Sex determination in *Strongyloides ratti*.

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ABSTRACT

The mechanism of sex determination and the developmental control of the life-cycle of the gastro-intestinal parasite, *Strongyloides ratti* was investigated using a combination of genetic and parasitological techniques. Parasitological analysis investigated the effects of intra- and extra-host factors on the development of the free-living phase of the life-cycle. An initial analysis of the distribution of infective stages among host faecal pellets showed that they were significantly overdispersed and well described by the negative binomial distribution. This overdispersion was found to occur over a wide range of infection intensities and to increase significantly during infection.

Further investigation, coupled with an artificial selection experiment, suggested the existence of two discrete developmental switches; an intra-host sex determination switch and an extra-host free-living female/directly developing iL3 developmental conversion. Analysis of the effects of host immunity on these developmental switches clarified the way in which the composition of the free-living phase varies over the course of an infection. Increasing host immunity results in a greater proportion of female larvae developing into free-living females rather than into directly developing iL3s. Further evidence suggested that the increase in the proportion of female larvae that develop into free-living females with increased host immunity is due to an increased temperature sensitivity of the free-living female/directly developing iL3 developmental conversion. Increasing host immunity also alters the sex ratio, resulting in a greater proportion of larvae developing into free-living males. In addition, increased parasitic female age appears to increases the proportion of larvae that develop into free-living males, but does not increase the proportion of female larvae that develop into free-living females.

Molecular genetic analysis of the free-living males and free-living females allowed the isolation of a number of putatively sex-linked markers. The sex-linkage of two of these, *Sr-mvP1* and *Sr-mvP2*, was confirmed by semi-quantitative PCR. A restriction fragment length polymorphism (RFLP) was subsequently identified in *Sr-mvP1*. Analysis of the number of alleles of this RFLP present within each stage of the life-cycle demonstrated that there is a consistent genetic difference between the free-living males and the free-living females. This is consistent with the hypothesised XX/X0 system of sex determination in *S. ratti*. Under this scenario, the intra-host sex determination switch occurs between X0 males and XX females. The extra-host free-living female/directly developing iL3 developmental conversion is thus a switch between alternative XX morphs.

Analysis of the inheritance of alleles of *Sr-mvP1* in the progeny of controlled crosses between free-living adults, and those of naturally mated free-living females, demonstrated that all progeny inherited the paternal X chromosome. In addition, it was further shown that all life-cycle stages, with the exception of the free-living males are XX, and thus genetically female. Sequence analysis of the two sex-linked markers and other molecules screened for sex-linkage suggested that a number were related to transposable elements. Further analysis indicated that one of these, *Sr-mvP2*, is present in multiple copies within the *S. ratti* genome. Sequence analysis of *Sr-mvP2* identified a number of stop codons, suggesting that the sequence represents a non-functional, degenerate element. However, other data suggests that *Sr-mvP2* may be active within the genome.

In conclusion, this thesis has used a combined parasitological and genetic approach to investigate the mechanism of sex determination of *S. ratti*. This understanding now allows a rational view of the *S. ratti* life-cycle to be presented. These findings are discussed in relation to previous studies of *Strongyloides spp.* and further questions raised by this work are discussed.

CHAPTER 1.

General introduction.

1.1 Sex determination

In dioecious species, those with two morphologically distinct sexes, reproduction itself must be preceded by sex determination. For those species that lack the ability to alter their sex during life, sex determination occurs when the developing zygote or embryo commits to development as one or the other sex. This makes sex determination one of the most crucial developmental decisions. The wealth of available information on the mechanisms of sex determination has identified two broad categories into which the majority of species fall. The first is genetic sex determination (GSD), in which the primary sex determination signal is genetic and sex is fixed at fertilisation. In the second category, environmental sex determination (ESD), environmental factors experienced by either the developing zygote or embryo act as the primary sex determination signal (Bull, 1985).

1.1.1 Genetic sex determination (GSD).

GSD systems are characterised by a consistent genetic difference between the sexes, the opposite to ESD systems in which there can be no such difference (Bull, 1985). The best understood of the various GSD systems are those found in the free-living nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and in humans. In both *D. melanogaster* and humans, normal individuals with two X chromosomes (XX) develop into females, and those with a single X chromosome and a single Y chromosome (XY) develop into males (Bridges, 1925; Ford *et al.* 1959; Jacobs & Strong, 1959; Welshons & Russell, 1959). A similar situation exists in *C. elegans*, except that there is no male-specific Y chromosome, with XX individuals developing into hermaphrodites and X0 individuals developing into males (Hodgkin, 1987).

However, there is an important difference between the mechanism of sex determination humans and that found in both *D. melanogaster* and *C. elegans*. In humans, it is the presence or absence of the small, heterochromatic, Y chromosome which determines sex (Ford *et al.* 1959; Jacobs & Strong, 1959; Welshons & Russell, 1959). The presence of a normal Y chromosome acting to force the embryonic gonads to differentiate into testis, which subsequently results in male development (Hacker *et al.* 1995). This form of sex determination is referred to as an XX/XY system, and is widespread in mammals. In contrast to this form of XX/XY system, sex in both *D. melanogaster* and *C. elegans* is determined by the ratio of X chromosomes to sets of autosomes (Parkhurst & Meneely, 1994). In these species, individuals with a single X chromosome and two sets of autosomes (X/A = 0.5) normally develop into males. Those with two X chromosomes and two sets of autosomes (X/A = 1) normally develop into females in *D. melanogaster* or into hermaphrodites in *C. elegans*. This form of sex determination is referred to as an XX/X0 system. Thus, the *D. melanogaster* Y chromosome is unrelated to sex determination, although it is required for male fertility (Bridges, 1925; Parkhurst & Meneely, 1994). Thus, the presence of a Y chromosome is, in itself, not diagnostic of a XX/XY sex determination system, where sex is determined by the presence or absence of the Y chromosome.

XX/XY systems of sex determination are widespread in mammals and are closely related to the mechanism of sex determination that is found in marsupials. A qualitatively similar situation is also found in birds, except that avian sex determination is referred to as a WZ/ZZ system. In this system, the presence of the W chromosome results in female development, with ZZ individuals developing into males. Thus, in both XX/XY and WZ/ZZ systems, sex is determined by the presence or absence of a single chromosome.

In addition to the XX/XY, WZ/ZZ and XX/X0 systems of sex determination outlined above, there are a number of other mechanisms by which GSD can occur, although they are comparatively rare among metazoans. Perhaps the most widespread of these other mechanisms is haplo-diploidy, in which females develop from fertilised eggs and males from unfertilised eggs. This method of sex

determination has been found in several insect groups, monogonant rotifers and the Oxyurid nematodes and is believed to have evolved on only twelve separate occasions (Adamson, 1989). Further examples of GSD mechanisms include the multi-factor systems that have been identified in certain species of fish. A good example of such a system is provided by the southern platyfish, *Xiphophorus maculatus*, in which sex determination is controlled by three commonly occurring factors (Belamy & Queal, 1951; Gordon, 1952). These factors are termed W, X and Y and result in three female genotypes (WX, XX and WY) and two male genotypes (XY and YY) (Belamy & Queal, 1951; Gordon, 1952).

1.1.2 Environmental sex determination (ESD).

The best understood ESD systems are those of reptiles, in which sex is commonly determined by the temperature at which eggs are incubated (Janzen & Paukstis, 1991). ESD has been demonstrated in three of the five major reptile lineages, being universal in the Crocodilians, common in turtles and rare amongst lizards (Bull, 1983). So far, three different relationships between egg incubation temperature and sex ratio have been found in reptiles. Two lizard species and one species of alligator produce females at low temperatures and males at high ones, while in many turtle species this situation is reversed (Bull, 1985). There are also a number of turtle species and a species of crocodile in which males are only produced at intermediate temperatures, with females produced at both high and low temperatures (Bull, 1985). Further examples of ESD systems have been identified in a number of teleost fish species (Francis & Barlow, 1993), for instance the Atlantic Silverside, *Menidia menidia* (Conover *et al.* 1992). In addition, ESD has been demonstrated in a number of nematode species, which will be discussed below.

In contrast to the wealth of genetic information about GSD, relatively little is known about the genes involved in ESD. However, it has recently been observed that incubation temperature in reptiles appears to act on genes coding for steroidogenic enzymes and sex steroid hormone receptors (Crews, 1996). In addition, it has been observed that the sex ratio of turtles can be manipulated both by

temperature and by sex steroid hormones. This suggests that incubation temperature in reptiles serves as the physiological equivalent of the sex steroid hormones of mammals (Crews, 1996).

1.1.3 Reproductive mode and sex determination.

The mechanism of sex determination in a species is intimately related to the reproductive mode (Triantaphyllou & Hirschmann, 1964). At a simplistic level, sex determination is simply the mechanism that allows an individual to 'choose' which sex to develop into, and this 'choice' is limited by the reproductive mode. The method of reproduction will also determine the inheritance of chromosomes, which is therefore also related to sex determination. Methods of reproduction can be divided into two classes, sexual and asexual. In this thesis the term sexual reproduction will be used exclusively to describe reproduction that involves both recombination and segregation at meiosis and the subsequent reconstitution of ploidy at fertilisation (syngamy) (Hughes, 1989). This is often seen as characteristic of species with two separate, morphologically distinct sexes, for example humans. However, many species that reproduce sexually do not fit this pattern, for example the yeast mating types and self fertilising hermaphrodites.

In comparison to sexual species, those that reproduce asexually do so by a far greater variety of methods. These methods can be divided into two groups characterised by the presence or absence of specialised gametes. Asexual agametic reproduction is considered to be the more primitive system, relying as it does on totipotent primordial cells that grow into an exact copy of the parent organism. In contrast, asexual gametic reproduction, or parthenogenesis, normally occurs by the development of unfertilised eggs and is therefore seen as a secondary adaptation of sexual reproduction. The previously discussed haplo-diploid system of sex determination represents a specific form of parthenogenesis termed arrhenotoky (Maynard Smith, 1978). With arrhenotoky, haploid males develop from unfertilised eggs and diploid females from fertilised eggs. However, for the purpose of this thesis, thelytokic parthenogenesis, the development of unfertilised eggs into females, requires further elaboration..

Thelytokic parthenogenetic reproduction commonly takes place by one of two separate methods, apomictic (ameiotic) parthenogenesis or automictic (meiotic) parthenogenesis (Maynard Smith, 1978; Hughes, 1989). In apomictic parthenogenesis, meiosis is suppressed and oogenesis occurs by a single mitotic maturation division (Hughes, 1989) (see Figure 1.1A). Thus, all progeny produced by apomictic parthenogenesis are, barring mutation, genetically identical to their mother. In contrast to apomictic parthenogenesis, automictic parthenogenesis is characterised by the meiotic production of eggs and a mechanism for restoring ploidy. The genetic consequences of automictic parthenogenesis are also more complex and progeny produced are not necessarily genetically different to their mother or siblings. The different genetic consequences of automictic parthenogenesis are dependent on the point at which the restitution of ploidy occurs. If endomitosis, the replication of the chromosome complement without subsequent nuclear division, occurs before oogenesis, then the meiotic process begins with twice the normal chromosome number (Hughes, 1989). If the subsequent formation of bivalents involves the pairing of sister chromosomes, then recombination will occur between identical chromatids and the further meiotic divisions will result in the production of genetically identical progeny (Maynard Smith, 1978; Hughes, 1989). Parthenogenesis of this form is known to occur in whiptail lizards (Cueller, 1971) and is shown in Figure 1.1B.

However, if the restitution of ploidy occurs during meiosis, by the suppression of either the first or second meiotic divisions, then progeny will differ genetically from their mother and siblings (Hughes, 1989) (see Figure 1.2A & B). Genetically dissimilar progeny can also be produced if the meiotic divisions are normal and the diploid chromosome number is restored by either endomitosis or the fusion of the first cleavage nuclei (Maynard Smith, 1978; Hughes, 1989). Thus, to avoid confusion, the terms functionally mitotic parthenogenesis and functionally meiotic parthenogenesis will be used to distinguish between the genetic consequences of the different types of parthenogenetic reproduction. Functionally mitotic parthenogenesis referring to both apomictic parthenogenesis (Figure 1.1A) and the form of automictic parthenogenesis that results in the production of genetically identical progeny (Figure 1.1B). Functionally meiotic parthenogenesis referring to the forms of automictic

parthenogenesis that result in the production of genetically distinct progeny (Figure 1.2A, B & C). Thus, in both cases, the terms functionally mitotic and functionally meiotic are used to denote the genetic consequences and not the mechanism of egg production.

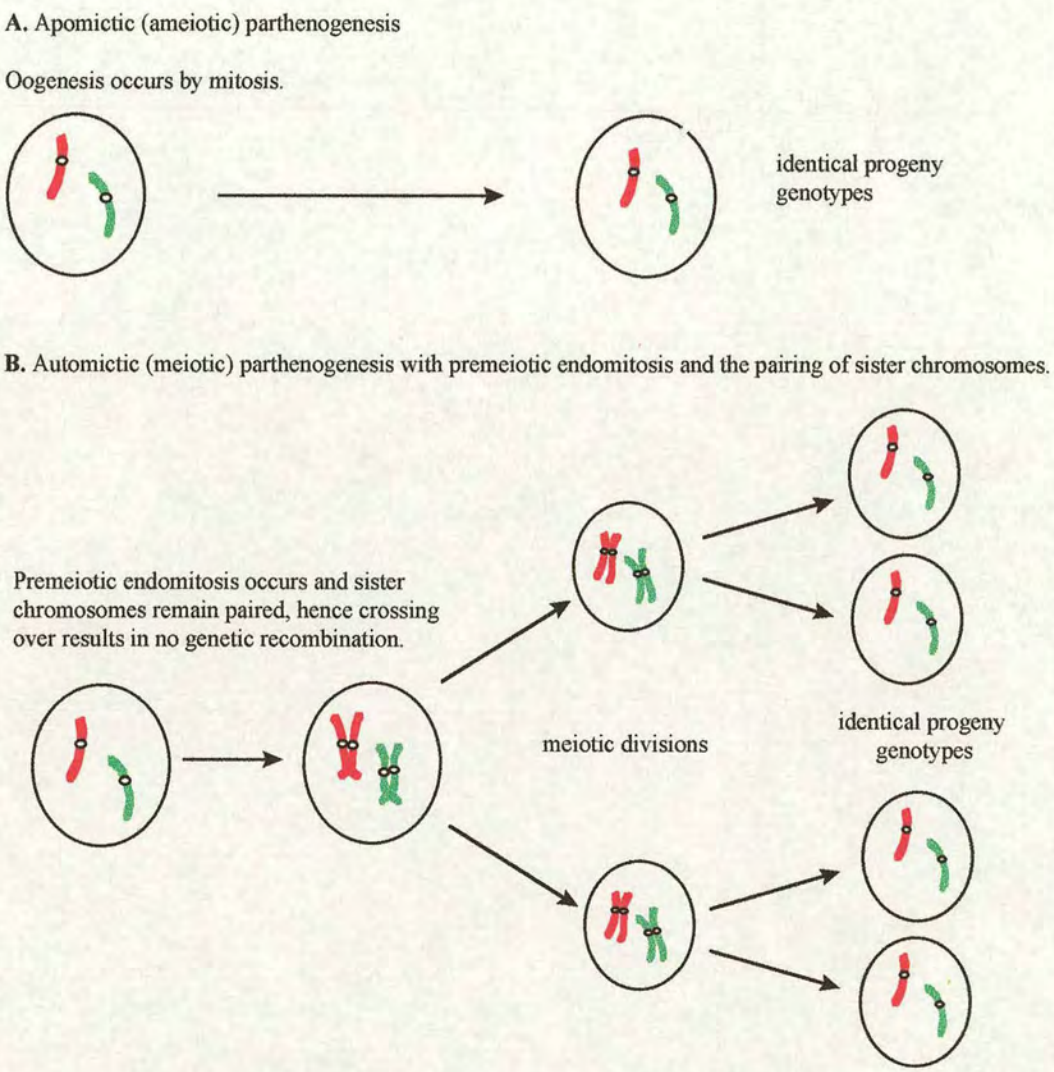
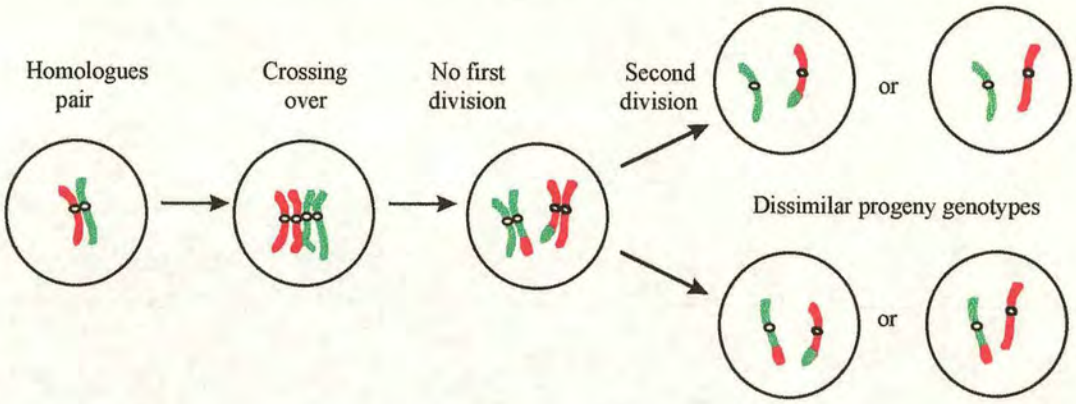


Figure 1.1 Functionally mitotic parthenogenesis, resulting in progeny that are genetically identical. A. Apomictic (ameiotic) parthenogenesis. B. Automictic (meiotic) parthenogenesis, with premeiotic endomitosis and the pairing of sister chromosomes. After Hughes, 1989.

A. Automictic (meiotic) parthenogenesis with suppression of the first meiotic division.



B. Automictic (meiotic) parthenogenesis with suppression of the second meiotic division.

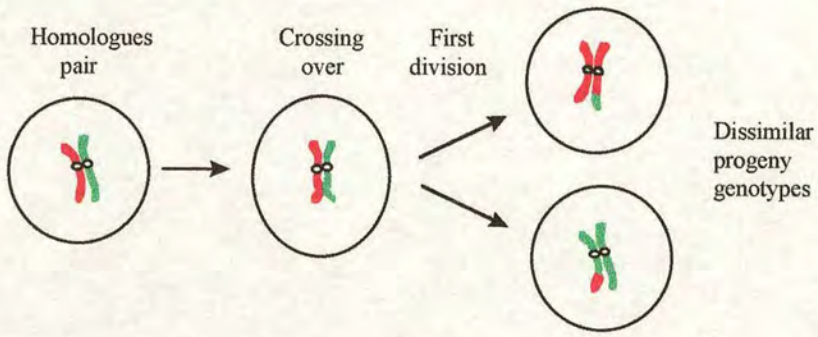


Figure 1.2 Functionally meiotic parthenogenesis, resulting in progeny that are not genetically identical. A. Automictic (meiotic) parthenogenesis with suppression of the first meiotic division. B. Automictic (meiotic) parthenogenesis with suppression of the second meiotic division. After Hughes, 1989.

1.2 *Strongyloides spp.*

Members of the nematode genus *Strongyloides* are intestinal parasites, and have been found in a wide range of mammals, reptiles and birds (Speare, 1989). There are 52 described species of *Strongyloides* (Speare, 1989), the majority of which have no direct or indirect effects on man. The three known natural human parasites are *Strongyloides stercoralis*, *S. fuelleborni fuelleborni* and *S. fuelleborni kellyi* (Viney *et al.* 1991). It has been estimated that over one hundred million people are infected with *S. stercoralis* (Power *et al.* 1994), with *S. fuelleborni* widespread in Africa (Ashford &

Barnish, 1989) and Papua New Guinea (Viney *et al.* 1991). *Strongyloides* infections in man are not normally pathogenic, however the effects are exacerbated in immuno-compromised individuals and can prove fatal (Grove, 1989). There is also increasing evidence that non-pathogenic intestinal helminth infections, such as *Strongyloides spp.*, in children cause what is termed 'failure to thrive' (Chan *et al.* 1994; Hadidjaja *et al.* 1998; Bundy *et al.* 1998). This is characterised by a reduction in growth rate and cognitive function. The long term affects of 'failure to thrive' are less clear, with some studies indicating that cognitive ability improves after removal of the parasites (e.g. Nokes *et al.* 1992a & b) and others indicating that treatment causes little or no improvement in cognitive function (Simeon *et al.* 1995; Watkins *et al.* 1996). In addition to humans, *S. fuelleborni* is capable of infecting a number of Old World primates and is considered to be an almost universal infection of wild primates (Ashford & Barnish, 1989). In contrast, *S. stercoralis* has been only isolated from cats and dogs in the wild (Sandground, 1928), with the latter providing the major experimental model for the species. The second other commonly used experimental model of strongyloidiasis is that of *S. ratti* in both rats and mice.

1.2.1 The life-cycle of *Strongyloides spp.*

The majority of *Strongyloides spp.* share the same, complex life-cycle. This life-cycle consists of both free-living and parasitic phases and is of particular interest due to the developmental plasticity of the free-living phase. The adult parasitic phase is found embedded in the mucosa of the small intestine and is thought to be composed solely of either parthenogenetic females or hermaphrodites. Detailed analysis of these parasitic adults in *S. ratti* (Chitwood & Graham, 1940; Bolla & Roberts, 1968), *S. papillosus* (Triantaphyllou & Moncol, 1977) and *S. ransomi* (Triantaphyllou & Moncol, 1977) found no trace of sperm. Hence, it was concluded that reproduction was parthenogenetic and hereafter, the worms of the parasitic phase are referred to as parasitic females. Parasitic males have been reported in *S. stercoralis* (Kreis, 1932; Faust, 1933), but these reports have largely been dismissed. The progeny of the parasitic females pass with the host faeces into the external environment and either develop through two larval stages into filariform infective third stage larvae (iL3) or through four larval stages to become rhabditiform free-living adult males and free-living adult females. The

development of the larval progeny of the parasitic females into iL3s is known as direct development, homogonic development or asexual development. In contrast, the development of the larval progeny of the parasitic females into free-living males and free-living females is known as indirect development, heterogonic development or sexual development. This generalised life-cycle of *Strongyloides spp.* is shown in Figure 1.3. For clarity, the terms direct and indirect will be used in this thesis to distinguish the developmental routes.

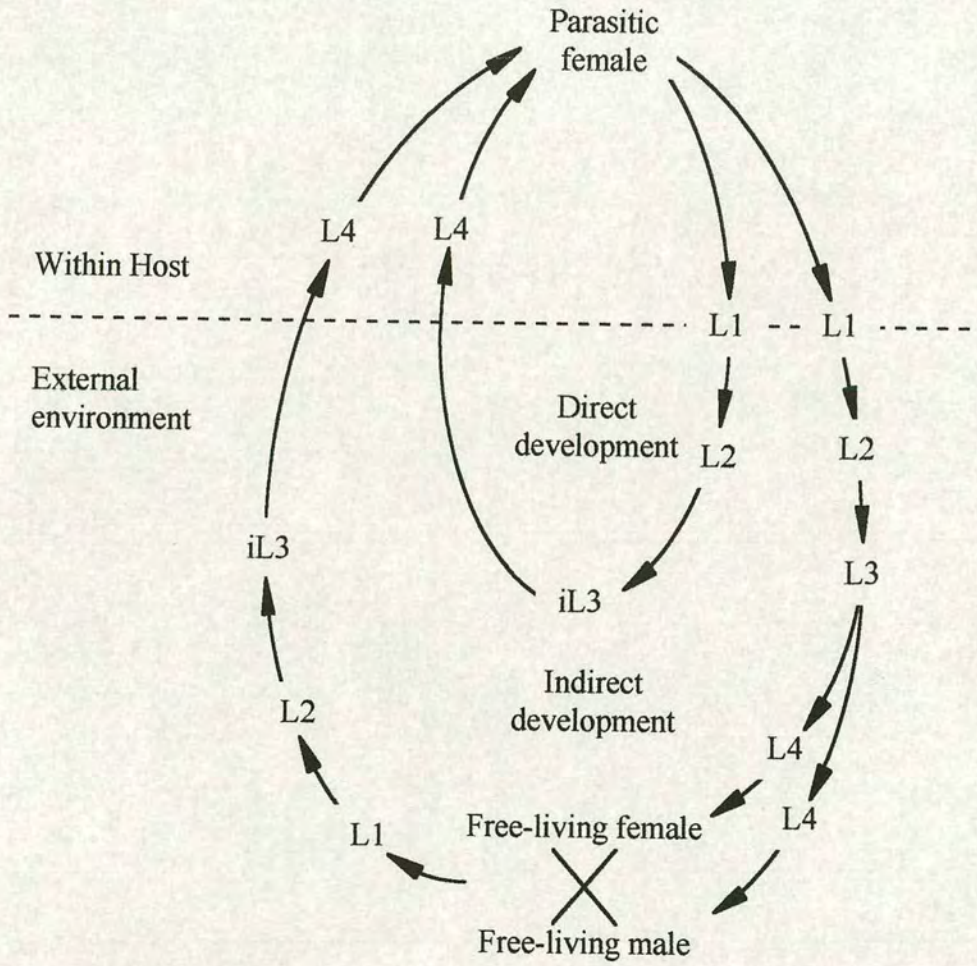


Figure 1.3 The generalised life-cycle of species in the genus *Strongyloides*, showing the two characteristic developmental routes. L denotes larval stage. iL3 denotes the filariform infective third stage larvae.

The free-living adults reproduce in the external environment and all of their progeny subsequently develop through two larval stages into iL3s. These iL3 progeny of the free-living adults are morphologically indistinguishable from the directly developing iL3 progeny of the parasitic females. Therefore, to avoid confusion, the iL3 progeny of the parasitic females will be referred to as 'directly developing iL3s' and those of the free-living adults as 'indirectly developing iL3s.' Both the directly and indirectly developing iL3s must subsequently locate a suitable host before continuing development. After a host has been infected, larvae migrate from the site of infection to the host small intestine, where they subsequently moult twice more, completing development as parasitic females. The main route of infection for the majority of *Strongyloides spp.* is thought to be skin penetration. However, patent infections have been produced from orally administered iL3s of *S. stercoralis* (Sandground, 1928), *S. papillosus* (Mönnig, 1930) and *S. ratti* (Sheldon, 1937d), with this route considered to be the main route of infection for natural populations of *S. papillosus* (Mönnig, 1930).

At present there are only two known exceptions to the typical *Strongyloides spp.* life-cycle (Figure 1.3). The first occurs in *S. planiceps*, in which the progeny of the free-living adults do not all develop into indirectly developing iL3s. Instead, some larvae develop into free-living males and free-living females (Yamada *et al.* 1991). In total, eleven, decreasingly fecund, generations of free-living males and free-living females have been obtained (Yamada *et al.* 1991). The production of further generations of free-living adults has also been reported in *S. simiae* (Beach, 1936), a chimpanzee strain of *Strongyloides* (Augustine, 1940), *S. fuelleborni* (Hanson *et al.* 1969) and in *S. stercoralis* (Attia *et al.* 1981). However, in the majority of these cases it is hard to exclude the possibility that the free-living generations are composed of a separate species of free-living nematode rather than free-living adults of *Strongyloides spp.*. It is indeed possible that the free-living species, *Rhabditis hominis*, which is easily cultured in a variety of media and has previously been diagnosed as *S. stercoralis* is the source of this confusion (Sandground, 1925a). The second exception to the life-cycle shown in Figure 1.3 is found in *S. stercoralis*, where the directly developing iL3 progeny of the parasitic females are capable of completing development within the host intestine and infecting the same host (Schad, 1989). This

phenomenon is termed autoinfection and is the main cause of the pathogenic effects of *S. stercoralis* in humans.

The existence of the two developmental routes in *Strongyloides* and the variation in the life-cycle between different *Strongyloides spp.* has led to speculation that the genus represents a transitional stage between free-living and parasitic life histories. For this reason, the life-cycle of *Strongyloides spp.* has been the subject of much study for nearly a hundred years. However, there is still considerable controversy over aspects of the biology and life history of *Strongyloides spp.*. In particular, the mechanism of sex determination in the genus is not understood and the reproductive mode of the free-living adults is unclear. The current view of the life-cycle is also unable to explain the complex manner in which the proportion of the parasitic female's progeny that develop into directly developing iL3s, free-living males and free-living females change as a result of alterations in the intra- and extra-host environment.

1.2.2 *Strongyloides ratti*

S. ratti was first described in 1925 (Sandground, 1925b) and is a widely distributed natural parasite of rats, with a life-cycle as shown in Figure 1.3. As in other *Strongyloides spp.*, the infective larvae of *S. ratti* penetrate the skin of the host and migrate through the tissues to the intestine. Oral infection of rats with *S. ratti* iL3s is possible but, in comparison to iL3s allowed to penetrate the skin, a much smaller fraction of the infective dose can be recovered from the small intestine (Sheldon 1937c). It is therefore likely that the main route of infection in natural populations is via skin penetration. Until recent years, it had been assumed that *Strongyloides spp.* migrated along the blood-lung route before reaching the intestine (Abadie, 1963). However, in a primary infection of *S. ratti*, it has been shown that the main migration route of the developing larvae is via the head and nasal areas (Tindall & Wilson, 1988; Bhopale *et al.* 1992). The first worms appear in the mucosa of the host small intestine approximately 34-36 hours after infection (Wertheim & Lengy, 1965; Tindall & Wilson, 1990), with the first eggs and larvae appearing in host faeces three to four days after infection (Sheldon, 1937a).

In laboratory infections of *S. ratti*, the number of parasitic females in the host intestine first rises to a peak and then declines with increasing time post infection (p.i.), with the majority of worms lost four to six weeks after infection. However, this rate of parasitic female loss appears to be dependent on the size of the initial infection; as infections with single iL3s are known to be patent until at least 7 months p.i. (Graham, 1939). Auto-infection, as occurs in *S. stercoralis*, has never been recorded for *S. ratti* although evidence of its occurrence has been sought (Carter & Wilson, 1989). In laboratory infections, it has been shown that only a small fraction of iL3s complete development, with between 21 and 27% of the infective dose reaching the small intestine and developing into parasitic females (Sheldon, 1937b; Moqbel & Wakelin, 1981; Dawkins & Grove, 1981). For single, directly developing iL3s, allowed to penetrate the skin, the proportion of patent infections produced varies between 18 and 32% (Graham, 1936 & 1938).

1.2.3 Sex determination in Strongyloides spp.

All work on the mechanisms of sex determination in *Strongyloides spp.* has been cytological and suggests that sex determination is chromosomal. Most studies have concluded that the free-living males and free-living females of a species have different chromosome numbers. The most extensive cytological observations have been made for *S. papillosus*, but *S. ratti*, *S. stercoralis* and *S. ransomi* have also been analysed in this manner. The conclusions of these studies are summarised in Table 1.1. Many of the observations that have been made of *S. papillosus* are contradictory, with different studies reaching different conclusions (Table 1.1). Initial work indicated that the parasitic females were triploid, with six chromosomes, and produced two types of eggs with different chromosome complements (Chang & Graham, 1957). Those eggs with two chromosomes were believed to develop into free-living males and those with four chromosomes into free-living females. Reproduction by the free-living adults was therefore assumed to involve the fusion of the free-living females' diploid pronuclei with the free-living males' haploid pronuclei, producing triploid zygotes that develop into indirectly developing iL3s and subsequently into parasitic females (Chang & Graham, 1957). However,

this work was not supported by later studies that found no chromosomal differences between the sexes (Zaffagnini, 1973; Triantaphyllou & Moncol, 1977). These studies identified two pairs of chromosomes, one pair being distinctly longer than the second, giving a karyotype of two long and two medium chromosomes (2L2M) (Zaffagnini, 1973; Triantaphyllou & Moncol, 1977).

Species	2n chromosome complement			Reference
	Parasitic female	F-l female	F-l male	
S. papillosus	6	4	2	Chang & Graham, (1957)
S. papillosus	4	4	-	Zaffagnini (1973)
S. papillosus	4	4	4	Triantaphyllou & Moncol (1977)
S. papillosus	4	4	5	Albertson *et al.* (1979)
S. stercoralis	6	6	5	Hammond & Robinson (1994)
S. ransomi	4	4	4	Triantaphyllou & Moncol (1977)
S. ratti	6	6	5	Nigon & Roman (1952)
S. ratti	6	6	5	Abe & Tanaka (1965)
S. ratti	6	6	5	Bolla & Roberts (1968)

Table 1.1 The 2n chromosome complement of the parasitic female, free-living female (F-l female) and free-living male (F-l male) life-cycle stages of the *Strongyloides spp.* in which the mechanism of sex determination has been analysed cytologically. - indicates that no observations were made.

A further study of the eggs of *S. papillosus* parasitic females demonstrated two distinct karyotypes (Albertson *et al.* 1979). The first of these was the same as that previously described, 2L2M (Zaffagnini, 1973; Triantaphyllou & Moncol, 1977), and the second was of a single long chromosome, three medium chromosomes and one short chromosome (L3MS) (Albertson *et al.* 1979). It was proposed that this difference in the oocyte karyotype resulted from chromatin diminution of one of the long chromosomes, with the central portion being deleted to form the third medium and the short chromosome (Albertson *et al.* 1979). In animals infected with a number of parasitic females, it was

found that about half (47%) of the eggs had the L3MS karyotype and that similar percentage of the progeny developed into free-living males. Thus, it was concluded that eggs with the L3MS karyotype developed into free-living males. It was subsequently shown that individual parasitic females produced only a single type of egg, either 2L2M or L3MS, but never both. Thus, the most recent karyotype proposed for *S. papillosus* is that the free-living males are 2n=5 and both the parasitic and free-living females are 2n=4 (Albertson *et al.* 1979).

As in *S. papillosus,* the diploid chromosome number of both the parasitic and free-living females of *S. ransomi* is thought to be four (Triantaphyllou & Moncol, 1977). However, it is not known if a second karyotype, analogous to the L3MS karyotype of *S. papillosus,* exists in S. *ransomi.* In contrast, the diploid chromosome number of *S. ratti* free-living females of is believed to be six and that of the free-living males five (Nigon & Roman, 1952; Abe & Tanaka, 1965; Bolla & Roberts, 1968). Cytological studies of *S. stercoralis* have produced similar results to those of *S. ratti,* concluding that the diploid chromosome number of the free-living females is six and that of the males five (Hammond & Robinson, 1994).

The general conclusion of these cytological studies is that sex in *Strongyloides spp.*, is determined by a chromosomally based GSD system. As can be seen from Table 1.1, all of the cytological studies, except that by Triantaphyllou and Moncol (1977), have concluded that there is a chromosomal difference between the free-living males and free-living females. It has further been suggested that sex determination in *S. ratti* and *S. stercoralis* is an XX/X0 system (Triantaphyllou & Moncol, 1977). The 2L2M karyotype of *S. papillosus* is also postulated to be the result of an X chromosome to autosome translocation in such an XX/X0 system (Triantaphyllou & Moncol, 1977). Therefore, the elimination of the X chromosome portion from one of the long chromosomes would result in an egg of the L3MS karyotype that was functionally X0 and which would develop into a free-living male. These conclusions would suggest that sex determination in *Strongyloides spp.* is similar to that in *C. elegans*, where the hermaphrodites are XX and the males X0 (Hodgkin, 1987).

1.2.4 Reproduction in Strongyloides spp.

In common with the studies of sex determination, the majority of the studies of reproductive mechanisms in *Strongyloides spp.* have also been cytological. To date, *S. ratti* is the only species in which reproduction has been analysed using genetic techniques (Viney *et al.* 1993; Viney, 1994). This has lead to a clear consensus about reproduction in the parasitic phase of *Strongyloides*, with both cytological (Chitwood & Graham, 1940; Nigon & Roman, 1952; Bolla & Roberts, 1968) and genetic analyses (Viney, 1994) supporting the same conclusion. However, there is still considerable controversy over the way in which the free-living adults reproduce, since the genetic analysis of reproduction in *S. ratti* (Viney *et al.* 1993) directly conflicts with the cytological studies on this species (Nigon & Roman, 1952; Bolla & Roberts, 1968).

As previously mentioned, the parasitic phase of all *Strongyloides spp.* is believed to be female only and is believed to reproduce by parthenogenesis (Chitwood & Graham, 1940; Bolla & Roberts, 1968; Triantaphyllou & Moncol, 1977). This conclusion is supported by a genetic analysis of reproduction in the parasitic females of *S. ratti* (Viney 1994). This demonstrated that parasitic females, heterozygous for a restriction fragment length polymorphism (RFLP), produced progeny that were also all heterozygous for this RFLP (Viney, 1994). Thus, there was no allelic segregation and all progeny were genetically identical both to their mother and to their siblings. From this it was concluded that reproduction by the parasitic females is functionally mitotic (Viney, 1994).

The cytological studies of *Strongyloides spp.* have nearly all concluded that reproduction in the free-living adults occurs primarily by meiotic parthenogenesis and pseudogamy (Nigon & Roman, 1952; Triantaphyllou & Moncol, 1977; Albertson *et al.* 1979; Hammond & Robinson, 1994). In pseudogamy, sperm are only required to activate the eggs and the sperm nucleus does not fuse with that of the egg. Therefore, in pseudogamous reproduction, true fertilisation does not occur and the free-living males have no genetic input in the next generation. However, is has been acknowledged in studies of *S. ratti* (Nigon & Roman, 1952), *S. papillosus* (Zaffagnini, 1973) and *S. stercoralis*

(Hammond & Robinson, 1994), that the occurrence of rare fertilisation could not be excluded. Further support for the idea that reproduction is pseudogamous comes from the observation that the free-living adults of *S. ransomi* and those of *S. papillosus* can mate and produce viable progeny (Triantaphyllou & Moncol, 1977). This suggests that either reproduction is pseudogamous and that the egg activating mechanism is conserved between species, or that the *S. ransomi* and *S. papillosus* populations used in this study were not in fact separate species. Only a single cytological study of *S. papillosus* has suggested that reproduction in the free-living adults is normally sexual (Chang & Graham, 1957). However, this study did not involve any observations of reproduction between the free-living adults, and the assumption that reproduction was sexual was made purely to rationalise the life-cycle with the observed chromosome numbers in the free-living males, free-living females and parasitic females (Chang & Graham, 1957).

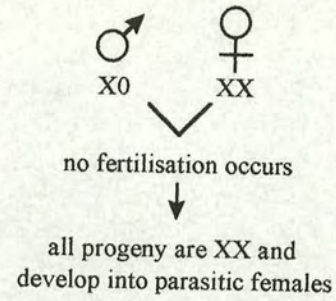
In contrast to the earlier cytological studies, a recent genetic analysis of reproduction between the free-living adults of *S. ratti* has demonstrated that the free-living males can make a genetic contribution to the next generation (Viney *et al.* 1993). This study used genetic fingerprinting to genotype the parents and progeny of crosses between free-living males and free-living females. In crosses between free-living males and free-living females of different parasite lines (unrelated free-living adults), results were consistent with normal sexual reproduction and genetic exchange. However, in crosses between free-living males and free-living females from the same parasite line (related free-living adults) it could only be demonstrated that allelic segregation had occurred, a result consistent with both functionally meiotic parthenogenesis and with sexual reproduction (Viney *et al.* 1993). The first of these findings therefore directly conflicts with the earlier cytological observations that reproduction in the free-living adults is by meiotic parthenogenesis and pseudogamy. Several hypotheses have been put forward to explain this conflict, but none provide very satisfying answers. One possibility is that different *Strongyloides spp.* have different reproductive modes. However, it is unlikely that *S. ratti* is alone among the many *Strongyloides spp.* in reproducing sexually (Viney *et al.* 1993). Alternatively, different strains of *S. ratti* may have different reproductive modes (Hammond & Robinson, 1994), but this is also unlikely as sexual reproduction has been demonstrated between

different parasite lines (Viney *et al.* 1993). A third possibility is that sexual reproduction occurs only between unrelated free-living adults and that related free-living adults reproduce by mitotic parthenogenesis and pseudogamy (Viney *et al.* 1993; Hammond & Robinson, 1994).

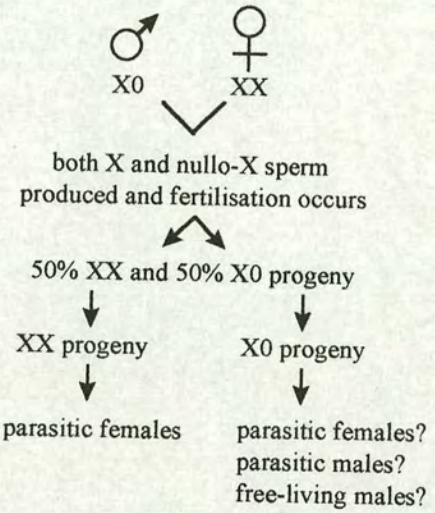
The finding that unrelated free-living adults of *S. ratti* reproduce sexually (Viney *et al.* 1993) raises a further problem. It is not clear how this finding can be reconciled with the conclusions of the cytological studies of this species (Nigon & Roman, 1952; Abe & Tanaka, 1965; Bolla & Roberts, 1968). As previously mentioned, all progeny of *S. ratti* free-living adults are known to develop into indirectly developing iL3s, which are subsequently believed to develop into parasitic females. However, cytological observations of the germline of free-living males have shown the existence of cells that appear to have 2 or 3 chromosomes in both *S. ratti* (Nigon & Roman, 1952) and *S. stercoralis* (Hammond & Robinson, 1994). If, as postulated (Nigon & Roman, 1952; Hammond & Robinson, 1994), these cells are sperm, then the findings are consistent with the Mendelian segregation of X chromosomes in spermatogenesis.

If reproduction does occur by meiotic parthenogenesis and psuedogamy, then there is no problem and all progeny will be XX (Figure 1.4A). However, sexual reproduction between an XX female and an X0 male would be expected to produce equal numbers of XX and X0 progeny (Figure 1.4B). It would then be expected that the genetically male X0 progeny would develop into either free-living males or into parasitic males. As neither second generation free-living males or parasitic males have ever been observed in *S. ratti*, it is unlikely that X0 progeny, if produced, could develop by these routes. In contrast, if the development of an X0 individual into a parasitic female was possible, then it would be expected that all the progeny of that individual would be also X0 and hence develop into free-living males. Again, this phenomenon has not been observed in *S. ratti*, which suggests that none of the progeny of the free-living adults are X0. A similar argument can be made for reproduction between the free-living adults of *S. stercoralis*, and the absence of X0 progeny from matings between free-living males and free-living females has been used to support the idea that reproduction is by meiotic parthenogenesis and pseudogamy (Hammond & Robinson, 1994).

A.
Meiotic parthenogenesis and psuedogamy



B.
Sexual reproduction with both XX and X0 progeny



C.
Sexual reproduction with only XX progeny

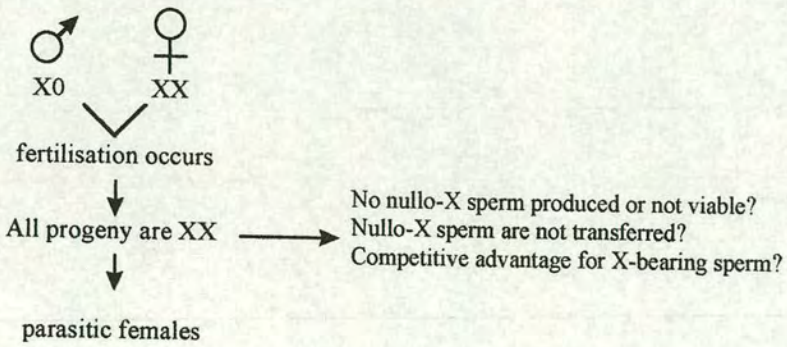


Figure 1.4 Various hypotheses about the method and genetic consequences of reproduction between free-living males and free-living females of *S. ratti*. A. Reproduction is by meiotic parthenogenesis and psuedogamy. B. Reproduction is sexual, with the Mendelian segregation of X chromosomes in spermatogenesis. C. Reproduction is sexual and the majority of progeny are XX.

The development of these putative X0 progeny of the free-living adults may however explain the reports of parasitic males in *S. stercoralis* (Kreis, 1932; Faust, 1933). The development of X0 progeny into parasitic females may also be a possible explanation for the finding that the parasitic females of *S. papillosus* produce only a single type of egg (Albertson *et al.* 1979). Therefore, in addition to the fact that the proposed XX/X0 GSD system has not been proved, both the method by which the free-living adults reproduce and the chromosome complement of their progeny are unclear.

A third option is that all progeny of the free-living adults are XX and do develop into parasitic females (Figure 1.4C). Such a situation could arise by a number of mechanisms. If the reports of cells containing two chromosomes in the male germline (putatively nullo-X) were either incorrect, or represented observations of sperm precursor cells that were not capable of completing development, then all functional sperm would contain an X chromosome. It is also possible, but biologically unlikely, that only sperm containing an X chromosome are transferred from the free-living male to the free-living female. A third possibility is that X-bearing sperm have a competitive advantage over nullo-X sperm and preferentially fertilise the oocytes of the free-living female, as is known to occur in the free-living nematode species *C. briggsae* (LaMunyon & Ward, 1997).

1.2.5 Environmental influences on the Strongyloides spp. *life-cycle*

Development in the free-living phase of *Strongyloides spp.* is, as previously mentioned, commonly viewed as a choice between the direct and indirect developmental routes. However, it is known that the proportion of the parasitic females' progeny that develop into directly developing iL3s, free-living males and free-living females varies in response to environmental factors. These factors can be divided into two classes: those acting within the host on parasitic females and their eggs (intra-host factors); and those acting outside the host on eggs and developing larvae (extra-host factors).

At a general level, the main intra-host factor is the immune status of the host. This has profound effects on both the location of the parasitic females within the host intestine (Moqbel &

Denham, 1977; Uchikawa *et al.* 1989) and on their ultrastructural morphology (Moqbel & McLaren, 1980). In addition, the proportion of the progeny of the parasitic females that develop by the indirect route has been observed to increase over the course of infection in pig *Strongyloides* (Varju, 1966), *S. ransomi* (Moncol & Triantaphyllou, 1978), *S. stercoralis* (Shiwaku *et al.* 1988) and *S. ratti* (Viney *et al.* 1992). Further, it has been noted in the studies of *S. ransomi* (Moncol & Triantaphyllou, 1978) and *S. ratti* (Viney *et al.* 1992) that the proportion of the larval progeny of the parasitic females that developed into free-living males increased over the course of infection. However, when changes that occur in the composition of the free-living phase are considered, it has proved particularly difficult to separate the effects of the host immune status from those of parasitic female age. Only a limited number of studies have experimentally investigated the effect of host immunity on the composition of the free-living phase. A study of *S. stercoralis* in immunosuppressed and normal dogs did not detect any difference in the proportion of larvae that developed by the direct and indirect routes (Shiwaku *et al.* 1988). In contrast, another study demonstrated that immunosuppression results in a decreased proportion of the larval progeny of *S. ratti* parasitic females developing by the indirect route (Gemmill *et al.* 1997).

In contrast to the limited number of intra-host factors that affect the composition of the free-living phase of *Strongyloides spp.*, a large number of extra-host factors have been identified. These factors and their effects on the composition of the free-living phase are shown in Table 1.2. The general conclusion that has been reached by these studies is that development by the indirect route is favoured by harsh intra-host conditions and favourable extra-host conditions (Schad, 1989). However, as can be seen from Table 1.2, the majority of studies that investigated the effect of changes in extra-host factors on the composition of the free-living phase of *Strongyloides spp.* reached similar conclusions. The first of these is that the proportion of larvae that develop into free-living males is unaffected by changes in extra-host factors. Further, it is apparent that changes in extra-host factors that result in an increased proportion of larvae developing into free-living females also result in a decreased proportion of larvae developing into directly developing iL3s (Table 1.2). This suggests that the free-living females and the directly developing iL3s may represent alternative female morphs.

This idea is supported by morphological observations of *S. papillosus* which indicate that larvae that develop into free-living females are indistinguishable from those that develop into directly developing iL3s until the early second larval stage (Albertson *et al.* 1979). At this point, the vulval precursor cells in larvae that develop into free-living females swell prior to cell division (Albertson *et al.* 1979). In contrast, first stage larvae that subsequently develop into free-living males can be differentiated from other newly hatched larvae by the appearance of the B cell posterior to the anus (Albertson *et al.* 1979). In larvae that develop into free-living males, this cell is swollen and the nucleus contains a prominent nucleolus, whereas in larvae that develop either into free-living females or into directly developing iL3s, the cell is insignificant.

Species	Extra-host factor	Effect on the free-living phase *	Reference
S. ratti	Increased CO_2 concentrations	Increased free-living females Decreased directly developing iL3s	Taylor & Weinstein, 1990
S. ratti	Increased fatty acid concentration	Decreased free-living females Increased directly developing iL3s	Minematsu *et al.* 1989
S. ratti	Increased temperature	Increased free-living adults Decreased directly developing iL3s	Viney, 1996
S. stercoralis	Increased temperature and increased faecal dilution	Increased free-living females Decreased directly developing iL3s	Shiwaku *et al.* 1988
S. ransomi	pH below 5.9 or above 7.2	Decreased free-living females Increased directly developing iL3s	Moncol & Triantaphyllou, 1978
S. fuelleborni	Increased pH	Increased free-living females Decreased directly developing iL3s	Premvati, 1958
S. fuelleborni	Decreased O_2 concentration	Increased free-living females Decreased directly developing iL3s	Hanson *et al.* 1975
S. planiceps	Low population density	Increased free-living females Decreased directly developing iL3s	Arizona, 1976
S. papillosus	Increased temperature	Increased free-living females Decreased directly developing iL3s	Nwaorgu, 1983

Table 1.2 Extra-host factors that have been shown to alter the composition of the free-living phase of *Strongyloides spp.* and their effects. * The effect on the composition of the free-living phase is shown as the change in the proportion of larvae that develop into that morph in comparison to the controls.

