Rapid Ligation Buffer was vortexed vigorously before each use.

	Sample Reaction	**Positive Control**	**Negative control**
2X Rapid Ligation Buffer	10µl	5µl	5µl
pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	7µl (at 1ng/µl)	-	-
Control Insert DNA	-	2µl	-
T4 DNA Ligase (3 Weiss units/µl)	2µl	1µl	1µl
Deionized water	-	1µl	3µl
Final volume	**20µl**	**10µl**	**10µl**

The reactions were mixed by pipetting, and incubated at room temperature for 1 hour, and then at 4°C overnight.

2.4.4.2 Transformation protocol

1. The tubes containing the ligation reactions were centrifuged to collect the contents at the bottom
2. 2µl of each ligation reaction was placed into a sterile 1.5ml tube on ice
3. A tube of *Eschericia coli* JM109 High Efficiency Competent Cells was removed from storage at -70°C and thawed on ice (about 5 mins)
4. The cells were mixed by very gently flicking the tube
5. 50µl of cells were carefully transferred into each tube containing a ligation reaction
6. The contents were mixed by gently flicking the tubes, and the tubes placed on ice for 20 mins
7. The cells were heat-shocked for 45-50 secs in a water bath at exactly 42°C (no shaking)
8. The tubes were immediately returned to ice for 20 mins
9. 950µl of room temperature SOC medium was added to the transformed cells
10. The tubes were incubated for 1.5 hrs at 37°C with shaking (~150 rpm)

11. 200µl, 100µl and 50µl aliquots of the sample transformation cultures and 100µl of each control was plated onto separate, pre-dried LB Ampicillin/IPTG/X-Gal plates at room temperature using a sterile glass spreader.
12. The plates were incubated overnight (16-24 hrs) at 37°C

2.4.4.3 Screening Transformants for Inserts

1. Plates were screened for colonies containing inserts: these are generally white because of disruption of the ß-galactosidase coding sequence of the pGEM®-T Easy Vector by PCR product insertion – this renders bacteria unable to convert X–Gal in the plates into a blue substance. However, with short, in-frame inserts, disruption may be incomplete and recombinant colonies can be pale blue.
2. White colonies were picked from the sample plates and gridded onto an LB/Ampicillin agar plate using disposable sterile inoculating loops, to create a plate containing only likely recombinant clones
3. Colonies were incubated at 37°C overnight
4. A small portion of each colony from this plate was then picked using a sterile inoculating loop and transferred into a separate microfuge tube containing 100µl ddH_20.
5. Tubes were heated at 95°C for 5 minutes to lyse the bacterial cells and release the DNA
6. 10µl PCR amplification reactions were set up for each lysate, using primers M13 forward and reverse (which are specific for sites on either side of the cloning site of the vector), as set out below:

Reagent	**Volume**
DNA	1.0µl (of colony lysate)
dNTP	1.6µl (at 1.25mM)
Promega buffer	1.0µl
Promega *Taq* DNA polymerase (in storage buffer B)	0.05µl (at 5U/µl)
Primers M13 (forward and reverse)	0.4µl (at 25pmol/µl)
ddH_20	5.95µl
Total volume	**10µl**

Amplification was performed in a Biometra UNO PCR machine programmed for: 95°C for 5 mins; 30 cycles of [95°C for 30 secs, 59°C for 30 secs and 72°C for 1.5 mins]; 72°C for 5 mins.

Each reaction (the entire 10µl) was mixed with 3µl of loading dye and run out on a 1% agarose gel containing ethidium bromide.

Recombinant colonies containing the insert were those which yielded a PCR product of approximately 1000bp length, representing the ~700bp insert plus vector sequence lying between the primer binding sites and the cloning site. Positive recombinant colonies were picked using a sterile inoculating loop and transferred into universal tubes containing LB/Ampicillin broth. 3ml bacterial cultures were grown overnight by incubation at 37°C, with shaking (300 rpm).

2.4.4.4 Miniprep protocol

To purify plasmid DNA from the recombinant cultures, an alkaline lysis/ DNA binding column method (Perfectprep Plasmid Mini Kit from Eppendorf) was used, following the protocol below:

1. Bacteria were pelleted by centrifuging the universal containers for 5 mins at 5 000 rpm
2. The supernatant was discarded
3. The bacterial pellets were completely resuspended by adding 100µl of Buffered RNase Solution (Solution 1) and vortexing vigorously
4. The resuspended bacteria were transferred to 1.5ml tubes
5. Bacteria were lysed by adding 300µl Alkaline lysis solution (Solution II) and mixing several times by repeated gentle inversion of the tube, until the solution cleared
6. The bacterial lysate was neutralised by adding 300µl Neutralization solution (Solution III) and immediately mixing thoroughly by vigorous inversion of the tube until a granular, whitish precipitate (containing proteins, bacterial chromosomal DNA and large RNA) formed
7. The neutralized lysate was centrifuged for 8 mins at 13 000 rpm to precipitate the bacterial cell material

8. The cleared supernatant was transferred to a (supplied) spin column in a 2ml collection tube.
9. 50µl DNA Binding Matrix Suspension was added (having mixed it vigorously first), and mixed vigorously by pipetting up and down
10. The tube was left to stand for 2 mins, and then centrifuged for 1 min at 13 000 rpm
11. The filtrate was decanted, and the spin column replaced in the collection tube
12. 600µl Dilution Purification Solution was added to the spin column and centrifuged for 1 min at 13 000 rpm to wash the DNA binding matrix
13. The filtrate was decanted and the spin column replaced in the collection tube
14. A further 600µl of Dilution Purification Solution, pre-heated to 70°C, was added, and centrifuged for 1 min at 13 000 rpm
15. The filtrate was decanted and the spin column replaced in the collection tube
16. The tube was centrifuged for a further 2 mins at 13 000 rpm to thoroughly dry the DNA binding matrix
17. The spin column was transferred to a fresh collection tube and 80µl of ddH_20 added directly to the DNA binding matrix in the spin column
18. The tubes were left to stand for 2 mins, and then centrifuged for 1 min at 13 000 rpm
19. The used spin column was discarded and the eluted plasmid DNA retained
20. The plasmid DNA was stored at -20°C until use.

5µl of plasmid DNA from each sample was mixed with 2µl loading dye and run out on a 1% agarose gel to confirm the presence, quality and yield of the DNA. The concentration of plasmid DNA in each was determined to be approximately 30ng/µl by comparison of the intensity of the plasmid DNA bands with a pGEM standard marker.

2.4.5 SEQUENCING

Overview

Dideoxy chain-termination sequencing (Sanger, 1977) was carried out using the Applied Biosystems (ABI) PRISM® BigDye™ Terminator Version 2 Cycle Sequencing Kit and an ABI 377.96XL automated DNA sequencer.

Dye-terminator systems for DNA sequencing use fluorescently labelled ddNTPs to tag the 3' terminal position of primer-mediated extension products from the sequencing reactions. Each of the four ddNTPs in the ABI PRISM® BigDye™ Terminator kits is labelled with a different dichlororhodamine acceptor dye linked to a fluorescein donor dye. The cycle sequencing reaction produces a "ladder" of extension products all starting at the same initiation point and terminating in a fluorescently labelled ddNTP. The donor dye, when excited, transfers energy to the acceptor dyes which each have a different emission spectrum. Four chain-extension reactions can therefore be carried out in a single tube, and the products of the reactions loaded in a single lane for gel electrophoresis. Cycle sequencing uses a cyclical primer extension reaction to linearly increase the quantity of sequencing reaction products, and so amplify the fluorescent signals and increase the sensitivity of the sequencing, in addition to decreasing the quantity of template required.

2.4.5.1 ABI PRISM® BigDye™ Terminator Cycle Sequencing protocol

M13 primers (forward and reverse) were used separately to sequence all (13) clones, starting from the vector either side of the cloning site. The cycle sequencing reactions were set up as follows:

	Per Reaction
Primer (M13 F or R) at 1.6pmol/µl	2.0µl
DNA (250ng)	8.3µl
Terminator Ready Reaction Mix	4.0µl
Dilution Buffer	4.0µl
ddH_20	1.7µl
Total volume	**20µl**

Amplification was carried out in a GeneAmp PCR System 9700 thermal cycler programmed for 25 cycles (with rapid thermal ramp speed of 1°C/sec) of [96°C for 10 secs; 50°C for 5 secs; 60°C for 4 mins].

2.4.5.2 Removal of Excess Dye Terminators by Ethanol Precipitation

Following cycle sequencing, it is necessary to completely remove any unincorporated dye terminators before analysing the samples, as excess dye would obscure data in the early part of the sequence. This is achieved by ethanol precipitation.

1. The entire contents of each sequencing reaction was transferred into separate 1.5ml tubes containing 50µl of 95% ethanol and 2µl of 3M sodium acetate, pH 5.2
2. The tubes were vortexed briefly and placed at room temperature for 15 mins
3. The tubes were then centrifuged for 30 mins at 13 000 rpm
4. The supernatant was aspirated without disturbing the pellet. (NB. It is extremely important that all the ethanol was removed at this stage, otherwise the "dye blobs" would not be removed in the later wash steps)
5. The pellet was rinsed with 100µl 70% ethanol and centrifuged for 10 mins at 13 000 rpm
6. The ethanol was then aspirated
7. The tubes were then placed with lids open in a heating block at 90°C for 1 min to dry the pellet
8. The samples were stored at –20°C prior to use.

Samples were run on an ABI 377.96XL automated sequencer by staff of the NHM sequencing facility. The results were entered into Sequencher v3.1.1 software where the vector sequence was stripped. For each of the 13 recombinant templates, the forward and reverse reads were aligned, and by comparing the chromatograms, ambiguities were resolved, and any discrepancies checked. A consensus sequence was generated for each recombinant template, and these were then aligned to produce an "all template" consensus (Table 8.1).

2.4.6 DESIGN OF SPECIFIC PRIMERS FOR THE CLONED SEQUENCE

"Primer Express" software was used to design forward and reverse primers to the resulting consensus sequence. The chosen forward and reverse primers differed by less than 1°C in their melting temperature (61°C and 60.3°C), with a GC content of 63% and 50% and a length of 19bp and 22bp, respectively. The length of the fragment amplified ('amplicon') was 701bp. The sequences of the forward and reverse primers are given below.

	Primer names	**5' to 3' sequence**
Forward	Sh700f	GCGCAGTCACAGTGAAGCG
Reverse	Sh700r	CAGTTGAGGAGAAAACCTTCGG

2.4.7 PCR AMPLIFICATION OF THE Sh700 SEQUENCE WITH SPECIFC PRIMERS

The primers were tested for female-specificity in PCR amplification reactions using male and female DNA from the original mixture of *S. haematobium* isolates, and, separately, from individual isolates from S. Africa, Nigeria, Cameroon, Senegal and Zambia. All DNA extractions were carried out using the phenol/chloroform protocol in section 2.4.1 (adapted from the phenol/chloroform protocol in section 2.3.1).

25µl PCR reactions were set up as below:

Reagent	**Volume**
DNA	1µl at 25ng/µl
dNTP	4µl (at 1.25mM)
Promega buffer	2.5µl
Promega *Taq* DNA polymerase	0.25µl (at 5U/µl)
Primer (Sh700f and r)	1µl of each (at 30 pmol/µl)
dH_20	15.25µl
Total volume	**25µl**

Amplification was performed in a Biometra UNO PCR machine programmed for: 95°C for 5 mins; 30 cycles of [95°C for 15 sec, 60°C for 15 sec and 72°C for 1 min]; 72°C for 5 mins. The annealing temperature was chosen as 60°C, as this is less than 5°C of the melting temperature of primers Sh700 f and r.

After amplification, 20µl of each reaction was mixed with 3µl of loading dye and run on a 1% agarose gel containing ethidium bromide.

2.5 BUFFERS AND REAGENTS

2.5.1 FOR LIVE MATERIAL PROTOCOLS

0.85% saline (for perfusion and snail infections)

8.5g sodium chloride

Dissolved in 200ml ddH_20

ddH_20 to a final volume of 1L

Citrate saline (for perfusion)

7.5g sodium citrate

8.5g sodium chloride

Dissolved in 200ml ddH_20

ddH_20 to a final volume of 1L

2.5.2 FOR DNA EXTRACTION

1M Tris-HCl pH 8.0

121.1g Tris-base

Dissolved in 800ml ddH_20

Adjusted the pH to 8.0 by adding concentrated HCl

dd H_20 to a final volume of 1 L

Autoclaved

DNA Extraction Buffer 1

50 ml 1M Tris-HCl pH 8.0 (final concentration 50mM)

20ml 5M NaCl (final concentration 100mM)

10ml 0.5M EDTA (final concentration 50mM)

ddH_20 to a volume of 500ml

Autoclaved

DNA Extraction Buffer 2 (+ 1% SDS)

250ml DNA Extraction Buffer 1

2.5g sodium dodecyl sulphate (SDS)

70% Ethanol

70ml Ethanol 100% v/v AnalR

30ml ddH_20

Mixed and stored at 4°C

Proteinase K Digestion Buffer

10ml 1M Tris-HCl pH 8.0 (final concentration 10mM)

3ml 5M NaCl (final contration 15mM)

ddH_20 to a final volume of 100ml

Proteinase K 10mg/ml

25mg of Proteinase K from *Tritarachium album* (supplied as powder, from Roche)

1.25ml Proteinase K Digestion Buffer

Aliquoted in 50µl volumes and stored frozen

DNAse-free RNAse 10mg/ml

20mg bovine pancreatic RNAse A (supplied as powder)

Dissolved in 2ml ddH_20

Heated to 100°C for 15 mins

Aliquoted in 100µl volumes and stored frozen

Phenol

Supplied (from Sigma) saturated with 10mM Tris-HCl pH 8.0, 1mM EDTA

Added equilibration buffer (supplied) to adjust the phenol phase to pH 7.9 ± 0.2

Shook and allowed phases to separate

Stored at 2-8°C

"Chloroform-IAA"

Mixed 24 parts chloroform with 1 part isoamyl alcohol

Stored at 4°C

TE Buffer, pH 8.0

0.6055g Tris-base (final concentration 10mM)

1.861g EDTA (final concentration 1mM)

Dissolved in 450 ddH_20 and adjust pH to 8.0

ddH_20 to a final volume of 500ml

Autoclaved

2.5.3 FOR PCR REACTIONS

10X PCR Buffer

670mM Tris-HCl (pH 8.8)

20mM $MgCl_2$

dNTP

(Supplied by Amersham Pharmacia Biotech as separate 100mM solutions of each dNTP)

10µl of each of the 4 solutions at 100mM

760µl ddH_20

***Taq* DNA Polymerase 10X Reaction Buffer with $MgCl_2$ (Promega)**

***Taq* DNA Polymerase (Promega)**

Supplied as 5U/µl in Storage Buffer B

MasterAmp™ *Taq* DNA Polymerase (Cambio)

Supplied as 5U/µl in a storage buffer

2.5.4 FOR GEL ELECTROPHORESIS

EDTA (0.5M, pH 8.0)

Dissolved 93.05g of EDTA (disodium salt) in 450ml double distilled H_20 (ddH_20) and adjusted to pH 8.0 with NaOH

ddH_20 to a final volume of 500ml

Autoclaved

TAE Buffer x50

Dissolved 242g of Tris-base (MW 121.1) in approximately 800ml ddH_20

Added 57.1ml glacial acetic acid

Added 100ml 0.5M EDTA, pH 8.0

ddH_20 to a final volume of 1 lL

A working concentration of 1x TAE buffer was used to make agarose gels, and as the electrophoresis buffer.

Agarose gels

All agarose gels were made with 1x TAE buffer prepared from 50X stock

Added agarose (Agarose 1, from Amresco) to the desired volume of 1xTAE buffer (1g agarose per 100ml of buffer for a 1% gel, 2g per 100ml for a 2% gel etc.)

Heated in microwave until agarose had dissolved

Cooled to 60°C and added ethidium bromide (2.5µl stock at 10mg/ml per 50ml of buffer)

Poured the gel at approximately 55°C

Electrophoresis buffer

See TAE buffer

Gel-loading dye

40g sucrose

0.02g bromophenol blue

0.02g xylene cyanol

ddH_20 to 100ml

Stored at 4°C

DNA Size and Quantitation Markers

Hyperladder I (Bioline), 200 - 10 000bp

pGEM standard at 200ng/µl (Promega)

2.5.5 FOR IEF

2.5.5.1 IEF SEPARATION

Ampholine mixtures

pH 3.5-9.5 (for AcP)

Preblended, from Amersham Pharmacia Biotech

pH 4-7.5 (for G6PDH)

33ml preblended ampholine, pH 4-6 (from Amersham Pharmacia Biotech)

+ 15ml preblended ampholine, pH 5-7 (from Amersham Pharmacia Biotech)

+ 60ml preblended ampholine, pH 5-8 (from Amersham Pharmacia Biotech)

10% Ammonium persulphate

10g of ammonium persulphate (powder)

100ml ddH_20

Anode Electrolyte (1M H_3P0_4 from Sigma)

Cathode Electrolyte (1M NaOH)

11.5g of 85.2% pure NaOH powder

100mls ddH_20

0.1% Triton-X-100

0.1ml Triton-X-100 solution

ddH_20 to a final volume of 100ml

2.5.5.2 AcP DEVELOPMENT

0.1M sodium acetate, pH 5.0 (AcP Development Buffer)

Dissolved 27.2g of sodium acetate trihydrate in 500 ml ddH_20

Adjusted pH to 5.0 using concentrated HCl

ddH_20 to a final volume of 2L

10% $MnCl_2$

10g of $MnCl_2.4H_20$ powder

100ml ddH_20

AcP Enzyme Development Mixture

Added 4ml of 10% $MnCl_2$ to 50ml of sodium acetate, pH 5

Warmed to 50°C

Dissolved 0.2g of α-naphthyl acid phosphate (α-NAP) and 0.1g of Fast Blue RR (both from Sigma) in the warmed buffer

Filtered through filter paper

2.5.5.3 G6PDH DEVELOPMENT

0.2M Tris-HCl pH 8.0 (G6PDH Development Buffer)

48.44g Tris-base

Dissolved in 800ml ddH_20

Adjusted the pH to 8.0 by adding concentrated HCl

dd H_20 to a final volume of 2L

Autoclaved

10% $MgCl_2$

10g of $MgCl_2.6H_20$ powder

100ml ddH_20

Phenazine methosulphate (PMS) (100mg/ml)

2g PMS (powder)

20ml ddH_20

Thiazolyl blue (MTT) (50mg/ml)

1g MTT (powder)

20 ml ddH_20

G6PDH Enzyme Development Mixture

1.5ml 10% $MgCl_2$

0.02g G6P (glucose-6-phosphate, monosodium salt, ≈ 98%, Sigma)

0.015g NADP (ß-Nicotinamide adenine dinucleotide 3'phosphate sodium salt, ≈ 90%, Sigma)

Dissolved the above in 50ml Tris-HCl pH 8.0

Added 300µl MTT (at 50mg/ml) and 100µl PMS (at 100mg/ml)

2.5.5.4 GEL FIXING/ WASHING

Stop solution (4% glycerol, 5% acetic acid)

40ml glycerol

50ml glacial acetic acid

ddH_20 to a final volume of 1L

Wash solution (3% glycerol)

30ml glycerol

50ml glacial acetic acid

Added ddH_20 to a final volume of 1L

2.5.6 FOR PROTOCOLS IN CHAPTER 8

Ampicillin stock solution (20mg/ml)

Dissolved 1g ampicillin in 50ml ddH_20

Filter-sterilised

Dispensed in 5ml aliquots and stored frozen

X-Gal solution (2% w/v)

Added 0.2g X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) to 10ml dimethyl formamide

Dispensed in 2ml aliquots and stored frozen

IPTG stock solution (100mM)

Added 1.2g IPTG (isopropyl-ß- thiogalactopyranoside) powder (dioxane-free, from Promega) to 50ml ddH_20

Filter-sterilised

Dispensed into 5ml aliquots and stored frozen

(**NB.** IPTG is a reported carcinogen: the powder, stock solutions, working reagents and waste was handled accordingly).

LB broth

10g Bacto®-tryptone (pancreatic digest of casein, from Difco)

5g yeast extract (from Merck)

5g NaCl

Dissolved in 800µl ddH_20

ddH_20 to a final volume of 1 L

Autoclaved

Stored at 4°C

LB/ampicillin broth

Added 5ml of ampicillin stock solution at 20mg/ml to 1L of LB broth to give a final ampicillin concentration of 100µg/ml

Autoclaved

(**NB.** If adding freshly autoclaved broth, it was allowed to cool below 60°C before adding ampicillin).

LB ampicillin/ X-Gal/ IPTG plates

Added 15g Agar to 1 L of LB broth

Autoclaved

Cooled to 50-60°C and added the following:

Ampicillin:	5ml of stock solution (final concentration 100µl/ml)
X-Gal:	2ml of stock solution (final concentration 0.004%)
IPTG:	5ml of stock solution (final concentration 0.5mM)

Poured approximately 25ml into 90mm (diameter), single vented petri dishes

Allowed to set

Stored the plates inverted at 4°C for up to 1 month

Pre-warmed plates and dried agar surface before use

2M Mg^{2+} stock

20.33g $MgCl_2.6H_20$

24.65g $MgSO_4.7H_20$

Added ddH_20 to a final volume of 100ml

Autoclaved

SOC medium

20g Bacto®-tryptone (pancreatic digest of casein, from Difco)

5g yeast extract (from Merck)

10ml 1M NaCl

2.5ml 1M KCl

10ml 2M Mg^{2+} stock

Dissolved in 1 litre ddH_20

Autoclaved

Added 2ml of 1M glucose (filter-sterilised) to 100ml of media prior to use

3M Sodium acetate, pH 5.2

Dissolved 40.81g sodium acetate trihydrate in 80ml ddH_20

Adjusted pH to 5.2 using glacial acetic acid

ddH_20 to a final volume of 100ml

Autoclaved

CHAPTER 3

Mating interactions between *S. mansoni* and *S. margrebowiei*

CHAPTER 3

MATING INTERACTIONS BETWEEN *S. MANSONI* AND *S. MARGREBOWIEI*

INTRODUCTION

S. mansoni is a medically important parasite of humans belonging to the *S. mansoni* group of schistosomes, and has a wide distribution throughout much of Africa south of the Sahara. *S. margrebowiei* is parasitic in bovines, chiefly lechwe (*Kobus leche*) and puku (*K. vardoni*), and belongs to the *S. haematobium* group of schistosomes. *S. margrebowiei* has a far more restricted distribution than *S. mansoni*, and is mainly found in the area extending from the Chobe Game Reserve in north-east Botswana (Pitchford, 1974) through the eastern Caprivi Strip into south-western Zambia (Le Roux, 1933) and southern Zaire (van den Berghe, 1934). However, there have been some reports of *S. margrebowiei* outside this area, such as in Chad (Graber, 1969, 1978), Mali (Lapierre & Hien, 1973) and possibly in north-east Zaire (Walkiers, 1928).

The distribution of *S. margrebowiei* and *S. mansoni* overlaps within the Chobe National Park, Botswana, but because they utilise different definitive hosts, these 2 species are unlikely to co-exist within any single individual.

Thus, the laboratory model of mating interactions between *S. margrebowiei* and *S. mansoni* presented below is "theoretical" in that the species used do not interact in nature, and as such, it is the first of its kind to be studied. It is hoped that the information the model provides will help substantiate and/ or add to existing knowledge of schistosome mating interactions, particularly those between species from different evolutionary groups.

RESULTS

Experiment 1: Simultaneous infections

Table 3.1 summarizes the worms obtained from each mouse, and how they paired. Four types of pairing were found: 2 homospecific (*S. mansoni* ♂ x *S. mansoni* ♀ and *S. margrebowiei* ♂ x *S. margrebowiei* ♀), and 2 heterospecific (*S. mansoni* ♂ x *S. margrebowiei* ♀ and *S. margrebowiei* ♂ x *S. margrebowiei* ♀). There was also a surplus of unpaired male worms of both species. More unusually, 4 multiple-worm pairs were obtained in total, involving 3 or more worms of the same or different species in the following arrangements: 1 *S. margrebowiei* male paired with 2 *S. margrebowiei* females; 1 *S. margrebowiei* male paired with 1 female of each species; 1 *S. mansoni* male paired with 3 *S. margrebowiei* females; and 2 *S. mansoni* males paired with 1 *S. margrebowiei* female.

Table 3.1 Data from mice simultaneously infected with 150 cercariae of *Schistosoma mansoni* (Ms) and 150 cercariae of *S. margrebowiei* (Mg).

Mouse	Ms♂	Mg♂	Ms♂x Ms♀	Mg♂x Mg♀	Ms♂x Mg♀	Mg♂x Ms♀	Multiple pairings (♂:♀)
1	1	1	3	5	4	3	
2	9	10	7	21	8	9	
3	16	1	7	6	2	1	
4	1	0	9	5	6	0	2Ms : 1Mg
5	8	1	6	6	9	1	
6	3	7	6	11	1	1	
7	2	1	11	3	10	2	1Mg:1Ms+1Mg 1Mg : 2 1Ms:3Mg
8	4	2	3	2	1	1	
9	2	3	10	15	3	3	
10	26	2	0	6	5	1	
Total:	**72**	**28**	**62**	**80**	**49**	**22**	

Overall, there were twice as many homospecific pairs (142) as heterospecific pairs (71). To test whether this difference was indicative of any species preference, the Mantel-Haenszel test was carried out on the proportions of males of both species paired homospecifically and heterospecifically. The result of the test, which incorporated a Yates-style correction, was highly significant ($\chi^2 = 23.210$, $P < 0.001$), indicating that both species have a strong preference for homospecific pairing.

Of paired *S. mansoni* males, 55.9% formed homospecific pairs, and 44.1% heterospecific pairs. Of paired *S. margrebowiei* males, 78.4% paired homospecifically, and 21.6% heterospecifically, indicating that *S. margrebowiei* has a stronger homospecific mate preference than *S. mansoni.*

The proportion of paired males among *S. mansoni* was 60.7%, whereas that for *S. margrebowiei* was 78.5%. This difference was statistically significant according to the Mantel-Haenszel test (χ^2 = 7.990, P < 0.01), with *S. margrebowiei* males being more successful than *S. mansoni* males at pairing.

Experiment 2: infections with S. mansoni *and 2 weeks later with* S. margrebowiei

Four types of pairing were obtained in this experiment (Table 3.2), of which the homospecific *S. mansoni* ♂ x *S. mansoni* ♀ pairing was the most common, and was the only type of pairing in mice 1, 3, 4, 7 and 8. One multiple-worm pair was obtained from mouse 8, consisting of 1 *S. mansoni* male paired with 2 *S. mansoni* females.

Table 3.2. Data from mice infected first with 150 cercariae of *Schistosoma mansoni*, and 2 weeks later with 150 cercariae of *S. margrebowiei.*

Mouse	Ms♂	Mg♂	Ms♂x Ms♀	Mg♂x Mg♀	Ms♂x Mg♀	Mg♂x Ms♀	Multiple pairings (♂:♀)
1	9	0	13	0	0	0	
2	2	1	4	1	0	2	
3	12	0	10	0	0	0	
4	17	1	8	0	0	0	
5	4	9	11	0	0	1	
6	7	3	9	0	2	0	
7	14	0	7	0	0	0	
8	5	0	8	0	0	0	1Ms : 2Ms
9	1	3	10	0	1	1	
10	4	5	3	2	1	4	
Total:	**75**	**22**	**83**	**3**	**4**	**8**	

The proportion of paired males among *S. mansoni* was 53.7%, whereas that for *S. margrebowiei* was 33.3%. This difference was statistically significant according to a

Mantel-Haenszel test ($\chi^2 = 9.494$, $P < 0.01$), with *S. mansoni* males being more successful than *S. margrebowiei* males at pairing.

Since *S. mansoni* was introduced first, it is assumed that all *S. mansoni* females were mated with *S. mansoni* males before *S. margrebowiei* worms were ready to pair. However, mice 2, 5, 9 and 10 contained up to 4 *S. margrebowiei* ♂ x *S. mansoni* ♀ heterospecific pairs, leaving in each case at least 1 unpaired *S. mansoni* male. There was a statistically significant difference in the proportions of males from both species which paired with *S. mansoni* females ($\chi^2 = 10.380$, $P < 0.005$) with *S. mansoni* males being better than *S. margrebowiei* males at pairing with *S. mansoni* females. In other words, despite the occurrence of several *S. margrebowiei* ♂ x *S. mansoni* ♀ pairings, overall *S. margrebowiei* males are unable to outcompete *S. mansoni* males for *S. mansoni* females to any significant extent.

Four heterospecific *S. mansoni* ♂ x *S. margrebowiei* ♀ pairs were obtained in total, in each case leaving an excess of single *S. margrebowiei* males. Two of these 4 pairs may have involved *S. mansoni* males which had been actively displaced from their original homospecific *S. mansoni* partners by *S. margrebowiei* males, as indicated by the presence of 2 corresponding *S. margrebowiei* ♂ x *S. mansoni* ♀ pairs. These displaced *S. mansoni* males had managed to re-pair with a heterospecific (*S. margrebowiei*) female. There was no significant difference between the proportions of males of both species paired with *S. margrebowiei* females, indicating that *S. mansoni* males are just as good at pairing with *S. margrebowiei* females as *S. margrebowiei* males are.

Experiment 3: infections with S. margrebowiei *and 2 weeks later with* S. mansoni

Two homospecific and 2 heterospecific types of pairing were obtained in this experiment (Table 3.3), of which the homospecific *S. margrebowiei* ♂ x *S. margrebowiei* ♀ pairing was the most common. Two multiple-worm pairs were obtained, 1 from mouse 3, consisting of a *S. mansoni* male paired with 2 *S. margrebowiei* females, and the other from mouse 9, consisting of a *S. margrebowiei* male paired with 1 female of each species.

Table 3.3. Data from mice infected first with 150 cercariae of *Schistosoma margrebowiei*, and 2 weeks later with 150 cercariae of *S. mansoni*.

Mouse	Ms♂	Mg♂	Ms♂x Ms♀	Mg♂x Mg♀	Ms♂x Mg♀	Mg♂x Ms♀	Multiple pairings (♂:♀
1	5	0	6	8	0	1	
2	4	3	10	9	3	0	
3	6	0	1	11	5	0	1Ms : 2Mg
4	1	1	6	14	3	1	
5	4	0	5	3	8	1	
6	1	6	0	8	1	0	
7	0	10	0	4	0	4	
8	0	13	0	4	0	3	
9	7	0	1	7	0	0	1Mg:1Mg+1Ms
10	1	2	5	9	3	3	
Total:	**29**	**35**	**34**	**77**	**23**	**13**	

The proportion of paired males among *S. margrebowiei* was 72.0%, whereas that for *S. mansoni* was 66.3%. This difference was statistically significant according to the Mantel-Haenszel test ($\chi^2 = 11.624$, $P < 0.005$), with *S. margrebowiei* males being more successful than *S. mansoni* males at pairing.

Despite the assumption that all *S. margrebowiei* females would have been paired with *S. margrebowiei* males prior to the introduction of *S. mansoni*, 23 heterospecific *S. mansoni* ♂ x *S. margrebowiei* ♀ pairs were obtained in total from mice 2, 3, 4, 5, 6 and 10. The data show that in 10 of these cases there was at least one non-homospecifically paired *S. margrebowiei* male for each *S. mansoni* ♂ x *S. margrebowiei* ♀ pair, which is assumed to have been actively displaced from its homospecific partner by a competitor *S. mansoni* male. The remaining 13 *S. mansoni* ♂ x *S. margrebowiei* ♀ pairs arose from there being a deficit of *S. margrebowiei* males for the *S. margrebowiei* females to pair with. However, a Mantel-Haenszel test comparing the proportions of males of both species paired with *S. margrebowiei* females showed that overall *S. margrebowiei* males were significantly better able to pair with *S. margrebowiei* females than *S. mansoni* males were ($\chi^2 = 25.943$, $P < 0.001$).

Data from mice 1, 4, 5, 7, 8, and 10 show that 46% of the heterospecific *S. margrebowiei* ♂ x *S. mansoni* ♀ pairs formed leaving an excess of *S. mansoni* males which *S. margrebowiei* males had out-competed. The remaining 54% arose from there being a deficit of *S. mansoni* males for the *S. mansoni* females to pair with. All 5 of the *S. margrebowiei* ♂ x *S. mansoni* ♀ pairs recovered from mice 4, 5, and 10 may have involved *S. margrebowiei* males which had been actively displaced from their original homospecific *S. margrebowiei* partners by *S. mansoni* males, as indicated by the presence of 5 corresponding *S. mansoni* ♂ *S. margrebowiei* ♀ pairs. These displaced *S. margrebowiei* males had managed to re-pair with a heterospecific (*S. mansoni*) female. The difference between the proportions of males of both species paired with *S. mansoni* females was not significant, indicating that *S. margrebowiei* males are just as good as *S. mansoni* males at pairing with *S. mansoni* females.

Experiment 4: infections with S. mansoni *and 3 weeks later with* S. margrebowiei

In this experiment, no homospecific *S. margrebowiei* pairs were obtained (Table 3.4), and there was only 1 heterospecific *S. margrebowiei* ♂ x *S. mansoni* ♀ pair. In contrast, 104 homospecific *S. mansoni* pairs were obtained in total, and there were 25 heterospecific *S. mansoni* ♂ x *S. margrebowiei* ♀ pairs. One multiple-worm pair was recovered from mouse 4, consisting of 2 *S. mansoni* males paired with 1 *S. mansoni* female.

Table 3.4. Data from mice infected first with 150 cercariae of *Schistosoma mansoni*, and 3 weeks later with 150 cercariae of *S. margrebowiei*.

Mouse	Ms♂	Mg♂	Ms♂x Ms♀	Mg♂x Mg♀	Ms♂x Mg♀	Mg♂x Ms♀	Multiple pairings (♂:♀)
1	13	5	8	0	0	1	
2	12	8	8	0	7	0	
3	13	4	11	0	5	0	
4	7	0	9	0	1	0	2Ms :1Ms
5	16	0	4	0	2	0	
6	6	1	15	0	6	0	
7	10	1	12	0	2	0	
8	11	0	14	0	0	0	
9	14	1	11	0	1	0	
10	8	4	12	0	1	0	
Total:	**110**	**24**	**104**	**0**	**25**	**1**	

Only 4% of all *S. margrebowiei* males were paired, compared with 54.0% of *S. mansoni* males. This difference was statistically significant (χ^2 = 18.485, P < 0.001), with *S. mansoni* males proving better at pairing than *S. margrebowiei* males.

The single *S. margrebowiei* ♂ x *S. mansoni* ♀ pair was recovered from mouse 1 along with 13 surplus *S. mansoni* males, all of which are assumed to have been outcompeted by the *S. margrebowiei* male. Mantel-Haenszel analysis revealed that the difference between the proportions of males of both species paired with *S. mansoni* females was highly significant (χ^2 = 13.520, P < 0.001) with *S. mansoni* males being more successful than *S. margrebowiei* males at pairing with *S. mansoni* females.

Data from mice 2, 3, 4, 5, 6, 7, 9 and 10 show that for 60% of the heterospecific *S. mansoni* ♂ x *S. margrebowiei* ♀ pairs formed, at least one corresponding unpaired *S. margrebowiei* male was also obtained, which the paired *S. mansoni* males had out-competed. The remaining 40% resulted from there being a deficit of *S. margrebowiei* males for the *S. margrebowiei* females to pair with. The difference in proportions of males of both species paired with *S. margrebowiei* females was statistically significant (χ^2 = 5.066, P < 0.05), with *S. mansoni* males being more successful than *S. margrebowiei* males at pairing with *S. margrebowiei* females.

Experiment 5: infections with S. margrebowiei *and 3 weeks later with* S. mansoni

Four types of pairing were obtained in this experiment (Table 3.5), the most common of which was the homospecific *S. margrebowiei* ♂ x *S. margrebowiei* ♀ pairing (140 pairs compared with 19 pairs in total for the other 3 types of pairing). Two multiple-worm pairs were recovered, both from mouse 4, 1 of which consisted of a *S. margrebowiei* male paired with 2 *S. margrebowiei* females, and the other of 2 *S. margrebowiei* males paired with 1 *S. margrebowiei* female.

The proportion of paired males among *S. margrebowiei* was 84.5%, whereas that for *S. mansoni* was 27.3%. This difference was highly significant (χ^2 = 40.725, P < 0.001), with *S. margrebowiei* males proving better than *S. mansoni* males at pairing.

Table 3.5. Data from mice infected first with 150 cercariae of *Schistosoma margrebowiei*, and 3 weeks later with 150 cercariae of *S. mansoni*.

Mouse	Ms♂	Mg♂	Ms♂x Ms♀	Mg♂x Mg♀	Ms♂x Mg♀	Mg♂x Ms♀	Multiple pairings (♂:♀)	
1	0	1	1	14	1	1		
2	4	1	0	21	0	0		
3	2	0	0	25	0	0		
4	4	7	0	9	1	0	2Mg :1Mg	1Mg :2Mg
5	4	2	0	15	0	0		
6	4	4	1	2	3	3		
7	5	3	0	5	0	1		
8	1	3	2	18	0	0		
9	1	4	0	14	1	1		
10	7	2	2	17	0	1		
Total:	**32**	**27**	**6**	**140**	**6**	**7**		

Despite the assumption that all *S. margrebowiei* females would have been paired with *S. margrebowiei* males prior to the establishment of *S. mansoni*, 6 *S. mansoni* ♂ x *S. margrebowiei* ♀ pairs were recovered from mice 1, 4, 6 and 9 in total. In each case, there was 1 unpaired *S. margrebowiei* male per *S. mansoni* ♂ x *S. margrebowiei* ♀ pair, which is assumed to have been actively displaced by the paired *S. mansoni* male. However, Mantel-Haenszel analysis showed that overall, *S. margrebowiei* males remained significantly better than *S. mansoni* males at pairing with *S. margrebowiei* females (χ^2 = 45.995, $P < 0.001$).

Data from mice 1, 6, 7, 9 and 10 show that 86% (6/7) of the heterospecific *S. margrebowiei* ♂ x *S. mansoni* ♀ pairs formed left an unpaired *S. mansoni* male which the paired *S. margrebowiei* male had out-competed. The remaining 14% (1 pair) resulted from a deficit of *S. mansoni* males for the *S. mansoni* female to pair with. All 5 of the *S. margrebowiei* ♂ x *S. mansoni* ♀ pairs recovered from mice 1, 6, and 9 may have involved *S. margrebowiei* males which had been actively displaced from their original homospecific *S. margrebowiei* partners by *S. mansoni* males, as indicated by the presence of 5 corresponding *S. mansoni* ♂ *S. margrebowiei* ♀ pairs. These displaced *S. margrebowiei* males had managed to re-pair with a heterospecific (*S. mansoni*) female. There was no

significant difference in the proportions of males of both species paired with *S. mansoni* females, suggesting that *S. margrebowiei* males are just as good as *S. mansoni* males at pairing with *S. mansoni* females.

DISCUSSION

The data from the 5 experiments show that in mixed infections of *S. mansoni* and *S. margrebowiei* homospecific and heterospecific pairs readily form, confirming previous observations that there are no physiological barriers preventing the meeting and pairing of two different species in the same definitive host (Southgate *et al.*, 1982; Tchuem Tchuenté *et al.*, 1995, 1996 *b*; Webster *et al.*, 1999). Furthermore, the fact that all heterospecifically paired females were mature confirms earlier findings that maturation and reproductive stimulation of females by males is not species-specific (Khalil & Mansour, 1995; Southgate *et al.*, 1998).

Previous models of mating interactions between two species from different evolutionary groups involved mixed infections of schistosome species which occur sympatrically in nature and are therefore able to co-infect the same human host, e.g. the *S. intercalatum/ S. mansoni* model (Tchuem Tchuenté *et al.*, 1993, 1994, 1995) and the S. *haematobium/ S. mansoni* model (Webster *et al.*, 1999). In such situations, where competition exists between co-infecting schistosomes there exists the risk of partial or total exclusion of one species by the other, and indeed, *S. mansoni* is seen to be restricting the distribution of *S. intercalatum* in Africa (Southgate *et al.*, 1982, 1998; Tchuem Tchuenté *et al.*, 1993, 1994, 1995, 1996 *a*, 1997 *a*) and *S. mansoni* is progressively replacing *S. haematobium* in the Fayoum, Egypt (Abdel-Wahab *et al.*, 1993; Webster *et al.*, 1999).

Data from mice simultaneously infected with both *S. mansoni* and *S. margrebowiei* (experiment #1), revealed a significant prevalence of homospecific pairs, indicating the existence of a specific mate preference system for both species, as previously described for the *S. intercalatum/ S. mansoni* and the *S. haematobium/ S. mansoni* models (Tchuem Tchuenté *et al.*, 1993; Webster *et al.*, 1999).

However, *S. margrebowiei* exhibited a greater homospecific mate preference than *S. mansoni*. This gives *S. margrebowiei* a competitive advantage over *S. mansoni* for, by maintaining a stronger homospecific mate preference, *S. margrebowiei* is helping to ensure its reproductive viability as any offspring of mating between the two species will be of greatly reduced fitness. This is because these species are from different evolutionary lineages, and such is the phylogenetic difference between them that they cannot hybridise,

instead producing matroclonal eggs of very low viability, by parthenogenesis (Jourdane *et al.*, 1995; Khalil & Mansour, 1995; Southgate & Rollinson, 1987; Southgate *et al.*, 1982, 1995; Tchuem Tchuenté *et al.*, 1994). In the *S. margrebowiei*/ *S. mansoni* mating model it is assumed that all eggs seen in heterospecifically-paired females were of parthenogenetic origin. For male worms, a heterospecific pairing which leads to parthenogenesis is a reproductive "dead end": the reproductive disadvantages of a tendency to form heterospecific pairs are postulated as one reason why *S. haematobium*, which has been shown to be competitively dominant to *S. mansoni* (Webster *et al.*, 1999) is being replaced by *S. mansoni* in the Fayoum, Egypt (Abdel-Wahab *et al.*, 1993; Webster *et al.*, 1999).

Statistical analysis of the data in experiment #1 also showed *S. margrebowiei* males were better than *S. mansoni* males at pairing with females of either species, again indicating that *S. margrebowiei* has the competitive edge over *S. mansoni* in simultaneous infections.

However, in sequential infections, it seems that the competitive dominance of one species over the other was mainly determined by which species was the first to enter and establish itself in the host (species 1), and which was the species of reinfection (species 2). In all 4 sequential experiments (#2-5), species 1 had the competitive advantage over species 2 in terms of the following: firstly, a greater ability to pair with with 'species 1 females'; secondly, pairing more readily with females of either species. Thirdly, 'species 1 males' were at least as good as 'species 2 males' at pairing with 'species 2 females'.

Consideration of the types of mating competition involved in sequential infections may help in finding an explanation for the advantages conferred on species 1.

The sex ratio of *S. margrebowiei* was consistently less male-biased than that for *S. mansoni* (except in experiment #2, where the low recovery of *S. margrebowiei* females resulted in a male:female ratio of 4.7:1 for *S. margrebowiei* compared with 1.8:1 for *S. mansoni*), in accordance with previously reported sex ratios for *S. margrebowiei* (Southgate & Knowles, 1977). Nevertheless, there were at least as many males as females of both species, and therefore the assumption that all females of species 1 were paired with 'species 1 males' by the time species 2 commenced pairing is a reasonable assumption. Consequently, in order for 'species 2 males' to pair with ''species 1 females'', they must have first separated the

female from her species 1 male partner. That worm pairs can dissociate and change of mate take place has been demonstrated in recent studies of schistosome mating interactions (Tchuem Tchuenté *et al.*, 1995, 1996 *b*; Pica-Mattoccia *et al.*, 2000). In mixed infections of *S. mansoni* and *S. intercalatum* it has been observed that competitively dominant *S. mansoni* males actively pull away paired *S. intercalatum* or paired *S. mansoni* females from *S. intercalatum* males (Tchuem Tchuenté *et al.*, 1995). It can therefore be postulated that in the *S. margrebowiei*/ *S. mansoni* model unpaired 'species 2 males' are similarly able to pull paired 'species 1 females' out from the gynaecophoric canal of 'species 1 males' and pair with them. The newly displaced 'species 1 males' may then in turn start competing for females afresh. In sequential infections, where the number of heterospecifically paired 'species 1 males' was less than or equal to the number of heterospecifically paired 'species 2 males' recovered from a mouse, all heterospecifically paired 'species 1 males' from that mouse can be assumed to have re-paired heterospecifically, having been actively displaced from their original homospecific females by competitor 'species 2 males'. This is the case in experiment #2, mice 9 and 10; experiment #3, mice 4, 5 and 10; experiment #5, mice 1, 6, and 9. It is likely that this "active competition" for paired females is more energy-consuming than competition for unpaired females, and so it is not surprising that 'species 2 males' appeared unable to outcompete 'species 1 males' for 'species 1 females' to any statistically significant extent in experiments #2-5.

If mice had been culled 8 weeks or more after final patency, then it is possible that the number of species 2 ♂ x species 1 ♀ pairs may have been much greater, since Tchuem Tchuente *et al.,* (1995) described the phenomenon of change of mate "as a progressive process, requiring up to 8 weeks" to happen. In these experiments however, it was not possible to delay the culling of mice any longer due to the pathogenicity of such a large worm burden. Unlike paired worms, which migrate from the liver to the mesenteric veins (or to the vesical venous plexus in the case of *S. haematobium*), unpaired worms remain in the liver or the hepatic portal vein, so that the question arises as to where change of mate takes place. Pica-Mattoccia *et al.* (2000), suggested that it may be necessary for paired worms to periodically return to the liver and hepatic portal system, in order for them to move from one branch of the mesenteric veins to another, so bringing unpaired and paired worms together and providing the opportunity for mate changing.

When considering the data showing 'species 1 males' to be significantly better at pairing (that is, at pairing with females) than 'species 2 males', it should be noted that this result may be largely due to the greater ability of 'species 1 males' to pair with 'species 1 females', since the worm return of 'species 2 females' (and males) is so much lower than that of 'species 1 females'. The worm return of *S. margrebowiei* females in experiment #2 is particularly low (7 worms). However, in all sequential experiments 'species 1 males' were shown to be at least equally as able to pair with 'species 2 females' as 'species 2 males', and therefore, on balance, this does indicate superiority of 'species 1 males' pairing ability.

With regard to the relative competitive mating abilities of *S. margrebowiei* and *S. mansoni* in sequential infections, it seems that they are fairly evenly matched, both enjoying the same competitive advantages when they form the first infection, and the same disadvantages when they form the second. The only exception to this was in experiment #4 when species 1 (*S. mansoni*) males outcompeted species 2 (*S. margrebowiei*) males for species 2 (*S. margrebowiei*) females, whereas in all other sequential infections, species 1 and 'species 2 males' were equally able to pair with 'species 2 females'.

Statistical analysis of the data found no significant differences in the abilities of males of the two species to form heterospecific pairs (rather than not mating at all), again suggesting that neither species is competitively dominant to the other in sequential infections. Furthermore, both *S. margrebowiei* and *S. mansoni* males, when they were the second infection, were able to "actively compete" for 'species 1 females' by pulling them away from 'species 1 males' and pairing with them. In mixed infections of *S. haematobium* and *S. mansoni* where *S. haematobium* emerged as competitively dominant irrespective of whether it was the first or second infection, the ability to actively pull away paired females was almost exclusively confined to *S. haematobium* males, and they had a greater ability to form heterospecific pairs than *S. mansoni* males (Webster *et al.*, 1999).
Thus it seems that the timing of sequential mixed infections is a crucial determinant of mating success only where the co-infecting species are evenly matched in their competitive mating abilities. In that situation, the species which forms the first infection will have the competitive mating advantage over the second species.

The other marked advantage which species 1 has over species 2 in sequential infections is that it has a much higher worm return, suggesting that species 1 confers some degree of resistance to reinfection. In mice exposed to both species simultaneously (experiment #1) the worm returns were nearly identical: 17.9% and 17.5% for *S. mansoni* and *S. margrebowiei* respectively, showing that there is no difference between the two species in their infectivity or ability to develop in mice. In sequential experiments, however, the worm return of the second species was up to 7 times less than that of the first.

That an initial infection can confer resistance against subsequent challenge infections whilst adult worms of the first infection survive and remain active is well documented, and has been termed "concomitant immunity" (Smithers & Terry, 1969). This resistance may cross the species barrier to varying degrees and protect against a different schistosome species (Smithers & Doenhoff, 1982; Rollinson *et al.*, 1990; Tchuem Tchuenté *et al.*, 1996 *a*): a phenomenon known as "heterologous immunity". Resistance to reinfection by the same, or a different species in this manner has the obvious biological advantage of preventing overcrowding of parasites, thus extending the survival of both host and schistosome.

It has been strongly argued that the onset of protective immunity is at patency, stimulated by the presence of eggs in tissues and egg-associated pathology, and peak immunity is reached 4-6 weeks later (Smithers & Terry, 1965; Pearce & McLaren, 1983; Dean, 1983). However, it is known that immune attrition is most active against later stages of schistosomula of the challenge infection, such as lung-stage worms, 6-13 days after penetration (Smithers & Doenhoff, 1982). This means that in the sequential *S. margrebowiei*/ *S. mansoni* experiments, by the time the first infection reaches patency (at 35 days post infection) the schisotomula of the second infection will be 21 and 14 days old in 2- and 3-week time lag experiments, respectively, and by that time may be largely refractory to immune killing. Therefore the protective immunity against the second infection in this model may be effected before the onset of patency in the first infection.

Some workers have acknowledged the existence of a degree of protective immunity in experiments where egg-laying and associated pathology are absent. For example, a study by Tchuem Tchuenté *et al.* (1996 *a*) showed that a unisexual infection of *S. mansoni* gave

cross protection against *S. intercalatum* cercariae of the opposite sex only 10 days after the initial infection with *S. mansoni.* They attributed this phenomenon to "the immunization process" as the time interval between immunizing and challenge infections was too short to allow cross-protection induced by liver pathology. In a study by Miller & Smithers (1980), mice immunized via an abdominal skin site with irradiated cercariae, and challenged via the same site 4 weeks later showed nearly 60% immunity against the second infection only 5 days post-challenge. It was observed that local inflammation persisted despite the 4 week interval since immunization which was suggested as reason for the rapidity and high levels of attrition of the challenge infection. By analogy, therefore, it may be postulated that in the sequential *S. margrebowiei/ S. mansoni* infections, cutaneous inflammation induced by the initial infection at the sites of penetration may have persisted up to the time of challenge with the second infection (2 or 3 weeks later), resulting in rapid killing of a large proportion of the penetrating schistosomula, well before the onset of patency (the same areas of the mice would have been exposed for each infection).

If the challenge infection in the *S. margrebowiei/ S. mansoni* experiments had been delayed until after patency of the first infection, it is a matter of speculation as to whether the consequent egg-induced immunity would equal or outweigh that induced in prepatency by the putative mechanisms outlined above.

The resistance induced by *S. mansoni* against *S. margrebowiei* attained its highest level in mice challenged 2 weeks post infection (experiment #2) whereas that induced by *S. margrebowiei* against *S. mansoni* attained the same high level only when challenge was 3 weeks post infection (experiment #5). That *S. mansoni* may be slightly more immunogenic than other species was indicated by the non-reciprocity of cross-immunity induced by *S. mansoni* against *S. intercalatum* in the experiments of Tchuem Tchuenté *et al.* (1996 *a*). Nevertheless, the final levels of cross-protection induced by *S. margrebowiei* and *S. mansoni* were identical, with the worm returns of the second infection being 6 to 7 times less than that of the first infection.

Smithers & Doenhoff (1982) observed that the degree of cross-protection induced by one species against the other does not seem to be related to the phylogenetic distance between the two species. Certainly the non-reciprocity of cross immunity in the *S. mansoni* / *S.*

intercalatum model on the one hand but the almost full reciprocity of cross-protection in the *S. margrebowiei/ S. mansoni* model seems to confirm this observation, as in both models the species are from different phylogenetic groups. These authors also suggest that the development or the severity of the immunizing infection in the host may be the more important factor in determining the degree of cross-protection. This may indeed explain the high reciprocity of cross-protection between *S. margrebowiei* and *S. mansoni*, as the nearly identical worm returns of both species in experiment #1 indicates that both species were equally able to infect and develop in mouse hosts.

Pitchford (1976) refers to preliminary work which indicates that an infection with *S. margrebowiei* greatly reduces the development of *S. mansoni* in the multimammate mouse *Mastomys*. This same author also suggests that *S. margrebowiei*, together with *S. leiperi* (another "lechwe schistosome") might, through heterologous immunity, be preventing the development of *S. mansoni*, *S. haematobium* and *S. mattheei* in Eastern Caprivi. Evidence for this comes from studies of schoolchildren in areas of only *S. margrebowiei* and *S. leiperi* endemicity in Eastern Caprivi: these children had positive schistosomal intradermal and complement fixation tests but were negative for schistosome eggs in urine or stools (Pitchford & Wolstenholme, 1977). Given that the distributions of *S. mansoni*, *S. haematobium* and *S. mattheei* had little or no overlap with the lechwe schistosomes in Eastern Caprivi, this suggests that previous exposure of the children to lechwe schistosomes affords some protection against infection with the human schistosomes. The immunological observations from the *S. margrebowiei/ S. mansoni* mating model lend some support to this hypothesis.

Another feature of the *S. margrebowiei/ S. mansoni* experiments which deserves comment is the recovery of up to 4 multiple-worm pairs, involving either homospecific or heterospecific partners, from each experiment. Multiple matings have previously been observed amongst Schistosomatidae: Armstrong (1965) reported the occupation of the gynaecophoric canal of *Heterobilharzia americana* by 29 females of *S. mansoni*; and Tchuem Tchuenté *et al.* (1993) obtained males of *S. intercalatum* paired with 2-3 females of *S. intercalatum* or *S. mansoni* from experiments where *S. mansoni* males were absent. Basch (1991) observed that in cultures of *S. mansoni* it was common to find single females clasped by 2 males, one at either end. Furthermore, single male worms in culture have been

shown to clasp both cotton and alginate fibres (Basch & Nicolas, 1989), suggesting that the clasping by males is a thigmotactic process rather than one dependent on chemotactic (pheromonal) signals from females. Hence the occurrence of male-female pairing between different species, and, in the case of the *H. americana-S. mansoni* interaction, between individuals from different genera.

In experiment #1, mouse 4, all *S. margrebowiei* males are paired homospecifically with *S. margrebowiei* females, leaving one surplus *S. margrebowiei* female which is clasped by 2 of the excess *S. mansoni* males. In mouse 7, two *S. margrebowiei* females are paired with one *S. margrebowiei* male, despite the availability of an unpaired *S. margrebowiei* male. Another multiple pairing involved a *S. margrebowiei* male paired with one female from each species, and it is a matter of speculation as to which female it may have paired with first of all, since mice were infected with both species simultaneously, and all females would be available for pairing at the same time. Interestingly, in mouse 8, one *S. mansoni* male was found to be mated with three *S. margrebowiei* females, despite the availability of two single *S. margrebowiei* males.

Both species, therefore, are able to form these multiple-worm pairs, either involving two or more males paired with one female, or one male paired with two or more females. In experiment #5, mouse 4, two multiple pairs were obtained, containing a total of three *S. margrebowiei* males and three *S. margrebowiei* females between them, but distributed unequally as one pairing of two *S. margrebowiei* males x one *S. margrebowiei* female; and one of one *S. margrebowiei* male x two *S. margrebowiei* females, so highlighting intra-*S. margrebowiei* competition between males for females.

In the sequential experiments, all multiple pairings involved only males of species 1 (except in experiment #3, mouse 3, where there was a deficit of *S. margrebowiei* males), and only females of species 1 , again, except in experiment #3 (mouse 9), where a *S. margrebowiei* male was paired with one female from each species. In this latter pairing, since *S. margrebowiei* was the first infection, it is likely that the male was first paired homospecifically with the *S. margrebowiei* female, and later managed to pair with a *S. mansoni* female as well, outcompeting 7 unpaired *S. mansoni* males in so doing. It seems, therefore, that the ability to form multiple pairs is another mating advantage conferred upon

species 1. However, the advantages are mainly at an individual rather than a species level, as when there is a female deficit, the mating of several females by a male will be to the reproductive detriment of unpaired males of the same species. It was also observed that whilst single males can mate multiple females when there is either a male or female deficit, the pairing of single females with more than one male only occurred when there was a female deficit. This supports previous observations that the male is the more active sex in making the choice of mate (Tchuem Tchuenté *et al.*, 1993, 1996 *b*).

S. margrebowiei worms, having bovine rather than human hosts, are much larger than *S. mansoni* worms, hence have more body musculature and a larger gynaecophoric canal in which to clasp females. It is therefore perhaps surprising that *S. margrebowiei* males are not significantly dominant to *S. mansoni* males in terms of their competitive mating abilities. There is some slight suggestion of mating dominance of *S. margrebowiei*, by virtue of it having a stronger homospecific mate preference and a greater ability to pair than *S. mansoni* in simultaneous infections, but it is not as obvious as the dominance of *S. mansoni* over *S. intercalatum* (Tchuem Tchuenté *et al.*, 1993) and of *S. haematobium* over *S. mansoni* (Webster *et al.*, 1999) in the two previous models of mating between species from different phylogenetic groups.

In all three models the species are reproductively isolated from one another, firstly by having a specific mate preference system, and secondly by their inability to hybridise and produce offspring of high viability. The heterologous cross-protection observed between *S. intercalatum* and *S. mansoni* (Tchuem Tchuenté *et al.*, 1996 *a*) and here between *S. margrebowiei* and *S. mansoni* also serves as an isolating mechanism by acting to minimise reinfection of the host by a different species. However, *S. margrebowiei* and *S. mansoni* are reproductively isolated to a greater extent than the species in the other two models by their utilisation of different definitive hosts. So effective is reproductive isolation by the host, it is a little surprising that there should be such a high degree of immunological cross-protection between *S. margrebowiei* and *S. mansoni*, if it is indeed primarily a reproductive isolating mechanism. Indeed, in many species belonging to the same group and therefore capable of hybridisation, they may lack any isolating mechanisms other than that of host specificity. More likely the benefits of heterologous immunity are several, serving, for

example, to prevent superinfection so as to ensure the long-term survival of the schistosomes and the host.

This model of mating between *S. margrebowiei* and *S. mansoni* indicates that one of the species used in inter-group mating models will not necessarily be significantly competitively dominant to the other, and that the timing of the infections and sequence in which the different species enter the host may prove a more crucial determinant in the outcome of the mating interactions than hitherto realised.

CHAPTER 4

Mating interactions between *S. intercalatum* (Zaire strain) and *S. mansoni*

CHAPTER 4
MATING INTERACTIONS BETWEEN *S. INTERCALATUM* (ZAIRE STRAIN) AND *S. MANSONI*

INTRODUCTION

Two distinct strains of *S. intercalatum* are currently recognized: the Lower Guinea and Zaire (Democratic Republic of Congo) strains, which differ in their geographical distribution and a number of biological characteristics such as intermediate host specificity, prepatent periods, isoenzyme profiles and chronobiological cercariae shedding patterns (Wright *et al.*, 1972, 1979 *b*; Pagès & Théron, 1990). In addition, recent data have shown considerable molecular divergence between the strains (Pagès *et al.*, 2001 *a, b*). There is some uncertainty as to the origin of the two strains. The first reports of mesenteric terminal-spined schistosomiasis were in 1923, by Chesterman from Yakusu, near Stanleyville (Kisangani) in the Democratic Republic of Congo (Chesterman, 1923), and by Clapier from Libreville in Gabon (Clapier, 1923), but it was Fisher (1934) who officially described *S. intercalatum* as a new species from the Stanleyville region. A study by Deschiens & Delas (1969) suggested that the parasite was spreading northwards into Cameroon with immigrant manual workers. These authors assumed that the Stanleyville focus was the true origin of the *S. intercalatum* parasite, and that its outward spread led to its divergence into two different strains through geographical isolation. However, Browne (1969) noted that there was no evidence that the Stanleyville focus was older than the Libreville one, and Wright *et al.* (1972) interpreted the numerous biological differences between the strains as indicative that they had been separated for some time. Indeed, more recent studies incorporating the molecular divergence between the strains confirm that their differentiation from one another is ancient, and probably took place at the same time as speciation of all the other *S. haematobium*-group schistosomes (Wright *et al.*, 1972; Desprès *et al.*, 1992; Pagès *et al.*, 2001 *a, b*). Wright *et al.* (1972) postulated that, as the two strains are unable to utilize each other's snail host (*B. africanus* group snails for the Zaire strain, and *B. forskalii* group snails for the Lower Guinea strain), this parasite originated in an ancestral bulinid snail of forest regions and the two strains diverged with separate lines of snails derived from the common stock.

Of the two, it is the Lower Guinea strain which has the wider distribution, occuring in Gabon, Cameroon, Nigeria, Equatorial Guinea and Sao Tomé, whereas the Zaire strain appears confined to the Democratic Republic of Congo (Tchuem Tchuenté *et al.,* 1997 *a*). However, in comparison to the extremely wide distribution of their definitive (human) and intermediate hosts across Africa, the distribution of both strains appears to be highly restricted (Tchuem Tchuenté *et al.,* 1996 *a*, 1997 *a*).

One biological reason postulated for this concerns the tendency of *S. intercalatum* cercariae, in contrast to other African schistosomes, to form non-infective aggregates in response to small temperature changes. The aggregates are formed by the release of the adhesive post-acetabular gland secretion which causes the cercariae to stick together. It has been suggested that *S. intercalatum* originated in streams within tropical rainforest areas, and only comparatively recently spread into water bodies in more open areas of savannah. Here cercariae would experience greater diurnal temperature changes, and thereby aggregate more readily, so impeding invasion of the definitive host and limiting its spread (Southgate, 1978).

Other factors possibly restricting the distribution of *S. intercalatum* concern mating interactions of *S. intercalatum* with other schistosome species with which it is sympatric. For example, the introduction of *S. haematobium* by immigrants in 1968 into the town of Loum, Cameroon where *S. intercalatum* (Lower Guinea) was indigenous led to the large-scale replacement of *S. intercalatum* by *S. haematobium* through hybridisation (Southgate, 1978; Tchuem Tchuenté *et al.,* 1997 *b*). *S. haematobium* and *S. intercalatum* are closely related (both belonging to the *S. haematobium* group) and will hybridize both in nature and in the laboratory when they co-parasitize the same individuals (Wright *et al.,* 1974; Southgate *et al.,* 1976; Wright & Southgate, 1976; Tchuem Tchuenté *et al.,* 1997 *a*, *b*). Hybrid offspring of *S. haematobium* ♂ x *S. intercalatum* ♀ crosses are viable (Southgate *et al.,* 1976), and several generations of backcrossing with parental species (introgressive hybridisation) may cause the emergence of a new strain of *S. haematobium*, as at Loum. Key to the replacement of *S. intercalatum* was the competitive dominance of *S. haematobium* over *S. intercalatum* (Lower Guinea) with regard to pairing in mixed infections, as demonstrated by Southgate *et al.* (1982).

A possible past history of hybridization between *S. intercalatum* and *S. haematobium* in the Dogon country of Mali has been implicated in the apparently sudden cessation of *S. intercalatum* infection in the region (De Clercq *et al.*, 1994).

S. intercalatum is also found sympatrically in Africa with *S. mansoni* (Tchuem Tchuenté *et al.*, 1993) and, like *S. haematobium*, *S. mansoni* is thought to be limiting the distribution of *S. intercalatum* for several reasons.

Firstly, as demonstrated by Tchuem Tchuenté *et al.* (1996 *a*) in mixed infections of *S. mansoni* and *S. intercalatum* (Lower Guinea) in rodents, infection with *S. mansoni* induces a significant degree of cross-protection against subsequent infection with *S. intercalatum*, but infection with *S. intercalatum* does not cross-protect against reinfection with *S. mansoni*. In nature *S. mansoni*-induced immunity against *S. intercalatum* in infected individuals will make it difficult for *S. intercalatum* to become established in areas where *S. mansoni* is already present.

Secondly, similar to the situation with *S. haematobium*, the competitive dominance of *S. mansoni* over *S. intercalatum* in mixed infections may limit the distribution of *S. intercalatum* (Tchuem Tchuenté *et al.*, 1996 *a*). In the experiments of Tchuem Tchuenté *et al.* (1993, 1994, 1996 *a*) using *S. intercalatum* (Lower Guinea) in mixed infections with *S. mansoni*, not only was *S. mansoni* shown to be better at forming pairs than *S. intercalatum*, but also maintained a stronger homospecific mate preference. As *S. mansoni* and *S. intercalatum* cannot hybridize, being from different evolutionary groups, offspring of heterospecific pairings would be parthenogenetic and of low viability, and so homospecific pairing is preferable. It is likely that as *S. intercalatum* has only recently become sympatric with other schistosome species, pre-zygotic reproductive isolating traits such as competitive pairing and strong homospecific mate preference have not been strongly selected for (Tchuem Tchuenté *et al.*, 1995; Southgate *et al.*, 1998; Pagès *et al.*, 2001 *b*). Using data from the Tchuem Tchuenté *et al.* (1993) investigation into mating interactions between *S. intercalatum* (Lower Guinea) and *S. mansoni*, Tchuem Tchuente *et al.* (1996 *a*) used a mathematical model of mating probabilities in these mixed infections to suggest that the basic transmission rate of *S. intercalatum* could be reduced by a factor of 5 in areas of

sympatry with *S. mansoni*, independent of the effect of *S. mansoni*-induced cross-protection against *S. intercalatum*.

A third reason for *S. mansoni* outcompeting and so limiting the distribution of *S. intercalatum* concerns the fact that *S. mansoni* populations will always be greater in nature than those of *S. intercalatum*, by virtue of its greater cercarial productivity; and its greater worm burden and shorter patency period in definitive hosts (Loker, 1983).

Given the wide distribution of both *S. haematobium* and especially *S. mansoni* across much of Africa, all of the above immunological and competitive mating factors make it very difficult for *S. intercalatum* to spread. Indeed the relatively new foci of *S. intercalatum* reported in Sao Tomé and Equatorial Guinea corrrespond to situations where both *S. haematobium* and *S. mansoni* are absent (Tchuem Tchuenté *et al.*, 1996 *a*; Jourdane *et al.*, 2001).

So far, all work on mating interactions of *S. intercalatum* with *S. mansoni* and *S. haematobium* has used the Lower Guinea strain of *S. intercalatum*. Given the clear biological differences between the two strains outlined above, together with the fact that they cannot utilize each other's intermediate snail host, there is currently much debate over whether or not the two strains are in fact separate species (Jourdane *et al.*, 2001; Pagès *et al.*, 2001 *a*). Therefore, it cannot necessarily be assumed that the Zaire strain will interact in the same way as the Lower Guinea strain with other species of schistosome.

The aim of this study is to elucidate the mating interactions of *S. intercalatum* (Zaire) with *S. mansoni*, to see which (if any) species emerges as competitively dominant, and more specifically if *S. mansoni* could be limiting the distribution of *S. intercalatum* (Zaire) through competitive dominance as appears to be the case for *S. intercalatum* (Lower Guinea). It should also provide further insight into the differences between the two strains of *S. intercalatum*.

RESULTS

Experiment 1: Infections with S. intercalatum *(Zaire) and 10 days later with* S. mansoni

Table 4.1 summarizes the worms recovered from each mouse and how they paired. Two homospecific and 2 heterospecific types of pairing were obtained, and there was a surplus of unpaired males of both species. Three multiple worm pairs were obtained in total, 1 from mouse 4, mouse 5 and mouse 6. In all cases these pairings consisted of 1 male paired with 2 females: in mice 4 and 5 all worms involved were *S. mansoni*, and in mouse 6, they were all *S. intercalatum* worms.

Table 4.1. Data from mice infected first with 150 cercariae of the Zaire strain of *S. intercalatum* (Int), and 10 days later with 150 cercariae of *S. mansoni* (Ms) (so that both species reach patency simultaneously).

Mouse	Ms♂	Int♂	Ms♂ x Ms♀	Int♂ x Int♀	Ms♂ x Int♀	Int♂ x Ms♀	Multiple pairings (♂:♀)
1	5	1	7	5	8	0	
2	4	0	13	17	14	0	
3	4	0	21	8	6	0	
4	10	0	7	7	3	0	1 Ms : 2 Ms
5	2	0	10	12	2	0	1 Ms : 2 Ms
6	0	0	11	15	0	1	1 Int : 2 Int
7	2	0	7	12	20	7	
8	8	2	7	6	10	2	
9	0	0	8	4	4	2	
10	6	3	4	15	10	5	
Total:	**41**	**6**	**95**	**101**	**77**	**17**	

Overall, 196 homospecific pairs and 94 heterospecific pairs were obtained. To test whether this difference was indicative of any species preference, the Mantel-Haenszel test was carried out on the proportions of males of both species paired homospecifically and heterospecifically. The result was highly significant ($\chi^2 = 51.101$, $P < 0.001$), showing that both species have a strong preference for homospecific mating.

Of paired *S. intercalatum* males, 85.6% formed homospecific pairs, and 14.4% heterospecific pairs, whereas 55.2% of paired *S. mansoni* males formed homospecific pairs,

and 44.8% heterospecific pairs. This indicates that *S. intercalatum* has the stronger homospecific mate preference. However, it should be noted that the sex ratio of males: females for *S. intercalatum* in this experiment was 0.8:1, and this female bias may have influenced this result (see later discussion).

The proportion of all *S. intercalatum* males which were paired was 95.2%, whereas that for *S. mansoni* males was 80.8%. This difference was statistically significant according to Mantel-Haenszel analysis ($\chi^2 = 11.394$, $P < 0.005$), with *S. intercalatum* males being more successful than *S. mansoni* males at pairing. Again, it should be noted that this result may have been influenced by the female bias of the *S. intercalatum* sex ratio in this experiment.

Experiment 2: infections with S. mansoni *and 1 week later with* S. intercalatum *(Zaire)*
Two homospecific and 2 heterospecific types of pairing were obtained in this experiment (Table 4.2) together with 1 multiple-worm pair from mouse 1, which consisted of 1 *S. intercalatum* male paired with 1 *S. intercalatum* female and 1 *S. mansoni* female. The proportion of males which formed pairs was 87.4% for *S. mansoni* males and 74.2% for *S. intercalatum* males. Mantel-Haenszel analysis showed this difference to be highly significant ($\chi^2 = 4.744$, $P < 0.05$), with *S. mansoni* males being better able to form pairs than *S. intercalatum* males.

The sex ratio of males:females in this experiment was 1.3:1 for *S. mansoni*, and therefore the assumption that all *S. mansoni* females would have initially been paired with *S. mansoni* males, leaving an excess of *S. mansoni* males which may compete with *S. intercalatum* males for *S. intercalatum* females, is a reasonable one.

However, despite this assumption a total of 9 heterospecific *S. intercalatum* ♂ x *S. mansoni* ♀ pairs were obtained from mice 1, 3, 5, 7 and 9 (Table 4.2). In mice 1, 3, 5 and 7, there was at least 1 unpaired *S. mansoni* male for each *S. intercalatum* ♂ x *S. mansoni* ♀ pair, which the *S. intercalatum* males had outcompeted (Table 4.2). In mouse 9, which yielded 2 *S. intercalatum* ♂ x *S. mansoni* ♀ pairs but only 1 unpaired *S. mansoni* male, 1 of these 2 pairs may have resulted from there being 1 fewer *S. mansoni* males than *S. mansoni* females: thus an *S. intercalatum* male paired with the extra *S. mansoni* female. A Mantel-

Haenszel test carried out on the proportions of males of both species paired with *S. mansoni* females returned a highly significant result (χ^2 =30.069, P < 0.001), indicating that *S. mansoni* males were better overall at pairing with *S. mansoni* females than were *S. intercalatum* males. Therefore, *S. intercalatum* males are unable to outcompete *S. mansoni* males for *S. mansoni* females to any significant extent.

Table 4.2. Data from mice infected first with 150 cercariae of *S. mansoni* (Ms) and 1 week later with 150 cercariae of *S. intercalatum*, Zaire (Int) (so that *S. mansoni* reaches patency 2 weeks before *S. intercalatum*).

Mouse	Ms♂	Int♂	Ms♂ x Ms♀	Int♂ x Int♀	Ms♂ x Int♀	Int♂ x Ms♀	Multiple pairings (♂:♀)
1	5	0	10	9	0	1	1 Int:1 Int + 1 Ms
2	0	1	9	7	7	0	
3	2	2	14	5	10	2	
4	1	1	14	6	0	0	
5	4	13	17	13	1	3	
6	1	0	11	8	0	0	
7	4	0	16	8	10	1	
8	4	7	14	0	4	0	
9	1	1	15	7	0	2	
Total:	**22**	**25**	**120**	**63**	**32**	**9**	

Data from mice 2, 3, 5, 7 and 8 indicate that 21 of the 32 heterospecific *S. mansoni* ♂ x *S. intercalatum* ♀ pairs obtained in total arose from there being a deficit of *S. intercalatum* males for the *S. intercalatum* females to pair with. Four of the 32 pairings may have involved *S. mansoni* males that had been actively displaced from their original homospecific *S. mansoni* ♀ partners by competitor *S. intercalatum* males, as indicated by the presence of a corresponding *S. intercalatum* ♂ x *S. mansoni* ♀ pair for each of the 4 *S. mansoni* ♂ x *S. intercalatum* ♀ pairings. These displaced males may have managed to re-pair with a heterospecific female. For each of the remaining 7 heterospecific *S. mansoni* ♂ x *S. intercalatum* ♀ pairs at least 1 unpaired *S. intercalatum* male was also obtained which is assumed to have been outcompeted by the *S. mansoni* males for the *S. intercalatum*

females. The difference in the proportions of males of each species paired with *S. intercalatum* females was significant according to Mantel-Haenszel analysis ($\chi^2 = 5.041$, $P < 0.05$) indicating that overall, *S. intercalatum* males are better than *S. mansoni* males at pairing with *S. intercalatum* females.

Experiment 3: infections with S. intercalatum *(Zaire) and 4 weeks later with* S. mansoni

Table 4.3 summarizes the worms obtained from each mouse and how they paired. Four main types of pairing were found: 2 homospecific and 2 heterospecific.There were no multiple-worm pairs. The proportion of males which formed pairs was 98.6% for *S. intercalatum*, and 82.6% for *S. mansoni*. The results of a Mantel-Haenszel test carried out on these proportions was not significant, and therefore males of each species do not significantly differ in their ability to form pairs.

The sex ratio of males:females was 1.7:1 for *S. intercalatum* in this experiment, and therefore the assumption that all *S. intercalatum* females would have initially been paired with *S. intercalatum* males, leaving an excess of *S. intercalatum* males which may compete with *S. intercalatum* males for *S. intercalatum* females, is a reasonable one.

Nevertheless, 12 heterospecific *S. mansoni* ♂ x *S. intercalatum* ♀ pairs were obtained in total from mice 2, 3, 4, 6, 8 and 10. Three of the *S. mansoni* ♂ x *S. intercalatum* ♀ pairs resulted from a deficit of *S. intercalatum* males for the females to pair with in mouse 2. There was at least 1 unpaired or non-homospecificially paired *S. intercalatum* male for each of the 9 remaining pairs, indicating that these *S. intercalatum* males had been outcompeted for *S. intercalatum* females by the *S. mansoni* males. However, Mantel-Haenszel analysis revealed a highly significant difference in the proportions of males of each species paired with *S. intercalatum* females, ($\chi^2 = 20.487$, $P < 0.001$), with *S. intercalatum* males being better able to do so than *S. mansoni* males. Therefore, overall, *S. mansoni* males are unable to outcompete *S. intercalatum* males for *S. intercalatum* females to any significant extent.

Table 4.3. Data from mice infected first with 150 cercariae of *S. intercalatum* (Int), and 4 weeks later with 150 cercariae of *S. mansoni* (Ms) (so that *S. intercalatum* reaches patency 3 weeks before *S. mansoni*).

Mouse	Ms♂	Int♂	Ms♂ x Ms♀	Int♂ x Int♀	Ms♂ x Int♀	Int♂ x Ms♀
1	1	11	2	5	0	7
2	1	0	9	1	4	1
3	0	10	6	27	3	2
4	1	0	8	11	2	2
5	6	0	5	20	0	0
6	3	3	7	6	1	1
7	4	3	15	14	0	6
8	1	8	9	8	1	1
9	5	17	9	8	1	10
10	2	1	8	5	0	7
Total:	**24**	**53**	**78**	**105**	**12**	**37**

Heterospecific *S. intercalatum* ♂ x *S. mansoni* ♀ pairs were obtained from all mice in this experiment except mouse 6. Seventeen out of the 37 pairs recovered in total arose from there being a deficit of *S. mansoni* males compared with *S. mansoni* females. Eight out of the 37 were obtained along with a *S. mansoni* ♂ x *S. intercalatum* ♀ pair. Therefore, the 8 heterospecifically paired *S. intercalatum* males may have been those which had been actively displaced from their original *S. intercalatum* female partners by competitor *S. mansoni* males, and which subsequently managed to re-pair with heterospecific females. For each of the remaining 12 heterospecific *S. intercalatum* ♂ x *S. mansoni* ♀ pairs at least 1 unpaired *S. mansoni* male was also obtained which is assumed to have been outcompeted by the *S. intercalatum* males for the *S. mansoni* females. The difference in the proportions of males of each species paired with *S. mansoni* females was highly significant according to Mantel-Haenszel analysis (χ^2 =15.503, $P < 0.001$), indicating that *S. intercalatum* males are unable to outcompete *S. mansoni* males for *S. mansoni* females to any significant extent.

DISCUSSION

In all 3 experiments both homospecific and heterospecific pairs were obtained, and all females recovered were paired and contained eggs, thereby confirming that pairing and reproductive stimulation of females is possible between different species of schistosome, even where they belong to different evolutionary groups (Tchuem Tchuenté *et al.*, 1994; Khalil & Mansour, 1995; Southgate *et al.*, 1998).

Mice infected simultaneously with *S. intercalatum* (Zaire) and *S. mansoni* (experiment #1) yielded a significant preponderance of homospecific pairs over heterospecific, indicating the presence of a specific mate preference system, as demonstrated for the *S. intercalatum* (Lower Guinea)/ *S. mansoni*; *S. haematobium*/ *S. mansoni*; and *S. margrebowiei*/ *S. mansoni* models of mating between schistosomes from different evolutionary groups (Tchuem Tchuenté *et al.*, 1993; Webster *et al.*, 1999; Cosgrove & Southgate, 2002).

In this model, *S. intercalatum* (Zaire) exhibited a significantly greater homospecific mate preference than *S. mansoni*, in contrast to *S. intercalatum* (Lower Guinea) which had a lesser homospecific mate preference than *S. mansoni* (Tchuem Tchuenté *et al.*, 1993). However, it should be borne in mind that the *S. mansoni* isolate used by Tchuem Tchuenté *et al.* (1993) originated from Brazil, whereas the isolate used in these experiments came from Senegal, so it is conceivable that some of the differences in the data between Tchuem Tchuenté *et al.* (1993) and those presented here may possibly be correlated with the different isolates of *S. mansoni*. On the other hand, the work of Fletcher *et al.* (1981) mitigates against this idea, as they demonstrated, using isoenzymes, a relative lack of genetic divergence between Old and New World isolates of *S. mansoni*. The reproductive disadvantage of heterospecific pairing in yielding offspring of reduced viability has been mentioned earlier, but parthenogenetic offspring are particularly undesirable for *S. intercalatum* males in mixed infections with *S. mansoni*. This is because the *S. mansoni* ♂ x *S. intercalatum* ♀ cross yields sterile eggs, whilst the *S. intercalatum* ♂ x *S. mansoni* ♀ cross yields some parthenogenetic eggs of low viability, which may produce a small number of *S. mansoni* worms (Wright & Southgate, 1976). Therefore, the (albeit small) advantage of parthenogenesis in mixed infections (in allowing the propagation of some

germ plasm) is enjoyed only by (female) *S. mansoni*, and therefore provides a further incentive for *S. intercalatum* to avoid heterospecific pairing.

However, the sex ratio of *S. intercalatum* (Zaire) was female-biased in this experiment (0.8:1 males:females), which may have had a significant influence on the results by increasing the chances of *S. intercalatum* (Zaire) being able to pair homospecifically over those of *S. mansoni* whose sex-ratio was male-biased (as sex ratios of schistosomes in nature usually are). The excess single *S. intercalatum* females would then be available for the excess single *S. mansoni* males to pair with, so increasing the proportion of heterospecifically paired *S. mansoni* males.

Similarly, the female-bias of the *S. intercalatum* (Zaire) infection may have influenced the finding that *S. intercalatum* (Zaire) males were better at pairing than *S. mansoni* males in experiment #1, which again differs from the *S. intercalatum* (Lower Guinea)/ *S. mansoni* mating model where *S. mansoni* males were equally good at pairing as *S. intercalatum* (Lower Guinea) males (Tchuem Tchuenté *et al.*, 1993). In the simultaneous *S. intercalatum* (Lower Guinea)/*S. mansoni* experiments of Tchuem Tchuenté *et al.* (1993), equal numbers of males and females of each species were used.

Although there are indications from pairing ability and the strength of homospecific mate preference in experiment #1 that *S. intercalatum* (Zaire) has the competitive advantage over *S. mansoni* in simultaneous infections (which is the reverse of the situation with the Lower Guinea strain of *S. intercalatum*) the possible influence of the female-biased sex ratio of *S. intercalatum* (Zaire) must be taken into account.

One possible reason for there being more females than males of *S. intercalatum* (Zaire) in experiment #1 and not in experiments #2 or #3 is that the *S. intercalatum* (Zaire) infections for experiment #1 were carried out very soon after the infected snails (laboratory *B. wrighti*) reached patency (45 days after miracidial infection). Not all snails will start to shed at the same time, and it may have been possible that some of the early shedders were shedding only female cercariae. Frandsen (1977) reported that snails shedding only female *S. intercalatum* (Zaire) cercariae shed for only 32 days, whereas snails shedding only male cercariae shed for up to 119 days. Therefore, by the time the *S. intercalatum* (Zaire)

infections for experiments #2 and #3 were carried out (over one month later), some infected snails may produce fewer female cercariae, hence restoring the usual sex balance of more males to females. However, it should be noted that there is some debate on whether male cercariae have a shorter prepatent period in the snail host than females: male cercariae of an Iranian and a Mauritian strain of *S. haematobium* matured faster in *Bulinus* than female cercariae (Wright & Bennett, 1967; Wright & Knowles, 1972), whereas no difference was found between the maturation time of male and female cercariae of *S. intercalatum* (both strains) (Frandsen, 1977), and of *S. mansoni* (Liberatos, 1987).

In this model of mating interactions, several multiple-worm pairs were recovered. In experiment #1 the 3 multiple worm pairs recovered in total all consisted of 1 male paired with 2 homospecific females. It would appear that in pairing with 2 *S. mansoni* females, 1 *S. mansoni* male outcompeted 10 and 2 unpaired *S. mansoni* males in mice 4 and 5, respectively. In mouse 6, 1 *S. intercalatum* (Zaire) male outcompeted 1 other *S. intercalatum* (Zaire) male in pairing with 2 *S. intercalatum* (Zaire) females. It is likely that the outcompeted *S. intercalatum* (Zaire) male is the male partner of the single heterospecific *S. intercalatum* (Zaire) ♂ x *S. mansoni* ♀ pair recovered from mouse 6: i.e. it had managed to re-pair with a surplus *S. mansoni* female. All 3 multiple-worm pairs from experiment #1 demonstrate intraspecific male-male competition for females, and it is apparent that both *S. mansoni* and *S. intercalatum* (Zaire) males are able to pair with multiple females.

In experiment #2, where *S. mansoni* reached patency in mice 2 weeks before *S. intercalatum* (Zaire), 1 multiple worm pair was recovered from mouse 1, consisting of an *S. intercalatum* (Zaire) male paired with 1 female of each species. This pairing may have arisen by a homospecifically paired *S. intercalatum* (Zaire) male pairing with an additional *S. mansoni* female, outcompeting 5 unpaired *S. mansoni* males in the process and demonstrating the involvement of interspecific male-male competition in multiple pairings.

Tchuem Tchuenté *et al.* (1993) obtained *S. intercalatum* (Lower Guinea) males paired with 2-3 females of *S. intercalatum* (Lower Guinea) or *S. mansoni* in experiments where *S. mansoni* males were absent. Therefore it appears that both strains of *S. intercalatum* are able to pair homospecifically or heterospecifically with more than one female.

That multiple pairings are not reported more frequently from models of mating interactions between different species of schistosome may in part be due to the fact that multiple pairs could easily become separated when recovering worms from rodents by perfusion so that one or more of the participant worms is classified as unpaired.

Statistical analysis of data from experiment #2 shows that *S. mansoni* males are significantly better at pairing than *S. intercalatum* (Zaire) males. This might be expected, given that homospecific *S. mansoni* pairing would have taken place initially in the absence of competition from *S. intercalatum* males. Also, *S. mansoni* males were better overall than *S. intercalatum* males at pairing with *S. mansoni* females. Again, this is as expected since the pairing of "species 2" (*S. intercalatum*) males with "species 1" (*S. mansoni*) females is a much more active process involving the males pulling away homospecifically paired "species 1" females from their partners (except for where there is an excess of *S. mansoni* females over *S. mansoni* males as for one *S. intercalatum* ♂ x *S. mansoni* ♀ pair in mouse 9). That several *S. intercalatum* ♂ x *S. mansoni* ♀ pairs were recovered, however, shows that *S. intercalatum* males had the capability to "actively compete" for females in this manner. It also indicates that *S. intercalatum* (Zaire) males may be more competitive than *S. intercalatum* (Lower Guinea) males, as, in experiment 7 of the Tchuem Tchuenté *et al.* (1993) study, mice infected with male and female *S. mansoni* and male *S. intercalatum* (Lower Guinea) yielded only homospecific *S. mansoni* ♂ x *S. mansoni* ♀ pairs.

As mentioned in chapter 3 for the *S. margrebowiei/ S. mansoni* model of mating interactions, it is possible that in sequential infections (experiments #2 and #3), the number of males of the second infection paired with females of the first infection may have been higher had the worms been perfused from animals more than 8 weeks post-final patency since Tchuem Tchuenté *et al.* (1995) described the phenomenon of change of mate 'as a progressive process, requiring up to 8 weeks' to happen. However, in these experiments, it was not possible to delay the culling of mice any longer due to the pathogenicity of the worms.