In *S. ratti*, it has been found that changes in the concentration of fatty acids (Minematsu *et al.* 1989) or the carbon dioxide concentration (Taylor & Weinstein, 1990) do not affect the proportion of larvae that develop into free-living males (Table 1.2). However, these experiments are unsatisfactory since the starting populations of worms comprised only a proportion of the total population. The starting populations in these experiments were derived from either first stage larvae isolated from fresh faeces (Minematsu *et al.* 1989) or eggs isolated from the host gut (Taylor & Weinstein, 1990), but in *S. ratti*, both eggs and newly hatched larvae are passed with the host faeces (Sandground, 1925b). It is therefore possible that analysis of the total worm population would give different results. Another general criticism of the studies summarised in Table 1.2 is that differential mortality is not considered. Thus, as the number of eggs and larvae in the starting population is unknown, it is not clear if the resulting changes in the composition of the free-living phase are a consequence of a developmental switch or of changes in the mortality of the different morphs.

These studies do however suggest that the view of the *Strongyloides spp.* life-cycle as a choice between direct and indirect development (Figure 1.3) may be inaccurate. If this is the case, then the current view of the life-cycle may be acting to obscure the nature of the developmental events or switches that occur within the life-cycle. For example, increased host immunity is known to increase the proportion of the progeny of the parasitic females that develop by the indirect route (Gemmill *et al.* 1997). However, if the free-living females and the directly developing iL3s are alternative female morphs, then consideration of the free-living males and free-living females together would be inappropriate. This is a case as an increase in the proportions of either free-living females or free-living males would cause the proportion of larvae that developed by the indirect route to increase.

If the development of larvae into free-living males is unaffected by extra-host factors then this would be consistent with GSD in the free-living phase of *Strongyloides spp.*, as suggested by the cytological studies. In contrast, if the development of larvae into free-living males is affected by extra-host environmental conditions, then it is likely that sex is determined by an ESD system.

1.3 Sex determination in other nematode species

Parallels between *Strongyloides spp.* and other nematodes can, and have, been drawn. In general, the infective third stage larvae found in many parasitic nematode species have been considered analogous to the developmentally arrested third stage dauer larvae found in many free-living nematodes (Hotez *et al.* 1993). In addition, the dauer pathway of *C. elegans* itself has also been compared to the direct development of the progeny of the parasitic females in *S. ratti* (Viney, 1996). However, these and other comparisons are limited as there are no documented examples of species with the same complex life-cycle as *Strongyloides spp.*. The alternation of a dioecious sexual generation and a parthenogenetic female-only generation is known in a number of other animal parasitic species (e.g. *Rhabdias fuelleborni*) (Anya, 1976; Poinar & Hansen, 1983). In contrast, there are no known examples of species in which a single mitotic parthenogen can produce progeny that develop into both dioecious adults and further mitotic parthenogens. There is however one group of nematode species that may be comparable to *Strongyloides.* These are the Tylenchid nematodes, an order parasitic on insect species. Recent molecular phylogenetic analysis of the Nematoda, based on analysis of the sequence of the small subunit ribosomal RNA genes, has indicated that these species can be grouped with the cephalobes, aphelenchids and the various *Strongyloides spp.* into a well defined clade (Blaxter *et al.* 1998). This is interesting as the Tylenchids have a great variety of different life-cycles and reproductive modes, with apparently closely related species having notably different life-histories. Such life-cycles include both sexual and parthenogenetic reproduction and in many cases are characterised by the infection of the insect host by females that have been inseminated in the external environment (Remillet & Laumond, 1991). Therefore, before the specific aims of this work are discussed, it is important to place what is known of *Strongyloides spp.* in context with what is known of reproduction and sex determination in other nematode species.

As previously discussed, the mechanism of sex determination within a species is dependent on the reproductive mode, and within the phylum Nematoda all forms of gametic reproduction, except cross-fertilising hermaphroditism, have been found (Poinar & Hansen, 1983). It is not therefore

surprising that almost every documented form of sex determination has also been found in nematodes (Goldstein, 1981). The majority of nematode species have two distinct sexes and reproduce sexually, but it appears that parthenogenesis and self-fertilising hermaphroditism have evolved on a number of separate occasions within the phylum (Poinar & Hansen, 1983). With the exception of work carried out on *C. elegans* and *C. briggsae* the majority of studies of nematode sex determination have, like those of *Strongyloides spp.*, been exclusively cytological. Thus, in most cases all that is known is the chromosome number, which varies greatly between species, for instance, 2n = 2 in *Diploscapter coronata* and 2n = 54 in *Meloidogyne arenaria* (Triantaphyllou, 1971).

As previously discussed, there are two main categories of sex determination, with the primary signal either environmental (ESD) or genetic (GSD) in nature. At a broad level, GSD is believed to be more common in animal parasitic and free-living nematodes and ESD in plant parasitic nematodes (Goldstein, 1981), but little is known about sex determination in the majority of species. In many dioecious nematodes, it has been found that the males have one less chromosome than the females. For instance, in a list of 37 dioecious species that had been analysed cytologically, 27 were believed to have X0 males (Walton, 1940), suggesting that XX/X0 GSD systems may be common among nematodes. However, in species where the chromosome number of males and females is believed to be the same and no sex chromosomes have been identified, sex may be determined by an ESD system (Triantaphyllou, 1971).

The best characterised of the nematode XX/X0 GSD systems is that of *C. elegans*, which was briefly described earlier. Natural populations of the species, when analysed in the laboratory, are composed mostly of self-fertilising hermaphrodites and a small proportion of males. Normally, males comprise less than 0.5% of the progeny of a hermaphrodite (Wood, 1988) and arise by non-disjunction of the X chromosome (Hodgkin *et al.* 1979). *C. elegans* hermaphrodites are regarded as modified females, as the hermaphrodite gonad produces a small number of sperm during the fourth larval stage and then exclusively produces oocytes (Hodgkin, 1988). It has proved possible experimentally to disable spermatogenesis in the hermaphrodites and hence produce true females (Doniach, 1986). As it

is also known that a number of other *Caenorhabditis* species are composed solely of males and female, this is believed to be the ancestral state of *C. elegans* (Hodgkin, 1988). As described previously, sex in *C. elegans* is dependent on the ratio of X chromosomes to sets of autosomes, the X/A ratio (Hodgkin, 1987). At a molecular level, the X/A ratio is believed to be determined by the number of primary numerator and denominator elements. Numerator elements are essentially 'counted' to determine the X chromosome dose, with the denominator elements predicted to allow the number of sets of autosomes to be determined. To date, two numerator sites have been identified, feminizing locus on X (*fox-1*) (Hodgkin *et al.* 1994) and signal element on X (*sex-1*) (Carmi *et al.* 1998). Duplication of either of these genes in otherwise normal X0 worms is sufficient to cause feminization.

The result of the X/A ratio is a discrete, hierarchical and relatively simple cascade of negatively regulated genes (Hodgkin, 1984) (see Figure 1.5). This gene cascade controls two separate systems, the sexual differentiation of body tissues and X chromosome dosage compensation (Villeneuve & Meyer, 1990). The initial steps in these two pathways rely on the action of the same genes (Figure 1.5), but the end effector systems are entirely unrelated (Miller *et al.* 1988). Of the genes known to be involved in the pathway, only six control both sex determination and dosage compensation.

Among nematodes other GSD systems have been described, although again all studies have been of a exclusively cytological nature. Analysis of the Oxyurida has shown for all species examined to date that sex determination is a haplo-diploid system (Adamson, 1989). There are also a number of nematode species in which male-specific Y chromosomes have been identified, such as *Ascaris lumbricoides* (Goldstein, 1981), *Brugia malayi* (Sakaguchi *et al.* 1983; Underwood & Bianco, 1999), *Onchocerca volvulus* (Sakaguchi *et al.* 1983) and *Baylisascaris transfuga* (Mutafova, 1995). However, it is not known if these represent examples of XX/XY sex determination systems (male-determining Y chromosome) or if, like in *D. melanogaster*, the Y chromosome is unrelated to sex determination and sex determination is actually an XX/X0 system. It is also possible that XX/XY GSD systems are more common than is currently recognised among nematodes, as the Y chromosome may

not be easily distinguished from the X chromosome by cytology. This may mean that species possessing XX/XY GSD systems have been erroneously described as having no specialised sex chromosomes (Triantaphyllou, 1973).

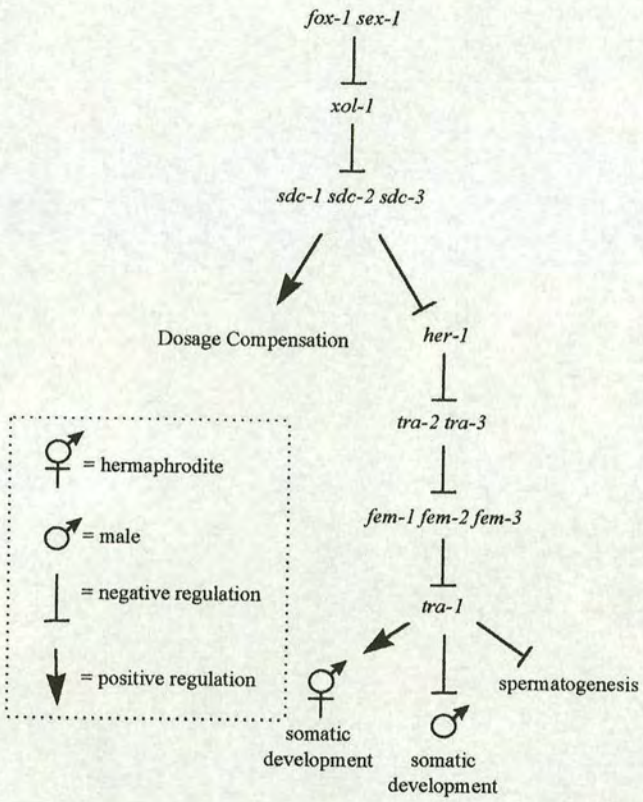


Figure 1.5 The gene cascade that controls sex determination and dosage compensation in *C. elegans*, after Hodgkin (1990).

The existence of ESD systems have been demonstrated in only a relatively small number of nematode species. In the Mermithida (e.g. *Paramermis contorta* and *Mermis nigrescens*), a nematode family parasitic on insects, every species examined to date has had an ESD system (Poinar, 1979). In these species, eggs are ingested by the insects and all parasite growth occurs within the host. The sex of developing larvae is determined in part by the parasite load and in part by the size, sex and nutritional status of the host (Peterson, 1977; Blackmore & Charnov, 1989). Thus, large, well fed hosts with low parasite loads favour female development and *vice versa* (Peterson, 1977). As in many other species (Peters, 1983), the reproductive success of female mermithids is size-dependent, greater

size resulting in increased egg production and hence greater reproductive success. In contrast, the reproductive success of male mermithids is not dependent on their body size. Hence, the ability to develop into an adult female when conditions are favourable and into a male when conditions are unfavourable provides a substantial fitness benefit for the worms.

Other examples of ESD in nematodes include the plant parasitic Heteroderidae (Triantaphyllou, 1973) and the free-living Diplogasteridae (Clark, 1978). In the Heteroderidae, the chromosome complement of males and females has been shown, by electron microscopy, to be identical (Goldstein, 1981). This indicates that *Heterodera spp.* do not have specialised sex chromosomes. There is also a report of ESD in the nematode *Echinomermella matsi*, a parasite of the sea urchin *Strongylocentrotus droebachiensis* (Stein *et al.* 1996). It appears that in this species, the presence of adult females within a sea urchin cause newly invading larvae to develop into males. This study did not however completely rule out the possibility that the observed changes in the numbers of males and females were a result of differential mortality. In general, it must be noted that the possibility of differential mortality rates between the sexes present the same problems when attempting to identify ESD systems as they do when attempting to draw general conclusions about developmental switches. For instance, if consistently female biased sex ratios were observed within a hypothetical population, then this could be taken as indicative of an ESD system. However, the same situation could equally be due to increased male mortality in the environment in which the population was observed. This uncertainty would still occur if the chromosome complement of the males and females of this hypothetical population were found, by cytology, to be the same. In this case, the possibility of an undetected XX/XY or ZW/ZZ GSD system could not be discounted.

It is clear from the preceding review that there is no consistent sex determination mechanism among the nematodes. However, it is apparent that XX/X0 GSD systems are relatively common. The other GSD systems that have been observed in nematodes, XX/XY and haplo-diploidy, appear to be rare or confined to particular taxonomic groups. Therefore, as suggested by the cytological analyses, it is a reasonable hypothesis that sex determination in *Strongyloides spp.* may be an XX/X0 GSD system.

1.4 Summary and aims

The preceding review of what is known of reproduction and sex determination in *Strongyloides spp.* in relation to other nematode species has indicated the questions to be addressed in this thesis. Further study of *S. ratti* is particularly appropriate as the limited genetic analysis that has been carried out on *Strongyloides spp.* has been on this species. It is therefore important to further this work and rationalise our understanding of the species. Cytological studies of the number and behaviour of chromosomes have proved to be very successful in the study of sex determination, but are limited by the chromosomal behaviour being studied and also by the size of the genome. Further cytological analysis is not appropriate in *S. ratti* as conclusions from such studies would be subject to the same criticism as the previous studies. In addition, conclusions drawn from cytological studies cannot be extrapolated to explain the genetics of a species. Thus, a novel approach to the analysis of sex determination in this species is required. This thesis takes a combined approach using both parasitological and genetic analyses to determine the mechanism of sex determination and to rationalise the life-cycle of *S. ratti*. The main questions to be addressed are:

i. To investigate and test genetically the hypothesis that sex determination in *S. ratti* is an XX/X0 system. Such an XX/X0 system of sex determination has been proposed by cytological studies of *S. ratti* (Nigon & Roman, 1952; Bolla & Roberts, 1968), and the hypothesis is supported by the fact that this method of sex determination is common among nematodes.

ii. To determine the inheritance of the putative X chromosome and hence to determine the genetic sex of the indirectly developing iL3 progeny of unrelated free-living adults. Genetic analysis has shown that reproduction between unrelated free-living adults is sexual (Viney *et al.* 1993), but, if sex determination in *S. ratti* is an XX/X0 system, then the sex of the indirectly developing iL3s is unclear.

iii. To investigate the effect of intra- and extra-host factors on the development of the free-living morphs of *S. ratti* and to relate this to the mechanism of sex determination.

iv. To determine the method of reproduction between related free-living adults. Cytological analyses of reproduction between free-living adults of *S. ratti* have concluded that reproduction is by meiotic parthenogenesis and pseudogamy (Nigon & Roman, 1952). However, it has been demonstrated that unrelated free-living adults reproduce sexually (Viney *et al.* 1992).

CHAPTER 2.

Investigation of the distribution of worms of the free-living phase of *Strongyloides ratti* among the faecal pellets of rats.

I would like to acknowledge and thank Dr S. Paterson who performed the statistical analysis reported in this Chapter.

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A copy of this paper can be found in Appendix 4.

CHAPTER 2.

Investigation of the distribution of worms of the free-living phase of *Strongyloides ratti* among the faecal pellets of rats.

ABSTRACT

The production and the distribution of worms of the free-living phase of *Strongyloides ratti* both among the faecal pellets of its host and over time is described. It is shown that both the number of nematodes produced and the mean number of nematodes per faecal pellet peaks each day between midnight and 6.00 am. In addition, the distribution of nematodes among faecal pellets is overdispersed and well described by the negative binomial distribution. This overdispersion increases during the course of infection and occurs over a range of infection intensities. These results raise important questions regarding the accurate quantification of helminth egg counts from faecal samples and indicate that for hosts infected with *S. ratti* it is impossible to standardise the number of worms in faecal cultures. In addition, the overdispersion of nematodes among faecal pellets may have a role in the generation of the overdispersed distribution of helminth parasites within their host population. The causes of the overdispersion of adult parasites are poorly understood, but heterogeneity in the distribution of infective stages within the environment has been implicated as a possible factor. Overdispersion of nematodes among faecal pellets is likely to increase the spatial heterogeneity of the infective stages in the environment and thus may contribute to the generation of overdispersion of adult parasitic stages.

2.1 Introduction

The progeny of parasitic females of *S. ratti* develop into one of three distinct morphs: free-living adult males, free-living adult females and directly developing infective third stage larvae (iL3s). It is known that the extra-host environment encountered by the larval progeny of the parasitic females has a significant effect on the proportion of free-living males, free-living females and directly developing iL3s that develop. A number of studies have identified extra-host factors that effect the proportion of directly developing iL3s (Minematsu *et al.* 1989; Taylor & Weinstein, 1990; Viney, 1996). It has been suggested that this change in development represents a developmental switch between the free-living female and the directly developing iL3 morphs (Minematsu *et al.* 1989; Taylor & Weinstein, 1990). This suggestion assumes that the proportion of the larval progeny of the parasitic females that develop into free-living males is unaffected by extra-host factors and that the free-living females and directly developing iL3s are alternative female morphs. However, it is not known if the free-living male, free-living female and directly developing iL3 progeny of the parasitic females differ in their ability to tolerate different extra-host environmental conditions, although it has been suggested that this is the case (Schad, 1989). If this is the case, then analysis of the free-living phase of *S. ratti* is problematic. Hypothetically, if larvae destined to develop into free-living females have a lower tolerance for changes in temperature in comparison to other larvae, the mortality rate of larvae destined to develop into free-living females will differ if populations of worms are compared at two different temperatures. In contrast, the mortality rate of all other larvae will be the same across the two environments. Such a situation would result in changes in the number of free-living females at the two temperatures and hence changes in the proportions of larvae that developed into free-living females, free-living males and directly developing iL3s. Thus, unless differential mortality can be excluded, analysis of such data is problematic and firm conclusions cannot be drawn.

In order to exclude differential mortality as an explanation for the observed changes in the composition of the free-living phase of *S. ratti* in response to changes in the extra-host environment, both the number of eggs and larvae in the starting population and the number of worms that survive to

maturity must be known. A number of studies have investigated the effects of changes in the extra-host environment on starting populations composed of known numbers of eggs (Taylor & Weinstein, 1990) or of larvae (Minematsu *et al.* 1989). However, as previously discussed (Chapter 1), this is unsatisfactory as both eggs and newly hatched larvae of *S. ratti* are passed with the host faeces (Sandground, 1925b). Thus, these observations have only analysed the effect of extra-host conditions on a subset of the whole population. However, when the effect of changes in the extra-host environment on the proportion of each morph that develop in faecal cultures is analysed, it is not possible to know the initial number of eggs and larvae present. It would therefore not be possible to detect any changes that occurred in the number of worms that survive to maturity between the different treatments. However, if we could produce replicate, standardised, faecal cultures that contained equal numbers of worms then the role of differential mortality in the changes observed in the composition of the free-living phase could be fully investigated.

It is known that there is considerable day-to-day variation in the total worm output of animals infected with *S. ratti* (Sheldon, 1937a). In order to estimate the total daily worm output, the number of worms that developed from single, weighed, faecal pellets was calculating and then this figure was multiplied by the weight of the total daily faecal output (Sheldon, 1937a). However, there has been no investigation into the source of this variability and it is unclear if it represents heterogeneity in the distribution of eggs and larvae among host faecal pellets, temporal variation in the reproductive output of the parasitic adults, differences in culture conditions or temporal changes in host physiology.

There will be important diagnostic implications if the distribution of eggs, and hence developing nematodes, is heterogeneous between host faecal pellets. The diagnosis and quantification of the intensity of helminth infections is typically performed by determining egg counts from faecal samples. This sample is often only a small proportion of the total faecal output. The combination of small faecal samples and a heterogeneous distribution of eggs in the faeces may lead to very inaccurate results. Most studies of human helminth infections analyse single stool specimens on consecutive days, weeks or months (Hall, 1981; Sinniah, 1982) and thus detailed comparisons of the distribution of eggs

among human stools are rare. However, it has been found that eggs of *Schistosoma japonicum* were overdispersed both within (Yu *et al.* 1998) and between (Ye *et al.* 1998) single human stools. In contrast, only slight overdispersion ($k = 2.59$) of *S. japonicum* eggs was observed between stools by Ross *et al.* (1998). The distribution of eggs of *Trichuris trichiura* and *Ascaris lumbricoides* within sub-samples of individual stools was also found to be overdispersed (Hall, 1981: Ye *et al.* 1997). However, the relevance of studies involving the distribution of eggs within single human stools to the distribution of helminth infective stages in rodent faeces is unclear.

Therefore, before any parasitological analysis of the life-cycle of *S. ratti* can be undertaken, the distribution of eggs and newly hatched larvae among the host faecal pellets was investigated. Here, the distribution of worms of the free-living phase of *S. ratti* between faecal pellets as a function of infection intensity, throughout the course of infection and through a 24 hour period, is investigated. These results are discussed in relation to the proposed parasitological analysis of the *S. ratti* life-cycle and to the generation of spatial heterogeneity of helminth infective stages in the environment.

2.2 Materials and Methods

Parasites and maintenance

S. ratti was maintained by serial passage in size-matched, six week old, female Wistar rats (Bantin & Kingman, UK) which were used in all experiments. Food and water were provided *ad libitum* and animals were held in a 12 hour light/dark cycle, with the dark cycle commencing at 18.30. All infections were by subcutaneous injection. Inocula of 100 and 500 infective third stage larvae (iL3s) were prepared by dilution, and inocula of 10 iL3s by counting individual larvae under a dissecting microscope. Faeces from infected animals were collected as described elsewhere (Viney *et al.* 1993). Faecal pellets were cultured by placing individual pellets in the wells of a 24 well culture dish (Nunc) with a small quantity of distilled water, and maintained at 19°C. An animal infected with *S. ratti* passes eggs and newly hatched larvae in faeces (Sandground, 1925b). After three days at 19°C in faecal culture, the progeny of the parasitic females develop into directly developing iL3s, free-living males or free-living females, but the progeny of the free-living adults have not yet developed into indirectly developing iL3s. Therefore, the total number of directly developing iL3s, free-living males and free-living females present in cultured faeces after three days was used as a direct measure of the number of eggs and larvae passed in fresh faeces. This was determined by repeatedly rinsing each culture well with distilled water, which was collected and made up, with distilled water, to 10 mls. The number of worms present in an aliquot of 10 or 20% of this volume, see below, was then determined. Here, as it is the distribution of the worms of the free-living phase among the host faecal pellets that is under investigation, the number of directly developing iL3s, free-living males and free-living females that developed in faecal cultures is referred to as the number of nematodes.

Terminology used in describing lines of S. ratti

The terminology used in the description of *S. ratti* lines conforms to a standard nomenclature for parasitic nematodes (Bird & Riddle, 1994) and supersedes that previously used. For each line used

in this thesis, the prefix ED denotes the laboratory designation, that of Dr. M. Viney. The suffix Heterogonic or Homogonic used in the description of several lines denotes that the parasite line is under laboratory selection for that developmental route (Viney, 1996). Note that heterogonic and homogonic development are otherwise referred to in this thesis as indirect and direct development, respectively. To avoid ambiguity, the following terms are also used specifically in this thesis:

i. Isofemale line - This term denotes a population of parasites derived from a single parasitic female and maintained by serial passage (Viney, 1994). The genetic homogeneity of an isofemale line is dependent on the method used to passage the line. If only directly developing iL3s are used in the passage of the line then, barring mutation, all individuals will be genetically identical. If the indirectly developing iL3 progeny of the free-living adults, or all iL3s that develop in culture (*i.e.* both directly developing iL3s and indirectly developing iL3s), are used in the passage of the line, then individuals cannot be considered genetically identical. This is because reproduction by the free-living adults involves genetic recombination. *i.e.* reproduction is either by functionally meiotic parthenogenesis or is sexual.

ii. Isolate - This term denotes a line of *S. ratti* derived from multiple iL3s collected from a wild-caught host (Fisher, 1997) and is used to distinguish lines derived from multiple iL3s from isofemale lines. As they may be derived from more than one parasitic female, isolates may be genetically heterogeneous.

2.2.1 Distribution of nematodes among faecal pellets through an infection.

Three rats were infected with 10, 100 or 500 iL3s of isofemale line ED335. Faeces were collected overnight between 18.00 and 09.00, on days 5, 10, 15 and 20 p.i., cultured and the number of nematodes present in 20% of the total volume determined as described above. For the faeces collected on day 5 p.i. each pellet was weighed.

2.2.2 Distribution of nematodes among faecal pellets through a 24 hour period.

Four rats were infected with 500 iL3s of isofemale line ED248. Faeces were collected on day 5 p.i. in four consecutive, six hour collection periods, *i.e.* period 1, 18.00-00.00; period 2, 00.00-06.00; period 3, 06.00-12.00 and period 4, 12.00-18.00. Faecal pellets were cultured and the number of nematodes present in 10% of the total volume determined, as described above.

Statistical analysis

The distribution of nematodes among faecal pellets was fitted to Poisson and negative binomial distributions. For the negative binomial distribution, k was estimated by iterative scoring and maximum likelihood using moment estimates as initial values (Bliss & Fisher, 1953). The standard error of k was estimated as the square root of the inverse of the Fisher information at the maximum likelihood estimate of k. (Bliss & Fisher, 1953; McCullagh & Nelder, 1989). Goodness of fit of observed data to both the Poisson and negative binomial distributions was investigated by fitting generalised linear models (GLMs) with a log link function to the data and examining the relative deviances from the models using Pearson's χ^2 statistic (McCullagh & Nelder, 1989). GLM fitting was performed in Matlab using glmlab, a publicly available routine (http://mathworks.com), modified to accommodate the negative binomial distribution.

GLMs were fitted to a negative binomial error distribution by estimating k within each round of the iteratively reweighted least squares algorithm. This method allows the log likelihood of the model to be maximised over both k and the regression coefficients (β) (Lawless, 1982). Log likelihoods for groups of independent models were calculated by summing log likelihoods. To test whether the addition of extra parameters (β or k) significantly improves the goodness of fit, the likelihood ratios of different models were compared to each other (Venables & Ripley, 1997). Calculations were performed in Matlab v5.2 (Mathworks Inc., Natick, MA) using code converted from S-plus (Mathsoft, Cambridge, MA) (Venables, available at: ftp://lib.stat.cmu.edu).

2.3 Results

2.3.1 Distribution of nematodes among faecal pellets through an infection.

The number of nematodes per faecal pellet plotted against faecal pellet weight is shown in Figure 2.1. For each infection dose a GLM, using a negative binomial error distribution, of the number of nematodes per faecal pellet on day 5 p.i. and including the weight of each faecal pellet was constructed. For all infection doses there was no association between the number of nematodes per faecal pellet and the weight of the faecal pellet (null deviance = 34.75, Δdeviance = 0.15, $p = 0.70$, null deviance = 30.62, Δdeviance = 0.53, $p = 0.47$ and null deviance = 42.97, Δdeviance = 1.14, $p = 0.29$ for the 10, 100 and 500 iL3 infections, respectively).

The distribution of the number of nematodes per faecal pellet was significantly overdispersed compared with the Poisson distribution but was well described by the negative binomial distribution (Table 2.1). The negative binomial distribution is characterised by two parameters, the mean and an inverse measure of the degree of overdispersion, k (Hunter & Quenouille, 1952; Bliss & Fisher, 1953). Values of k approaching 20 indicate a random or Poisson distribution; lower values of k indicate increasing levels of overdispersion. On day 20 p.i., for all infection doses, there were insufficient faecal pellets containing nematodes to derive estimates of k or to fit the Poisson and negative binomial distributions. The fit of the Poisson and negative binomial distributions to the data from the 10 iL3 infection on day 5 p.i. is shown in Figure 2.2. To investigate whether the degree of overdispersion differed between infection doses or between days p.i. a single GLM with a common k was constructed containing all explanatory variables and interactions and compared with individual GLMs that contain the same number of data points and regression coefficients, but that were split by *Day p.i.* or *Infection Dose* and fitted separately. This allowed k to be estimated separately for each day p.i. and each infection dose and the significance of separate ks to be determined by examining the log likelihood ratios (Shaw *et al.* 1998) (Table 2.2). These estimated values of k for the different infection doses and

days p.i. are shown in Figure 2.3. This shows that k differs between different days p.i. (Table 2.2 & Figure 2.3A) and between different infection doses (Table 2.2 & Figure 2.3B).

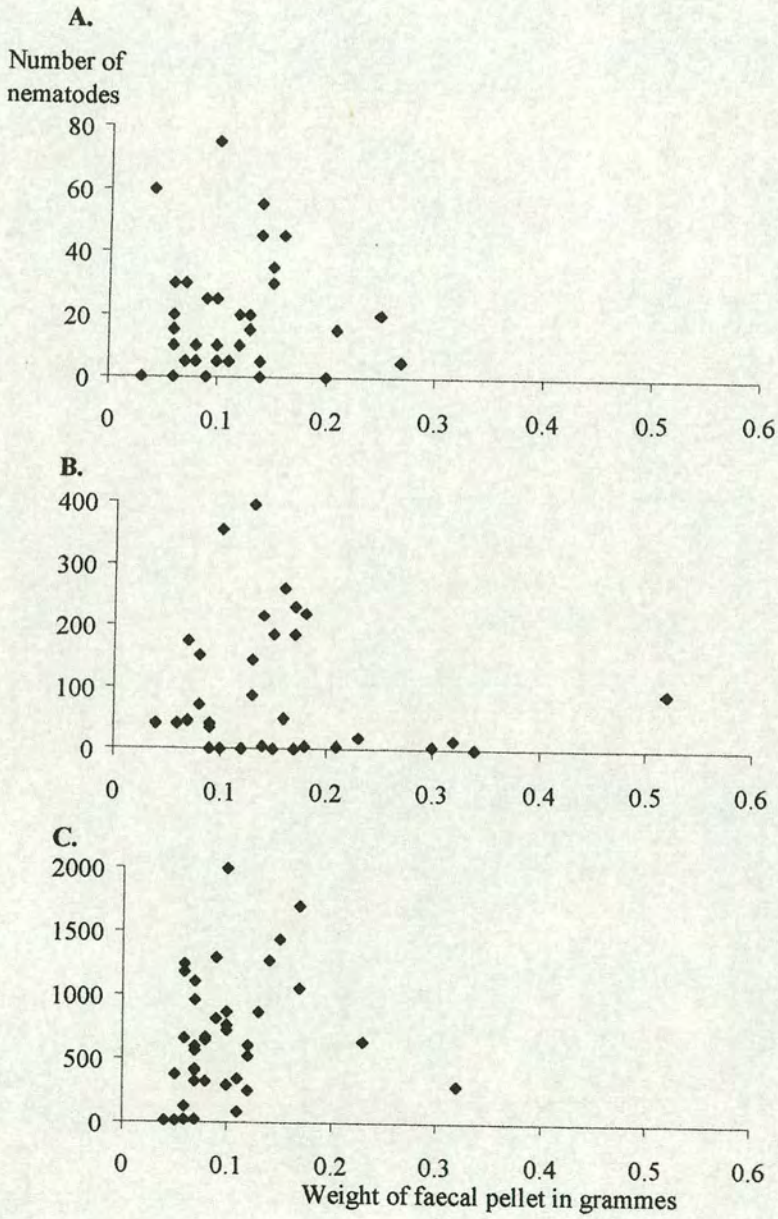


Figure 2.1 Scatterplots of the weight of a faecal pellet against the number of nematodes that developed from that faecal pellet for the (A) 10 iL3, (B) 100 iL3 and (C) 500 iL3 infections on day 5 p.i..

	Number of		No. of nematodes per faecal pellet			Poisson distribution		Negative binomial distribution	
Day p.i.	pellets	nematodes	Mean	Variance	*k*	Deviance	*p* value	Deviance	*p* value
A									
5	43	725	16.9	308	1.35	141.57	<0.01	34.75	0.74
10	43	180	4.2	100	0.24	112.00	<0.01	8.74	>0.99
15	39	485	12.4	3995	0.05	559.66	<0.01	5.24	>0.99
B									
5	33	3070	93.0	12053	0.47	812.55	<0.01	30.62	0.49
10	48	750	15.6	956	0.25	375.67	<0.01	20.56	>0.99
15	32	410	12.8	1814	0.12	331.33	<0.01	8.21	>0.99
C									
5	38	25405	668.6	234170	1.22	2791.56	<0.01	42.97	0.20
10	48	13015	271.1	113281	0.43	3592.07	<0.01	47.79	0.40
15	44	210	4.8	259	0.09	183.56	<0.01	5.63	>0.99

Table 2.1 Analysis of the goodness of fit of the number of nematodes per faecal pellet relative to the Poisson and negative binomial distributions for the (A) 10, (B) 100 and (C) 500 iL3 infections in 2.3.1.

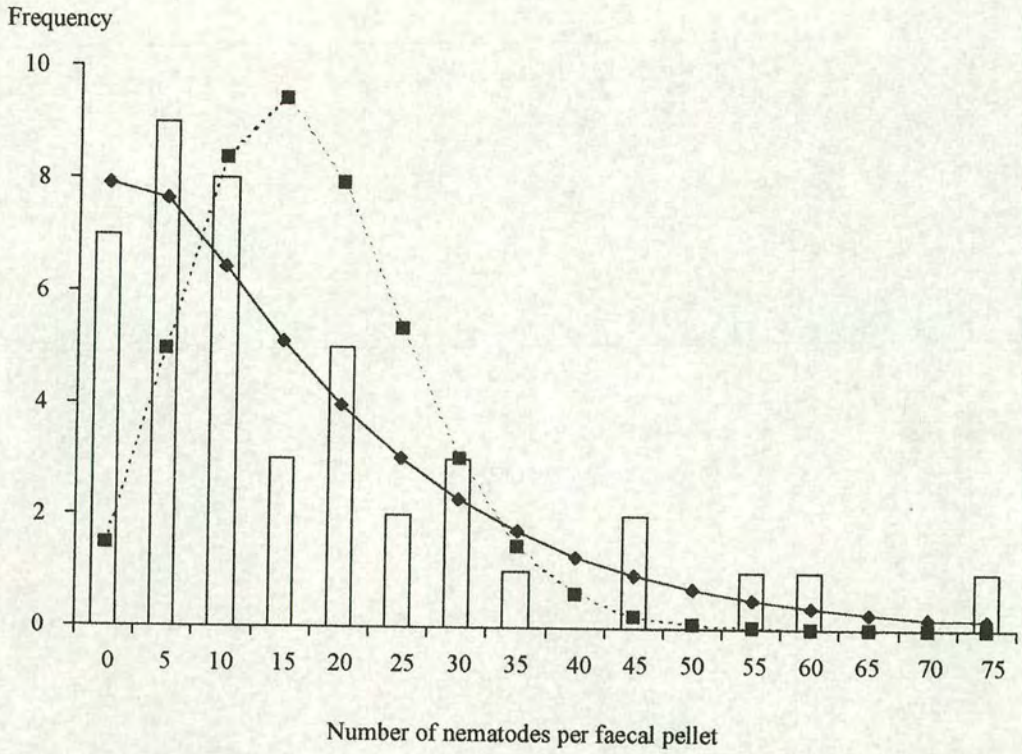


Figure 2.2 The frequency distribution of the number of nematodes per faecal pellet from the 10 iL3 infection on day 5 p.i. and the fitted Poisson (■) and negative binomial (◆) distributions.

Model	df	2 x log likelihood	χ^2	Δdf	*p* value
Common *k* for all treatments.					
Terms: *Intercept* + *Infection Dose* + *Day p.i.* + *Infection Dose•Day p.i.*	358	65192			
Separate *k*s for days p.i..					
Terms: *Intercept* & *Infection Dose*	356	65275	83.68	2	< 0.001
Separate *k*s for infection doses.					
Terms: *Intercept* & *Day p.i.*	356	65201	9.20	2	0.01

Table 2.2 Analysis of *k* between infection doses and days p.i. by comparison of a GLM with a common *k* for all groups with separate GLMs split by *Infection Dose* or by *Day p.i.*.

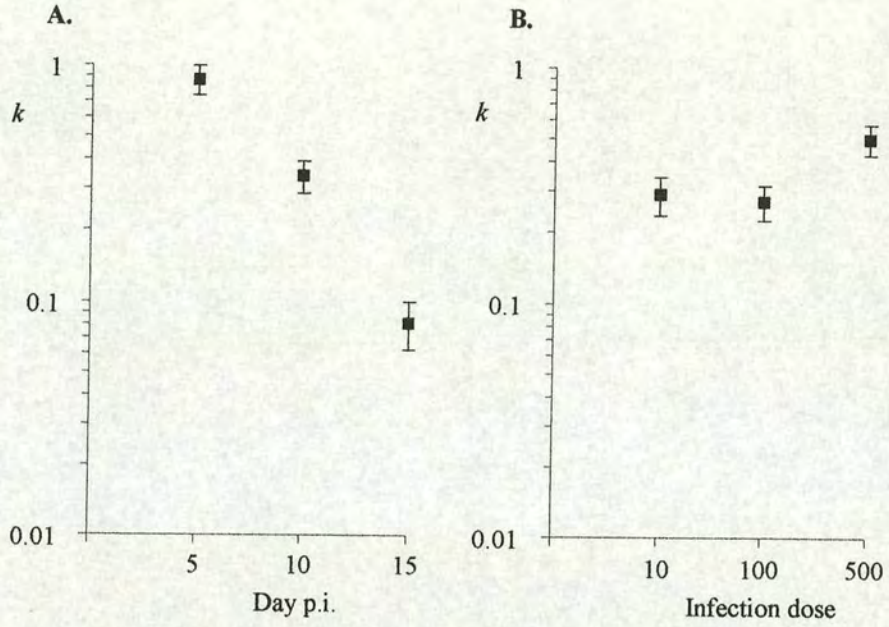


Figure 2.3 The estimated values of k from GLMs (Table 2.2) (A) on days 5, 10 and 15 days p.i. for all infection doses combined and (B) for the 10, 100 and 500 iL3 infection doses for days 5, 10 and 15 p.i. combined. Error bars are $\pm$ 1 S.E..

2.3.2 Distribution of nematodes among faecal pellets through a 24 hour period.

The number of faecal pellets produced by each rat during each six hour collection period was analysed by fitting a GLM to these data, assuming Poisson errors. *Rat* and *Collection Period* were fitted as main effects and significance tested by examining deviance residuals. This identified a significant effect of *Collection Period* on the number of faecal pellets produced (Δdeviance = 47.73, Δ df = 3, $p < 0.001$) and showed that there was no significant effect of *Rat* on the number of faecal pellets produced (Δdeviance = 2.50, Δdf = 3, $p = 0.475$, null model: deviance = 75.45, residual df = 15). Comparison of parameter estimates showed that the number of faecal pellets produced in collection period 2 (00.00-06.00) was significantly greater than in other collection periods (Δdeviance = 28.07, Δdf = 1, $p < 0.001$).

The distribution of nematodes between faecal pellets, for these data combined for all rats and all collection periods, was significantly overdispersed relative to the Poisson distribution (deviance = 2359, df = 207, $p < 0.001$), but was well described by the negative binomial distribution (deviance = 184.2, df = 206, $p = 0.86$) with an overall mean and k of 114 and 0.744, respectively. For the faecal collection period comparable to *2.2.1* (collection periods 1 and 2 combined), for all four rats combined, the distribution of nematodes was overdispersed with a mean and k of 128 and 0.836, respectively. To investigate whether the degree of overdispersion differed between rats and collection periods, a GLM with a common k for all groups was compared with individual GLMs to which k values were fitted separately for *Collection Period* and for *Rat* (Table 2.3). This showed that there was no significant difference in k between rats or between collection periods.

Model	df	2 x log likelihood	χ^2	Δdf	p value
Common k for all treatments.					
Terms: *Intercept* + *Rat* + *Collection Period* + *Rat•Collection Period*	191	8527	-	-	-
Separate ks for collection period.					
Terms: *Intercept* & *Rat*	188	8531	3.63	3	0.30
Separate ks for rat.					
Terms: *Intercept* & *Collection Period*	188	8531	3.67	3	0.30

Table 2.3 Analysis of k between rats and collection periods by comparison of a GLM with a common k for all groups with separate GLMs split by *Rat* or by *Collection Period*.

A GLM with a normal error distribution of the natural log transformed number of nematodes passed by each rat showed that there was no significant variation between rats in the number of nematodes passed by each rat over the entire 24 hour period, but that the mean number of nematodes passed in each six hour collection period did vary ($\chi^2 = 7.91$, df = 3, $p = 0.048$). The parameter estimates of the mean number of nematodes produced in each six hour collection period is shown in Figure 2.4A. This shows that the greatest number of nematodes were passed in collection period 2 (00.00-06.00).

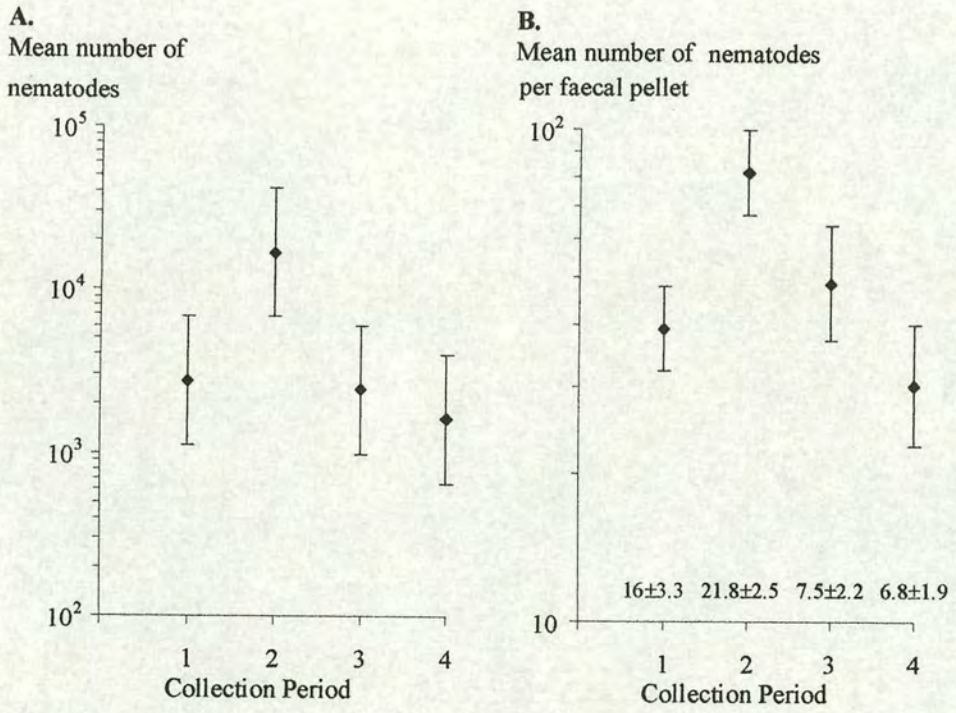


Figure 2.4 A. The mean number of nematodes over the four six hour collection periods. Error bars are ± 1 S.E.. B. The mean number of nematodes per faecal pellet over the four six hour collection periods. Error bars are ± 1 S.E.. Also shown are the mean number of faecal pellets per collection period ± 1 S.E..

The number of nematodes per faecal pellet was examined by fitting a GLM with a negative binomial error structure to the observed data (Table 2.4). The error structure required k to be

estimated from the data and was assumed to be constant between time periods and rats which, as shown in Table 2.3, is a valid assumption. In Models 1 and 3, collection periods 3 and 4 were combined since this did not result in a significant increase in deviance (Δ deviance = 1.86, df = 1, p = 0.173). *Collection Period* is significantly associated with the mean number of nematodes per faecal pellet when considered as a factor (Model 1) or as a quadratic function (Model 2) (Table 2.4). The highest mean number of nematodes per faecal pellet is in collection period 2 (00.00-06.00) (Figure 2.4B). Model 4 shows that there is no significant difference between *Rat* in the mean number of nematodes per faecal pellet when considered as a main effect (Table 2.4). However, there is a significant interaction between the factors *Rat* and *Collection Period* (Table 2.4). The relationship between the number of nematodes per faecal pellet and the number of faecal pellets produced within each time period is shown in Figure 2.5.

Model	Term	Residual df	2 x log likelihood	χ^2	Δdf	p value
Null		206	8469.51	-	-	-
Model 1	*Collection Period* (factor; periods, 1, 2 & (3 + 4))	204	8489.38	19.88	2	<0.001
Model 2	*Collection Period* (polynomial)	204	8489.10	19.60	2	<0.001
Model 3	*Collection Period* (factor; periods, 1, 2 & (3 + 4))	-	-	20.59	2	<0.001
	Rat (factor)	-	-	6.13	3	0.105
	Rat × *Collection Period*	195	8523.40	27.89	6	<0.001

Table 2.4 The results of GLM analyses of the mean number of nematodes per faecal pellet.

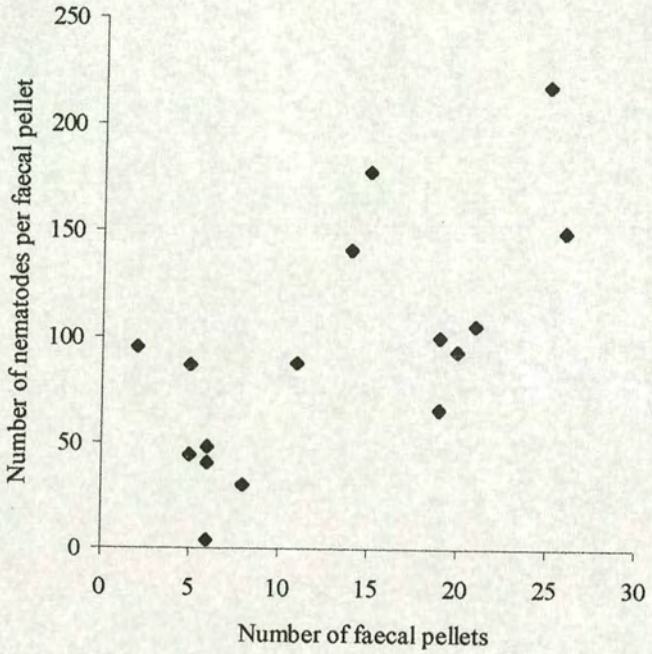


Figure 2.5 A scatterplot of the number of nematodes per faecal pellet against the number of faecal pellets produced within each time period.

2.4 Discussion

In both experiments, the distribution of the worms of the free-living phase of *S. ratti* among host faecal pellets was significantly overdispersed and well described by the negative binomial distribution. Variation in the weight of faecal pellets did not explain the distribution of nematodes among faecal pellets and thus the observed overdispersion is not a trivial function of faecal pellet weight. The distribution of the nematodes among host faecal pellets was overdispersed for the three infection doses used. A reduction in the observed degree of overdispersion was found for the 500 iL3 infection dose, which may be a consequence of the very large number of eggs, and hence greater density, in the faeces. For the two isofemale lines used, for comparable infection doses (500 iL3s) and during comparable sampling periods (day 5 p.i. and collection periods 1 and 2), the mean production of nematodes was different, but the overdispersion among the faecal pellets was similar. Thus, ED335 and ED248 had a mean and k of 669 and 1.22 (Table 2.1) and 128 and 0.836, respectively.

The causes of this overdispersion of nematodes among faecal pellets may be due to heterogeneity in the timing of egg production and egg laying or due to the behaviour of the eggs in the intestinal lumen. If the production and laying of eggs by parasitic females varies over time and varies similarly for cohorts of worms, then this may lead to the generation of an overdispersed distribution of nematodes among faecal pellets. Furthermore, the eggs of *S. ratti* are laid in the intestinal epithelium and thus are not immediately liberated into the intestinal lumen. It is not known how eggs enter the intestinal lumen, but it may well be due to epithelial abrasion. Such a process may lead to the liberation of clumps of eggs into the intestinal lumen which may also generate an overdispersed distribution. In *S. ratti*, it has also been reported that some eggs are laid in strings of varying lengths, held together with a mucus secreted by the parasitic female (Sandground, 1925b). This would lead to some clumping of the eggs and may also generate an overdispersed distribution of nematodes among faecal pellets. However, this phenomenon has only been reported for *Strongyloides spp.* and as there is evidence that the progeny of the parasitic phases of other nematode species are overdispersed among faecal pellets, this cannot be the general basis of the generation of heterogeneity in egg distribution.

There was no significant difference in the degree of overdispersion of the nematodes between the four collection periods throughout a 24 hour period (Table 2.3). In contrast to the consistency of overdispersion across a 24 hour period, the degree of overdispersion significantly increased during infection (Table 2.2 and Fig 2.3A). During the course of an infection with *S. ratti*, rats become increasingly immune and lose intestinal worms (Moqbel & McLaren, 1980). As shown above, an infection dose of 500 iL3s results in a decreased level of overdispersion, but there is no change in the degree of overdispersion between the 10 iL3 and 100 iL3 infection doses. Thus, the increased overdispersion later in an infection is unlikely to be a simple function of the reduced numbers of parasitic females. It is therefore probable that the greater overdispersion later in infection is a result of increasing heterogeneity in the egg production by the parasitic females rather than a reduction in the number of parasitic females *per se*. However, it is unclear if this increased overdispersion is a consequence of the age of the parasitic females or a response to increasing host immunity. It has been shown that increasing host immunity reduces the number of eggs *in utero* and results in changes to the ultrastructural morphology of *S. ratti* parasitic females (Moqbel & McLaren, 1980). It may be that these functional and morphological changes in the parasitic females are the cause of the increased heterogeneity of egg production over infection. In addition, over the course of infection the distribution of parasitic females within the small intestine changes. In the initial stages of infection, the majority of the parasitic females are located in the quarter of the small intestine immediately below the pyloric sphincter, but as the infection progresses they move to the posterior of the small intestine (Moqbel & Denham, 1977). This may reduce the opportunity for the eggs to be dispersed in the lumenal contents, with the result that the overdispersion of nematodes among the faecal pellets is increased.