It can be assumed that the excess of unpaired *S. mansoni* males in experiment #2 competed with unpaired *S. intercalatum* (Zaire) males for *S. intercalatum* (Zaire) females because the *S. mansoni* infection in this experiment was male-biased (1.3:1 males:females). Neither type of pairing involves "active competition" of the kind described above, instead involving "passive" heterospecific pairing by *S. mansoni* males, and "passive" homospecific pairing by *S. intercalatum* (Zaire) males. Overall, *S. intercalatum* (Zaire) males were significantly better than *S. mansoni* males at pairing with *S. intercalatum* (Zaire) females.

Experiment 6 in the Tchuem Tchuenté *et al.* (1993) study, using mice infected with males of both species but with only *S. intercalatum* (Lower Guinea) females, provides a directly comparable test of the relative abilities of males to pair with with *S. intercalatum* (Lower Guinea) females. No significant difference in this ability was found, again indicating that the Zaire strain of *S. intercalatum* is slightly more competitive in its interactions with *S. mansoni* than the Lower Guinea strain: *S. mansoni* is beaten to *S. intercalatum* (Zaire) females by *S. intercalatum* (Zaire) males, but is equally as good as *S. intercalatum* (Lower Guinea) males at pairing with *S. intercalatum* (Lower Guinea) females.

Statistical analysis of data from experiment #3, where *S. intercalatum* (Zaire) was the first infection and commenced pairing three weeks before *S. mansoni* was ready to do so, showed that there was no significant difference in the ability of males of both species to form pairs. In other words, when *S. intercalatum* (Zaire) has the advantage of being the first species to enter the host, it is only as good as *S. mansoni* at forming pairs, whereas when *S. mansoni* was the initial infection (experiment #2) it was significantly better at pairing than *S. intercalatum* (Zaire) males. Therefore in sequential infections, *S. mansoni* males appear to have the competitive edge over *S. intercalatum* (Zaire) males with regard to their ability to form pairs.

Despite the recovery in this experiment of 12 *S. mansoni* ♂ x *S. intercalatum* (Zaire) ♀ pairs, 9 of which were due to *S. mansoni* males actively pulling away paired *S. intercalatum* (Zaire) females from their homospecific partners, overall *S. intercalatum* (Zaire) males were significantly better at pairing with *S. intercalatum* (Zaire) females than *S. mansoni* males. A comparable test for the relative ability of *S. mansoni* and *S. intercalatum* (Lower Guinea) males to pair with *S. intercalatum* (Lower Guinea) females, involving "active"

competition on the part of the *S. mansoni* males and "passive" competition from the *S. intercalatum* (Lower Guinea) males was carried out by Tchuem Tchuenté *et al.* (1995) using mice infected first with males and females of *S. intercalatum* (Lower Guinea) and later reinfected with *S. mansoni* males. Surprisingly, the experiment revealed *S. mansoni* males as significantly better able than *S. intercalatum* (Lower Guinea) males to pair with female *S. intercalatum* (Lower Guinea: an as yet unique example of "active" competition winning over more "passive" competition to a statistically significant extent.

The sex ratio of *S. intercalatum* (Zaire) was male-biased in experiment #3 (1.7:1 males:females), so that excess unpaired *S. intercalatum* (Zaire) males would have been available for "passive" competition with *S. mansoni* males for *S. mansoni* females. Statistical analysis of the data revealed that overall *S. mansoni* males were better at pairing with *S. mansoni* females than were *S. intercalatum* (Zaire) males. Tchuem Tchuenté *et al.* (1993) carried out a comparable test for the relative ability of males to pair with *S. mansoni* females using mice infected with males of *S. intercalatum* (Lower Guinea) and *S. mansoni* but with only *S. mansoni* females. A similar result was obtained: male *S. mansoni* were better at pairing with female *S. mansoni* than male *S. intercalatum* (Lower Guinea).

As seen with the sequential infections in the *S. margrebowiei*/ *S. mansoni* model in chapter 3, the worm return of each species in sequential infections can be used to determine whether the initial infection induces immunological cross-protection against reinfection by the second species. In experiment #2, with *S. mansoni* as the initial infection the worm return of *S. mansoni* is 22.4% whilst that of *S. intercalatum* (Zaire) is 14.4%: 64.3% of the *S. mansoni* return. In experiment #3, with *S. intercalatum* (Zaire) as the initial infection, the worm return of *S. intercalatum* (Zaire) is 20.8% whilst that of *S. mansoni* is 15.3%: 73.6% of the *S. intercalatum* (Zaire) return. Therefore there does appear to be a similar degree of heterologous cross-protection induced by the initial infection against the second infection, in both situations. This differs from the results of Tchuem Tchuenté *et al.* (1996 *a*) who found that the cross-protection induced by *S. mansoni* against *S. intercalatum* (Lower Guinea) was not reciprocal, thus highlighting a possibly important immunological difference between the Zaire and Lower Guinea strains of *S. intercalatum*. However, when comparing degrees of cross-protection in experiments #2 and #3, it should be noted that the time interval between the initial and challenge infections differed (being 1 week and 4

weeks, respectively), which may have resulted in differing levels of immunity. As with the heterologous immunity observed in the Tchuem Tchuenté *et al.* (1996 *a*) study and the *S. margrebowiei*/ *S. mansoni* model in chapter 3, the cross-protection observed in experiments #2 and #3 is likely to result from the immunization process itself rather than to egg-induced immunity, because the first infection would not have reached patency by the time of reinfection with the second species in either of the experiments.

When comparing the results of this present study (using the Zaire strain of *S. intercalatum*) with those of Tchuem Tchuenté *et al.* (1993, 1995) (using the Lower Guinea strain) it becomes apparent that *S. mansoni* does not appear to be as competitively dominant to the Zaire strain of *S. intercalatum* as it is to the Lower Guinea strain, as evidenced by observations that the homospecific mate preference of *S. mansoni* is stronger than that of *S. intercalatum* (Lower Guinea) but weaker than that of *S. intercalatum* (Zaire); that *S. mansoni* males are equally good as *S. intercalatum* (Lower Guinea) males but worse than *S. intercalatum* (Zaire) males both at pairing in simultaneous infections and pairing with *S. intercalatum* females in sequential experiments with *S. mansoni* as the initial infection; and that *S. mansoni* males are better than *S. intercalatum* (Lower Guinea) males but worse than *S. intercalatum* (Zaire) males at pairing with *S. intercalatum* females in sequential infections with *S. intercalatum* as the initial infection. Indeed, in this present study, *S. intercalatum* (Zaire) appears to be competitively dominant to *S. mansoni* in simultaneous infections (experiment #1) by having a stronger homospecific mate preference and greater pairing ability, although the aforementioned female-bias of the *S. intercalatum* (Zaire) infection in this experiment casts doubt on the validity of these results.

However, it is experiment #2, with *S. mansoni* as the initial infection, which most closely resembles the situation in nature, that of *S. intercalatum* encountering pre-established infections of *S. mansoni* in its attempt to spread. The competitive dominance of *S. mansoni* in this situation was demonstrated by its greater ability to form pairs and its ability to retain homospecific pairing by outcompeting *S. intercalatum* males for *S. mansoni* females. In the light of this, then, it can be suggested that the competitive dominance of *S. mansoni* might be a contributory factor to the restricted distribution of the Zaire strain of *S. intercalatum*.

Given the above, it might be predicted that *S. intercalatum* (Zaire) would be competitively dominant to *S. intercalatum* (Lower Guinea) in mixed infections of the two. Interestingly, a recent study by Pagès *et al.* (2001 *b*) found mating between the two strains to be random, with no specific mate preference system in mice infected simultaneously with both strains. In contrast to many species within the *S. haematobium* group which have assortative mating as a pre-zygotic mating barrier but lack the post-zygotic mating barrier of hybrid infertility, there appears to be no assortative mating barrier between the strains of *S. intercalatum.* However, hybrids between the two strains are of reduced viability in the F_1 generation, and are sterile beyond the F_2 generation (Frandsen, 1978; Pagès *et al.,* 2001 *a*). Pagès *et al.* (2001 *b*) note that strong assortative mating tends to evolve when there are strong contact zones between different species, and therefore the geographical isolation of the Zaire and Lower Guinea strains of *S. intercalatum*, not usually so marked for other species of the *S. haematobium* group, may explain why the post-zygotic barrier of hybrid infertility is not reinforced by strong homospecific mate preference.

However, this lack of assortative mating does not exclude the possibility of one of the strains being more competitive at pairing than the other, since in the *S. haematobium*/ *S. intercalatum* (Lower Guinea) mating model (Southgate *et al.,* 1982), mating was similarly random with no specific mate preference system, yet *S. haematobium* was competitively dominant to *S. intercalatum* (Lower Guinea) in terms of pairing ability.

If the Zaire strain is indeed more competitive than the Lower Guinea strain, this then begs the question as to why the Zaire strain is restricted to the Democratic Republic of Congo whilst the Lower Guinea strain is more widespread. One important reason suggested by Tchuem Tchuenté *et al.* (1997 *a*) concerns the poor compatibility of *S. intercalatum* (Zaire) with many potential intermediate hosts (Wright *et al.,* 1972; Frandsen, 1979 *c*) in contrast to *S. intercalatum* (Lower Guinea). Apart from *B. wrighti*, which is a universal host for all schistosomes within the *S. haematobium* group (Wright & Knowles, 1972; Wright *et al.*, 1972; Southgate & Knowles, 1975; Frandsen 1979 *a*, 1979 *b*, 1979 *c*), *S. intercalatum* (Zaire) is only compatible with a very few isolates of *B. globosus*: for example, those from Zaire and Zambia, but not those from Cameroon, Togo, and the Ivory Coast (Tchuem Tchuenté *et al.*, 1997 *a*; Frandsen *et al.,* 1978). In addition, factors responsible for the reduction in prevalence (from 30% to less than 4% as reported by Tchuem Tchuenté *et al.*,

1997 *b*) of *S. intercalatum* (Zaire) in the Kinshasa focus between 1987 and 1997 may play a role in restricting the overall distribution of the Zaire strain. For example, these authors found evidence of natural hybridisation between *S. intercalatum* (Zaire) and *S. haematobium* in the area causing a decline in the transmission of pure *S. intercalatum* (Zaire). They also suggest that the intermediate host of the Zaire strain, *B. globosus*, may have been disappearing from the area, perhaps in the manner of *Biomphalaria camerunensis* in the early 1980s which led to the decline of *S. mansoni* transmission in the region in spite of the replacement of *B. camerunensis* by *B. pfeifferi*, another potential snail host for *S. mansoni* (De Clercq, 1987). For this reason, the reduced prevalence of *S. intercalatum* (Zaire) in Kinshasa cannot be blamed on its interactions with *S. mansoni*. Other factors implicated in the regional decline of the Zaire strain include the increasing socio-economic status of the human population and sanitation improvements such as the provision of piped water reducing the extent of water contact by humans (Tchuem Tchuenté *et al.*, 1997 *a*).

In summary, the mating model presented here describes further differences between the Zaire and Lower Guinea strains of *S. intercalatum*, and allows the prediction that the Zaire strain may be competitively dominant to the Lower Guinea strain to be made. There is evidence to suggest that *S. mansoni* may hinder the spread of *S. intercalatum* (Zaire) to areas where *S. mansoni* is already present, both through its competitiveness at mating and by the induction of immunological cross-protection against *S. intercalatum* (Zaire), but to a lesser extent than for *S. intercalatum* (Lower Guinea). Nevertheless, *S. intercalatum* (Zaire) is more restricted in its distribution than *S. intercalatum* (Lower Guinea), which highlights the great importance of setting observations from laboratory models of mating interactions in a wider context incorporating other factors and influences on the transmission of schistosomes in the field.

CHAPTER 5

Mating interactions between *S. margrebowiei* and *S. bovis*

CHAPTER 5

MATING INTERACTIONS BETWEEN *S. MARGREBOWIEI* AND *S. BOVIS*

INTRODUCTION

Schistosoma bovis and *S. margrebowiei* both belong to the *S. haematobium* group of schistosomes, and are the causative organisms of bovine schistosomiasis.

S. bovis utilises cattle as its primary definitive host, but has been reported as a naturally-acquired infection in kobs, horses, donkeys, sheep, goats, camels, dromedaries, pigs, 7 species of antelope and 2 rodents (Pitchford, 1977 *a*). The few reports which have suggested human infection with *S. bovis* (or *S. margrebowiei*) have not been substantiated (Pitchford, 1977 *a*; Rollinson & Southgate, 1987). *S. bovis* has a wide geographical distribution, being found in Spain; a number of Mediterrannean islands such as Corsica, Sicily and Sardinia; the Middle-East; North Africa and 12 African countries south of the Sahara (Moné *et al.*, 2000). Southgate & Knowles (1975) identified 2 biologically different "forms" of *S. bovis*, one form encompassing *S. bovis* in the Mediterrannean, North Africa and the Middle East which is transmitted exclusively by *Bulinus truncatus*, and the other form encompassing *S. bovis* in Kenya which is transmitted by 3 groups of *Bulinus*: the *truncatus/tropicus*, *forskalii* and *africanus* groups. More recently Moné *et al.* (2000) have applied the latter form more widely to *S. bovis* occurring in countries south of the Sahara, and have separated Iberian *S. bovis* from *S. bovis* from elsewhere in the Mediterrannean, as *S. bovis* in Iberia is transmitted by *Planorbarius metidjensis* as well as *B. truncatus*.

As described in chapter 3, *S. margrebowiei* has a fairly restricted distribution, especially in comparison to the distribution of its intermediate and definitive hosts, and is found mainly in the area extending from the Chobe Game Reserve in Botswana, through the Caprivi Strip into south-western Zambia and southern Zaire (Pitchford, 1976). *S. margrebowiei* is naturally transmitted by bulinid snails of the *B. forskalii* and *B. truncatus/tropicus* groups (Wright *et al.*, 1979 *a*; Southgate *et al.*, 1985) and has a wide spectrum of definitive hosts, which includes cattle, goats, buffalo, impala, roan and sable antelope, blue wildebeest, cobs, waterbuck, horses and zebra (Pitchford, 1977 *a*). However, according to Pitchford (1976) the primary definitive hosts of *S. margrebowiei* are lechwe (*Kobus leche*) and puku

(*K. vardoni*). It is the somewhat limited distribution of these primary definitive hosts which Pitchford (1976) suggests as the reason for the restricted distribution of *S. margrebowiei*. However, Southgate & Knowles (1977) suggest an alternative reason based on the behaviour of *S. margrebowiei* cercariae. Similar to cercariae of *S. intercalatum*, *S. margrebowiei* cercariae will form aggregates in response to small increases in temperature, which are non-infective to definitive hosts. Therefore it is possible that, as postulated for *S. intercalatum* (which also has a restricted distribution), transmission of *S. margrebowiei* is limited to areas which are subject to little temperature fluctuation (Southgate & Knowles, 1977).

S. bovis and *S. margrebowiei* are sympatric in the Katanga Province of southern Zaire (van den Berghe, 1934) and in Chad (Graber, 1969, 1978), but mixed infections of the two species have only been reported from the latter country, in Defassa waterbuck (*K. defassa*) and kobs (*K. kob*). However, given the wide definitive host spectrum for both species, and modern animal husbandry methods leading to increasingly mobile animal populations, there exists the possibility that these two species may come into contact with one another more in the future, increasing the chances of their co-parasitizing the same definitive hosts.

In the following study, the mating interactions between these two species in mixed infections in mice are investigated. Theoretically, *S. bovis* and *S. margrebowiei* are able to hybridise where they co-exist within the same definitive host, since they belong to the same evolutionary group (Wright & Southgate, 1976; Wright & Ross, 1980; Jourdane & Southgate, 1992). The only previous study of mating interactions between two bovine schistosome species, *S. bovis* and *S. curassoni*, which are able to hybridise and produce fertile hybrid progeny, demonstrated the absence of assortative mating between these species (Rollinson *et al.*, 1990). Assortative mating is an important pre-zygotic mating barrier which acts to reduce the likelihood of hybridisation taking place, and it is the aim of the study presented here to establish the existence or otherwise of this mating barrier between *S. bovis* and *S. margrebowiei* and to determine their relative competitive mating abilities.

RESULTS

Experiment 1: Simultaneous infections

The percentage worm return of *S. margrebowiei* worms in this experiment was extremely low (3.7%), with no *S. margrebowiei* worms recovered at all from mice 1-5. Comparisons between *S. margrebowiei* and *S. bovis* can therefore be made using data from mice 6, 7, and 8 only. The sex ratio was male-biased for both species.

Table 5.1 summarizes the worms recovered from each mouse and how they paired. Both types of homospecific pairs were obtained, but only mouse 8 contained any heterospecific pairs: 2 *S. margrebowiei* ♂ x *S. bovis* ♀ pairs and 1 *S. bovis* ♂ x *S. margrebowiei* ♀ pair. A surplus of unpaired males of both species was obtained from mice 6-8, and 1 multiple worm pair consisting of a *S. bovis* male paired with 2 *S. bovis* was recovered from mouse 5.

Table 5.1. Data from mice infected with 150 cercariae of *S. bovis* (Bo), and 10 days later with 150 cercariae of S. *margrebowiei* (Mg) (so that both reach patency simultaneously).

Mouse	Mg♂	Bo♂	Mg♂x Mg♀	Bo♂x Bo♀	Mg♂x Bo♀	Bo♂x Mg♀	Multiple pairings (♂:♀)
1	0	0	0	8	0	0	
2	0	5	0	6	0	0	
3	0	3	0	10	0	0	
4	0	2	0	3	0	0	
5	0	0	0	5	0	0	1Bov: 2Bov
6	3	0	8	6	0	0	
7	2	2	4	8	0	0	
8	3	3	5	2	2	1	
Total:	**8**	**15**	**17**	**48**	**2**	**1**	

The proportions of males paired homospecifically and heterospecifically were compared using a Mantel-Haenszel test, and the result was highly significant ($\chi^2 = 19.377$, $P < 0.001$), showing that both species have a strong preference for homospecific mating.

Of paired *S. bovis* males from mice 6-8, 16/17 (94.1%) formed homospecific pairs, compared with 17/19 (89.5%) of paired *S. margrebowiei* males which paired

homospecifically. The strength of the preference for homospecific partners is therefore very similar for males of the two species.

The proportion of all *S. bovis* males which were paired was 70.4% whereas that for *S. margrebowiei* males was 76.6%. According to Mantel-Haenszel analysis, this difference was not significant, suggesting that both species are equally good at pairing.

Experiment 2: infections with S. margrebowiei, *and 2 weeks later with* S. bovis

Two homospecific and two heterospecific types of pairing were obtained in this experiment (Table 5.2) together with one multiple-worm pair from mouse 7, which consisted of 1 *S. margrebowiei* male paired with 2 *S. margrebowiei* females. There was a surplus of unpaired worms of both species, and the sex ratio of both species was male-biased, with 1.7 males per female.

Table 5.2. Data from mice infected with 150 cercariae of *S. margrebowiei* (Mg), and 2 weeks later with 150 cercariae of *S. bovis* (Bo) (so that *S. margrebowiei* reaches patency 3-4 weeks earlier than *S. bovis*).

Mouse	Mg♂	Bo♂	Mg♂x Mg♀	Bo♂x Bo♀	Mg♂x Bo♀	Bo♂x Mg♀	Multiple pairings (♂:♀)
1	14	5	4	2	0	0	
2	15	9	18	7	0	0	
3	3	1	14	4	1	0	
4	5	0	13	8	0	0	
5	9	1	11	1	1	1	
6	13	5	11	1	3	2	
7	0	2	7	0	0	0	1Mg: 2Mg
8	7	4	15	4	1	1	
9	10	0	10	0	0	0	
Total:	**76**	**27**	**103**	**27**	**6**	**4**	

The proportion of males which formed pairs was 58.9% for *S. margrebowiei* and 46.5% for *S. bovis*. Mantel-Haenszel analysis showed this difference not to be significant, and therefore there is no significant difference in the ability of the two species to form pairs.

As the sex ratio of both species was male-biased in this experiment (with 1.7 males per female), all *S. margrebowiei* females should initially have been paired with *S. margrebowiei* males, leaving an excess of *S. margrebowiei* males which may have competed with *S. bovis* males for *S. bovis* females.

However, a total of 4 heterospecific *S. bovis* ♂ x *S. margrebowiei* ♀ pairs were obtained from mice 5, 6, and 8 (Table 5.2). All of these pairs had at least one corresponding unpaired *S. margrebowiei* male, which is therefore assumed to have been actively displaced from its homospecific pairing by the competitor *S. bovis* male. However, a Mantel-Haenszel test carried out on the proportions of males of both species paired with *S. margrebowiei* females returned a highly significant result (χ^2 = 16.014, P < 0.001), indicating that overall *S. margrebowiei* males were better at pairing with *S. margrebowiei* females than *S. bovis* males were, despite the occurrence of the 4 *S. bovis* ♂ x *S. margrebowiei* ♀ pairs.

Mice 3, 5, 6 and 8 yielded a total of 6 heterospecific *S. margrebowiei* ♂ x *S. bovis* ♀ pairs. Four out of these 6 may have involved the 4 *S. margrebowiei* males that had been actively displaced from their original homospecific *S. margrebowiei* partners (described above) by *S. bovis* males, as indicated by the presence of the 4 corresponding *S. bovis* ♂ x *S. margrebowiei* ♀ pairs for each of the 4 *S. margrebowiei* ♂ x *S. bovis* ♀ pairs. These displaced *S. margrebowiei* males had managed to re-pair with a heterospecific (*S. bovis*) female. For each of the 2 remaining heterospecific *S. margrebowiei* ♂ x *S. bovis* ♀ pairs at least one unpaired *S. bovis* male was also obtained which is assumed to have been outcompeted by the *S. margrebowiei* males for the *S. bovis* females. The difference in the proportions of males of each species paired with *S. bovis* females was highly significant according to Mantel-Haenszel analysis (χ^2 = 22.115, P < 0.001), indicating that overall *S. bovis* males were better than *S. margrebowiei* males at pairing with *S. bovis* females.

Experiment 3: infections with S. bovis, *4 weeks before* S. margrebowiei

Two types of homospecific and 2 types of heterospecific pairing were obtained in this experiment (Table 5.3), together with 9 multiple-worm pairs, consisting of a *S. margrebowiei* male paired with 2-4 females, either of *S. bovis*, or of a mixture of *S. bovis* and *S. margrebowiei*. There was a surplus of unpaired male worms of both species.

Table 5.3. Data from mice infected with 150 cercariae of *S. bovis* (Bo), and 4 weeks later with 150 cercariae of S. *margrebowiei* (Mg) (so that *S. bovis* reaches patency 2-3 weeks earlier than *S. margrebowiei*).

Mouse	Mg♂	Bo♂	Mg♂x Mg♀	Bo♂x Bo♀	Mg♂x Bo♀	Bo♂x Mg♀	Multiple pairings (♂:♀)			
1	1	1	8	11	0	0	1Mg: 4Bov	1Mg:3Bov+ 1Mg		
2	1	0	9	7	1	1				
3	0	0	14	3	2	0				
4	0	1	7	8	2	0	1Mg:1Bo+ 1Mg	1Mg:1Bov+ 1Mg	1Mg:1Bov+ 1Mg	1Mg:1Bov 1Mg
5	4	0	11	6	3	0	1Mg:2Bov	1Mg:1Bov+ 1Mg		
6	4	0	5	7	2	1				
7	5	0	7	13	5	1				
8	4	0	0	5	6	0	1Mg:2Bov			
Total:	**19**	**2**	**61**	**60**	**21**	**3**				

The proportions of males which formed pairs was 96.9% for *S. bovis* and 81.2% for *S. margrebowiei* . Mantel-Haenszel analysis showed this difference to be significant (χ^2 = 8.128, P < 0.005), with *S. bovis* males being better able to form pairs than *S. margrebowiei* males. However, it should be noted that this result may be influenced by the fact that the sex ratio of *S. bovis* in this experiment was female-biased (with 1.5 *S. bovis* females per *S. bovis* male), making it easier for *S. bovis* males to achieve a homosexual pairing.

Similarly, the female-biased *S. bovis* sex ratio means that not all *S. bovis* females will initially have been paired with *S. bovis* males: there will have been an excess of unpaired *S. bovis* females available to pair with *S. margrebowiei* males (there were 1.6 *S. margrebowiei* males per *S. margrebowiei* female). Indeed, data from mice 3, 4, 5, 6, 7 and 8 indicate that 17 out of the 21 heterospecific *S. margrebowiei* ♂ x *S. bovis* ♀ pairs obtained in total in this experiment arose from a deficit of *S. bovis* males for the *S. bovis* females to pair with. However, data from mice 2, 4, 6 and 7 indicate that the *S. bovis* females from the remaining 4 *S. margrebowiei* ♂ x *S. bovis* ♀ pairs probably were originally paired homospecifically with *S. bovis* males, but the latter were actively displaced by competitor *S. margrebowiei* males. The evidence for this comes from the

recovery of one corresponding non-homospecifically paired *S. bovis* male for each of these 4 pairings. The difference in the proportions of males of each species paired with *S. bovis* females was highly significant according to Mantel-Haenszel analysis ($\chi^2 = 22.716$, $P < 0.001$), indicating that overall *S. bovis* males are better than *S. margrebowiei* males at pairing with *S. bovis* females.

Data from mice 2, 6, and 7 indicate that all 3 of the *S. bovis* ♂ x *S. margrebowiei* ♀ pairs obtained in total involved *S. bovis* males which had initially been displaced from homospecific pairings with *S. margrebowiei* females by *S. bovis* males (as described above), but had managed to re-pair heterospecifically. Evidence for this comes from the recovery of one corresponding *S. margrebowiei* ♂ x *S. bovis* pairing for each of the 3 *S. bovis* ♂ x *S. margrebowiei* ♀ pairs. According to Mantel-Haenszel analysis, the difference in the proportions of males of each species paired with *S. margrebowiei* females was not significant: however, given that 61 homospecifically paired *S. margrebowiei* females were obtained in this experiment as opposed to just 3 heterospecifically paired *S. margrebowiei* females, this lack of significance is likely to result from the lack of data for *S. bovis* ♂ x *S. margrebowiei* ♀ pairings rather than from *S. bovis* males being equally as good as *S. margrebowiei* males at pairing with *S. margrebowiei* females.

DISCUSSION

The data from all 3 experiments show that in mixed infections with *S. bovis* and *S. margrebowiei* both homospecific and heterospecific pairs are formed. All females recovered were paired and ovigerous, once again demonstrating that pairing and reproductive stimulation of females can take place between different species of schistosome (Khalil & Mansour, 1995; Southgate *et al.*, 1998).

In experiment #1, where all worms were ready to form pairs at the same time and therefore had a free choice of mate from either species, data from the only 3 mice which sustained infections with *S. margrebowiei* as well as *S. bovis* showed that, given the choice of mate, both species have a strong preference for homospecific partners. This is in contrast to a previous study of mating interactions between bovine schistosomes, *S. bovis* and *S. curassoni* (Rollinson *et al.*, 1990), where no such specific mate preference was shown to exist: mating between the 2 species appeared to be random.

In this simultaneous infection, there appeared to be little difference between *S. bovis* and *S. margrebowiei* in terms of the strength of this homospecific preference, nor was there a significant difference in their ability to form pairs.

In experiment #2, where mice were infected initially with *S. margrebowiei* and later with *S. bovis* so that *S. margrebowiei* worms would form pairs before *S. bovis* males were ready to do so, *S. bovis* males were equally as good as *S. margrebowiei* males at forming pairs. Given that *S. margrebowiei* enjoys a time advantage over *S. bovis* in being the first infection and therefore able to form pairs initially in the absence of any competition from *S. bovis*, the fact that *S. bovis* is still equally as good at pairing as *S. margrebowiei* indicates that *S. bovis* males might be slightly more competitive than *S. margrebowiei* males. So, too, might the results of the Mantel-Haenszel analysis which showed that *S. margrebowiei* males are unable to outcompete *S. bovis* males for *S. bovis* females to any significant extent. In the model of mating interactions between *S. margrebowiei* and *S. mansoni*, (chapter 3) where neither species appeared to be competitively dominant to the other, males of the species which formed the first infection did better those of the second infection in terms of overall pairing ability; pairing with its own females, and were at least as good as males of the second species at pairing with females of the second species. Apart from *S.*

margrebowiei males proving better than *S. bovis* males at pairing with *S. margrebowiei* females, experiment #2 does not conform to this model of "no competitive dominance" for the reasons given above, suggesting, on the contrary that *S. bovis* males may have the competitive edge over *S. margrebowiei* males.

Indeed, this experiment has strong parallels with one from the *S. intercalatum* (Zaire strain)/ *S. mansoni* mating model (chapter 4) whereby mice were initially infected with *S. intercalatum* (Zaire) and later with *S. mansoni.* Although *S. intercalatum* (Zaire) males were better than *S. mansoni* males at pairing with *S. intercalatum* (Zaire) females, *S. mansoni* males (just as for *S. bovis* males) were equally as good as *S. intercalatum* (Zaire) males at forming pairs, and were better than *S. intercalatum* (Zaire) males at pairing with *S. mansoni* females. The reciprocal sequential infection whereby *S. mansoni* formed the first infection provided further evidence that *S. mansoni* was more competitive than *S. intercalatum* (Zaire) males as *S. mansoni* males proved better than *S. intercalatum* (Zaire) males at forming pairs and at pairing with *S. mansoni* females, that *S. mansoni* appeared to be dominant to *S. intercalatum* (Zaire).

Unfortunately however, although *S. bovis* males proved better than *S. margrebowiei* males at pairing with *S. bovis* females in mice infected firstly with *S. bovis* in experiment #3, it is difficult to gather further evidence of the competitive dominance of *S. bovis* over *S. margrebowiei* from experiment #3, owing to the female-bias of the *S. bovis* infection in this experiment. This female-bias casts doubt on the validity of finding that *S. bovis* males were statistically better than *S. margrebowiei* males at pairing, because it makes it easier for *S. bovis* males rather than *S. margrebowiei* males to achieve desirable homospecific pairings. Similarly, the finding that there was no significant difference in the ability of males of either species to pair with *S. margrebowiei* females cannot be interpreted as *S. bovis* males being equally as good as *S. margrebowiei* males at pairing with *S. margrebowiei* females, because clearly this lack of significance results from a lack of data for *S. bovis* ♂ x *S. margrebowiei* ♀ pairings. This lack of data itself results from the female-bias of the *S. bovis* infection, as it means that most *S. bovis* males will have been able to achieve a homospecific pairing, and are therefore unlikely to pair with a *S. margrebowiei* female.

However, by analogy with the sequential infections from the *S. intercalatum* (Zaire) / *S. mansoni* mating model, we may predict that, were the *S. bovis* infection to have been male-biased in experiment #3, we may indeed have found *S. bovis* males to be more competitive at pairing than *S. margrebowiei* males. To be certain of this, however, this experiment would need to be repeated and the usual male-bias of schistosome infections obtained. Interestingly, *S. bovis* was found to be less competitive than *S. curassoni* at pairing in the simultaneous infection experiments carried out by Rollinson *et al.* (1990).

The recovery in experiments #2 and #3 of several pairings of "species 2 males" paired with "species 1 females" together with at least one unpaired "species 1 male" for each such pairing to indicate that a true displacement had occurred, demonstrates that males of both species have the ability to compete for paired females by actively pulling them away from their partners.

Males of both species also appeared to be able to pair with more than one female: for example, one *S. bovis* male was found paired with 2 *S. bovis* females in mouse 5 in experiment #1, probably because there was one surplus *S. bovis* female compared with *S. bovis* males in this mouse. Similarly, there was one more *S. margrebowiei* female than *S. margrebowiei* males in experiment #2, mouse 7, which may explain why one *S. margrebowiei* male was found paired with 2 *S. margrebowiei* females. The large number (9) of heterospecific multiple pairs recovered from experiment #3 is probably also due to the female-bias of the *S. bovis* infection in this experiment resulting in a surplus of *S. bovis* females. Interestingly, the male worm from all 9 multiple-worm pairs in experiment #3 was always a *S. margrebowiei* male, 6 of which appear to have paired homospecifically and then paired with an additional surplus *S. bovis* female. The remaining 3 multiple-worm pairs appear to have arisen from *S. margrebowiei* males which had not gained a homospecific partner pairing with 2 or 4 surplus *S. bovis* females. It is interesting that no multiple worm pairs involving *S. bovis* males paired with more than one *S. bovis* female were recovered in this experiment, and suggests that *S. margrebowiei* males are slightly better at forming multiple-worm pairs than *S. bovis* males. If so, then a possible explanation is afforded by the larger size of *S. margrebowiei* male worms compared with *S. bovis* males, hence they have a larger gynaecophoric canal in which to grasp multiple females.

The strong homospecific preference demonstrated for both species in experiment #1 represents an important pre-zygotic isolating mechanism which will serve to reduce the chances of these two species hybridising should they come into contact with one another in the field. No laboratory studies on the exact nature of *S. bovis*/ *S. margrebowiei* hybrids have been carried out, however, and it is therefore not known whether any post-zygotic isolating mechanisms are in place. For example, Taylor (1970) discovered strong post-zygotic isolation between *S. bovis* and *S. mattheei* in the form of low viability of hybrid F_1 miracidia, which is probably why there is little evidence of hybridisation between these 2 species in the field, even though they co-exist in the same area or within the same definitive host in Chad (Graber, 1969; Birgi & Graber, 1969; Delpy *et al.*, 1972), and in Kenya and Tanzania (Dinnik & Dinnik, 1965). Christensen *et al.* (1983) suggest that information so far available does not point towards hybridization between bovine schistosomes occurring to the extent of becoming important to the clinical picture in the bovine host. These authors point out that suggestions of natural hybridisation between *S. bovis* and *S. leiperi* (Dinnik & Dinnik, 1965) and *S. mattheei* and *S. leiperi* (Pitchford, 1974) have not been substantiated.

However, the Rollinson *et al.* (1990) study on *S. bovis*/ *S. curassoni* does provide evidence of natural interactions between these 2 schistosomes in cattle in West Africa in the form of heterospecific pairings, intrauterine eggs of indeterminate shape, and worms with acid phosphatase isoenzyme profiles identical to those of laboratory-produced F_1 hybrids. As mentioned above, these species were shown in laboratory studies to lack the pre-zygotic isolating barrier of assortative mating, and furthermore, to lack the post-zygotic barrier of hybrid infertility: viable laboratory hybrids were produced up to the F_2 generation for the *S. curassoni* ♂ x *S. bovis* ♀ cross, and to the F_4 generation for the *S. bovis* ♂ x *S. curassoni* ♀ cross.

The question therefore arises for *S. bovis*/ *S. curassoni* as to how they maintain their species identity whilst co-parasitizing the same definitive host. Rollinson *et al.* (1990) suggest the involvement of heterologous immunity in this, as there is growing evidence that such immunity plays a role in interactions between bovine schistosomes and other schistosomes

species or even trematodes of a different genus. For example, Yagi *et al.* (1986) demonstrated that cross-genus protection was induced in zebu cattle by *S. bovis* against *Fasciola gigantica*, and *vice versa*. Wright *et al.* (1979 *a*) suggested that *S. leiperi* may have a suppressive effect on *S. margrebowiei* in lechwe in the Lochinvar National Park, Zambia where these two species are sympatric, as it seems that once *S. leiperi* is established in an animal, further infection with *S. margrebowiei* does not occur. As discussed in chapter 3, Pitchford (1976, 1977 *b*) suggests that the "lechwe schistsomes" *S. margrebowiei* and *S. leiperi* might, through heterologous immunity, prevent the development of *S. mattheei* (parasitic in bovines and humans), and of the human parasites *S. haematobium* and *S. mansoni*. This latter hypothesis is supported by the immunological evidence in chapter 3 for *S. margrebowiei*-induced cross-protection against *S. mansoni* in mice (and *vice versa*).

As for the involvement of heterologous immunity in the *S. bovis*/ *S. margrebowiei* model presented here, it appears from the worm return data that *S. margrebowiei* induces a significant degree of cross-protection against *S. bovis* when *S. margrebowiei* is the first infection (in experiment #2 the worm return of *S. margrebowiei* was 26.1% whilst that of *S. bovis* was 6.7%) but in the reciprocal situation in experiment #3 *S. bovis* fails to induce any cross-protection against *S. margrebowiei* (in experiment #3 the worm return of *S. bovis* was 15.0% whilst that of *S. margrebowiei* was 13.5%). As discussed for heterologous immunity in the *S. margrebowiei*/ *S. mansoni* model (chapter 3; Cosgrove & Southgate, 2002), protection is not induced by eggs of the first infection, as the first infection would not have reached patency by the time mice were reinfected with the second species. Instead, it is possible that factors associated with the initial immunization process itself, such as persistent skin inflammation might be responsible for the protection. However, the very poor worm return of *S. margrebowiei* in experiment #1, and the female-bias of the *S. bovis* infection in experiment #3 does cast some doubt on the validity of worm return data from the experiments in this *S. bovis*/ *S. margrebowiei* model.

In summary, therefore, *S. bovis* and *S. margrebowiei* appear to be reproductively isolated to some extent by strong assortative mating, and there is some suggestion that pre-patent heterologous cross-protection, indicated for *S. margrebowiei* against *S. bovis* but possibly not *vice versa*, may play a role in the interactions between these two species. That *S. bovis*

might be slightly more competitive than *S. margrebowiei* at mating is also indicated by these experiments, but whether this would confer any advantage in the natural situation to *S. bovis* is not yet known, and may depend partly on whether hybrids of both male/female combinations are equally viable. Laboratory crosses between *S. bovis* and *S. margrebowiei*, to determine the viability of the hybrid progeny and so to establish whether the post-zygotic isolating mechanism of hybrid inviability operates between these species, have yet to be performed.

CHAPTER 6

Mating interactions between *S. haematobium* and *S. intercalatum* (Lower Guinea strain)

CHAPTER 6

MATING INTERACTIONS BETWEEN *S. HAEMATOBIUM* AND *S. INTERCALATUM* (LOWER GUINEA STRAIN)

INTRODUCTION

S. haematobium and *S. intercalatum* belong to the *S. haematobium* group of schistosomes and are thought to have originated from a common stock, diverging through geographical isolation into two separate species. It has been postulated that *S. intercalatum* evolved as a parasite of primates in forest areas, and *S. haematobium* as a parasite of hominoid ancestors of man in savannah areas (Wright *et al.,* 1972). Being from the same evolutionary group, these two species are able to hybridise (Wright *et al.,* 1974; Southgate *et al.,* 1976, 1998; Mutani *et al.,* 1985; Pagès & Théron, 1990), and there have been numerous reports of the occurrence of natural hybridisation in humans between *S. haematobium* and the Lower Guinea strain of *S. intercalatum*: in Gabon (Burchard & Kern, 1985; Zwingenberger *et al.,* 1990); possibly in Mali (De Clercq *et al.,* 1994) and in Cameroon (Wright *et al.,* 1974; Southgate *et al.,* 1976; Ratard & Greer, 1991; Tchuem Tchuenté *et al.,* 2001). So far, there has only been one report of natural hybridisation between *S. haematobium* and the Zaire strain of *S. intercalatum*, in Kinshasa, Democratic Republic of Congo (Tchuem Tchuenté *et al.,* 1997 *a*): most likely a reflection of the fact that *S. intercalatum* (Zaire) is confined to this one country, whereas the distribution of *S. intercalatum* (Lower Guinea) is more widespread.

The first, and perhaps the most remarkable occurrence of natural hybridisation between *S. haematobium* and *S. intercalatum* (Lower Guinea) was reported by Wright *et al.* (1974) in the town of Loum, Cameroon. As described in chapter 4, *S. haematobium* was introduced by immigrants between 1968-1972 into this area of *S. intercalatum* endemicity, where recent forest clearance had allowed an intermediate host for *S. haematobium*, *B. truncatus*, to become locally established. In 1974, Wright *et al.* observed eggs intermediate in size and shape between *S. haematobium* and *S. intercalatum*, and found that most individuals were passing eggs in the urine (typical of *S. haematobium* infections) and not in the faeces (as with *S. intercalatum* infections). This was not due to any decline in the populations of *B. forskalii*, the intermediate host for *S. intercalatum* (Lower Guinea) as this snail was present

in more sites in the River Mbette (the main river running through Loum) than *B. truncatus* in a survey carried out by Tchuem Tchuenté *et al.* (1997 *b*).

Wright & Southgate (1976) demonstrated that hybrid offspring of *S. haematobium* ♂ x *S. intercalatum* ♀ crosses are viable, whereas offspring of the reciprocal *S. intercalatum* ♂ x *S. haematobium* ♀ crosses are not. Because *S. haematobium* eggs are passed in the urine and it is the male which carries the female to the oviposition site, so too would eggs from viable hybrids be passed in the urine. Thus the hybrids most resemble *S. haematobium.*

By 1976, the swing from intestinal to urinary schistosomiasis at Loum was already apparent: *S. haematobium* (and its hybrid) was progressively replacing *S. intercalatum* (Southgate *et al.,* 1976). Today, a recent epidemiological study has shown that *S. intercalatum* has been completely replaced by *S. haematobium* over a period of less than thirty years (Tchuem Tchuenté *et al.,* 1997 *b*).

Several factors implicated in this replacement were highlighted by Southgate *et al.* (1976). Firstly, parasites transmitting eggs via urine (in this case *S. haematobium* and the hybrid) are at an advantage to those transmitting eggs via faeces (e.g. *S. intercalatum*) in areas such as Loum where improved sanitation involving the use of latrines means that eggs in faeces do not reach water in a fresh condition. In contrast, urinary eggs are more likely to be voided directly into water, for example, by bathing children. Secondly, *B. truncatus* (the snail host of *S. haematobium* at Loum) is much larger than *B. forskalii* (the snail host of *S. intercalatum* (Lower Guinea), and therefore has the capacity to produce more cercariae. Thus populations of *S. haematobium* cercariae will be greater in nature than populations of *S. intercalatum* cercariae, placing *S. haematobium* at an advantage. Furthermore, the viable hybrids themselves exhibit heterosis and have several advantages over their parental species: they are able to use both *B. truncatus* and *B. forskalii* as intermediate hosts, but have greater infectivity for *B. truncatus*; they have greater fecundity and are more infective to definitive hosts (Wright & Southgate, 1976).

However, the chief means by which *S. haematobium* effected the replacement of *S. intercalatum* was through introgressive hybridisation (Southgate *et al.,* 1976). As

mentioned in chapter 4, this involved generations of backcrossing of hybrids with the parental species (of which parental *S. haematobium* may have been in the majority as explained above) which led, when coupled with the greater ability of *S. haematobium* males to pair with females (Southgate *et al.,* 1982), to the progressive dilution of the *S. intercalatum* genome into that of *S. haematobium* (Jourdane *et al.,* 2001).

Southgate *et al.* (1982) demonstrated the greater competitiveness of *S. haematobium* over *S. intercalatum* (Lower Guinea) with regard to pairing ability, in a laboratory model of mating interactions between these two species in hamsters. Hamsters were either infected simultaneously, or infected first with *S. intercalatum* and then with *S. haematobium.* Surprisingly, mating between the two species appeared to be random: no specific mate preference system was demonstrated for either species. It has been suggested that *S. haematobium* and *S. intercalatum* had not evolved strong assortative mating because they have only fairly recently come into contact with one another, largely through the incursions of man into the rainforest (Southgate *et al.,* 1982; Pagès *et al.,* 2001 *b*).

In both the simultaneous infections and infections with *S. intercalatum* before *S. haematobium* which Southgate *et al.* (1982) carried out, *S. haematobium* males were clearly shown to be better at pairing with females of either species than *S. intercalatum* males. Given the usual natural bias of schistosome sex ratios towards males (Liberatos, 1987; Mitchell *et al.,* 1990; Jourdane *et al.,* 2001) it is clear how the competitiveness of *S. haematobium* males can reduce the reproductive success of *S. intercalatum.*

Southgate *et al.* (1982) did not carry out a sequential infection involving hamsters infected first with *S. haematobium* and later with *S. intercalatum,* because the reverse situation, of *S. intercalatum* being established in hosts prior to infection with *S. haematobium,* most closely reflected the situation in the field at the time. However, as the replacement of *S. intercalatum* by *S. haematobium* progressed, it is likely that younger individuals would initially have been infected with *S. haematobium* prior to any subsequent infection with *S. intercalatum.* The study presented here was undertaken in order to complete the model of *S. haematobium/ S. intercalatum* mating interactions by infecting hamsters initially with *S. haematobium* and subsequently with *S. intercalatum.* This should provide further insight into the process by which *S. haematobium* replaced *S. intercalatum* in such a short period

of time, and allow us to ascertain the likelihood of *S. intercalatum* re-establishing itself in Loum in the future, in the face of pre-established *S. haematobium* infections.

RESULTS

Both types of homospecific and both types of heterospecific pairing were obtained in this experiment (Table 6.1), together with 1 homosexual pair from hamster 11 consisting of 2 male *S. intercalatum* worms (one smaller male inside the gynaecophoric canal of the other). The proportion of males which formed pairs was 90.4% for *S. haematobium* males, and 65.4% for *S. intercalatum* males. Mantel-Haenszel analysis showed this difference to be significant ($\chi^2 = 11.931$, $P < 0.005$), with *S. haematobium* males being better at pairing than *S. intercalatum* males. The sex ratio of males: females in this experiment was 2.2:1 for *S. haematobium* and 1.2:1 for *S. intercalatum*.

Table 6.1. Data from hamsters infected first with 150 cercariae of *S. haematobium* (Hm) and 4 weeks later with 150 cercariae of the Lower Guinea strain of *S. intercalatum* (Int), so that *S. haematobium* reaches patency approximately 2 weeks before *S. intercalatum*)

Hamster	Hm♂	Int♂	Hm♂ xHm♀	Int♂ xInt♀	Hm♂ xInt♀	Int♂ xHm♀	Homosexual pairings (♂: ♂)
1	1	1	2	2	4	0	
2	0	2	0	1	5	0	
3	1	1	0	0	0	0	
4	0	0	0	1	0	0	
5	0	1	0	0	1	0	
6	1	2	0	0	3	0	
7	0	0	0	1	0	1	
8	0	0	2	2	2	0	
9	0	0	1	3	0	0	
10	0	1	2	0	5	0	
11	0	5	0	6	1	1	1 Int : 1 Int
12	0	0	0	4	0	1	
13	0	0	2	4	1	0	
14	0	0	3	0	3	0	
15	0	3	2	8	1	2	
16	0	3	1	3	0	1	
17	1	9	2	2	2	0	
18	0	0	0	7	0	0	
19	1	0	2	3	0	0	
Total:	**5**	**28**	**19**	**47**	**28**	**6**	

Although all *S. haematobium* females should have been paired with *S. haematobium* males before *S. intercalatum* males commenced pairing, a total of 6 heterospecific *S. intercalatum* ♂ x *S. haematobium* ♀ pairs were obtained from hamsters 7, 11, 12, 15 and 16. However, 4 out of these 6 pairs resulted from there being a deficit of *S. haematobium* males for the *S. haematobium* females to pair with: in each case the extra females were mated by an *S. intercalatum* male. For 2 out of the 6 pairings (one from hamster 11, one from hamster 15) a corresponding non-homospecifically paired *S. haematobium* male was also obtained, indicating that this male had been displaced from its original homospecific female partner by a competitor *S. intercalatum* male. A Mantel-Haenszel test carried out on the proportions of males of both species paired with *S. haematobium* females returned a significant result ($\chi^2 = 8.987$, $P < 0.005$), indicating that overall *S. haematobium* males were better than *S. intercalatum* males at pairing with *S. haematobium* females.

Data from hamsters 1, 2, 5, 6, 8, 10, 11, 13-15 and 17 indicate that 17 out of the 28 heterospecific *S. haematobium* ♂ x *S. intercalatum* ♀ pairs obtained in total resulted from there being a deficit of *S. intercalatum* males for the *S. intercalatum* females to pair with. Two of the 28 pairings may have involved *S. haematobium* males that had been actively displaced from their original homospecific *S. haematobium* ♀ partners by competitor *S. intercalatum* males, as described above. These displaced *S. haematobium* males had managed to re-pair with a heterospecific female. For each of the remaining 9 heterospecific *S. haematobium* ♂ x *S. intercalatum* ♀ pairs at least one unpaired *S. intercalatum* male was also obtained which is assumed to have been outcompeted by the *S. haematobium* males for the *S. intercalatum* females. The difference in the proportions of males of each species paired with *S. intercalatum* females was significant according to Mantel-Haenszel analysis ($\chi^2 = 7.072$, $P < 0.01$) indicating that overall, *S. haematobium* males are better than *S. intercalatum* males at pairing with *S. intercalatum* females.

DISCUSSION

In this experiment, both homospecific and heterospecific pairs were obtained, and all females recovered were paired and contained eggs.

The low worm return (2.7%) of *S. haematobium* from hamsters in this experiment highlights the difficulty which *S. haematobium* presents with regard to its maintenance in laboratory rodents. *S. haematobium* cannot be maintained in mice, and it is the necessary use of hamsters in this study which is probably responsible for the poor worm return of *S. intercalatum* (5.4%), which develops more readily in mice (Frandsen, 1977). It is somewhat surprising, however, that the worm return of *S. intercalatum* should be higher than that of *S. haematobium*, as previous studies indicate that the reverse is usually the case (Wright & Knowles, 1972; Wright *et al.,* 1972; Southgate *et al.,* 1976).

The recovery of one homosexual pairing between two *S. intercalatum* males from hamster 11 (in which there were no available females for them to pair with) is interesting. Male-male pairing has often been noted in schistosomes in unisex infections or situations where there are few or no available females, mainly for *S. mansoni* (Giovannola, 1936; Paraense & Malheiros Santos, 1949; Vogel, 1947; Basch & Gupta, 1988). As observed for the homosexual pair obtained in this study, the male partner clasped within the gynaecophoric canal of the other tends, as a rule, to be smaller (Vogel, 1947; Armstrong, 1965; Basch 1991). Armstrong (1965) working on *S. mansoni* observed that these smaller males were also sexually immature. In addition, this author noted that homosexual male pairs remained in or near the liver, indicating that the mere presence of a schistosome in the gynaecophoric canal is insufficient to stimulate migration from the liver to the mesenteric veins, a finding supported by Smith & Chappell (1990).

The two *S. intercalatum* ♂ x *S. haematobium* ♀ pairs recovered from hamsters 11 and 15 demonstrate the ability of *S. intercalatum* males to "actively compete" for *S. haematobium* females by pulling them away from their homospecific *S. haematobium* partners. Data from the Southgate *et al.* (1982) experiment where *S. intercalatum* was the initial infection and *S. haematobium* the subsequent infection reveal that *S. haematobium* males were similarly able to "actively compete" with *S. intercalatum* males for *S. intercalatum* females.

In demonstrating that *S. haematobium* males are better than *S. intercalatum* males at pairing with females of either species when *S. haematobium* is the initial infection, the experiment presented here confirms the competitive dominance of *S. haematobium* over *S. intercalatum* as observed by Southgate *et al.* (1982). It therefore seems that at all stages throughout the progressive replacement of *S. haematobium* at Loum, i.e. whether co-parasitized hosts were initially infected with *S. intercalatum*, or with *S. haematobium*, or with both species simultaneously, the reproductive success of *S. intercalatum* was severely compromised by the competitive mating of *S. haematobium*, leading to its eventual exclusion from the region.

However, it should be noted that whilst recent field data (based largely on biological and morphological criteria) indicates that introgressive hybridisation between *S. haematobium* and its hybrid have progressed to the point of emergence of a new strain of pure *S. haematobium* such that only *S. haematobium* exists now at Loum (Tchuem Tchuenté *et al.*, 1997 *b*), there has been some recent suggestion that not all traces of *S. intercalatum* genes have been lost from the local schistosome population.

For example, a mathematical model constructed by Morand *et al.* (2002), of the interactions between *S. haematobium* and *S. intercalatum*, which assumes a male-biased sex ratio (as is usual in schistosome populations) and incorporates "recombinations" and compatibility levels with the snail hosts *B. truncatus* and *B. forskalii*, predicts the loss of *S. intercalatum* but the persistence of the hybrids together with *S. haematobium*. Therefore, in the population of hybrids, some remnant of the *S. intercalatum* genome is predicted to exist, although the authors acknowledge that the model may be oversimplified and that increasing the complexity of the model (e.g. by assuming that compatibility with intermediate hosts is controlled by several loci instead of one) may cause the complete disappearance of these hybrids. However, the prediction of persistent *S. intercalatum* genes in the schistosome population at Loum is supported by very recent molecular data (Webster *et al.*, in press).

Undeniably, however, there is no longer transmission of pure *S. intercalatum* at Loum, and the results of the study presented here indicate that *S. intercalatum*, in the absence of any other factors, will be unable to re-establish itself in a population of human hosts already infected with *S. haematobium*.

As discussed in chapter 4, introgressive hybridisation with *S. haematobium* is thought to be one of the key factors limiting the distribution of *S. intercalatum* in Africa, and there appear to be more areas of sympatry between *S. intercalatum* and *S. haematobium* than between *S. intercalatum* and *S. mansoni* in Africa (Morand *et al.*, 2002).

However, the competitiveness of *S. haematobium* over *S. intercalatum* and introgressive hybridisation may not always prove advantageous as a survival strategy for *S. haematobium* when other ecological or socio-economic factors have a large role to play in the transmission of schistosomiasis in a particular area. For example, in 1986 Ratard & Greer observed hybridisation between *S. haematobium* and *S. intercalatum* in the village of Kinding Ndjabi, 200km north of Yaoundé, Cameroon, and outside the hybrid zone of Loum (Ratard & Greer, 1991). Strangely, the only aquatic snail found was *B. forskalii*, the snail host of *S. intercalatum*, and therefore the origin of the *S. haematobium* in the village was (and still is) unknown. When the village was re-surveyed in 1999, Tchuem Tchuenté *et al.* (2001) discovered that the transmission of schistosomiasis had completely ceased. Mostly this was attributed to the installation of a water pump in the village in 1989, which dramatically reduced the water contact of the villagers with natural water-bodies. However, it was also thought that introgressive hybridisation between the species, together with the competitive mating abilities of *S. haematobium*, may have led to a *S. haematobium*-like parasite population which was less compatible with *B. forskalii*, thereby contributing to the local decline of schistosomiasis.

In addition, Zwingenberger *et al.* (1990), in a study concerning a focus of *S. haematobium*/ *S. intercalatum* hybridisation in Lambaréné, Gabon, noted that even when the snail hosts of both species are present, optimal conditions for the transmission of *S. haematobium* and *S. intercalatum* may be mutually exclusive, because transmission of *S. haematobium* is optimised in sites receiving full sunshine, whereas *S. intercalatum* cercariae form non-infective aggregates in water exceeding 25°C.

Therefore, whilst introgressive hybridisation and the competitive mating ability of *S. haematobium* over *S. intercalatum*, which the studies of Southgate *et al.* (1976, 1982) have identified (and the results of this study confirmed) as crucial determinants of the

replacement of *S. intercalatum* by *S. haematobium* at Loum, undoubtedly play a role in restricting the distribution of *S. intercalatum* in Africa, this role may itself be limited by the fact that *S. intercalatum*/ *S. haematobium* interactions are largely confined to the boundaries between rainforest and savannah.

CHAPTER 7

Mating interactions between *S. haematobium* and *S. mansoni*

CHAPTER 7

MATING INTERACTIONS BETWEEN *S. HAEMATOBIUM* AND *S. MANSONI*

INTRODUCTION

S. mansoni and *S. haematobium* are from two different evolutionary groups which bear their names, and are respectively the causative organisms of intestinal and urinary schistosomiasis in man. They are widely distributed across Africa, and mixed foci of these two species are numerous, occuring in over 30 African countries (Doumenge *et al.,* 1987; Fig 1.1). The construction of hydroelectric dams and irrigation schemes have increased the contact areas between these species, usually as a result of alterations to the local ecology (such as changes in water salinity) allowing snail hosts to become established.

For example, the Aswan High Dam in Egypt (built in the 1960s); the dams at Diama in Senegal (1985) and Manantali in Mali (1989), and the Lagdo Dam in North Cameroon (1982) all resulted in the introduction of *S. mansoni* into regions where *S. haematobium* was already established (Abdel-Wahab *et al.,* 1993; Southgate, 1997; Cunin *et al.,* 2000). Evidence that these two species interact with one another in human hosts in mixed foci has come from observations of the ectopic elimination of *S. mansoni* eggs in the urine, by Blair (1965) in Zimbabwe; Amin (1972) in Sudan; Woldemichael & Wondimagegnehu (1985) in Ethiopia; Ratard *et al.* (1990) in North Cameroon and Ernould (1996) in the Senegal River Basin. As it is the male schistosome which carries the female to the egg-laying site after pairing in the hepatic portal vein, the implication from these observations was that *S. haematobium* males were dominating *S. mansoni* males by pairing heterospecifically with *S. mansoni* females and carrying them to the urinary oviposition site for *S. haematobium.* Ratard *et al.* (1990) noted that when *S. haematobium* was the heavier infection, more *S. mansoni* eggs were passed in the urine than when the infection with *S. mansoni* predominated. Similar observations were made more recently by Cunin *et al.* (2000, personal communication) in the aftermath of the 1992 irrigation of the area surrounding the Lagdo Dam in North Cameroon, which led to a significant increase in the prevalence of *S. mansoni* in the Bénoué river basin. These authors reported the ectopic elimination of eggs of both species, but predominantly of *S. mansoni* eggs in urine, together with a reduction in *S. mansoni* morbidity, probably due to the diversion of *S. mansoni* eggs from intestinal organs towards the urinary tract. All these observations suggested that the tendency for *S.*

mansoni males to form heterospecific pairs was less than that for *S. haematobium*. Indeed the study by Webster *et al.* (1999) which examined mating interactions between *S. haematobium* and *S. mansoni* in laboratory hamsters, largely supported these field observations, establishing that *S. mansoni* had the stronger homospecific mate preference of the two species, and that *S. haematobium* males were more competitive at pairing heterospecifically than *S. mansoni* males.

The work presented here forms a valuable extension of the Webster *et al.* (1999) study, by examining the mating interactions between *S. haematobium* and *S. mansoni* in experiments where a single sex of *S. mansoni* is introduced into hamsters pre-infected with male and female *S. haematobium*. Snails infected with only one miracidium will give rise to clonal populations of cercariae of a single sex (Cort, 1921). However, as male and female cercariae are morphologically identical, in contrast to the adult worms, there exists the problem of identifying cercarial sex. There are two methods for determining the sex of *S. mansoni* cercariae, both of which were used in the experiments in this chapter. The first method uses the PCR technique to amplify a *S. mansoni* female-specific marker, called W1. The W1 sequence, first identified by Webster *et al.* (1989), is present as a highly reiterated set of tandem repeats on the female W chromosome, and its routine use in *S. mansoni* sex determination has been described by Gasser *et al.* (1991) and Dias Neto *et al.* (1993). The second method involves infecting control mice with cercariae shed from unimiracidially infected snails, and waiting approximately 35 days for the development of the adult worms before culling the mice to reveal the sex. This method relies on the short life cycle of *S. mansoni* to increase the chance of the snails surviving and continuing shedding up to, and after the culling of the control mice. It is this factor of snail survival which makes it impractical to work with separate sexes of *S. haematobium* cercariae, as the prepatent period for *S. haematobium* (approximately 60 days in hamsters) is far longer than that for *S. mansoni*, and no equivalent of the *S. mansoni* W1 marker has yet been identified for *S. haematobium*. Thus the experimental infections below involve mixed-sex *S. haematobium*, and unisex *S. mansoni*.

The experiments should allow us to re-examine the issue of whether it is the male or female which makes the choice of mate in schistosome infections, and the phenomenon of change of mate, which challenges the long-held belief that schistosomes "pair for life".

RESULTS

Experiment 1: infections with male and female S. haematobium, *and 8 weeks later with male* S. mansoni

Both homospecific *S. haematobium* ♂ x *S. haematobium* ♀ pairs and heterospecific *S. mansoni* ♂ x *S. haematobium* ♀ pairs were recovered from hamsters in this experiment (Table 7.1), and there was a surplus of unpaired males of both species. Eleven homosexual pairings were obtained in total, which either consisted of 2 *S. mansoni* males (1 such pair was recovered from hamster 4, 1 from hamster 5 and 3 from hamster 6) or of 1 *S. mansoni* male and 1 *S. haematobium* male (1 pair of this type was recovered from hamster 4, 2 from hamster 5, 2 from hamster 6 and 1 from hamster 7). The sex ratio of males:females for *S. haematobium* in this experiment was 1.3:1.

Table 7.1. Data from hamsters infected with 200 mixed male and female cercariae of *S. haematobium* (Hm) and 8 weeks later with 100 male cercariae of *S. mansoni* (Ms) (so that *S. haematobium* reaches patency 5 weeks before *S. mansoni*).

Hamster	Hm♂	Ms♂	Hm♀	Hm♂ xHm♀	Ms♂ xHm♀	Homosexual pairings (♂: ♂)	
						2 Ms ♂	1Hm ♂ + 1Ms ♂
1	2	12	0	3	2		
2	1	12	0	4	1		
3	3	16	0	4	2		
4	2	8	0	1	1	1	1
5	1	14	0	4	0	1	2
6	1	14	0	2	0	3	2
7	1	6	0	0	0	0	1
8	2	18	0	4	2		
9	2	19	0	5	2		
Total:	**15**	**119**	**0**	**27**	**10**		

Given the male-biased sex ratio for *S. haematobium* in this experiment, it is assumed that all *S. haematobium* females would have been paired with *S. haematobium* males prior to the introduction of *S. mansoni* males. However, 10 *S. mansoni* ♂ x *S. haematobium* ♀ pairs were recovered in this experiment, all of which had at least 1 corresponding unpaired *S.*

haematobium male. These pairs are therefore assumed to have arisen from the *S. mansoni* males actively displacing *S. haematobium* males by pulling away their *S. haematobium* female partners and mating with them in place of the *S. haematobium* males.

The proportion of males which formed pairs was 64.3% for *S. haematobium* and 7.8% for *S. mansoni*. This difference was highly significant according to Mantel-Haenszel analysis ($\chi^2 = 51.959$, $P < 0.001$), with *S. haematobium* males proving far better than *S. mansoni* males at pairing with *S. haematobium* females. Therefore, whilst *S. mansoni* males were able to actively displace some *S. haematobium* males from their *S. haematobium* female partners, overall they were far worse than *S. haematobium* males at pairing with *S. haematobium* females.

Experiment 2: infections first with mixed male and female S. haematobium *and 8 weeks later with female* S. mansoni

In this experiment, both homospecific *S. haematobium* ♂ x *S. haematobium* ♀ pairs and heterospecific *S. haematobium* ♂ x *S. mansoni* ♀ pairs were obtained, and there was an excess of unpaired females of both species (Table 7.2). Eight multiple-worm pairs were also recovered in total, from hamsters 5, 7, 8 and 10, each consisting of a *S. haematobium* male paired with 2 females: either 1 female from each species (3 pairs: 1 from hamster 7, one from hamster 8 and 1 from hamster 10); 2 *S. haematobium* females (1 pair: from hamster 10); or 2 *S. mansoni* females (4 pairs: 1 from hamster 5, 2 from hamster 7, 1 from hamster 8). The sex ratio of males:females for *S. haematobium* in this experiment was 1.4:1. All paired females of both species were sexually mature.

Three stunted and sexually immature unpaired *S. haematobium* females were recovered in total from this experiment, from hamsters 2, 9, and 10 (1 from each). The remaining 12 unpaired *S. haematobium* females were sexually mature. Five out of 20 unpaired *S. mansoni* females recovered were sexually mature: the remainder were stunted and sexually immature. All immature worms were identified by the Sm107 technique (chapter 2, sections 2.2.1 and 2.2.5).

Table 7.2. Data from hamsters infected with 200 mixed male and female cercariae of *S. haematobium* and 8 weeks later with 100 female cercariae of *S. mansoni* (so that *S. haematobium* reaches patency 5 weeks before *S. mansoni*).

Hamster	Hm♂	Hm♀	Ms♀	Hm♂ xHm♀	Hm♂ xMs♀	Multiple pairings (♂: ♀)		
1	0	2	2	1	6			
2	0	1	1	0	0			
3	0	0	1	0	1			
4	0	1	6	1	4			
5	0	4	3	0	3	1Hm: 2Ms		
6	0	0	3	1	0			
7	0	1	3	3	6	1Hm: 2Ms	1Hm: 2Ms	1Hm: 1Hm+1Ms
8	0	2	1	1	2	1Hm: 1Hm+1Ms	1Hm: 2Ms	
9	0	2	0	1	1			
10	0	2	0	0	1	1Hm: 2Hm	1Hm: 1Hm+1Ms	
Total:	**0**	**15**	**20**	**8**	**24**			

The proportion of females which were paired was 70.0% for *S. haematobium* and 55.0% for *S. mansoni*. According to Mantel-Haenszel analysis, this difference was not statistically significant. It should be noted when interpreting this result, however, that the low worm return of both *S. haematobium* and *S. mansoni* in this experiment (3.4% and 5.5%, respectively) may have contributed to this lack of significance.

It is assumed that all *S. haematobium* females would have been paired with *S. haematobium* males prior to the introduction of *S. mansoni* females because of the male-biased sex ratio for *S. haematobium* in this experiment. However, 24 *S. haematobium* ♂ x *S. mansoni* ♀ pairs were obtained in total, 11 of which were recovered along with at least 1 corresponding unpaired *S. haematobium* female which had presumably previously been paired but had been replaced by the *S. mansoni* females. The remaining 13 *S. haematobium* ♂ x *S. mansoni* ♀ pairs arose from there being a deficit of *S. haematobium* females for the *S. haematobium* males to pair with: the excess males paired with available *S. mansoni* females instead.

DISCUSSION

Experiment #1 demonstrates the greater ability of *S. haematobium* males to pair with *S. haematobium* females compared with *S. mansoni* males which have been introduced into a pre-established infection of male and female *S. haematobium*. Given that *S. haematobium* males are initially able to pair with conspecific females in the absence of any competition from *S. mansoni* in this experiment, whereas *S. mansoni* males have to actively pull away paired *S. haematobium* females in order to achieve a heterospecific pairing, this result may not be considered surprising. However, a similar experiment was carried out by Tchuem Tchuenté *et al.* (1995) where *S. mansoni* males were introduced into a pre-established infection of male and female *S. intercalatum* (Lower Guinea strain) and here, it was *S. mansoni* males which had the greater pairing ability, such was the competitive dominance of *S. mansoni* over *S. intercalatum*. This, together with the fact that homospecific pairing is not necessarily the preferred option for *S. haematobium* males (as indicated by the simultaneous infection of hamsters with *S. haematobium* and *S. mansoni* carried out by Webster *et al.* (1999) in which 64% of paired *S. haematobium* males had heterospecific partners) suggests it is reasonable to interpret the results of experiment #1 as further evidence for the competitive dominance of *S. haematobium* males over *S. mansoni* males, first observed by Webster *et al.* (1999).

All of the 11 male homosexual pairs recovered in this experiment (Table 7.1) involved *S. mansoni* males paired either homospecifically (5 pairs) or heterospecifically (6 pairs), with one of the male partners being held in the gynaecophoric canal of the other. This is consistent with the fact that (as mentioned in chapter 6) male homosexual pairing has most often reported been for *S. mansoni*, in unisex infections or (as in this experiment) where there is a shortage of available females (Giovannola, 1936; Paraense & Malheiros Santos, 1949; Vogel, 1947; Basch & Gupta, 1988). Armstrong (1965) demonstrated that older males in unisexual infections will grasp newer, sexually undifferentiated worms on re-infection with male cercariae, and by so doing impede their development. It would therefore have been valuable to have observed whether it was the *S. haematobium* male or the *S. mansoni* male partner which was clasped within the gynaecophoric canal of the other male in the 6 heterospecific homosexual pairings obtained in this experiment: the implication being that the older unpaired *S. haematobium* worms may perhaps have clasped the younger *S. mansoni* males as a means of inhibiting their development. Unfortunately it

was not possible to determine this before the partners of the heterospecific homosexual pairs had separated: separation of the partners of homosexual pairs occurs more rapidly than for heterosexual pairs, as observed by Vogel (1947). However, the recovery of both homospecific and heterospecific homosexual pairs in this experiment confirm observations by Armstrong (1965) that the clasping of a schistosome within the gynaecophoric canal of a male is neither sex-specific nor species-specific. Indeed, Armstrong (1965) obtained homosexual pairings between different genera such as *S. mansoni*/ *Schistosomatium douthitti* and *Heterobilharzia americana*/ *S. mansoni,* and Basch & Nicolas (1989) observed the clasping of cotton and alginate fibres by single *S. mansoni* males in culture indicating the involvement of thigmotactic rather than chemotactic (pheromonal) processes in clasping by males (Basch, 1991).

Before discussing the implications arising from the Mantel-Haenszel test result in experiment #2 which indicates that there is no significant difference between the proportions of *S. haematobium* males paired heterospecifically or homospecifically, several interesting and/or anomalous features of the pairings obtained in this experiment will be discussed.

Firstly, the low worm return of both species in this experiment must be remembered, as it might have contributed to the result of the Mantel-Haenszel test. The low return of *S. haematobium* (3.4%) highlights the difficulty which *S. haematobium* presents with regard to its maintenance in laboratory rodents. The low return of *S. mansoni* females (5.5%) in this experiment in comparison with the 16.1% return of *S. mansoni* males in experiment #1, is in accordance with the results of Liberatos (1987) who obtained a 16% worm return for male *S. mansoni* and a 9% worm return for female *S. mansoni* from mice infected with equal numbers of male and female *S. mansoni* cercariae. There is therefore some suggestion of greater infectivity of male cercariae, at least for *S. mansoni*.

Two unpaired and immature *S. haematobium* females were recovered from hamsters 9 and 10 (one from each). These females are likely never to have been paired owing to a deficit of *S. haematobium* males compared with *S. haematobium* females in these hamsters: hamster 9 yielded 3 *S. haematobium* females and only 2 *S. haematobium* males; hamster 10 yielded 5 *S. haematobium* males and only 3 *S. haematobium* females. The reason why 2 immature

S. haematobium females were not recovered from hamster 10 is probably because one *S. haematobium* male was found paired with 2 *S. haematobium* females.

Five unpaired but gravid *S. mansoni* females were recovered in total from hamsters 3, 4, 7 and 8, which requires some explanation as the implication is that these *S. mansoni* males had previously been paired. That they were discarded by males in preference for *S. haematobium* females is unlikely (and impossible in mouse 3 which contained no paired *S. haematobium* females) as the *S. haematobium* females themselves would have to have been previously discarded by a *S. haematobium* male. That the gravid unpaired *S. mansoni* females had been discarded by males in preference for other *S. mansoni* females is a possiblility, because, although all paired *S. mansoni* females recovered in this experiment were sexually mature, Popiel *et al.* (1984) demonstrated that intrauterine eggs of *S. mansoni* can retain a normal appearance for up to 10 days following separation from a male worm, before they start to degenerate: 10 days is longer than the (approximately) 1 week period necessary for the replacement *S. mansoni* females to reach sexual maturity (Clegg, 1959). However, evidence against the unpaired gravid *S. mansoni* females having been discarded by a *S. haematobium* male in preference for other *S. mansoni* females is provided by Tchuem Tchuenté *et al.* (1996 *b*) who argue that in experiments with an excess of females, paired males would have no need to change partners for another female of the same species.

An alternative explanation for the origin of these gravid unpaired *S. mansoni* females is that they were once part of a multiple-worm pairing with a *S. haematobium* male, but became separated during perfusion of the hamsters. For example, the two unpaired gravid *S. mansoni* females from hamster 7 may have originally been part of any of the 3 multiple-worm pairs obtained along with them, whilst the unpaired gravid *S. mansoni* female from hamster 8 may have originated from either of 2 multiple-worm pairs. No multiple-worm pairs were recovered from hamsters 3 and 4, but the gravid unpaired *S. mansoni* females from these hamsters could have originally shared a *S. haematobium* male with another female (of *S. mansoni* for hamster 3 or of *S. haematobium* or *S. mansoni* for mouse 4), which would appear as a normal male:female pairing if one of the *S. mansoni* females partners had become separated.

If females did become separated from multiple-worm pairs in this way, then some of the apparently "normal" pairs should be treated with some caution as they may originally have been "multiples". In addition, this may suggest that females from multiple-worm pairings are more easily separated than those from normal pairs. A possible explanation for this might be that the females of multiple-pairings must share the surface of the gynaecophoric canal (which contains the spines necessary for males to grip onto females) between them, so reducing the surface area available to each, and resulting in a looser male grasp of the female.

In experiment #2, all females of *S. haematobium* should initially be paired with *S. haematobium* males, because of the male-biased sex ratio of the *S. haematobium* infection. Therefore, any *S. haematobium* ♂ x *S. mansoni* ♀ pairs, which have a corresponding unpaired *S. haematobium* female are evidence that a change of mate has taken place. Depending on whether this mate change is carried out mainly by the male or the female partner, these *S. haematobium* ♂ x *S. mansoni* ♀ pairings, of which 11 were obtained in this experiment, will have arisen either by the *S. haematobium* male discarding its *S. haematobium* female partner in preference for a *S. mansoni* female, or by *S. mansoni* females actively displacing the *S. haematobium* females. Therefore the result of the Mantel-Haenszel test, which indicates that there is no significant difference between the proportions of *S. haematobium* males paired heterospecifically or homospecifically, may either be interpreted as *S. haematobium* males having no preference for homospecific females over heterospecific females, or as females of both species being equally good at pairing with *S. haematobium* males.

Even though sexual selection theory (Sutherland, 1985; Tchuem Tchuenté *et al.*, 1996 *b*) predicts that it is the sex which is usually in the minority which makes the choice of mate (i.e. females in the case of schistosome infections), Tchuem Tchuenté *et al.* (1993, 1995, 1996 *b*) have argued that it is the male and not the female which chooses, and is the active partner in the process of mate changing. In their experiment on mate change in mixed infections of *S. mansoni* and *S. intercalatum* (Lower Guinea strain) Tchuem Tchuenté *et al.* (1995) reported that there was a far greater change to homospecific partners when *S. mansoni* females were introduced into a pre-established heterospecific infection of *S.*

mansoni males and *S. intercalatum* females than the reverse case where *S. mansoni* males were introduced into a pre-established heterospecific infection of *S. intercalatum* males and *S. mansoni* females. In the latter situation, if *S. mansoni* females were discarding their heterospecific *S. intercalatum* male partners, rather than *S. mansoni* males pulling them away from the *S. intercalatum* males, it would be expected that they would have done so more readily given the strong homospecific mate preference of *S. mansoni* first established by Tchuem Tchuenté *et al.* (1993). Similarly, in experiment #2, given the competitiveness of *S. haematobium* over *S. mansoni* at pairing, as demonstrated by Webster *et al.* (1999) and confirmed by experiment #1 (above), little or no change from homospecific to heterospecific pairs might have been expected if females were responsible for the change of mate, rather than a similar proportion of homospecific and heterospecific pairs. Furthermore, schistosome males have actually been observed in a tug-of-war for females by Armstrong (1965), for example, who recovered one *Heterobilharzia americana* male which was in the process of pulling away a *S. mansoni* female from a *S. mansoni* male (from a mouse infected with both these species plus *Schistosomatium douthitti*): the anterior of the female was clasped by the *H. americana* male, and the posterior by the male *S. mansoni* worm.

The observation that unpaired females remain immature in monospecific infections with an excess of female worms was cited by Combes *et al.* (1987) as evidence that change of mate did not occur in schistosome infections because, if change of mate was taking place, all the unpaired females should be brought to maturity in rotation. However, Tchuem Tchuenté *et al.* (1995, 1996 *b*) argued that on the contrary, this was not only consistent with the phenomenon of change of mate, but that it provided further support for mate choice by males. This is because absence of mate change in monospecific infections where females are in excess is due to the fact that paired males do not require a change of partner, whereas in monospecific infections with an excess of male worms (as is usual for schistosome infections), mate changing does occur because males compete for scarce females and may effect a change of mate. The latter was demonstrated by Pica-Mattoccia *et al.* (2000) using drug-resistant and drug-susceptible strains of *S. mansoni* to infect mice, initially with male and female cercariae of one of the strains, and later with males of the other strain. Any pairs containing a male of the second infection which also had a corresponding unpaired male of the first infection were taken as evidence that a change of mate had taken place. 20.8% of

all pairs recovered from their experiments fulfilled these criteria, demonstrating that change of mate does indeed occur in monospecific infections with an excess of males.

Mate choice by males is also predicted where there is variation in the quality of females (Tchuem Tchuenté *et al.,* 1996 *b*): in schistosomes there is little difference between paired and unpaired males, but unpaired females are highly stunted and sexually immature whilst paired females are sexually mature and therefore more fecund. In addition, a homospecific female will be of higher "quality" than a heterospecific female in terms of being better able to propagate the germ plasm of the male into the next generation.

Therefore, it would appear that the 11 cases where *S. mansoni* females replaced *S. haematobium* females as partners to *S. haematobium* males were brought about by *S. haematobium* males discarding the *S. haematobium* females in preference for *S. mansoni* females. Hamster 5 yielded an unpaired *S. haematobium* female which had a discernible ovary and had therefore once been paired, but no corresponding *S. haematobium* ♂ x *S. mansoni* ♀ pair was obtained with it. However, a multiple-worm pair consisting of a *S. haematobium* male paired with 2 *S. mansoni* females was obtained, and therefore it would appear that the *S. haematobium* male had discarded the *S. haematobium* female in preference for 2 *S. mansoni* females.

This apparent discarding of *S. haematobium* females for *S. mansoni* females presents us with considerable difficulties, not least because it runs contrary to the findings of Tchuem Tchuenté *et al.* (1995) that change of mate (between *S. intercalatum* and *S. mansoni*) was always from heterospecific to homospecific pairings, with the obvious reproductive advantage of allowing the reconstitution of fertile pairs. Indeed it is very difficult to find an explanation as to why a *S. haematobium* male would want to abandon a homospecific female for a heterospecific one with which it has no chance of reproductive success: any eggs produced would be matroclonal and of parthenogenetic origin (Jourdane *et al.,* 1995; Khalil & Mansour, 1995; Southgate & Rollinson, 1987; Southgate *et al.,* 1982, 1995; Tchuem Tchuenté *et al.,* 1994). Webster *et al.* (1999) found that 64% of paired *S. haematobium* males partnered *S. mansoni* females in hamsters infected simultaneously with both species. Astonishingly, this appears to suggest not just a lack of preference for

homospecific females, but a preference for *S. mansoni* female partners, as do the results of experiment #2 presented here. To substantiate these surprising observations, however, it would be necessary to repeat this experiment using larger numbers of schistosomes, as the worm return from this experiment was very low.. Unfortunately for purposes of comparison, no other studies of mixed infections of *S. haematobium* with schistosomes from a different evolutionary group have been carried out: the 2 previous studies of *S. haematobium* in mixed infections have involved *S. intercalatum* (Southgate *et al.*, 1982) and *S. mattheei* (Southgate *et al.*, 1995), both of which belong to the *S. haematobium* group. *S. haematobium* did not exhibit any specific mate preference in infections with *S. intercalatum*, but was shown to have a strong homospecific mate preference in infections with *S. mattheei*.

Webster *et al.* (1999) suggested that this lack of assortative mating by *S. haematobium*, given that it leads to heterospecific pairing and offspring of greatly reduced fitness, is contributory to its progressive exclusion by *S. mansoni* over a period of 15-20 years in the Fayoum oasis in Egypt: an area previously endemic only for *S. haematobium.* In addition, whilst the female *S. mansoni* partners of heterospecifically paired *S. haematobium* males experience the same reproductive disadvantages as *S. haematobium*, the higher numbers of females per male; the greater worm burden and shorter reproductive cycle of *S. mansoni* compared with *S. haematobium* all act to reduce the impact of competitive pairing by *S. haematobium* males on the overall reproductive success of *S. mansoni.*

However, whilst the implication from the above is that replacement of *S. haematobium* by *S. mansoni* is favoured in mixed foci of the two species, this is somewhat countered by the findings of Cunin (personal communication), which show that *S. mansoni* has made no inroads into the prevalence of *S. haematobium* at Douloumi, a non-irrigated village in the Benoué river basin in North Cameroon, despite the introduction of *S. mansoni* into the area 10 years previously. This indicates that some other factor, probably environmental, may be needed to shift the epidemiological balance towards *S. mansoni.* Indeed, in seven neighbouring villages to Douloumi, all of which had irrigation, the marked spread of *S. mansoni* in this zone was apparent, with the prevalence of *S. mansoni* eggs in urine greater than 5%. Here it seems that the environmental factor responsible for this spread of *S. mansoni*, and for its likely increase in prevalence in future, is that of irrigation, with its

associated changes in snail fauna. Similarly, irrigation is probably responsible for facilitating the increase in prevalence and intensity of *S. mansoni* in the Senegal River Basin over and above that of *S. haematobium* (Southgate, 1997).

We may therefore speculate that environmental factors are mainly responsible for the replacement of *S. haematobium* by *S. mansoni* in the Fayoum region of Egypt: certainly it is the view of Abdel-Wahab *et al.* (1993) that the main reason for this replacement is the reversal in the relative abundance of the snail vectors for these species, with populations of *Biomphalaria alexandrina* (the snail host for *S. mansoni*) flourishing and populations of *Bulinus truncatus* (the snail host for *S. haematobium*) in sharp decline following the construction of the Aswan Dam in the 1960s.

Thus, whilst laboratory experiments on mating interactions between different species of schistosome provide some indication of the likely consequences of interactions between the species involved in the field, other factors, usually environmental, may play as great, or an even greater part in determining the overall epidemiological outcome.

CHAPTER 8

Identification and characterization of a possible female-specific marker for *S. haematobium*

CHAPTER 8

IDENTIFICATION AND CHARACTERIZATION OF A POSSIBLE FEMALE-SPECIFIC MARKER FOR *S. HAEMATOBIUM*

INTRODUCTION

Schistosome larval stages do not have morphologically distinct male and female sexes, in contrast to the adult worms. There exists, therefore, the problem of identifying cercarial sex, for example, in order to perform schistosome mating experiments such as those in the preceeding chapter using cercariae of a single, known sex. Traditionally, experiments of this nature have relied on infecting control rodents with cercariae shed from unimiracidially infected snails, and waiting for the development of the adult worms in order to determine which sex each snail is infected with. However, as mentioned in chapter 7, for species with very long pre-patent periods such as *S. haematobium*, the chances of snails surviving and/or continuing to shed cercariae after the *S. haematobium* in control hamsters has reached patency are greatly reduced. Clearly, a molecular technique for cercarial sexing using DNA extracted from single or pooled cercariae would be preferable. In schistosomes, it is the female which is the heterogametic sex (ZW as opposed to the male ZZ) and a number of molecular studies have identified female-specific markers located on the female W chromosome of *S. mansoni* (Spotila *et al.,* 1987, 1989; Walker *et al.,* 1989; Webster *et al.,* 1989; Dias Neto *et al.,* 1993; Drew & Brindley, 1995; Grevelding, 1999), one of which, "W1" (Webster *et al.,* 1989; Dias Neto *et al.,* 1993) was used with success to distinguish between male and female *S. mansoni* cercariae in chapter 7. Barral *et al.* (1993) reported the discovery of a RAPD (Randomly Amplified Polymorphic DNA) marker called B5-700 which was specific to females belonging to the *S. haematobium* group of schistosomes, and therefore diagnostic for sex for species within this group. The RAPD technique, which uses a single "randomly chosen" oligonucleotide primer and low-stringency PCR conditions to generate multiple amplification products from loci distributed throughout the genome (Williams *et al.,* 1990; Welsh & McClelland, 1990), is a powerful method for screening populations and individuals for sequence diversity and for generating molecular markers from specific regions of the genome without any requirement for a detailed genetic map (Edwards, 1998). However the RAPD technique is prone to poor reproducibility (Penner *et al.,* 1993; Skroch & Nienhuis, 1995), and, despite the use of the same oligonucleotide primer and close adherence to the standardised protocols given by Barral *et al.* (1993) it has

unfortunately not been possible to generate the sex-specific B5-700 marker (identified by these authors) in samples processed in the laboratories of the Natural History Museum (results not shown).

So great would be the potential practical use of a valid, reproducible female-specific marker for *S. haematobium* (for the reasons given above) that in the work described below, a further attempt was made to identify such a marker by first screening male and female *S. haematobium* genomic DNA with 50 separate RAPD oligonucleotide primers, and then, with the aim of overcoming the problems of RAPD reproducibility, by sequencing any female-specific RAPD markers identified and designing specific primer pairs for the sequences obtained. To ensure as wide an application as possible for any such diagnostic marker identified, the genomic DNA screened comprised a mixture of 4 different *S. haematobium* isolates, from South Africa, Nigeria, Cameroon and Senegal.

RESULTS

Of the 50 RAPD primers screened (Fig 8.1*a* and *b*) only 1, AB10-18, yielded a PCR product which was present only in the female mixture of *S. haematobium* isolates, and not the male. This fragment was approximately 700bp in size (Fig 8.2, lane 2).