To analyse the distribution of nematodes among faecal pellets with greater precision, faeces were sampled in four, equal, six hour periods. This showed that the production of faecal pellets is similar between rats and is at its greatest between midnight and 06.00 am. Behavioural studies of rats have found that they are most active at night, with peak foraging behaviour occurring two to three hours after dark (Barnett *et al.* 1975). This increased activity is accompanied by a higher body

temperature and increased metabolic activity (Honma & Hirochige, 1978). The dark period of the study animals commenced at 18.30 and hence a peak in faecal output between 00.00 and 06.00 is not unexpected and is likely to result from peak feeding activity a number of hours earlier. In addition to the peak in faecal output between 00.00 and 06.00, the greatest number of nematodes passed also occurred during this period. There was also a significant difference between collection periods in the mean number of nematodes per faecal pellet (Figure 2.4B). Thus, the observed peak in the number of nematodes passed between 00.00 and 06.00 is due to a combination of the peak in faecal production and the peak in mean number of nematodes per faecal pellet. Parasitic females of *S. ratti* lie buried in the mucosa of the small intestine where eggs are deposited (Dawkins, 1989). Therefore, it is likely that the liberation of eggs from the mucosa is related to, or dependent on, gut activity. Thus, a peak in the number of nematodes passed by a host would be expected to coincide with a peak in faecal production, as was observed. However, a peak in the mean number of nematodes per faecal pellet was also observed in the same time period. This strongly suggests that the production of eggs by parasitic females varies through time. These data support previous suggestions that such variation in egg production occurs in *S. ratti* (Sandground, 1928). The findings also agree well with a study of *Aspiculuris tetraptera* which found that over a 24 hour period egg production peaked at the same time as the peak in faecal output (Phillipson, 1974). Furthermore, this peak in *A. tetraptera* egg production was found to be due to an increase in the mean number of eggs per faecal pellet. For *S. ratti*, there is probably a delay between the time at which eggs are laid by parasitic females and their liberation into the intestinal lumen. Thus the peak egg production of a parasitic female is likely to occur before the peak faecal production of the host and may coincide with the peak in rat activity that occurs two to three hours after dark (Barnett *et al.* 1975). The analysis of the mean number of nematodes per faecal pellet also identified a significant interaction between the factors *Rat* and *Collection Period* (Table 2.4). Biologically this implies that there is variation between different rat and time period combinations in the number of nematodes per faecal pellet. The significance of this finding is unclear, given the limited number of animals used and the small number of faecal pellets produced in some time periods.

These observations have important practical consequences for parasitological studies of *S. ratti*. For example, when determining the number of nematodes per gram of faeces from an infected animal, the result obtained will be heavily dependent on the proportion of faecal output considered and over what time period faeces were collected. Thus, for *S. ratti*, to obtain an accurate measure of the number of nematodes in faeces, a substantial proportion of the daily faecal output must be collected and treated as a whole. It is likely that these observations may explain the extreme variation in the daily worm output that have been observed in studies that have extrapolated from single faecal pellets (Sheldon, 1937a). The observation of considerable variation in daily egg output seen in other helminth infections (Hall, 1981) suggests that the overdispersion of worms of the free-living phase of *S. ratti* reported here may also occur in other helminth systems. These results also suggest that extreme care may have to be taken when relying on faecal egg counts to determine either the existence or intensity of helminth infections. The fact that the worms of the free-living phase of *S. ratti* are overdispersed among the faecal pellets of the host and that there is a peak in the production of nematodes between 00.00 and 06.00 also indicate that there is no accurate method of standardising the number of *S. ratti* larvae and eggs in faecal cultures.

These observations may also be important in the generation of the overdispersion of adult helminth parasites in their host population. This overdispersion is a common observation in natural helminth infections in which a minority of hosts harbour the majority of the parasites (Hudson & Dobson, 1995), and is normally well described by the negative binomial distribution. Generally, helminth species have values of k between 0.1 and 1 (Hudson & Dobson, 1995). It has been suggested that important factors in the generation of helminth overdispersion are differences in host susceptibility (Gregory *et al.* 1990; Wakelin, 1996) and variable rates of encounter between host and parasite (Crofton, 1971; Anderson & Gordon, 1982).

Differences in host immune function have been implicated in the generation of helminth overdispersion from observations that found increased variability in *Heligmosomoides polygyrus* burdens following repeated exposure compared with a single infection (Keymer & Hiorns, 1989;

Gregory *et al.* 1990). However, differences in host susceptibility to helminth infection *per se* are not observed since helminth species are not normally found to covary within hosts (Poulin, 1996). This may be due to the fact that different immune mechanisms mediate responses to different helminth species (Maizels & Holland, 1998). The rate of encounter between host and parasite will depend on the spatial distribution of parasite infective stages in the environment and on the behaviour of the host (Shaw & Dobson, 1998). An even distribution of the infective stages of *Hymenolepis diminuta* has been shown to produce an overdispersed distribution in *Tribolium confusum*. However, increasing the heterogeneity of the distribution of the infective stages within the environment increased the overdispersion, but did not affect the mean intensity of infection (Keymer & Anderson, 1979). Furthermore, the overdispersed distribution of *Howardula aoronymphium* among species of *Drosophila* was found to be due to variation in the exposure to infective stages (Jaenike, 1994). The overdispersion of the worms of the free-living phase of *S. ratti* between faecal pellets identified here may contribute to the spatial heterogeneity of the infective stages in the environment. However, spatial environmental heterogeneity will also depend on the defecation behaviour of rats, about which little is known. Spatial heterogeneity of the infective stages in the environment may in turn be involved in the production of overdispersed distributions of adult parasites.

CHAPTER 3

The control of morph development in the free-living phase of the parasitic nematode *Strongyloides ratti*.

I would like to acknowledge Dr A.W. Gemmill and Dr M.E. Viney for allowing me to use the data for the immune manipulation experiments and the incubation temperature and day post infection experiments, respectively. In both cases, these data have been published previously (Gemmill et al. *1997 and Viney, 1996, respectively) and the analysis of this work presented here represents a separate analysis and new interpretation of these data.*

CHAPTER 3

The control of morph development in the free-living phase of the parasitic nematode *Strongyloides ratti*.

ABSTRACT

The life-cycle of the parasitic nematode *Strongyloides ratti* is complex. The progeny of the parasitic females form a free-living phase and can develop into three distinct morphs:- directly developing infective third stage larvae (iL3s), or free-living adult males and females. The proportion of each morph of the free-living phase varies dependent on both intra- and extra-host factors. Evidence is presented which suggests that this variation is the result of at least two discrete developmental switches. The first is a sex determination event that occurs inside the host; the second is a developmental conversion between alternative female morphs, namely the free-living females and directly developing iL3s. In the experiments reported here, the free-living females/directly developing iL3 switch is mediated by the incubation temperature of the faecal cultures. The host immune status affects both of these two developmental switches, with increased immunity causing a greater proportion of larvae to develop into free-living males and a greater proportion of female larvae to develop into free-living females. The greater proportion of female larvae that develop into free-living females appears to be mediated by an increased sensitivity of the free-living female/directly developing iL3 developmental conversion to incubation temperature. These findings clarify the developmental basis of this complex life-cycle and demonstrate how such life-cycles can result from the combination of multiple, simple developmental switches.

3.1 Introduction

Strongyloides ratti has a complex life-cycle with both parasitic and free-living phases. The parasitic phase is female only and is found in the host intestine. Reproduction by these parasitic females is functionally mitotic (Viney, 1994) and progeny pass with the host faeces into the external environment, where subsequent development occurs (Sandground, 1925b). The larval progeny of the parasitic females either moult through two larval stages into directly developing infective third stage larvae (iL3s), termed direct development, or through four larval stages into free-living adult males and free-living adult females, termed indirect development. The free-living phase is composed of these free-living males, free-living females and directly developing iL3s. The free-living adults reproduce in the external environment and all their progeny develop into indirectly developing iL3s (Viney *et al.* 1993). The directly developing iL3s and the indirectly developing iL3 progeny of the free-living adults then penetrate the skin of a host and subsequently develop to maturity as parasitic females.

The proportion of the *S. ratti* parasitic females' progeny that develop into directly developing iL3s, free-living males or free-living females varies in a complex manner (see Chapter 1). It is known that the proportion of directly developing iL3s varies between isofemale lines (Viney *et al.* 1992) and can be altered by selection (Viney, 1996). In addition, it is affected by both the incubation temperature of faecal cultures (Viney, 1996) and the host immune status (Gemmill *et al.* 1997). Thus, in *S. ratti* the proportion of larvae that develop directly versus indirectly is affected by both genetic and environmental factors. The environmental factors can be divided into two classes: intra-host factors acting both on the parasitic females and their progeny; and extra-host factors acting solely on the progeny. In *S. ratti*, the concentration of fatty acids (Minematsu *et al.* 1989) and carbon dioxide and oxygen concentrations (Taylor & Weinstein, 1990) do not affect the proportion of larvae that develop into free-living males. In these experiments, changes in the proportion of larvae that developed by the direct and indirect routes occurred due to changes in the proportions of directly developing iL3s and free-living females (Minematsu *et al.* 1989; Taylor & Weinstein, 1990). Similar observations have also

been made for *S. stercoralis* (Shiwaku *et al.* 1988) and *S. ransomi* (Moncol & Triantaphyllou, 1978) (see Chapter 1 for review).

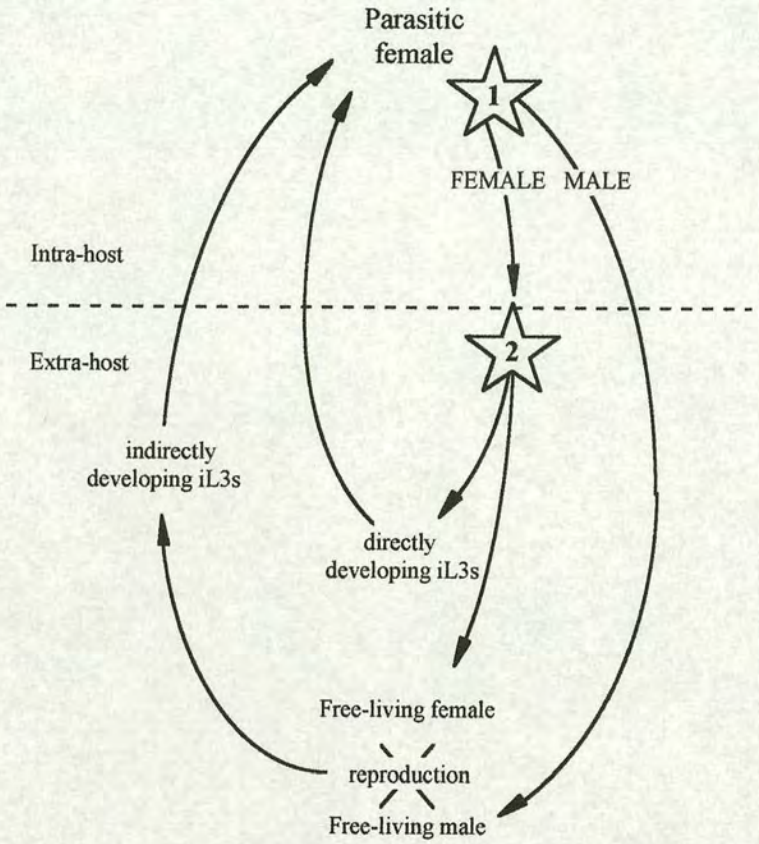


Figure 3.1 Postulated life-cycle of *S. ratti* indicating the proposed way in which two discrete developmental switches act at different life-cycle stages. 1. Intra-host sex determination switch. 2. Extra-host free-living female/directly developing iL3 switch.

These observations suggest that the progeny of the parasitic females are either male or female, and that male progeny develop into free-living males, while female progeny develop either into directly developing iL3s or into free-living females (Minematsu *et al.* 1989; Taylor & Weinstein, 1990). In such a scenario, the proportion of larvae that develop into free-living males would, barring differential mortality, be unaffected by extra-host factors. Further, if the directly developing iL3s and the free-living females are alternative female morphs, it is anticipated that the developmental switch between them would be dependent on extra-host factors. If so, changes in extra-host factors would affect the

relative proportion of directly developing iL3s and free-living females, but not the proportion of free-living males. This postulated life-cycle for *S. ratti* is shown in Figure 3.1. This view of the life-cycle is also consistent with the findings of the cytological studies of *S. ratti*, which concluded that the diploid chromosome number of both the parasitic and free-living females is six while that of the free-living males is five (Nigon & Roman, 1952; Bolla & Roberts, 1968).

However, it is not clear if this view of the life-cycle is correct. Previous experiments on *S. ratti*, which suggested that the proportion of larvae that developed into free-living males was unaffected by changes in the extra-host environment, were not conclusive (see Chapter 1). In particular it is possible that previous results are a consequence of differential mortality. The ideal method by which to address this question would be to analyse the effect of changes in extra-host factors on the number of worms that develop, using replicate, standardised, faecal cultures containing equal numbers of worms. This would allow both the number of eggs and larvae in the starting population and the number of worms that survive to maturity to be determined. Previous work has demonstrated that the worms of the free-living phase of *S. ratti* are overdispersed among the faecal pellets of the host and the production of infective stages is not constant over time (Chapter 2). Therefore, it is not possible to produce replicate faecal cultures that contain the same number of worms. However, the distribution of infective stages among the host faecal pellets is well described by the negative binomial distribution (Chapter 2). This distribution is characterised by two parameters, the mean and an inverse measure of the degree of overdispersion, k (Bliss & Fisher, 1953). Thus, the total number of worms that survive to maturity in faecal cultures composed of many faecal pellets would allow the mean number of worms to be compared. While this would not preclude the possibility that any changes in the composition of the free-living phase were a result of differential mortality, it would indicate that any differential mortality that was occurring was of an extremely complex form. For instance, if results showed the presence of a free-living female/directly developing iL3 switch, as postulated in Figure 3.1. Then, for this to be a consequence of differential mortality, the number of larvae destined to develop into free-living females which died at 19°C would have to be equal to the number of larvae destined to develop into directly developing iL3s which died at 25°C.

If the proposed model of the *S. ratti* life-cycle (Figure 3.1) is correct, it is still unclear how it accounts for the variation in the composition of the free-living phase that results from changes in intra-host factors. In laboratory infections of *S. ratti*, the number of parasitic females in the host intestine first rises to a peak and then declines with time, with the majority of parasites expelled from the host four to six weeks post infection (p.i.). During this time, the proportion of the parasitic females' progeny that develop into directly developing iL3s falls while the proportion that develop indirectly into both free-living males and free-living females rises (Viney *et al.* 1992). In common with many other species of nematode parasites, infection with *S. ratti* elicits a strong protective immune response from the host (Sheldon 1936b; Moqbel & Denham, 1977; Olsen & Schiller, 1978). Comparison of *S. ratti* infections in normal hosts and those with altered immunocompetence has shown that this change in the proportion of larvae that develop via the indirect route is primarily an effect of the host immune response (Gemmill *et al.* 1997). Similar observations had previously been made for *S. ransomi* (Moncol & Triantaphyllou, 1978) and for *Strongyloides* in pigs (Varju, 1966). In contrast, a study of *S. stercoralis* in immunosuppressed and normal dogs did not detect any difference in the proportions of larvae that developed by the direct and indirect routes (Shiwaku *et al.* 1988). However, although the idea that the directly developing iL3s and the free-living females are alternative female morphs has previously been suggested (Minematsu *et al.* 1989; Taylor & Weinstein, 1990), there has been no investigation into the effects of the host immune status on this developmental switch.

Here this model of the *S. ratti* life-cycle (Figure 3.1) is tested by manipulating the incubation temperature of faecal cultures. This allows the proposed model of the life-cycle to be investigated in a natural system to determine if the apparent changes in the composition of the free-living phase in response to extra-host environmental factors are a consequence of differential mortality or represent a sequence of developmental switches as shown in Figure 3.1. Further, the effect of changes in host immune status on these two developmental switches are investigated. Analysis of these data allows the effect of both intra- and extra-host factors on the developmental route of the progeny of the parasitic females to be investigated. In so doing, the developmental basis of the complex life-cycle of *S. ratti* is clarified.

3.2 Materials and Methods

Parasites and maintenance

All parasite lines were maintained by serial passage in female Wistar rats. Randomly bred, size matched (100-150g) female Wistar rats (Bantin & Kingman, UK) were used in all experiments unless otherwise stated, with food and water provided *ad libitum*. Unless otherwise stated, all infections were prepared by dilution in saline (0.8% w/v NaCl) and administered by subcutaneous injection. Faeces from experimental animals were collected and cultured as described by Viney *et al.* (1992). Briefly, this entails placing a number of faecal pellets (6-8) in a 5 cm diameter watch glass within a 9 cm diameter petri dish. Distilled water is then added under the watch glass and around the faecal mass (Viney *et al.* 1992). All faecal cultures were maintained at 19°C unless otherwise stated. The total number of worms and the proportions of each morph (directly developing iL3s, free-living males and free-living females) in faecal cultures, after three days at 19°C or two days at 25°C, were determined as described by Viney (1996), unless otherwise stated. Briefly, in order to determine the total number of worms in a faecal culture, the faecal mass and the floor of the petri dish are repeatedly rinsed with distilled water, which is then collected. The number of worms present in a known fraction of the total volume is then determined. In order to determine the proportions of each morph, repeated samples of freshly agitated liquid were observed under a binocular microscope. Except where noted, all proportion data was angular transformed prior to analysis.

3.2.1 Incubation temperature

a. Incubation temperature and morph development

Twelve rats were infected with 500 indirectly developing iL3s of isofemale line ED321 Heterogonic, an isofemale line derived from ED5 Heterogonic (Viney, 1996). Faeces were collected on day 8 p.i. and an equal number of cultures from each animal maintained at 19°C and 25°C. The total

number of worms and the proportion of directly developing iL3s, free-living males and free-living females were determined for each culture. For each rat, the total number of worms and the proportion of each morph at each incubation temperature was calculated by combining the data from all cultures in that temperature treatment. The effects of incubation temperature on the transformed proportion of each morph and on the total number of worms in faecal cultures maintained at 19°C and 25°C from each animal were determined by paired *t*-tests.

b. Incubation temperature and the total number of worms

The total number of worms in faecal cultures maintained at 19°C and 25°C was measured, as described above, for a further eight groups of rats (n = 11, 11, 10, 11, 9, 12, 8 & 10 animals, respectively) each infected with 500 indirectly developing iL3s of isofemale line ED321 Heterogonic. The effect of incubation temperature on the total number of worms was investigated by determining the difference in the total number of worms in cultures maintained at 19°C and 25°C. This was calculated as: (number at 25°C - number at 19°C)/((number at 25°C + number at 19°C)/2). The difference in total number of worms between 19°C and 25°C for rats in these eight groups of animals and those from *3.2.1a* (9 groups, 94 rats in total) were compared by ANOVA to determine if the effect of incubation temperature on the total number of worms differed between groups of rats. The difference in total number of worms between faecal cultures maintained at 19°C and 25°C for all animals (n = 94) was then analysed by a one sample *t*-test to determine if the difference in total number of worms between 19°C and 25°C differed from zero.

3.2.2 Incubation temperature and day p.i.

These data were collected during the course of previously published experiments (Viney, 1996). Two rats were infected with 500 iL3s of isofemale line ED5 Homogonic (Viney, 1996). Faeces were sampled throughout the infection and equal numbers of faecal cultures maintained at 19°C and 25°C. For each culture, the proportion of larvae that developed into free-living males, free-living

females and directly developing iL3s was determined. For the free-living males, free-living females and directly developing iL3s, the effect of incubation temperature was calculated as: the proportion that developed in cultures maintained at 25°C - the proportion that developed in cultures maintained at 19°C. 95% confidence limits on these differences in proportions were calculated as described by Samuals (1989). The effect of day p.i. on the difference in the proportion of larvae that developed into each morph in cultures maintained at 19°C and 25°C was investigated by least squares regression.

3.2.3 Immunity

These data were collected during the course of previously published experiments (Gemmill *et al.* 1997). Faeces were sampled throughout the infection and the proportion of larvae that developed into each morph was determined as described above.

a. Previous exposure

Three rats were infected percutaneously (Tindall & Wilson, 1988) with 10 iL3s of isofemale line ED 5 Heterogonic, with the dose determined by counting individual iL3s under a binocular microscope. Infections were monitored to confirm patency. Three control animals were treated comparably, but not infected. On days 27 and 28 p.i. all animals were treated with 0.11 ml of a 17.6% w/v thiabendazole suspension (Thibenzole, MSD AGVET) by oral intubation to clear remaining parasites. Thirty five days after initial infection, all animals were challenged by percutaneous infection with 250 iL3s.

b. Congenitally hypothymic (nude) rats

Congenitally hypothymic rats (n = 4) (homozygous for HsdHan:NZNU-rnu^{N}) and heterozygous, thymus intact, control animals (n = 4) (HsdHan:NZNU-rnu^{N}/ +) (Harlan, UK), were infected with 500 iL3s of isofemale line ED5 Heterogonic.

c. Whole-body γ-irradiation

Three rats were exposed to 6.5 Grays from a ^{137}Caesium γ-emitting source three days prior to infection with 500 iL3s of isofemale line ED5 Heterogonic. Three non-irradiated control animals were treated comparably.

d. Corticosteroids

i. Six rats were infected with 500 iL3s of isofemale line ED5 Heterogonic and monitored from day 6 p.i. onwards. On day 8 p.i., animals were arbitrarily assigned to treatment or control groups (n = 3 in each). Experimental animals were treated with 10 mg/kg mean body weight of betamethasone (Betsolan, Pitman Moore) by subcutaneous injection on days 8 through 13 p.i. (inclusive). Control animals received an equal volume of sterile water.

ii. The above experiment was repeated, except that the animals were monitored from day 7 p.i. onwards and were arbitrarily assigned to treatment or control groups (n = 3 in each) on day 18 p.i.. In this experiment, treated animals received 10 mg/kg mean body weight of betamethasone by subcutaneous injection on days 18 through 27 p.i. (inclusive).

Statistical analysis

To investigate the effect of previous exposure to *S. ratti* (*3.2.3a*), a generalised linear models (GLM) were fitted to the proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females. The proportion of larvae that developed into free-living males was calculated as: (the number of larvae that developed into free-living males) / (total number of worms counted). The proportion of female larvae that developed into free-living females was calculated as: (the number of larvae that developed into free-living females) / (the number of larvae that developed into free-living females + the number of larvae that developed into directly developing

iL3s). *Treatment* (previously exposed vs naive), *Day p.i.*, *Rat* within *Treatment* and *Rat* within *Treatment* by *Day p.i.* were then fitted to these models and significance calculated by determined by analysis of variance.

The effect of the other immune manipulations (*3.2.3b-d*, above) on the two postulated developmental switches was investigated by analysing the proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females. The proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females was calculated as described above. For each animal, the slope of the least squares regression line of the angular transformed proportion against day p.i. was calculated. The slopes of the regression lines for the immune manipulated animals were then compared by unpaired t-tests to the slopes of the regression lines for the control animals. The slopes of the regression lines for the immune manipulated animals were also compared, by a one sample t-test, to zero.

3.3 Results

3.3.1 Incubation temperature

a. Incubation temperature and morph development

The proportion of the parasitic females progeny that developed into directly developing iL3s and into free-living females were significantly different between the 19°C and 25°C temperature treatments ($t_{11\ d.f.} = -9.94$, $p < 0.0001$ and $t_{11\ d.f.} = 7.30$, $p < 0.0001$, respectively) (Figure 3.2). In contrast, the proportion of larvae that developed into free-living males did not differ significantly between the two temperature treatments ($t_{11\ d.f.} = -1.28$, $p = 0.227$) (Figure 3.2). There was no significant difference in the total number of worms that developed in cultures maintained at 19°C and 25°C ($t_{11\ d.f.} = 0.64$, $p = 0.53$) (mean ± S.E. of 6640 ± 875 at 19°C and 5940 ± 624 at 25°C).

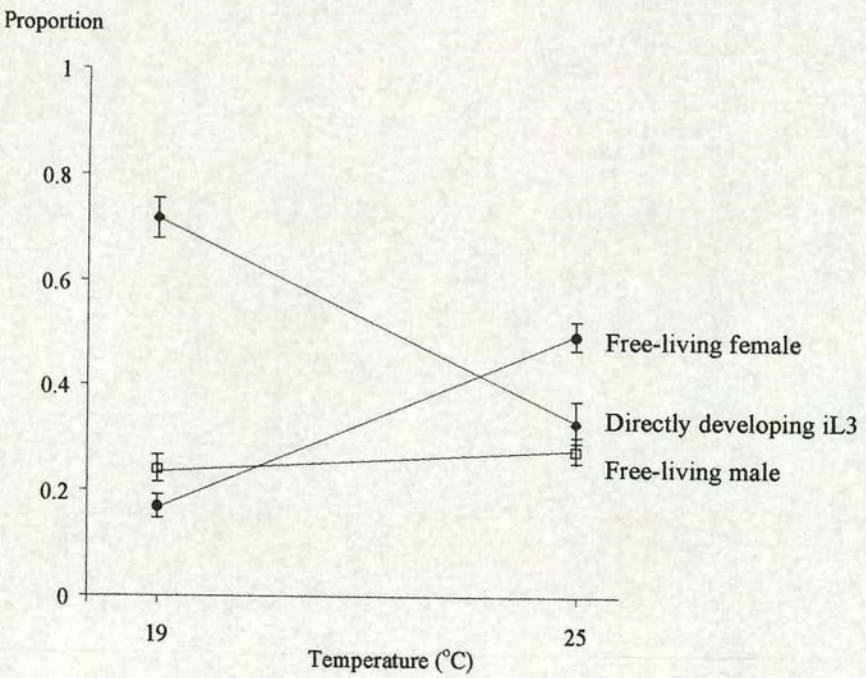


Figure 3.2 The effect of incubation temperature on the mean proportion of the parasitic females progeny that developed into free-living males (□), free-living females (●) and iL3s (◆) in faecal cultures maintained at 19°C and 25°C for twelve rats. Error bars are ± 1 S.E.

b. Incubation temperature and the total number of worms

Comparison of the nine groups of animals indicated that the effect of incubation temperature on the difference in the total number of worms that developed in faecal cultures maintained at 19°C and 25°C was the same in all groups of animals ($F_{93\ d.f.} = 0.84, p = 0.572$). Thus, data for all animals was combined and analysed to determine if incubation at 19°C or at 25°C caused a difference in the total number of worms. Analysis of the difference in the total number of worms between temperature treatments for all rats showed that the difference in the total number of worms between 19°C and 25°C was not significantly different from zero ($t_{93\ d.f.} = 0.08, p = 0.94$).

3.3.2 Incubation temperature and day p.i.

For each morph, the difference in the proportion of larvae that develop in cultures at 19°C and 25°C through infection for two rats is shown in Figure 3.3. Regression analysis of the difference in the proportion of larvae that developed into free-living males between 19°C and 25°C showed there was no significant effect of day p.i. ($t = 0.63, p = 0.57$ and $t = 0.64, p = 0.58$ for rats A and B, respectively). In contrast, as the infection progressed there was a significant increase in the difference between the proportion of larvae that developed into free-living females at 19°C and 25°C in rat A, but not in rat B ($t = 7.16, p = 0.006$ and $t = 2.66, p = 0.08$ for rats A and B, respectively). A significant increase in the difference between the proportion of larvae that developed into directly developing iL3s at 19°C and 25°C was observed in rat B, but not in rat A ($t = 3.34, p = 0.04$ and $t = 2.02, p = 0.14$ for rats A and B, respectively).

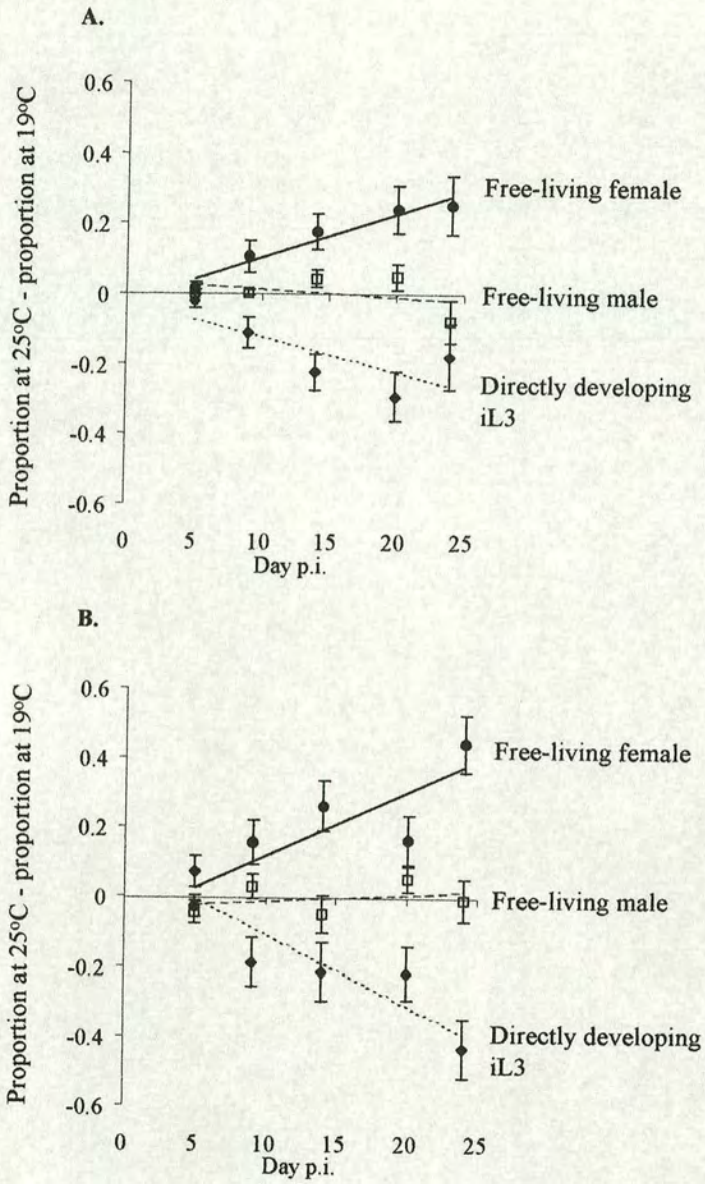


Figure 3.3 The difference in the proportion of free-living males (□), free-living females (●) and directly developing iL3s (◆) that developed in faecal cultures maintained at 19°C and 25°C over the course of infection, for two rats (A and B). Error bars are 95% confidence intervals for the differences between the proportions. Best fit regression lines are shown.

3.3.3 Immunity

a. Previous exposure

The proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females through an infection in the previously exposed and naive animals is shown in Figure 3.4. Analysis of the proportion of larvae that developed into free-living males identified significant effects of *Treatment* ($F_{1,4\ d.f.} = 40.47$, $p = 0.003$) and *Day p.i.* ($F_{4,16\ d.f.} = 12.28$, $p < 0.001$). In contrast, there was no significant *Treatment* by *Day p.i.* interaction ($F_{4,4\ d.f.} = 0.32$, $p > 0.5$). Analysis of the proportion of female larvae that developed into free-living females identified a significant effect of *Day p.i.* ($F_{4,16\ d.f.} = 3.45$, $p = 0.03$), with no significant effect of *Treatment* ($F_{1,4\ d.f.} = 4.04$, $p = 0.12$) or a *Treatment* by *Day p.i.* interaction ($F_{4,4\ d.f.} = 0.32$, $p > 0.5$) identified.

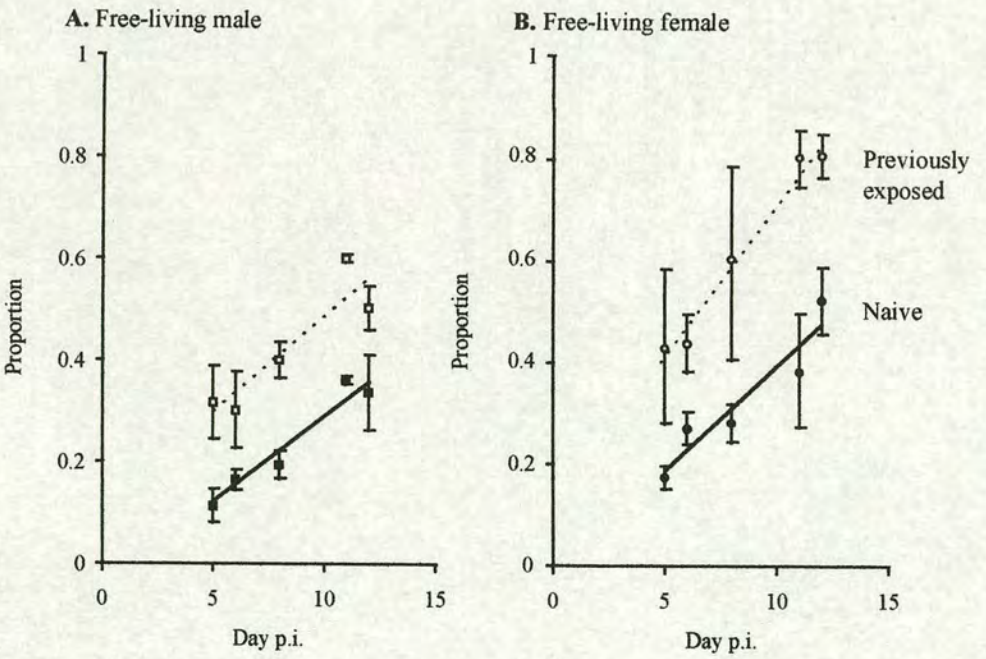


Figure 3.4 (A) The proportion of larvae that develop into free-living males and (B) the proportion of female larvae that develop into free-living females for previously exposed (open symbols) and naive animals (solid symbols). Plotted values are means of three rats (± 1 S.E.) and best fit regression lines for all data from each group are shown (····· and —— for the previously exposed and naive animals, respectively).

b. Congenitally hypothymic (nude) rats

The proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females through an infection in the hypothymic and intact animals is shown in Figure 3.5. The slopes of the regression lines calculated from the proportion of larvae that developed into free-living males from the hypothymic and intact animals were significantly different ($t_{6\ d.f.} = 4.70, p = 0.003$). The slopes of the regression lines calculated from the proportion of female larvae that developed into free-living females from the hypothymic and intact animals were also significantly different ($t_{6\ d.f.} = 4.45, p = 0.004$). In the hypothymic animals, the slopes of the regression lines calculated from the proportion of larvae that developed into free-living males were significantly different from zero ($t_{3\ d.f.} = 3.90, p = 0.03$), but those calculated from the proportion of female larvae that developed into free-living females were not ($t_{3\ d.f.} = 0.17, p = 0.88$).

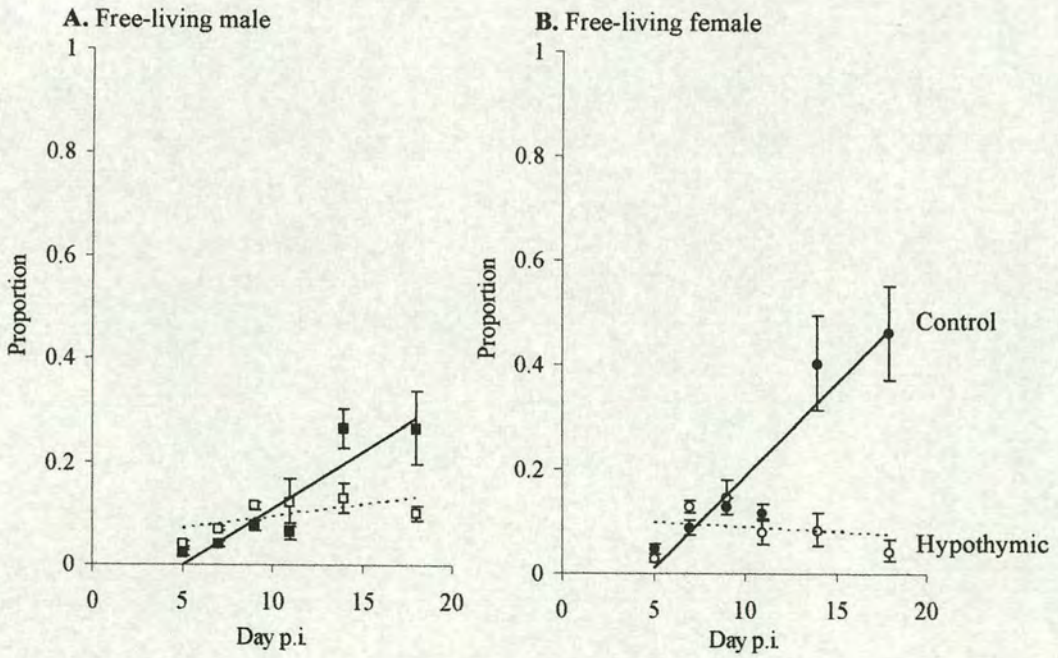


Figure 3.5 (A) The proportion of larvae that develop into free-living males and (B) the proportion of female larvae that develop into free-living females for hypothymic (open symbols) and intact animals (solid symbols). Plotted values are means of four rats (± 1 S.E.) and best fit regression lines for all data from each group are shown (····· and —— for the hypothymic and intact animals, respectively).

c. Whole body γ-irradiation

The proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females through an infection in the irradiated and non-irradiated animals is shown in Figure 3.6. The slopes of the regression lines calculated from the proportion of larvae that developed into free-living males from the irradiated and non-irradiated animals were significantly different ($t_{4\ d.f.} = 3.64$, $p = 0.02$). The slopes of the regression lines calculated from the proportion of female larvae that developed into free-living females from the irradiated and non-irradiated animals were also significantly different ($t_{4\ d.f.} = 3.24$, $p = 0.03$). In the irradiated animals, the slopes of the regression lines calculated from both the proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females were not significantly different from zero ($t_{2\ d.f.} = 0.52$, $p = 0.66$ and $t_{2\ d.f.} = 1.57$, $p = 0.26$, respectively).

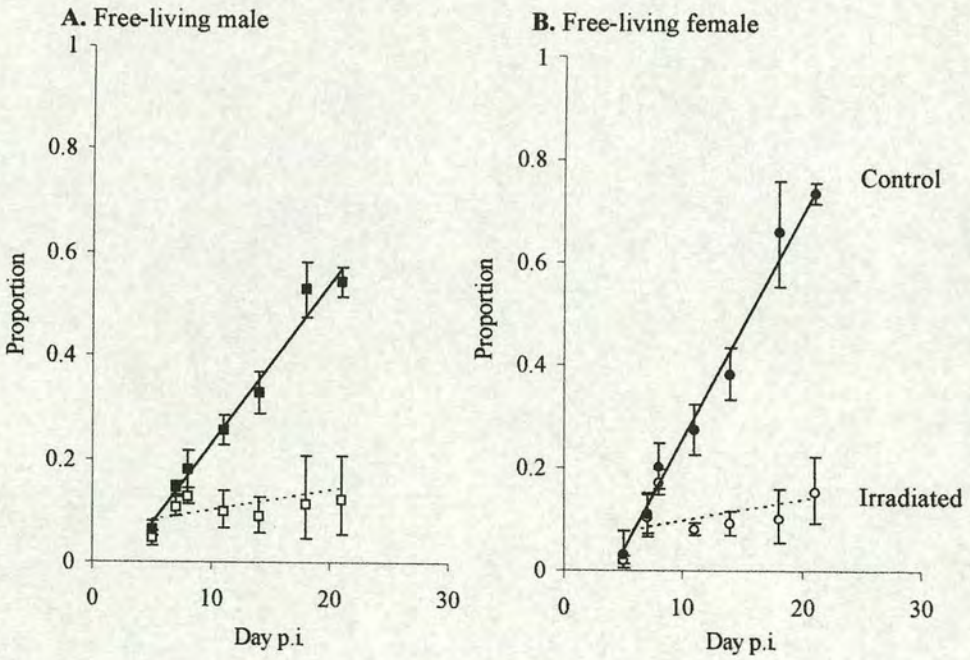


Figure 3.6 (A) The proportion of larvae that develop into free-living males and (B) the proportion of female larvae that develop into free-living females for irradiated (open symbols) and non-irradiated animals (solid symbols). Plotted values are means of three rats (± 1 S.E.) and best fit regression lines for all data from each group are shown (····· and —— for the irradiated and non-irradiated animals, respectively).

d. Corticosteroids

i. The proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females through an infection in the corticosteroid treated and untreated animals is shown in Figure 3.7. The slopes of the regression lines calculated from the proportion of larvae that developed into free-living males from the corticosteroid treated and untreated animals were significantly different ($t_{4\,d.f.} = 4.13$, $p = 0.02$). The slopes of the regression lines calculated from the proportion of female larvae that developed into free-living females from the corticosteroid treated and untreated animals were also significantly different ($t_{4\,d.f.} = 4.65$, $p = 0.01$). In the corticosteroid treated animals, the slopes of the regression lines calculated from both the proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females were not significantly different from zero ($t_{2\,d.f.} = 1.99$, $p = 0.19$ and $t_{2\,d.f.} = 1.78$, $p = 0.21$, respectively).

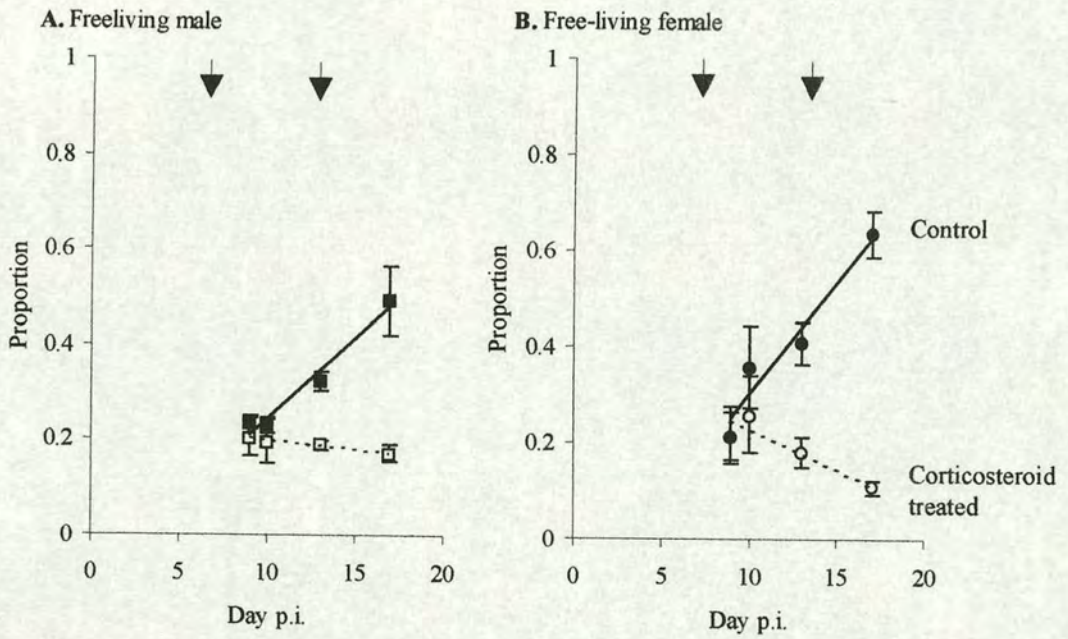


Figure 3.7 (A) The proportion of larvae that develop into free-living males and (B) the proportion of female larvae that develop into free-living females for corticosteroid treated (open symbols) and untreated animals (solid symbols). Plotted values are means of three rats (± 1 S.E.) and best fit regression lines for all data from each group are shown (····· and —— for the corticosteroid treated and untreated animals, respectively). Arrows indicate the start and end of the period of corticosteroid treatment.

ii. The proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females through an infection in the corticosteroid treated and untreated animals is shown in Figure 3.8. The slopes of the regression lines calculated from both the proportion of larvae that developed into free-living males and the proportion of female larvae that developed into free-living females from the corticosteroid treated and untreated animals were not significantly different ($t_{4\,d.f.} = 2.20$, $p = 0.09$ and $t_{4\,d.f.} = 2.42$, $p = 0.07$, respectively). In the corticosteroid treated animals, the slopes of the regression lines calculated from both the proportion of larvae that developed into free-living males and those calculated from the proportion of female larvae that developed into free-living females were not significantly different from zero ($t_{2\,d.f.} = 3.20$, $p = 0.09$ and $t_{2\,d.f.} = 2.86$, $p = 0.10$, respectively).

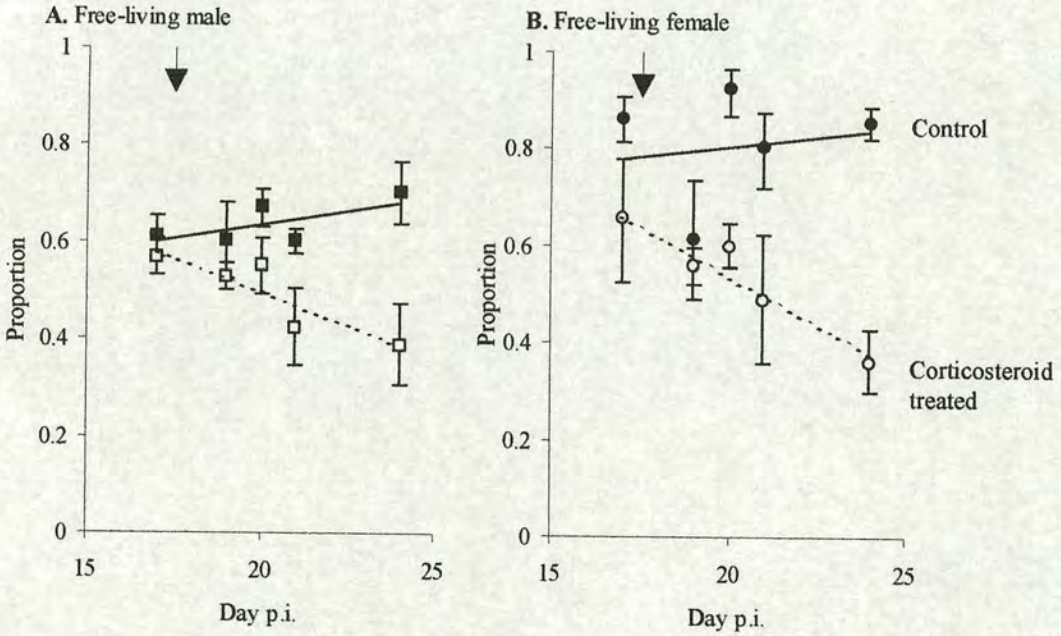


Figure 3.8 (A) The proportion of larvae that develop into free-living males and (B) the proportion of female larvae that develop into free-living females for corticosteroid treated (open symbols) and untreated animals (solid symbols). Plotted values are means of three rats (± 1 S.E.) and best fit regression lines for all data from each group are shown (····· and —— for the treated and untreated animals, respectively). Arrows indicate the point at which corticosteroid treatment began.

3.4 Discussion

This study has attempted to resolve the developmental events that occur in the life-cycle of *S. ratti* and to elucidate the developmental histories of the three alternative free-living morphs. Previous studies have suggested that the proportion of larvae that develop into free-living males is unaffected by extra-host environmental conditions and that the free-living females and directly developing iL3s are alternative female morphs (Minematsu *et al.* 1989; Taylor & Weinstein, 1990). This suggests a modified life-cycle for *S. ratti* containing two discrete developmental switches (Figure 3.1). Here this model of the life-cycle has been tested by manipulating the incubation of faecal cultures. This has demonstrated that the proportion of the progeny of the parasitic females that develop into free-living males is constant between faecal cultures maintained at 19°C and 25°C (Figure 3.2). In contrast, with the same temperature treatments, the proportion of larvae that develop into free-living females is highly negatively correlated with the proportion of larvae that develop into directly developing iL3s (Figure 3.2). It was also shown in this experiment that there is no change in the total number of worms of the fee-living phase that develop in cultures incubated at 19°C and 25°C.

It is biologically unlikely that these data are the result of differential mortality. Such a scenario would, as previously explained, indicate that any differential mortality was of an extremely complex and unlikely form. Therefore, the most parsimonious explanation for these findings is that the changes in the proportion of larvae that develop into free-living females and directly developing iL3s are not a trivial consequence of changes in the total number of worms that develop at 19°C and 25°C. This strongly suggests that the proportion of larvae that develop into free-living males is unaffected by incubation temperature and that there is a developmental switch between the free-living female and directly developing iL3 morphs. This free-living female/directly developing iL3 switch represents a form of phenotypic plasticity termed a developmental conversion, a threshold response that leads to a developmental switch between different phenotypes (Smith-Gill, 1983). These data agree with the conclusions of the previous studies of *S. ratti* (Minematsu *et al.* 1989; Taylor & Weinstein, 1990) and are consistent with the modified view of the life-cycle postulated here (Figure 3.1).

The proportion of larvae that develop into free-living males at 19°C and 25°C has also been shown to be constant over the course of an infection (Figure 3.3). In contrast, the difference in the proportion of larvae that develop into free-living females and directly developing iL3s between the two temperatures increases over the course of infection (Figure 3.3). This result suggests that the sensitivity of the free-living female/directly developing iL3 developmental conversion to incubation temperature increases over the course of infection. It is however unclear if this apparent change in the sensitivity of the free-living female/directly developing iL3 switch is a result of the host immune response or is a product of increasing parasitic female age. It would therefore be informative to replicate the incubation temperature and day p.i. experiment using both immunosuppressed and normal animals.

A possible analogy for the free-living female/directly developing iL3 switch in *S. ratti* can be found in the free-living nematode *Caenorhabditis elegans*. In *C. elegans*, second stage larvae can, dependent on environmental conditions, develop into the environmentally resistant third stage dauer larvae. This dauer/non-dauer switch is known to be controlled by a combination of signals, the most important of which are concentration of dauer pheromone, the food availability and the temperature at which the larvae develop (Golden & Riddle, 1984a & b). However, from the experiments reported here, it is unclear which extra-host factor is controlling the switch between the free-living female and directly developing iL3 morphs. The switch may be directly controlled by the incubation temperature or by a separate extra-host factor that is itself altered by temperature. Such a caveat is warranted as the results of a number of experiments on other *Strongyloides spp.* suggest the proportions of larvae that develop into free-living females and directly developing iL3s vary in response to such factors as food availability (Arizona, 1976; Shiwaku *et al.* 1988) and pH (Premvati, 1958; Moncol & Triantaphyllou, 1978). Control of the switch by the bacterial type and density of the faecal culture would not be surprising, as such factors are known to have a profound effect on the growth rate of free-living nematode species (Venette & Ferris, 1998). However, it is also possible that the free-living female/directly developing iL3 switch may even be mediated by host factors present in faecal cultures that degrade in a temperature dependent manner. However, there are important differences between

the dauer/non-dauer switch in *C. elegans* and the free-living female/directly developing iL3 developmental conversion in *S. ratti*. Firstly, increased temperature results in a greater number of *C. elegans* larvae developing via the dauer pathway, the opposite situation to that observed in *S. ratti*, where increased temperature results in a greater proportion of larvae developing into free-living females (Figure 3.2). Secondly, in *C. elegans*, both males and hermaphrodites can enter the dauer pathway, whereas in *S. ratti* it is apparent that only female progeny of the parasitic females can switch developmental routes. Thus, in *S. ratti* the male progeny of the parasitic females may have lost the ability to initiate "dauer-like" development.

It has previously been shown for *S. ratti* that changes in host immunity alter the proportion of larvae that develop by the indirect route (Gemmill *et al.* 1997). Here, the effects of host immune status on the developmental switches postulated in Figure 3.1 were analysed. From these experiments it is clear that, unlike incubation temperature, host immune status affects both the proportion of larvae that develop into free-living males and the proportion of female larvae that develop into free-living females. In the comparison of the naive and previously exposed animals, the significant effect of *Day p.i.* indicates that an increasing proportion of larvae develop into free-living males over the course of infection in both groups of animals (Figure 3.4A) and that an increasing proportion of female larvae develop into free-living females (Figure 3.4B). In addition, analysis of the proportion of larvae that developed into free-living males identified a significant effect of *Treatment*, indicating that in a greater proportion of larvae developed into free-living males in the previously exposed animals. However, no significant difference between the previously infected and naive animals could be detected in the proportion of female larvae that develop into free-living females. This result is slightly surprising, as previous exposure to *S. ratti* it is known to result in a strong protective immune response (Sheldon, 1937b; Moqbel & Denham, 1977; Olsen & Schiller, 1978). In addition, the changes to the ultrastructural morphology of *S. ratti* parasitic females that result from increasing host immunity are known to occur earlier in secondary infections (Moqbel & McLaren, 1980). However, the absence of any significant effect may be due to the small number of animals infected.

The immunological effects of the γ-irradiation (*3.3.3 b.*), thymus deficiency (*3.3.3 c.*) and the first of the two corticosteroid experiments (*3.3.3d.i*) are similar. In each case, the immune manipulation acts to block the development of a normal immune response to *S. ratti*. The results obtained from this group of experiments were also broadly consistent (Figures 3.5, 3.6 & 3.7). In all three experiments, the way in which the proportion of larvae that developed into free-living males changed over the course of infection differed between the control and the immunosuppressed animals. A similar effect was also observed on the proportion of female larvae that developed into free-living females. This indicates that both the proportion of larvae that develop into free-living males and the proportion of female larvae that develop into free-living females are dependent on the immunological state of the host. The three experiments were also consistent in that there was no observed change in either the proportion of female larvae that developed into free-living females or the proportion of larvae that developed into free-living males over the course of infection in the immunosuppressed animals. This implies that the changes that occur in the composition of the free-living phase over the course of a normal infection are solely due to the host immune response.

In contrast to the results of the first corticosteroid experiment, the comparison of animals treated with corticosteroids from day 18 p.i. with untreated controls (Figure 3.8) found no significant effects on either the proportion of larvae that develop into free-living males or the proportion of female larvae that develop into free-living females. The two corticosteroid experiments differed in that corticosteroid treatment was initiated at an earlier point in the first experiment and that the infection was therefore monitored over a longer period in the second experiment. It has been observed that the treatment of animals infected with *S. ratti* with corticosteroids reverses the decrease in parasitic female size that is caused by the developing immune response (Moqbel & Denham, 1978). In addition, treatment of *S. ratti* infected animals with corticosteroids halts the movement of the parasitic females from the anterior to the posterior small intestine, but it does not reverse this migration if it has already occurred (Moqbel & Denham, 1978). It may therefore be that the anterior to posterior migration did not occur in the corticosteroid treated animals from the first experiment due to the earlier start of corticosteroid treatment, but did occur in the second experiment. This possible difference in the

distribution of parasitic females between the two experiments may be the cause of the differences in the results.

Taken together the results of the immune manipulations indicate that both the proportion of larvae that develop into free-living males and the proportion of female larvae that develop into free-living females are affected by host immune status. Unfortunately, the consequences of the immunosuppressive agents used in these experiments are not fully understood (Jacobson, 1982). No general conclusions can therefore be drawn about the specific components of the immune system that trigger the observed developmental changes in *S. ratti*. Such understanding would have to derive from more specific methods of immunosuppression such as monoclonal antibodies directed against specific components of the immune system.

The findings reported here are consistent with the existence of two distinct developmental switches in the life-cycle of *S. ratti*. An intra-host male/female sex determination switch, which cytological studies suggest is genetic (Nigon & Roman, 1952; Bolla & Roberts, 1968) and a subsequent extra-host free-living female/directly developing iL3 developmental conversion. The change in the proportion of female larvae that develop into free-living females as a consequence of the host immune response (Figures 3.5, 3.6 & 3.7) may represent a third developmental switch. However, a more parsimonious explanation is that, as suggested by Figure 3.3, increased host immunity increases the sensitivity of the extra-host free-living female/directly developing iL3 developmental conversion to temperature. Even with this caveat, these findings support the life-cycle of *S. ratti* shown in Figure 3.1, but do not rule out the possibility that further developmental switches exist within the life-cycle. However, the life-cycle shown in Figure 3.1 is sufficient to explain the way in which the proportion of larvae that develop into free-living males, free-living females and directly developing iL3s vary as a consequence of intra- and extra-host factors and is consistent with the XX/X0 system of sex determination that has been postulated for *S. ratti* (Nigon & Roman, 1952; Bolla & Roberts, 1968). These findings also demonstrate how complex life-cycles, such as that of *S. ratti*, can be explained by combinations of simple developmental switches.

CHAPTER 4.

Sex determination in *Strongyloides ratti* I.

A. Attempt to isolate *S. ratti* homologues of the *Caenorhabditis elegans* sex determination genes *fox-1* and *xol-1*.

B. Identification of anonymous sex-linked DNA fragments in *S. ratti*.

ABSTRACT

The mechanism of sex determination in *Strongyloides ratti* is unclear, but cytological evidence suggests that it may be an XX/X0 system. Any genetic analysis of sex determination in *S. ratti* requires this XX/X0 hypothesis to be tested, and hence requires the isolation of X-linked DNA markers. Two approaches to the isolation of such markers were attempted.

a. Isolation of *S. ratti* homologues of the X-linked *Caenorhabditis elegans* sex determination genes *fox-1* and *xol-1* by low stringency PCR. This approach proved unsuccessful and is discussed in relation to the evolution of sex determination genes and nematode phylogeny.

b. Anonymous genomic DNA fragments were hybridised to dot blots of *S. ratti* free-living male and free-living female DNA. This approach identified three putatively sex-linked markers, apparently present at a higher copy number in free-living females than in free-living males. The sex-linkage of two of these markers, *Sr-mvP1* and *Sr-mvP2*, was confirmed by semi-quantitative PCR. These findings are consistent with the proposed XX/X0 system of sex determination. Southern blotting indicated there is only a single copy of *Sr-mvP1* within the *S. ratti* genome, but that the *S. ratti* genome contains multiple copies of *Sr-mvP2*. These findings are discussed in relation to sex determination in *S. ratti*.

4.1 Introduction

The life-cycle of *S. ratti* is shared with the majority of other *Strongyloides spp.* and has two adult generations, a female only parasitic generation and a dioecious free-living generation. The eggs of the parasitic female either develop through two larval stages into directly developing infective third stage larvae (iL3), or develop through four larval stages into free-living females and free-living males. Parasitological studies (Chapter 3) indicate that the life-cycle of *S. ratti* contains two discrete developmental switches, the first an intra-host male/female sex determination switch, and the second an extra-host free-living female/directly developing iL3 developmental conversion. It has also been shown that extra-host environmental factors do not alter the proportion of larvae that develop into free-living males (Chapter 3), a result that suggests sex determination in *S. ratti* is a GSD system. To date, all investigation of sex determination in *Strongyloides spp.* has been cytological. The studies of *S. ratti* have concluded that the diploid chromosome number of the free-living females is six, and that of the free-living males is five (Nigon & Roman, 1952; Abe & Tanaka, 1965; Bolla & Roberts, 1968). These results are consistent with an XX/X0 system of sex determination in *S. ratti* (see Chapter 1 for review).

A possible approach to the investigation of the hypothesis that sex determination in *S. ratti* is an XX/X0 system is to molecularly identify X chromosomes and to subsequently follow their inheritance through the life-cycle. Rather than attempting to isolate mutations in X-linked genes that result in a visible phenotype, molecular markers on the X chromosome were sought. There are a number of possible approaches to the isolation of X-linked markers in species thought to have an XX/X0 system of sex determination. Perhaps the most logical of these would be to use previously mapped and characterised polymorphisms or genes. Unfortunately, there has only been limited genetic analysis of *Strongyloides spp.* and, to date, there is no information available on the linkage of any of the sequences that have been isolated. Therefore, other possible approaches were attempted. The first of these was to isolate *S. ratti* homologues of X-linked genes from other nematode species and the second to isolate anonymous *S. ratti* DNA fragments that differed in their copy number between the free-living males and free-living females.

As the best genetically characterised nematode species is the free-living species *Caenorhabditis elegans*, an attempt was made to isolate *S. ratti* homologues of X-linked *C. elegans* genes. Sex determination in *C. elegans* is an XX/X0 system (see Chapter 1), but, as in all species with chromosomal differences between the sexes, the X chromosome of *C. elegans* contains many genes. The majority of these genes are unrelated to sex determination and X-linkage is not required for their correct expression. Thus, most X-linked genes are only present on the X chromosome by chance. However, in order to function correctly a number of the genes that control sex determination in *C. elegans* need to be X-linked. One of these is the *C. elegans* sex determination gene feminizing locus on X (*fox-1*). This gene is one of the two primary numerator elements that have been identified and plays a crucial early role in sex determination in *C. elegans*. Therefore, *fox-1* must be X-linked in order to function (Hodgkin *et al.* 1994). If an *S. ratti* homologue of *fox-1* was identified, and the gene function was conserved, the gene would also be present on the *S. ratti* X chromosome. In addition, an attempt was made to isolate an *S. ratti* homologue of another *C. elegans* sex determination gene, X0 lethal (*xol-1*). While *xol-1* is X-linked in *C. elegans*, this linkage is not required for gene function. However, as the isolation of *S. ratti* homologues of either *xol-1* or *fox-1* would provide a crucial first step in determining the molecular biology of sex determination in *S. ratti*, these two genes where chosen as targets for this work. If this approach led to the isolation of *S. ratti* genes related to either *xol-1* or *fox-1*, then the temporal expression pattern could be determined by mRNA analysis of different life-cycle stages. In addition, attempts could be made to alter the expression of the *S. ratti* genes, using RNA interference techniques, mutagenesis or the creation of transgenic strains with the gene of interest under the control of a heat-shock promoter.

In the attempt to amplify *S. ratti* homologues *xol-1* and *fox-1* a low-stringency polymerase chain reaction (PCR) approach was chosen. Amplifying gene homologues from related species by low-stringency or degenerate PCR has proved effective in a number of cases. In particular, PCR has recently been used to obtain sequences related to the human sex determination gene *sry* from a number of other mammalian species (Coriat *et al.* 1993). However, this technique is entirely dependent on the

conservation of either amino acid or nucleotide sequence between the genes. As no homologues of the *C. elegans* genes *xol-1* or *fox-1* have yet been isolated there is, unfortunately, no information on areas of conserved sequence. *S. ratti* homologues of the *C. elegans* small subunit ribosomal RNA (SSU rRNA) gene (Fisher, 1997) and an actin gene (Viney, 1994) have been isolated. However, the conservation of the nucleotide sequence of these genes between species is high, with the SSU rRNA genes representing one of the most highly conserved sequences yet identified. In the work reported here, in addition to the attempt to isolate *S. ratti* homologues of *xol-1* and *fox-1*, fragments of the *C. elegans xol-1* and *fox-1* genes were hybridised to Southern blots of both *C. elegans* and *S. ratti* genomic DNA. This was done to determine if DNA sequences related to these two genes are present in *S. ratti*.

The second approach employed in the identification of sex-linked and X-linked markers in *S. ratti* was to isolate anonymous DNA fragments that differed in their dose between the free-living males and free-living females. This was done by hybridising random fragments of *S. ratti* genomic DNA to dot blots of free-living male DNA and free-living female DNA. The ratio of the signal from the free-living male DNA to the signal from the free-living female DNA could then be used to identify sex-linked DNA fragments. The rationale was that if there is a chromosomal difference between the free-living males and free-living females, then sex-linked DNA fragments would produce a different ratio from that obtained from non-sex linked fragments. For instance, if sex determination is an XX/X0 system, then an autosomal DNA fragment would be present at an equal dose in the free-living males and free-living females and would be expected to yield a free-living male:free-living female ratio of 1:1. In contrast, an X-linked DNA fragment would be present at a higher copy number in the XX free-living females than in the X0 free-living males and would be expected to yield a free-living male:free-living female ratio of 1:2.

This approach would allow a large number of fragments to be screened rapidly and, in contrast to the first approach, no similarity to known sequences was required. In addition, this approach would also have the advantage of allowing the proposed XX/X0 system of sex determination

to be refuted if enough random DNA fragments were shown not to differ in their free-living male:free-living female ratio. Cytological studies have suggested that the chromosome complement of the free-living females is 2n = 6 and that of the free-living males 2n = 5 (Nigon & Roman, 1952; Bolla & Roberts, 1968). This would suggest that, unless the X chromosome in *S. ratti* is significantly smaller than the autosomes, a result not found during the cytological analyses of *S. ratti* (Nigon & Roman, 1952; Abe & Tanaka, 1965; Bolla & Roberts, 1968), approximately one third of random fragments would be X-linked. Analysis of the *C. elegans* genome has shown that the X chromosome is in fact slighter larger than the autosomes, with the X chromosome approximately 18 million base pairs (Mbp) in length and the average size of the autosomes approximately 16 Mbp (The *C. elegans* Sequencing Consortium, 1998). It is therefore a valid assumption that the X chromosome of *S. ratti* is significantly less than half the size of the autosomes.

In addition to their role in the identification of putatively X-linked DNA fragments in *S. ratti*, both of the primary screens described above are likely to result in the generation of a number of novel, uncharacterised DNA fragments. At present, relatively little is known of the genetics and genomics of parasitic nematodes and the generation of more sequence information from such species has been highlighted as an important future goal in nematology (Blaxter, 1998). Recently, this situation has started to change, with a number of new studies initiated to specifically address this problem. For example, recent work on the human filarial nematode *Brugia malayi* has resulted in the generation of 16,500 expressed sequence tags (ESTs) (Blaxter *et al.* 1999). Further generation of such data is important as it will allow a clearer picture of both gene and genome evolution in nematodes to be ascertained. To date, there has only been very limited genomic analysis of *Strongyloides spp.*. Two partial sequences from the human infective species, *S. stercoralis*, have been reported (Putland *et al.* 1993; Harrop *et al.* 1995) and a single study has systematically examined expressed sequences in this species (Moore *et al.* 1996). This study involved the generation and analysis of 57 unique ESTs from both the filariform and rhabditiform larvae of *S. stercoralis* (Moore *et al.* 1996).

The two approaches outlined above would, if successful, result in the isolation of putatively

sex-linked DNA fragments. However, neither approach would, *per se*, test the hypothesis that the sex determination in *S. ratti* is an XX/X0 system. In order to confirm the putative sex-linkage of any markers identified in the primary screens, their dose in the free-living males versus the free-living females should also be determined by another method. Here, this was done by semi-quantitative PCR. Semi-quantitative PCR is a versatile technique that allows the relative abundance of one locus with respect to another to be determined. The technique relies on the exponential increase in PCR products during the early stages of the reaction, where the number of copies doubles in each cycle. In semi-quantitative PCR, the locus of interest is simultaneously amplified with another locus, to yield the starting ratio between the two fragments. Confirmation of sex-linkage would indicate that there is a consistent genetic difference between the free-living males and females of *S. ratti* and be consistent with an XX/X0 system of sex determination. However, neither of the two primary screens or the secondary screen would determine the actual copy number of any sex-linked marker. Determination of copy number is vital for any sex-linked marker to be used in the analysis of X chromosome inheritance. Confirmation that the markers are single copy can be done by Southern blotting, at which point, single copy, sex-linked markers can be considered linked to the putative X chromosome of *S. ratti*. Polymorphisms identified in such markers would then allow sex determination and X chromosome inheritance in *S. ratti* to be analysed.

4.2 Isolation of *S. ratti* homologues of the *C. elegans* sex determination genes *fox-1* and *xol-1*

4.2.1 Materials and Methods

Worms and maintenance

Isofemale line ED321 Heterogonic (Viney, 1996) was used for all experiments and maintained by serial passage in randomly bred, size-matched, six week old female Wistar rats (Bantin & Kingman, UK). All experimental inoculations were by subcutaneous injection of 500 iL3s. Faeces from infected animals were collected and cultured as described previously (Chapter 3). Cultures were incubated at 19°C for three days to allow development of the free-living males, free-living females and directly developing iL3s. To obtain worms to be used in the preparation of genomic DNA, worms that had migrated away from the faecal mass and out of the watch glass were collected by pipetting from the water in the petri dish. The majority of these worms were directly developing iL3s, but free-living males and free-living females were also present. Worms were rinsed repeatedly in distilled water, transferred to 0.5 ml microfuge tubes, frozen in liquid nitrogen and stored at -20°C.

C. elegans N2 strain worms were obtained from Dr M. Blaxter (University of Edinburgh) and grown on 3 cm plastic petri dishes containing NGM agar* and seeded with OP50 strain *E. coli* (Wood, 1988). Worms were washed from plates with distilled water, rinsed in distilled water, transferred to 0.5 ml microfuge tubes, frozen in liquid nitrogen and stored at -20°C.

DNA extraction

S. ratti genomic DNA was prepared from approximately 25,000 mixed stage worms. These were frozen in liquid nitrogen and physically disrupted using polypropylene micro-homogenisers

* see Appendix 1.

(Biomedix), with this entire procedure repeated four times. The disrupted worm debris was incubated in a 1.5 ml microfuge tube with 0.5 ml TNESST* with SDS (Sigma) and sodium deoxycholate (Sigma) added to final concentrations of 0.3% w/v and 0.15% w/v, respectively, in the presence of 200 µg of Proteinase K (Roche Molecular Biochemicals). The reaction was then incubated at 55°C for 4 hours. After incubation, 0.5 ml of TE* equilibrated phenol was added, the tube was repeatedly inverted until the contents were thoroughly mixed and then centrifuged for 10 minutes at 13000×g. The aqueous layer was then transferred to a clean 1.5 ml microfuge tube and 0.5 ml of phenol : chloroform : isoamyl-alcohol (25:24:1) was added. The tube was repeatedly inverted until the contents were thoroughly mixed and then centrifuged for 10 minutes at 13000×g. The aqueous layer was then transferred to a clean 1.5 ml microfuge tube and the phenol : chloroform : isoamyl -alcohol (25:24:1) extraction repeated. The aqueous layer was then transferred to a clean 1.5 ml microfuge tube and 0.5 ml chloroform : isoamyl-alcohol (24:1) added, inverted several times and centrifuged for 5 minutes at 13000×g. The aqueous layer was then transferred to a clean 1.5 ml microfuge tube and two volumes of 100% ethanol and a one-tenth volume of 3M sodium acetate added. This was held at -20°C for approximately 12 hours (overnight) and then centrifuged for 30 minutes at 13000×g and the supernatant removed. The pellet was then rinsed briefly in 70% v/v ethanol, dried and resuspended in 50 µl distilled water. *C. elegans* genomic DNA was extracted from approximately 10,000 mixed stage worms as described above for *S. ratti* genomic DNA.

PCR amplification

The sequence of the *C. elegans* gene *xol-1* was obtained from Genbank and that of *fox-1* was obtained from Hodgkin *et al.* (1994). The sequence of the primers designed to these genes and the expected products are shown in Table 4.1. The primers designed to the sequence of *fox-1* were designed to flank the RNP domains, allowing these to be used in the identification of putative *S. ratti* homologues. The first of the two sets of primers designed to *xol-1*, P1 and P3, was designed to amplify a segment of one of the gene exons and the second set of primers, P5 and P3, were designed to span

* see Appendix 1.

one of the introns. PCR was then used in an attempt to amplify bands from *S. ratti* genomic DNA, with *C. elegans* genomic DNA as a positive control. The specificity of PCR reactions is dependent on the annealing temperature and the concentration of magnesium chloride in the reaction. The three primer combinations were tested in the following PCR reactions:- 1 minute denaturing at 95°C, 1 minute annealing varied between 45°C and 55°C, in 2°C intervals and 2 minute extension at 70°C for 45 cycles. All reactions were done in 50 µl volume, with primers at a final concentration of 100 nM, in 1 ×$MgCl_2$ free PCR buffer (Promega), $MgCl_2$ added to a final concentration of 1.5 mM, 0.75 µM each of dATP, dTTP, dCTP and dGTP (Roche Molecular Biochemicals), and *Taq* DNA polymerase (Promega) at 200 units ml^{-1} final concentration. Magnesium concentrations also were varied between 1 and 4µM in 0.5µM increments. Approximately 5 ng/µl of *S. ratti* or *C. elegans* genomic DNA was used as template for these PCR reactions. 5µl of each PCR reaction was electrophoresed in 1% w/v agarose gels containing ethidium bromide (0.5 µg/ml) in 1 × TAE buffer*. PCR reactions that resulted in the amplification of fragments from *S. ratti* genomic DNA were ligated into plasmids, which were subsequently transformed into chemically competent *E. coli* using the TA cloning kit (Invitrogen) and following the manufacturer's instructions. Plasmids containing the amplified fragments were then sequenced using the M13 forward and reverse primers and the dye terminator cycle sequencing ready reaction kit (Perkin Elmer) following the manufacturers' instructions. Sequencing reactions were run on an ABI prism 377 automated sequencer (Perkin Elmer).

Sequence	Primer	Sequence (5' to 3')	Expected product / bp
xol-1	P1	ACC CAG AAG ATT TCA CAC C	418
	P3	CTA GAG GTA AAC ACA ATG TG	
xol-1	P5	TT ATG AAC TCT GTA ATC GCC	650
	P3	CTA GAG GTA AAC ACA ATG TG	
fox-1	P6	GAT CCC AGC ACT AGC TCC GG	348
	P7	GTG CGG CAA GAG CTG ATT GG	

Table 4.1 The primer sequence and the size of the expected products for the primers designed for the *C. elegans* genes *xol-1* and *fox-1*.

* see Appendix 1.

Southern blotting

C. elegans and *S. ratti* genomic DNA was incubated overnight at 37°C with 20 units of one of the restriction endonucleases, *Hind*-III, *Xba*-I, *Dra*-I, *Bgl*-II or *Hind*-II (Roche Molecular Biochemicals) in a total volume of 20µl, with the appropriate incubation buffer at a 1× concentration. Digested DNA was electrophoresed in 1% w/v agarose gels, containing ethidium bromide (0.5 µg ml^{-1}), in 1 × TAE buffer and blotted onto Hybond N^+ (Amersham) as per the manufacturers' instructions. Blots were pre-hybridised for one hour in 7-10 ml of pre-hybridisation solution*.

To prepare probes, fragments of the *C. elegans xol-1* and *fox-1* genes that had been successfully amplified from *C. elegans* genomic DNA were purified of *Taq* and unincorporated nucleotides using PCR spin columns (Qiagen), following the manufacturer's instructions. Approximately 5 mg of this purified PCR product was radiolabelled with 1.85 MBq $\alpha^{32}P$ dCTP using a random primer labelling kit (Roche Molecular Biochemicals), following the manufacturers' instructions. 0.9 mg of sheared herring sperm DNA was then added, and the probe denatured by a 10 minute incubation in a boiling water bath and snap cooled by a 10 minute incubation in ice. This probe mixture was then added to the pre-hybridised blot and allowed to hybridise overnight at 65°C or 45°C.

Following hybridisation, the hybridisation solution was removed and blots were washed briefly in 100 ml of 3 × SSC*/0.1% w/v SDS*. Blots were then washed twice, at the temperature at which they had been incubated overnight, in 100 ml of 3 × SSC/0.1% w/v SDS for 30 minutes. Blots that had been hybridised at 65°C were then washed again, at 65°C, in 100 ml of 0.1×SSC/0.1% w/v SDS for 30 minutes. After washing, blots were covered in Saran wrap and exposed to XAR 5 film (Kodak) at -70°C for 24 hours. Blots were stripped of hybridised probe by washing twice in 500 ml of boiling 0.1% w/v SDS, then exposed to XAR 5 film (Kodak) at -70°C for 24 hours to confirm that hybridised probe had been removed.

* see Appendix 1.

Sequence analysis

Sequence fragments were assembled, where possible, using the GCG software (Wisconsin Package Version 10.0, Genetics Computer Group, Madison). Further sequence analysis and modification was done using the AssemblyLIGN programme (Oxford Molecular Group). The full sequence of each fragment or the sequence of the 3' and 5' ends of the fragment were then compared the sequence of the *C. elegans* genes *fox-1* and *xol-1*, using the GCG software. Sequences were also compared to existing sequences in Genbank (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) and to the *C. elegans* genomic, expressed sequence tag (EST) and peptide databases (Sanger Centre, http://www.sanger. ac.uk/Projects/C_elegans/blast_ server.shtml) using gapped Blastn and Blastx search programs (Gish & States, 1993; Altschul *et al.* 1997). All sequence analysis was last completed on 2/6/1999 and the results of these comparisons are shown in Appendix 2.

4.2.2 Results

PCR amplification

i. xol-1

Primers P1 and P3 (Table 4.1), amplified a single fragment of approximately 420 bp from *C. elegans* genomic DNA, corresponding with the size predicted from the sequence of the *C. elegans xol-1* gene sequence (Table 4.1). In contrast, fragments of approximately 1000 bp, 500 bp and 350 bp were amplified from *S. ratti* genomic DNA (see Figure 4.1A). Varying the reaction conditions did not produce any bands that corresponded in size to that predicted for the *C. elegans* gene fragment, with more stringent conditions giving no amplification from *S. ratti* genomic DNA. The three fragments amplified from *S. ratti* genomic DNA with the primers P1 and P3 (Figure 4.1A) were sequenced. This resulted in the isolation of five unique sequences and indicated that the PCR amplified fragment of approximately 500 bp was actually composed of three separate sequences of 508 bp, 507 bp and 476 bp. The primers P5 and P3 (Table 4.1), amplified a single fragment of approximately 650 bp from *C. elegans* genomic DNA. This size corresponds with that predicted from the sequence of the *C. elegans xol-1* gene sequence (Table 4.1). However, it was not possible to amplify any fragments from *S. ratti* genomic DNA using these primers (data not shown).

ii. fox-1.

The primers P6 and P7 (Table 4.1), did not amplify any fragments from *C. elegans* genomic DNA. When these primers were used with *S. ratti* genomic DNA, numerous fragments were observed that ranged in size from approximately 700 bp to approximately 50 bp (Figure 4.1B). Varying the reaction conditions either resulted in no amplification from *S. ratti* DNA or production of the numerous fragments shown in Figure 4.1B. Amplified fragments were cloned and individual clones were sequenced. This resulted in the sequencing of ten fragments amplified from *S. ratti* genomic DNA, ranging in size from 517 bp to 94 bp.

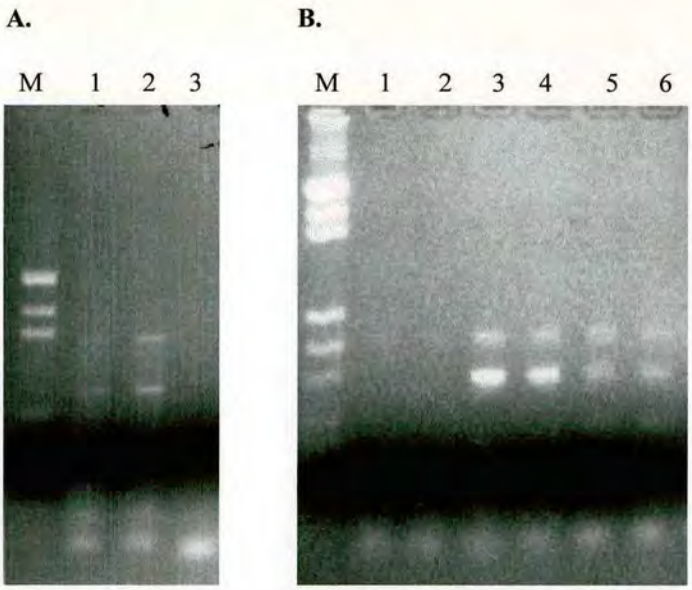


Figure 4.1 A. Fragments amplified from *S. ratti* genomic DNA with the primers P1 and P3, Lanes 1 and 2 show amplification from *S. ratti* genomic DNA, lane 3 shows the -ve control. B. Fragments amplified from *S. ratti* genomic DNA with the primers P6 and P7, Lanes 3 - 6 show amplification from *S. ratti* genomic DNA, lane 1 and 2 show -ve controls. M denotes molecular weight markers.

Sequence analysis.

Comparison of both the nucleic acid and the predicted amino acid sequences of the fifteen fragments amplified from *S. ratti* genomic DNA with the primers designed to the *C. elegans fox-1* and *xol-1* genes indicated that none of the *S. ratti* fragments were similar to either *fox-1* or *xol-1*. The DNA sequence obtained for the fragments and further analysis of the sequences is shown in Appendix 2.

Southern blotting.

A Southern blot containing *S. ratti* and *C. elegans* genomic DNA was probed with a 650 bp fragment of the *C. elegans xol-1* gene, which had been amplified from *C. elegans* genomic DNA with

the primers P5 and P3 (Table 4.1). Hybridisation was carried out at both at 65°C and 45°C. The hybridisation at 45°C is shown in Figure 4.2A. There were no differences in the hybridisation of the probe to the *C. elegans* genomic DNA at 45°C and 65°C (data not shown). There was no hybridisation of the *C. elegans xol-1* gene fragment to *S. ratti* DNA at either hybridisation temperature (Figure 4.3A). Figure 4.2B shows the same blot probed with a 572 bp fragment of the *S. ratti* LSU rRNA gene at 65°C. In this case, the probe hybridised to both the *C. elegans* and *S. ratti* DNA, indicating that *S. ratti* DNA is present on the blot.

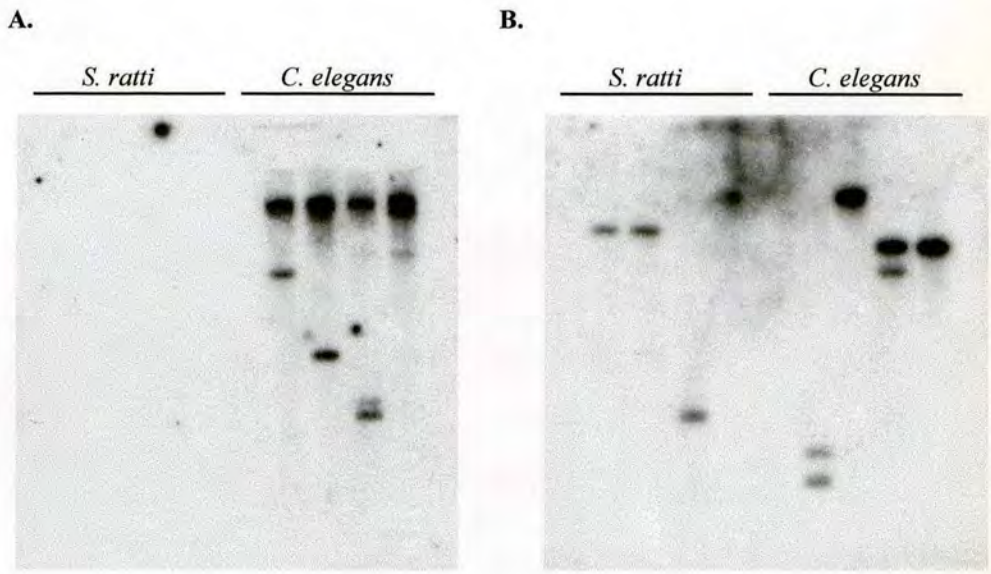


Figure 4.2 Southern blot of *S. ratti* and *C. elegans* genomic DNA A. Probed with a 650 bp fragment of the *C. elegans xol-1* gene at 45°C B. The same blot subsequently probed with a 572 bp fragment of the *S. ratti* LSU rRNA gene at 65°C.

4.3 Identification of anonymous sex-linked DNA fragments in *S. ratti*

4.3.1 Materials and Methods

Parasites and maintenance

Isofemale line ED321 Heterogonic (Viney, 1996) was used for all experiments, and was maintained as described in 4.2.1. Faeces from infected animals were collected and cultured as described previously (Chapter 3). Worms to be used in the preparation of genomic DNA were collected as described previously (4.2.1). To collect worms of known sex for the extraction of free-living male and free-living female genomic DNA, worms were removed from around the faecal mass of cultures that had been maintained at 19°C for three days, rinsed repeatedly in distilled water and placed in a petri dish containing distilled water. Free-living males and free-living females were identified by microscopy and pipetted into 0.5 ml microfuge tubes. When each tube contained either 100, 150 or 200 worms, these were frozen in liquid nitrogen and stored at -20°C. To collect worms for single worm PCR preparations, single worms, collected and identified as described above, were individually pipetted, in 5 µls of water, into microfuge tubes and frozen at -20°C.

DNA extraction

Genomic DNA was extracted from mixed stage worms as previously described (4.2.1). Free-living male and free-living female DNA was isolated by the same method from the frozen free-living males and free-living females. For the single worm PCR preparations, 14.0 µg of proteinase K (Roche Molecular Biochemicals) and 0.5 µls of 10× PCR buffer without magnesium chloride ($MgCl_2$) (Promega) were added to each microfuge tube. Tubes were subsequently placed in a -70°C freezer for 30 minutes, then maintained at 55°C for 90 minutes and 95°C for 30 minutes. Single worm PCR preparations were then stored at -20°C until required.

Dot blots

The genomic DNA, extracted as previously described, from 2500 free-living male worms and 2500 free-living female worms was divided equally, by volume, into five aliquots for each sex and blotted onto Hybond N+ (Amersham) using a Bio-Dot microfilter (Bio-Rad) following the manufacturers' instructions. Probes (see below) were labelled, hybridised to blots and the blots washed as previously described for Southern blots (4.2.1). After washing, labelled blots were exposed to a Phosphorimager screen (Molecular Dynamics) for four hours and the resulting signal quantified using the analysis software Imagequant (Molecular Dynamics). In each case, the signal obtained from each aliquot of free-living male and free-living female DNA was quantified and a series of background values were taken. The use of a Phosphorimager in this situation has several advantages over film, chiefly that accurate quantification of intensity is possible over a wide range of intensities and that the screens are not easily saturated. Blots were stripped of hybridised probe as described for Southern blots (4.2.1) and then exposed to a Phosphorimager screen for four hours to obtain a background value for subsequent probes. To calculate the free-living male to free-living female ratio for each probe, the mean signal obtained from the five aliquots of free-living male DNA was divided by the mean signal obtained from the five aliquots of free-living female DNA. The mean signal obtained from the five aliquots of free-living male and free-living female DNA was calculated as the sum of the signals from each point minus the sum of the background (if any) due to previous probes from those points, minus the background from that probe, divided by the number of observations.

To analyse the results of the dot blotting, the probability that the observed data would occur by chance was calculated. This probability was determined by the equation: $(1 - x)^n$, where n is equal to the number of fragments hybridised to the blots and x is the probability of a fragment being X-linked. The number of fragments which had to be shown not to be X-linked in order to reject the hypothesis that sex determination in *S. ratti* is an XX/X0 system with 99% confidence (*i.e.* $p < 0.01$) is therefore $0.01 = (1- x)^n$. Rearranging this equation gives $n = \log(0.01)/\log(1 - x)$. Therefore, if the X chromosome is the same size as the autosomes, the chance of a random fragment being X-linked will

be $^1/_3$. Therefore, n = log(0.01)/log(1 - $^1/_3$) = 11.36. Thus, if no sex-linked fragments were identified out of 12 random fragments then the hypothesis that the *S. ratti* X chromosome is the same size as the autosomes can be rejected with 99% probability. If the X chromosome is half the size of the autosomes, the chance of a random fragments being X-linked will be $^1/_6$, and n = log(0.01)/log(1 - $^1/_6$) = 25.26. Therefore, 26 fragments must be screened and shown not to be X-linked before that hypothesis can be rejected.

Random genomic DNA markers

The random genomic DNA markers screened against the dot blots came from a number of sources. DNA fragments isolated from a pZErO (Invitrogen) library made specifically as a source of markers; a microsatellite library constructed and donated by Dr M. Fisher; a number of other markers isolated by Dr M. Fisher; and a cDNA library constructed and donated by Dr M. Viney. The pZErO library was constructed from *S. ratti* genomic DNA, prepared as previously described and hence consisting of DNA from directly developing iL3s. DNA was digested to completion with the restriction endonuclease *EcoR*-1 (Promega) in a total volume of 20 µl, with the appropriate incubation buffer at a 1 × concentration. 5 µl was electrophoresed in a 1% w/v agarose gel containing ethidium bromide (0.5 µg/ml) in 1 × TAE buffer to confirm digestion. Digested DNA was then ligated into the pZErO plasmid (Invitrogen), which was transformed into chemically competent Top10 F' cells as per manufacturer's instructions (Invitrogen). Transformed cells were selected on IPTG/Kanamycin agar plates*, colonies picked and grown in LB/Kanamycin medium*. The size of each insert was then determined by PCR from the M13 forward and M13 reverse primer sites.

Semi-quantitative PCR.

Primers for the marker 27, subsequently named *Sr-mvP1*, were supplied by Dr M. Fisher. The 5' and 3' ends of the marker pZ-2, subsequently named *Sr-mvP2*, were sequenced as previously

* see Appendix 1.

described (4.2.1) and PCR primers were then designed to these sequences. In both cases, the other locus used was xol.22/XP1, a marker isolated in the attempt to amplify *S. ratti* homologues of the *C. elegans* sex determinations *fox-1* and *xol-1* (4.2.1). XP1 had previously been sequenced (4.2.2) and primers were designed to this sequence. To optimise PCR reaction conditions, each locus was amplified from *S. ratti* genomic DNA using various reaction conditions, as described in 4.2.1. Success was judged by the reproducibility of the amplification and by the intensity of the amplified fragment as visualised after electrophoresis in 1% w/v agarose gels containing ethidium bromide (0.5 μg/ml) in 1 × TAE buffer. The two loci PCRs were then optimised in the same manner, first using *S. ratti* genomic DNA and then single worm PCR preparations. After optimisation, semi-quantitative radioactive PCR was performed on ten free-living males and ten free-living females using the optimised conditions shown in Table 4.2. All semi-quantitative PCR reactions were carried out in a Hybaid touchdown thermal cycler without oil. Radioactive PCRs were electrophoresed in 1.5% (w/v) agarose gels in 1× TBE* buffer, without ethidium bromide, and dried onto gel drying film (Promega). Dried gels were exposed to a Phosphorimager screen for four hours and the signal from each band and a measure of the background quantified using the Imagequant analysis software (Molecular Dynamics). The ratio of the putatively sex-linked fragment to XP1 was calculated for each free-living male and free-living female as: (the signal from the putatively sex-linked fragment - the signal from the background) / (the signal from XP1 - the signal from the background).

Southern blotting

S. ratti genomic DNA was incubated overnight at 37°C with 20 units of one of the restriction endonucleases, *Hin*d-III, *Xba*-I, *Dra*-I, *Bgl*-II or *Hin*d-II, as previously described. Probes used were purified, PCR amplified, fragments of 27/*SrmvP1* and pZ-2/*Sr-mvP2*. Blots were made, pre-hybridised and probes labelled as previously described (4.2.1).

* see Appendix 1.

Locus	Primer sequence (5' to 3')	Expected size / bp	PCR conditions
Sr-mvP1	F CGT ATC TTG CGA TGA TCA TC R GAT ATC AGT TGC AAA ACT ACC	1230	95°C 1min, 54°C 1min, 72°C 3mins for 40 cycles and 10min at 70°C
Sr-mvP2	F CCA TAG AAG GTA TGT ATT A R CTA TCT TGA AAC AGA ACC C	1300	95°C 1min, 52°C 1min, 70°C 2mins for 40 cycles and 10min at 70°C
XP1	F ACT TGG ACA TTT CGA ATT GG R TAA GGA AAC TCC CAT CTG G	795	95°C 1min, 52°C 1min, 70°C 2mins for 40 cycles and 10min at 70°C
Sr-mvP1 and XP1	*Sr-mvP1* F and R XP1 F and R	1230 & 795	95°C 1min, 54°C 1min, 72°C 3mins for 20 cycles and 10min at 70°C
Sr-mvP2 and XP1	*Sr-mvP2* F and R XP1 F and R	1300 & 795	95°C 1min, 52°C 1min, 70°C 2mins for 20 cycles and 10min at 70°C

Table 4.2 Primers, expected products and the optimised amplification conditions for the putatively sex-linked markers *Sr-mvP1* and *Sr-mvP2* and the autosomal control XP1. All reactions were done in 50 µl volume, with primers at a final concentration of 100 nM, in 1 × $MgCl_2$ free PCR buffer (Promega), with $MgCl_2$ at 1.5 mM final concentration, 0.75 µM each of dATP, dTTP, dCTP and dGTP (Roche Molecular Biochemicals) and Taq DNA polymerase (Promega) at 200 units ml^{-1} final concentration. For the semi-quantitative radioactive PCRs, 0.01 MBq of α^{32}P dCTP was added to each reaction.

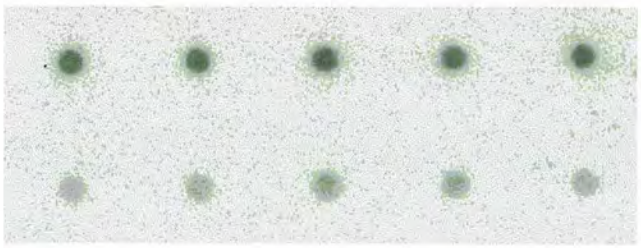
4.2.2 Results

Dot blots

In total, three dot blots of free-living male and free-living female genomic DNA were used. The free-living male/free-living female ratio was calculated for a total of 22 unique markers, see Figure 4.3 for examples of these hybridisations. The name, source and free-living male/free-living female ratio of these markers are shown in Table 4.3 and the corrected free-living male/free-living female ratios are shown in Figure 4.4. The corrected ratio was calculated by obtaining the mean free-living male/free-living female ratio of those markers not considered to be sex-linked on a blot and dividing all the ratios obtained from that blot by this figure. This calculation acts to correct for differences in the amounts of free-living male and free-living female DNA present on each blot and allows the ratios produced from different blots to be more accurately compared. Note that the number of probes used against each of the dot blots gives a total of 24 probes, rather than 22, as two of the probes were used against more than one blot (see Table 4.3).

Of the 22 markers, 19 did not produce ratios that would be expected for sex-linked sequences. The remaining three markers were identified as putatively sex linked since the free-living male/free-living female ratio produced suggested that the sequences were present at a lower copy number in free-living males than in free-living females. The three putative sex-linked markers identified were 27 (Fisher, 1997), pZ2 and pZ4. In accordance with the standard terminology for parasitic nematodes (Bird & Riddle, 1994) these markers were renamed *Sr-mvP1*, *Sr-mvP2* and *Sr-mvP3*, respectively. *Sr* indicating the species (*S. ratti*), *mv* indicating the laboratory designation (Mark Viney) and *P* indicating that the markers are physical sites within the *S. ratti* genome. Note that the putatively sex-linked marker 27/*Sr-mvP1* was one of the probes that were hybridised to two of the dot blots, giving two different corrected free-living male/free-living female ratios (Table 4.3).

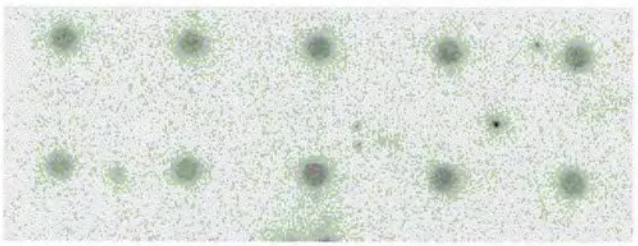
A.



Free-living female genomic DNA

Free-living male genomic DNA

B.



Free-living female genomic DNA

Free-living male genomic DNA

Figure 4.3 A dot blot of *S. ratti* free-living male and free-living female genomic DNA. A. Probed with pZ-2/*Sr-mvP2* at 65°C. B. Probed with BSP-8, a previously identified polymorphic loci (Fisher, 1997) at 65°C.

Blot	Probe	Source	M/F ratio	Comments
1	300596.01	cDNA	0.884	
	300596.15	cDNA	1.065	
	XP1	*fox-1/xol-1*	1.170	
	280696.06	cDNA	1.125	
	fox6.8	*fox-1/xol-1*	-	
	300596.30	cDNA	-	
	280696.15	cDNA	0.991	
	280696.07	cDNA	0.950	
	280696.11	cDNA	0.904	
	CM2	random	-	
	27 (*Sr-mvP1*)	MF	0.624	Putatively sex-linked
	16S	MF	1.311	Mitochondrial sequence
	300596.14	cDNA	1.029	
	B1B	random	-	
	Q13	MF	-	
	xol23	*fox-1/xol-1*	-	
2	300596.21	cDNA	2.616	
	300596.19	cDNA	2.469	
2	300596.18	cDNA	2.502	
	CM6	random	-	
	BSP-8	MF	2.547	
	18S	MF	2.315	
	300596.20	cDNA	2.679	
	Q14	MF	-	
	fox1.5	*fox-1/xol-1*	-	
3	27 (*Sr-mvP1*)	MF	0.714	Putatively sex-linked
	BSP-8	MF	0.938	
	pZ-1	pZErO	1.394	
	pZ-2 (*Sr-mvP2*)	pZErO	0.235	Putatively sex-linked
	pZ-4 (*Sr-mvP3*)	pZErO	0.532	Putatively sex-linked
	pZ-7	pZErO	1.893	
	pZ-8	pZErO	1.397	
	RP8	random	-	
	pZ-5	pZErO	0.950	
	pZ-11	pZErO	-	

Table 4.3 The name, source, and free-living male/free-living female ratio for the markers screened against the *S. ratti* dot blots. MF denotes those markers donated by M.C. Fisher, cDNA denotes those markers donated by M.E. Viney, pZErO denotes the markers isolated from the pZErO library, *fox-1/ xol-1* denotes markers isolated during the attempt to isolate *S. ratti* homologues of the *C. elegans* sex determination genes *xol-1* and *fox-1* and random denotes markers isolated during preliminary studies. - indicates that the marker did not successfully hybridise to the dot blots.

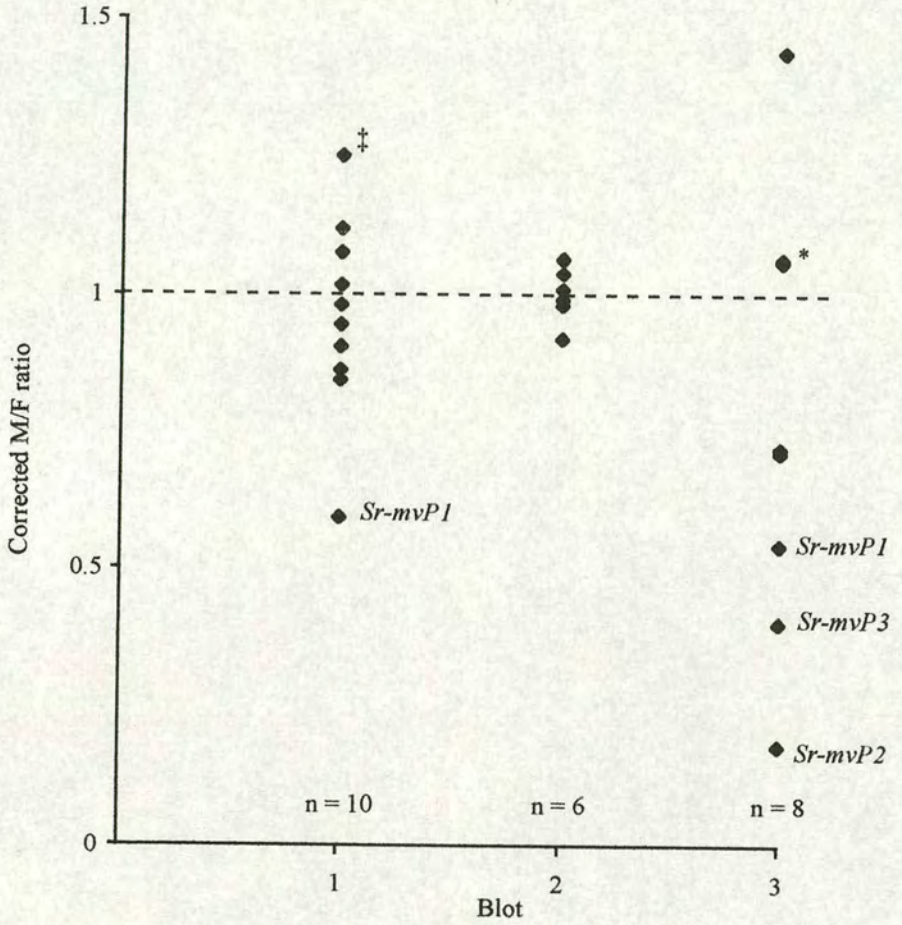


Figure 4.4 The corrected free-living male/free-living female (M/F) ratio for the three dot blots. The ratios obtained for the three putatively sex linked markers *Sr-mvP1*, *Sr-mvP2* and *Sr-mvP3* are indicated. n indicates the number of probes used against each dot blot that produced quantifiable signals. The dashed line indicates a corrected M/F ratio of one, a ratio that would be expected for markers present at equal copy number in the free-living males and the free-living females. The point marked by a * represents the corrected M/F ratios of two markers used against that blot. The point marked by a ‡ indicates the mitochondrial 16S marker.