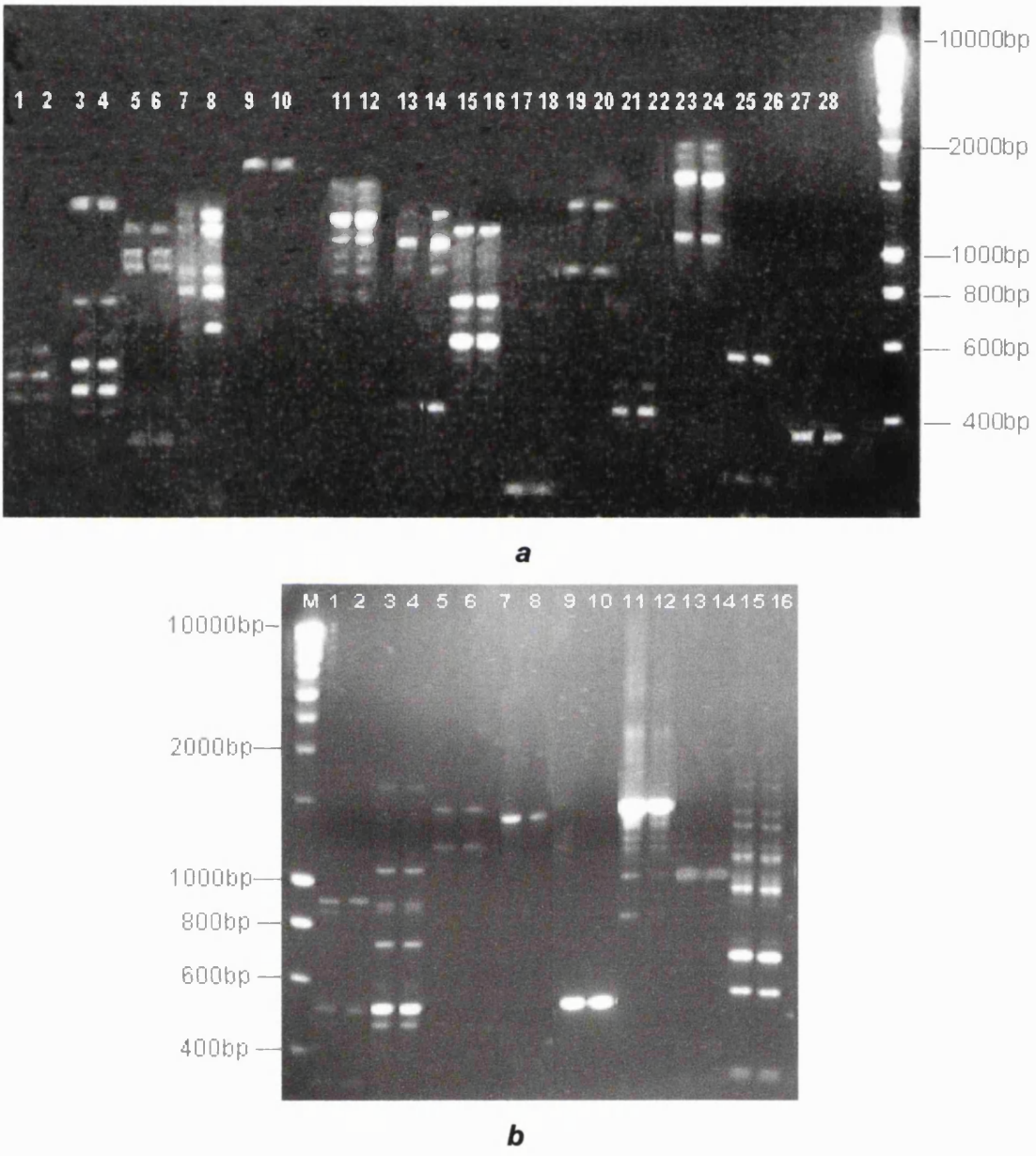


Fig 8.1 Two samples of agarose gels on which PCR products generated by different RAPD primers were screened for female-specific genomic markers. ***a.*** Screen of 14 different RAPD primers: AB11-15; AB 11-14; AB11-6; AB11-5; AB10-16; AB10-14; AB10-4; AB10-2; AB11-16; AB9-14; AB9-13; AB9-6; AB9-5; AB 11-17. Lanes 1, 3, 5, 7, etc., ♂ DNA; Lanes 2, 4, 6, 8 etc., ♀ DNA. ***b.*** Screen of 8 different RAPD primers: AB11-11; AB10-1; AB11-10; AB11-1; AB10-19; AB10-11; AB9-9; AB10-8. Lanes 1, 3, 5, 7, etc., ♂ DNA; Lanes 2, 4, 6, 8 etc., ♀ DNA. Lane M, size marker (Bioline, "Hyperladder I").

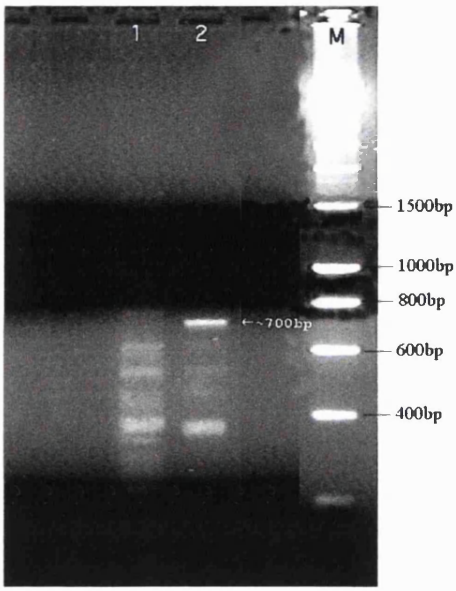


Fig. 8.2 Agarose gel showing the ~700bp fragment (Sh700) generated by primer AB10-18 from female *S. haematobium* DNA (lane 2) but not from male *S. haematobium* DNA (lane 1)

This was therefore potentially a diagnostic marker for distingushing between the sexes of *S. haematobium*. In order to check that it was a discrete marker and not made up of several, similar-sized, co-migrating fragments, it was necessary to clone this PCR product, (called Sh700) and sequence multiple clones. Prior to cloning, the purity of the 700bp band recovered from agarose gels (chapter 2, section 2.4.3) was confirmed by running an aliquot on an agarose gel. A single band of approximately 700bp in size was observed, confirming successful isolation of fragment Sh700 (Fig. 8.3, lane 1)

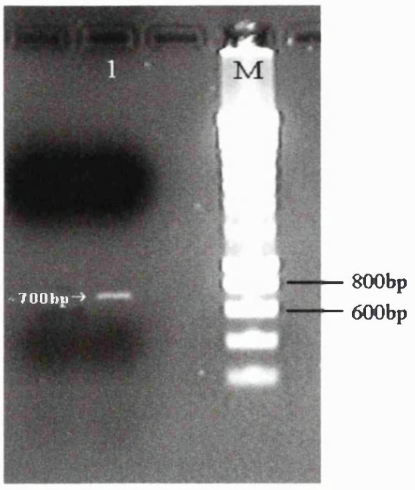


Fig 8.3. Agarose gel confirming recovery of the Sh700 fragment following gel extraction (lane 1); lane M, size marker

Blue/white screening of colonies of *E. coli* (strain JM109) transformed with pGEM®-T Easy plasmid ligated to Sh700, revealed 16 recombinant clones (13 white and 3 pale blue: as the latter sometimes contain inserts). PCR amplification from crude bacterial lysates of of all 16 clones with primers M13F + M13R gave PCR products of approximately 1000bp for all 13 white colonies (Fig 8.4), indicating that these colonies contained the insert. Products of approximately 250bp were obtained for the 3 pale blue colonies (lanes 4, 7, and 14 in Fig 8.3), indicating the absence of the insert in these colonies.

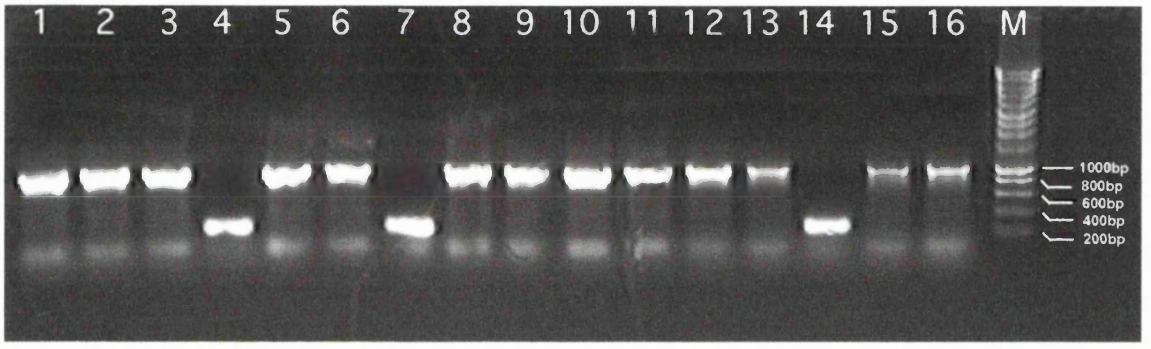


Fig 8.4. Result of PCR amplification across the inserts in 16 recombinant clones of *E. coli* (strain JM109) which had been transformed with a "pGEM T-Easy/Sh700" construct.

Samples of plasmid DNA purified from broth cultures raised from the 13 recombinant colonies were shown to contain supercoiled plasmid DNA with a concentration of approximately 30ng/µl, and to be free of bacterial genomic DNA or RNA contamination when run out on a 1% agarose gel with 1µl of pGEM marker at 200ng/µl. (Fig 8.5)

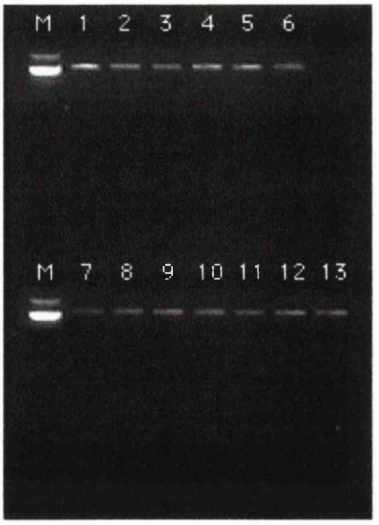


Fig 8.5. Agarose gel showing purified plasmid DNA samples from 13 recombinant clones containing fragment Sh700 (lanes 1-13); and a pGEM standard quantitiation marker (lanes M)

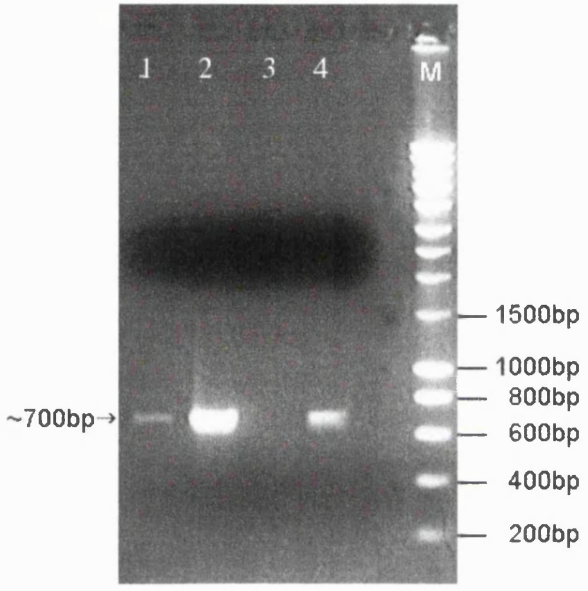
Sequencing of the individual recombinant clones and subsequent sequence analysis using Sequencher v 3.1.1. software revealed a 710bp consensus sequence for the insert (Table 8.1).

Table 8.1 Consensus sequence for all 13 recombinant clones

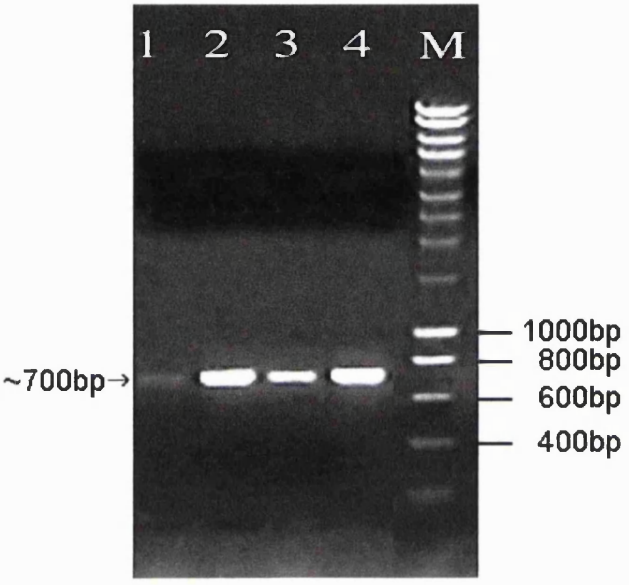
```
1     GTTGCGCAGT CACAGTGAAG CGTGGGATTA TCTGTTCAGA
41    CAAACAAAGG CTCTCAACAT TCAATATAAG ATAATAGGGA
81    ATATAACGCT TATACAAAAT AAGGAAGTGA GTGAATACTT
121   GAACAATACT GTCTTGGCAT ATGCTTGGTT GTTTGGGGTC
161   AACTCTTAAC CAACGAACCT AAGCTGTTAC AAATTCTCGT
201   AAAACTCACA TGGCAAATGC ATTTTCTCGA AACTCGACCT
241   CGTGCTCMCA TACCACCAAA TTCTGATGGC ACCAGAAGAC
281   ACCGGGACAA ACAGCCAYCA TCACACCCCT TGGACYGTCT
321   AAGTTTCTGA GAATACCATT CGGATTGAAA AATGCCGCCC
361   AGACATTCTG ACGATTCATG GATGATGTTA CAAGAGGCTA
401   AGACTTCGTA TTCGCATACA CAGACAAGGT TKTGATCGCA
441   AGCTCATCAA TAGAAGAGCA CGCCCAGCAT GTTCAGATCC
481   TCTTTGAACG TTTTAAGAAA CGTGGCGTTG TTATCAATCC
521   TTCTAAATGT ATATTCACCG TCCCAGCCTT AGAGTTTTTA
561   GGACATTACG TTGACTCTCA AGGCATCAAA CCATTTTCAG
601   AARAAGCAGA AGCCATAGTA AATTTTTCTG AGCCTAAGTT
641   GATAAAGGCA TTACGCCGGT TCCYGAGGAT GTGCAACTTC
681   TACCGAAGGT TTTCTCCTCA ACTGCGCAAC
```

PCR amplification of male and female DNA of the original mixture of *S. haematobium* isolates and of individual South African, Nigerian, Senegalese, Cameroonian and Zambian isolates, using primers designed to the sequence, yielded a ~700bp PCR product for all male and female pure and mixed isolates tested (Fig 8.6 *a-c*), with the exception of the original male mixture of *S. haematobium* isolates (Fig 8.6 *a*, Lane 3) which yielded no PCR product.

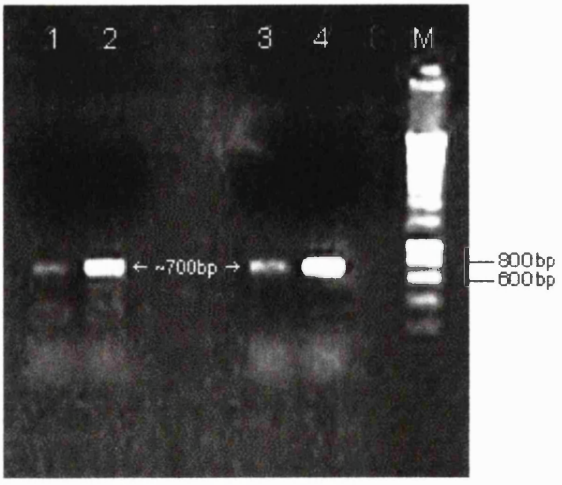
Fig 8.6. PCR screening of *S. haematobium* DNA using primers designed to the Sh700 sequence. **A.** Lane 1, Cameroonian male; Lane 2, Cameroonian female; Lane 3, Mixed-isolate male; Lane 4, Mixed-isolate female, Lane M, size marker **B.** Lane 1, South African male; Lane 2, South African female; Lane 3, Nigerian male; Lane 4, Nigerian female; Lane M, size marker **C.** Lane 1, Senegalese male ; Lane 2, Senegalese female ; Lane 3, Zambian male ; Lane 4, Zambian female; Lane M, size marker.



a



b



c

Therefore, despite the results of the initial RAPD screen, this DNA sequence does not appear to be female-specific in *S. haematobium* after all. However, there does appear to be a quantitative difference between the yield of the Sh700f/r PCR product in males and females: Table 8.2 (below) shows crude estimates of the relative yield of Sh700f/r PCR product for the male and female isolates tested, obtained by comparing the intensity of bands on the gels in Fig 8.6 with those of the internal marker lane. The yield from female isolates ranges from 40-200ng, whilst that from male isolates ranges from 0-100ng, and from 20% to 50% of the yield from females of the same isolate.

Table 8.2: Crude estimates of the relative yield of PCR product from male and female *S. haematobium* isolates.

***S. haematobium* isolate**	**Yield of Sh700f/rPCR product from 25ng template DNA**		**Male yield as a percentage of female yield**
	MALE	FEMALE	
Cameroon	20ng	100ng	20%
Nigeria	100ng	200ng	50%
South Africa	20ng	200ng	10%
Senegal	20ng	100ng	20%
Zambia	40ng	200ng	20%
Original mixture	-	40ng	-

In an attempt to eliminate the ~700 bp band from the male PCR profiles, PCR amplification was repeated, using male and female DNA from the Zambian isolate of *S. haematobium*, at a range of different annealing temperatures within 5°C above and below the melting temperatures of primers Sh700f and r. The temperatures chosen were 56.3°C; 57.9°C; 60.1°C; 61.2°C; 62.2°C; and 63.7°C, and an Eppendorf Thermocycler Gradient machine was used, as it allows a temperature gradient to be set up across the heating block, so that a range of different annealing temperatures can be tested in a single PCR "run". Reaction products were mixed with loading dye and run on a 1% agarose gel containing ethidium bromide (Fig 8.7). Changing the annealing temperature did not result in the disappearance of the male PCR product, the yield of which remained 20%-30% lower than that of females (Fig. 8.7).

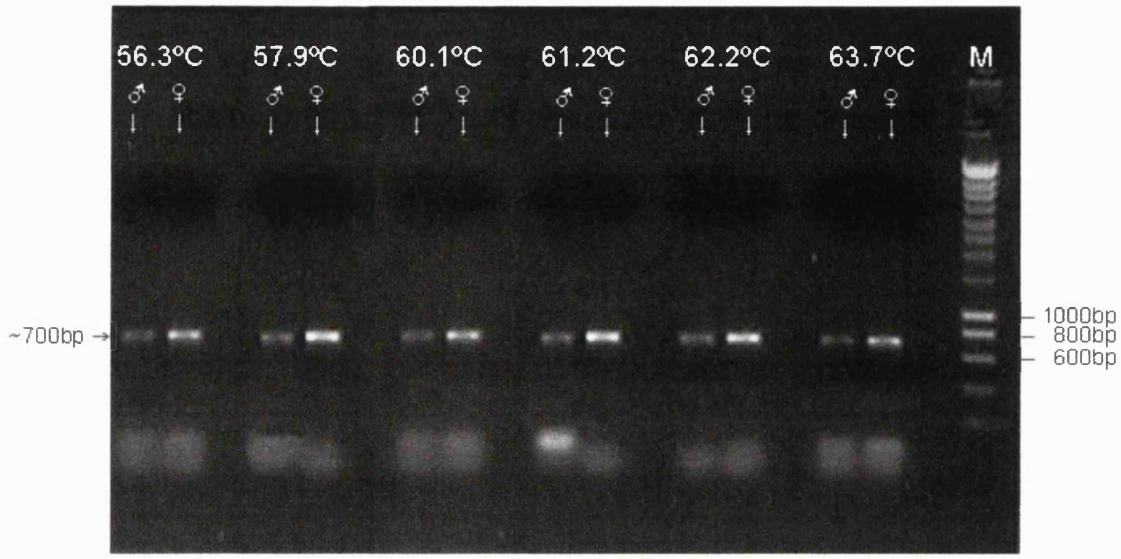


Fig 8.7. PCR amplification of the Sh700 fragment from males and females of the Zambian isolate at a range of different annealing temperatures. Lane M, size marker ("Hyperladder I")

DISCUSSION

The results indicate that the ~700bp PCR product "Sh700" identified, cloned and sequenced from the original female mixture of *S. haematobium* isolates as a potential tool for distinguishing between the sexes of *S. haematobium* is not specific to females as was initially hoped. For reasons unknown, Sh700 repeatedly failed to amplify from the original male mixed-isolate sample, both with primer AB10-18 (which gave a false indication of the diagnostic potential of this marker) and with the primers specifically designed to the cloned Sh700 fragment. In contrast, when male DNA from each of the constituent isolates of the original mixed-isolate sample was amplified individually with the specifically-designed primers (they had never been amplified with primer AB10-18) 20-100ng of the ~700bp product was yielded. The presence of good-quality DNA in the original male mixture had been confirmed by agarose gel analysis and by successful PCR amplification with primers targetted to the ITS1 region of the ribosomal RNA gene. The problem of reproducibility with the RAPD technique has already been mentioned, but if the RAPD technique was at fault for the initial absence of the band from the male mixed-isolate sample with primer AB10-18, it cannot explain its continued absence with the specifically-designed primers. The answer may partly lie with the under-representation of the Nigerian isolate in the original mixture. This isolate gave the highest male yield of Sh700 out of all isolates tested (Table 8.2), but only 5 Nigerian worms were available for use as opposed to 15 worms of the other isolates. Nevertheless, the complete lack of Sh700 from the male mixed-isolate sample still remains to be explained, given its presence in males (and females) of all individual isolates tested.

The results of the study do indicate quantitative differences between the yield of Sh700 from male and female *S. haematobium*. However, this is of limited practical application as a means of distinguishing between the sexes because for the small number of isolates tested, there was overlap in the Sh700 yield range for males and females (0-100ng for males, 40-200ng for females, Table 8.2).

There remains, however, a strong argument for continuing to search for a female-specific marker for *S. haematobium* (and other species of schistosome) using the methods employed above, as its practical use as a diagnostic tool for identifying cercarial sex would be invaluable. However, given the problems encountered in this study, it is to be

recommended that extensive verification of any putative female-specific RAPD markers generated is carried out prior to cloning and sequencing, using a greater variety of male and female *S. haematobium* specimens and isolates.

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All 5 mating models presented in chapters 3-7 yielded heterospecific pairs, the females of which were sexually mature and contained eggs. This confirms, for a broad range of schistosome species, that there are no physiological barriers preventing either the pairing of different species (even where the species belong to different evolutionary groups) or the reproductive stimulation of females by heterospecific males (Southgate *et al.,* 1982, 1998; Tcheum Tchuenté *et al.,* 1993, 1995, 1996 *b*; Khalil & Mansour, 1995).

However, in simultaneous infections where the choice of mate is maximised for each species, all species in the *S. margrebowiei/ S. mansoni*; *S. intercalatum* (Zaire)/ *S. mansoni* and *S. margrebowiei/ S. bovis* mating models (chapters 3, 4, and 5, respectively) exhibited a preference for homospecific partners. Thus for these models, as for those of *S. intercalatum* (Lower Guinea)/ *S. mansoni* (Tchuem Tchuenté *et al.,* 1993); *S. haematobium/ S. mattheei* (Southgate *et al.,* 1995) and *S. haematobium/ S. mansoni* (Webster *et al.,* 1999), specific "choice of mate" acts as an important pre-zygotic reproductive isolating mechanism, helping to maintain the genetic identity of co-infecting species. It is of note that this isolating mechanism is in place for all models of mating interactions between species from different evolutionary groups (the *S. haematobium* and *S. mansoni* groups), most probably having been selected for in order to reduce the production of parthenogenetic offspring (which result from heterospecific pairings between distantly related species and are of very low fitness). However, there is some suggestion, from the data in the Webster *et al.* (1999) study and chapter 7, that in mixed infections with *S. mansoni*, *S. haematobium* does not preferentially mate with same-species partners when given the choice (whereas *S. mansoni* does): a finding which requires further substantiation (see below). Whilst a specific mate preference system exists for the *S. haematobium/ S. mattheei* (Southgate *et al.,* 1995) and *S. margrebowiei/ S. bovis* mating models (chapter 5), other models of mating interactions between species belonging to the *S. haematobium* group such as *S. haematobium/ S. intercalatum* (Lower Guinea) (Southgate *et al.,* 1982), *S. bovis/ S. curassoni* (Rollinson *et al.,* 1990) and the "cryptic species" *S. intercalatum* (Lower Guinea)/ *S. intercalatum* (Zaire) (Pagès *et al.,* 2001 *b*) showed random mating, with no preference for same-species partners. For the two strains of *S. intercalatum*, their

marked geographical isolation from one another has been suggested as an explanation for their lack of assortative mating, as it renders additional isolating mechanisms unnecessary. Jiggins & Mallet (2000) suggest that strong assortative mating evolves when there are strong contact zones between species, and certainly the zone of contact between *S. bovis*/ *S. curassoni* (which have only recently been found to be sympatric in Nigeria and Senegal: Rollinson *et al.*, 1990); and *S. haematobium*/ *S. intercalatum* (Lower Guinea) (which Zwingenberger *et al.*, 1990 suggest may have largely mutually exclusive distributions) is not particularly strong. However, neither is the zone of contact between *S. margrebowiei* and *S. bovis* very strong: two species for which a specific mate preference system does exist (chapter 5).

It might be expected that the combinations of *S. haematobium*-group species which lack assortative mating are reproductively isolated by hybrid infertility, but whilst this is true for the Cameroon and Zaire strains of *S. intercalatum*, hybrids of *S. haematobium* ♂ x *S. intercalatum* ♀ crosses are viable as are those from both parental crosses of *S. bovis* and *S. curassoni*.

Reinforcement of assortative mating by the post-zygotic isolating mechanism of heterologous immunity is indicated for the models in chapters 3-5, by worm return data from sequential infections. For the *S. margrebowiei*/ *S. mansoni* and *S. intercalatum* (Zaire)/ *S. mansoni* models the cross-protection is reciprocal, whereas there is some suggestion that in the *S. margrebowiei*/ *S. bovis* mating model (chapter 5), *S. margrebowiei*-induced cross protection against *S. bovis* is not reciprocal. The reciprocity of cross-protection in the *S. intercalatum* (Zaire)/ *S. mansoni* model represents an important immunological difference between the Zaire and Lower Guinea strains of *S. intercalatum*, as the latter strain failed to induce cross-protection against *S. mansoni* in the experiments of Tchuem Tchuenté *et al.* (1996 *a*). In nature, it is likely that the cross-protection induced by pre-established *S. mansoni* infections against *S. intercalatum* plays a part in restricting the distribution of both strains of *S. intercalatum* (Tchuem Tchuenté *et al.*, 1996 *a*; chapter 4), and that cross-protection induced by pre-established *S. margrebowiei* infections may prevent *S. mansoni* from becoming established areas of *S. margrebowiei* endemicity (Pitchford, 1976, 1977 *b*; chapter 3).

The cross-protection observed in chapters 3-5, and in the study by Tchuem Tchuenté *et al.* (1996 *a*), is effected against the challenge species prior to worms of the initial infection reaching patency, contrary to previous studies which have indicated that heterologous immunity is induced by eggs of the first infection. It is possible that the "immunization process" itself, with the possible involvement of skin inflammation, is responsible for this "pre-patent" heterologous immunity.

The existence of mating competition and change of mate in mixed infections was demonstrated in all 5 mating models in chapters 3-7. In sequential infections with an excess of males, males of all species were able to actively pull paired females away from their partners and pair with them, as previously observed for mixed infections of *S. haematobium*/ *S. mansoni* (Webster *et al.*, 1999) and *S. intercalatum* (Lower Guinea)/ *S. mansoni* (Tchuem Tchuenté *et al.*, 1995) and for monospecific infections of *S. mansoni* (Pica-Mattoccia *et al.*, 2000). These findings contrast the long-held belief that schistosomes "pair for life", and are in accordance with males being the more active partner in making the choice of mate (Tchuem Tchuenté *et al.*, 1995, 1996 *b*).

Homospecific and heterospecific multiple-worm pairs were obtained from all mating models except *S. haematobium*/ *S. intercalatum* (Lower Guinea) (chapter 6), confirming previous observations of multiple pairings by Armstrong (1965). The multiple-worm pairs obtained either consisted of 2 males paired with one female, (a clear illustration of male-male competition) or of 1 male paired with multiple females. Whilst certain multiple-worm pairs of the latter type formed when females were in excess (e.g. experiment #2, chapter 6), others were obtained together with unpaired males, suggesting the involvement of male-male competition. Homosexual (male) pairings were obtained from mixed infections of *S. haematobium*/ *S. intercalatum* (Lower Guinea) (chapter 6) and *S. haematobium*/ male *S. mansoni* (experiment #1, chapter 7), where males were in excess. These pairings consisted of one male clasped by another male (homospecific or heterospecific), and confirm previous observations by Armstrong (1965) that the clasping of schistosomes within the gynaecophoric canal of males is neither sex- nor species-specific.

The mating models in chapters 3-7 provide insight into the relative competitive mating abilities of the species involved. In mixed infections of *S. margrebowiei* and *S. mansoni* (chapter 3), neither species appears to be competitively dominant to the other, and the data suggest that where this is the case in sequential infections, the most successful species in terms of worm return and pairing ability will be the first species to infect the host. In mixed infections of *S. intercalatum* (Zaire) and *S. mansoni* (chapter 4), *S. mansoni* appears to have the slight competitive edge over *S. intercalatum* overall, most crucially in sequential infections where *S. mansoni* is the first species to infect the host. This latter scenario best resembles the situation in the field, with the restricted distribution of *S. intercalatum* (Zaire) being at least partially explained by the reproductive disadvantage it faces when trying to spread to areas where *S. mansoni* is already established. However, the competitive dominance of *S. mansoni* over the Zaire strain of *S. intercalatum* does not appear to be as pronounced as it is over the Lower Guinea strain, highlighting further important differences between the two strains and informing the debate over whether they should be re-classified as separate species. Mixed infections of *S. margrebowiei* and *S. bovis* (chapter 5) indicated that *S. bovis* might be slightly competitively dominant to *S. margrebowiei*, although further work needs to be done on this mating model to substantiate this (see below). The infections in chapter 6, with *S. haematobium* as the first infection and *S. intercalatum* (Lower Guinea) as the second, confirm the competitive dominance of *S. haematobium* over *S. intercalatum* first identified by Southgate *et al.* (1982), and suggest that *S. intercalatum* (Lower Guinea) is unlikely to spread to or become re-established in areas where *S. haematobium* is already present. Data from mice infected initially with both sexes of *S. haematobium* and subsequently with male *S. mansoni* (chapter 7) confirm the competitive dominance of *S. haematobium* males over *S. mansoni* males, first established by Webster *et al.* (1999).

Few obvious patterns of competitive dominance emerge from the mating models in chapters 3-7 and in the literature. *S. mansoni*, for example, is more competitive than both strains of *S. intercalatum*; as competitive as *S. margrebowiei*, but less competitive than *S. haematobium*. *S. margrebowiei* is as competitive as *S. mansoni*, but probably less competitive than *S. bovis*. However, we may perhaps generalise that *S. intercalatum* (Lower Guinea) tends to be less competitive than other species, being dominated in mixed infections by both *S. mansoni* and *S. haematobium* (Tchuem Tchuenté *et al.*, 1993;

Southgate *et al.*, 1982), whereas *S. haematobium* tends to be more competitive than other species, dominating over *S. intercalatum* and *S. mansoni*, and over *S. mattheei* in sequential (but not simultaneous) infections (Southgate *et al.*, 1982; Webster *et al.*, 1999; Southgate *et al.*, 1995).

Nevertheless, for mixed infections involving new combinations of species, it would be difficult to predict with certainty which (if any) species would prove to be the more competitive. Thus, the need to examine such combinations in the laboratory remains.

However, it is important to remember that competitive mating dominance is only one factor influencing the outcome of interactions between different species of schistosome in the field. Reproductive isolating mechanisms such as assortative mating, heterologous immunity and hybrid infertility determine the extent (and duration) of such interactions, whilst environmental factors, often associated with human interference, may play a significant role in maintaining the epidemiological balance or shifting it towards one or other of the interacting species. For example, improvements in sanitation such as increased usage of latrines may favour schistosome species transmitted via the urine rather than faeces, as the latter are less likely to reach water in a fresh condition (Southgate *et al.*, 1976). Dams and irrigation schemes may lead to local reduction in water salinity, and deforestation may alter the temperature profile of water bodies, resulting in changes to the local snail fauna, with consequent influences upon schistosome transmission.

Laboratory models of mating interactions such as those presented in chapters 3-7 give some indication of the likely epidemiological consequences of mating between different schistosome species in nature, by determining the extent to which the species are reproductively isolated from one another by assortative mating and heterologous immunity, and by establishing the relative competitive mating abilities of the species involved. However, to gain a fuller understanding of these mixed foci and their implications for the biology and epidemiology of schistosome species, it is necessary to set this information in a wider context incorporating environmental influences on the transmission of schistosomes in the field.

SUGGESTIONS FOR FURTHER WORK

Below are some suggestions for future investigations to extend and build upon the work presented in the preceeding chapters.

It would be useful to elucidate more accurately the nature of the heterologous immunity observed in the infections in chapters 3-5 (and by Tchuem Tchuenté *et al.,* 1996 *a*), which is apparently effected prior to worms of the initial infection reaching patency, and hence not induced by schistosome eggs. Initial investigations could compare the levels of heterologous cross-protection induced by schistosome species pre- and post-patency, i.e. by altering the time interval between the initial and subsequent infections, and comparing the worm returns of the second infection. It is important that the cercarial numbers used for each infection is carefully chosen such that animal hosts will be able to sustain both infections for at least the time taken for the second infection to reach patency, whilst being certain that the parasite numbers will be sufficient for statistical significance.

Outbred (TO) mice were used throughout the work in this thesis, which is useful from an immunological point of view as it should reflect MHC variability in natural (human) populations. However, a recent study by Davies *et al.* (2001) demonstrates that schistosomes are highly sensitive to differences in host immune status, even to the point of slowing their development in less immunocompetent hosts in order to prolong the survival of host and parasite. Thus, an argument can be made for the use of inbred, genetically identical, strains of mice in studies of schistosome immunity to eliminate variation in host immune status. Mice of defined haplotype could help to pinpoint the effector mechanisms involved with heterologous immunity in schistosomes, e.g. by observing any deficiencies in this immune response in knockout mice, and/ or observing whether immunity can be "adoptively" transferred from immune mice to immunodeficient mice via particular lymphocyte populations.

Data from mixed infections of *S. margrebowiei* and *S. bovis* (chapter 5) indicated that *S. bovis* might be more competitive at pairing than *S. margrebowiei*. However, there was an extremely low worm return of *S. margrebowiei* in simultaneous infections, and the sex ratio of *S. bovis* was female-biased in sequential infections when *S. bovis* was the first

infection, and therefore it is necessary to repeat these experiments in order to substantiate the observations in chapter 5. In addition, it is important to determine the viability of *S. margrebowiei*/ *S. bovis* hybrids, carrying out the relevant crosses in the laboratory: hybrid inviability may influence the outcome of interactions between these two species in the field.

It would be valuable to follow up the prediction made in chapter 5 that the Zaire strain of *S. intercalatum* is more competitive at pairing than the Lower Guinea strain (based on the fact that *S. mansoni* appears to be less competitively dominant to the Zaire strain than to the Lower Guinea strain). Pagès *et al.* (2001 *b*) studied simultaneous mixed infections of the two strains, demonstrating the absence of a specific mate preference system, but the pairing abilities of the strains were not compared. Sequential mixed infections should be carried out to establish their relative competitive mating abilities, and to see whether there is any evidence for immunological cross-protection of one strain against the other. Further insight into differences between the two strains could be gained from studying mixed infections of the Zaire strain and *S. haematobium*, and by comparing the results to those from mixed infections of the Lower Guinea strain and *S. haematobium* (Southgate *et al.*, 1982; chapter 5).

Sequential infection experiments using male and female *S. haematobium* as the initial infection and female *S. mansoni* as the subsequent infection (experiment #2, chapter 7), yielded some surprising results, which appear to suggest that *S. haematobium* males have no preference for *S. haematobium* females over *S. mansoni* females, and in some instances will separate from a *S. haematobium* female in order to re-pair with a *S. mansoni* female. However, the worm return of both species in this experiment was extremely low, and it is important to repeat this experiment using substantially higher numbers of cercariae of both species (at least 400 mixed sex *S. haematobium* cercariae and at least 200 female *S. mansoni*) in order to substantiate these unexpected and puzzling findings. It would also be of value to conclusively establish whether *S. haematobium* has a homospecific mate preference in mixed infections with *S. mansoni*, using the Mantel-Haenszel test to analyse data from simultaneous infections of the two species in the Webster *et al.* (1999) study.

As discussed in chapter 8, it is important that the search for a female-specific marker for *S. haematobium* and species of schistosome other than *S. mansoni* is continued: once such a

marker is available, more complex studies of mating interactions between different species in mixed infections can be carried out, using different numbers of known sexes of each species. For example, the reciprocal experiments of those in chapter 7 could be extended by infecting hamsters with firstly with both sexes of *S. mansoni* and subsequently with a single, known sex of *S. haematobium*.

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APPENDIX OF STATISTICAL ANALYSES

APPENDIX OF STATISTICAL ANALYSES

Key to abbreviations:

m = male

f = female

Mg = *S. margrebowiei*

Ms = *S. mansoni*

Zr = *S. intercalatum* (Zaire)

Int = *S. intercalatum* (Lower Guinea)

Bo = *S. bovis*

Hm = *S. haematobium*

STATISTICAL ANALYSES OF DATA IN CHAPTER 3

Experiment 1

MsmXMsf	MsmXMgf	MgmXMsf	MgmXMgf	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
3	4	3	5	2.80	4.20	3.20	4.80	0.2	*
7	8	9	21	5.33	9.67	10.67	19.33	0.7	*
7	2	1	6	4.50	4.50	3.50	3.50	2.6	*
9	6	0	5	6.75	8.25	2.25	2.75	2.8	*
6	9	1	6	4.77	10.23	2.23	4.77	1.1	*
6	1	1	11	2.58	4.42	4.42	7.58	3.5	*
11	10	2	3	10.50	10.50	2.50	2.50	0.4	*
3	1	1	2	2.29	1.71	1.71	1.29	1.4	*
10	3	3	15	5.45	7.55	7.55	10.45	2.6	*
0	5	1	6	0.42	4.58	0.58	6.42	-0.9	**

Mantel-Haenszel uncorrected test	24.673	0.000
Mantel-Haenszel corrected test	23.210	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	1.43	
estd.s.e. of log odds	0.29	
empirical s.e. of log odds	0.44	
empirical test value	10.47	

MsmXf	Msm____	MgmXf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
7	1	8	1	7.06	0.94	7.94	1.06	-0.1	**
15	9	30	10	16.87	7.12	28.12	11.88	-0.6	**
9	16	7	1	12.12	12.88	3.88	4.12	-2.2	**
15	1	5	0	15.24	0.76	4.76	0.24	-0.1	**
15	8	7	1	16.32	6.68	5.68	2.32	-1.0	**
7	3	12	7	6.55	3.45	12.45	6.55	0.3	*
21	2	5	1	20.62	2.38	5.38	0.62	0.9	*
4	4	3	2	4.31	3.69	2.69	2.31	-0.3	**
13	2	18	3	12.92	2.08	18.08	2.92	0.0	*
5	26	7	2	9.30	21.70	2.70	6.30	-2.7	**

Mantel-Haenszel uncorrected test	8.825	0.003
Mantel-Haenszel corrected test	7.990	0.005
Yates correction, in effect		
mean log odds (adj by +0.5)	-0.58	
estd.s.e. of log odds	0.20	
empirical s.e. of log odds	0.34	
empirical test value	2.85	

Experiment 2

Msmxf	Msm____	MgmXf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
13	9	0	0	13.00	9.00	0.00	0.00	0.4	
4	2	3	1	4.20	1.80	2.80	1.20	-0.3	**
10	12	0	0	10.00	12.00	0.00	0.00	-0.2	
8	17	0	1	7.69	17.31	0.31	0.69	0.4	*
11	4	1	9	7.20	7.80	4.80	5.20	2.8	*
11	7	3	3	9.43	8.57	1.57	1.43	2.4	*
7	14	0	0	7.00	14.00	0.00	0.00	-0.7	
8	5	0	0	8.00	5.00	0.00	0.00	0.4	
11	1	1	3	9.00	3.00	3.00	1.00	2.9	*
4	4	6	5	4.21	3.79	5.79	5.21	-0.2	**

Mantel-Haenszel uncorrected test	10.949	0.001
Mantel-Haenszel corrected test	9.494	0.002
Yates correction, in effect		
mean log odds (adj by +0.5)	0.79	
estd.s.e. of log odds	0.24	
empirical s.e. of log odds	0.43	
empirical test value	3.46	

MsmXMsf	Msm____	MgmXMsf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
13	9	0	0	13.00	9.00	0.00	0.00	0.4	
4	2	2	1	4.00	2.00	2.00	1.00	0.1	*
10	12	0	0	10.00	12.00	0.00	0.00	-0.2	
8	17	0	1	7.69	17.31	0.31	0.69	0.4	*
11	4	1	9	7.20	7.80	4.80	5.20	2.8	*
9	7	0	3	7.58	8.42	1.42	1.58	2.2	*
7	14	0	0	7.00	14.00	0.00	0.00	-0.7	
8	5	0	0	8.00	5.00	0.00	0.00	0.4	
10	1	1	3	8.07	2.93	2.93	1.07	2.8	*
3	4	4	5	3.06	3.94	3.94	5.06	-0.1	**

Mantel-Haenszel uncorrected test	11.939	0.001
Mantel-Haenszel corrected test	10.380	0.001
Yates correction, in effect		
mean log odds (adj by +0.5)	0.81	
estd.s.e. of log odds	0.23	
empirical s.e. of log odds	0.40	
empirical test value	4.04	

MsmXMgf	Msm____	MgmXMgf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
0	9	0	0	0.00	9.00	0.00	0.00	-2.9	
0	2	1	1	0.50	1.50	0.50	1.50	-1.6	**
0	12	0	0	0.00	12.00	0.00	0.00	-3.2	
0	17	0	1	0.00	17.00	0.00	1.00	-2.5	
0	4	0	9	0.00	4.00	0.00	9.00	0.7	
2	7	0	3	1.50	7.50	0.50	2.50	0.8	*
0	14	0	0	0.00	14.00	0.00	0.00	-3.4	
0	5	0	0	0.00	5.00	0.00	0.00	-2.4	
1	1	0	3	0.40	1.60	0.60	2.40	1.9	*
1	4	2	5	1.25	3.75	1.75	5.25	-0.3	**

Mantel-Haenszel uncorrected test	0.086	0.770
Mantel-Haenszel corrected test	0.000	1.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-1.28	
estd.s.e. of log odds	4.36	
empirical s.e. of log odds	0.61	
empirical test value	4.36	

Experiment 3

MsmXf	Msm____	MgmXf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
6	5	9	0	8.25	2.75	6.75	2.25	-2.8	**
13	4	9	3	12.90	4.10	9.10	2.90	0.1	*
6	6	11	0	8.87	3.13	8.13	2.87	-3.1	**
9	1	15	1	9.23	0.77	14.77	1.23	-0.5	**
13	4	4	0	13.76	3.24	3.24	0.76	-1.1	**
1	1	8	6	1.12	0.88	7.87	6.12	-0.3	**
0	0	8	10	0.00	0.00	8.00	10.00	0.2	
0	0	7	13	0.00	0.00	7.00	13.00	0.6	
1	7	7	0	4.27	3.73	3.73	3.27	-4.3	**
8	1	12	2	7.83	1.17	12.17	1.83	0.1	*

Mantel-Haenszel uncorrected test	12.994	0.000
Mantel-Haenszel corrected test	11.624	0.001
Yates correction, in effect		
mean log odds (adj by +0.5)	-1.11	
estd.s.e. of log odds	0.31	
empirical s.e. of log odds	0.54	
empirical test value	4.26	