Semi-quantitative PCR

Analysis of the putatively sex-linked marker *Sr-mvP1* by semi-quantitative radioactive PCR showed that it is present at significantly higher copy number in free-living females than in free-living males. The quantified *Sr-mvP1*/XP1 ratio for the free-living males and free-living females from the semi-quantitative radioactive PCR of *Sr-mvP1* and XP1 is shown in Figure 4.5. Analysis by unpaired *t* test indicated that the *Sr-mvP1*/XP1 ratios of the free-living males were significantly different from those of the free-living females ($t = 4.53$, 17 d.f., $p < 0.001$).

Analysis of the putatively sex-linked marker, *Sr-mvP2*, by semi-quantitative radioactive PCR also showed that it is present at a higher copy number in free-living females than free-living males. The quantified *Sr-mvP2*/XP1 ratio for the free-living males and free-living females from the semi-quantitative radioactive PCR of *Sr-mvP2* and XP1 is shown in Figure 4.6. Analysis by unpaired *t* test indicates that there is a significant difference between the *Sr-mvP2*/XP1 ratios of the free-living males and those of the free-living females ($t = 3.13$, 17 d.f., $p = 0.006$).

Comparison of Figure 4.5 with Figure 4.6 indicates that the mean ratio for the sex-linked marker/XP1 differs between *Sr-mvP1* and *Sr-mvP2*. The mean ± S.E. of the *Sr-mvP1*/XP1 ratios calculated for the free-living males and free-living females were 0.62 ± 0.03 and 0.79 ± 0.03, respectively. In contrast, the mean ± S.E. of the *Sr-mvP2*/XP1 ratios for the free-living males and free-living females were 1.42 ± 0.14 and 2.08 ± 0.16, respectively.

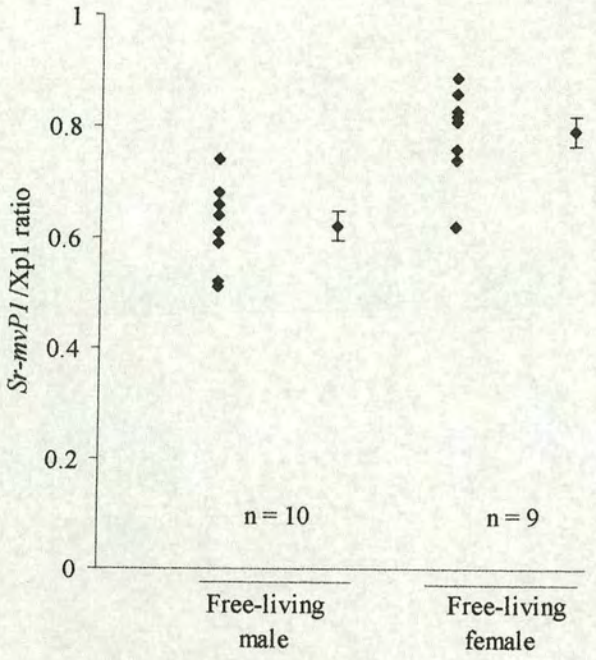


Figure 4.5 The *Sr-mvP1*/XP1 ratios and the mean ratio ± the S.E. for the free-living males and free-living females obtained from the 20 cycle semi-quantitative radioactive PCR of *Sr-mvP1* and XP1.

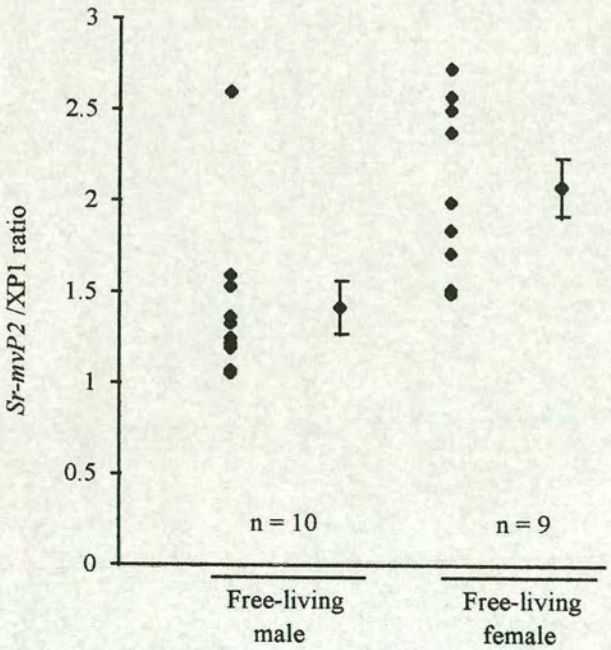


Figure 4.6 The *Sr-mvP2*/XP1 ratios and the mean ratio ± the S.E. for the free-living males and free-living females obtained from the 20 cycle semi-quantitative radioactive PCR of *Sr-mvP2* and XP1.

The hybridisation of the 1230 bp fragment of *Sr-mvP1* to a Southern blot of *S. ratti* genomic DNA is shown in Figure 4.7A. As can be seen, all lanes contain only a single band greater in size than 1400 bp, suggesting that the marker is single copy. In contrast, the hybridisation of the 1300 bp fragment of *Sr-mvP2* to the same Southern blot, after it had been stripped of previous probe, is shown in Figure 4.7A. As can be seen, lanes 2, 3 and 6 (Figure 4.7B) contain multiple bands that are greater in size than 1400 bp, suggesting that there are multiple copies of *Sr-mvP2* within the *S. ratti* genome.

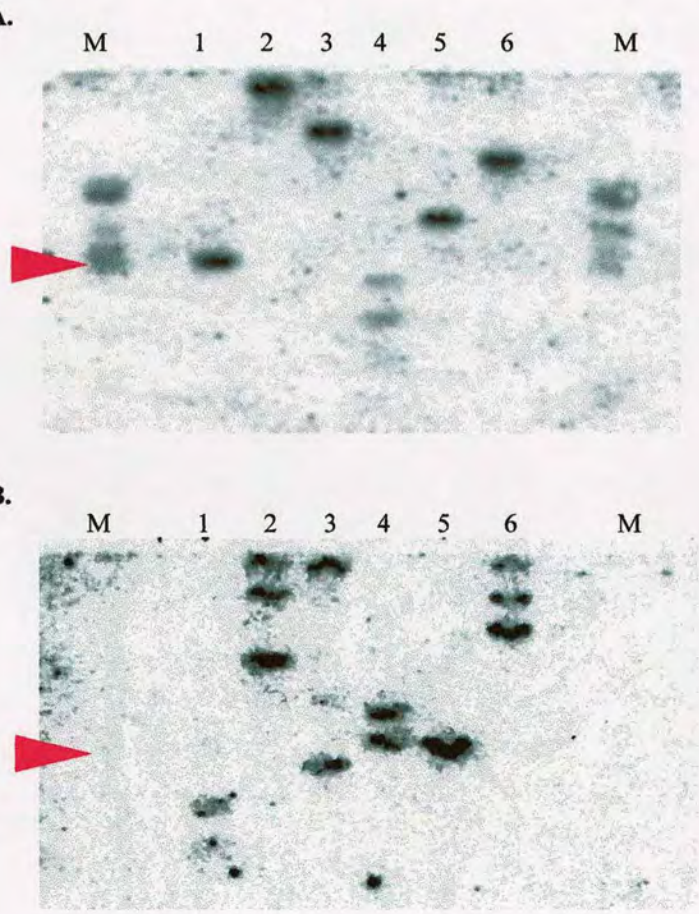


Figure 4.7 Southern blot of *S. ratti* genomic DNA. A. hybridised with the putatively sex-linked marker *Sr-mvP1* at 65°C. B. Hybridised with the putatively sex-linked marker *Sr-mvP2* at 65°C. M denotes molecular weight marker (visible only in A). The arrow indicates a fragment size of 1400 bp.

4.4 Discussion

The attempt to isolate *S. ratti* homologues of the X-linked *C. elegans* sex determination genes *fox-1* and *xol-1* by low stringency PCR was not successful. In the case of both sets of primers designed to the *C. elegans xol-1* gene, a fragment of the expected size could be amplified from *C. elegans* genomic DNA. However, the primers designed to amplify a fragment of *fox-1* failed to amplify any bands from *C. elegans* genomic DNA. The cause of this is unclear as, although the primers were designed to the sequence of the *fox-1* cDNA (Hodgkin *et al.* 1994), comparison of this cDNA with the genomic sequence indicates that the primers should amplify a single band of 519 bp. When *S. ratti* genomic DNA was used as PCR template, both the *xol-1* P1/P3 primers and the *fox-1* P6/P7 primers amplified multiple fragments (Figure 4.1A and 4.1B, respectively). In contrast, the *xol-1* P5/P3 primers did not amplify any fragments from *S. ratti* genomic DNA. However, comparison of the sequence of the fragments amplified from *S. ratti* genomic DNA with that of *xol-1* and *fox-1* showed that none of the sequenced fragments were homologous to either of the *C. elegans* genes.

The failure of the PCR based attempt to isolate *S. ratti* homologues of the *C. elegans* sex determination genes *xol-1* and *fox-1* could have a number of causes. At a mechanistic level, degenerate primers could have been designed to the amino acid sequence of the genes, rather than the non-degenerate primers that were used. However, such degenerate primers would have resulted in the amplification of a much larger number of fragments. In addition, the failure of the fragment of the *C. elegans xol-1* gene fragment to hybridise to *S. ratti* genomic DNA (Figure 4.2 A), suggests that this gene may not be present in *S. ratti* or that the nucleic acid sequence may be so diverged that amplification would not be possible even with degenerate primers. Such conclusions may be warranted in light of a number of recent studies. Firstly, a recent study has calculated the molecular phylogeny of nematodes (Blaxter *et al.* 1998) using the sequence of a fragment of the small subunit ribosomal RNA (SSU rRNA) gene. Previous phylogenetic trees for nematodes were based on morphology and, in parasitic nematodes, on the distribution of the parasites within host taxa. These trees have now been shown to be inaccurate, with the results of the analysis of the SSU rRNA gene sequences showing that

S. ratti and *C. elegans* are not, as had previously been believed, part of the same clade (Blaxter *et al.* 1998).

There is also evidence that suggests that genes involved in sex determination are evolving more rapidly than those involved in other genetic pathways (Kuwabara & Shah, 1994; De Bono & Hodgkin, 1996; Marin & Baker, 1998). In a study of gene evolution in nematode sex determination, the *C. briggsae* homologue of the *C. elegans* gene transformer-2 (*tra-2*) could not be identified by nucleotide homology. It was eventually identified as a result of cloning by synteny, an approach dependent on the conservation of gene linkage rather than sequence conservation (Kuwabara & Shah, 1994). Subsequent comparison of the *C. elegans tra-2* gene with *C. briggsae tra-2* gene, revealed that amino acid sequence similarity was unexpectedly low (43%), although there was a degree of conservation in the control regions (Kuwabara, 1996). This unusual degree of sequence divergence was also found when the *tra-1* genes from *C. elegans* and *C. briggsae* were compared. Here, there was only a 44% amino acid similarity between the two species, much lower than that for previously compared genes from the two species (De Bono & Hodgkin, 1996). These results led the authors to conclude that the evolution of sex determination in nematodes is both rapid and genetically complex (De Bono & Hodgkin, 1996). These findings agree well with theoretical work on the evolution of sex determination pathways. This work has suggested that the sex determination pathway in *C. elegans* arose in steps, with genes being added to the beginning of the pathway (Wilkins 1995). Thus, similarity in the sex determining pathways of different species is likely to be found not in the primary steps, but in the terminal regulators. In support of this idea, the male sexual regulatory gene, male abnormal-3 (*mab-3*) from *C. elegans* has been shown to be related to the *D. melanogaster* sexual regulatory gene doublesex (*dsx*) (Raymond *et al.*, 1998). Both genes encode proteins with a DNA-binding motif, the DM domain, and control sex-specific neuroblast differentiation and yolk protein gene transcription. It was found that the form of *dsx* found in male *D. melanogaster* can direct male-specific neuroblast differentiation in *C. elegans*.

Sequence analysis of the fragments obtained during the attempt to isolate *S. ratti* homologues of the *C. elegans* sex determination genes *fox-1* and *xol-1* (Appendix 2) indicated that a large proportion of the fragments isolated appear to be of bacterial origin (6/14 or 43%). This is probably due to the contamination of *S. ratti* genomic DNA with DNA from bacteria either in the intestines of the worms or adhering to their cuticles. Oddly, all of the bacterial sequences were obtained with the *fox-1* P6/P7 primers and all fragments amplified with the *xol-1* P1/P3 primers appear to be of nematode origin. The cause of this difference is unclear, as the same *S. ratti* template DNA was used for both sets of primers. Of the non-bacterial sequences isolated only two, xol.1b and xol.23, appear to be fragments of *S. ratti* genes (Table 2, Appendix 2). xol.23 is noteworthy, due to the high degree of similarity between the proteins from *C. elegans* and *X. laevis* and the conceptual amino acid translation of this sequence (Table 2, Appendix 2). Unfortunately, little is known of the function of the proteins that are related to xol.23, but a number of metalloprotease-disintegrins have been identified as important in early embryonic development (Yagami-Hiromasa, 1995; Alfandari *et al.* 1997). As the chromosomal location of sequences that were isolated in this experiment were not known, no attempt to determine the relative copy number of any of these fragments by semi-quantitative PCR was made.

In contrast to the failure of the attempt to isolate *S. ratti* homologues of the *C. elegans* genes *fox-1* and *xol-1*, the second approach used to isolate sex-linked DNA in *S. ratti* proved successful. In total, 33 unique, anonymous, DNA fragments were hybridised to dot blots of free-living male and free-living female DNA (Table 4.3). Of these 33 markers, the free-living male/free-living female ratio was calculated for a total of 22 unique sequences (Table 4.3). It is clear that the distribution of the corrected free-living male/free-living female ratios differed between the three dot blots (Figure 4.4). In particular, the corrected free-living male/free-living female ratios of markers hybridised to the third dot blot were more variable than those hybridised to the other two blots. On the basis of the free-living male/free-living female ratios calculated for the markers, several conclusions can be drawn. First it must be noted that, with the exception of the pZ-7, none of the 22 markers had a corrected free-living male/free-living female ratio that was conspicuously greater than one (Figure 4.4). It is therefore unlikely that any significant portion of the genome is present at a higher dose in the free-living males

than in the free-living females. It is however worth noting that the highest corrected free-living male / free-living female ratio from the first dot blot was obtained from the mitochondrial 16S marker (Figure 4.4). It was expected that the dose of this marker would not differ between the sexes, but this result suggests that the free-living males have a greater number of mitochondria than the free-living females. This difference may stem from differences in the behaviour of the sexes, if for instance the free-living males are more active than the free-living females, or may be the result of differences in germline physiology.

The second conclusion that can be drawn from the dot blotting experiments is that it is unlikely that there is any sex-specific DNA in *S. ratti*, as there is in mammals (male specific Y chromosomes) and birds (female specific W chromosomes). If such chromosomes existed, then it would be expected that any marker linked to them would only hybridise to a single sex, giving a free-living male/free-living female ratio either zero or infinity, a result which was not observed. Biologically it is unlikely that any male specific chromosomes exist in *S. ratti*, as the life-cycle suggests that any such chromosomes would have to be present in the parasitic females. In contrast, our current understanding of the *S. ratti* life-cycle does not preclude the existence of female specific chromosomes. However, iterative calculations indicate that there was a 95% chance of detecting any female specific chromosome composed of more than 12% of the haploid genome. These experiments do however indicate that three of the markers, 27/*Sr-mvP1*, pZ-2/*Sr-mvP2* and pZ-4/*Sr-mvP3*, appear to have a higher copy number in the free-living females than in the free-living males (Table 4.3 and Figure 4.5). This is consistent with an XX/X0 system of sex determination in *S. ratti* and suggests that, if sex determination is an XX/X0 system, these three markers may be X-linked.

The results of the dot blot experiments also suggest that only a relatively small fraction of the genome is present at a higher dose in the free-living females than in the free-living males. If this part of the genome is thought of as the X chromosome, then this suggests that the X chromosome in *S. ratti* is considerably smaller than the autosomes. Cytological studies suggests that the chromosome number of the free-living females is six, and that of the free-living males five (Nigon & Roman, 1952; Bolla &

Roberts, 1968). Thus, if the X chromosome is the same size as the autosomes, 33% of random sequences should be X-linked. However, in these experiments, if the mitochondrial 16S marker is discounted, only 14% (3/21) of the markers can be identified as putatively sex-linked. However, half of the markers used were isolated from a cDNA library and hence it is very likely that they represent functional genes. In *C. elegans* it is known that the X chromosome is, in Mbp, slightly larger than the autosomes, but has a lower gene density (The *C. elegans* sequencing consortium, 1998). Therefore, if the same lower gene density occurs in *S. ratti*, the X chromosome is likely to be larger than these results suggest. Thus, no firm conclusions concerning the relative size of the chromosome in *S. ratti* can be drawn, but the available data are consistent with an X chromosome that is slightly under half the size of the autosomes.

The sex-linkage of the markers 27/*Sr-mvP1* and pZ-2/*Sr-mvP2* was confirmed by semi-quantitative PCR (Figure 4.5 and Figure 4.6, respectively). This demonstrates that they are, as suggested by the dot blot experiments, present at a higher copy number in the free-living females than in the free-living males. This consistent genetic difference between the free-living males and the free-living females is consistent with the XX/X0 system of sex determination that was suggested for *S. ratti* by the early cytological studies (Nigon & Roman, 1952; Bolla & Roberts, 1968). This also suggests that 27/*Sr-mvP1* and pZ-2/*Sr-mvP2* may be X-linked sequences. However, Southern blot analysis indicates that there are multiple copies of pZ-2/*Sr-mvP2* within the *S. ratti* genome (Figure 4.7B). This result may indicate that there are that multiple copies of pZ-2/*Sr-mvP2* present on the putative X chromosome. As previously stated, the results of the semi-quantitative PCR support the conclusions drawn from the dot blot experiments, and indicate that pZ-2/*Sr-mvP2* is sex-linked. However, as only the original cloned fragment of pZ-2/*Sr-mvP2*, is known to contain the bindings site for the primers used in the semi-quantitative PCR, other copies of pZ-2/*Sr-mvP2* may not have been amplified. Thus, this result does not indicate how many copies of this marker are sex-linked. However, the free-living male/free-living female ratio for pZ-2/*Sr-mvP2* calculated from the dot blots (Table 4.3) was the lowest of any of the markers. This strongly suggests that the majority of the copies of pZ-2/*Sr-mvP2* are present at a higher copy number in the free-living females than in the free-living males. In contrast to

the situation observed for pZ-2/*Sr-mvP2*, Southern blotting (Figure 4.7A) suggests that there is only a single copy of 27/*Sr-mvP1* within the *S. ratti* genome.

The sequence analysis of the previously uncharacterised markers that were not identified as sex-linked when screened against the dot blots indicated that a number appear to be gene fragments (Table 5 and 6, Appendix 2). Of these, CM6 and pZ-8 are related by amino acid sequence to transposable elements (Table 6, Appendix 2) and pZ-1 appears to be a fragment of the *S. ratti* mitochondrial ATP synthase D chain protein. Comparison of the predicted amino acid sequence of pZ-1 with that of mitochondrial ATP synthase D chain proteins from *C. elegans* and *D. melanogaster* (Figure 2, Appendix 2), indicates that pZ-1 is, as would be expected, more closely related to the *C. elegans* gene. In addition, there is no apparent agreement between the exon structure of the *C. elegans* mitochondrial ATP synthase D chain protein and that of pZ-1. However, this is not surprising as there is only limited conservation of exon placement between *C. elegans* and *C. briggsae*. Of the DNA fragments that failed to hybridise to the dot blots of free-living male and free-living female DNA (Table 4.3), fox6.8 and fox1.5 had previously been shown to have no significant similarity to existing nucleic acid or amino acid sequences (Tables 1 and 2, Appendix 2). These sequences may therefore represent either non-coding *S. ratti* DNA or uncharacterised DNA sequences from another organism. In contrast, the fragment Q14 was shown to be of human origin (Table 5, Appendix 2), which explains why it did not hybridise to the dot blots.

Sequence analysis of the sex-linked markers, 27/*Sr-mvP1* and pZ-2/*Sr-mvP2*, and the putatively sex-marker pZ-4/*Sr-mvP3* showed that only 27/*Sr-mvP1* shows any significant similarity to published sequences at the nucleotide level (Table 5, Appendix 2). However, when the conceptual amino acid translation of 27/*Sr-mvP1* was analysed, no similar sequences were found (Table 6, Appendix 2). In contrast, comparison of the predicted amino acid sequence of pZ-2/*Sr-mvP2* and pZ-4/*Sr-mvP3* indicated that they show high levels of similarity to retrotransposons in *Ascaris lumbricoides* and *Bombyx mori*, respectively. The similarity of pZ-2/*Sr-mvP2* and pZ-4/*Sr-mvP3* to transposable elements means that out of the twenty three sequences analysed here, a total of five (22%)

are related to transposable elements (Tables 2 and 6, Appendix 2). These putative transposable elements will be discussed in Chapter 7.

The findings that the markers 27/*Sr-mvP1* and pZ-2/*Sr-mvP2* are sex linked in *S. ratti* indicates that there is a consistent genetic difference between the free-living males and free-living females. This is consistent with the XX/X0 system of sex determination in *S. ratti*, as suggested by the early cytological studies (Nigon & Roman, 1952; Bolla & Roberts, 1968). In addition these results have also shown that the approach of screening anonymous markers against dot blots of sex-specific DNA to identify sex-linked markers is effective. However, this study does not provide proof that sex determination in *S. ratti* is an XX/X0 system; for this, a molecular polymorphism in either 27/*Sr-mvP1* or pZ-2/*Sr-mvP2* is required. This would allow sex determination in *S. ratti* to be analysed and allow testing of the hypothesis that the sex-linkage reported here represents X-linkage. As the marker pZ-2/*Sr-mvP2* is present within the *S. ratti* genome in multiple copies it is not particularly suited to this type of analysis. It is possible to develop such multiple copy sequences into polymorphic markers, but, it is easier to identify polymorphic markers in single copy sequences such as 27/*Sr-mvP1*. Thus, 27/*Sr-mvP1* would, if polymorphic either within or between lines of *S. ratti*, be an ideal marker for the genetic analysis of sex determination in *S. ratti*.

CHAPTER 5.

Sex determination in *Strongyloides ratti* II.

a. Identification of RFLPs of the putatively X-linked marker *Sr-mvP1*.

b. Analysis of sex determination and reproduction in *S. ratti* using the RFLPs of the putatively X-linked marker *Sr-mvP1*.

ABSTRACT

The life-cycle of *Strongyloides ratti* is complex, and the genetics of the species is poorly understood. Cytological evidence suggests that sex determination is an XX/X0 system. This suggestion is supported by the finding that the free-living females have a consistently higher copy number of the sex-linked markers *Sr-mvP1* and *Sr-mvP2* than the free-living males. Here two alleles of a restriction length fragment polymorphism (RFLP) of *Sr-mvP1* are identified. Use of this RFLP to analyse both sex determination and reproduction in *S. ratti* demonstrates that the parasitic females, free-living females and directly developing iL3s can be heterozygous for the *Sr-mvP1* RFLP. In contrast, it is shown that free-living males can only have a single allele of the *Sr-mvP1* RFLP. This is consistent with the proposed XX/X0 system of sex determination and maps the *Sr-mvP1* to the *S. ratti* X chromosome. Analysis of reproduction between unrelated free-living males and free-living females demonstrates that all progeny inherit the paternal X chromosome and one of the two maternal X chromosomes. Therefore, the indirectly developing iL3s are XX and genetically female. In addition, it is shown that reproduction between related free-living adults is sexual.

5.1 Introduction

The life cycle of *Strongyloides ratti* is complex and contains both free living and parasitic phases. The parasitic generation is female only and reproduces by functionally mitotic parthenogenesis (Viney, 1994). The development of the progeny of the parasitic females is the consequence of two, discrete, developmental switches (Chapter 3). The first of these is a male/female sex determination switch and the second a free-living female/directly developing iL3 developmental conversion (Chapter 3) (Figure 5.1). Thus, the male progeny of the parasitic females all develop into free-living males, whereas the female progeny, depending on the extra-host environmental factors, develop into free-living females or directly developing infective third stage larvae (iL3s) (Chapter 3). In contrast to the developmental variation exhibited by the progeny of the parasitic females, all progeny of the free-living adults develop into indirectly developing iL3s. The mechanism of sex determination in *S. ratti* is not known, but cytological studies suggest that the free-living males and the free-living females differ in their chromosome number, with the free-living females believed to be 2n = 6 and the free-living males 2n = 5 (Nigon & Roman, 1952; Bolla & Roberts, 1968). These results suggest an XX/X0 system of sex determination in *S. ratti*, with the free-living females having two X chromosomes (XX) and the free-living males having only a single X chromosome (X0). As all indirectly and directly developing iL3s are thought to be capable of subsequent development into parasitic females, they are also believed to be both XX and female. Thus, with the exception of the free-living males, which are considered to be X0, all stages of the *S. ratti* are thought to be XX and female.

However, this proposed XX/X0 system of sex determination has not been tested genetically, but the opportunity to test this hypothesis is presented by the isolation of the sex-linked markers *Sr-mvP1* and *Sr-mvP2* (Chapter 5). The copy number of these two sex-linked markers has been shown to be higher in the free-living females than in the free-living males (Chapter 4). A result which is consistent with the hypothesis that sex determination in *S. ratti* is an XX/X0 system. However, in order to use either of these markers to analyse sex determination and to follow the inheritance of the putative X chromosomes through the life-cycle, a method of differentiating between different alleles is needed.

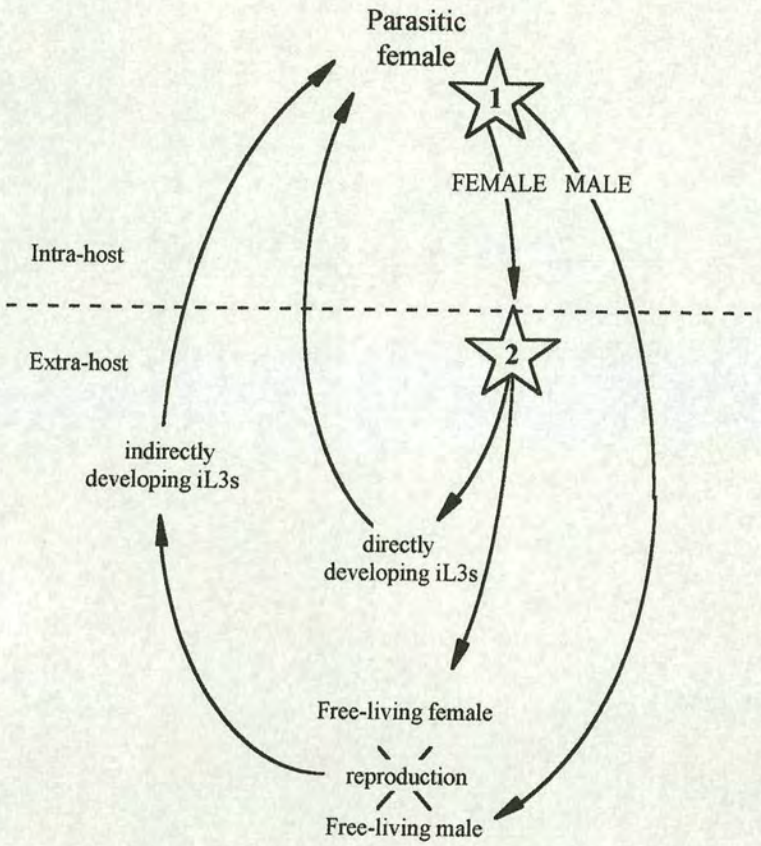


Figure 5.1 The life-cycle of *S. ratti*, indicating the two discrete developmental switches. 1. Intra-host sex determination switch. 2. Extra-host free-living female/directly developing iL3 developmental conversion.

Therefore, it is necessary to identify a polymorphism in one of these putatively X-linked markers. A commonly used method is that of restriction fragment length polymorphism (RFLP) analysis. This has proved useful in the genetic analysis of both populations and individuals. At a population level, this powerful molecular tool has recently been used to analyse the population genetic structure of *S. ratti* (Fisher & Viney, 1998). In situations more relevant to this study, RFLP analysis has shown that genetic recombination occurs in isolates of *Botryotinia fuckeliana*, a haploid, filamentous fungus (Giraud *et al.* 1997) and has also been used to determine the relative contributions of sexual and asexual reproduction to the genetic structure of populations of the pathogenic plant fungus, *Mycosphaerella graminicola* (Chen & McDonald, 1996). Southern blot analysis has indicated

that the marker *Sr-mvP2* is present in multiple copies within the *S. ratti* genome (Chapter 4). In contrast, Southern blot analysis suggested that there was only a single copy of *Sr-mvP1* (Chapter 4). Hence, it is possible that this marker is X-linked. Therefore, *Sr-mvP1* represents the marker that is most likely to be easily usable as a molecular marker on the putative *S. ratti* X chromosome.

In the course of a previous population genetic study of *S. ratti* (Fisher, 1997), an RFLP in *Sr-mvP1* had been unsuccessful sought by restriction enzyme screening (marker referred to as 27: Fisher, 1997). Therefore, in this study a DNA sequencing approach was taken and *Sr-mvP1* was sequenced from different populations and compared in order to identify changes in the DNA sequence predicted to alter restriction endonuclease recognition sites. Identification of an RFLP of *Sr-mvP1* would then allow the mechanism of sex determination in *S. ratti* to be investigated. As reproduction by *S. ratti* parasitic females is by functionally mitotic parthenogenesis (Viney, 1994), no genetic recombination occurs and all progeny are expected to be genetically identical. Therefore, all the directly developing iL3 and free-living female progeny parasitic females heterozygous for an RFLP of *Sr-mvP1* would also be expected to be heterozygous. If *Sr-mvP1* is X-linked and sex determination in *S. ratti* is an XX/X0 system, then the free-living male progeny of these heterozygous parasitic females would be expected have only a single allele of *Sr-mvP1*. If this was the case, then it would indicate that the *Sr-mvP1* is X-linked and would provide strong support for the proposed XX/X0 mechanism of sex determination.

If the proposed XX/X0 system of sex determination is supported then a further problem with the *S. ratti* life-cycle arises. This is the case, as it is not clear how reproduction between a putative X0 free-living males and a putative XX free-living female would result in progeny that are all apparently XX and female. If the free-living males are X0 and gamete production and chromosome segregation occur in a Mendelian fashion, then half of the free-living males' sperm would be expected to have no X chromosome. Thus, sexual reproduction between X0 free-living males and the XX free-living females would be expected to result in equal numbers of both X0 and XX progeny.

Such a situation occurs in *Caenorhabditis elegans*, where crosses between XX hermaphrodites and X0 males produce equal numbers of XX and X0 progeny (Hedgecock, 1976). However, in the closely related species *C. briggsae*, male sperm that carry an X chromosome have an increased competitive advantage and preferentially fertilise the oocytes of the hermaphrodite (LaMunyon & Ward, 1997). This preferential fertilisation results in a hermaphrodite biased progeny sex ratio, with up to 90% of the progeny developing into hermaphrodites during the initial period after fertilisation. As the number of X-bearing sperm decreases, more male progeny develop and the progeny sex ratio returns to approximately one-to-one (LaMunyon & Ward, 1997).

A further related problem is raised by the fact that the method by which the free-living males and free-living females reproduce is still disputed (discussed in Chapter 1). Cytological studies of *S. ratti* have concluded that reproduction in the free-living adults occurs primarily by meiotic parthenogenesis and pseudogamy (Nigon & Roman, 1952; Bolla & Roberts, 1968). In contrast, genetic analysis has shown that reproduction between free-living males and free-living females is sexual, with genetic input from the free-living males (Viney *et al.* 1993). It has been suggested that these conflicting results can be reconciled if the reproductive mode of the free-living adults differs between related and unrelated free-living males and free-living females. Hence, if reproduction between free-living males and free-living females of the same parasite line, related free-living adults, occurs by functionally meiotic parthenogenesis and pseudogamy and that between free-living males and free-living females of different parasite lines, unrelated free-living adults, is sexual (Viney *et al.* 1993; Hammond & Robinson, 1994). To date, genetic analyses of reproduction between related free-living adults have only been able to demonstrate that allelic segregation occurs and that the progeny are not genetically identical (Viney *et al.* 1993; Viney, 1994; Fisher, 1997). Thus, these are findings are consistent with both functionally meiotic parthenogenesis, where segregation occurs, and with sexual reproduction. Therefore, it is still unclear by which method related free-living adults reproduce.

Here the isolation of a RFLP of the sex-linked marker *Sr-mvP1* is described. Analysis of the inheritance of the alleles of this RFLP through the life-cycle allows the mechanism of sex determination

in *S. ratti* to be determined. Further analysis of the inheritance of the *Sr-mvP1* RFLP in controlled crosses between unrelated free-living adults of known *Sr-mvP1* genotype allows the sex of their indirectly developing iL3 progeny to be determined. In addition, the mechanism of reproduction between related free-living adults can be determined as the expected ratio of *Sr-mvP1* genotypes in the progeny of the free-living adults is dependant on the reproductive mode. These results are discussed in relation to previous cytological studies of sex determination and reproduction in *Strongyloides spp.*.

5.2 Identification of RFLPs of the putatively X-linked marker *Sr-mvP1*

5.2.1 Materials and Methods

Parasites and maintenance

S. ratti lines used in the identification of an RFLP of *Sr-mvP1* were: isofemale line ED5 Homogonic, isofemale line ED132 Homogonic and isolate ED204. Isofemale line ED5 Homogonic is derived from an isolate obtained in the United States (Viney *et al.* 1992) and isofemale line ED132 Homogonic is derived from an isolate originally obtained from Japan (Viney, 1996). Isolate ED204 is derived from *S. ratti* iL3s isolated from a wild rat population in Wiltshire (Fisher, 1997). Parasite lines were maintained and cultures made as previously described (see Chapter 3). Faecal cultures from animals infected with isofemale line ED5 Homogonic, isofemale line ED132 Homogonic and isolate ED204 were maintained at either, 19°C for three days or 25°C for two days. These incubations allowed the free-living females, free-living males and directly developing iL3s to develop to maturity. For each parasite line, single free-living males and directly developing iL3s were individually collected as previously described for single worm PCR preparations (Chapter 5).

Identification of an RFLP of Sr-mvP1

Individually collected worms were prepared as single worm PCR preparations (Chapter 5) and a 1,230 base pair (bp) fragment of *Sr-mvP1* was amplified from each worm using the *Sr-mvP1* F and *Sr-mvP1* R primers (Table 5.1). Amplification conditions were 40 cycles of 95°C for 1 minute, 54°C for 1 minute and 72°C for 3 minutes, followed by a 10 minute extension at 70°C. Reactions were performed in 50 µl volume, with primers at a final concentration of 100 nM, PCR buffer (Promega) at 1 × concentration and 0.75 µM each of dATP, dTTP, dCTP and dGTP. Reactions were then supplemented with $MgCl_2$ and *Taq* DNA polymerase (Promega) to 1.5 mM and 200 units ml^{-1} final concentrations, respectively. 5 µl of each PCR reaction was electrophoresed in 1% w/v agarose gels

containing ethidium bromide (0.5 μg ml^{-1}) in 1 × TAE buffer. The remainder of successful amplification reactions were purified using PCR spin columns (Qiagen). Purified *Sr-mvP1* PCR products were then directly sequenced, using the Dye terminator cycle sequencing ready reaction kit (Perkin Elmer) following the manufacturer's instructions and each of the six primers shown in Table 5.1. For each parasite line, both strands of *Sr-mvP1* were sequenced from four separate, purified, PCR reactions. Sequence fragments were assembled and compared using the AssemblyLIGN programme (Oxford Molecular Group).

Forward strand

Primer		Sequence	
Sr-mvP1 F	5'	CGT ATC TTG CGA TGA TCA TC	3'
Sr-mvP1 F.2	5'	CTT ATG TTG TCT TCA GTC TCC	3'
Sr-mvP1 F.3	5'	CAA CGT TAT TTT ACT ATC GC	3'

Reverse strand

Primer		Sequence	
Sr-mvP1 R	5'	GAT ATC AGT TGC AAA ACT ACC	3'
Sr-mvP1 R.3	5'	AGT GAT AAA AGT TTG GAG CTG	3'
Sr-mvP1 R.4	5'	TGC TTC ATA CAG AGG GAG C	3'

Table 5.1 The nucleotide sequence of the primers used in the amplification and sequencing of *Sr-mvP1*.

5.2.2 Results

Identification of an RFLP in Sr-mvP1

PCR amplified fragments of *Sr-mvP1* were successfully sequenced from four free-living males of isofemale line ED5 Homogonic and four free-living males from isofemale line ED132 Homogonic. It was not initially possible to obtain four free-living males from isolate ED204, so *Sr-mvP1* was sequenced from one free-living male and three directly developing iL3s. The sequences of *Sr-mvP1* from all of the free-living males from both isofemale line ED5 Homogonic and ED132 Homogonic were identical; this sequence of 1028 bp is shown in Figure 5.2.

```
1       CGATCTTGCG ATGATCATCT CCATCATCCA CTCGGTCAAG TTCAAATGCT
        GTGTCAATCT TCCAGATAAA ATCATCAAAT GTCTTTGTGT CTGAGTTGTA
101     ATGGTCAAAC TCAGTAACCT TTTCAATATA TATGCTTGTT TCAGCCATTT
        TAACTAACGT TTTTTCAGCA GGTATATCAG ATGCTTCACT ATTATAGTCT
201     TCCAGTGTTG GTGAACCAAT CCTTGGATAA TGTAGGTTTA AGTAGTCTTC
        TATCTTCTTT TGTGTATTGC TCCCTCTGTA TGAAGCAATT TCTTCAACCA
301     CTTTTGTAAC AGTTACTTCA CTAATATGTT CAAATCTCTT AAGTTGCAGG
        AGTACAGGTA GTATTCCACT AACTTTACTC ACTAATATCT TATGTGTCTC
401     AGTCTCCTTA TACAAATTGC TCATATACTA TGTCTGAGGT TACAGTAAAT
        ACCTTTTGTA TACTATATGG GTATCAATCA ATGACATATC CACCTTGCAG
501     TTCGATCCTA TCGATCATAT CAATAACTTT TGTCTGATGA AGTGGCAGTC
        TCAGCTCCAA ACTTTTATCA CTAAAAAACT CAGAAATTGT CAAATTCCTC
601     TTTCTATCAA ATTTATCCCT TTTTTACAAC GTTATTTTAC TATCGCAATA
        GTAAAATATT TATTTTTTGG TGACTTTAGA GTACTTTAAA TATGACAAAA
701     AATACAATAA AAATCAAAGA CTTACAATTA TATAAAGTAT CTTACTTAAA
        CAAAAGTCTC TCAGAACACC CAATCTATTA ATACTTTTAG TAGTGTTACT
801     TCGTTTAACC CTAAAATAAT GATAATAATA ATAATAATAA AGTATTCGGA
        AATACCAAAG AATACCTTAT GTTACGGCAT AGGCACGATA TAAAATATGA
901     AAGGATACTG CAATAATAAG AGATGAGATA CAATAACGAT AATAATAACT
        TAAATAAATT AAATAAAATG ATATTAACTT AGACAGACTA GCAATATGCA
1001    GCACACAGGT ATGGGTAGTT TTGCAACT
```

Figure 5.2 Sequence of 1028 bp of *Sr-mvP1* from the free-living males of isofemale lines ED5 Homogonic and ED132 Homogonic.

The sequence of *Sr-mvP1* from the single free-living male of isolate ED204 differed by a single nucleotide from the sequence of *Sr-mvP1* shown in Figure 5.2, a change of C to T at position 799. The sequences obtained from the each of the three directly developing iL3s of isolate ED204 contained a sequence ambiguity, either C or T, at position 799. This is consistent with the directly developing iL3s of isolate ED204 having two different alleles of *Sr-mvP1* and the single free-living

male having either a single allele of *Sr-mvP1* or two copies of the same allele. Sequence analysis indicated that position 795-799 in the sequence of *Sr-mvP1* from isofemale lines ED5 and ED132 is a recognition site for the restriction endonuclease *Mae*-III (Roche Molecular Biochemicals). The change of C to T at position 799 in the free-living male of isolate ED204 was predicted to disrupt this *Mae*-III recognition site. The region of *Sr-mvP1* sequence containing this change is shown in Figure 5.3. To conform to the standard nomenclature of parasitic nematodes (Bird & Riddle, 1994), the two alleles of Sr-mvP1 were designated *Sr-mvP1(1)* and *Sr-mvP1(2)*, the *Sr-mvP1(2)* allele resulting from the substitution of T for C at position 799 in isolate ED204.

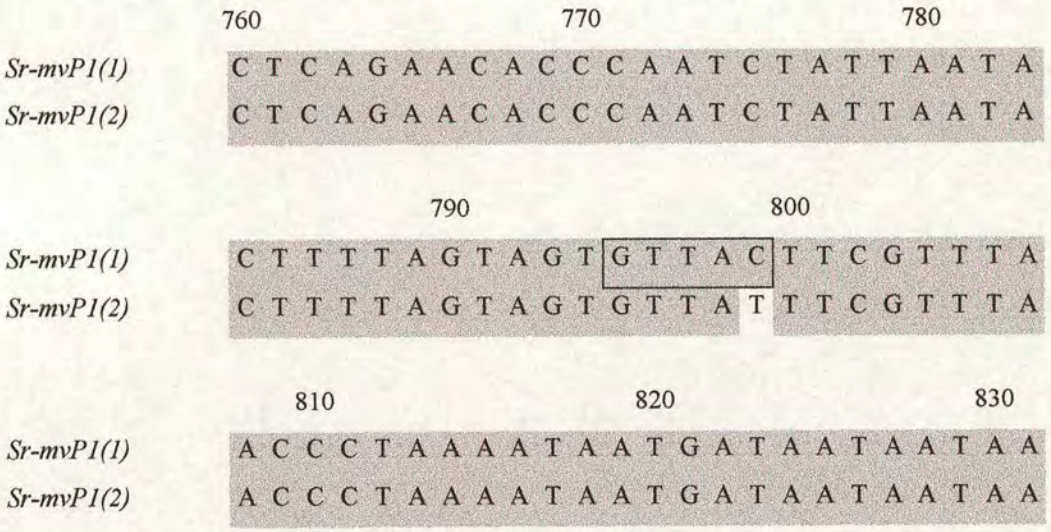


Figure 5.3 Comparison of a region of the sequence of the two alleles of *Sr-mvP1*. Sequence analysis indicates that isofemale lines ED5 Homogonic and ED132 Homogonic are homozygous for *Sr-mvP1(1)* and that the directly developing iL3s of isolate ED204 are heterozygous and of genotype *Sr-mvP1(1,2)*. The boxed area represents the recognition site for the restriction endonuclease *Mae*-III (↓GTNAC). Shaded regions show nucleotide identity. Sequence is numbered in relation to the consensus sequence of *Sr-mvP1* shown in Figure 5.2.

5.3 Analysis of sex determination and reproduction in *S. ratti* using RFLPs of the putatively X-linked marker *Sr-mvP1*

5.3.1 Materials and Methods.

Parasite lines and maintenance

Unless otherwise stated, parasite lines were maintained and cultures made as previously described (see Chapter 3). The origin of parasite lines used in the analysis of sex determination and reproduction in *S. ratti* is described below.

Confirmation of sequence analysis

Sequence analysis suggested the presence of an *Mae*-III RFLP of *Sr-mvP1* (5.2.2) in the lines studied. This observation was investigated by direct RFLP analysis. Sequence analysis indicated that the polymorphism identified in *Sr-mvP1* can be detected as a change in the restriction fragments produced by the restriction endonuclease *Mae*-III. In order to allow the two alleles to be distinguished, a smaller 398 bp fragment of *Sr-mvP1* was amplified using the *Sr-mvP1* F.3 and *Sr-mvP1* R primers (Table 5.1). The predicted *Mae*-III restriction map of the *Sr-mvP1* fragment amplified with these primers is shown in Figure 5.4.

In all cases where worms were genotyped for *Sr-mvP1*, they were collected and prepared as single worm PCR preparations, as previously described (Chapter 5). The PCR reaction was cycled 40 times through 95°C for 1 minute, 54°C 1 minute and 72°C for 1 minute. Reactions were performed in 50 µl volume, with primers at a final concentration of 100 nM, PCR buffer (Promega) at 1 × concentration and 0.75 µM each of dATP, dTTP, dCTP and dGTP. Reactions were supplemented with $MgCl_2$ and *Taq* DNA polymerase (Promega) to 1.5 mM and 200 units ml^{-1} final concentrations, respectively. 5 µl of the PCR reactions were electrophoresed on 1.5% w/v agarose gels in 1 × TAE

buffer containing ethidium bromide (0.5 μg ml^{-1}) to confirm amplification. 12 μl of successfully amplified fragments were then digested with two units of *Mae* III (Roche Molecular Biochemicals) in a 25 μl volume following the manufacturer's instructions.

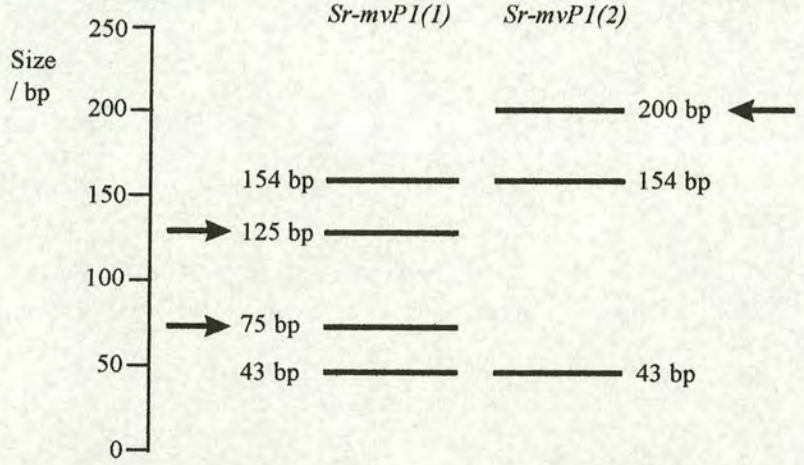


Figure 5.4 The predicted restriction fragments for the two alleles of *Sr-mvP1* produced after the 398 bp fragment of *Sr-mvP1* is digested with the restriction endonuclease *Mae*-III. Arrows denote the fragments that are characteristic of each allele.

Digested PCR products were visualised on 4% w/v Metaphor agarose gels (FMC Bioproducts) in 1 × TBE buffer containing ethidium bromide (0.5 μg ml^{-1}). Note that if only *Sr-mvP1(1)* is identified by this method, then the genotype will be given as *Sr-mvP1(1)*. This is the case as the homozygous genotype *Sr-mvP1(1,1)* and the hemizygous *Sr-mvP1(1,-)* would be indistinguishable by RFLP analysis and so should be considered together. The same situation will of course apply to *Sr-mvP1(2,2)* and *Sr-mvP1(2,-)*, so these will be referred to as *Sr-mvP1(2)*. To confirm the results of the earlier sequencing (5.2.2), the *Sr-mvP1* genotype of directly developing iL3s from isofemale line ED5 Homogonic and directly developing iL3s from isolate ED204 was determined using the restriction endonuclease *Mae*-III as described above.

Isolate ED204 is, as previously stated, derived from *S. ratti* iL3s isolated from a wild rat population in Wiltshire (Fisher, 1997). As it is not derived from a single parasitic female, it cannot be assumed to be genetically homogeneous. Therefore, a number of isofemale lines (Chapter 2) derived from single iL3s of isolate ED204 were created. In common with other of isolates of *S. ratti* from the United Kingdom, isolate ED204 develops almost exclusively by the direct route (Viney *et al.* 1992). Preliminary observations indicated that no free-living adults are observed until at least day 20 post infection (p.i.). Therefore, in order to increase the proportion of the larval progeny of the parasitic females that develop into free-living males and free-living females, selection for indirect development was also carried out. This was done by isolating gravid, naturally mated, free-living females from faecal cultures that had been maintained either at 19°C for three days or 25°C for two days. These free-living females were rinsed in distilled water and transferred to a watch glass containing approximately 300 µl of distilled water. The watch glass was enclosed within a petri dish and a damp piece of filter paper was placed in the base of the petri dish to prevent desiccation. Petri dishes were maintained at 19°C until progeny, if produced, had developed into indirectly developing iL3s. These indirectly developing iL3s were removed and used to initiate further isofemale lines. Attempts were also made to cross free-living adults from parasite lines derived from isolate ED204 with free-living adults from lines in which a greater proportion of larvae developed by the indirect route. Virgin free-living females were obtained by placing individual first or second stage larvae, which had been removed from faecal cultures maintained at 19°C for one day, in the wells of a 96 well microtitre plate that contained 100 µl of 0.25 mM NaCl. The microtitre plate was subsequently maintained at 19°C until larvae had developed to the L4 stage, when larvae that were developing into free-living females could be identified by microscopy. Free-living males were sexed by microscopy and isolated from faecal cultures that had been maintained at 19°C for three days. A virgin free-living female of one parasite line and a free-living male of the second line were then placed on NGM agar plates*. The plate was then maintained at 19°C and observed daily until either progeny were observed or the free-living adults had died. Progeny from

* see Appendix 1.

these crosses were then transferred daily to fresh agar plates until they had developed into indirectly developing iL3s, at which point they were removed and used to initiate isofemale lines.