MsmXMsf	Msm____	MgmXMsf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
6	5	1	0	6.42	4.58	0.58	0.42	-0.9	**
10	4	0	3	8.24	5.76	1.76	1.24	2.8	*
1	6	0	0	1.00	6.00	0.00	0.00	-1.5	
6	1	1	1	5.44	1.56	1.56	0.44	1.5	*
5	4	1	0	5.40	3.60	0.60	0.40	-0.9	**
0	1	0	6	0.00	1.00	0.00	6.00	1.5	
0	0	4	10	0.00	0.00	4.00	10.00	0.8	
0	0	3	13	0.00	0.00	3.00	13.00	1.3	
1	7	0	0	1.00	7.00	0.00	0.00	-1.6	
5	1	3	2	4.36	1.64	3.64	1.36	1.0	*

Mantel-Haenszel uncorrected test	2.271	0.132
Mantel-Haenszel corrected test	1.334	0.248
Yates correction, in effect		
mean log odds (adj by +0.5)	0.40	
estd.s.e. of log odds	0.26	
empirical s.e. of log odds	0.48	
empirical test value	0.70	

MsmXMgf	Msm____	MgmXMgf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
0	5	8	0	3.08	1.92	4.92	3.08	-5.2	**
3	4	9	3	4.42	1.58	7.58	4.42	-1.2	**
5	6	11	0	8.00	3.00	8.00	3.00	-3.3	**
3	1	14	1	3.58	0.42	13.42	1.58	-1.4	**
8	4	3	0	8.80	3.20	2.20	0.80	-1.3	**
1	1	8	6	1.12	0.88	7.87	6.12	-0.3	**
0	0	4	10	0.00	0.00	4.00	10.00	0.8	
0	0	4	13	0.00	0.00	4.00	13.00	1.1	
0	7	7	0	3.50	3.50	3.50	3.50	-5.4	**
3	1	9	2	3.20	0.80	8.80	2.20	-0.5	**

Mantel-Haenszel uncorrected test	28.113	0.000
Mantel-Haenszel corrected test	25.943	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-1.67	
estd.s.e. of log odds	0.32	
empirical s.e. of log odds	0.72	
empirical test value	5.35	

Experiment 4

MsmXf	Msm____	MgmXf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
8	13	1	5	7.00	14.00	2.00	4.00	0.8	*
15	12	0	8	11.57	15.43	3.43	4.57	3.0	*
16	13	0	4	14.06	14.94	1.94	2.06	2.4	*
10	7	0	0	10.00	7.00	0.00	0.00	0.3	
6	16	0	0	6.00	16.00	0.00	0.00	-0.9	
21	6	0	1	20.25	6.75	0.75	0.25	2.3	*
14	10	0	1	13.44	10.56	0.56	0.44	1.4	*
14	11	0	0	14.00	11.00	0.00	0.00	0.2	
12	14	0	1	11.56	14.44	0.44	0.56	1.0	*
13	8	0	4	10.92	10.08	2.08	1.92	2.7	*

Mantel-Haenszel uncorrected test	20.439	0.000
Mantel-Haenszel corrected test	18.485	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	1.32	
estd.s.e. of log odds	0.29	
empirical s.e. of log odds	0.40	
empirical test value	10.89	

MsmXMsf	Msm____	MgmXMsf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
8	13	1	5	7.00	14.00	2.00	4.00	0.8	*
8	12	0	8	5.71	14.29	2.29	5.71	2.4	*
11	13	0	4	9.43	14.57	1.57	2.43	2.0	*
9	7	0	0	9.00	7.00	0.00	0.00	0.2	
4	16	0	0	4.00	16.00	0.00	0.00	-1.3	
15	6	0	1	14.32	6.68	0.68	0.32	2.0	*
12	10	0	1	11.48	10.52	0.52	0.48	1.3	*
14	11	0	0	14.00	11.00	0.00	0.00	0.2	
11	14	0	1	10.58	14.42	0.42	0.58	0.9	*
12	8	0	4	10.00	10.00	2.00	2.00	2.6	*

Mantel-Haenszel uncorrected test	15.267	0.000
Mantel-Haenszel corrected test	13.520	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	1.12	
estd.s.e. of log odds	0.29	
empirical s.e. of log odds	0.38	

MsmXMgf	Msm____	MgmXMgf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
0	13	0	5	0.00	13.00	0.00	5.00	-0.9	
7	12	0	8	4.93	14.07	2.07	5.93	2.3	*
5	13	0	4	4.09	13.91	0.91	3.09	1.3	*
1	7	0	0	1.00	7.00	0.00	0.00	-1.6	
2	16	0	0	2.00	16.00	0.00	0.00	-1.9	
6	6	0	1	5.54	6.46	0.46	0.54	1.1	*
2	10	0	1	1.85	10.15	0.15	0.85	-0.3	*
0	11	0	0	0.00	11.00	0.00	0.00	-3.1	
1	14	0	1	0.94	14.06	0.06	0.94	-1.2	*
1	8	0	4	0.69	8.31	0.31	3.69	0.5	*

Mantel-Haenszel uncorrected test	6.632	0.010
Mantel-Haenszel corrected test	5.066	0.024
Yates correction, in effect		
mean log odds (adj by +0.5)	-0.39	
estd.s.e. of log odds	0.15	
empirical s.e. of log odds	0.53	
empirical test value	0.53	

Experiment 5

MsmXf	Msm____	MgmXf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
2	0	15	1	1.89	0.11	15.11	0.89	-0.7	*
0	4	21	1	3.23	0.77	17.77	4.23	-4.9	**
0	2	25	0	1.85	0.15	23.15	1.85	-5.5	**
1	4	9	7	2.38	2.62	7.62	8.38	-1.3	**
0	4	15	2	2.86	1.14	12.14	4.86	-4.0	**
4	4	5	4	4.24	3.76	4.76	4.24	-0.2	**
0	5	6	3	2.14	2.86	3.86	5.14	-3.0	**
2	1	18	3	2.50	0.50	17.50	3.50	-1.2	**
1	1	15	4	1.52	0.48	14.48	4.52	-1.2	**
2	7	18	2	6.21	2.79	13.79	6.21	-3.1	**

Mantel-Haenszel uncorrected test	43.258	0.000
Mantel-Haenszel corrected test	40.725	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-2.52	
estd.s.e. of log odds	0.38	
empirical s.e. of log odds	0.59	
empirical test value	18.45	

MsmXMsf	Msm____	MgmXMsf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
1	0	1	1	0.67	0.33	1.33	0.67	1.1	*
0	4	0	1	0.00	4.00	0.00	1.00	-1.1	
0	2	0	0	0.00	2.00	0.00	0.00	-1.6	
0	4	0	7	0.00	4.00	0.00	7.00	0.5	
0	4	0	2	0.00	4.00	0.00	2.00	-0.6	
1	4	3	4	1.67	3.33	2.33	4.67	-0.8	**
0	5	1	3	0.56	4.44	0.44	3.56	-1.6	**
2	1	0	3	1.00	2.00	1.00	2.00	2.5	*
0	1	1	4	0.17	0.83	0.83	4.17	0.0	**
2	7	1	2	2.25	6.75	0.75	2.25	-0.6	**

Mantel-Haenszel uncorrected test	0.043	0.836
Mantel-Haenszel corrected test	0.000	1.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-0.22	
estd.s.e. of log odds	1.07	
empirical s.e. of log odds	0.40	
empirical test value	0.30	

MsmXMgf	Msm____	MgmXMgf	Mgm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
1	0	14	1	0.94	0.06	14.06	0.94	-1.2	*
0	4	21	1	3.23	0.77	17.77	4.23	-4.9	**
0	2	25	0	1.85	0.15	23.15	1.85	-5.5	**
1	4	9	7	2.38	2.62	7.62	8.38	-1.3	**
0	4	15	2	2.86	1.14	12.14	4.86	-4.0	**
3	4	2	4	2.69	4.31	2.31	3.69	0.3	*
0	5	5	3	1.92	3.08	3.08	4.92	-2.8	**
0	1	18	3	0.82	0.18	17.18	3.82	-2.8	**
1	1	14	4	1.50	0.50	13.50	4.50	-1.2	**
0	7	17	2	4.58	2.42	12.42	6.58	-4.7	**

Mantel-Haenszel uncorrected test	48.866	0.000
Mantel-Haenszel corrected test	45.995	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-2.80	
estd.s.e. of log odds	0.40	
empirical s.e. of log odds	0.61	
empirical test value	20.80	

STATISTICAL ANALYSES OF DATA IN CHAPTER 4

Experiment 1

ZrmXZrf	ZrmXMsf	MsmXZrf	MsmXMsf	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
5	0	8	7	3.25	1.75	9.75	5.25	2.3	*
17	0	14	13	10.50	6.50	10.50	6.50	4.7	*
8	0	6	21	3.20	4.80	10.80	16.20	4.0	*
7	0	3	7	4.12	2.88	5.88	4.12	3.5	*
12	0	2	10	7.00	5.00	7.00	5.00	4.7	*
15	1	0	11	8.89	7.11	6.11	4.89	5.5	*
12	7	20	7	13.22	5.78	18.78	8.22	-0.5	**
6	2	10	7	5.12	2.88	10.88	6.12	0.6	*
4	2	4	8	2.67	3.33	5.33	6.67	1.2	*
15	5	10	4	14.71	5.29	10.29	3.71	0.2	*

Mantel-Haenszel uncorrected test	52.954	0.000
Mantel-Haenszel corrected test	51.101	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	2.61	
estd.s.e. of log odds	0.36	
empirical s.e. of log odds	0.67	
empirical test value	15.01	

ZrmXf	Zrm____	MsmXf	Msm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
5	1	15	5	4.62	1.38	15.38	4.62	0.3	*
17	0	27	4	15.21	1.79	18.79	2.21	2.2	*
8	0	27	4	7.18	0.82	27.82	3.18	1.0	*
7	0	10	10	4.41	2.59	12.59	7.41	2.7	*
12	0	12	2	11.08	0.92	12.92	1.08	1.6	*
16	0	11	0	16.00	0.00	11.00	0.00	0.4	
19	0	27	2	18.21	0.79	27.79	1.21	1.3	*
8	2	17	8	7.14	2.86	17.86	7.14	0.5	*
6	0	12	0	6.00	0.00	12.00	0.00	-0.7	
20	3	14	6	18.19	4.81	15.81	4.19	1.0	*

Mantel-Haenszel uncorrected test	12.629	0.000
Mantel-Haenszel corrected test	11.394	0.001
Yates correction, in effect		
mean log odds (adj by +0.5)	1.02	
estd.s.e. of log odds	0.29	
empirical s.e. of log odds	0.31	
empirical test value	10.82	

Experiment 2

ZrmXf	Zrm____	MsmXf	Msm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
10	0	10	5	8.00	2.00	12.00	3.00	2.4	*
7	1	16	0	7.67	0.33	15.33	0.67	-1.9	**
7	2	24	2	7.97	1.03	23.03	2.97	-1.2	**
6	1	14	1	6.36	0.64	13.64	1.36	-0.8	**
16	13	18	4	19.33	9.67	14.67	7.33	-1.2	**
8	0	11	1	7.60	0.40	11.40	0.60	0.8	*
9	0	26	4	8.08	0.92	26.92	3.08	1.2	*
0	7	18	4	4.34	2.66	13.66	8.34	-4.1	**
9	1	15	1	9.23	0.77	14.77	1.23	-0.5	**

Mantel-Haenszel uncorrected test	5.556	0.018
Mantel-Haenszel corrected test	4.744	0.029
Yates correction, in effect		
mean log odds (adj by +0.5)	-0.59	
estd.s.e. of log odds	0.25	
empirical s.e. of log odds	0.63	
empirical test value	0.87	

ZrmXZrf	Zrm____	MsmXZrf	Msm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
9	0	0	5	5.79	3.21	3.21	1.79	5.3	*
7	1	7	0	7.47	0.53	6.53	0.47	-1.1	**
5	2	10	2	5.53	1.47	9.47	2.53	-0.6	**
6	1	0	1	5.25	1.75	0.75	0.25	2.6	*
13	13	1	4	11.74	14.26	2.26	2.74	1.1	*
8	0	0	1	7.11	0.89	0.89	0.11	3.9	*
8	0	10	4	6.55	1.45	11.45	2.55	2.0	*
0	7	4	4	1.87	5.13	2.13	5.87	-2.7	**
7	1	0	1	6.22	1.78	0.78	0.22	2.7	*

Mantel-Haenszel uncorrected test	6.104	0.013
Mantel-Haenszel corrected test	5.041	0.025
Yates correction, in effect		
mean log odds (adj by +0.5)	1.46	
estd.s.e. of log odds	0.59	
empirical s.e. of log odds	0.86	
empirical test value	2.93	

ZrmXMsf	Zrm____	MsmXMsf	Msm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
1	0	10	5	0.69	0.31	10.31	4.69	0.5	*
0	1	9	0	0.90	0.10	8.10	0.90	-4.0	**
2	2	14	2	3.20	0.80	12.80	3.20	-1.8	**
0	1	14	1	0.88	0.13	13.13	1.87	-3.4	**
3	13	17	4	8.65	7.35	11.35	9.65	-2.7	**
0	0	11	1	0.00	0.00	11.00	1.00	-2.0	
1	0	16	4	0.81	0.19	16.19	3.81	-0.2	*
0	7	14	4	3.92	3.08	10.08	7.92	-3.9	**
2	1	15	1	2.68	0.32	14.32	1.68	-1.8	**

Mantel-Haenszel uncorrected test	32.579	0.000
Mantel-Haenszel corrected test	30.069	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-2.15	
estd.s.e. of log odds	0.38	
empirical s.e. of log odds	0.52	
empirical test value	17.35	

Experiment 3

ZrmXf	Zrm	MsmXf	Msm	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
12	11	2	1	12.38	10.62	1.62	1.38	-0.4	**
2	0	13	1	1.87	0.13	13.13	0.88	-0.6	*
29	10	9	0	30.87	8.13	7.12	1.87	-1.9	**
13	0	10	1	12.46	0.54	10.54	0.46	1.3	*
20	0	5	6	16.13	3.87	8.87	2.13	3.9	*
7	3	8	3	7.14	2.86	7.86	3.14	-0.1	**
20	3	15	4	19.17	3.83	15.83	3.17	0.5	*
9	8	10	1	11.54	5.46	7.46	3.54	-1.8	**
18	17	10	5	19.60	15.40	8.40	6.60	-0.6	**
12	1	8	2	11.30	1.70	8.70	1.30	0.9	*

Mantel-Haenszel uncorrected test	0.021	0.886
Mantel-Haenszel corrected test	0.000	1.000
Yates correction, in effect		
mean log odds (adj by +0.5)	0.12	
estd.s.e. of log odds	0.82	
empirical s.e. of log odds	0.53	
empirical test value	0.05	

ZrmXZrf	Zrm	MsmXZrf	Msm	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
5	11	0	1	4.71	11.29	0.29	0.71	0.4	*
1	0	4	1	0.83	0.17	4.17	0.83	0.0	*
27	10	3	0	27.75	9.25	2.25	0.75	-1.0	**
11	0	2	1	10.21	0.79	2.79	0.21	2.6	*
20	0	0	6	15.38	4.62	4.62	1.38	6.3	*
6	3	1	3	4.85	4.15	2.15	1.85	1.5	*
14	3	0	4	11.33	5.67	2.67	1.33	3.6	*
8	8	1	1	8.00	8.00	1.00	1.00	0.0	*
8	17	1	5	7.26	17.74	1.74	4.26	0.6	*
5	1	0	2	3.75	2.25	1.25	0.75	2.9	*

Mantel-Haenszel uncorrected test	22.499	0.000
Mantel-Haenszel corrected test	20.487	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	1.69	
estd.s.e. of log odds	0.36	
empirical s.e. of log odds	0.69	
empirical test value	5.94	

ZrmXMsf	Zrm	MsmXMsf	Msm	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
7	11	2	1	7.71	10.29	1.29	1.71	-0.9	**
1	0	9	1	0.91	0.09	9.09	0.91	-0.7	*
2	10	6	0	5.33	6.67	2.67	3.33	-4.0	**
2	0	8	1	1.82	0.18	8.18	0.82	-0.1	*
0	0	5	6	0.00	0.00	5.00	6.00	0.2	
1	3	7	3	2.29	1.71	5.71	4.29	-1.6	**
6	3	15	4	6.75	2.25	14.25	4.75	-0.6	**
1	8	9	1	4.74	4.26	5.26	4.74	-3.6	**
10	17	9	5	12.51	14.49	6.49	7.51	-1.1	**
7	1	8	2	6.67	1.33	8.33	1.67	0.4	*

Mantel-Haenszel uncorrected test	16.915	0.000
Mantel-Haenszel corrected test	15.503	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-1.21	
estd.s.e. of log odds	0.29	
empirical s.e. of log odds	0.47	
empirical test value	6.67	

STATISTICAL ANALYSES OF DATA IN CHAPTER 5

Experiment 1

MgmXMgf	MgmXBof	BomXMgf	BomXBof	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
0	0	0	8	0.00	0.00	0.00	8.00	2.8	
0	0	0	6	0.00	0.00	0.00	6.00	2.6	
0	0	0	10	0.00	0.00	0.00	10.00	3.0	
0	0	0	3	0.00	0.00	0.00	3.00	1.9	
0	0	0	5	0.00	0.00	0.00	5.00	2.4	
8	0	0	6	4.57	3.43	3.43	2.57	5.4	*
4	0	0	8	1.33	2.67	2.67	5.33	5.0	*
5	2	1	2	4.20	2.80	1.80	1.20	1.3	*

Mantel-Haenszel uncorrected test	22.525	0.000
Mantel-Haenszel corrected test	19.377	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	3.06	
estd.s.e. of log odds	0.65	
empirical s.e. of log odds	0.51	
empirical test value	36.45	

MgmXf	Mgm____	BomXf	Bom____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
0	0	8	0	0.00	0.00	8.00	0.00	-2.8	
0	0	6	5	0.00	0.00	6.00	5.00	-0.2	
0	0	10	3	0.00	0.00	10.00	3.00	-1.1	
0	0	3	2	0.00	0.00	3.00	2.00	-0.3	
0	0	5	0	0.00	0.00	5.00	0.00	-2.4	
8	3	6	0	9.06	1.94	4.94	1.06	-1.7	**
4	2	8	2	4.50	1.50	7.50	2.50	-0.6	**
7	3	3	3	6.25	3.75	3.75	2.25	0.8	*

Mantel-Haenszel uncorrected test	0.286	0.593
Mantel-Haenszel corrected test	0.042	0.838
Yates correction, in effect		
mean log odds (adj by +0.5)	-1.05	
estd.s.e. of log odds	1.96	
empirical s.e. of log odds	0.43	
empirical test value	6.07	

Experiment 2

MgmXf	Mgm___	BomXf	Bom___	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
4	14	2	5	4.32	13.68	1.68	5.32	-0.4	**
18	15	7	9	16.84	16.16	8.16	7.84	0.4	*
15	3	4	1	14.87	3.13	4.13	0.87	0.4	*
13	5	8	0	14.54	3.46	6.46	1.54	-1.9	**
12	9	2	1	12.25	8.75	1.75	1.25	-0.2	**
14	13	3	5	13.11	13.89	3.89	4.11	0.5	*
7	0	0	2	5.44	1.56	1.56	0.44	4.3	*
16	7	5	4	15.09	7.91	5.91	3.09	0.6	*
10	10	0	0	10.00	10.00	0.00	0.00	0.0	

Mantel-Haenszel uncorrected test	0.693	0.405
Mantel-Haenszel corrected test	0.447	0.504
Yates correction, in effect		
mean log odds (adj by +0.5)	0.41	
estd.s.e. of log odds	0.49	
empirical s.e. of log odds	0.55	
empirical test value	0.55	

MgmXMgf	Mgm____	BomXMgf	Bom___	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
4	14	0	5	3.13	14.87	0.87	4.13	1.2	*
18	15	0	9	14.14	18.86	3.86	5.14	3.1	*
14	3	0	1	13.22	3.78	0.78	0.22	2.5	*
13	5	0	0	13.00	5.00	0.00	0.00	0.9	
11	9	1	1	10.91	9.09	1.09	0.91	0.2	*
11	13	2	5	10.06	13.94	2.94	4.06	0.6	*
7	0	0	2	5.44	1.56	1.56	0.44	4.3	*
15	7	1	4	13.04	8.96	2.96	2.04	1.8	*
10	10	0	0	10.00	10.00	0.00	0.00	0.0	

Mantel-Haenszel uncorrected test	17.735	0.000
Mantel-Haenszel corrected test	16.014	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	1.64	
estd.s.e. of log odds	0.39	
empirical s.e. of log odds	0.48	
empirical test value	11.54	

MgmXBof	Mgm___	BomXBof	Bom___	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
0	14	2	5	1.33	12.67	0.67	6.33	-2.6	**
0	15	7	9	3.39	11.61	3.61	12.39	-3.2	**
1	3	4	1	2.22	1.78	2.78	2.22	-1.9	**
0	5	8	0	3.08	1.92	4.92	3.08	-5.2	**
1	9	1	1	1.67	8.33	0.33	1.67	-1.8	**
3	13	1	5	2.91	13.09	1.09	4.91	-0.1	*
0	0	0	2	0.00	0.00	0.00	2.00	1.6	
1	7	4	4	2.50	5.50	2.50	5.50	-1.6	**
0	10	0	0	0.00	10.00	0.00	0.00	-3.0	

Mantel-Haenszel uncorrected test	24.251	0.000
Mantel-Haenszel corrected test	22.115	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-1.99	
estd.s.e. of log odds	0.40	
empirical s.e. of log odds	0.65	
empirical test value	9.39	

Experiment 3

MgmXf	Mgm	BomXf	Bom	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
8	1	11	1	8.14	0.86	10.86	1.14	-0.3	**
10	1	9	0	10.42	0.58	7.58	0.42	-0.9	**
16	0	3	0	16.00	0.00	3.00	0.00	1.6	
9	0	8	1	8.50	0.50	8.50	0.50	1.2	*
14	4	6	0	15.00	3.00	5.00	1.00	-1.4	**
7	4	8	0	8.68	2.32	6.32	1.68	-2.3	**
12	5	14	0	14.26	2.74	11.74	2.26	-2.5	**
6	4	5	0	7.33	2.67	3.67	1.33	-2.0	**

Mantel-Haenszel uncorrected test	9.579	0.002
Mantel-Haenszel corrected test	8.128	0.004
Yates correction, in effect		
mean log odds (adj by +0.5)	-0.84	
estd.s.e. of log odds	0.27	
empirical s.e. of log odds	0.55	
empirical test value	2.32	

MgmXMgf	Mgm	BomXMgf	Bom	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
8	1	0	1	7.20	1.80	0.80	0.20	2.8	*
9	1	1	0	9.09	0.91	0.91	0.09	0.7	**
14	0	0	0	14.00	0.00	0.00	0.00	3.4	
7	0	0	1	6.12	0.88	0.88	0.13	3.8	*
11	4	0	0	11.00	4.00	0.00	0.00	0.9	
5	4	1	0	5.40	3.60	0.60	0.40	-0.9	**
7	5	1	0	7.38	4.62	0.62	0.38	-0.8	**
0	4	0	0	0.00	4.00	0.00	0.00	-2.2	

Mantel-Haenszel uncorrected test	0.771	0.380
Mantel-Haenszel corrected test	0.108	0.742
Yates correction, in effect		
mean log odds (adj by +0.5)	0.98	
estd.s.e. of log odds	1.11	
empirical s.e. of log odds	0.78	
empirical test value	1.57	

MgmXBof	Mgm	BomXBof	Bom	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
0	1	11	1	0.85	0.15	10.15	1.85	-3.1	**
1	1	7	0	1.78	0.22	6.22	0.78	-2.7	**
2	0	3	0	2.00	0.00	3.00	0.00	-0.3	
2	0	8	1	1.82	0.18	8.18	0.82	-0.1	*
3	4	6	0	4.85	2.15	4.15	1.85	-2.8	**
2	4	7	0	4.15	1.85	4.85	2.15	-3.3	**
5	5	13	0	7.83	2.17	10.17	2.83	-3.3	**
6	4	5	0	7.33	2.67	3.67	1.33	-2.0	**

Mantel-Haenszel uncorrected test	25.281	0.000
Mantel-Haenszel corrected test	22.716	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	-2.22	
estd.s.e. of log odds	0.44	
empirical s.e. of log odds	0.46	
empirical test value	23.51	

STATISTICAL ANALYSES OF DATA IN CHAPTER 6

HmmXf	Hmm	IntmXf	Intm	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
5	1	2	1	5.60	1.40	2.40	0.60	1.0	*
5	0	1	2	3.75	1.25	2.25	0.75	2.9	*
0	1	0	1	0.00	1.00	0.00	1.00	0.0	
0	0	1	0	0.00	0.00	1.00	0.00	-1.1	
1	0	0	1	0.50	0.50	0.50	0.50	2.2	*
3	1	0	2	2.00	2.00	1.00	1.00	2.5	*
0	0	2	0	0.00	0.00	2.00	0.00	-1.6	
4	0	2	0	4.00	0.00	2.00	0.00	0.6	
1	0	3	0	1.00	0.00	3.00	0.00	-0.8	
7	0	0	1	6.12	0.88	0.88	0.13	3.8	*
1	0	7	5	0.62	0.38	7.38	4.62	0.8	*
0	0	5	0	0.00	0.00	5.00	0.00	-2.4	
3	0	4	0	3.00	0.00	4.00	0.00	-0.3	
6	0	0	0	6.00	0.00	0.00	0.00	2.6	
3	0	10	3	2.44	0.56	10.56	2.44	0.8	*
1	0	4	3	0.63	0.38	4.37	2.62	0.8	*
4	1	2	9	1.87	3.12	4.12	6.87	2.4	*
0	0	7	0	0.00	0.00	7.00	0.00	-2.7	
2	1	3	0	2.50	0.50	2.50	0.50	-1.4	**

Mantel-Haenszel uncorrected test	13.845	0.000
Mantel-Haenszel corrected test	11.931	0.001
Yates correction, in effect		
mean log odds (adj by +0.5)	0.53	
estd.s.e. of log odds	0.14	
empirical s.e. of log odds	0.43	
empirical test value	1.49	

HmmXHmf	Hmm	IntmXHmf	Intm	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
2	1	0	1	1.50	1.50	0.50	0.50	1.6	*
0	0	0	2	0.00	0.00	0.00	2.00	1.6	
0	1	0	1	0.00	1.00	0.00	1.00	0.0	
0	0	0	0	*	*	*	*	0.0	
0	0	0	1	0.00	0.00	0.00	1.00	1.1	
0	1	0	2	0.00	1.00	0.00	2.00	0.5	
0	0	1	0	0.00	0.00	1.00	0.00	-1.1	
2	0	0	0	2.00	0.00	0.00	0.00	1.6	
1	0	0	0	1.00	0.00	0.00	0.00	1.1	
2	0	0	1	1.33	0.67	0.67	0.33	2.7	*
0	0	1	5	0.00	0.00	1.00	5.00	1.3	
0	0	1	0	0.00	0.00	1.00	0.00	-1.1	
2	0	0	0	2.00	0.00	0.00	0.00	1.6	
3	0	0	0	3.00	0.00	0.00	0.00	1.9	
2	0	2	3	1.14	0.86	2.86	2.14	1.9	*
1	0	1	3	0.40	0.60	1.60	2.40	1.9	*
2	1	0	9	0.50	2.50	1.50	7.50	3.5	*
0	0	0	0	*	*	*	*	0.0	
2	1	0	0	2.00	1.00	0.00	0.00	0.5	

Mantel-Haenszel uncorrected test	11.637	0.001
Mantel-Haenszel corrected test	8.987	0.003
Yates correction, in effect		
mean log odds (adj by +0.5)	1.09	
estd.s.e. of log odds	0.32	

HmmXIntf	Hmm	IntmXIntf	Intm	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
4	1	2	1	3.75	1.25	2.25	0.75	0.6	*
5	0	1	2	3.75	1.25	2.25	0.75	2.9	*
0	1	0	1	0.00	1.00	0.00	1.00	0.0	
0	0	1	0	0.00	0.00	1.00	0.00	-1.1	
1	0	0	1	0.50	0.50	0.50	0.50	2.2	*
3	1	0	2	2.00	2.00	1.00	1.00	2.5	*
0	0	1	0	0.00	0.00	1.00	0.00	-1.1	
2	0	2	0	2.00	0.00	2.00	0.00	0.0	
0	0	3	0	0.00	0.00	3.00	0.00	-1.9	
5	0	0	1	4.17	0.83	0.83	0.17	3.5	*
1	0	6	5	0.58	0.42	5.42	4.58	0.9	*
0	0	4	0	0.00	0.00	4.00	0.00	-2.2	
1	0	4	0	1.00	0.00	4.00	0.00	-1.1	
3	0	0	0	3.00	0.00	0.00	0.00	1.9	
1	0	8	3	0.75	0.25	8.25	2.75	0.2	*
0	0	3	3	0.00	0.00	3.00	3.00	0.0	
2	1	2	9	0.86	2.14	3.14	7.86	1.8	*
0	0	7	0	0.00	0.00	7.00	0.00	-2.7	
0	1	3	0	0.75	0.25	2.25	0.75	-3.0	**

Mantel-Haenszel uncorrected test	8.774	0.003
Mantel-Haenszel corrected test	7.072	0.008
Yates correction, in effect		
mean log odds (adj by +0.5)	0.18	
estd.s.e. of log odds	0.06	
empirical s.e. of log odds	0.45	
empirical test value	0.16	

STATISTICAL ANALYSES OF DATA IN CHAPTER 7

Experiment 1

HmmXHmf	Hmm____	MsmXHmf	Msm____	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
3	2	2	12	1.32	3.68	3.68	10.32	1.9	*
4	1	1	12	1.39	3.61	3.61	9.39	3.2	*
4	3	2	16	1.68	5.32	4.32	13.68	2.1	*
1	2	1	8	0.50	2.50	1.50	7.50	1.2	*
4	1	0	14	1.05	3.95	2.95	11.05	4.5	*
2	1	0	14	0.35	2.65	1.65	12.35	3.9	*
0	1	0	6	0.00	1.00	0.00	6.00	1.5	
4	2	2	18	1.38	4.62	4.62	15.38	2.6	*
5	2	2	19	1.75	5.25	5.25	15.75	2.8	*

Mantel-Haenszel uncorrected test	55.046	0.000
Mantel-Haenszel corrected test	51.959	0.000
Yates correction, in effect		
mean log odds (adj by +0.5)	2.64	
estd.s.e. of log odds	0.36	
empirical s.e. of log odds	0.36	
empirical test value	53.64	

Experiment 2

HmmXHmf	___Hmf	HmmXMsf	____Msf	fit[1]	fit[2]	fit[3]	fit[4]	rrv	fact
1	2	6	2	1.91	1.09	5.09	2.91	-1.5	**
0	1	0	1	0.00	1.00	0.00	1.00	0.0	
0	0	1	1	0.00	0.00	1.00	1.00	0.0	
1	1	4	6	0.83	1.17	4.17	5.83	0.4	*
0	4	3	3	1.20	2.80	1.80	4.20	-2.2	**
1	0	0	3	0.25	0.75	0.75	2.25	3.0	*
3	1	6	3	2.77	1.23	6.23	2.77	0.2	*
1	2	2	1	1.50	1.50	1.50	1.50	-1.0	**
1	2	1	0	1.50	1.50	0.50	0.50	-1.6	**
0	2	1	0	0.67	1.33	0.33	0.67	-2.7	**

Mantel-Haenszel uncorrected test	2.090	0.148
Mantel-Haenszel corrected test	1.370	0.242
Yates correction, in effect		
mean log odds (adj by +0.5)	-0.54	
estd.s.e. of log odds	0.37	
empirical s.e. of log odds	0.52	
empirical test value	1.06	

APPENDIX OF PUBLICATIONS

Mating interactions between *Schistosoma mansoni* and *S. margrebowiei*

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SUMMARY

Previous studies of mating interactions between schistosome species from different evolutionary lineages in mice showed that mating is not random: heterospecific pairs form but, given the choice, the preference is for homospecific partners. Mating competition and change of mate were also demonstrated. *Schistosoma mansoni* is a medically important parasite of humans belonging to a different evolutionary group from *S. margrebowiei*, a parasite of bovines of veterinary importance. Experiments were designed to investigate the mating behaviour of these two species in mice. Analysis of the data showed a preponderance of intraspecific pairs over interspecific, demonstrating a specific mate preference system for both species. The existence of mating competition between these species was indicated. Males of both species were able to actively compete for paired females by pulling them away from their partners, thus effecting a change of mate. Overall, neither species appeared to be competitively dominant to the other, and the data suggest that where this is the case in sequential infections, the most successful species in terms of worm return and ability to form pairs will be the first species to infect the host.

Key words: *Schistosoma mansoni*, *Schistosoma margrebowiei*, mating interactions, change of mate.

INTRODUCTION

Schistosomes are unusual amongst trematode worms in being dioecious rather than hermaphrodite. In the hepatic portal system of the definitive host, males and females must locate one another and form pairs in order for sexual maturation and mating to occur. Paired worms then migrate to the egg-laying site within the host (Armstrong, 1965; Wilson *et al.* 1978; Miller & Wilson, 1980; Webster, Southgate & Tchuem Tchuenté, 1999). Interestingly, this pairing is not always species specific: there appear to be no physiological barriers to interspecific pairing even between distantly related species from different evolutionary groups (Tchuem Tchuenté *et al.* 1993, 1995). These groups, of which there are 4 within the genus *Schistosoma*, differ in their biological life-cycles, intermediate host specificity and egg morphology (Rollinson & Southgate, 1987; Tchuem Tchuenté *et al.* 1993). In nature, interspecific pairing is known to occur in areas of sympatry between different species which are able to co-infect the same definitive host, potentially leading to hybridization in the case of closely related species (from the same group) or to parthenogenesis where the species are from different groups (Armstrong, 1965; Taylor, 1970; Jourdane & Southgate, 1992; Tchuem Tchuenté *et al.* 1994; Jourdane, Imbert-Establet & Tchuem Tchuenté, 1995; Southgate *et al.* 1998; Webster *et al.* 1999). Interspecific pairing may result in significant changes in epidemiology such as the progressive exclusion of a particular schistosome species from a given area (Tchuem Tchuenté *et al.* 1993, 1996*a*, 1997; Southgate *et al.* 1995, 1998; Southgate, 1997; Webster *et al.* 1999).

Initial studies of schistosome mating interactions in rodents infected with two or more different schistosome species suggested that choice of mate occurred by trial and error, and therefore mating was random and independent of species identity (Armstrong, 1965; Southgate *et al.* 1982; Rollinson *et al.* 1990). However, more detailed, recent studies have revealed the existence of specific mate preference systems in mixed infections of *Schistosoma intercalatum* and *S. mansoni* (Tchuem Tchuenté *et al.* 1993, 1994, 1995); *S. haematobium* and *S. mattheei* (Southgate *et al.* 1995) and *S. haematobium* and *S. mansoni* (Webster *et al.* 1999). Each time the mate preference was for intraspecific partners, thus establishing specific 'choice of mate' as an important pre-zygotic reproductive isolating mechanism, helping to maintain the genetic identity of co-infecting species (Tchuem Tchuenté *et al.* 1993).

The *S. intercalatum*/*S. mansoni* and *S. haematobium*/*S. mansoni* models, both involving species from different groups (the *S. haematobium* and *S. mansoni* groups), highlighted other important features of schistosome mating interactions. Mating competition was demonstrated, and males of one species emerged as competitively dominant to the other (Tchuem Tchuenté *et al.* 1993, 1994, 1995;

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Webster *et al.* 1999). Furthermore, this competition could involve exchange of partners, with unpaired males being able to pull paired females away from their male partners (Tchuem Tchuenté *et al.* 1995; Webster *et al.* 1999). This challenges the long-held belief that schistosomes 'pair for life'. It would be valuable to further characterize the mating interactions which occur between species from different groups. One application of this information may, for example, be to increase the accuracy with which the outcome of mating interactions between species from different groups may be predicted. To this end, experiments were conducted to examine the mating interactions between *S. mansoni* and *S. margrebowiei*, which belong to the *S. mansoni* and *S. haematobium* groups respectively.

S. mansoni, a parasite of humans, is widely distributed throughout much of Africa south of the Sahara, where *S. margrebowiei*, a parasite of bovines, has a more restricted distribution, but is found in Mali, Zaire, Zambia, Chad, Botswana and South West Africa (Ogbe, 1985). The distribution of *S. margrebowiei* and *S. mansoni* overlaps within the Chobe National Park, Botswana, but because they utilize different definitive hosts, they are unlikely to co-exist within any single individual. To date, there have only been a few, spurious reports of human infection with *S. margrebowiei* (Southgate & Knowles, 1977; Rollinson & Southgate, 1987).

Thus the *S. margrebowiei*/*S. mansoni* model of schistosome mating behaviour presented here is 'theoretical' in that the species used do not interact in nature and, as such, it is the first of its kind to be studied. It is hoped that the information the model provides will help substantiate and/or add to existing knowledge of schistosome mating interactions, particularly those between species from different evolutionary groups.

MATERIALS AND METHODS

Origin of species

The isolate of *S. mansoni* originated from Egypt and was introduced into the laboratory courtesy of Professor Mike Doenhoff (University of Bangor) where it was maintained for several years in *Biomphalaria glabrata* and albino TO mice. The isolate of *S. margrebowiei* originated from the Lochinvar National Park, Zambia and was isolated in 1991 by exposing laboratory-bred *Bulinus wrighti* to miracidia from the fresh faeces of *Kobus Leche* (lechwe). It was maintained in the laboratory in *B. natalensis* and albino TO mice.

Experimental infections

The experimental design was aimed at determining how male and female worms of both species interact in mixed infections in the vertebrate host, and whether a specific mate recognition system and mating competition exists between the two species. Mice were exposed individually by the paddling technique to a fixed number of male and female cercariae of both species in a series of 5 experiments. The experiments were of 2 types, the first of which (Exp. 1) involved the simultaneous infection of mice with 150 cercariae of *S. margrebowiei* and 150 cercariae of *S. mansoni*, thus allowing the schistosomes total choice of mate from either species. The second type (Exps 2–5) involved the sequential infection of mice: firstly with 150 cercariae of *S. mansoni*, and 2 weeks later with 150 cercariae of *S. margrebowiei* (Exp. 1) and *vice versa* (Exp.2), and repeated with a 3-week interval between infections (Exps 3 and 4). In sequential infections it is assumed that the first species will form homospecific pairs, and the usual male-bias of schistosome infections (Liberatos, 1987; Mitchell *et al.* 1990) should result in an excess of 'species 1 males' which may compete with 'species 2 males' for 'species 2 females'. Comparison of sequential experiments with 2-week and 3-week time-lags between infections should reveal any highly time-dependent features of mating interactions.

Mice were killed and worms collected by perfusion and dissection of the hepatic portal vein and mesenteric venous systems of each infected mouse: 45–50 days post-infection for Exp. 1 and 35–40 days post-reinfection for Exps 2–5.

Each pair and any unpaired worms recovered from the infected mice were segregated into individual containers and the pairs were separated. Individual females were taxonomically identified by microscopical examination of the morphometry and number of the intrauterine eggs: *S. mansoni* females produce single, lateral spined eggs whereas *S. margrebowiei* females produce several smaller, rounder eggs with a barely discernible terminal spine. Male worms were identified by microscopical examination of the morphometry of the dorsal tubercles: those of *S. mansoni* possess many spines whereas tubercles of *S. margrebowiei* are spineless. For immature (unpaired) males with poorly developed tubercles, identification was made by subjecting DNA extracted from each worm to PCR amplification of the first internal transcribed spacer (ITS1) region of ribosomal RNA (Kane *et al.* 1996). The resulting amplification product is larger for *S. margrebowiei* than for *S. mansoni* and therefore runs at a higher position on an agarose gel so differentiating between the species. Where the DNA yield from a number of tiny male worms was too low for identification using ITS1, a molecular marker called Sm107 was used instead. This marker, present only in *S. mansoni* (and therefore able to differentiate *S. mansoni* from *S. margrebowiei* worms), forms approximately one-tenth of the *S. mansoni* genome, and so can be

amplified from very small quantities of DNA (Hamburger *et al.* 1998).

The data were analysed using the Mantel–Haenszel test to evaluate the significance of the observed proportions (Mantel & Haenszel, 1959; Southgate *et al.* 1982; Webster *et al.* 1999).

RESULTS

Experiment 1: simultaneous infections

Table 1 summarizes the worms obtained from each mouse, and how they paired. Four types of pairing were found: 2 homospecific (*Schistosoma mansoni* ♂ × *S. mansoni* ♀ and *S. margrebowiei* ♂ × *S. margrebowiei* ♀), and 2 heterospecific (*S. mansoni* ♂ × *S. margrebowiei* ♀ and *S. margrebowiei* ♂ × *S. margrebowiei* ♀). There was also a surplus of unpaired male worms of both species. More unusually, 4 multiple-worm pairs were obtained in total, involving 3 or more worms of the same or different species in the following arrangements: 1 *S. margrebowiei* male paired with 2 *S. margrebowiei* females; 1 *S. margrebowiei* male paired with 1 female of each species; 1 *S. mansoni* male paired with 3 *S. margrebowiei* females; and 2 *S. mansoni* males paired with 1 *S. margrebowiei* female.

Overall, there were twice as many homospecific pairs (142) as heterospecific pairs (71). To test whether this difference was indicative of any species preference, the Mantel–Haenszel test was carried out on the proportions of males of both species paired homospecifically and heterospecifically. The result of the test, which incorporated a Yates-style correction, was highly significant ($\chi^2 = 23{\cdot}2$, $P < 0{\cdot}001$), indicating that both species have a strong preference for homospecific pairing.

Of paired *S. mansoni* males, 55·9% formed homospecific pairs, and 44·1% heterospecific pairs. Of paired *S. margrebowiei* males, 78·4% paired homospecifically, and 21·6% heterospecifically, indicating that *S. margrebowiei* has a stronger homospecific mate preference than *S. mansoni*. The proportion of paired males among *S. mansoni* was 60·7%, whereas that for *S. margrebowiei* was 78·5%. This difference was statistically significant according to the Mantel–Haenszel test ($\chi^2 = 8{\cdot}0$, $P < 0{\cdot}01$), with *S. margrebowiei* males being more successful than *S. mansoni* males at pairing.

Experiment 2: infections with S. mansoni *and 2 weeks later with* S. margrebowiei

Four types of pairing were obtained in this experiment (Table 2), of which the homospecific *S. mansoni* ♂ × *S. mansoni* ♀ pairing was the most common, and was the only type of pairing in mice 1, 3, 4, 7, 8. One multiple-worm pair was obtained from mouse 8, consisting of 1 *S. mansoni* male paired with 2 *S. mansoni* females.

The proportion of paired males among *S. mansoni* was 53·7%, whereas that for *S. margrebowiei* was 33·3%. This difference was statistically significant according to a Mantel–Haenszel test ($\chi^2 = 9{\cdot}5$, $P < 0{\cdot}01$), with *S. mansoni* males being more successful than *S. margrebowiei* males at pairing.

Since *S. mansoni* was introduced first, it is assumed that all *S. mansoni* females were mated with *S. mansoni* males before *S. margrebowiei* worms were ready to pair. However, mice 2, 5, 9 and 10 contained up to 4 *S. margrebowiei* ♂ × *S. mansoni* ♀ heterospecific pairs, leaving in each case an excess of single *S. mansoni* males. There was a statistically significant difference in the proportions of males from both species which paired with *S. mansoni* females ($\chi^2 = 10{\cdot}4$, $P < 0{\cdot}005$) with *S. mansoni* males better able to pair with *S. mansoni* females than *S. margrebowiei* males. In other words, despite the occurrence of several *S. margrebowiei* ♂ × *S. mansoni* ♀ pairings, overall *S. margrebowiei* males are unable to outcompete *S. mansoni* males for *S. mansoni* females to any significant extent.

Four heterospecific *S. mansoni* ♂ × *S. margrebowiei* ♀ pairs were obtained in total, in each case leaving an excess of single *S. margrebowiei* males. There was no significant difference between the proportions of males of both species paired with *S. margrebowiei* females, indicating that *S. mansoni* males are just as good at pairing with *S. margrebowiei* females as *S. margrebowiei* males are. There was no significant difference in the proportions of males of both species which were paired heterospecifically.

Experiment 3: infections with S. margrebowiei *and 2 weeks later with* S. mansoni

Two homospecific and 2 heterospecific types of pairing were obtained in this experiment (Table 3), of which the homospecific *S. margrebowiei* ♂ × *S. margrebowiei* ♀ pairing was the most common. Two multiple-worm pairs were obtained, 1 from mouse 3, consisting of a *S. mansoni* male paired with 2 *S. margrebowiei* females, and the other from mouse 9, consisting of a *S. margrebowiei* male paired with 1 female of each species.

The proportion of paired males among *S. margrebowiei* was 72·0%, whereas that for *S. mansoni* was 66·3%. This difference was statistically significant according to the Mantel–Haenszel test ($\chi^2 = 11{\cdot}6$, $P < 0{\cdot}005$), with *S. margrebowiei* males being more successful than *S. mansoni* males at pairing. Despite the assumption that all *S. margrebowiei* females would have been paired with *S. margrebowiei* males prior to the introduction of *S. mansoni*, 20 heterospecific *S. mansoni* ♂ × *S. margrebowiei* ♀ pairs were obtained in total from mice 2, 3, 4, 5, 6 and 10. The

Table 1. Data from mice simultaneously infected with 150 cercariae of *Schistosoma mansoni* (Ms) and 150 cercariae of *S. margrebowiei* (Mg)

Mouse	Ms♂	Mg♂	Ms♂ × Ms♀	Mg♂ × Mg♀	Ms♂ × Mg♀	Mg♂ × Ms♀	Multiple pairings (♂:♀)
1	1	1	3	5	4	3	
2	9	10	7	21	8	9	
3	16	1	7	6	2	1	
4	1	0	9	5	6	0	2Ms:1Mg
5	8	1	6	6	9	1	
6	3	7	6	11	1	1	
7	2	1	11	3	10	2	1Mg:1Ms+1Mg 1Mg:2Mg
8	4	2	3	2	1	1	1Ms:3Mg
9	2	3	10	15	3	3	
10	26	2	0	6	5	1	
Total	72	28	62	80	49	22	

Table 2. Data from mice infected first with 150 cercariae of *Schistosoma mansoni*, and 2 weeks later with 150 cercariae of *S. margrebowiei*

Mouse	Ms♂	Mg♂	Ms♂ × Ms♀	Mg♂ × Mg♀	Ms♂ × Mg♀	Mg♂ × Ms♀	Multiple pairings (♂:♀)
1	9	0	13	0	0	0	
2	2	1	4	1	0	2	
3	12	0	10	0	0	0	
4	17	1	8	0	0	0	
5	4	9	11	0	0	1	
6	7	3	9	0	2	0	
7	14	0	7	0	0	0	
8	5	0	8	0	0	0	1Ms:2Ms
9	1	3	10	0	1	1	
10	4	5	3	2	1	4	
Total	75	22	83	3	4	8	

Table 3. Data from mice infected first with 150 cercariae of *Schistosoma margrebowiei*, and 2 weeks later with 150 cercariae of *S. mansoni*

Mouse	Ms♂	Mg♂	Ms♂ × Ms♀	Mg♂ × Mg♀	Ms♂ × Mg♀	Mg♂ × Ms♀	Multiple pairings (♂:♀)
1	5	0	6	8	0	1	
2	4	3	10	9	3	0	
3	6	0	1	11	5	0	1Ms:2Mg
4	1	1	6	14	3	1	
5	4	0	5	3	8	1	
6	1	6	0	8	1	0	
7	0	10	0	4	0	4	
8	0	13	0	4	0	3	
9	7	0	1	7	0	0	1Mg:1Mg+1Ms
10	1	2	5	9	3	3	
Total	29	35	34	77	23	13	

data show that in 50% of cases there was 1 unpaired *S. margrebowiei* male for each *S. mansoni* ♂ × *S. margrebowiei* ♀ pairing, suggesting that the *S. mansoni* male of each pair had outcompeted the unpaired *S. margrebowiei* male for the *S. margrebowiei* female. The other 50% of *S. mansoni* ♂ × *S. margrebowiei* ♀ pairs arose from there being a deficit of *S. margrebowiei* males. However, a Mantel–Haenszel test comparing the proportions of males of both species paired with *S. margrebowiei* females showed that overall *S. margrebowiei* males were significantly better able to pair with *S. margrebowiei* females than *S. mansoni* males were ($\chi^2 = 25{\cdot}9$, $P < 0{\cdot}001$).

Table 4. Data from mice infected first with 150 cercariae of *Schistosoma mansoni*, and 3 weeks later with 150 cercariae of *S. margrebowiei*

Mouse	Ms♂	Mg♂	Ms♂ × Ms♀	Mg♂ × Mg♀	Ms♂ × Mg♀	Mg♂ × Ms♀	Multiple pairings (♂:♀)
1	13	5	8	0	0	1	
2	12	8	8	0	7	0	
3	13	4	11	0	5	0	
4	7	0	9	0	1	0	2Ms:1Ms
5	16	0	4	0	2	0	
6	6	1	15	0	6	0	
7	10	1	12	0	2	0	
8	11	0	14	0	0	0	
9	14	1	11	0	1	0	
10	8	4	12	0	1	0	
Total	110	24	104	0	25	1	

Data from mice 1, 4, 5, 7, 8 and 10 show that 46 % of the heterospecific *S. margrebowiei* ♂ × *S. mansoni* ♀ pairs formed leaving an excess of *S. mansoni* males which *S. margrebowiei* males had out-competed. The remaining 54 % arose from there being a deficit of *S. mansoni* males for the *S. mansoni* females to pair with. The difference between the proportions of males of both species paired with *S. mansoni* females was not significant, indicating that *S. margrebowiei* males are just as good as *S. mansoni* males at pairing with *S. mansoni* females.

There was no significant difference in the proportions of males of both species paired heterospecifically, i.e. in their abilities to form heterospecific pairs.

Experiment 4: infections with S. mansoni *and 3 weeks later with* S. margrebowiei

In this experiment, no homospecific *S. margrebowiei* pairs were obtained (Table 4), and there was only 1 heterospecific *S. margrebowiei* ♂ × *S. mansoni* ♀ pair. In contrast, 104 homospecific *S. mansoni* pairs were obtained in total, and there were 25 heterospecific *S. mansoni* ♂ × *S. margrebowiei* ♀ pairs. One multiple-worm pair was recovered from mouse 4, consisting of 2 *S. mansoni* males paired with 1 *S. mansoni* female.

Only 4 % of all *S. margrebowiei* males were paired, compared with 54·0 % of *S. mansoni* males. This difference was statistically significant ($\chi^2 = 18{\cdot}5$, $P < 0{\cdot}001$), with *S. mansoni* males proving better at pairing than *S. margrebowiei* males.

The single *S. margrebowiei* ♂ × *S. mansoni* ♀ pair was recovered from mouse 1 along with 13 surplus *S. mansoni* males, all of which are assumed to have been outcompeted by the *S. margrebowiei* male. Mantel–Haenszel analysis revealed that the difference between the proportions of males of both species paired with *S. mansoni* females was highly significant ($\chi^2 = 13{\cdot}5$, $P < 0{\cdot}001$) with *S. mansoni* males being more successful than *S. margrebowiei* males at pairing with *S. mansoni* females.

Data from mice 2, 3, 4, 5, 6, 7, 9 and 10 show that 60 % of the heterospecific *S. mansoni* ♂ × *S. margrebowiei* ♀ pairs formed left an unpaired *S. margrebowiei* male which the paired *S. mansoni* male had out-competed. The remaining 40 % resulted from a deficit of *S. margrebowiei* males for the *S. margrebowiei* females to pair with. The difference in proportions of males of both species paired with *S. margrebowiei* females was statistically significant ($\chi^2 = 5{\cdot}1$, $P < 0{\cdot}05$), with *S. mansoni* males being more successful than *S. margrebowiei* males at pairing with *S. margrebowiei* females.

The difference in proportions of males of both species paired heterospecifically was not significant according to Mantel–Haenszel analysis, suggesting equality of heterospecific pairing ability. However, the low numbers of *S. margrebowiei* males recovered in this experiment makes interpretation of this result difficult.

Experiment 5: infections with S. margrebowiei *and 3 weeks later with* S. mansoni

Four types of pairing were obtained in this experiment (Table 5), the most common of which was the homospecific *S. margrebowiei* ♂ × *S. margrebowiei* ♀ pairing (140 pairs compared with 19 pairs in total for the other 3 types of pairing). Two multiple-worm pairs were recovered, both from mouse 4, one of which consisted of a *S. margrebowiei* male paired with 2 *S. margrebowiei* females, and the other of 2 *S. margrebowiei* males paired with 1 *S. margrebowiei* female.

The proportion of paired males among *S. margrebowiei* was 84·5 %, whereas that for *S. mansoni* was 27·3 %. This difference was highly significant ($\chi^2 = 40{\cdot}7$, $P < 0{\cdot}001$), with *S. margrebowiei* males proving better than *S. mansoni* males at pairing.

Despite the assumption that all *S. margrebowiei* females would have been paired with *S. margrebowiei* males prior to the establishment of *S. mansoni*, 6 *S. mansoni* ♂ × *S. margrebowiei* ♀ pairs were recovered from mice 1, 4, 6 and 9 in total. In each case, there

Table 5. Data from mice infected first with 150 cercariae of *Schistosoma margrebowiei*, and 3 weeks later with 150 cercariae of *S. mansoni*

Mouse	Ms♂	Mg♂	Ms♂ × Ms♀	Mg♂ × Mg♀	Ms♂ × Mg♀	Mg♂ × Ms♀	Multiple pairings (♂:♀)
1	0	1	1	14	1	1	
2	4	1	0	21	0	0	
3	2	0	0	25	0	0	
4	4	7	0	9	1	0	2Mg:1Mg 1Mg:2Mg
5	4	2	0	15	0	0	
6	4	4	1	2	3	3	
7	5	3	0	5	0	1	
8	1	3	2	18	0	0	
9	1	4	0	14	1	1	
10	7	2	2	17	0	1	
Total	32	27	6	140	6	7	

was 1 unpaired *S. margrebowiei* male per *S. mansoni* ♂ × *S. margrebowiei* ♀ pair, assumed to have been out-competed by the paired *S. mansoni* male. However, Mantel–Haenszel analysis showed that overall, *S. margrebowiei* males remained significantly better than *S. mansoni* males at pairing with *S. margrebowiei* females ($\chi^2 = 25{\cdot}9$, $P < 0{\cdot}001$).

Data from mice 1, 6, 7, 9 and 10 show that 86% (6/7) of the heterospecific *S. margrebowiei* ♂ × *S. mansoni* ♀ pairs formed left an unpaired *S. mansoni* male which the paired *S. margrebowiei* male had out-competed. The remaining 14% (1 pair) resulted from a deficit of *S. mansoni* males for the *S. mansoni* female to pair with. There was no significant difference in the proportions of males of both species paired with *S. mansoni* females, suggesting that *S. margrebowiei* males are just as good as *S. mansoni* males at pairing with *S. mansoni* females. There was no significant difference in the proportions of males of both species paired heterospecifically, i.e. in their abilities to form heterospecific pairs.

DISCUSSION

The data from the 5 experiments show that in mixed infections of *Schistosoma mansoni* and *S. margrebowiei* homospecific and heterospecific pairs readily form, confirming previous observations that there are no physiological barriers preventing the meeting and pairing of two different species in the same definitive host (Southgate *et al.* 1982; Tchuem Tchuenté *et al.* 1995, 1996*b*; Webster *et al.* 1999). Furthermore, the fact that all heterospecifically paired females were mature confirms earlier findings that maturation and reproductive stimulation of females by males is not species-specific (Khalil & Mansour, 1995; Southgate *et al.* 1998).

Previous models of mating interactions between 2 species from different evolutionary groups involved mixed infections of schistosome species which occur sympatrically in nature and are therefore able to co-infect the same human host, e.g. the *S. intercalatum*/*S. mansoni* model (Tchuem Tchuenté *et al.* 1993, 1994, 1995) and the *S. haematobium*/*S. mansoni* model (Webster *et al.* 1999). In such situations, where competition exists between co-infecting schistosomes there exists the risk of partial or total exclusion of one species by the other and, indeed, *S. mansoni* is seen to be restricting the distribution of *S. intercalatum* in Africa (Southgate *et al.* 1982, 1998; Tchuem Tchuenté *et al.* 1993, 1994, 1995, 1996*a*, 1997) and *S. mansoni* is progressively replacing *S. haematobium* in the Fayoum, Egypt (Abdel-Wahab *et al.* 1993; Webster *et al.* 1999).

Data from mice simultaneously infected with both *S. mansoni* and *S. margrebowiei* (Exp. 1), revealed a significant prevalence of homospecific pairs, thus indicating the existence of a specific mate preference system for both species, as previously described for the *S. intercalatum*/*S. mansoni* and the *S. haematobium*/*S. mansoni* models (Tchuem Tchuenté *et al.* 1993; Webster *et al.* 1999).

However, *S. margrebowiei* exhibited a greater homospecific mate preference than *S. mansoni*. This gives *S. margrebowiei* a competitive advantage over *S. mansoni* for, by maintaining a stronger homospecific mate preference, *S. margrebowiei* is helping to ensure its reproductive viability as any offspring of mating between the two species will be of greatly reduced fitness. This is because these species are from different evolutionary lineages, and such is the phylogenetic difference between them that they cannot hybridize, instead producing matroclonal eggs of very low viability, by parthenogenesis (Jourdane *et al.* 1995; Khalil & Mansour, 1995; Southgate & Rollinson, 1987; Southgate *et al.* 1982, 1995; Tchuem Tchuenté *et al.* 1994). In the *S. margrebowiei*/*S. mansoni* mating model it is assumed that all eggs seen in females were of parthenogenetic origin. For male worms, a heterospecific pairing which leads to parthenogenesis is a reproductive 'dead end': the reproductive disadvantages of a tendency to form heterospecific pairs are postulated as one reason why *S. haematobium*, which has been

shown to be competitively dominant to *S. mansoni* (Webster *et al.* 1999) is being replaced by *S. mansoni* in the Fayoum, Egypt (Abdel-Wahab *et al.* 1993; Webster *et al.* 1999).

Statistical analysis of the data in Exp. 1 also showed that *S. margrebowiei* males were better than *S. mansoni* males at pairing with females of either species, again indicating that *S. margrebowiei* has the competitive edge over *S. mansoni* in simultaneous infections.

However, in sequential infections, it seems that the competitive dominance of one species over the other was mainly determined by which species was the first to enter and establish itself in the host (species 1), and which was the species of reinfection (species 2). In all 4 sequential experiments (2–5), species 1 had the competitive advantage over species 2 in terms of the following: firstly, a greater ability to pair with 'species 1 females'; secondly, pairing more readily with females of either species. Thirdly, 'species 1 males' were at least as good as 'species 2 males' at pairing with 'species 2 females'.

Consideration of the types of mating competition involved in sequential infections may help in finding an explanation for the advantages conferred on species 1.

The sex ratio of *S. margrebowiei* was found to be less male-biased than for *S. mansoni*, being closer to 1 (except in Exp. 2, where it was 4·7, owing to a very low recovery of *S. margrebowiei* females) in accordance with previously reported sex ratios for *S. margrebowiei* (Southgate & Knowles, 1977). Nevertheless, there were at least as many males as females of both species, and therefore the assumption that all females of species 1 were paired with 'species 1 males' by the time species 2 commenced pairing is a reasonable assumption. Consequently, in order for 'species 2 males' to pair with 'species 1 females', they must have first separated the female from her species 1 male partner. That worm pairs can dissociate and change of mate take place has been demonstrated in recent studies of schistosome mating interactions (Tchuem Tchuenté *et al.* 1995, 1996*b*; Pica-Mattoccia *et al.* 2000). In mixed infections of *S. mansoni* and *S. intercalatum* it has been observed that competitively dominant *S. mansoni* males actively pull away paired *S. intercalatum* or paired *S. mansoni* females from *S. intercalatum* males (Tchuem Tchuenté *et al.* 1995). It can therefore be postulated that in the *S. margrebowiei*/*S. mansoni* model unpaired 'species 2 males' are similarly able to pull paired 'species 1 females' out from the gynaecophoric canal of 'species 1 males' and pair with them. The newly displaced 'species 1 males' may then in turn start competing for females afresh. In sequential infections, where the number of heterospecifically paired 'species 1 males' was less than or equal to the number of heterospecifically paired 'species 2 males' recovered from a mouse, all heterospecifically paired 'species 1 males' from that mouse can be assumed to have repaired heterospecifically, having been actively displaced from their original homospecific females by competitor 'species 2 males'. This is the case in Exp. 2, mice 9 and 10; Exp. 3, mice 4, 5 and 10; Exp. 5, mice 1, 6 and 9.

It is likely that this 'active competition' for paired females is more energy-consuming than competition for unpaired females, and so it is not surprising that 'species 2 males' appeared unable to outcompete 'species 1 males' for 'species 1 females' to any statistically significant extent in Exps 2–5. If mice had been culled 8 weeks or more after reinfection, then it is possible that the number of species 2 ♂ × species 1 ♀ pairs may have been much greater, since Tchuem Tchuenté *et al.* (1995) described the phenomenon of change of mate 'as a progressive process, requiring up to 8 weeks' to happen. In these experiments, however, it was not possible to delay the culling of mice any longer due to the pathogenicity of such a large worm burden. Unlike paired worms, which migrate from the liver to the mesenteric veins (or to the vesicle venous plexus in the case of *S. haematobium*), unpaired worms remain in the liver or the hepatic portal vein, so that the question arises as to where change of mate takes place. Pica-Mattoccia *et al.* (2000) suggested that it may be necessary for paired worms to periodically return to the liver and hepatic portal system, in order for them to move from one branch of the mesenteric veins to another, so bringing unpaired and paired worms together and providing the opportunity for mate changing.

When considering the data showing 'species 1 males' to be significantly better at pairing (that is, at pairing with females) than 'species 2 males', it should be noted that this result may be largely due to the greater ability of 'species 1 males' to pair with 'species 1 females', since the worm return of 'species 2 females' (and males) is so much lower than that of 'species 1 females'. The worm return of *S. margrebowiei* females in Exp. 2 is particularly low (7 worms). However, in all sequential experiments 'species 1 males' were shown to be at least equally as able to pair with 'species 2 females' as 'species 2 males', and therefore, on balance, this does indicate superiority of 'species 1 males' pairing ability.

With regard to the relative competitive mating abilities of *S. margrebowiei* and *S. mansoni* in sequential infections, it seems that they are fairly evenly matched, both enjoying the same competitive advantages when they form the first infection, and the same disadvantages when they form the second. The only exception to this was in Exp. 4 when species 1 (*S. mansoni*) males outcompeted species 2 (*S. margrebowiei*) males for species 2 (*S. margrebowiei*) females, whereas in all other sequential infections, species 1 and 'species 2 males' were equally able to pair with 'species 2 females'.

Statistical analysis of the data found no significant differences in the abilities of males of the two species to form heterospecific pairs (rather than not mating at all), again suggesting that neither species is competitively dominant to the other in sequential infections. Furthermore, both *S. margrebowiei* and *S. mansoni* males, when they were the second infection, were able to 'actively compete' for 'species 1 females' by pulling them away from 'species 1 males' and pairing with them. In mixed infections of *S. haematobium* and *S. mansoni* where *S. haematobium* emerged as competitively dominant irrespective of whether it was the first or second infection, the ability to actively pull away paired females was almost exclusively confined to *S. haematobium* males, and they had a greater ability to form heterospecific pairs than *S. mansoni* males (Webster *et al.* 1999).

Thus it seems that the timing of sequential mixed infections is a crucial determinant of mating success only where the co-infecting species are evenly matched in their competitive mating abilities. In that situation, the species which forms the first infection will have the competitive mating advantage over the second species.

The other marked advantage which 'species 1' has over 'species 2' in sequential infections is that it has a much higher worm return, suggesting that 'species 1' confers some degree of resistance to reinfection. In mice exposed to both species simultaneously (Exp. 1) the worm returns were nearly identical: 17·9% and 17·5% for *S. mansoni* and *S. margrebowiei* respectively, showing that there is no difference between the two species in their infectivity or ability to develop in mice. In sequential experiments, however, the worm return of the second species was up to 7 times less than that of the first.

That an initial infection can confer resistance against subsequent challenge infections whilst adult worms of the first infection survive and remain active is well documented, and has been termed 'concomitant immunity' (Smithers & Terry, 1969). This resistance may cross the species barrier to varying degrees and protect against a different schistosome species (Smithers & Doenhoff, 1982; Rollinson *et al.* 1990; Tchuem Tchuenté *et al.* 1996*a*): a phenomenon known as 'heterologous immunity'. Resistance to reinfection by the same, or a different species in this manner has the obvious biological advantage of preventing overcrowding of parasites, thus extending the survival of both host and schistosome.

It has been strongly argued that the onset of protective immunity is at patency, stimulated by the presence of eggs in tissues and egg-associated pathology, and peak immunity is reached 4–6 weeks later (Smithers & Terry, 1967; Pearce & McLaren, 1983; Dean, 1983). However, it is known that immune attrition is most active against later stages of schistosomula of the challenge infection, such as lung-stage worms, 6–13 days after penetration (Smithers & Doenhoff, 1982). This means that in the sequential *S. margrebowiei*/*S. mansoni* experiments, by the time the first infection reaches patency (at 35 days post-infection) the schistosomula of the second infection will be 21 and 14 days old in 2- and 3-week time-lag experiments, respectively, and by that time may be largely refractory to immune killing. Therefore the protective immunity against the second infection in this model may be effected before the onset of patency in the first infection.

Some workers have acknowledged the existence of a degree of protective immunity in experiments where egg-laying and associated pathology are absent. For example, a study of Tchuem Tchuenté *et al.* (1996*a*) showed that a unisexual infection of *S. mansoni* gave cross-protection against *S. intercalatum* cercariae of the opposite sex only 10 days after the initial infection with *S. mansoni*. They attributed this phenomenon to 'the immunization process' as the time-interval between immunizing and challenge infections was too short to allow cross-protection induced by liver pathology. In a study by Miller & Smithers (1980), mice immunized via an abdominal skin site with irradiated cercariae, and challenged via the same site 4 weeks later showed nearly 60% immunity against the second infection only 5 days post-challenge. It was observed that local inflammation persisted despite the 4-week interval since immunization which was suggested as reason for the rapidity and high levels of attrition of the challenge infection. By analogy, therefore, it may be postulated that in the sequential *S. margrebowiei*/*S. mansoni* infections, cutaneous inflammation induced by the initial infection at the sites of penetration may have persisted up to the time of challenge with the second infection (2 or 3 weeks later), resulting in rapid killing of a large proportion of the penetrating schistosomula, well before the onset of patency (the same areas of the mice would have been exposed for each infection).

If the challenge infection in the *S. margrebowiei*/*S. mansoni* experiments had been delayed until after patency of the first infection, it is a matter of speculation as to whether the consequent egg-induced immunity would equal or outweigh that induced in pre-patency by the putative mechanisms outlined above.

The resistance induced by *S. mansoni* against *S. margrebowiei* attained its highest level in mice challenged 2 weeks post-infection (Exp. 2) whereas that induced by *S. margrebowiei* against *S. mansoni* attained the same high level only when challenge was 3 weeks post-infection (Exp. 5). That *S. mansoni* may be slightly more immunogenic than other species was indicated by the non-reciprocity of cross-immunity induced by *S. mansoni* against *S. intercalatum* in the experiments of Tchuem Tchuenté *et al.* (1996*a*). Nevertheless, the final levels of cross-protection induced by *S. margrebowiei*

and *S. mansoni* were identical with the worm returns of the second infection and 6 to 7 times less than that of the first.

Smithers & Doenhoff (1982) observed that the degree of cross-protection induced by one species against the other does not seem to be related to the phylogenetic distance between the two species. Certainly the non-reciprocity of cross-immunity in the *S. mansoni*/*S. intercalatum* model on the one hand but the almost full reciprocity of cross-protection in the *S. margrebowiei*/*S. mansoni* model seems to confirm this observation, as in both models the species are from different phylogenetic groups. These authors also suggest that the development or the severity of the immunizing infection in the host may be the more important factor in determining the degree of cross-protection. This may indeed explain the high reciprocity of cross-protection between *S. margrebowiei* and *S. mansoni*, as the nearly identical worm returns of both species in Exp. 1 indicates that both species were equally able to infect and develop in mouse hosts.

Another feature of the *S. margrebowiei*/*S. mansoni* experiments which deserves comment is the recovery of up to 4 multiple-worm pairs, involving either homospecific or heterospecific partners, from each experiment. Multiple matings have previously been observed amongst Schistosomatidae: Armstrong (1965) reported the occupation of the gynaecophoric canal of *Heterobilharzia americana* by 29 females of *S. mansoni*; and Tchuem Tchuenté *et al.* (1993) obtained males of *S. intercalatum* paired with 2–3 females of *S. intercalatum* or *S. mansoni* from experiments where *S. mansoni* males were absent. Basch (1991) observed that in cultures of *S. mansoni* it was common to find single females clasped by 2 males, one at either end. Furthermore, single male worms in culture have been shown to clasp both cotton and alginate fibres (Basch & Nicolas, 1989), suggesting that the clasping by males is a thigmotactic process rather than one dependent on chemotactic (pheromonal) signals from females. Hence the occurrence of male–female pairing between different species, and, in the case of the *H. americana*–*S. mansoni* interaction, between individuals from different genera.

In Exp. 1, mouse 4, all *S. margrebowiei* males are paired homospecifically with *S. margrebowiei* females, leaving 1 surplus *S. margrebowiei* female which is clasped by 2 of the excess *S. mansoni* males. In mouse 7, two *S. margrebowiei* females are paired with 1 *S. margrebowiei* male, despite the availability of an unpaired *S. margrebowiei* male. Another multiple pairing involved a *S. margrebowiei* male paired with 1 female from each species, and it is a matter of speculation as to which female it may have paired with first of all, since mice were infected with both species simultaneously, and all females would be available for pairing at the same time. Interestingly, in mouse 8, one *S. mansoni* male was found to be mated with 3 *S. margrebowiei* females, despite the availability of 2 single *S. margrebowiei* males.

Both species, therefore, are able to form these multiple-worm pairs, either involving 2 or more males paired with 1 female, or 1 male paired with 2 or more females. In Exp. 5, mouse 4, two multiple pairs were obtained, containing a total of 3 *S. margrebowiei* males and 3 *S. margrebowiei* females between them, but distributed unequally as 1 pairing of 2 *S. margrebowiei* males × 1 *S. margrebowiei* female; and 1 of 1 *S. margrebowiei* male × 2 *S. margrebowiei* females, so highlighting intra-*S. margrebowiei* competition between males for females.

In the sequential experiments, all multiple pairings involved only males of species 1 (except in Exp. 3, mouse 3, where there was a deficit of *S. margrebowiei* males), and only females of species 1, again, except in Exp. 3 (mouse 9), where a *S. margrebowiei* male was paired with 1 female from each species.

In this latter pairing, since *S. margrebowiei* was the first infection, it is likely that the male was first paired homospecifically with the *S. margrebowiei* female, and later managed to pair with a *S. mansoni* female as well, outcompeting 7 unpaired *S. mansoni* males in so doing. It seems, therefore, that the ability to form multiple pairs is another mating advantage conferred upon species 1. However, the advantages are mainly at an individual rather than a species level, as when there is a female deficit, the mating of several females by a male will be to the reproductive detriment of unpaired males of the same species. It was also observed that whilst single males can mate multiple females when there is either a male or female deficit, the pairing of single females with more than 1 male only occurred when there was a female deficit. This supports previous observations that the male is the more active sex in making the choice of mate (Tchuem Tchuenté *et al.* 1993, 1996*b*).

S. margrebowiei worms, having bovine rather than human hosts, are much larger than *S. mansoni* worms, hence, have more body musculature and a larger gynaecophoric canal in which to clasp females. It is therefore perhaps surprising that *S. margrebowiei* males are not significantly dominant to *S. mansoni* males in terms of their competitive mating abilities. There is some slight suggestion of *S. margrebowiei*'s mating dominance, by virtue of it having a stronger homospecific mate preference and a greater ability to pair than *S. mansoni* in simultaneous infections, but it is not as obvious as the dominance of *S. mansoni* over *S. intercalatum* (Tchuem Tchuenté, 1993) and of *S. haematobium* over *S. mansoni* (Webster *et al.* 1999) in the 2 previous models of mating between species from different phylogenetic groups. In all 3 models the species are reproductively isolated from one another,

firstly by having a specific mate preference system, and secondly by their inability to hybridize and produce offspring of high viability. The heterologous cross-protection observed between *S. intercalatum* and *S. mansoni* (Tchuem Tchuenté *et al.* 1996*a*) and here between *S. margrebowiei* and *S. mansoni* also serves as an isolating mechanism by acting to minimize reinfection of the host by a different species. However, *S. margrebowiei* and *S. mansoni* are reproductively isolated to a greater extent than the species in the other 2 models by their utilization of different definitive hosts. So effective is reproductive isolation by the host, it is a little surprising that there should be such a high degree of immunological cross-protection between *S. margrebowiei* and *S. mansoni*, if it is indeed primarily a reproductive isolating mechanism. Indeed, in many species belonging to the same group and therefore capable of hybridization, they may lack any isolating mechanisms other than that of host specificity. More likely the benefits of heterologous immunity are several, for example (as mentioned above) serving to prevent superinfection so as to ensure the long-term survival of the schistosomes and the host.

This model of mating between *S. margrebowiei* and *S. mansoni* indicates that one of the species used in inter-group mating models will not necessarily be significantly competitively dominant to the other, and that the timing of the infections and sequence in which the different species enter the host may prove a more crucial determinant in the outcome of the mating interactions than hitherto realised.

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Competitive mating interactions of Schistosoma haematobium and S. intercalatum (Lower Guinea strain)

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Running title: Interactions of Schistosoma haematobium / S. intercalatum (Lower Guinea)

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Abstract Schistosoma haematobium and S. intercalatum belong to the S. haematobium group of schistosomes and can hybridize in nature where they are sympatric and therefore able to co-infect the same human host. Hybridization and competitive mating interactions with S. haematobium have been implicated in restricting the distribution of S. intercalatum in Africa and in the remarkably rapid replacement of S. intercalatum by S. haematobium at Loum, Cameroon. Previous studies have demonstrated the greater pairing ability of S. haematobium over S. intercalatum in hamsters infected with both species simultaneously or infected first with S. intercalatum (Lower Guinea strain) and later with S. haematobium. The present study demonstrates the greater pairing ability of S. haematobium over S. intercalatum in hamsters infected first with S. intercalatum (Lower Guinea) and later with S. haematobium, and indicates that S. intercalatum is unlikely to spread to areas where S. haematobium is already established.

It has been shown that Schistosoma haematobium and the hybrid of S. haematobium and S. intercalatum have completely replaced S. intercalatum in a period of less than 30 years in Loum, Cameroon (Southgate & Rollinson, 1980; Tchuem Tchuenté et al. 1997). The chief means by which S. haematobium effected this replacement of S. intercalatum was through introgressive hybridisation (Southgate et al. 1976)

Southgate et al. (1982) demonstrated the greater competitiveness of S. haematobium over S. intercalatum (Lower Guinea) with regard to pairing ability, in a laboratory model of mating interactions between these two species in hamsters. Hamsters were either infected simultaneously, or infected first with S. intercalatum and then with S. haematobium. Surprisingly, mating between the two species appeared to be random: no specific mate preference system was demonstrated for either species. It has been suggested that S. haematobium and S. intercalatum had not evolved strong assortative mating because they have only fairly recently come into contact with one another, largely through the incursions of man into the rainforest (Southgate et al. 1982; Pagès et al. 2001).

In both the simultaneous infections and infections with S. intercalatum before S. haematobium which Southgate et al. (1982) carried out, S. haematobium males were clearly shown to be better at pairing with females of either species than S. intercalatum males. Given the usual natural bias of schistosome sex ratios towards males (Liberatos, 1987; Mitchell et al. 1990; Jourdane et al. 2001) it is clear how the competitiveness of S. haematobium males can reduce the reproductive success of S. intercalatum.

Southgate et al. (1982) did not carry out a sequential infection involving hamsters infected first with S. haematobium and later with S. intercalatum, because the reverse situation, of S. intercalatum being established in hosts prior to infection with S. haematobium, most closely reflected the situation in the field at the time. However, as the replacement of S. intercalatum by S. haematobium progressed, it is likely that younger individuals would initially be infected with S. haematobium prior to any subsequent infection with S. intercalatum. The study presented here was undertaken in order to complete the model of S. haematobium/ S. intercalatum mating interactions by infecting hamsters initially with S. haematobium and subsequently with S. intercalatum. This should provide further insight into the process by which S. haematobium replaced S. intercalatum, and allow us to ascertain the likelihood of S. intercalatum re-establishing itself in Loum, in the face of pre-established S. haematobium infections.

The Lower Guinea strain of S. intercalatum used in this study was isolated from 26 naturally infected Bulinus forskalii collected in Edea, Cameroon in 1998. It was maintained for 2 years in laboratory-bred B. wrighti and albino TO mice. Eggs of S. haematobium were isolated from urine collected from the population of the village of Barombi Mbo, Cameroon in 2000. Laboratory-bred B. wrighti were exposed to the miracidia hatched from the eggs, and the isolate was passaged once in the laboratory through albino TO mice and B. wrighti prior to use in this study.

The experimental design was aimed at determining the interactions between males and females of S. haematobium and S. intercalatum in sequential infections in hamsters with S. haematobium as the first infection and S. intercalatum as the second. Taking into account the differences in patency of the two species in hamsters (approximately 60 days for S. haematobium and approximately 45 days for S. intercalatum (Loker, 1983)) hamsters were exposed individually by the paddling technique to 150 cercariae of S. haematobium, and 4 weeks later to 150 cercariae of S. intercalatum (Zaire strain) so that S. haematobium would reach patency approximately 2 weeks earlier than S. intercalatum. It is assumed that S. haematobium will form homospecific pairs before S. intercalatum and the usual male-bias of schistosome infections (Liberatos, 1987; Mitchell et al. 1990) should result in an excess of S. haematobium males which may compete with S. intercalatum males for S. intercalatum females. Hamsters were killed and worms collected by perfusion and dissection of the hepatic portal vein and mesenteric venous systems of each infected hamster, 13-16 weeks post reinfection. Each pair and any unpaired worms recovered from infected hamsters were segregated into individual containers and the pairs were separated. Individual females were taxonomically identified by microscopic examination of the morphology of intrauterine eggs. Since the taxonomic identity of the male worms could not be determined by morphometry, individual males were examined for glucose-6-phosphate-dehydrogenase (G6PD) activity using the isoelectric focusing technique

(Wright et al. 1979). Worms of the isolates of both species used in this study are monomorphic for G6PD but differ in their pI values producing an identifiable profile which can be used to differentiate the two species (Southgate et al. 1982). Each male and female worm within each pair and any unpaired worms were identified and recorded for each hamster. The data were analysed using the Mantel-Haenszel test to evaluate the significance of the observed proportions (Mantel and Haenszel, 1959; Southgate et al. 1982; Webster et al. 1999).

Both types of homospecific and both types of heterospecific pairing were obtained in this experiment (Table 1), together with one homosexual pair from hamster 11 consisting of two male S. intercalatum worms (one smaller male inside the gynaecophoric canal of the other). The proportion of males which formed pairs was 90.4% for S. haematobium males, and 65.4% for S. intercalatum males. Mantel-Haenszel analysis showed this difference to be significant ($\chi^2 = 11.931$, $P < 0.005$), with S. haematobium males being better at pairing than S. intercalatum males. The sex ratio of males: females in this experiment was 2.2:1 for S. haematobium and 1.2:1 for S. intercalatum.

Although all S. haematobium females should have been paired with S. haematobium males before S. intercalatum males commenced pairing, a total of 6 heterospecific S. intercalatum ♂ x S. haematobium ♀ pairs were obtained from hamsters 7, 11, 12, 15 and 16. However, 4 out of these 6 pairs resulted from there being a deficit of S. haematobium males for the S. haematobium females to pair with, in each case the extra females were mated by an S. intercalatum male. For 2 out of the 6 pairings (one from hamster 11, one from hamster 15) a corresponding non-homospecifically paired S. haematobium male was also obtained, indicating that this male had been displaced from its original homospecific female partner by a competitor S. intercalatum male. A Mantel-Haenszel test carried out on the proportions of males of both species paired with S. haematobium females returned a significant result ($\chi^2 = 8.987$, $P < 0.005$), indicating that overall S. haematobium males were better than S. intercalatum males at pairing with S. haematobium females.

Seventeen out of the 28 heterospecific S. haematobium ♂ x S. intercalatum ♀ pairs obtained in total resulted from there being a deficit of S. intercalatum males for the S. intercalatum females to pair with. Two of the 28 pairs may have involved S. haematobium males that had been actively displaced from their original homospecific S. haematobium ♀ partners by competitor S. intercalatum males, as described above. These displaced S. haematobium males had managed to re-pair with a heterospecific female. For each of the remaining 9 heterospecific S. haematobium ♂ x S. intercalatum ♀ pairs at least one unpaired S. intercalatum male was also obtained which is assumed to have been outcompeted by the S. haematobium males for the S.

intercalatum females. The difference in the proportions of males of each species paired with S. intercalatum females was significant according to Mantel-Haenszel analysis ($\chi^2 = 7.072$, $P < 0.01$) indicating that overall, S. haematobium males are better than S. intercalatum males at pairing with S. intercalatum females.

The two S. intercalatum ♂ x S. haematobium ♀ pairs recovered from hamsters 11 and 15 demonstrate the ability of S. intercalatum males to "actively compete" for S. haematobium females by pulling them away from their homospecific S. haematobium partners. Data from the Southgate et al. (1982) experiment where S. intercalatum was the initial infection and S. haematobium the subsequent infection reveal that S. haematobium males were similarly able to "actively compete" with S. intercalatum males for S. intercalatum females.

In demonstrating that S. haematobium males are better than S. intercalatum males at pairing with females of either species when S. haematobium is the initial infection, the experiment presented here confirms the competitive dominance of S. haematobium over S. intercalatum as observed by Southgate et al. (1982). It therefore seems that at all stages throughout the progressive replacement of S. haematobium at Loum, i.e. whether co-parasitized hosts were initially infected with S. intercalatum, or with S. haematobium, or with both species simultaneously, the reproductive success of S. intercalatum was severely compromised by the competitive mating of S. haematobium, leading to its eventual exclusion from the region.

A mathematical model constructed by Morand et al. (2002), of the interactions between S. haematobium and S. intercalatum, assumes a male-biased sex ratio (as is usual in schistosome populations) and incorporates "recombinations" and compatibility levels with the snail hosts B. truncatus and B. forskalii, predicts the loss of S. intercalatum but the persistence of the hybrids together with S. haematobium. Therefore, in the population of hybrids, some remnant of the S. intercalatum genome is predicted to exist, although the authors acknowledge that the model may be oversimplified and that increasing the complexity of the model (e.g. by assuming that compatibility with intermediate hosts is controlled by several loci instead of one) may cause the complete disappearance of these hybrids. However, the prediction of persistent S. intercalatum genes in the schistosome population at Loum is supported by very recent molecular data (Webster et al. in press).

Undeniably, however, there is no longer transmission of pure S. intercalatum at Loum, and the results of the study presented here indicate that S. intercalatum, in the absence of any other factors, will be unable to re-establish itself in a population of human hosts already infected with S. haematobium.

Introgressive hybridisation with S. haematobium is thought to be one of the key factors limiting the distribution of S. intercalatum in Africa, which is highly restricted in comparison to the wide distribution of its potential snail hosts (Southgate, 1978; Jourdane et al. 2001). A possible past history of hybridization between S. intercalatum and S. haematobium in the Dogon country of Mali has been implicated in the apparently sudden cessation of S. intercalatum infection in the region (De Clercq et al. 1994). Morand et al. (2002) observed that there appear to be more areas of sympatry between S. intercalatum and S. haematobium than between S. intercalatum and S. mansoni in Africa, and both Tchuem Tchuenté et al. (1996) and Jourdane et al. (2001) note that the relatively new foci of S. intercalatum (Lower Guinea) described from São Tomé and Equatorial Guinea, correspond to situations where S. haematobium (and S. mansoni) are absent.

Therefore, whilst introgressive hybridisation and the competitive mating ability of S. haematobium over S. intercalatum, which the studies of Southgate et al. (1976, 1982) have identified (and the results of this study confirmed) as crucial determinants of the replacement of S. intercalatum by S. haematobium at Loum, undoubtedly play a role in restricting the distribution of S. intercalatum in Africa, this role may itself be limited by the fact that S. intercalatum/ S. haematobium interactions are largely confined to the boundaries between rainforest and savannah.

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Table 1. Data from hamsters infected first with 150 cercariae of S. haematobium (Hm) and 4 weeks later with 150 cercariae of the Lower Guinea strain of S. intercalatum (Int), so that S. haematobium reaches patency approximately 2 weeks before S. intercalatum)

Hamster	Hm♂	Int♂	Hm♂xHm♀	Int♂xInt♀	Hm♂xInt♀	Int♂xHm♀	Homosexual pairings (♂:♂)
1	1	1	2	2	4	0	
2	0	2	0	1	5	0	
3	1	1	0	0	0	0	
4	0	0	0	1	0	0	
5	0	1	0	0	1	0	
6	1	2	0	0	3	0	
7	0	0	0	1	0	1	
8	0	0	2	2	2	0	
9	0	0	1	3	0	0	
10	0	1	2	0	5	0	
11	0	5	0	6	1	1	1 Int : 1 Int
12	0	0	0	4	0	1	
13	0	0	2	4	1	0	
14	0	0	3	0	3	0	
15	0	3	2	8	1	2	
16	0	3	1	3	0	1	
17	1	9	2	2	2	0	
18	0	0	0	7	0	0	
19	1	0	2	3	0	0	
Total:	**5**	**28**	**19**	**47**	**28**	**6**	

Interactions between *Schistosoma intercalatum* (Zaire strain) and *S. mansoni*

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Running title: Interactions *Schistosoma intercalatum* (Zaire)/*S. mansoni*

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ABSTRACT

Schistosoma mansoni and *S. intercalatum*, two schistosomes from different evolutionary lineages, are parasitic in humans and therefore able to co-infect the same host where they occur sympatrically in Africa. Previous studies of mating interactions between these species in mice, using the Lower Guinea strain of *S. intercalatum*, have demonstrated the competitive dominance of *S. mansoni* over *S. intercalatum* in terms of pairing ability, which is potentially an important mechanism restricting the distribution of *S. intercalatum* in Africa. The study presented here examines the mating interactions in mice between *S. mansoni* and the Zaire (Democratic Republic of Congo) strain of *S. intercalatum*, which differs from the Lower Guinea strain in many biological characteristics. Analysis of the data showed a preponderance of intraspecific pairs over interspecific, demonstrating a specific mate preference system for both species. Mating competition between these species and the ability of males of both species to effect a change of mate by pulling paired females away from their partners was indicated. Comparisons are made between the competitive mating abilities of both strains of *S. intercalatum* relative to those of *S. mansoni*, with the data suggesting that *S. mansoni* is competitively dominant to *S. intercalatum* (Zaire) in sequential infections but to a lesser extent than for *S. intercalatum* (Lower Guinea). Additional factors which may contribute to the confinement of *S. intercalatum* (Zaire) to the Democratic Republic of Congo are discussed.