These attempts to increase the production of free-living males and free-living females by the various isofemale lines derived from isolate ED204 resulted in the creation of a large number of parasite lines (see Appendix 3). The parasite lines used in the analysis of sex determination and reproductive mode in *S. ratti* are shown in Figure 5.5 In all cases the *Sr-mvP1* genotype of each isofemale line shown in Appendix 3 and Figure 5.5 was determined, as described above, for at least 4 directly developing iL3s.

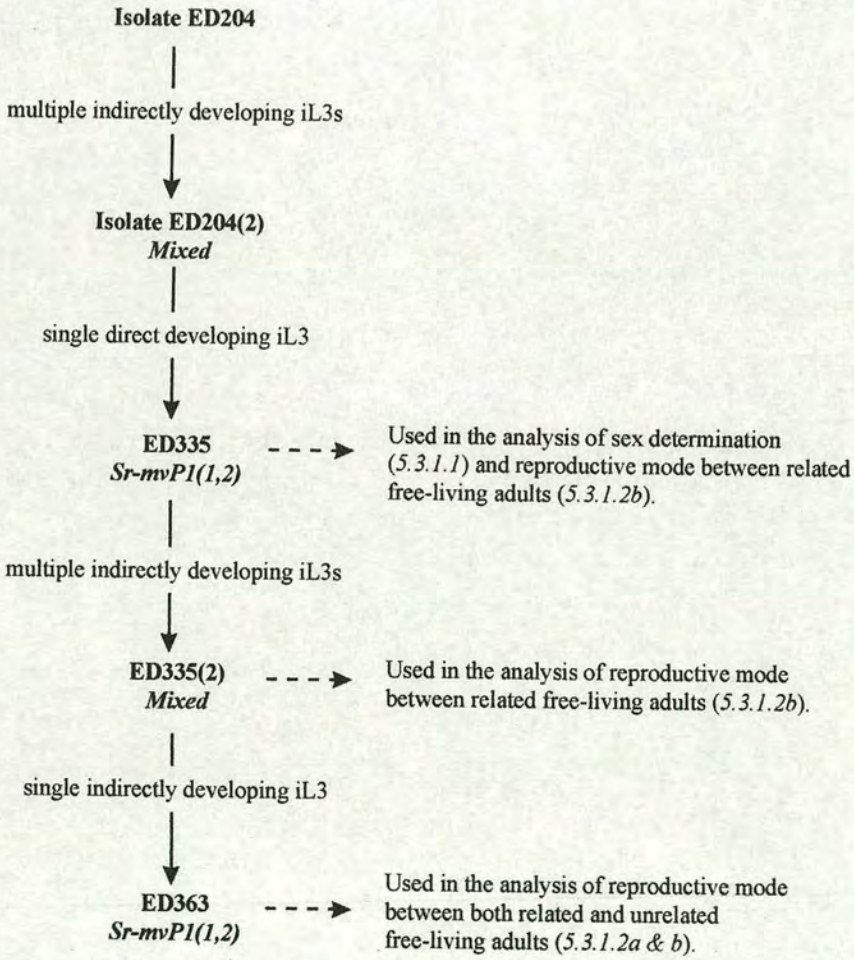


Figure 5.5 The ancestry of parasite lines used in the analysis of sex determination and reproduction in *S. ratti*. The *Sr-mvP1* genotype, where known, of each parasite line is given, with *Mixed* denoting that the line was composed of parasitic females of multiple *Sr-mvP1* genotypes.

5.3.1.1 Sex determination in S. ratti

Sr-mvP1 *in different life-cycle stages of an isofemale line*

Two rats were each infected with the 100 directly developing iL3 progeny of isofemale line ED335, a line derived from isolate ED204 (Figure 5.5). Faeces were collected at regular intervals from day 5 p.i. until day 30 p.i and faecal cultures were made as previously described (Chapter 3). Cultures were maintained at 19°C for two days, at which time larvae that are developing into free-living males or free-living females have reached the fourth larval stage (L4) and sex can be determined microscopically. Larvae that had developed into L4 free-living males, L4 free-living females and larvae that had developed into directly developing iL3s were removed and genotyped for *Sr-mvP1* as described above. To determine the genotype of parasitic females, rats were sacrificed and the small intestine removed, cut open longitudinally and the gut contents removed and discarded. The intestine was then incubated in 50 ml of saline (0.85% w/v NaCl) at 37°C for 90 minutes to allow the migration of the parasitic females from the mucosa into the saline. After incubation, the intestine was discarded and the saline transferred to a petri dish. Individual parasitic females were then removed, by pipetting, from the saline, rinsed repeatedly in distilled water and genotyped for *Sr-mvP1* as described above.

5.3.1.2 Reproduction between free-living males and free-living females

a. Controlled crosses between unrelated free-living males and free-living females

Controlled crosses were made between isofemale line ED321 Heterogonic, genotype *Sr-mvP1(1)*, and isofemale line ED363, genotype *Sr-mvP1(1,2)*. Virgin free-living females of isofemale line ED363 and free-living males of isofemale line ED321 Heterogonic were isolated as described above. Five virgin free-living females and ten free-living males were then placed on an NGM agar plate. The plates were then maintained at 19°C for a further four days to allow the free-living males and free-living females to mate and any resulting progeny to develop. All worms were then washed from the

plates with distilled water, washed again in distilled water and any progeny genotyped for *Sr-mvP1* as described above. In addition, ten directly developing iL3s from each of the parental isofemale lines (ED321 Heterogonic and ED363) were also genotyped for *Sr-mvP1* as described above. The observed ratio of progeny genotypes from controlled crosses were compared, by χ^2 test, to the ratio of progeny genotypes that would be expected if both XX and X0 progeny are possible and to the ratio that would be expected if only XX progeny are possible.

b. Naturally mated free-living females

Faecal cultures from animals infected with either isolate ED204, isofemale line ED335 or ED335(2) (Figure 5.5), were maintained at either 19°C for three days or 25°C for two days. Gravid free-living females that had naturally mated within faecal cultures were then removed and placed in a petri dish as described above. Petri dishes were maintained at 19°C and observed daily until either the free-living female had died or three days had elapsed, whichever occurred first. At this point, the free-living female and any resulting progeny were individually collected and genotyped for *Sr-mvP1*, as described above.

The observed ratios of progeny genotypes from the naturally mated free-living females were compared, by χ^2 test, with the ratios that would be expected if progeny were produced by functionally meiotic parthenogenesis or by sexual reproduction.

5.3.2 Results

Confirmation of sequence analysis

The predicted distribution of the fragments produced by digesting the 398 bp region of *Sr-mvP1* that is amplified with the primers *Sr-mvP1*F and *Sr-mvP1*R.3 with *Mae*-III is shown in Figure 5.4. The successfully amplified PCR product from four directly developing iL3s from isofemale line ED5 Homogonic and five directly developing iL3s from isolate ED204 were digested with *Mae*-III. The resulting distribution of restriction fragments are shown in Figure 5.6. This demonstrates that the directly developing iL3s of isolate ED204 are heterozygous for the *Sr-mvP1* RFLP, with genotype *Sr-mvP1(1,2)* and that the directly developing iL3s of isofemale line ED5 Homogonic are homozygous, with genotype *Sr-mvP1(1)*.

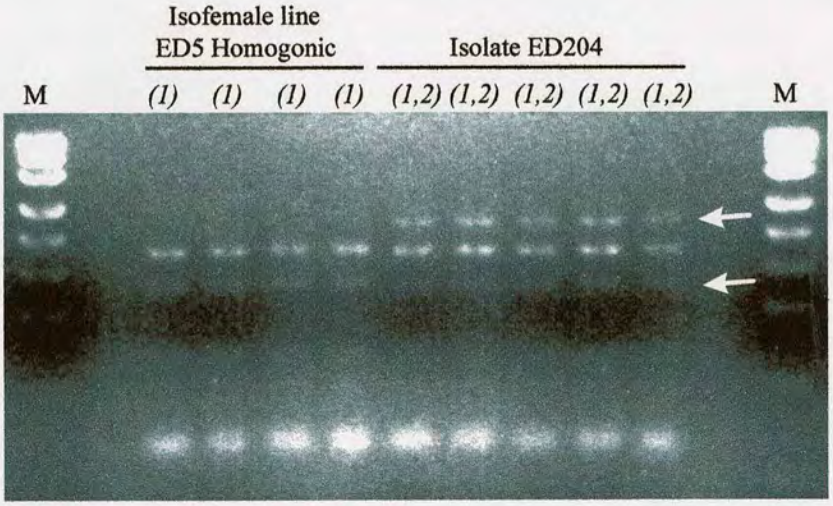


Figure 5.6 The restriction fragments produced when the 398 bp fragment of *Sr-mvP1* amplified from directly developing iL3s of isolate ED204 and isofemale line ED5 Homogonic are digested with *Mae*-III. Arrows denote the fragments that are characteristic of each allele.

5.3.2.1 Sex determination in S. ratti

Sr-mvP1 in different life-cycle stages of an isofemale line

The *Sr-mvP1* genotypes of free-living males, free-living females, directly developing iL3s and parasitic females of isofemale line ED335 were determined and are shown in Table 5.2. All of the parasitic females, free-living females and directly developing iL3s were heterozygous for the RFLP of *Sr-mvP1*, with genotype *Sr-mvP1(1,2)* (Table 5.2). In contrast, all of the free-living males had only a single allele of *Sr-mvP1* (Table 5.2 and Figure 5.7), with genotypes of either *Sr-mvP1(1)* or *Sr-mvP1(1,2)*.

		Sr-mvP1 genotype		
Morph	Number genotyped	*Sr-mvP1(1)*	*Sr-mvP1(1,2)*	*Sr-mvP1(2)*
Parasitic female	10	0	10	0
Directly developing iL3	10	0	10	0
Free-living female	10	0	10	0
Free-living male	20	13	0	7

Table 5.2 The *Sr-mvP1* genotypes of worms from isofemale line ED335.

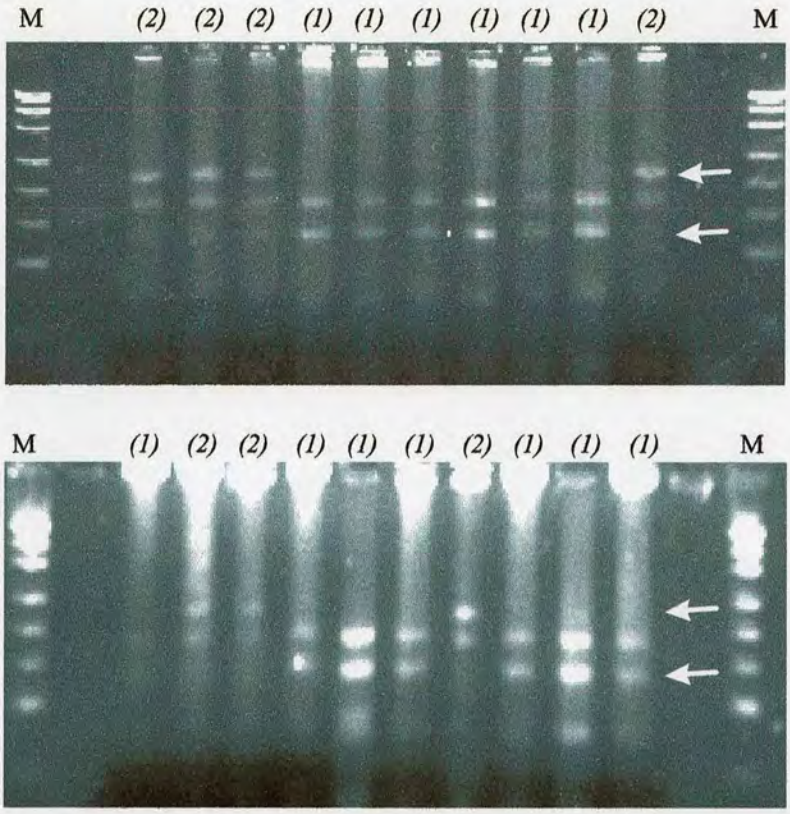


Figure 5.7 The *Sr-mvP1* genotypes of twenty free-living male progeny of heterozygous, *Sr-mvP1(1,2)*, parasitic females. Arrows indicate bands characteristic of each allele of *Sr-mvP1*. M denotes molecular weight marker.

5.3.2.2 Reproduction between free-living males and free-living females

a. Controlled crosses between related free-living males and free-living females

Two out of the twenty group matings between virgin free-living females of isofemale line ED363 and free-living males of isofemale line ED321 Heterogonic that were initiated resulted in progeny. In the first mating six progeny were produced and five were successfully genotyped for *Sr-mvP1*. In the second mating, 34 progeny were produced and 25 were successfully genotyped for *Sr-mvP1*. The progeny genotypes are shown in Table 5.3 and the genotypes of ten of the progeny from the second mating are shown in Figure 5.8A. Note that in Figure 5.8A, while the genotypes are difficult to

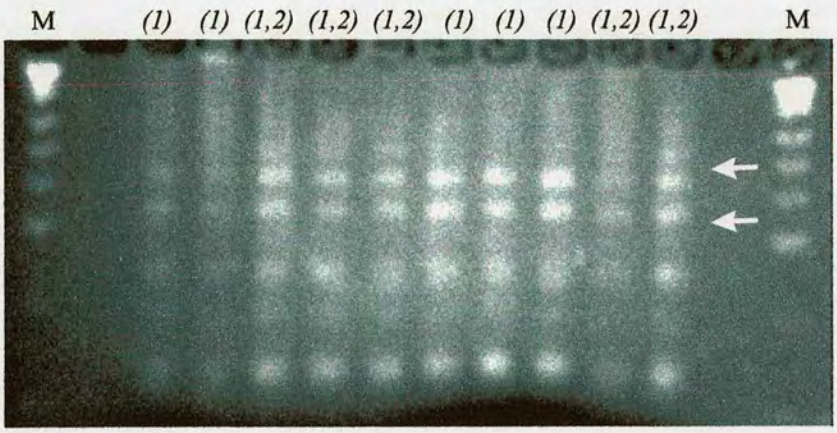
distinguish, the smaller of the marked bands is present in all of the progeny, indicating that all progeny have inherited the *Sr-mvP1(1)* allele. The *Sr-mvP1* genotype was also determined for 10 directly developing iL3s from isofemale line ED363 and 10 directly developing iL3s from isofemale line ED321 Heterogonic. All 10 directly developing iL3s from isofemale line ED363 had both alleles of *Sr-mvP1* and were therefore heterozygous, with genotype *Sr-mvP1(1,2)*. All 10 directly developing iL3s from isofemale line ED321 Heterogonic had only the first allele of *Sr-mvP1* and were of genotype *Sr-mvP1(1)*. The genotypes of five of the directly developing iL3s from each parental line are shown in Figure 5.8B.

Mating	Number of progeny produced	Number successfully genotyped	*Sr-mvP1* genotype		
			Sr-mvP1(1)	*Sr-mvP1(1,2)*	*Sr-mvP1(2)*
1	6	5	2	3	0
2	34	25	11	14	0

Table 5.3 The *Sr-mvP1* genotypes of the progeny from of controlled cross between ED363, genotype *Sr-mvP1(1,2)*, and ED321 Heterogonic, genotype *Sr-mvP1(1)*.

The progeny genotype ratio that would be expected from a cross between an *Sr-mvP1(1,2)* free-living female and an *Sr-mvP1(1,-)* free-living male if both XX and X0 progeny are possible is two *Sr-mvP1(1)* : one *Sr-mvP1(1,2)* : one *Sr-mvP1(2)* and if only XX progeny are possible is one *Sr-mvP1(1)* : one *Sr-mvP1(1,2)* : zero *Sr-mvP1(2)*. The observed progeny genotypes ratios for the first mating were not analysed as the sample size was too small. However, the ratio of progeny genotypes observed in the second cross between isofemale line ED363 and isofemale ED321 Heterogonic (Table 5.3) were significantly different from that expected if the X chromosome is inherited in a Mendelian manner and X0 progeny are possible ($\chi^2 = 16.04$, 2 d.f., $p < 0.001$). However, the observed ratio of progeny genotypes is not significantly different from that expected if the X chromosome is inherited in a non-Mendelian manner and only XX progeny are possible ($\chi^2 = 0.36$, 2 d.f., $p > 0.5$).

A.



B.

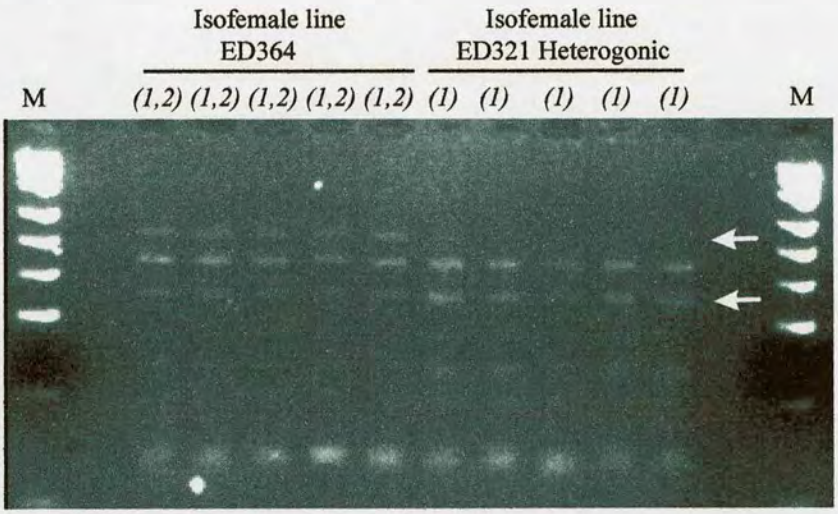


Figure 5.8 A. *Sr-mvP1* genotypes of ten progeny from the second controlled cross between virgin free-living females of isofemale line ED363 and free-living males of isofemale line ED321 Heterogonic. B. *Sr-mvP1* genotypes of five directly developing iL3s of isofemale line ED363 and five directly developing iL3s of isofemale line ED321 Heterogonic. Arrows indicate bands characteristic of each allele of *Sr-mvP1*. M denotes molecular weight marker.

b. Progeny of naturally mated free-living females

In total twelve naturally mated free-living females and their progeny were genotyped for *Sr-mvP1*. The *Sr-mvP1* genotypes of two of these naturally mated free-living females and their progeny are shown in Figure 5.11. Seven of these of free-living females were from isofemale line ED335, genotype *Sr-mvP1(1,2)*, and the other five were either from isolate ED204 or from ED335(2), lines of mixed *Sr-mvP1* genotype (Figure 5.5).

The *Sr-mvP1* genotypes of the free-living females and their progeny are shown in Table 5.4. The *Sr-mvP1* genotype was also determined for 6 directly developing iL3s from isofemale line ED335, all of which were shown to have both alleles of *Sr-mvP1* and therefore to be of genotype *Sr-mvP1(1,2)*. With free-living females of genotype *Sr-mvP1(1,2)* and assuming that only XX progeny are produced, functionally meiotic parthenogenesis would be expected to give a progeny genotype ratio of one *Sr-mvP1(1)*: two *Sr-mvP1(1,2)*: one *Sr-mvP1(2)*. In contrast, sexual reproduction between a free-living female of genotype *Sr-mvP1(1,2)* and a single male, of either genotype *Sr-mvP1(1)* or *Sr-mvP1(2)*, would give progeny genotype ratios of one *Sr-mvP1(1)*: one *Sr-mvP1(1,2)*: zero *Sr-mvP1(2)* or zero *Sr-mvP1(1)*: one *Sr-mvP1(1,2)*: one *Sr-mvP1(2)*, respectively. Again assuming that only XX progeny are produced. For each of the naturally mated free-living females, the observed ratios of progeny genotypes were then compared, by χ^2 test, to that expected if reproduction was by functionally meiotic parthenogenesis or by sexual reproduction (Table 5.4). This analysis indicates that only naturally mated free-living female 2 produced progeny of a genotype ratio that was significantly different from what would be expected if reproduction was sexual (Table 5.4). In contrast, the observed ratios of progeny genotypes were significantly different from those that would be expected if reproduction occurred by functionally meiotic parthenogenesis in five out of the twelve naturally mated free-living females analysed (Table 5.4).

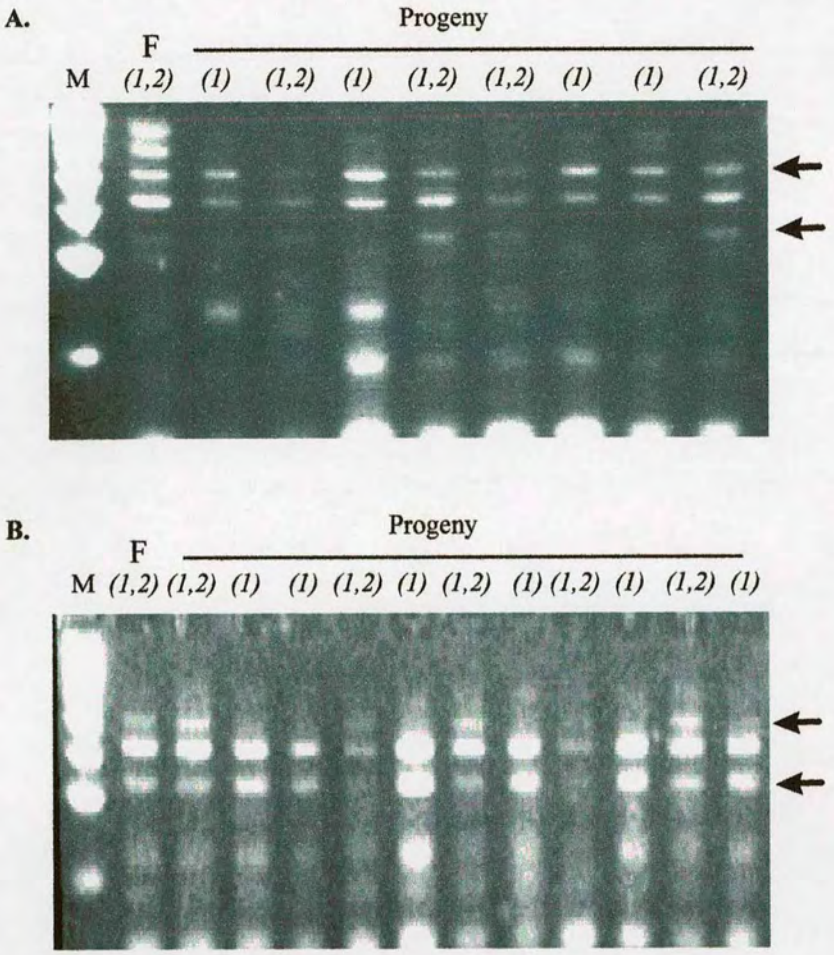


Figure 5.9 The *Sr-mvP1* genotypes of two naturally mated free-living females (F) and their progeny (P) (Free-living females 1 and 10 from Table 5.4 are shown in A and B, respectively). Arrows indicate the bands characteristic of each allele of *Sr-mvP1*. M denotes molecular weight marker.

Free-living female	Parasite line	Parasite line genotype	FL female genotype	Progeny genotype			Inferred paternal genotype	Functionally meiotic parthenogenesis	Sexual reproduction
				1	1,2	2		χ^2	χ^2
1	ED335	1,2	1,2	0	4	4	2,0	4.00	0.00
2	ED335	1,2	1,2	1	3	3	2,0 and 1,0	1.29	∞*
3	ED335	1,2	1,2	0	4	3	2,0	2.71	0.14
4	ED335	1,2	-	0	3	3	2,0	3.00	0.00
5	ED335	1,2	1,2	12	4	0	1,0	22.00*	4.00
6	ED335	1,2	1,2	10	8	0	1,0	11.33*	0.22
7	ED335	1,2	1,2	5	8	0	1,0	4.54	0.69
8	Isolate ED204	*mixed*	1,2	0	5	0	1,0 or 2,0	5.00	5.00
9	Isolate ED204	*mixed*	1,2	0	4	6	2,0	7.65*	0.40
10	ED335(2)	*mixed*	1,2	6	5	0	1,0	6.63*	0.09
11	ED335(2)	*mixed*	1,2	0	6	3	2,0	3.00	1.00
12	ED335(2)	*mixed*	1,2	5	2	0	1,0	8.43*	1.29

Table 5.4 The *Sr-mvP1* genotypes of the progeny of naturally mated free-living females. Also shown is the *Sr-mvP1* genotype of the free-living female (FL female), if known, and that of the parasite line from which they were isolated. - denotes that the genotype could not be determined. *mixed* denotes that the parasite line was not of a single *Sr-mvP1* genotype. The results of the comparison of the observed progeny genotype ratios with the ratios expected if progeny were produced by functionally meiotic parthenogenesis and sexual reproduction. * denotes significance at $p < 0.05$.

5.4 Discussion

The nucleotide sequence of the putatively X-linked marker *Sr-mvP1* was determined for isofemale line ED5 Homogonic, isofemale line ED132 Homogonic and isolate ED204. A single base pair change in the sequence of *Sr-mvP1* was identified in ED204, which was predicted to alter a recognition site for the restriction endonuclease *Mae*-III (Figure 5.3). It was subsequently shown that this base pair change defined a RFLP of *Sr-mvP1*, with isofemale line ED5 Homogonic shown to be homozygous, *Sr-mvP1(1)*, and isolate ED204 shown to be heterozygous, *Sr-mvP1(1,2)* at this locus (Figure 5.8). The isolation of these two alleles of *Sr-mvP1* subsequently allowed the hypothesis that sex determination in *S. ratti* is an XX/X0 system to be tested.

Analysis of the RFLP of *Sr-mvP1* in different life-cycle stages of an isofemale line indicated that the free-living female and directly developing iL3 progeny of parasitic females heterozygous for the *Sr-mvP1* RFLP are also heterozygous (Table 5.2). This finding agrees with a study of *S. ratti* which demonstrated that there was no allelic segregation of an RFLP of the actin gene in the progeny of single parasitic females (Viney, 1994). In this study, the free-living females were isolated at the L4 stage, when they are not sexually mature. Hence, sperm transfer from the free-living males to the free-living females will not have occurred. Therefore, no PCR amplification of *Sr-mvP1* from sperm present in free-living females could have occurred and result in the inaccurate determination of the *Sr-mvP1* genotype. In contrast, it was shown that the free-living male progeny of parasitic females heterozygous for the RFLP of *Sr-mvP1* have only a single allele of the RFLP (Table 5.2 and Figure 5.7). Thus, the free-living males are either hemizygous, with only a single copy of *Sr-mvP1* or homozygous, with two copies of the same allele. As reproduction in the parasitic phase of *S. ratti* is functionally mitotic, no genetic segregation occurs (Viney, 1994). This implies that the free-living males, with only a single allele of *Sr-mvP1* (Table 5.2 and Figure 5.7), must be hemizygous and hence X0. This indicates that the free-living males genotyped as *Sr-mvP1(1)* are actually *Sr-mvP1(1,-)* and not *Sr-mvP1(1,1)* and similarly for the free-living males that were genotyped *Sr-mvP1(2)*. These results are consistent with previous cytological studies of *S. ratti* (Nigon & Roman, 1952; Bolla & Roberts, 1968) and strongly

support the proposed XX/X0 system of sex determination. In addition, these results allow *Sr-mvP1* to be mapped to the *S. ratti* X chromosome.

The mechanism by which the free-living males of *Strongyloides spp.* are produced is not known. Previous cytological analysis of the production of free-living males by the parasitic females of *S. ransomi* and *S. papillosus* attributed the process to 'an anomalous cytological mechanism' (Triantaphyllou & Moncol, 1977). Reproduction by *S. ratti* parasitic females is functionally mitotic (Viney, 1994), but their free-living male progeny are X0, indicating that an X chromosome is lost during their production. A similar situation occurs in certain aphid species, were XX female aphids produce X0 males by a functionally mitotic process (Blackman, 1978). This involves the normal mitotic separation and replication of the autosomes and a form of modified meiosis that only affects the X chromosomes, resulting in the deletion of one X chromosome from the egg (Blackman, 1978). It is also possible that the X chromosome loss may occur by chromatin diminution, as has reported for *S. papillosus* (Albertson *et al.* 1979). Another possibility is that the production of free-living males in *Strongyloides spp.* is related to male production in *C. elegans.* In this case, XX hermaphrodites produce X0 males due to the loss of an X chromosome during gametogenesis. This occurs by a process termed X chromosome non-disjunction, the rate of which is increased by a number of mutations in the high incidence of male (*him*) genes (Hodgkin *et al.* 1979; Broverman & Meneely, 1994). The production of free-living males by the *S. ratti* parasitic females may therefore be a consequence of a similar mechanism and possibly even the action of homologous genes. However, since no segregation occurs in the parasitic phase of *S. ratti*, it seems likely that the mechanism by which free-living males are produced in *S. ratti* differs from that in *C. elegans.*

In *S. ratti*, all progeny of the free-living males and free-living females develop into indirectly developing iL3s. These indirectly developing iL3s are all believed to be able to develop into parasitic females and hence should be XX females. Reproduction between unrelated free-living males and free-living females of *S. ratti* has previously been shown to involve both genetic recombination and genetic input from the free-living males (Viney *et al.* 1993). However, if gamete production and chromosome

segregation in the germline of the free-living males occur in a Mendelian fashion, then half the zygotes of the free-living adults should have only a single X chromosome. Analysis of the *Sr-mvP1* genotype of the progeny from a controlled cross between unrelated free-living males of genotype *Sr-mvP1(1)* and virgin free-living females of genotype *Sr-mvP1(1,2)* indicated that of the twenty five progeny, eleven were of genotype *Sr-mvP1(1)* and fourteen were of genotype *Sr-mvP1(1,2)* (Table 5.3 and Figure 5.8). The ratio of progeny genotypes from the second of the two controlled crosses (Table 5.3) was shown to be significantly different from that expected if gamete production and X chromosome inheritance are Mendelian and both XX and X0 progeny are possible. In contrast, this ratio of progeny genotypes was not significantly different from that expected if X chromosome segregation in gamete production is non-Mendelian and only XX progeny are possible. As it has been shown that *S. ratti* free-living males have only a single allele of *Sr-mvP1* (Table 5.2 and Figure 5.8), this indicates that the paternal genotype in this cross was *Sr-mvP1(1,-)* and that all progeny inherit the single paternal X chromosome. This indicates that all of the indirectly developing iL3 progeny of unrelated free-living adults are XX and female.

The findings reported here agree with the previous genetic analysis of reproduction between unrelated free-living males and free-living females (Viney *et al.* 1993) and directly conflict with previous cytological studies of *S. ratti* (Nigon & Roman, 1952; Bolla & Roberts, 1968). One way in which this conflict can be resolved is if reproduction between closely related worms occurs by pseudogamy and functionally meiotic parthenogenesis and reproduction between unrelated worms is sexual (Viney *et al.* 1993; Hammond & Robinson, 1994). Other explanations have been put forward but, as discussed in Chapter 1, they do not provide very satisfying answers. If reproduction between related free-living adults did occur by functionally meiotic parthenogenesis, it would be predicted that the progeny of naturally mated free-living females would inherit the alleles of *Sr-mvP1* in a simple Mendelian fashion. The naturally mated free-living females analysed here were genotyped for *Sr-mvP1* and, with the exception of naturally mated free-living female 4, were shown to be of genotype *Sr-mvP1(1,2)* (Table 5.4). In addition, six directly developing iL3s from isofemale line ED335 were also shown to be of genotype *Sr-mvP1(1,2)*. As this isofemale line was passaged solely by use of directly

developing iL3s (Figure 5.5), the parasitic females and free-living females would also be of this genotype (Table 5.2). Therefore, the genotype of free-living females one to seven can be independently confirmed as *Sr-mvP1(1,2)*. However, free-living females eight to twelve were not derived from parasite lines that had been maintained solely by use of directly developing iL3s (Figure 5.5). Hence, their genotype could not be confirmed and the possibility that *Sr-mvP1* had been amplified from sperm cannot be discounted. For free-living females of genotype *Sr-mvP1(1,2)* functionally meiotic parthenogenesis would be expected to result in a one *Sr-mvP1(1)*: two *Sr-mvP1(1,2)*: one *Sr-mvP1(2)* ratio of progeny genotypes. However, as can be seen in Table 5.4 only a single naturally mated free-living female (free-living female 2) produced progeny with all three *Sr-mvP1* genotypes. Analysis showed that the observed ratio of progeny genotypes were significantly different from those that would be expected if reproduction occurred by functionally meiotic parthenogenesis in five out of the twelve cases (Table 5.4). In contrast, the observed ratio of progeny genotypes were significantly different from those that would be expected if reproduction was sexual for only a single free-living female (Table 5.4). Even if only the naturally mated free-living females from isofemale line ED335 are considered, only a single group of progeny genotypes is inconsistent with sexual reproduction. In contrast, two of the seven naturally mated free-living females from isofemale line ED335 produced progeny genotype ratios that differed significantly from those that would be expected if reproduction occurred by functionally meiotic parthenogenesis (Table 5.4). In addition, it must be noted that the naturally mated free-living females may have mated with free-living males of more than one genotype. Thus, the ratio of progeny genotypes produced by naturally mated free-living female 2 (Table 5.4), may be a consequence of multiple mating rather than meiotic parthenogenesis. Therefore, the most parsimonious explanation for these findings is that reproduction between related free-living adults is sexual.

This apparent inheritance of the paternal X chromosome by all progeny of the free-living adults (Table 5.3 and Figure 5.8) is intriguing, and the mechanism by which it occurs is unclear. It is possible that only X-bearing sperm are produced, as occurs in certain aphid species where reproduction between XX females and X0 males results in the production of only XX progeny (Blackman, 1987). However, cells with 2 and 3 chromosomes, which were assumed to be sperm, have

been observed in the germline of free-living males of both *S. ratti* (Nigon & Roman, 1952) and *S. stercoralis* (Hammond & Robinson, 1994). These observations are consistent with the Mendelian segregation of X chromosomes during spermatogenesis, but it must be noted that the cells shown to have 2 chromosomes may have been immature or non-functional. It is therefore possible that sperm precursor cells which lack an X chromosome cannot complete development. It is unlikely that only X-bearing sperm are transferred from the free-living male to the free-living female, as this would be difficult to explain biologically. Other possible hypotheses are that sperm which lack an X chromosome are unable to fertilise oocytes, for instance if an active gene required either for fertilisation or sperm motility was X-linked, or that if fertilisation does occurs, the resulting X0 chromosome complement is lethal to the developing embryo. It is also possible that some of the progeny of the free-living adults are not female. As previously discussed, crosses between XX hermaphrodites and X0 males of *C. briggsae* result in approximately 90% XX hermaphrodite progeny during the first six hours after fertilisation (LaMunyon & Ward, 1997). i.e. an approximately ten-fold advantage. This advantage steadily decreases as X-bearing sperm are depleted and by 24 hours after fertilisation, approximately 25% of the total number of progeny produced by the hermaphrodite are male (LaMunyon & Ward, 1997). Unfortunately, as only small numbers of progeny were produced by each individual *S. ratti* free-living female, the possibility that X-bearing sperm have a competitive advantage cannot be discounted.

The demonstration of sexual reproduction between both related and unrelated worms of *S. ratti* conflicts with cytological studies of *S. ratti* (Nigon & Roman, 1952; Bolla & Roberts, 1968), *S. papillosus* (Zaffagnini, 1973; Triantaphyllou & Moncol, 1977; Albertson *et al.* 1979), *S. ransomi* (Triantaphyllou & Moncol, 1977) and *S. stercoralis* (Hammond & Robinson, 1994). These cytological studies all concluded that reproduction between the free-living adults occurred by pseudogamy and meiotic parthenogenesis, with the free-living males making no genetic input into the next generation. As discussed previously (Chapter 1), all of the cytological studies of reproduction in the free-living phase of *Strongyloides spp.* produced similar results. In brief, these were that the sperm pronucleus degenerates, only a single polar body is given off by the dividing oocyte and that the chromosome complement is restored by the fusion of the egg pronucleus with the second polar body (Nigon &

Roman, 1952; Triantaphyllou & Moncol, 1977; Hammond & Robinson, 1994). As it is now clear that reproduction between the free-living males and free-living females of *S. ratti* is sexual in crosses between both related (Table 5.3) and unrelated worms (Viney *et al.* 1993; Table 5.4) the cytological observations of *S. ratti* are obviously incorrect. It is therefore possible that, as all the cytological studies of reproduction have shown similar results, all *Strongyloides spp.* are, like *S. ratti*, reproducing sexually. The alternative, that *S. ratti* is alone among the many *Strongyloides spp.* in reproducing sexually, would suggest that the evolution of reproductive mode in the genus was occurring very rapidly.

These observations complement the previous parasitological analysis of the *S. ratti* life-cycle (Chapter 3). It is now clear that the intra-host sex determination switch is a chromosomally based GSD switch, resulting in the production of XX female and X0 male larvae. This supports the finding that the proportion of larvae that develop into free-living males is independent of extra-host factors. The extra-host free-living female/directly developing iL3 developmental conversion is also supported as analysis of the X-linked RFLP in *Sr-mvP1* confirms that the two morphs are both XX and female. That all the progeny of the free-living adults inherit the paternal X chromosome, are XX and female, rationalises this part of life-cycle. In addition, the finding that reproduction between free-living adults of *S. ratti* is sexual in all crosses is particularly important and may indicate that reproduction in the free-living phase of other *Strongyloides spp.* is also sexual.

CHAPTER 6.

Analysis of putative transposable elements present in the *Strongyloides ratti* genome and implications of the sex-linkage of such elements.

ABSTRACT

Transposable elements have been identified in all species in which they have been sought. However, only a limited number have been found in parasitic nematodes. Preliminary molecular work on *S. ratti* has identified a number of sequences similar to transposable elements. One of these, *Sr-mvP2*, exhibits similarity to a large, 4700 base pair, sequence-specific retrotransposon, termed R4, initially identified in *Ascaris lumbricoides* and subsequently found in *Parascaris equorum*. In *A. lumbricoides*, R4 is present in multiple copies, with a number inserted in the large subunit ribosomal RNA (LSU rRNA) genes. It is apparent that *Sr-mvP2* is sex-linked in *S. ratti*, with a higher copy number in free-living females than in free-living males. Further analysis of *Sr-mvP2* in *S. ratti* indicates that there are multiple copies of the element within the genome and that there appear to be differences in the copy number and position of *Sr-mvP2* between laboratory lines of *S. ratti*. Sequence analysis of the region of the LSU rRNA gene of *S. ratti* in which R4 inserts in *A. lumbricoides* demonstrates that the 13 base pair region of nucleotide sequence in which the element is found in *A. lumbricoides* and *P. equorum* is altered in *S. ratti*. However, Southern blotting suggests that the element is present in a fraction of the LSU rRNA genes of *S. ratti*. Analysis of the other sequences that are related to transposable elements indicates that they are likely to represent non-functional elements. These findings are discussed in relation to the genomic distribution and the sex-linkage of transposable elements.

6.1 Introduction

Transposable elements are mobile DNA elements and are, in all likelihood, present within the genome of every eukaryote. For example, it has been shown that the *Tc1* family of transposable elements is almost ubiquitous in animal genomes, with most species having multiple representatives of the family (Avancini *et al.* 1996). The most common group of transposable elements are the retrotransposons, mobile elements that exist as RNA and require the action of a reverse transcriptase to insert into the host DNA (Varmus, 1983). A subclass of these elements are the non-LTR retrotransposons, characterised by the lack the of long terminal repeats (LTRs) that define elements such as *copia* and *Ty1* (Burke *et al.* 1993). The best known of the non-LTR retrotransposons are the LINE-1 elements of mammals (Hutchinson *et al.* 1989).

The copy number of transposable elements is known to vary widely, from single copy elements to those present in the thousands (e.g. mariner-like elements in insects: Robertson & Lampe, 1995). In some species, transposable elements can form a substantial fraction of the genome. For instance, recent evidence indicates that the maize genome has doubled in size over the past three million years solely as a consequence of the accumulation of retrotransposons (SanMiguel & Bennetzen, 1998; SanMiguel *et al.* 1998). The presence of so many transposable elements, fragments of these elements and pseudogenes in most genomes makes identification of true transposable elements problematic. The role of transposable elements is also unclear and it is not known if they are purely selfish DNA parasites or if they are important in the evolution of complex genomes and gene regulation (Lönnig & Saedler, 1997). There is now considerable evidence to suggest that in a number of cases, ancient transposable element insertions now serve in the regulation of gene expression (for review, see Britten, 1996 and Britten, 1997). There is also increasing evidence that these elements also play a role in the repair of double stranded breaks in DNA (Teng *et al.* 1996: Moore & Haber, 1996). In contrast, at an individual level, the mutagenic effects of transposable elements are normally deleterious. For instance, *Tc1* is believed to be responsible for the majority of spontaneous mutations in *C. elegans* (Eide & Anderson, 1985).

The distribution of many transposable elements within the genome is not random, with elements known to insert into specific sequences and specific chromosomal regions. In addition, it has been shown that chromosomes and chromosomal regions that do not recombine, or have limited recombination in comparison to other areas of the genome, accumulate transposable elements. This phenomenon has been shown for the male determining region of chromosome III in the midge, *Chironomus thummi* (Kraemer & Schmidt, 1993), the neo-Y chromosome of *Drosophila miranda* (Steinemann & Steinemann, 1998), and the W chromosomes of *Bombyx mori* and *B. mandarina* (Abe *et al.* 1998). While it is unclear if the accumulation of transposable elements is a result of the non-random insertion of the elements or a consequence of reduced elimination of elements present in such areas, the accumulation is believed to be linked to the degeneration of non-recombining chromosomes.

As in other species, a number of transposable elements have been found in nematodes. In particular, sequences related to the *Tc* transposable elements of *C. elegans* are widespread among free-living nematodes (Abad *et al.* 1991). However, only a limited number of transposable elements have been identified in parasitic nematodes. These include a member of the *Tc1*-family of transposable elements found in *Haemonchus contortus* (Hoekstra *et al.* 1999), a mariner-like element (*mle-1*) found in *Trichostrongylus colubriformis* (Wiley *et al.* 1997) and a number of different elements that have been identified in *Ascaris lumbricoides*. One of the transposon-like elements, *Tas*, that has been identified in *A. lumbricoides* is present in approximately 50 copies per genome (Aeby *et al.* 1986; Felder *et al.* 1994), another is a large, 4700 base pair (bp), sequence-specific non-LTR retrotransposon, termed R4 (Back *et al.* 1984; Neuhaus *et al.* 1987). R4 was initially identified in *A. lumbricoides* and has subsequently been found in the closely related species *Parascaris equorum* (Burke *et al.* 1995). In addition, a Y-linked sequence in *Brugia malayi*, TOY (Tag on Y), has been found that exhibits significant similarity with a family of reverse transcriptase-like genes from *C. elegans* (Underwood & Bianco, 1999). While the sequence of TOY indicates that the gene is non-functional, multiple hybridisation bands have been found in Southern blots, suggesting that the TOY sequence may be the relic or fragment of a transposable element (Underwood & Bianco, 1999).

Genetic analysis of sex determination in the parasitic nematode *Strongyloides ratti* has resulted in the identification of five DNA fragments that are related by amino acid sequence to transposable elements (Chapter 4). Two of these sequences, *Sr-mvP2* and *Sr-mvP3*, have also been identified as putatively sex-linked in *S. ratti*, with the sex-linkage of *Sr-mvP2* subsequently confirmed by semi-quantitative PCR (Chapter 4). Further analysis of another sex-linked marker in *S. ratti* (Chapter 5) has shown that the species has an XX/X0 system of sex determination. This suggests that other sex-linked markers identified are also X-linked. However, as *Sr-mvP2* has also been shown to be present in multiple copies within the genome of isofemale line ED321 Heterogonic (Chapter 4), it is not clear how many of these copies are X-linked. Previous analysis of the sequence of *Sr-mvP2* has indicated that it is similar to the R4 element of *A. lumbricoides* (Appendix 2).

The genome of *A. lumbricoides* contains multiple copies of the R4 element, with approximately twenty found to be inserted within copies of the large subunit ribosomal RNA (LSU rRNA) genes. These genes are among the most highly conserved regions of sequence yet found and are also present at relatively high copy number. For instance, *A. lumbricoides* has approximately one hundred rRNA repeats. Sequence-specific transposable element insertions within the rRNA genes are known to be almost ubiquitous in insects (Jakubczak *et al.* 1991: Besansky *et al.* 1992). The nucleotide sequence of these retrotransposons and their insertion sites within the LSU rRNA genes has allowed the elements to be grouped into two main families, R1 and R2 (Burke *et al.* 1993). There is also evidence to suggest that there is a third, rarer, group of elements termed the R3 insertions (Kerrebrock *et al.* 1989). The R4 element of *A. lumbricoides* and the homologue from *P. equorum* share the same insertion site within the LSU rRNA gene and it has been suggested that they define a fourth group of LSU rRNA specific elements (Burke *et al.* 1995). However, due to the close phylogenetic relationship between *A. lumbricoides* and *P. equorum*, the presence of R4 within the LSU rRNA genes may represent an ancestral insertion event rather than conservation of the insertion site within the LSU rRNA gene.

Previous results do not indicate if *Sr-mvP2* is an active transposable element or if it is inserted in the LSU rRNA gene. In addition, although it is apparent from the sex-linkage of *Sr-mvP2* that at least one copy of *Sr-mvP2* is present on the X chromosome, the location of other copies of the sequence is unclear. Here, the sequence of *Sr-mvP2* and its distribution within the *S. ratti* genome are investigated. The sequences of the other *S. ratti* markers that are related to transposable elements are also more fully investigated.

6.2 Materials and Methods

Parasites and maintenance

Methods for the maintenance, culture and collection of *S. ratti* are as previously described (Chapter 4). Methods for the extraction of DNA are as previously described (Chapter 5). The isofemale lines ED321 Heterogonic, ED5 Homogonic, ED132 Homogonic and ED248 Heterogonic were used to compare the of copy number of *Sr-mvP2* and to assess possible mobility of the putative transposable element within laboratory lines of *S. ratti*. ED321 Heterogonic and ED5 Homogonic are isofemale lines derived from ED5 (Viney, 1996) and have been separated for approximately 100 generations. ED132 Homogonic and ED248 Heterogonic are isofemale lines of ED132 (Viney, 1996) and have been separated for approximately 30 generations. The isofemale line ED321 Heterogonic was used for all other experiments.

Sequencing of Sr-mvP2

The entire length of the *Sr-mvP2* genomic DNA fragment was sequenced, as described in Chapter 5, using the M13 forward and reverse primers and the *S. ratti* specific primers shown in Table 6.1. Both strands of the marker were sequenced from purified plasmid and sequence fragments were assembled using the AssemblyLIGN programme (Oxford Molecular Group).

Forward strand			
*Sr-mvP2*F	5'	CCA TAG AAG GTA TGT ATT A	3'
*Sr-mvP2*F.2	5'	CAA TAG AAA TTG TCT ATT GCC	3'
Reverse strand			
*Sr-mvP2*R	5'	CTA TCT TGA AAC AGA ACC C	3'
*Sr-mvP2*R.2	5'	GTT AAA GAA GGG AAT CAT GC	3'

Table 6.1 The sequence of the primers used for the sequencing of *Sr-mvP2*.

Sequence analysis

The sequences that showed significant similarity to previously published amino acid sequences, CM6, pZ-8, xol.1b, *Sr-mvP3* and *Sr-mvP2* (Chapter 5), were analysed for coding potential and compared to previously characterised transposable elements. Comparisons were carried out using the GCG sequence analysis program (Wisconsin Package Version 10.0, Genetics Computer Group, Madison).

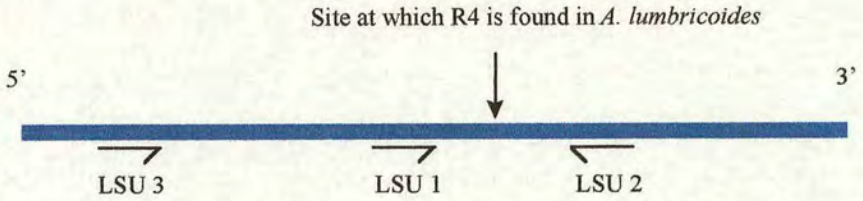
PCR amplification of S. ratti *sequences*

To determine if *Sr-mvP2* is present within the LSU rRNA genes of *S. ratti*, an attempt was made to amplify, by PCR, a fragment spanning the insertion site using *Sr-mvP2* specific primers (Table 6.1) in conjunction with nematode specific LSU rRNA gene primers (Table 6.2). In addition, a degenerate primer originally used to amplify the 3'-half of the R4 elements from *P. equorum* (Burke *et al.* 1995), was also used (Table 6.2). A schematic representation of the primer location is shown in Figure 6.1. To obtain the 3' end of *Sr-mvP2*, the primer LSU 1 was used in conjunction with either RT 1 or *Sr-mvP2*F.2 (Table 6.1). To obtain the *S. ratti* LSU rRNA sequence spanning the site at which R4 is found in *A. lumbricoides*, the primers LSU 2 and LSU 3 were used (Burke *et al.* 1995). To obtain the 5' end of *Sr-mvP2*, the primer LSU 2 was used with *Sr-mvP2*R.2 (Table 6.1).

Primer name	Sequence
LSU 1	5' - GCC AGA TTA GAG TCA AGC TC - 3'
LSU 2	5' - CTA AGT CGA CTG CCC AGT GCT CTG AAT GTC - 3'
LSU 3	5' - AAG AGC CGA CAT CGA AGG ATC - 3'
RT 1	5' - TTY TWY ATG GAY GAY NT - 3'

Table 6.2 The sequence of the primers used in the attempt to amplify from *Sr-mvP2* to the LSU rRNA gene in *S. ratti*. The sequence of the primers shown here is taken from (Burke *et al.* 1995).

A. Uninterrupted LSU rRNA gene



B. Interrupted LSU rRNA gene and predicted primer alignment.

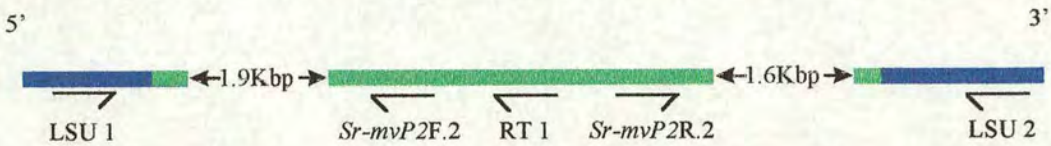


Figure 6.1 A schematic representation of the primer locations and the postulated organisation of the LSU rRNA genes and the putative retrotransposon *Sr-mvP2*.

The PCR reaction conditions as described by Burke *et al.* (1995) were used (35 cycles of 95°C for 1 minute, 55°C for 1 minute and 70°C for 2 minutes). In addition, further reactions were carried out with a 3 minute extension time and the annealing temperature varied in 5°C increments between 45°C and 55°C. All reactions were done in 50 µl volume, with primers at a final concentration of 100 nM, PCR buffer (Promega) at 1 × concentration, 1.5 mM $MgCl_2$ final concentration, 0.75 µM of dATP, dTTP, dCTP and dGTP (Roche Molecular Biochemicals), and *Taq* DNA polymerase (Promega) at 200 units ml^{-1} final concentration. In addition, biased primer concentrations were also used, with the primer specific for the large subunit rRNA gene at a final concentration of 25 nM and the primers specific for the *S. ratti* sequence or the degenerate RT primer at a final concentration of 200 nM. Successfully amplified PCR fragments were purified and directly sequenced using the PCR amplification primers, as previously described (Chapter 5).

Varying the reaction conditions and changing the primer combinations did not result in the amplification of any fragments.

PCR amplification of the region of the *S. ratti* LSU rRNA gene, with the primers LSU 2 and LSU 3, resulted in the amplification of a single band of approximately 600 bp. When sequenced and analysed (NCBI Blastn search), the 572 bp of novel sequence (Figure 6.8) was found to be more than 90% identical at the nucleotide level to the *C. elegans* LSU rRNA gene. Thus, the degenerate LSU rRNA primers successfully amplified a fragment of the LSU rRNA gene from *S. ratti*. Comparison of this *S. ratti* sequence with that of the LSU rRNA sequences of *A. lumbricoides*, *C. elegans* and *D. melanogaster* shows that the *S. ratti* sequence is more diverged from the others than would be expected (Figure 6.9). In addition, it is apparent that three of the base pair differences found in the *S. ratti* LSU rRNA gene fragment fall within the 13 bp region in which the R4 element is found in *A. lumbricoides* and *P. equorum* (Figure 6.9).

```
1       TCATTGGGAA ACCAATTGAA GCTCTTGTAA ACGGCGGGAG TAACTATGAC TCTCTTAAGG
61      TAGCCAAATG CCTCGTCGGG TAATTTCCGA CGCGCACGAA TGGATTAACG AGATTCCTAC
121     TGTCCCTATC TACCATTCAG CGAAATCACA GCCAAGGGAA CGGGCTTGGC AAAAACAGCG
181     GGGAAAGAAG ACCCTGTTGA GCTTGACTCT AGCTAGGCAT TGTGAAGTGT CATATGAGGT
241     GTAGCATAGG TGGGAGTTAT TTTATAACGA TCTTGAAATA CCACTACTTA TATTGATATT
301     TTACTTAGTT GATTAAATGG AAATTTTATA TATTAATTTA TATAATAATT TTTGTATTAA
361     ATTAATATCT TTGAGATATT TAATAATCCA CGTCAATGAC CATGTCTGGC GGGGAGTTTG
421     ACTGGGGCGG TACATCTATC AAACTGTAAC GTAGGTGTCC TAAGGCAAAC TCAAAGAGGA
481     CAGAAACCTC TTGTAGAGTA AAAGGGCAAA AGTTTGCTTG ATCATGATTT TCAGTACGAA
541     TACAGACCCT GAAAGGGTGG CCTATCGATC CT
```

Figure 6.8 Sequence of a 572 bp fragment of the *S. ratti* LSU rRNA gene that spans the point at which the R4 element is found in *A. lumbricoides* and *P. equorum*. The coloured region represents the sequence shown in Figure 6.9.

Southern blotting

General methods for the extraction of genomic DNA, restriction endonuclease digestion of DNA, Southern blotting and the preparation of radioactively labelled probes were as described previously (Chapter 5). In all cases, hybridisations were carried out at 65°C. After hybridisation, blots were rinsed briefly in 3 × SSC/0.1% w/v SDS, washed twice, at 65°C, in 100 ml of 3 × SSC/0.1% w/v SDS for 30 minutes and washed again, at 65°C, in 100 ml of 0.1×SSC/0.1% w/v SDS for 30 minutes.

i. The distribution and copy number of Sr-mvP2 *in different isofemale lines of* S. ratti

To compare the distribution and copy number of *Sr-mvP2* between isofemale lines of *S. ratti*, a Southern blot containing genomic DNA from the isofemale lines ED321 Heterogonic, ED5 Homogonic, ED248 Heterogonic and ED132 Homogonic was made. DNA from each isofemale line had digested with the restriction endonucleases *Hind*-II, *Sin*-I, *Msp*-I, *Nci*-I or *Eco*R-V (Roche Molecular Biochemicals). Purified, PCR amplified, DNA from the cloned *Sr-mvP2* fragment was labelled and hybridised to this blot.

ii. Localisation of Sr-mvP2 *to the LSU rRNA genes of* S. ratti

A Southern blot containing genomic DNA from isofemale line ED5 Homogonic which had been digested with the restriction endonucleases *Mun*-I (*Mfe*-1), *Taq*-I and both *Mun*-I and *Taq*-I (Roche Molecular Biochemicals) was made. A purified and labelled, PCR amplified, 572 bp fragment of the *S. ratti* LSU rRNA gene that spans the site at which R4 is found in *A. lumbricoides*, was hybridised to this blot. *Taq*-1 removes 4 bp from the 5' end of this fragment of the LSU rRNA gene and *Mun*-1 removes 17 bp from the 3' end. Thus, if there are no insertions within this region of the LSU rRNA gene then only a single band will be identified in the genomic DNA digested with both enzymes. In addition, purified and labelled, PCR amplified DNA from the cloned *Sr-mvP2* fragment was hybridised to the blot.

6.3 Results

Sequence analysis

i. CM6

The 725 bp marker CM6 has previously been shown to be similar to a short, 308 amino acid, bacterial OrfB insertion element from *Caulobacter crescentus* (U39501) (Chapter 5). Comparison of the conceptual amino acid translation of CM6 with the amino acid sequence of the *C. crescentus* insertion element IS511 (Mullin *et al.* 1997) and the amino acid sequence of a 281 amino acid OrfB element from *Xanthomonas campestris* (U77781) is shown in Figure 6.2. The similarity of CM6 to the insertion elements is limited to the short, 168 bp, region that is shown in Figure 6.2. Analysis of the rest of the conceptual amino acid translation of CM6 shows that, in addition to the stop codon shown in Figure 6.2, the sequence shown below is flanked by further stop codons.

```
Sr  G P P Q V V R X D N G P E F I S E A X R T F * E D
Cc  G R P G M I V S D N G T E F T S T A I L A W A E D
Xc  G L P Q V I R T D N G K E F C G K A M V A W A H D

Sr  - Q - V G X V Y V P P G Q p W K N G Y I E S F N N
Cc  H R - V A W H Y I A P G K p T Q N G F V E S F N G
Xc  - R G V Q L R L I Q P G K p N Q N A Y V E S F N G

Sr  R V R D E X L N
Cc  R M R D E L L N
Xc  R V R D E C L N
```

Figure 6.2 Alignment of the conceptual translation of the CM6 (*Sr*) with the amino acid sequences of the *C. crescentus* insertion element IS511 (*Cc*) and the OrfB element from *X. campestris* (*Xl*). Amino acid identity is indicated by heavy shading and amino acid similarity by light shading. - denotes a gap to allow alignment. * denotes a stop codon. X denotes that the nucleotide sequence could not be translated at that point.

ii. pZ-8

The conceptual amino acid translation of the available sequence of pZ-8 has been shown to exhibit significant similarity to a *Ciona intestinalis* sequence (COS41.3) which is itself similar to a reverse transcriptase (Chapter 5). Further analysis of pZ-8 indicated that all similarity was related to a 400 bp region of a 460 bp open reading at the 5' end of the pZ-8 sequence. This region of the *S. ratti* sequence was similar to the 526-648 amino acid region of the 1209 amino acid sequence of the *C. intestinalis* element. Comparison of the conceptual amino acid translation of pZ-8 with that of the predicted reverse transcriptase from *C. intestinalis* is shown in Figure 6.3.

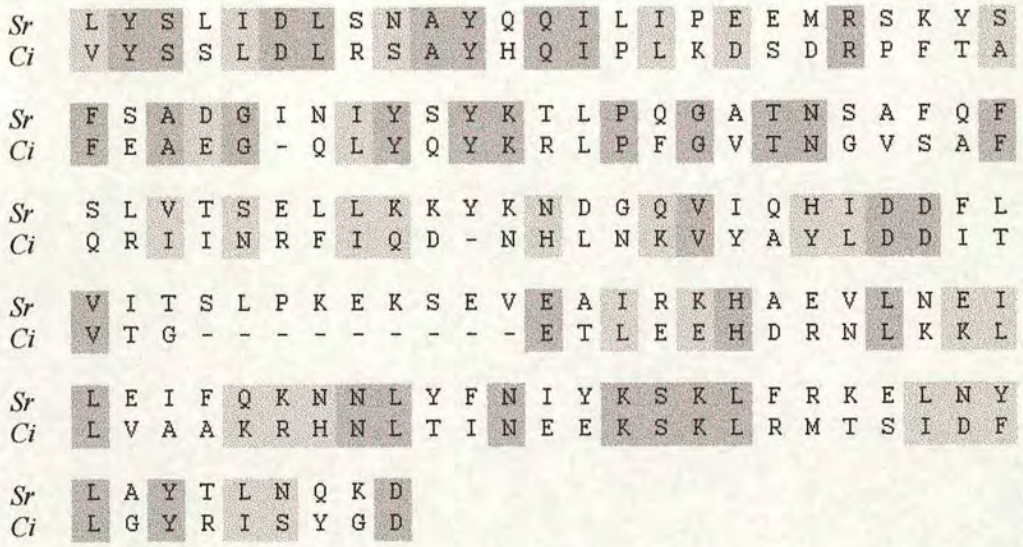


Figure 6.3 Alignment of the conceptual translation of the pZ-8 (*Sr*) with the amino acid sequence of the predicted reverse transcriptase, COS41.3, from *C. intestinalis* (*Ci*). Amino acid identity is indicated by heavy shading and amino acid similarity by light shading. - denotes a gap to allow alignment.

iii. xol.1b

The predicted amino acid translation of the 509 bp xol.1b fragment was previously shown to be similar to a 1150 amino acid *Pol* protein from *Drosophila melanogaster* and to an 1180 amino acid *Pol* protein from *D. virilis* (Chapter 5). Both of these previously characterised transposable elements were similar to the same predicted reading frame of xol.1b. Further analysis indicated that this open

reading frame spanned, with the exception of two stop codons, the entire length of the xol.1b sequence. However, the similarity of xol.1b to the two *Drosophila* elements is limited to a 218 bp region at the 3' end of the sequence (nucleotide position 18-236). This region corresponds to the 457-529 and 500-572 amino acid regions in the *D. melanogaster and D. virilis* elements, respectively. Comparison of the conceptual amino acid translation of xol.1b with the *Pol* proteins from *D. melanogaster* and *D. virilis* is shown in Figure 6.4.

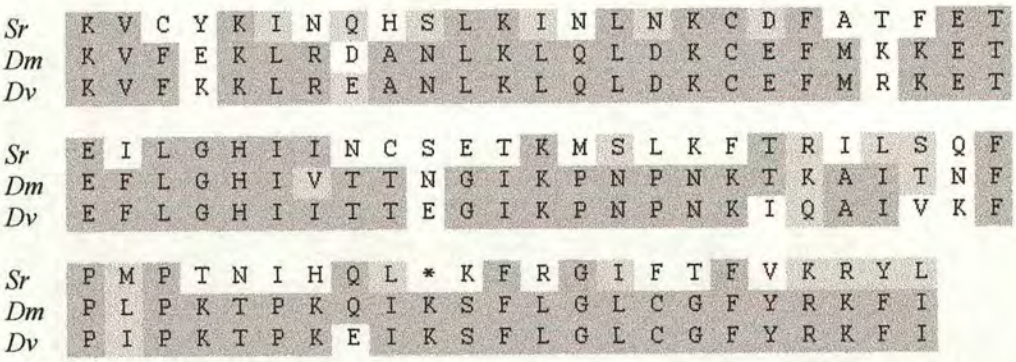


Figure 6.4 Alignment of the conceptual translation of the xol.1b (*Sr*) with the amino acid sequence of the *Pol* proteins from *D. melanogaster* (*Dm*) and from *D. virilis* (*Dv*). Amino acid identity is indicated by heavy shading and amino acid similarity by light shading. * denotes a stop codon.

iv. Sr-mvP3

The predicted amino acid translation of this sequence is related to a 960 amino acid retrotransposon from *Bombyx mori* (U07847) and a 1221 amino acid retrotransposon from *C. elegans* (T19C9.7) (Chapter 5). Analysis indicated that, with the exception of four stop codons, the translation frame of the *Sr-mvP3* sequence that is similar to the sequence of the *B. mori* and *C. elegans* transposable elements is open for the entire length of the sequence. A comparison of the conceptual translation of *Sr-mvP3* to the reverse transcriptases from *B. mori* and *C. elegans* is shown in Figure 6.5. The conceptual translation of *Sr-mvP3* shown in Figure 6.5 represents nucleic acids 7-750, out of the 773 bp of sequence available for this marker. This region of sequence corresponds to positions 744-965 and 640-713 in the amino acid sequences of the *B. mori* and *C. elegans* retrotransposons, respectively.

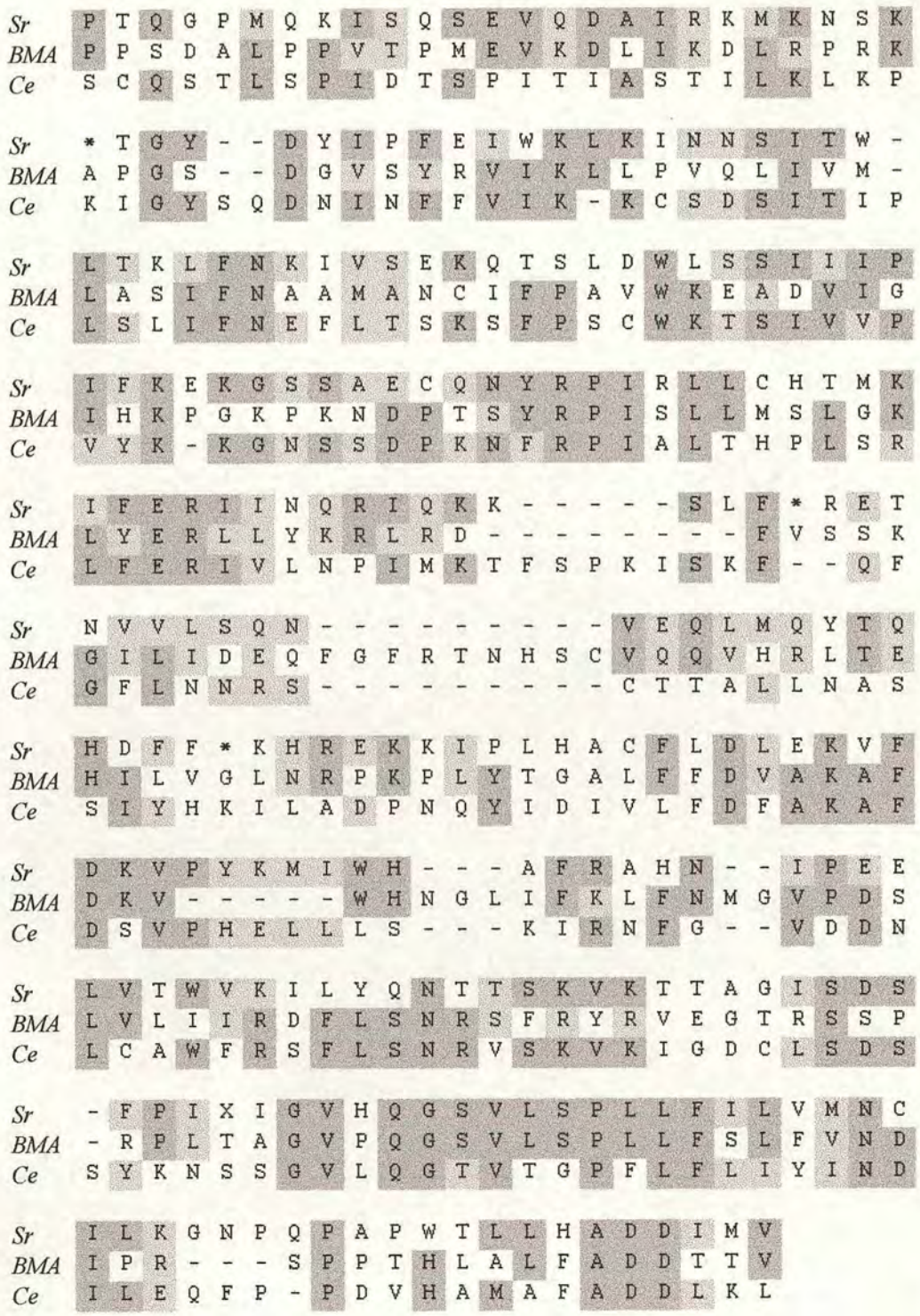


Figure 6.5 Alignment of the conceptual translation of the *Sr-mvP3* (*Sr*) with the amino acid sequences of the *B. mori* reverse transcriptase U07847 (*BMA*) of the *C. elegans* reverse transcriptase T19C9.7 (*Ce*). Amino acid identity is indicated by heavy shading and amino acid similarity by light shading. - denotes a gap to allow alignment. * denotes a stop codon. X denotes that the nucleotide sequence could not be translated at that point.

The full length sequence of *Sr-mvP2* from the original cloned DNA fragment was obtained and is shown in Figure 6.6. Analysis of this sequence by Blastx or Blastn search of Genbank (NCBI) again showed that, as in Chapter 5, there were no similar nucleic acid sequences present in the database, but that the conceptual amino acid translation is most similar to the amino acid sequence of the R4 element from *A. lumbricoides*. A comparison of the conceptual translation of *Sr-mvP2* with the amino acid sequence of the *A. lumbricoides* R4 element is shown in Figure 6.7. Further analysis of the conceptual translation of *Sr-mvP2* indicated that there were no open reading frames that spanned the whole of the fragment and that in order to allow the alignment shown in Figure 6.7, the translation of *Sr-mvP2* contained a number of frame shifts.

```
1       CAAACTTTTT GATCTACTTA TCATAGTATC TCTAGTTAAG AATGTTTTTT ATCTTACCAC
61      TTATGATTTT TGTGAACTCA TTTATATCAT CATAATACAT ACCTTCTATG GTAATACCTG
121     ACTTTCTTAA TGAATATTCA ACATTGAACT TTTTCTATTA GTGTTGTGTA CTGTCTTATT
181     GGAGTGAACT TTCCTTCTTT AAGTTCATGC TGTATGCTTA ATTCTTTAAT CTCTTCGTTT
241     ATAAGGTTAA TCCAGAGATT CGCCATGTGT TTTACTATAG CTTGATAAGC TACGCTTTCT
301     AGTGATCTTA AGCCTAGACT ACCTTTCTCT GGTGTGCAAT ACAGTCTTGC AATAGAAATT
361     GTCTATTGCC TTACTTCCTA TTTGTTCACT GTCAGTTATA TTACCTGCAA GGATCTTTAT
421     CACTAGCTTG TCTAGTCTTT TACACAAGCT AGGTAGTATT CCTTGCTTGA CCGCTGGTAA
481     AGCATGTGCT ACTGCATACC CGATTGCAGG TGCTATTGAC GAGTTAAACC ATTTTATTTT
541     TTGACCTATA TTTAATTTGG TTTTAAACAC TTGGCATACA CTGCCTAGTA TTTTCTTTTC
601     CAAACTCTTT TCTAGCGTTT CTACACATAT GCTTCTACCT AACTTAGGTA TACCTAAGTA
661     TTTGTAGCAG TCTGTCAATT TACTAATGTT TTCTAACATT ACGTTATCTA ACTTATTGTT
721     GTATAGGACT CCACATTTCT TTGCGTTTAC GTAGAAACCA AGCTGAAGGG CAACAGATTC
781     AAAAATGACT TTGAATCTTT TGATTGTTGC ATTATCCATA GCAAAGATTT TCATGTCGTC
841     AACGAAAGAT ATGTGATTGG TCATAGCTGT TTCTGACATC TTTTTTATGT GTTTAGAGTC
901     AGGAGTATCA GCAACCGTTT CTCCTTCCTC ATGTCCTATA CTTGACTCAT TTTGAAACTT
961     ATGGTTGAGT TGGACCAATT AATCAGTTGG TAAGCTGTTG ATGTTCTTAC CTATTGGTAA
1021    CATAGACTCT TTATTTATCT GGTAGCTAAT TATATCCATA CCAAGGACGA ATAGACAGGG
1081    TGTTAATGCA CAACCTTGCA TGATTCCCTT CTTTAACGTT TACCCTATTG TCTTTCATGC
1141    TTTTTGTCTT ATCCACATTG GATATGGTAA AACTTCTTGA CCCTAGCATA CCTTGGATGT
1201    ATTTTGTGAT ACTAGCTGGT AGATTCAATG CTCCCACAGT TTTCCTCATG ACTGAGTGGA
1261    ATACTGAATC AAAAGCTTTT TTCATATCTA CATACAATAC GCTGGTAGCT TTTGGGTTCT
1321    GTTTCAAGAT AGAGTAATTT ACCATCGTAG CGTGTAGTGT TTCAGGTATT CCCTTGAACG
1381    CTGCACACTG ATTTGTGGCT ATATATTGGA AGCAGTTTTT ATATATG
```

Figure 6.6 Nucleotide sequence of 1427 bp of *Sr-mvP2*.

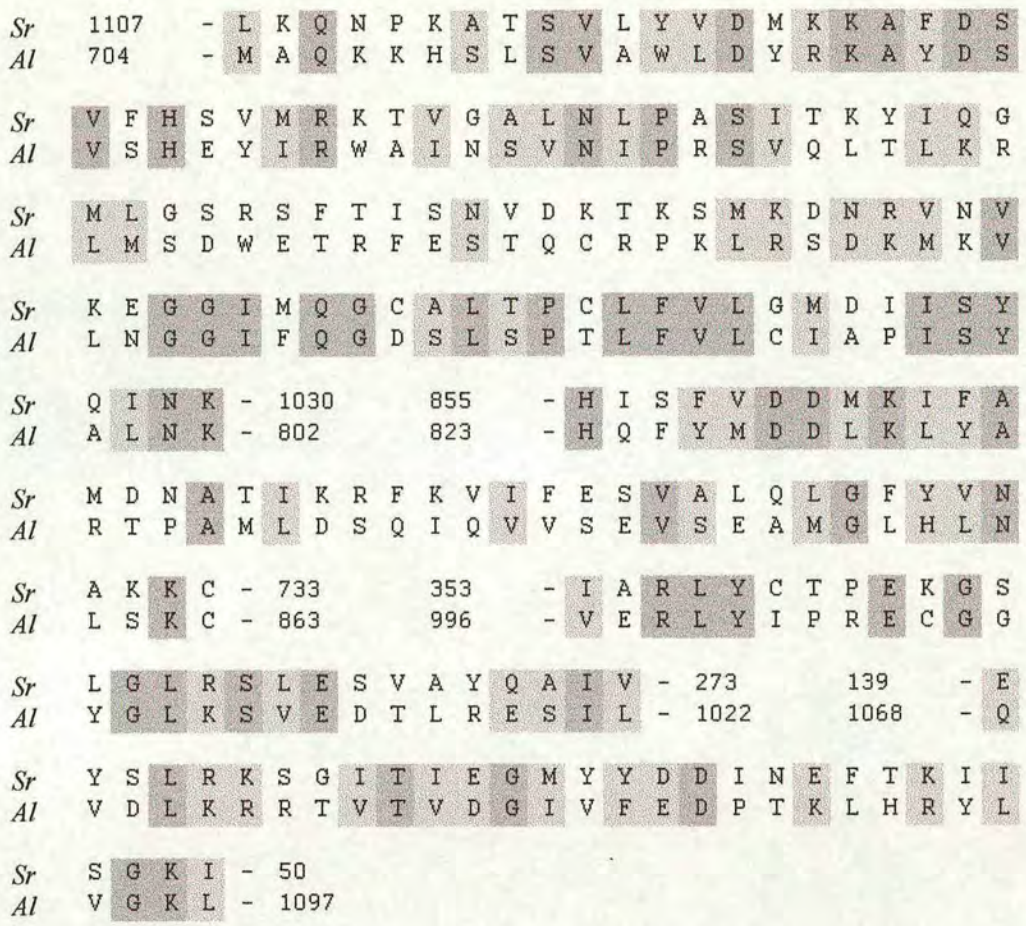


Figure 6.7 Alignment of the conceptual translation of the *Sr-mvP2* (*Sr*) with the amino acid sequence of the *A. lumbricoides* R4 element (*Al*). Amino acid identity is indicated by heavy shading and amino acid similarity by light shading. - denotes a gap to allow alignment. The *Sr-mvP2* sequence is numbered in relation to the nucleotide sequence shown in Figure 6.6 and the sequence of R4 is numbered in relation to the amino acid position.

PCR amplification of S. ratti *sequences*

The attempt to amplify, by PCR, from the LSU rRNA gene of *S. ratti* to the insertion site of the putative retrotransposon, *Sr-mvP2*, was not successful. No amplified fragments were detected from PCR reactions using the primer LSU 1 in conjunction with either RT 1 or *Sr-mvP2*F.2. Attempts to amplify the 5' end of *Sr-mvP2*, with the primers LSU 2 and *Sr-mvP2*R.2 were also unsuccessful.

Varying the reaction conditions and changing the primer combinations did not result in the amplification of any fragments.

PCR amplification of the region of the *S. ratti* LSU rRNA gene, with the primers LSU 2 and LSU 3, resulted in the amplification of a single band of approximately 600 bp. When sequenced and analysed (NCBI Blastn search), the 572 bp of novel sequence (Figure 6.8) was found to be more than 90% identical at the nucleotide level to the *C. elegans* LSU rRNA gene. Thus, the degenerate LSU rRNA primers successfully amplified a fragment of the LSU rRNA gene from *S. ratti*. Comparison of this *S. ratti* sequence with that of the LSU rRNA sequences of *A. lumbricoides*, *C. elegans* and *D. melanogaster* shows that the *S. ratti* sequence is more diverged from the others than would be expected (Figure 6.9). In addition, it is apparent that three of the base pair differences found in the *S. ratti* LSU rRNA gene fragment fall within the 13 bp region in which the R4 element is found in *A. lumbricoides* and *P. equorum* (Figure 6.9).

```
1       TCATTGGGAA ACCAATTGAA GCTCTTGTAA ACGGCGGGAG TAACTATGAC TCTCTTAAGG
61      TAGCCAAATG CCTCGTCGGG TAATTTCCGA CGCGCACGAA TGGATTAACG AGATTCCTAC
121     TGTCCCTATC TACCATTCAG CGAAATCACA GCCAAGGGAA CGGGCTTGGC AAAAACAGCG
181     GGGAAAGAAG ACCCTGTTGA GCTTGACTCT AGCTAGGCAT TGTGAAGTGT CATATGAGGT
241     GTAGCATAGG TGGGAGTTAT TTTATAACGA TCTTGAAATA CCACTACTTA TATTGATATT
301     TTACTTAGTT GATTAAATGG AAATTTTATA TATTAATTTA TATAATAATT TTTGTATTAA
361     ATTAATATCT TTGAGATATT TAATAATCCA CGTCAATGAC CATGTCTGGC GGGGAGTTTG
421     ACTGGGGCGG TACATCTATC AAACTGTAAC GTAGGTGTCC TAAGGCAAAC TCAAAGAGGA
481     CAGAAACCTC TTGTAGAGTA AAAGGGCAAA AGTTTGCTTG ATCATGATTT TCAGTACGAA
541     TACAGACCCT GAAAGGGTGG CCTATCGATC CT
```

Figure 6.8 Sequence of a 572 bp fragment of the *S. ratti* LSU rRNA gene that spans the point at which the R4 element is found in *A. lumbricoides* and *P. equorum*. The coloured region represents the sequence shown in Figure 6.9.

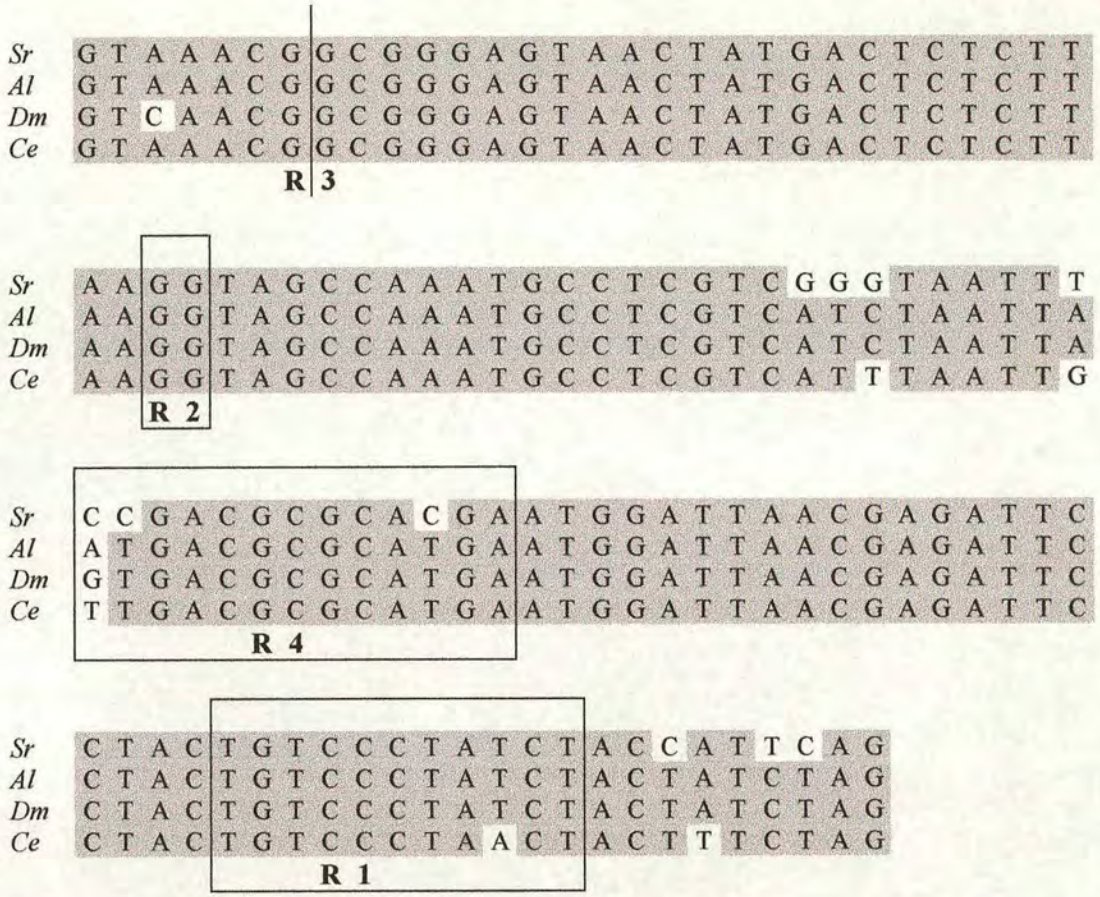


Figure 6.9 Comparison of the conserved region of the LSU rRNA gene that spans the R1, R2, R3 and R4 insertion sites from *S. ratti* (*Sr*), *A. lumbricoides* (*Al*), *D. melanogaster* (*Dm*) and *C. elegans* (*Ce*). Boxed areas represent the insertion sites of the R1, R2 and R4 elements, the insertion point of the R3 elements is represented by a line (Burke *et al.* 1995). Shaded regions show nucleotide identity. The *A. lumbricoides*, *D. melanogaster* and *C. elegans* sequences were obtained from Genbank (http://www.ncbi.nlm.nih.gov/).

Southern blotting.

i. Comparison of the distribution of Sr-mvP2 *in different isofemale lines of* S. ratti

Southern blot analysis of the copy number and distribution of *Sr-mvP2* in different laboratory lines of *S. ratti* (Figure 6.10A-D) confirms the earlier finding that this sequence is present in multiple copies within the genome. It also indicates that there are less than 10 copies of *Sr-mvP2* within the *S. ratti* genome and that there are no large differences in the copy number of *Sr-mvP2* between the isofemale lines. However, comparison of the banding pattern produced from the different isofemale lines indicates that there are a number of differences in the pattern (comparison of Figure 6.10 A-D), consistent with differences in the distribution of *Sr-mvP2* between isofemale lines.

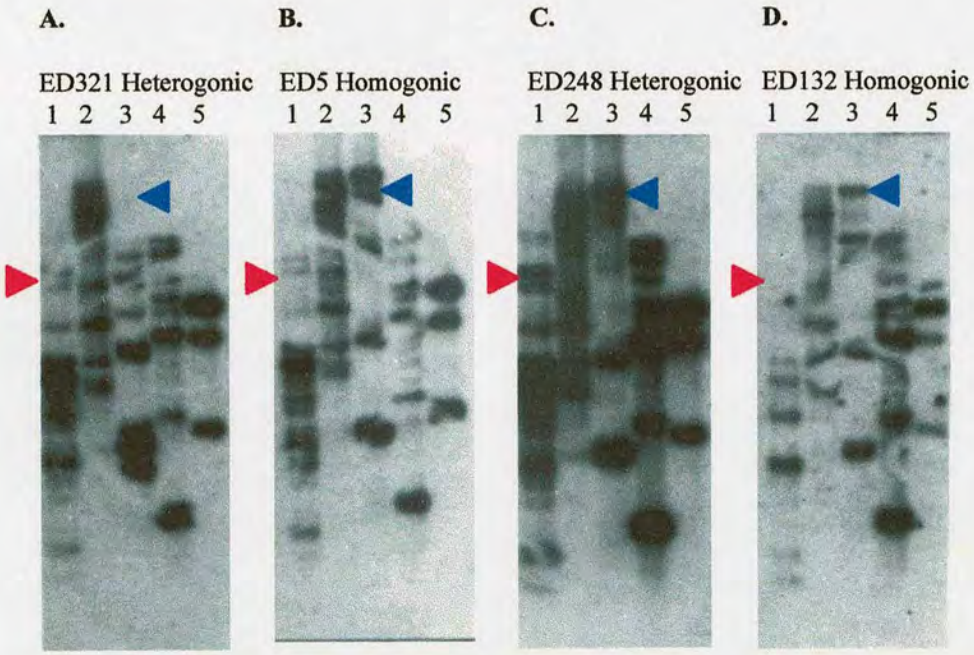


Figure 6.10 Comparison of the copy number and distribution of *Sr-mvP2* in the isofemale lines ED321 Heterogonic (A), ED5 Homogonic (B), ED248 Heterogonic (C) and ED132 Homogonic (D). Coloured arrows indicate differences in the hybridisation patterns between these isofemale lines. Note that the lines ED321 Heterogonic and ED5 Homogonic are both derived from ED5 and ED248 Heterogonic and ED132 Homogonic are both derived from ED132.

A comparison of the hybridisation of the 572 bp fragment of the *S. ratti* LSU rRNA and that of the *Sr-mvP2* fragment to the *Taq*-1, *Mun*-1 and both *Taq*-I and *Mun*-I digested genomic DNA from isofemale line ED5 Homogonic is shown in Figure 6.11A and Figure 6.11B, respectively. Hybridisation of the LSU rRNA gene fragment to the double digested DNA (Figure 6.11A, lane 3) indicates the presence of a single dark band and two larger, faint bands. Hybridisation of the *Sr-mvP2* fragment to the double digested DNA (Figure 6.11B, lane 3) indicates the presence of two dark bands. Comparison of Figure 6.11A and Figure 6.11B indicates that the larger of the two *Sr-mvP2* bands and the largest of the LSU rRNA bands are localised to the same region of the blot. This is consistent with the presence of *Sr-mvP2* is present within a subset of the *S. ratti* LSU rRNA genes.

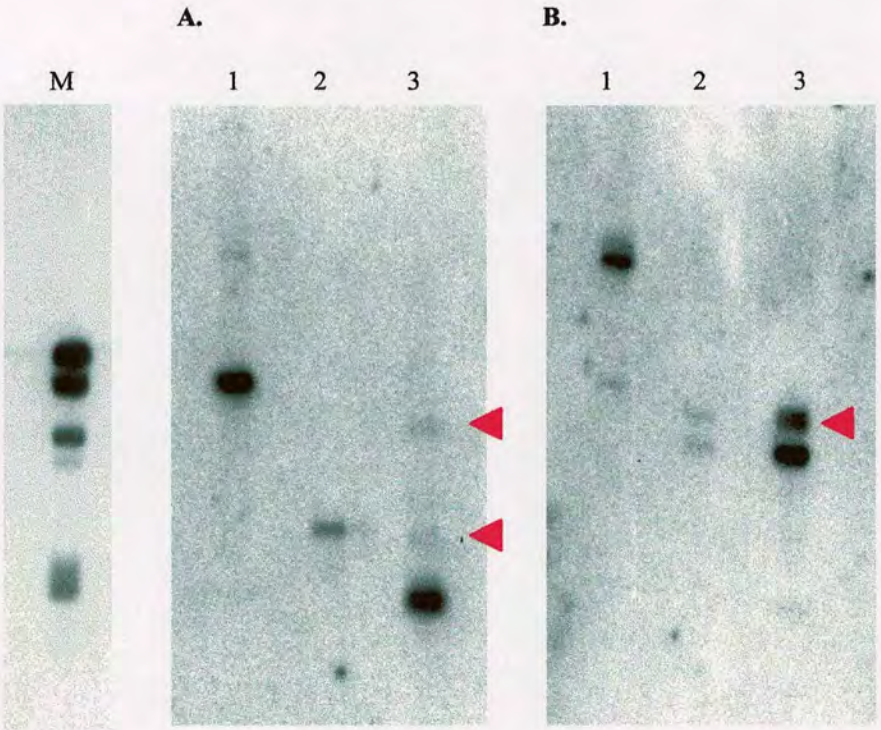


Figure 6.11 A. Hybridisation of the *S. ratti* LSU rRNA gene fragment to genomic DNA from isofemale line ED5 Homogonic, digested with *Mun*-1 (Lane 1), *Taq*-1 (Lane 2) or *Taq*-1 and *Mun*-1 (Lane 3). Arrows indicate the multiple faint bands present in Lane 3. B. Hybridisation of *Sr-mvP2* to the same blot. Arrow indicates the band in Lane 3 that localises to the same point of the blot as the larger of the two bands marked in Figure 6.11A. M denotes molecular weight marker.

In total, including the sequence of *Sr-mvP1* (Chapter 6), *Sr-mvP2* and the *S. ratti* LSU rRNA gene and the *S. ratti* sequences obtained in both the attempt to isolate *S. ratti* homologues of the *C. elegans* sex determination genes *xol-1* and *fox-1* (Chapter 5A) and in the isolation of the sex-linked markers (Chapter 5B), 9983 bp of sequence were obtained of which 9944 bp were unambiguously determined. This sequence was 65% AT (6471/9944).

6.4 Discussion

Analysis of the conceptual amino acid sequence of the *S. ratti* DNA fragments which were previously shown to have similarity to transposable elements indicates that none are likely to be active elements. This is the case because all of the sequences are either incomplete, and may only be fragments of transposable elements, or are punctuated with stop codons and hence are unlikely to be expressed.

Further characterisation of *Sr-mvP2* has confirmed that it is present within the *S. ratti* genome in multiple copies. Comparison of the distribution of *Sr-mvP2* in the genomes of isofemale lines ED321 Heterogonic, ED5 Homogonic, ED132 Homogonic and ED248 Heterogonic (Figure 6.10A-D) indicates that the copy number of *Sr-mvP2* is approximately the same in the different lines. That the copy number of *Sr-mvP2* should be similar between ED321 Heterogonic and ED5 Homogonic (Figure 6.10A & B) and between ED248 Heterogonic and ED132 Homogonic is not surprising as they are derived from the same isofemale lines, ED5 and ED132, respectively (Viney, 1996). However, that the copy number of *Sr-mvP2* is similar in all four of the isofemale lines examined is interesting as they were originally isolated from widely separated locations; ED5 from the United States and ED132 from Japan. This could imply that the majority of the copies of *Sr-mvP2* are non-functional and relatively ancient or could indicate that the Japanese and American populations of *S. ratti* are closely related. However, it is also apparent that there are a number of differences in the distribution between the different lines (Figure 6.10A-D). In particular, a number of bands appear to be absent in ED321 Heterogonic (Figure 6.10A, lane 3), that are present in the other lines (Figure 6.10B-D, lane 3). It also appears that there are bands absent in ED132 Homogonic (Figure 6.10D, lane 1), that are present in the other lines (Figure 6.10A-C, lane 1). One explanation for this is that a number of transposition events have occurred in the recent past. In contrast, it is also possible that these differences are the result of mutation or recombination and do not indicate that *Sr-mvP2* is an active transposable element.

As previously noted, a number of the copies of the *A. lumbricoides* R4 element are found in the LSU rRNA genes. It is therefore possible that copies of *Sr-mvP2* are also inserted within these genes. However, attempts to amplify, by PCR, from *Sr-mvP2* to the *S. ratti* LSU rRNA gene were unsuccessful. This failure may simply have been caused by the fact that the fragment whose amplification was attempted was large. Comparison of the conceptual translation of *Sr-mvP2* with the amino acid sequence of R4 (Figure 6.7) indicates that the 5' and 3' fragments should be approximately 1.9 Kbp and 1.6 Kbp, respectively. However, this failure may suggest that *Sr-mvP2* is not present in the LSU rRNA genes of *S. ratti*. In addition, sequencing of the *S. ratti* LSU rRNA gene showed that the sequence of the site at which R4 is found in both *A. lumbricoides* and *P. equorum* is altered in *S. ratti* (Figure 6.9). This may suggest if this region of the LSU rRNA gene was the insertion site, that *Sr-mvP2* would not be able to insert into that position. However, it is equally possible that the insertion of *Sr-mvP2* would not be blocked by this sequence change, or that the element could insert at another site.

Analysis of the hybridisation of the *S. ratti* LSU rRNA gene to the *Taq*-I and *Mun*-I digested DNA (Figure 6.11A Lane 3) indicates that there are three size classes of band. Digestion with these endonucleases should have resulted in only a single size class in such a hybridisation, as these enzymes cut within the sequence of the *S. ratti* LSU rRNA gene shown in Figure 6.8. This suggests that there is an insertion within this region of a subset of the *S. ratti* LSU rRNA genes. Comparison of the hybridisation of the LSU rRNA gene with that of the hybridisation of *Sr-mvP2* (Figure 6.11 Lane 3) indicates that the largest of the *Sr-mvP2* bands is localised to the same region of the blot as the largest of the LSU rRNA bands. This may indicate that *Sr-mvP2* is present within a subclass of the *S. ratti* LSU rRNA genes. However, in combination with the failure of the PCR attempt to amplify from the LSU rRNA gene to *Sr-mvP2*, it is unclear if these bands represent fragments of *Sr-mvP2*, entire *Sr-mvP2* elements or sequences unrelated to *Sr-mvP2*. In *A. lumbricoides,* no truncated fragments of R4 have been identified within the LSU rRNA genes and studies of the distribution of the R1 and R2 elements of insects have shown that the only are a small number are found inserted outside the LSU rRNA genes and that it is these copies that are normally non-functional (Jakubczak *et al.* 1991).

Therefore, the significance of these findings is unclear, but it is possible *Sr-mvP2*, or a fragment of *Sr-mvP2*, is present within a subclass of the LSU rRNA genes in *S. ratti*. An obvious method by which to resolve this question would be to construct a library of *Mun*-1 and *Taq*-1 digested DNA that had been size selected to remove the most common band and then to identify and sequence the two larger bands shown in lane 3 of Figure 6.11A. The relevance of these findings to the sex-linkage of *Sr-mvP2* (Chapter 5) is also unclear. In previous work, a number of rRNA fragments were hybridised to the dot blots of free-living male and free-living female DNA and did not produce results consistent with sex-linkage (Chapter 5). This may imply that the copies of *Sr-mvP2* that are not found within the LSU rRNA genes are X-linked.

CHAPTER 7.

General discussion and future directions.

The aim of this thesis was to resolve the developmental events that occur in the life-cycle of *S. ratti* and to investigate the mechanism of sex determination in the species. A combined approach was taken, using both genetic and parasitological analyses. This proved successful and has allowed a more detailed and consistent view of the *S. ratti* life-cycle to be demonstrated (Figure 7.1). Parasitological analysis of the events that occur during the development of the progeny of the parasitic females demonstrated the existence of at least two discrete developmental switches. An intra-host sex determination switch and an extra-host free-living female/directly developing infective third stage larva (iL3) developmental conversion. Genetic analysis subsequently showed that the intra-host sex determination switch is between X0 male progeny and XX female progeny. This clarifies the basis of the extra-host free-living female/directly developing iL3 developmental conversion which is therefore a switch between alternative female morphs of the same genotype. This life-cycle is able to explain the complex way in which the proportion of directly developing iL3s, free-living females and free-living males change in response to intra- and extra-host environmental factors. In addition, it is able to accommodate the results of previous parasitological (Minematsu *et al.* 1989; Taylor & Weinstein, 1990) and cytological (Nigon & Roman, 1952; Bolla & Roberts, 1968) analyses of *S. ratti*.

7.1 Environmental effects on the *S. ratti* life-cycle

The progeny of *S. ratti* parasitic females comprise the free-living phase and can develop into directly developing iL3s, free-living males and free-living females. The relative proportions of each of these morphs varies in a complex manner, both over the course of infection and as consequence of the environment in which larvae develop (Viney, 1996; Gemmill *et al.* 1997). It has previously been

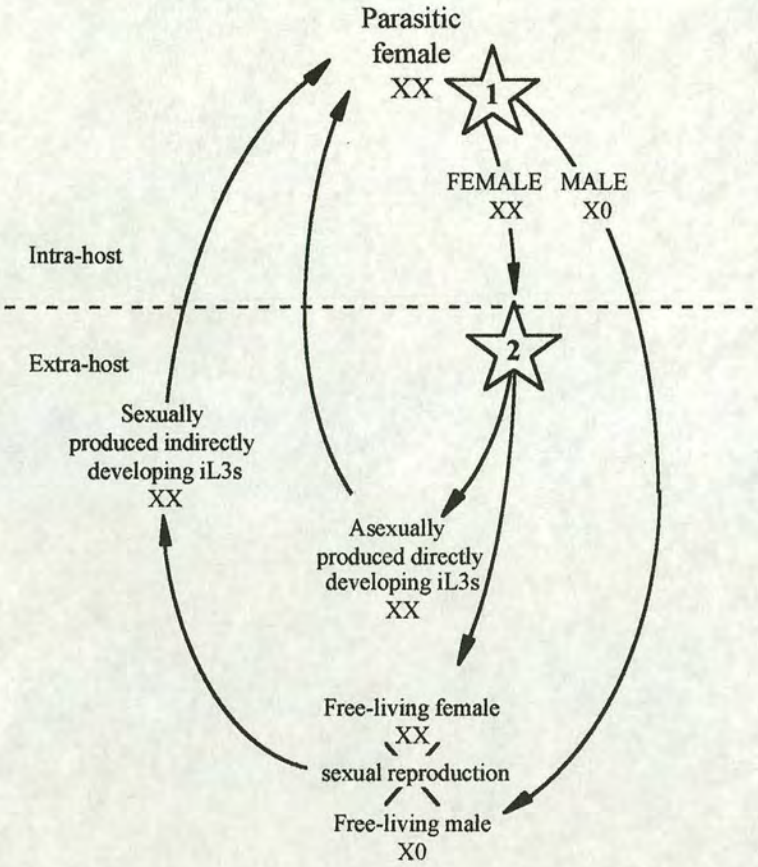


Figure 7.1 The life-cycle of *S. ratti*, indicating the two discrete developmental switches which act at different life-cycle stages and the X chromosome complement of the different life-cycle stages. 1. Intra-host sex determination switch between XX females and X0 males. 2. Extra-host free-living female/directly developing iL3 developmental conversion.

postulated that this variation in the composition of the free-living phase in response to extra-host factors is a consequence of changes in the proportion of larvae that develop into free-living females and directly developing iL3s (Minematsu *et al.* 1989; Taylor & Weinstein, 1990). Temperature shift experiments (Chapter 3) demonstrated that the incubation temperature of faecal cultures affected the proportion of larvae that developed into free-living females and into directly developing iL3s, but not the proportion of larvae that developed into free-living males. Most importantly, it was shown that this change in the composition of the free-living phase cannot easily be explained by changes in the number

of worms that develop at each temperature. Thus, the variation produced by changes in the extra-host environment is unlikely to be a result of differential morph mortality. These findings are consistent with the existence of an intra-host sex determination switch and an extra-host developmental conversion between alternative female morphs.