INTRODUCTION

Two distinct strains of *S. intercalatum* are currently recognized: the Lower Guinea and Zaire (Democratic Republic of Congo) strains, which differ in their geographical distribution and a number of biological characteristics such as intermediate host specificity, prepatent periods, isoenzyme profiles and chronobiological cercariae shedding patterns (Wright *et al.*, 1972, 1979; Pagès & Théron, 1990). In addition, recent data have shown considerable molecular divergence between the strains (Pagès *et al.*, 2001 a, b). There is some uncertainty as to the origin of the two strains. The first reports of mesenteric terminal-spined schistosomiasis were in 1923, by Chesterman from Yakusu, near Stanleyville (Kisangani) in the Democratic Republic of Congo (Chesterman, 1923), and by Clapier from Libreville in Gabon (Clapier, 1923), but it was Fisher (1934) who officially described *S. intercalatum* as a new species from the Stanleyville region. A study by Deschiens & Delas (1969) suggested that the parasite was spreading northwards into Cameroon with immigrant manual workers. These authors assumed that the Stanleyville focus was the true origin of the *S. intercalatum* parasite, and that its outward spread led to its divergence into two different strains through geographical isolation. However, Browne (1969) noted that there was no evidence that the Stanleyville focus was older than the Libreville one, and Wright *et al.* (1972) interpreted the numerous biological differences between the strains as indicative that they had been separated for some time. Indeed, more recent studies incorporating the molecular divergence between the strains confirm that their differentiation from one another is ancient, and probably took place at the same time as speciation of all the other *S. haematobium*-group schistosomes (Wright *et al.*, 1972; Desprès *et al.*, 1992; Pagès *et al.*, 2001 a, b). Wright *et al.* (1972) postulated that, as the two strains are unable to utilize each other's snail host (*B. africanus* group snails for the Zaire strain, and *B. forskalii* group snails for the Lower Guinea strain), this parasite originated in an ancestral bulinid snail of forest regions and the two strains diverged with separate lines of snails derived from the common stock.

Of the two, it is the Lower Guinea strain which has the wider distribution, occuring in Gabon. Cameroon, Nigeria, Equatorial Guinea and Sao Tomé, whereas the Zaire strain appears confined to the Democratic Republic of Congo (Tchuem Tchuenté *et al.*, 1997 a). However, in comparison to the extremely wide distribution of their definitive (human) and intermediate hosts across Africa, the distribution of both strains appears to be highly restricted (Tchuem Tchuenté *et al.*, 1996, 1997 a).

One biological reason postulated for this concerns the tendency of *S. intercalatum* cercariae, in contrast to other African schistosomes, to form non-infective aggregates in response to small temperature changes. The aggregates are formed by the release of the adhesive post-acetabular gland secretion which causes the cercariae to stick together. It has been suggested that *S. intercalatum* originated in streams within tropical rainforest areas, and only comparatively recently spread into water bodies in more open areas of savannah. Here cercariae would experience greater diurnal temperature changes, and thereby aggregate more readily, so impeding invasion of the definitive host and limiting its spread (Southgate, 1978).

Other factors possibly restricting the distribution of *S. intercalatum* concern mating interactions of *S. intercalatum* with other schistosome species with which it is sympatric. For example. the introduction of *S. haematobium* by immigrants in 1968 into the town of Loum, Cameroon where *S. intercalatum* (Lower Guinea) was indigenous led to the large-scale replacement of *S. intercalatum* by *S. haematobium* through hybridisation (Southgate, 1978; Tchuem Tchuenté *et al.*, 1997 b). *S. haematobium* and *S. intercalatum* are closely related (both belonging to the *S. haematobium* group) and will hybridize both in nature and in the laboratory when they co-parasitize the same individuals (Wright *et al.*, 1974; Southgate *et al.*, 1976; Wright & Southgate, 1976; Tchuem Tchuenté *et al.*, 1997 a, b). Hybrid offspring of *S. haematobium* ♂ x *S. intercalatum* ♀ crosses are viable (Southgate *et al.*, 1976), and several generations of backcrossing with parental species (introgressive hybridisation) may cause the emergence of a new strain of *S. haematobium*, as at Loum . Key to the replacement of *S. intercalatum* was the competitive dominance of *S. haematobium* over *S. intercalatum* (Lower Guinea) with regard to pairing in mixed infections, as demonstrated by Southgate *et al.* (1982).

S. intercalatum is also found sympatrically in Africa with *S. mansoni* (Tchuem Tchuenté *et al.*, 1993) and, like *S. haematobium*, *S. mansoni* is thought to be limiting the distribution of *S. intercalatum* for several reasons.

Firstly, as demonstrated by Tchuem Tchuenté *et al.* (1996) using mixed infections of *S. mansoni* and *S. intercalatum* (Lower Guinea) in rodents, infection with *S. mansoni* induces a significant degree of cross-protection against subsequent infection with *S. intercalatum*, but infection with *S. intercalatum* does not cross-protect against reinfection with *S. mansoni*. In nature *S. mansoni*-induced immunity against *S. intercalatum* in infected individuals will make it difficult for *S. intercalatum* to become established in areas where *S. mansoni* is already present.

Secondly, similar to the situation with *S. haematobium*, the competitive dominance of *S. mansoni* over *S. intercalatum* in mixed infections may limit the distribution of *S. intercalatum* (Tchuem Tchuenté *et al.*, 1996). In the experiments of Tchuem Tchuenté *et al.* (1993, 1994, 1996) using *S. intercalatum* (Lower Guinea) in mixed infections with *S. mansoni*, not only was *S. mansoni* shown to be better at forming pairs than *S. intercalatum*, but also maintained a stronger homospecific mate preference. As *S. mansoni* and *S. intercalatum* cannot hybridize, being from different evolutionary groups, offspring of heterospecific pairings would be parthenogenetic and of very low viability, and so homospecific pairing is preferable. It is likely that as *S. intercalatum* has only recently become sympatric with other schistosome species, pre-zygotic reproductive isolating traits such as competitive pairing and strong homospecific mate preference have not been strongly selected for (Tchuem Tchuenté *et al.*, 1995; Southgate *et al.*, 1998; Pagès *et al.*, 2001 b). Using data from the Tchuem Tchuenté *et al.* (1993) investigation into mating interactions between *S. intercalatum* (Lower Guinea) and *S. mansoni*, Tchuem Tchuente *et al.* (1996) used a mathematical model of mating probabilities in these mixed infections to suggest that the basic transmission rate of *S. intercalatum* could be reduced by a factor of 5 in areas of sympatry with *S. mansoni*, independent of the effect of *S. mansoni*-induced cross-protection against *S. intercalatum*.

A third reason for *S. mansoni* outcompeting and so limiting the distribution of *S. intercalatum* concerns the fact that *S. mansoni* populations will always be greater in nature than those of *S. intercalatum*, by virtue of its greater cercarial productivity; its greater worm burden and shorter patency period in definitive hosts (Loker, 1983).

Given the wide distribution of both *S. haematobium* and especially *S. mansoni* across much of Africa, all of the above immunological and competitive mating factors make it very difficult for *S. intercalatum* to spread. Indeed the relatively new foci of *S. intercalatum* reported in Sao Tomé and Equatorial Guinea corrrespond to situations where both *S. haematobium* and *S. mansoni* are absent (Tchuem Tchuenté *et al.*, 1996; Jourdane *et al.*, 2001).

So far, all work on mating interactions of *S. intercalatum* with *S. mansoni* and *S. haematobium* has used the Lower Guinea strain of *S. intercalatum*. Given the clear biological differences between the two strains outlined above, together with the fact that they cannot utilize each other's intermediate snail host, there is currently much debate over whether or not the two strains are in fact separate species

(Jourdane *et al.*, 2001; Pagés *et al.*, 2001 a). Therefore, it cannot necessarily be assumed that the Zaire strain will interact in the same way as the Lower Guinea strain with other species of schistosome.

The aim of this study was to elucidate the mating interactions of *S. intercalatum* (Zaire) with *S. mansoni*, to see which (if any) species emerged as competitively dominant, and more specifically if *S. mansoni* could limit the distribution of *S. intercalatum* (Zaire) through competitive dominance as appears to be the case for *S. intercalatum* (Lower Guinea). It should also provide further insight into the differences between the two strains of *S. intercalatum*.

MATERIALS AND METHODS

Origin of species

The isolate of *S. intercalatum* (Zaire strain) used in these experiments originates from Kinshasa, Democratic Republic of Congo, where an autochthonous focus of *S. intercalatum* was described in 1987 by De Clercq. Tchuem Tchuenté *et al.* (1997 a) noted a possible PGM isoenzyme difference between this isolate and the original one from Kisangani described by Fisher (1934), but it utilizes the same snail host (*B. globosus*) and remains distinct from the Lower Guinea strain. It was isolated in 1994 from faeces of infected primary school children in Kinshasa by L. A. Tchuem Tchuenté and maintained in the laboratory at the University of Perpignan in *B. globosus* from Zambia and Swiss OF1 mice for several years. It was introduced into the laboratories of the Natural History Museum in 2001 courtesy of L. A. Tchuem Tchuenté where it was maintained in laboratory-bred *B. wrighti* from Oman, and albino TO mice for two cycles prior to its use in this study.

The isolate of *S. mansoni* originated from Richard Toll, Senegal and was isolated from wild-caught *Biomphalaria pfeifferi* in 1993. It has been maintained in laboratory-bred *B. pfeifferi*, *B. glabrata* and albino TO mice to date.

Experimental infections

The experimental design was aimed at determining how male and female worms of both species interact in mixed infections in the vertebrate host, and whether a specific mate recognition system and mating competition exists between the two species. Mice were exposed individually by the paddling technique to a fixed number of male and female cercariae of both species in three experiments. These

were of two types, the first of which (experiment 1) involved the simultaneous infection of mice with 150 cercariae of *S. intercalatum* (Zaire) and 10 days later with 150 cercariae of *S. mansoni*, to ensure that both species reached patency simultaneously and therefore had total choice of mate from either species. The second type of experiment (experiments 2 and 3) involved the sequential infection of mice, with the time interval between infections adjusted according to the differences in patency of the two species in mice (approximately 45 days for *S. intercalatum* and approximately 35 days for *S. mansoni* (Loker, 1983)) so that the first species would reach patency 2-3 weeks before the second. Mice in experiment 2 were infected firstly with 150 cercariae of *S. mansoni* and then with 150 cercariae of *S. intercalatum* (Zaire) one week later, whilst mice from experiment 3 were infected first with 150 cercariae of *S. intercalatum* (Zaire) and 4 weeks later with 150 cercariae of *S. mansoni*. In sequential infections it is assumed that the first species will form homospecific pairs, and the usual male-bias of schistosome infections (Liberatos, 1987; Mitchell *et al.*, 1990) should result in an excess of "species 1 males" which may compete with "species 2 males" for "species 2 females".

Mice were killed and worms collected by perfusion and dissection of the hepatic portal vein and mesenteric venous systems of each infected mouse 6-8 weeks post reinfection. Each pair and any unpaired worms recovered from infected mice were segregated into individual containers and the pairs were separated. Individual females were taxonomically identified by microscopic examination of the morphometry and number of the intrauterine eggs: *S. mansoni* females have single lateral spined eggs *in utero*, whereas *S. intercalatum* females produce several eggs which have a conical posterior end tapering to a terminal posterior spine. Since the taxonomic identity of the male worms could not be determined by morphometry, individual male worms were examined for glucose-6-phosphate-dehydrogenase (G6PD) activity using the isoelectric focusing technique (Wright *et al.*, 1979; Fletcher *et al.*, 1981; Wright & Ross, 1983). Male worms of the isolates of both species used in this study are monomorphic for G6PD but differ in their pI values, producing an identifiable profile which enabled distinction between the two species of schistosome. Each male and female worm within each pair and any unpaired worms were identified and recorded for each mouse. The data were analysed using the Mantel-Haenszel test to evaluate the significance of the observed proportions (Mantel & Haenszel, 1959; Southgate *et al.*, 1982; Webster *et al.*, 1999).

RESULTS

Experiment 1: Infections with S. intercalatum *(Zaire) and10 days later with* S. mansoni

Table 1 summarizes the worms recovered from each mouse and how they paired. Two homospecific and two heterospecific types of pairing were obtained, and there was a surplus of unpaired males of both species. Three multiple worm pairs were obtained in total, one from mouse 4, mouse 5 and mouse 6. In all cases these pairings consisted of one male paired with two females: in mice 4 and 5 all worms involved were *S. mansoni*, and in mouse 6, they were all *S. intercalatum* worms.

Overall, 196 homospecific pairs and 94 heterospecific pairs were obtained. To test whether this difference was indicative of any species preference, the Mantel-Haenszel test was carried out on the proportions of males of both species paired homospecifically and heterospecifically. The result was highly significant ($\chi^2 = 51.101$, $P < 0.001$), showing that both species have a strong preference for homospecific mating.

Of paired *S. intercalatum* males, 85.6% formed homospecific pairs, and 14.4% heterospecific pairs, whereas 55.2% of paired *S. mansoni* males formed homospecific pairs, and 44.8% heterospecific pairs. This indicates that *S. intercalatum* has the stronger homospecific mate preference. However, it should be noted that the sex ratio of males: females for *S. intercalatum* in this experiment was 0.8:1, and this female bias may have influenced this result (see later discussion).

The proportion of all *S. intercalatum* males which were paired was 95.2%, whereas that *S. mansoni* males was 80.8%. This difference was statistically significant according to Mantel-Haenszel analysis ($\chi^2 = 11.394$, $P < 0.005$), with *S. intercalatum* males being more successful than *S. mansoni* males at pairing. Again, it should be noted that this result may have been influenced by the female bias of the *S. intercalatum* sex ratio in this experiment.

Experiment 2: infections with S. mansoni *and 1 week later with* S. intercalatum *(Zaire)*

Two homospecific and two heterospecific types of pairing were obtained in this experiment (Table 2) together with one multiple-worm pair from mouse 1, which consisted of one *S. intercalatum* male paired with one *S. intercalatum* female and one *S. mansoni* female. The proportion of males which formed pairs was 87.4% for *S. mansoni* males and 74.2% for *S. intercalatum* males. Mantel-Haenszel

analysis showed this difference to be highly significant ($\chi^2 = 4.744$, $P < 0.05$), with *S. mansoni* males being better able to form pairs than *S. intercalatum* males.

The sex ratio of males:females in this experiment was 1.3:1 for *S. mansoni*, and therefore the assumption that all *S. mansoni* females would have initially been paired with *S. mansoni* males, leaving an excess of *S. mansoni* males which may compete with *S. intercalatum* males for *S. intercalatum* females, is a reasonable one.

However, despite this assumption a total of 9 heterospecific *S. intercalatum* ♀ x *S. mansoni* ♀ pairs were obtained from mice 1, 3, 5, 7 and 9 (Table 2). In mice 1, 3, 5 and 7. there was at least one unpaired *S. mansoni* male for each *S. intercalatum* ♂ x *S. mansoni* ♀ pairing, which the *S. intercalatum* males had outcompeted (Table 2). In mouse 9, which yielded two *S. intercalatum* ♂ x *S. mansoni* ♀ pairs but only one unpaired *S. mansoni* male, one of these two pairs may have resulted from there being one fewer *S. mansoni* males than *S. mansoni* females: thus an *S. intercalatum* male paired with the extra *S. mansoni* female. A Mantel-Haenszel test carried out on the proportions of males of both species paired with *S. mansoni* females returned a highly significant result ($\chi^2 = 30.069$, $P < 0.001$), indicating that *S. mansoni* males were better overall at pairing with *S. mansoni* females than were *S. intercalatum* males. Therefore, *S. intercalatum* males are unable to outcompete *S. mansoni* males for *S. mansoni* females to any significant extent.

Data from mice 2, 3, 5, 7 and 8 indicate that 21 of the 32 heterospecific *S. mansoni* ♂ x *S. intercalatum* ♀ pairs obtained in total arose from there being a deficit of *S. intercalatum* males for the *S. intercalatum* females to pair with. Four of the 32 pairings may have involved *S. mansoni* males that had been actively displaced from their original homospecific *S. mansoni* ♀ partners by competitor *S. intercalatum* males, as indicated by the presence of a corresponding *S. intercalatum* ♂ x *S. mansoni* ♀ pair for each of the four *S. mansoni* ♂ x *S. intercalatum* ♀ pairings. These displaced males may have managed to re-pair with a heterospecific female. For each of the remaining 7 heterospecific *S. mansoni* ♂ x *S. intercalatum* ♀ pairs at least one unpaired *S. intercalatum* male was also obtained which is assumed to have been outcompeted by the *S. mansoni* males for the *S. intercalatum* females. The difference in the proportions of males of each species paired with *S. intercalatum* females was significant according to Mantel-Haenszel analysis ($\chi^2 = 5.041$, $P < 0.05$) indicating that overall, *S. intercalatum* males are better than *S. mansoni* males at pairing with *S. intercalatum* females.

Experiment 3: infections with S. intercalatum *(Zaire) and 4 weeks later with* S. mansoni

Table 3 summarizes the worms obtained from each mouse and how they paired. Four main types of pairing were found: two homospecific and two heterospecific.There were no multiple-worm pairs. The proportion of males which formed pairs was 98.6% for *S. intercalatum*, and 82.6% for *S. mansoni*. The results of a Mantel-Haenszel test carried out on these proportions was not significant, and therefore males of each species do not significantly differ in their ability to form pairs.

The sex ratio of males:females was 1.7:1 for *S. intercalatum* in this experiment, and therefore the assumption that all *S. intercalatum* females would have initially been paired with *S. intercalatum* males, leaving an excess of *S. intercalatum* males which may compete with *S. intercalatum* males for *S. intercalatum* females, is a reasonable one.

Nevertheless, 12 heterospecific *S. mansoni* ♂ x *S. intercalatum* ♀ pairs were obtained in total from mice 2, 3, 4, 6, 8 and 10. Three of the *S. mansoni* ♂ x *S. intercalatum* ♀ pairs resulted from a deficit of *S. intercalatum* males for the females to pair with in mouse 2. There was at least one unpaired or non-homospecificially paired *S. intercalatum* male for each of the 9 remaining pairs, indicating that these *S. intercalatum* males had been outcompeted for *S. intercalatum* females by the *S. mansoni* males. However, Mantel-Haenszel analysis revealed a highly significant difference in the proportions of males of each species paired with *S. intercalatum* females, (χ^2 = 20.487, P < 0.001), with *S. intercalatum* males being better able to do so than *S. mansoni* males. Therefore, overall, *S. mansoni* males are unable to outcompete *S. intercalatum* males for *S. intercalatum* females to any significant extent.

Heterospecific *S. intercalatum* ♂ x *S. mansoni* ♀ pairs were obtained from all mice in this experiment except mouse 6. Seventeen out of the 37 pairs recovered in total arose from there being a deficit of *S. mansoni* males compared with *S. mansoni* females. Eight out of the 37 were obtained along with a *S. mansoni* ♂ x *S. intercalatum* ♀ pair. Therefore, the 8 heterospecifically paired *S. intercalatum* males may have been those actively displaced from their original *S. intercalatum* female partners by competitor *S. mansoni* males, and which subsequently managed to re-pair with heterospecific females. For each of the remaining 12 heterospecific *S. intercalatum* ♂ x *S. mansoni* ♀ pairs at least one unpaired *S. mansoni* male was also obtained which is assumed to have been outcompeted by the *S. intercalatum* males for the *S. mansoni* females. The difference in the proportions of males of each species paired with *S. mansoni* females was highly significant according to Mantel-Haenszel analysis

(χ^2 =15.503, P < 0.001), indicating that *S. intercalatum* males are unable to outcompete *S. mansoni* males for *S. mansoni* females to any significant extent.

DISCUSSION

In all 3 experiments both homospecific and heterospecific pairs were obtained, and all females recovered were paired and contained eggs, thereby confirming that pairing and reproductive stimulation of females is possible between different species of schistosome, even where they belong to different evolutionary groups (Tchuem Tchuenté *et al.*, 1994; Khalil & Mansour, 1995; Southgate *et al.*, 1998).

Mice infected simultaneously with *S. intercalatum* (Zaire) and *S. mansoni* (experiment 1) yielded a significant preponderance of homospecific pairs over heterospecific, indicating the presence of a specific mate preference system, as demonstrated for the *S. intercalatum* (Lower Guinea)/*S. mansoni*; *S. haematobium*/*S. mansoni*; and *S. margrebowiei*/*S. mansoni* models of mating between schistosomes from different evolutionary groups (Tchuem Tchuenté *et al.*, 1993; Webster *et al.*, 1999, Cosgrove & Southgate, 2002).

In this model, *S. intercalatum* (Zaire) exhibited a significantly greater homospecific mate preference than *S. mansoni*, in contrast to *S. intercalatum* (Lower Guinea) which had a lesser homospecific mate preference than *S. mansoni* (Tchuem Tchuenté *et al.*, 1993). However, it should be borne in mind that the *S. mansoni* isolate used by Tchuem Tchuenté *et al.* (1993) originated from Brazil, whereas the isolate used in these experiments came from Senegal, so it is conceivable that some of the differences in the data between Tchuem Tchuenté *et al.* (1993) and those presented here may possibly be correlated with the different isolates of *S. mansoni*. On the other hand, the work of Fletcher *et al.* (1981) mitigates against this idea, as they demonstrated, using isoenzymes, a relative lack of genetic divergence between Old and New World isolates of *S. mansoni*. The reproductive disadvantage of heterospecific pairing in yielding offspring of reduced viability has been mentioned earlier, but parthenogenetic offspring are particularly undesirable for *S. intercalatum* males in mixed infections with *S. mansoni*. This is because the *S. mansoni* ♂ x *S. intercalatum* ♀ cross yields sterile eggs, whilst the *S. intercalatum* ♂ x *S. mansoni* ♀ cross yields some parthenogenetic, matroclonal eggs of low viability, which, being matroclonal, may produce a small number of *S. mansoni* worms (Wright & Southgate, 1976). Therefore, the (albeit small) advantage of parthenogenesis in mixed infections (in allowing the

propagation of some germ plasm) is enjoyed only by (female) *S. mansoni*, and therefore provides a further incentive for *S. intercalatum* to avoid heterospecific pairing.

However, the sex ratio of *S. intercalatum* (Zaire) was female-biased in this experiment (0.8:1 males:females), which may have had a significant influence on the results by increasing the chances of *S. intercalatum* (Zaire) being able to pair homospecifically over those of *S. mansoni* whose sex-ratio was male-biased (as sex ratios of schistosomes in nature usually are). The excess single *S. intercalatum* females would then be available for the excess single *S. mansoni* males to pair with, so increasing the proportion of heterospecifically paired *S. mansoni* males.

Similarly, the female-bias of the *S. intercalatum* (Zaire) infection may have influenced the finding that *S. intercalatum* (Zaire) males were better at pairing than *S. mansoni* males in experiment 1, which again differs from the *S. intercalatum* (Lower Guinea)/*S. mansoni* mating model where *S. mansoni* males were equally good at pairing as *S. intercalatum* (Lower Guinea) males (Tchuem Tchuenté *et al.*, 1993).
In the simultaneous *S. intercalatum* (Lower Guinea)/*S. mansoni* experiments of Tchuem Tchuenté *et al.* (1993), equal numbers of males and females of each species were used.

Although there are indications from pairing ability and the strength of homospecific mate preference in experiment 1 that *S. intercalatum* (Zaire) has the competitive advantage over *S. mansoni* in simultaneous infections, which is the reverse of the situation with the Lower Guinea strain of *S. intercalatum*, the possible influence of the female-biased sex ratio of *S. intercalatum* (Zaire) must be taken into account.

One possible reason for there being more females than males of *S. intercalatum* (Zaire) in experiment 1 and not in experiments 2 or 3 is that the *S. intercalatum* (Zaire) infections for experiment 1 were carried out very soon after the infected snails (laboratory *B. wrighti*) reached patency (45 days after miracidial infection). Not all snails will start to shed at the same time, and it may have been possible that some of the early shedders were shedding only female cercariae. Frandsen (1977) reported that snails shedding only female *S. intercalatum* (Zaire) cercariae shed for only 32 days, whereas snails shedding only male cercariae shed for up to 119 days. Therefore, by the time the *S. intercalatum* (Zaire) infections for experiments 2 and 3 were carried out (over one month later), some infected snails may produce fewer female cercariae, hence restoring the usual sex balance of more males to females.

However, it should be noted that there is some debate on whether male cercariae have a shorter prepatent period in the snail host than females: Male cercariae of an Iranian and a Mauritian strain of *S. haematobium* matured faster in *Bulinus* than female cercariae (Wright & Bennett, 1967; Wright & Knowles, 1972), whereas no difference was found between the maturation time of male and female cercariae of *S. intercalatum* (both strains) (Frandsen, 1977); and of *S. mansoni* (Liberatos, 1987).

In this model of mating interactions, several multiple-worm pairs were recovered. In experiment 1 the 3 multiple worm pairs recovered in total all consisted of 1 male paired with 2 homospecific females. It would appear that in pairing with 2 *S. mansoni* females, 1 *S. mansoni* male outcompeted 10 and 2 unpaired *S. mansoni* males in mice 4 and 5 respectively. In mouse 6, 1 *S. intercalatum* (Zaire) male outcompeted 1 other *S. intercalatum* (Zaire) male in pairing with 2 *S. intercalatum* (Zaire) females. It is likely that the outcompeted *S. intercalatum* (Zaire) male is the male partner of the single heterospecific *S. intercalatum* (Zaire) ♂ x *S. mansoni* ♀ pair recovered from mouse 6: i.e. it had managed to re-pair with a surplus *S. mansoni* female. All 3 multiple-worm pairs from experiment 1 demonstrate intraspecific male-male competition for females, and it is apparent that both *S. mansoni* and *S. intercalatum* (Zaire) males are able to pair with multiple females.

In experiment 2, where *S. mansoni* reached patency in mice 2 weeks before *S. intercalatum* (Zaire), 1 multiple worm pair was recovered from mouse 1, consisting of an *S. intercalatum* (Zaire) male paired with 1 female of each species. This pairing may have arisen by a homospecifically paired *S. intercalatum* (Zaire) male pairing with an additional *S. mansoni* female, outcompeting 5 unpaired *S. mansoni* males in the process and demonstrating the involvement of interspecific male-male competition in multiple pairings.

Tchuem Tchuenté *et al.* (1993) obtained *S. intercalatum* (Lower Guinea) males paired with 2-3 females of *S. intercalatum* (Lower Guinea) or *S. mansoni* in experiments where *S. mansoni* males were absent. Therefore it appears that both strains of *S. intercalatum* are able to pair homospecifically or heterospecifically with more than one female.

That multiple pairings are not reported more frequently from models of mating interactions between different species of schistosome may in part be due to the fact that multiple pairs could easily become separated when recovering worms from rodents by perfusion so that one or more of the participant worms is classified as unpaired.

Statistical analysis of data from experiment 2 shows that *S. mansoni* males are significantly better at pairing than *S. intercalatum* (Zaire) males. This might be expected, given that homospecific *S. mansoni* pairing would have taken place initially in the absence of competition from *S. intercalatum* males. Also, overall, *S. mansoni* males were better than *S. intercalatum* males at pairing with *S. mansoni* females. Again, this is as expected since the pairing of species 2 (*S. intercalatum*) males with species 1 (*S. mansoni*) females is a much more active process involving the males pulling away homospecifically paired species 1 females from their partners (except for where there is an excess of *S. mansoni* females over *S. mansoni* males as for one *S. intercalatum* ♂ x *S. mansoni* ♀ pair in mouse 9). That several *S. intercalatum* ♂ x *S. mansoni* ♀ pairs were recovered, however, shows that *S. intercalatum* males had the capability to "actively compete" for females in this manner. It also indicates that *S. intercalatum* (Zaire) males may be more competitive than *S. intercalatum* (Lower Guinea) males, as in experiment 7 of the Tchuem Tchuenté *et al.* (1993) study, mice infected with male and female *S. mansoni* and male *S. intercalatum* (Lower Guinea) yielded only homospecific *S. mansoni* ♂ x *S. mansoni* ♀pairs.

It should be noted that in sequential infections (experiments 2 and 3), the number of males of the second infection paired with females of the first infection may have been higher had the worms been perfused from animals more than 8 weeks post-reinfection, since Tchuem Tchuenté *et al.* (1995) described the phenomenon of change of mate 'as a progressive process, requiring up to 8 weeks' to happen. However, in these experiments, it was not possible to delay the culling of mice any longer due to the pathogenicity of the worms.

Because the *S. mansoni* infection in this experiment was male-biased (1.3:1 males:females), it can be assumed that the excess of unpaired *S. mansoni* males competed with unpaired *S. intercalatum* (Zaire) males for *S. intercalatum* (Zaire) females. Neither types of pairing involve "active competition" of the kind described above, instead involving "passive" heterospecific pairing by *S. mansoni* males, and "passive" homospecific pairing by *S. intercalatum* (Zaire) males. Overall, *S. intercalatum* (Zaire) males were significantly better at pairing with *S. intercalatum* (Zaire) females.

Experiment 6 in the Tchuem Tchuenté *et al.* (1993) study, using mice infected with males of both species but with only *S. intercalatum* (Lower Guinea) females, provides a directly comparable test of the relative abilities of males to pair with with *S. intercalatum* (Lower Guinea) females. No significant difference in this ability was found, again indicating that the Zaire strain of *S. intercalatum* is slightly

more competitive in its interactions with *S. mansoni* than the Lower Guinea strain: *S. mansoni* males are outcompeted for *S. intercalatum* (Zaire) females by *S. intercalatum* (Zaire) males, but is equally good as *S. intercalatum* (Lower Guinea) males at pairing with *S. intercalatum* (Lower Guinea) females.

Statistical analysis of data from experiment 3, where *S. intercalatum* (Zaire) was the first infection and commenced pairing three weeks before *S. mansoni* was ready to do so. showed that there was no significant difference in the ability of males of both species to form pairs. In other words, when *S. intercalatum* (Zaire) has the advantage of being the first species to enter the host, it is only as good as *S. mansoni* at forming pairs, whereas when *S. mansoni* was the initial infection (experiment 2) it was significantly better at pairing than *S. intercalatum* (Zaire) males. Therefore in sequential infections, *S. mansoni* males appear to have the competitive edge over *S. intercalatum* (Zaire) males with regard to their ability to form pairs.

Despite the recovery in this experiment of 12 *S. mansoni* ♂ x *S. intercalatum* (Zaire) ♀ pairs, 9 of which were due to *S. mansoni* males actively pulling away paired *S. intercalatum* (Zaire) females from their homospecific partners, overall *S. intercalatum* (Zaire) males were significantly better at pairing with *S. intercalatum* (Zaire) females than *S. mansoni* males. A comparable test for the relative ability of *S. mansoni* and *S. intercalatum* (Lower Guinea) males to pair with *S. intercalatum* (Lower Guinea) females, involving active competition on the part of the *S. mansoni* males and "passive" competition from the *S. intercalatum* (Lower Guinea) males was carried out by Tchuem Tchuenté *et al.* (1995) using mice infected first with males and females of *S. intercalatum* (Lower Guinea) and later reinfected with *S. mansoni* males. Surprisingly, the experiment revealed *S. mansoni* males as significantly better able to pair with female *S. intercalatum* (Lower Guinea) than *S. intercalatum* (Lower Guinea) males: an as yet unique example of "active" competition winning over more "passive" competition to a statistically significant extent.

The sex ratio of *S. intercalatum* (Zaire) was male-biased in experiment 3 (1.7:1 males:females), so that excess unpaired *S. intercalatum* (Zaire) males would have been available for "passive" competition with *S. mansoni* males for *S. mansoni* females. Statistical analysis of the data revealed that overall *S. mansoni* males were better at pairing with *S. mansoni* females than were *S. intercalatum* (Zaire) males. Tchuem Tchuenté *et al.* (1993) carried out a comparable test for the relative ability of males to pair with *S. mansoni* females using mice infected with males of *S. intercalatum* (Lower Guinea) and *S.*

mansoni but with only *S. mansoni* females. A similar result was obtained: male *S. mansoni* were better at pairing with female *S. mansoni* than male *S. intercalatum* (Lower Guinea).

As seen with the sequential infections in the *S. margrebowiei/S. mansoni* model (Cosgrove & Southgate, 2002), the worm return of each species in sequential infections can be used to determine whether the initial infection induces immunological cross-protection against reinfection by the second species. In experiment 2, with *S. mansoni* as the initial infection the worm return of *S. mansoni* is 22.4% whilst that of *S. intercalatum* (Zaire) is 14.4%, which is 64.3% of the *S. mansoni* return. In experiment 3, with *S. intercalatum* (Zaire) as the initial infection. the worm return of *S. intercalatum* (Zaire) is 20.8% whilst that of *S. mansoni* is 15.3%, which is 73.6% of the *S. intercalatum* (Zaire) return. Therefore there does appear to be a similar degree of heterologous cross-protection induced by the initial infection against the second infection, in both situations. This differs from the results of Tchuem Tchuenté *et al.* (1996) which found that the cross-protection induced by *S. mansoni* against *S. intercalatum* (Lower Guinea) was not reciprocal, thus highlighting a possibly important immunological difference between the Zaire and Lower Guinea strains of *S. intercalatum*. However, when comparing degrees of cross-protection in experiments 2 and 3, it should be noted that the time interval between the initial and challenge infections differed (being 1 week and 4 weeks, respectively), which may have resulted in differing levels of immunity. As with the heterologous immunity observed in the Tchuem Tchuenté *et al.* (1996) study and the *S. margrebowiei/S. mansoni* model of Cosgrove & Southgate (2002), the cross-protection observed in experiments 2 and 3 is likely to result from the immunization process itself rather than to egg-induced immunity, because the first infection would not have reached patency by the time of reinfection with the second species in either of the experiments.

When comparing the results of this present study (using the Zaire strain of *S. intercalatum*) with those of Tchuem Tchuenté *et al.* (1993, 1995) (using the Lower Guinea strain) it appears that *S. mansoni* is not as competitively dominant to the Zaire strain of *S. intercalatum* as it is to the Lower Guinea strain, as evinced by observations that the homospecific mate preference of *S. mansoni* is stronger than that of *S. intercalatum* (Lower Guinea) but weaker than that of *S. intercalatum* (Zaire); that *S. mansoni* males are equally good as *S. intercalatum* (Lower Guinea) males but worse than *S. intercalatum* (Zaire) males both at pairing in simultaneous infections and pairing with *S. intercalatum* females in sequential experiments with *S. mansoni* as the initial infection; and that *S. mansoni* males are better than *S. intercalatum* (Lower Guinea) males but worse than *S. intercalatum* (Zaire) males at pairing with *S. intercalatum* females in simultaneous infections with *S. intercalatum* as the initial infection. Indeed, in

this present study, *S. intercalatum* (Zaire) appears to be competitively dominant to *S. mansoni* in simultaneous infections (experiment 1) by having a stronger homospecific mate preference and greater pairing ability, although the aforementioned female-bias of the *S. intercalatum* (Zaire) infection in this experiment casts doubt on the validity of these results.

However, it is experiment 2, with *S. mansoni* as the initial infection, which most closely resembles the situation in nature, that of *S. intercalatum* encountering pre-established infections of *S. mansoni* in its attempt to spread. The competitive dominance of *S. mansoni* in this situation was demonstrated by its greater ability to form pairs and its ability to retain homospecific pairing by outcompeting *S. intercalatum* males for *S. mansoni* females. In the light of this, then, it can be suggested that the competitive dominance of *S. mansoni* might be a contributory factor to the restricted distribution of the Zaire strain of *S. intercalatum*.

Given the above, it might be predicted that *S. intercalatum* (Zaire) would be competitively dominant to *S. intercalatum* (Lower Guinea) in mixed infections of the two. Interestingly, a recent study by Pagès *et al.* (2001 b) found mating between the two strains to be random, with no specific mate preference system in mice infected simultaneously with both strains. In contrast to many species within the *S. haematobium* group which have assortative mating as a pre-zygotic mating barrier but lack the post-zygotic mating barrier of hybrid infertility, there appears to be no assortative mating barrier between the strains of *S. intercalatum* but hybrids between the two strains are of reduced viability in the F_1 generation, and are sterile beyond the F_2 generation (Frandsen, 1978; Pagès *et al.*, 2001 a). Pagès *et al.* (2001 b) note that strong assortative mating tends to evolve when there are strong contact zones between different species, and therefore the geographical isolation of the Zaire and Lower Guinea strains of *S. intercalatum*, not usually so marked for other species of the *S. haematobium* group, can explain why the post-zygotic barrier of hybrid infertility is not reinforced by strong homospecific mate preference.

However, this lack of assortative mating does not exclude the possibility of one of the strains being more competitive at pairing than the other, since in the *S. haematobium*/ *S. intercalatum* (Lower Guinea) mating model (Southgate *et al.*, 1982), mating was similarly random with no specific mate preference system, yet *S. haematobium* was competitively dominant to *S. intercalatum* (Lower Guinea) in terms of pairing ability.

If the Zaire strain is indeed more competitive than the Lower Guinea strain, this then begs the question as to why the Zaire strain is restricted to the Democratic Republic of Congo whilst the Lower Guinea strain is more widespread. One important reason suggested by Tchuem Tchuenté *et al.* (1997) concerns the poor compatibility of *S. intercalatum* (Zaire) with many potential intermediate hosts (Wright *et al.*, 1972; Frandsen, 1979 c) in contrast to *S. intercalatum* (Lower Guinea). Apart from *B. wrighti*, which is a universal host for all schistosomes within the *S. haematobium* group (Wright & Knowles, 1972; Wright *et al.*, 1972; Southgate & Knowles, 1975; Frandsen 1979 a, 1979 b, 1979 c) *S. intercalatum* (Zaire) is only compatible with a very few isolates of *B. globosus*: for example, those from Zaire and Zambia, but not those from Cameroon, Togo, and the Ivory Coast (Tchuem Tchuenté *et al.*, 1997; Frandsen *et al.*, 1978). In addition, factors responsible for the reduction in prevalence (from 30% to less than 4% as reported by Tchuem Tchuenté *et al.*, 1997 a) of *S. intercalatum* (Zaire) in the Kinshasa focus between 1987 and 1997 may play a role in restricting the overall distribution of the Zaire strain. For example, these authors found evidence of natural hybridisation between *S. intercalatum* (Zaire) and *S. haematobium* in the area causing a decline in the transmission of pure *S. intercalatum* (Zaire). They also suggest that the intermediate host of the Zaire strain, *B. globosus*, may have been disappearing from the area, perhaps in the manner of *Biomphalaria camerunensis* in the early 1980s which led to the decline of *S. mansoni* transmission in the region in spite of the replacement of *B. camerunensis* by *B. pfeifferi*, another potential snail host for *S. mansoni* (De Clercq, 1987). For this reason, the reduced prevalence of *S. intercalatum* (Zaire) in Kinshasa cannot be blamed on its interactions with *S. mansoni*. Other factors implicated in the regional decline of the Zaire strain include the increasing socio-economic status of the human population and sanitation improvements such as the provision of piped water reducing the extent of water contact by humans (Tchuem Tchuenté *et al.*, 1997 a).

In summary, the mating model presented here describes further differences between the Zaire and Lower Guinea strains of *S. intercalatum*, and allows the prediction that the Zaire strain may be competitively dominant to the Lower Guinea strain to be made. There is evidence to suggest that *S. mansoni* may hinder the spread of *S. intercalatum* (Zaire) to areas where *S. mansoni* is already present both through its competitiveness at mating and by the induction of immunological cross-protection against *S. intercalatum* (Zaire), but to a lesser extent than for *S. intercalatum* (Lower Guinea). Nevertheless, *S. intercalatum* (Zaire) is more restricted in its distribution than *S. intercalatum* (Lower Guinea), which highlights the great importance of setting observations from laboratory models of

mating interactions in a wider context incorporating other factors and influences on the transmission of schistosomes in the field.

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Table Captions

Table 1. Data from mice infected first with 150 cercariae of the Zaire strain of *S. intercalatum* (Int), and 10 days later with 150 cercariae of *S. mansoni* (Ms) (so that both species reach patency simultaneously).

Table 2. Data from mice infected first with 150 cercariae of *S. mansoni* and 1 week later with 150 cercariae of *S. intercalatum*, Zaire (so that *S. mansoni* reaches patency 2 weeks before *S. intercalatum*).

Table 3. Data from mice infected first with 150 cercariae of *S. intercalatum*, and 4 weeks later with 150 cercariae of *S. mansoni* (so that *S. intercalatum* reaches patency 3 weeks before *S. mansoni*).

Table 1.

Mouse	Ms♂	Int♂	Ms♂xMs♀	Int♂xInt♀	Ms♂xInt♀	Int♂xMs♀	Multiple pairings (♂:♀)
1	5	1	7	5	8	0	
2	4	0	13	17	14	0	
3	4	0	21	8	6	0	
4	10	0	7	7	3	0	1Ms : 2Ms
5	2	0	10	12	2	0	1Ms : 2Ms
6	0	0	11	15	0	1	1Int : 2Int
7	2	0	7	12	20	7	
8	8	2	7	6	10	2	
9	0	0	8	4	4	2	
10	6	3	4	15	10	5	
Total:	**41**	**6**	**95**	**101**	**77**	**17**	

Table 2.

Mouse	Ms♂	Int♂	Ms♂xMs♀	Int♂xInt♀	Ms♂xInt♀	Int♂xMs♀	Multiple pairings (♂:♀)
1	5	0	10	9	0	1	1 Int: 1 Int + 1 Ms
2	0	1	9	7	7	0	
3	2	2	14	5	10	2	
4	1	1	14	6	0	0	
5	4	13	17	13	1	3	
6	1	0	11	8	0	0	
7	4	0	16	8	10	1	
8	4	7	14	0	4	0	
9	1	1	15	7	0	2	
Total:	**22**	**25**	**120**	**63**	**32**	**9**	

Table 3.

Mouse	Ms♂	Int♂	Ms♂xMs♀	Int♂xInt♀	Ms♂xInt♀	Int♂xMs♀
1	1	11	2	5	0	7
2	1	0	9	1	4	1
3	0	10	6	27	3	2
4	1	0	8	11	2	2
5	6	0	5	20	0	0
6	3	3	7	6	1	1
7	4	3	15	14	0	6
8	1	8	9	8	1	1
9	5	17	9	8	1	10
10	2	1	8	5	0	7
Total:	**24**	**53**	**78**	**105**	**12**	**37**