Previous studies that had considered the effects of changes in host immune status and the age of the parasitic female on the composition of the free-living generation of *S. ratti* had treated the life-cycle as a choice between direct and indirect development (e.g. Graham, 1939; Viney *et al.* 1992; Gemmill *et al.* 1997). Analysis of the effects of host immunity on the intra-host sex determination switch and the extra-host developmental conversion clarifies the way in which the composition of the free-living phase varies over the course of the infection (Chapter 3). Increased host immunity and increased parasitic female age were shown to result in a greater proportion of larvae developing into free-living males. Increased host immunity also resulted in a greater proportion of female larvae developing into free-living females, with results suggesting that this is mediated by an increase in the temperature sensitivity of the free-living female/directly developing iL3 developmental conversion.

It was also demonstrated that the distribution of the infective stages of *S. ratti* among host faecal pellets was significantly overdispersed and well described by the negative binomial distribution (Chapter 2). This overdispersion was shown to occur over a wide range of infection intensities and to increase significantly during infection. As these observations may have important practical consequences for parasitological studies of helminth infections, it would be informative to replicate this study in other host/parasite systems. In addition, it would be of great interest to determine the mechanism by which the observed overdispersion is produced and to determine if the increase over the course of infection is related to the age of the parasitic female or to the host immune status. It was also found that the mean number of infective stages per faecal pellet peaked between 00.00 and 06.00. The cause of this peak is unknown, but it would be interesting to determine if it is a product of the host behaviour, gut physiology or a form of cyclic egg production or release by the parasitic females. Preliminary experiments to determine if *S. ratti* parasitic females could be maintained in culture were

not successful, but if such a culture system could be developed then this would be the obvious method to determine if an underlying cycle in egg production exists.

7.2 Sex determination in *S. ratti*

To analyse the genetics of sex determination in *S. ratti*, a number of sex-linked markers were isolated. The work presented in Chapter 5 demonstrated that the technique of screening dot blots of male and female genomic DNA with anonymous DNA fragments was an effective and novel method for the isolation of sex-linked DNA fragments in species with XX/X0 sex determination systems. An obvious extension of this technique would be to screen very large fragments of the genome (i.e. bacterial artificial chromosomes - BACs) against such dot blots. This would enable the X chromosome of species with XX/X0 or XX/XY systems of sex determination to be mapped rapidly. Use of this initial screening technique in *S. ratti* resulted in the isolation of three novel, putatively sex-linked markers, *Sr-mvP1*, *Sr-mvP2* and *Sr-mvP3*. The sex-linkage of two of these markers, *Sr-mvP1* and *Sr-mvP2*, was subsequently confirmed by semi-quantitative PCR. Sequence analysis of the three markers indicated that they showed no nucleotide similarity with published sequences, but that both *Sr-mvP2* and *Sr-mvP3* showed high levels of amino acid similarity to retrotransposons in *Ascaris lumbricoides* and *Bombyx mori*, respectively. The results of the dot blotting also indicated that it is very unlikely that there is any sex specific DNA in *S. ratti*, as there is in other nematode species (Sakaguchi *et al.* 1983; Mutafova, 1995; Underwood & Bianco, 1999), mammals (male specific Y chromosomes) and birds (female specific W chromosomes).

The sex-linked marker *Sr-mvP1* was shown to be single copy and hence, the sex-linkage indicates that the marker is X-linked. As such *Sr-mvP1* represents the first mapped marker in *S. ratti* (Chapter 6). A restriction fragment length polymorphism (RFLP) was subsequently identified in *Sr-mvP1* and this allowed the inheritance of X chromosomes in the *S. ratti* life-cycle to be studied. Analysis of the inheritance of the RFLP of *Sr-mvP1* demonstrated that the free-living males have a single X chromosome, and that all other stages of the life-cycle have two X chromosomes. This is

consistent with the proposed XX/X0 system of sex determination that has been postulated for *S. ratti*. Analysis of the progeny of crosses between free-living males and virgin free-living females showed that all progeny are XX and female, inheriting the single paternal X chromosome (Chapter 6).

Further analysis of the sex-linked, putative retrotransposon *Sr-mvP2* showed that it was present in multiple copies within the *S. ratti* genome and may to be mobile within laboratory lines (Chapter 7). It was also shown that *Sr-mvP2* may be present within a subset of the large subunit ribosomal RNA (LSU rRNA) genes of *S. ratti*. The highest amino acid similarity of *Sr-mvP2* is to the R4 element of *A. lumbricoides*, a retrotransposon present in a number of the LSU rRNA genes of *A. lumbricoides*. The available evidence from the screen of random DNA fragments against the dot blots of free-living male and free-living female DNA suggests that the rRNA gene repeats of *S. ratti* are not X-linked (Chapter 5). Therefore, the most parsimonious explanation for these findings is that a number of copies or fragments of *Sr-mvP2* within the *S. ratti* genome are inserted in the LSU rRNA genes and that the majority of the other copies of *Sr-mvP2* are X-linked.

7.3 Reproduction in the free-living adults of *S. ratti*

The other major finding of this study is that reproduction between related free-living adults of *S. ratti* is sexual and involves genetic input from the free-living males (Chapter 6). It had previously been shown that reproduction between unrelated worms was sexual (Viney *et al.* 1993). However, the possibility that reproduction between related worms occurs by functionally meiotic parthenogenesis could not be excluded (Viney *et al.* 1993). This problem, as previously discussed (Chapter 1), arises because cytological studies of *S. ratti* (Nigon & Roman, 1952; Bolla & Roberts, 1968), *S. papillosus* (Zaffagnini, 1973; Triantaphyllou & Moncol, 1977; Albertson *et al.* 1979), *S. ransomi* (Triantaphyllou & Moncol, 1977) and *S. stercoralis* (Hammond & Robinson, 1994) all concluded that reproduction between the free-living adults was by meiotic parthenogenesis and pseudogamy. One way in which the observations of Viney *et al.* (1993) could be reconciled with those of the cytological studies is if reproduction between closely related worms was by pseudogamy and meiotic parthenogenesis and that

between unrelated worms was sexual (Viney *et al.* 1993; Hammond & Robinson, 1994). However, analysis of the progeny of naturally mated free-living females indicated that reproduction is sexual (Chapter 6). These results conclusively demonstrate that reproduction in the free-living adult generation of *S. ratti* is true sexual reproduction. As discussed previously (Chapter 1), all of the cytological studies of reproduction in the free-living phase of *Strongyloides spp.* produced similar results. As it is now clear that reproduction between the free-living males and free-living females of *S. ratti* is sexual in crosses between both related and unrelated worms, the cytological observations of *S. ratti* are obviously incorrect. It is therefore possible that, as all the cytological studies of reproduction have shown similar results, all *Strongyloides spp.* are, like *S. ratti*, reproducing sexually. The other alternative, that *S. ratti* is alone among the many *Strongyloides spp.* in reproducing sexually is unlikely and would suggest that the evolution of reproductive mode in the genus was occurring very rapidly.

The mechanism of X chromosome inheritance in the free-living phase of *S. ratti* is intriguing. All progeny of the free-living adults inherit the paternal X chromosome, but the mechanism by which this occurs is unknown. Cytological observations of the germline of free-living males have shown the existence of cells with 2 and 3 chromosomes in *S. ratti* (Nigon & Roman, 1952) and *S. stercoralis* (Hammond & Robinson, 1994). These were taken to be sperm, which would suggest that spermatogenesis involves the normal Mendelian inheritance of chromosomes. However, it is clear that reproduction between XX free-living females and X0 free-living males does not produce equal numbers of XX and X0 progeny. The most likely mechanism by which this could occur is that either a gene required for fertilisation or for sperm motility is X-linked, so only the X-bearing sperm would be capable of fertilising oocytes, or that only sperm precursors that contain an X chromosome are capable of completing development. It is also possible that X-bearing sperm have a competitive advantage over sperm that do not carry an X chromosome, as is the case in *C. briggsae* (LaMunyon & Ward, 1997). Preliminary experiments to determine the X chromosome complement of sperm in the free-living males proved unsuccessful. It would therefore be of great interest to determine the mechanism by which this pattern of X chromosomes inheritance is generated.

7.4 The life-cycle of *S. ratti*

As shown in the Chapter 3, the free-living female/free-living male ratio in *S. ratti* is to a large extent environmentally determined, with the proportion of larvae that develop into free-living females affected by both intra- and extra-host factors, and the proportion of larvae that develop into free-living males affected solely by intra-host factors. Thus, the variation in the proportion of larvae that develop into free-living males and in the proportion of female larvae that develop into free-living females results from a combination of both environmental and genetic processes. Therefore, there is no obvious relationship between the proportion of free-living males and the proportion of free-living females, as is generally the case in species with a chromosomal method of sex determination. This lack of a one-to-one sex ratio is due to the fact that the production of free-living males in *S. ratti* is by X chromosome loss during mitotic parthenogenesis. As discussed in Chapter 5, mutations in the high incidence of male (*him*) genes of *C. elegans* result in increased rates of male production (Hodgkin *et al.* 1979). These mutations cause an increased rate of chromosome non-disjunction, with a number specifically affecting the X chromosome (Broverman & Meneely, 1994). The production of the free-living males by the *S. ratti* parasitic females may therefore be a consequence of a similar mechanism and possibly even the action of the homologous genes. This hypothesis could be tested experimentally as it would be expected that parasitic females that did not produce any free-living male progeny, for example at the beginning of infection, would not be expressing these genes. Thus, the use of molecular techniques such as differential display would allow the genes expressed by parasitic females producing progeny that only developed into directly developing iL3s to be compared with the genes expressed by parasitic females producing progeny that developed into free-living males, free-living females and directly developing iL3s.

The mechanism of X chromosome inheritance in crosses between free-living males and free-living females also suggests that any factor that increased the production of free-living males would be most favoured if it was X-linked. This is the case as all progeny of the free-living males inherit the paternal X chromosome and hence all X-linked genes. In contrast autosomal genes are only inherited

by half of the progeny. This observation is important as the lack of a one-to-one sex ratio indicates that the determinates of male formation are X-linked, and exempts *S. ratti* from the constraints of Fisherian sex ratio theory (Fisher, 1930) as the inheritance of the X chromosome in the species is not Mendelian. Thus, it is possible that the production of free-living males in *S. ratti* is analogous to the various meiotic drive mechanisms that produce biased sex ratios in *Drosophila* species.

This study also suggests an explanation for the finding that the genetic structure of *S. ratti* populations in the United Kingdom is consistent with it being a single interbreeding population (Fisher & Viney, 1998). A low level of sexual recombination is sufficient to bring allele frequencies into Hardy-Weinberg equilibrium (Maynard Smith, 1989). However, it is worth noting that a combination of the increased proportion of larvae that develop into free-living adults in response to host immunity (Chapter 3) and the increased overdispersion of infective stages among faecal pellets over time (Chapter 2) would result in greatly increased chances for mating.

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APPENDIX 1.

Commonly used solutions and media.

LB medium: 10 g/l Bacto®-tryptone, 5 g/l yeast extract, 5g/l NaCl. pH adjusted to 7.5.

LB agar plates: 15 g/l agar in LB medium. For IPTG/Kanamycin LB agar plates, isopropyl-β-D-thiogalactopyranoside (IPTG) and Kanamycin are added after the solution is autoclaved, at final concentrations of 0.1 mM and 30 μg/ml, respectively.

LB/Kanamycin medium: LB medium with 30 μg/ml Kanamycin.

NGM agar plates: 3 g/l NaCl, 22 g/l agar, 2.5 g/l peptone. After solution is autoclaved, $CaCl_2$, $MgSO_4$, KPO_4, cholesterol and nystatin are added at final concentrations of 1 mM, 1 mM and 25 mM, 5 μg/ml and 100 U/ml, respectively.

TAE buffer (50×): 0.20 M Tris-base, 57.1 ml/l glacial acetic acid, 0.05 M disodium ethylendiaminetetraacetate (EDTA) (pH 8.0).

TE: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

TBE buffer (10×): 0.09 M Tris-base, 0.09 M boric acid, 0.002 M EDTA.

Pre-hybridisation solution: 100g/l dextran sulphate solution (sodium salt, MW ~ 500,000, Sigma) and 58g/l NaCl.

SSC (10×): 3M NaCl, 0.3M sodium citrate (adjusted to pH 7.0).

TNESST: 10 mM Tris-HCl pH 7.4, 60 mM NaCl, 10 mM EDTA (pH 8.0), 0.15 mM spermidine, 0.15 mM spermine and 0.5% v/v Triton X-100.

APPENDIX 2.

2.1 Sequence analysis of fragments obtained in the attempt to isolate S. ratti *homologues of the* C. elegans *genes* fox-1 *and* xol-1

Comparison of the nucleic acid sequence of the fifteen DNA fragments to existing nucleic acid sequences in Genbank and the *C. elegans* genomic and EST databases indicated that only four, xol.22, fox1.3, fox5.3 and fox5.7, showed any significant similarity to existing nucleic acid sequences (Table 1). Of these fragments, fox1.3, fox5.3 and fox5.7 were found to be identical to *Escherichia coli* genomic DNA sequences (>95% identical, unresolved nucleotides in the sequence of fox1.3, fox5.3 and fox5.7 preventing complete identity). These sequences are therefore of *E. coli* origin. These *E. coli* sequences seem to represent portions of various *E. coli* proteins (Table 2) and are hence not discussed further. Seven further sequences showed no significant similarity to existing amino acid sequences, after conceptual translation (Table 2). Of these seven, xol.1a, fox1.5, fox6.2, fox6.8 and fox6.6 had also shown no significant similarity to existing nucleic acid sequences and are therefore not discussed further. In contrast, the fragments xol.22 (XP1) and xol.2a which showed significant nucleotide similarity to existing *C. elegans* sequences (Table 1), did not show any significant similarity to existing protein sequences. Of the remaining sequences, fox2.2, fox3.1 and fox3.3 all showed significant similarity to bacterial proteins and no significant similarity to proteins from *C. elegans*. This suggests that these sequences may also be of bacterial rather than nematode origin. Thus, only two of the sequences, xol.1b and xol.23 are likely to represent fragments of coding DNA from *S. ratti*. The first of these, xol.1b, appears to be related to a transposable element and is discussed further in Chapter 7. The second, xol.23 is discussed below. The DNA sequences obtained for the fragments which were not of bacterial origin are shown in Table 3.

	Database					
Marker	Genbank	*p*	*C. elegans* genomic	*p*	*C. elegans* EST	*p*
xol.1a	-	-	-	-	-	-
xol.1b	-	-	-	-	-	-
xol.22 (XP1)	-	-	Y18D10A	$3.8e^{-37}$	CEMSH70F	$4.1e^{-11}$
xol.23	-	-	-	-	-	-
xol.2a	-	-	-	-	-	-
fox1.3	*Escherichia coli*	e^{-158}	-	-	-	-
fox1.5	-	-	-	-	-	-
fox2.2	-	-	-	-	-	-
fox3.1	-	-	-	-	-	-
fox3.3	-	-	-	-	-	-
fox5.3	*Escherichia coli*	e^{-146}	-	-	-	-
fox5.7	*Escherichia coli*	e^{-140}	-	-	-	-
fox6.2	-	-	-	-	-	-
fox6.8	-	-	-	-	-	-
fox6.6	-	-	-	-	-	-

Table 1 The name and any similarity to existing nucleic acid sequences as assessed by Blastn searches of non-redundant sequences in Genbank (NCBI) and the *C. elegans* genomic and EST databases (Sanger Centre) for the markers used in the identification of sex-linked DNA in *S. ratti*. - denotes that no significant similarity was identified.

Marker	Database			
	Non-redundant sequences in Genbank	*p*	*C. elegans* Wormpep	*p*
xol.1a	-	-	-	-
xol.1b	Pol protein *Drosophila melanogaster*	$4e^{-09}$	T23E7.1 T03F1.4	$2.2e^{-05}$ $6.5e^{-05}$
xol.22 (XP1)	-	-	-	-
xol.23	AF032383 metalloprotease-disintegrin from *Xenopus laevis*	$2e^{-12}$	C04A11.4 zinc metalloprotease	$3.2e^{-14}$
xol.2a	-	-	-	-
fox1.3	*E. coli* fumarate hydratase	$2e^{-61}$	-	-
fox1.5	-	-	-	-
fox2.2	Hypothetical *E. coli* protein	$9e^{-9}$	-	-
fox3.1	Phosphoglycerate dehydrogenase from *Edwardsiella ictaluri*	$5e^{-33}$	-	-
fox3.3	Trimethylamine dehydrogenase from *Hyphomicrobium spp.*	$4e^{-13}$	-	-
fox5.3	*E. coli* Rfe protein	e^{-52}	-	-
fox5.7	Hypothetical *E. coli* protein	e^{-57}	-	-
fox6.2	-	-	-	-
fox6.8	-	-	-	-
fox6.6	-	-	-	-

Table 2 The name and any similarity to existing amino acid sequences as assessed by Blastx searches of non-redundant sequences in Genbank (NCBI) and the *C. elegans* wormpep database (Sanger Centre) for the markers used in the identification of sex-linked DNA in *S. ratti*. - denotes that no significant similarity was identified.

Name	Sequence
xol.22 (XP1)	AAAGTGAAATAAGAAATAACATTTATAAATATAAAAAGCAACAATATAATAATCAAAAGATAAAAAGCATATTTTAA TAAAATAAGTACAGTTTTTCAAAAAAGAAAGACATAGAGTAAAAGAAAATTGATAAAGTAGAGAAAAAAAAAATTGG GCAGTTTATGTTTAATCTTTATAGAAAAGAAGTAAAGAAAATTATAAAAATATTAACTTGAACATTTCGAATTGGTT TTAGATTTCAAGAACACAGACAAATTTATTTTATTAAAAATATGTTACTATCTATACNATTGAAAGACACTTATGAT GTTTACTATTAAAAATAGTTTAAAAAAAGGAGGAACAGAAATAATTTTTAATGTTCCCACAATTTAATACACTTCAA TATGTTATTATAAACGAATTTTTTTTTATAAATATAAAAAGTTAATATTTAATATTACCTTCTTTTAATGTTTAAAT TAAATTTATTTGAGATTAATATTTTTATTTGGGAGATAATACCCTTTAAATAAATTGGGACCTTATTTAAAAGGGAA GTATAAAAATCCAATATAAAATAAAAATTTAATAAATGTTAAAAAGATTTTCTACGGATATATTTTAATCCACTAAT AGATATATATTATATAATCTACTTTTCCCAGATAATTTTACACAATAATATGATAACCATTTAAAAAAATTGCTAAA CTTGAGATGTGGCAATGAATAATAATTAATTTAAATAAATAAAAAAAAAATTACCGTAAAAATTAATATTTAATAGAT TAAAAAAAATAATATTATTTGAGGTAAATTTAAATGTTAAATTTTGAGAAAAATATATAGTAAAATAACTGGCAATA TTTAATAGTGAAATAGAAACCCAAATGTATGATTAACTTCTATTCAAGATATATTGGAATATCATTCATTTTATGAC TAAATTTACTGGTTTTTATCATTCTTCAAATTTATGATTCAATAAACAGTTGATTGTATTTACCAGATGGGAGTTTC CTTATTATTATCGTAGATGTGTTGT (1026 bp)
xol.1b	ACAAAGAAGAAGTCTTCAAGGTATGCTATAAAATCAACCAACATTCTCTTAAAATAAATCTCAATAAATGTGATTTT GCAACGTTTGAAACAGAAATTTTAGGCCACATTATCAATTGTTCAGAAACAAAAATGTCTCTCAAGTTCACTCGAAT TCTCTCTCAATTTCCAATGCCTACAAACATTCACCAACTTTAAAAATTTAGAGGTATATTTACCTTTGTAAAGAGGT ATCTTTATGATACCATGGACGAAAAGCGCGTCATTGATCAATACATTGGAAATACGTCCAAAATCAAAGAAAAAAAT CCTACAACAATTTTCCTACAAGTACAAGAAGCTATTAAATGCATTCAAAATAAATCTCATTACACCAAACACCTAGC ACATATTAATTATCAATTACCATTTTGTTTATCTGTAGATGCTTCCTTTCAAAACACTGGAGTTGCTTTTTTCAACC TGCTTCTATCGAAACCTCCAATGTACAAAACCTTTTAAGTCGAAGTC (509 bp)
xol.1a	GTGCATACCAAATTGACGTTCCCAATCTACATACATTTTGCCATACATATATTGGTATGTCGATACCGAACGGTAAC TGTTGGTTTGTGAACTCACATCTCCATACTTGTCATAAGCTTCGTTGCCTTGTTGGGTTATGCTGTATTGAAATACT TGTGCCTGTTTGGTGCGAACGATGGCATTGCGCACTTGTGATGAGATGTTTCCCGTTGCACCAACGGAAAGTCCTTT CACCAATTTGTCGAAGTCATATTTCAAATTAACAGTTCCTACGACATCGCGGGTATTGCTTGATATATATCCCGAAC CAGTGGTCTGTGCATAGAGATTTTGTGTATACGAAGCGTTACCACCATACGTTCCGTTGGGATTTAAGACGGGATAA GCATTGTTGGGAGTCTGCCATACGTTGCTCAAAAGGTCGCTGTATCCTGTCCCGAAATACCTCCGGGTTGGTTCCCC TCTTCTATACGTCCCATCAAGGACATACTTACCTTAAATTCTCTGT (508 bp)
xol.23	TTAAATTATGGGGTGCTGAAATACATAATGGTGTTGGTATATGTTATAAAAAACTTAATATTGTGGGAAACGAATAT GGAAATTGTGGTTATAAAAATGATACATTTTTTAAATGTAAAGAACAAGATGTTTTTTGTGGTTCATTACAATGTGA TAATCTTAATAATCAACCAATATTTGGTGATCCATATTCTATACGTAGTGTAATGTCATGGGCAAAAGAANGAGATA AAACTACAACATGTAGAACATTTAGAACTACGTATCCCTATAAATCTTAAAGATAGAGATCCTGGTATGGTTCCTGA TGGTTCTAGATGTGGTAATGATAAATTCTGTGTTATGAGCACAATGTTAAACAAAAAAGTGATGTTATTGCAACTTT ACCATTTTGTGATCCGAGTGGTTGTAATAATTTAAGAATATGTTATAATGTTTGGAAATTGTCCATTGTNAATATGG TTATNGTGGTACAG (476 bp)
xol.2a	TCAGAACTAACATTTTGAAGTTGGTAGGCATTTAACATTGTGAAGATTTGAGAGTTAGGAACGACATAATCAATTCT AAATTCTTTAAATCCAATATTATCTTTAATTTTTTTTAAATTTTCTTTTGTTTCTTTGTTTTGTTTCCATCCTTTAT CTTTACTGGCTATTGTGTACCAACTTCCATAATCGTATGCTTTTACAGGTTCTGTTGCAATTTGGAGAGCAACTTCT TTTTGTTTCTCATCTTGAAATTATTGCTGTTCTAATCTATTTTTTTCTTTTTGAGAATTTGCGAGTTGTAAAGCACC ATAATCTATTGTGGTAATTGTTTTATGCTCATATACATTTCCACT (353 bp)
fox1.5	CGGTCAAACGGTTGTGGACGTGCCGCCAGTGATCCGGCGAGGCATCGGCCGGATCGAGGCCGAGCGCATCCACGGCG TCGATGGCTGCAAGCGCGCGCTGCACCTTGGGCCAGCTATCGAGGCGCAACAGGATTTCGCCGCCGGGTCGGACGAA GGGCAATGTCTGGAACGGTTCGCCCCGGCCGATGGCGCGCACAATGTCGATGCGCGAAACGACCGTGCCATGCTCGC CGCTCGCCCATCGCACAAAGGCGAAAACGCTGCTCGGTGCGAAGCCGACGACACTGCGGCGGCGGTCGATGATCTGT TCGTAGCTCTTGTGGCCGAACCGTATCCAGTGCTCAACCTTGCGTTTTCTCNAAAGGTCAGCTCNAANCAAATTTTT GTTAAAAGGGGGCCCCGGGCCCCTTCCGGGGAANCGGAAANGGGCCCTTCCCCCCCTTTCCNGGGGGGGGGCCNAAA AAAATTCCNTTGTTCCCTCCCCCCGGAAAACTTNNTTTCNNGGGGTTTTAAACCC (517 bp)
fox6.2	TTTGGCGTGGGTGCTTATTAAGTCACTGGGGCTTGATGCAGCGATCGGTACCGGTATGTTCACCGGCGCGCTGACCT CGACTCCCGGTATGGCAGCGGTCGTGGAATTGATTGAAGGAATCGATCCAAGCCTTGCCGGTGAACCTGTTATTGGT TATTCCTTGGCATATC (170 bp)
fox6.8	CTCTGTTGTCTCCTGCTGCTAGCACGCGCTTTGCTGCAGCAAATGCTTGCGCCGTATCAAATATCCCGGCTATTGCA CTAATAATTGCACCAAACTTGNTTAGCGAAACGCCAGTTCCCGCCGCGCCCTTAGCCCCGGGTATTGACCTCAGCAC GGGT (158 bp)
fox6.6	GCGGCAAGCTTAATTCACACCTGTAATCCGAGGGTGAAAAAAGAAGCTAAGGATCCAGGTTCGCCTGCTTTCTATAT ACTCTGCAGTTAAAGTC (94 bp)

Table 3 Sequences amplified from *S. ratti* genomic DNA in attempt to isolate the *S. ratti* homologue of the *C. elegans* sex determination gene *fox-1* and *xol-1*. N denotes that the nucleotide sequence could not be determined.

The predicted amino acid sequence of the *S. ratti* DNA fragment *xol.23* is most similar to a portion of the 952 amino acid *C. elegans* zinc metalloprotease, CE07906. This gene is composed of 19 exons, spread out over nearly 7500 bp, which result in a spliced DNA of 2859 bp. Analysis of the sequence of xol.23 indicates that the predicted translated protein does not confirm to the exon structure of the *C. elegans* zinc metalloprotease (Figure 1).

Figure 1 Alignment of the conceptual translation of the open reading frame in the *S. ratti* marker xol.23 (*Sr*) with the amino acid sequences of the zinc metalloprotease (CE07906) from *C. elegans* (*Ce*) and the metalloprotease-disintegrin from *Xenopus laevis* (*Xl*). Amino acid identity is indicated by heavy shading and amino acid similarity by light shading. - denotes a gap inserted to allow sequence alignment. gap denotes a larger break in the sequence alignment. The numbers refer to the nucleotide sequence of xol.23.

2.2 Sequence analysis of fragments obtained in the attempt to isolate anonymous sex-linked DNA fragments in S. ratti

Of the uncharacterised markers used in the isolation of anonymous sex-linked DNA fragments in *S. ratti*, the complete sequence of six fragments was obtained, with partial sequence obtained for a further three markers. No sequence data was obtained for four of the markers. The size of the fragments and the amount of available sequence data is shown in Table 4. Comparison of these sequences to existing nucleic acid and amino acid sequences are shown in Table 5 and Table 6, respectively. Of the nine markers for which sequence data is available, Q14, pZ-8, pZ-5 and 27/*Sr-mvP1* showed significant similarity to published nucleic acid sequence. The very high similarity of Q14 to human genomic DNA sequence (>95% identical) and the lack of any similarity to non-human nucleic acid sequences indicates that this clone is a fragment of human DNA rather than *S. ratti* DNA. The other three sequences showed similarity to *C. elegans* genomic DNA or EST sequences only. The remaining five sequences, CM2, CM6, pZ-1, pZ-4/*Sr-mvP3* and pZ-2/*Sr-mvP2* showed no similarity to either *C. elegans* or non-*C. elegans* nucleic acid sequences. When the conceptual translations of these sequences were compared to published amino acid sequences, five showed significant similarity, with only the human sequence Q14, pZ-5 and 27/*Sr-mvP1* showing no similarity to published sequences. Of the five that showed significant similarity to existing sequences, four, CM6, pZ-4/*Sr-mvP3*, pZ-8 and pZ-2/*Sr-mvP2* appear to be related to various transposable elements and are discussed further in Chapter 7. Further analysis of the sequence of CM2 supported the finding that the conceptual translation is more closely related to the bacterial glyceraldehyde-3-phosphate dehydrogenase (GPD) (49% identity over a 148 amino acid region) than to the GPD sequence of *C. elegans* (43% identity over a 90 amino acid region). It is therefore likely that CM2 is of bacterial origin. The final sequence, pZ-1, is discussed below. The available nucleic acid sequence for the *S. ratti* markers is shown in Table 7. Note that the sequences of the sex-linked markers 27/*Sr-mvP1* and pZ-2/*Sr-mvP2* are given in Chapters 6 and 7, respectively.

Marker	Size in bp	Amount of available sequence in base pairs
CM6	725	725
pZ-1	234	234
pZ-4/*Sr-mvP3*	773	773
pZ-5	466	466
CM2	approx. 1000	3' - 437 and 5' - 328
Q14	approx. 600	3' - 412 and 5' - 460
pZ-8	approx. 2000	3' - 490 and 5' - 457
pZ-7	approx. 400	none available
pZ-11	approx. 2100	none available
RP8	approx. 400	none available
Q13	approx. 1100	none available
27/*Sr-mvP1*	approx. 1230	1028 (sequenced from PCR product)
pZ-2/*Sr-mvP2*	1427	1427

Table 4 The name, size and the amount of available nucleic acid sequence for the markers used in the identification of sex-linked DNA in *S. ratti*. Approx. denotes that the fragment has only been sized by PCR and hence that the given size is only approximate. Note that the sequence of 27/*Sr-mvP1* was obtained from cloned and sequenced PCR product and hence does not contain the original 3' and 5' ends of the sequence.

	Database					
Marker	Genbank	*p*	*C. elegans* genomic	*p*	*C. elegans* EST	*p*
CM2	-	-	-	-	-	-
CM6	-	-	-	-	-	-
Q14	AL022324 Human sequence	e^{-139}	-	-	-	-
pZ-1	-	-	-	-	-	-
pZ-4 / *Sr-mvP3*	-	-	-	-	-	-
pZ-8	-	-	H28G03	$1.6e^{-24}$	-	-
pZ-5	-	-	Y39B6	$2.7e^{-14}$	-	-
27 / *Sr-mvP1*	-	-	AL033123	$1.4e^{-13}$	-	-
pZ-2 / *Sr-mvP2*	-	-	-	-	-	-

Table 5 The name and any similarity to existing nucleic acid sequences as assessed by Blastn searches of non-redundant sequences in Genbank (NCBI) and the *C. elegans* genomic and EST databases (Sanger Centre) for the markers used in the identification of sex-linked DNA in *S. ratti*. - denotes that no significant similarity was identified.

Marker	Database: Non-redundant sequences in Genbank	*p*	Database: *C. elegans* Wormpep	*p*
CM2	AF058302 bacterial glyceraldehyde-3-phosphate dehydrogenase (GPD)	$2e^{-34}$	K10B3.7 GPD-3 K10B3.8 GPD-2	$9e^{-5}$ $9e^{-5}$
CM6	U39501 bacterial OrfB insertion element	$1e^{-7}$	-	-
Q14	-	-	-	-
pZ-1	C06H2.1 ATP synthase D chain from *C. elegans*	$3e^{-16}$	C06H2.1 ATP synthase D chain	$3.6e^{-14}$
pZ-4 / *Sr-mvP3*	U07847 reverse transcriptase from *Bombyx mori*	$9e^{-19}$	T19C9.7 reverse transcriptase K01G12.1 reverse transcriptase	$1.5e^{-18}$ $1.5e^{-18}$
pZ-8	COS41.3 POL-like protein from *Ciona intestinalis*	$1e^{-8}$	-	-
pZ-5	-	-	-	-
27 / *Sr-mvP1*	-	-	-	-
pZ-2 / *Sr-mvP2*	R4 retrotransposon from *Ascaris lumbricoides*	$2e^{-10}$	-	-

Table 6 The name and any similarity to existing amino acid sequences as assessed by Blastx searches of non-redundant sequences in Genbank (NCBI) and the *C. elegans* wormpep database (Sanger Centre) for the markers used in the identification of sex-linked DNA in *S. ratti*. - denotes that no significant similarity was identified.

Name	Sequence
CM6	CCTTGACAACCATCCGGCACAACAGGTTTCCTTGGTCTGCTGGGTCATCGAGCTGTCCCAGCGTAGTCATAGCTCACGGTA CCCGTGTCGACGACGCTAGCGGTGTGGCCGCCGCCCGAATGCTGGTAGTTCAGTAGAAACTCGCCGCCGGTTGCGTCCACG TGGAGTGCACCATCGTTTTCCAGATCAACGCTGCCGTTGTTAGATGCTGCGCNTNTTTTTTATTGGTCCGCTGCACTTCGT TCCAAGCCCGCGGGTGGCGCTCAACGGTGGGAATATGCATCCCGAGGGTGTTGTCAAGTTGTCTGGAGTACAGCAGTTTTG GGGTGCGGCAAGGCATCGCACGGGGANGTCGGTCAGGGANCGTGCTCCAAGAAACAATGGGGCTCTCGCAGCGAATCNCCT GCAGAATCGTCGGGCAACCGCGATCGANCCAACGGAAAAACTTGCGAAAAAACACCCCCGACGATCCCGACTGAACATTGT CGAGAGATCCATCCCGGCAGAGGATCTNGTCGCCGCGANGAAGAAGGCGTTCGCANTTTGGGGCGGCCCACCGCAGGTCGT GCGGTGNGACAACGGGCCCGAGTTCATCTCCGAGGCANTGAGGACGTTTTGAGAGGATCAGGTNGGTATNGTATACGTTCC ACCTGGTCAGCCGTGGAAGAACGGGTACATCGAGTCGTTCAACAACCGTGTTCGGGACGAATGNTTGAACATGAACG
pZ-1	AAAATTGAAGGCTATCACTTCTACTGTTAGCGCCACGTAAGTTTTTCATTTTACTTTAGCATAATTTTCTTTAGCGTAAAT GAGCTTCCAGCAACTTTACCAAAAATTGATTTTACCGATCTTAAAAAAAAAAATGCCAGGATATGCCTCAATTCTTGATTCT CTTCAAAAACAATATGAAACTATAAAAATTCCAAAAGGAACTATTCCAGAGGAATATAATAAACAAATTAAT
pZ-4/ *Sr-mvP3*	GTCTGCTACCATTATGTCATCTGCATGTAGTAAAGTCCATGGGGCTGGTTGGGGGTTTCCTTTCAGGATACAATTCATCAC TAGGATGAATAATAGTGGTGATAAAACTGATCCTTGATGGACACCTATTNTGATNGGGAAGCTGTCTGATATGCCTGCCGT AGTTTTTACTTTGCTAGTTGTATTTTGGTAGAGTATTTTCACCCAGGTTACTAGTTCTTCAGGAATGTTATGTGCTCGAAA AGCATGCCATATCATTTTGTAAGGGACTTTATCGAATACTTTTTCTAGGTCTAAGAAACATGCGTGTAAAGGTATCTTCTT TTCACGGTGTTTTCAAAAAAAATCGTGTTGCGTGTATTGCATCAGTTGTTCTACATTTTGGGATAAAACCACATTGGTTTC TCTTTAGAACAGCGATTTTTTCTGAATTCTTTGGTTTATGATTCTTTCAAAGATTTTCATAGTGTGGCATAAGAGTCGGAT AGGTCTATAGTTTTGGCACTCTGCCGATGAACCTTTTTCTTTAAAAATAGGTATAATTATTGAAGATAGCCAATCTAATGA GGTTTGTTTTTCTGATACAATTTTGTTGAAGAGTTTTGTCAGCCATGTTATTGAGTTATTTATCTTTAATTTCCATATTTC AAACGGGATATAATCATAGCCTGTTTATTTGGAGTTCTTCATCTTTCTAATTGCATCTTGTACTTCAGATTGGCTTATTTT TTGCATAGGGCCTTGTGTAGGATTTATTTCTTGAATTAGGTCGT
CM2	TCAGCAAGGTGATTTTTCAAATTAATATTCTATTTCAATCCAAAAATTAAACCTAAAAAACGTGCAATAAAAAAAGAGCCT CAAATGAGGCCCTTTTTTCAGCTCAATACTAATTATGCATTAGTTTCAGCAGGAACTTTTGGATAGCTTACACCGCCCATT TGTTCAGCAATACGCAATACCTGGCAGCTATAACCCACTTCATTATCGTACCAAACATAAGCAGTTAAGCGGTTGCCTGAA GTGATAGTCGCTTGCGCGTCAAATACACCTGCAGTACGTGAACCAATAAAGTCAGAAGATACAACTTCCAGTCGAGTTTAG TATAACCAATTTGACCTTGAAGGTTAGAATTGATAGAGATTTGGCGAATGTTTCCATCACTCGTCACATCACTCTTGACCA ATCAGTTAGATCGCAGAATACTTGGTGTAGGT---TAGGATCACTTGGATGACTGACCGTTTGCATGATTGTTCGTTTTTC ATAACAGAGATCGTACCCGCGAATGGACCATGGATTGAGTCACGACGCAGATACAGACGCACGTTTTTCAAGGTCACCTCA GATGATTTACGAACGTACGATTGCTTTCAGGTTCAAGCCACGGCCTAGACCTGACTGGCTGATCATCAAACGTGCAAGAAT ACGACCGATACGACCGAAGCCATAAAGTACAACGTCTTTACCTTCAACTGAAGTTGCGCTGCCTTCAAGAGATTTCACAGC ATCAGCAACGAATGCGTTAACATCACCACCTTTTGCTTT
pZ-8	ATTCTGTTTCTTTAAAATAAAATAATCTCCAACTTTAAATTCATTTGATTTAAATATTTGATTCTTTTTGGATAATTTTCT ATATAAGTCTGTAGTATATACTGCAGCGTATAATTCTTCTTGCTTATGCATCATTGGGATTACATATTTTGAAATGTCGTA GTTGTAATTCTCCAAATTAATGTTTTCTATATTTCTTAAATTATTTTCTTTAAACATAAATATTTCAAAAGCGTTGAAATT ATTAGATTGTGTTCCTTTATTTAAAAAGTAAGTTGCCATTCCTAATAGATCTTTGAATTTAATTTCTTCATTTTTCTCTTG TCTAATTTTACTAAATGTTTCTTCTATTAATTTCAGGTATAATTCGGCAATTCTATTTCCATGAGTATAAGGCGCGACACT TATTAATTTTATCTGTGATATTTCAAAGAATCTTTTAGCTTCGTTACTATTTAAATATTGGCATTATCCACAACTATAATC GTAG---AATACTTTTTAAAGAAGGTGTATATCTTTTTGGTTTAAGGTATATGCTAAATAATTTAATTCTTTTCTAAATAG TTTGGACTTATAAATATTAAAATATAAATTATTCTTCTGAAATATTTCCAATATTTCGTTAAGTACTTCAGCATGTTTTCT TATAGCTTCTACTTCAGATTTTTCTTTTGGTAATGAAGTTATTACTAAGAAATCGTCGATATGTTGTATTACTTGACCATC ATTCTTATATTTCTTTAATAATTCACTTGTTACTAATGAAAATTGAAATGCTGAATTTGTAGCTCCTTGTGGTAATGTTTT ATAAGAATAAATATTTATTCCATCCGCACTAAANGAATATTTGGATCTCATTTCCTCTGGTATCAGTATTTGTTGATATGC ATTACTGAGGTCTATTAAACTATATAAAATATTTTCTCCTCCTTTTATTCTTTTGAATT
pZ-5	AATTCCAAAATTCTATAGTATCAATGCTGAAATGAAAAAAAAATTATTAGAATAAATTTAGACATCTTACACAATAACATC ATAATATACTTTATTATGTTTTACTTTATTGTTTTTTTTATCTTGAAATATGATAAATAATATACCATTATCAAAATTTGT AAATATATTGTTATTATAATTACCAGAACAATATCCTATTAAATTTTCTAATTGTGTAGTTTCTTCATGTAATAGTTTTAA AAATGTTCCTACATCCCAATTATATTTAACAGCAAAATCACAAGTTGGTGATAAAACCCATGTTAAATGTGAATCATATTT AGGATGAATGACAGCTAATTTATTAACTTTACAATCATCTAATTAAAACTTAATAATGTTAAAATAAAATAAAACATACTA CTTTGAAAAATANGAAATTGATAAACTTTTTTTCCATTAATTTTTGAAAAAAAAATTAAAT

Table 7 The available nucleic acid sequences for the markers used against the dot blots of free-living female and free-living male DNA (Chapter 5).: No sequence data is available for the markers pZ-7, pZ-11, RP8 and Q13. The sequence of the putatively sex-linked markers *Sr-mvP1* and *Sr-mvP2* are given in Chapters 6 and 7, respectively. The dashed line in the sequence of CM2 and pZ-8 denotes a gap in the sequence.

pZ-1.

The short (234 bp) *S. ratti* genomic clone contains a single open reading frame that extends from nucleic acid position 31, of the sequence shown in Table 4.7, and codes for 68 amino acids. Figure 2 shows a comparison of this predicted sequence with the sequence of the *C. elegans* and *Drosophila melanogaster* mitochondrial ATP synthase D chain proteins, obtained from the Sanger Centre (http://www.sanger.ac.uk) and NCBI (http://www.ncbi.nlm.nih.gov/), respectively. Analysis of this gene in *C. elegans* has shown that it is found on chromosome V and the final 191 amino acid protein is formed from four exons (Sanger Centre). The open reading frame in the *S. ratti* gene fragment pZ-1 corresponds to amino acid positions 34 to 94 of the *C. elegans* sequence. Thus, the start of the putative *S. ratti* exon does not coincide with the exon structure of the *C. elegans* gene, in which the second exon runs from amino acid position 19 to 122.

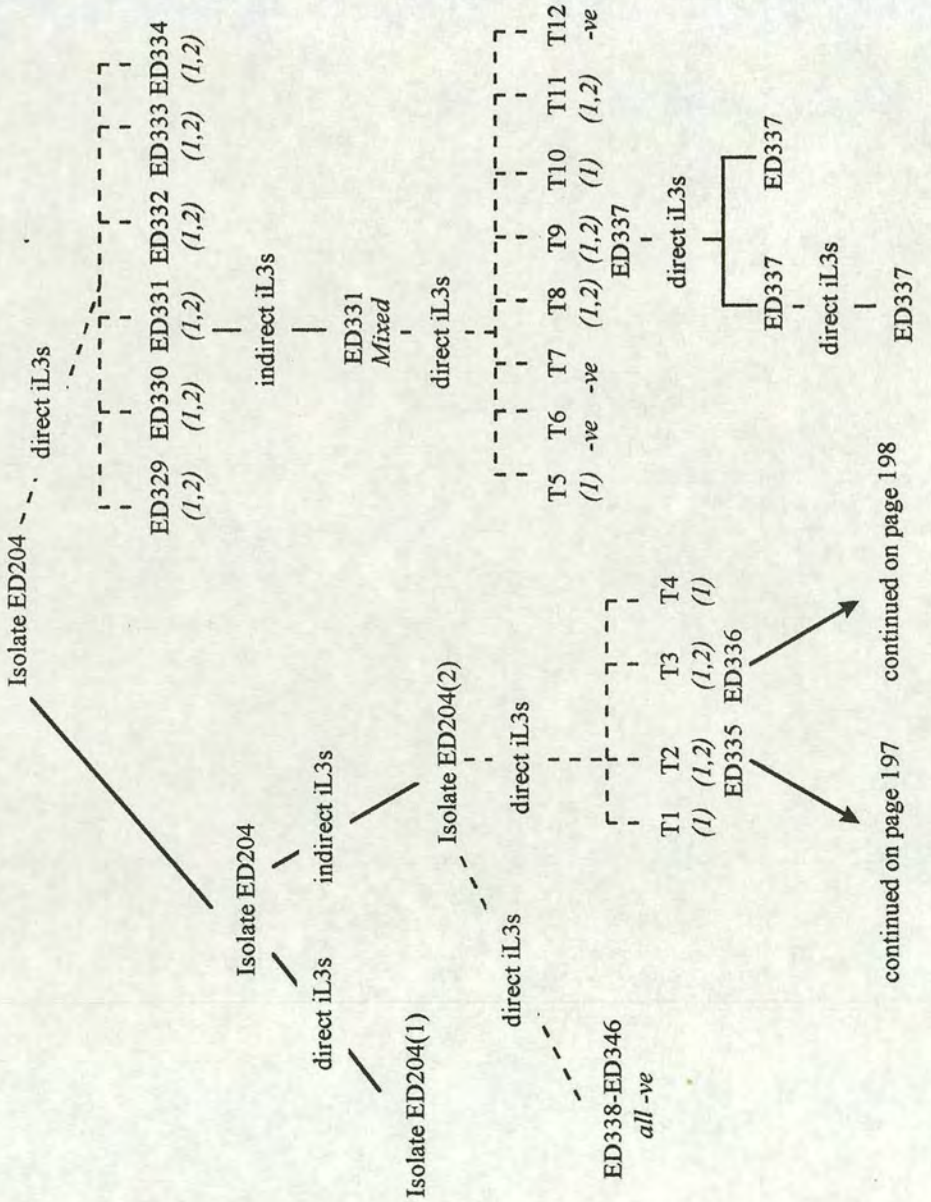
```
Sr   R H V S F S F Y F S I I F F S V N E L P A T L P K I
Ce   K G V S G T F Q - - - - - S A V S Q L P A D L P K I
Dm   K T K S D I Y V - - - - - R A V L A N P E C P P Q I

Sr   D F T D L K K K M P G Y A S I L D S L Q K Q Y E T I
Ce   D F A A L K K A L P A H S A V L D S L Q K Q Y E S V
Dm   D W A N Y K K L V P V - A G L V D S F Q K Q Y E A L

Sr   K I P K - - - - - - G T I P E E Y - - - N K Q I N
Ce   K I P Y - - - - - - G E V P A E Y - - - L K E V D
Dm   K V P Y P Q D K V S S Q V D A E I K A S Q N E I D
```

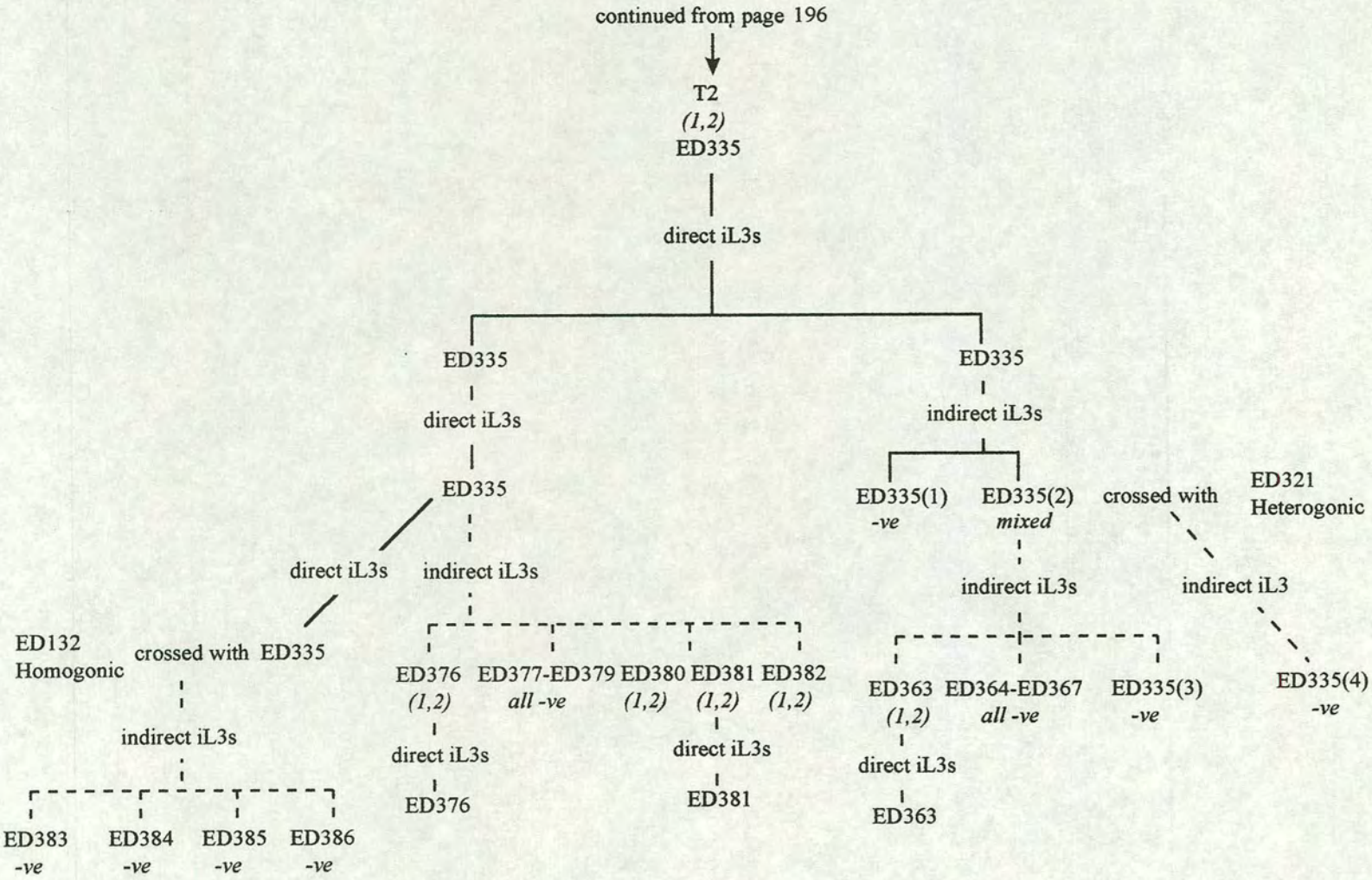
Figure 2 Alignment of the conceptual translation of the open reading frame in the *S. ratti* marker pZ-1 (*Sr*) with the amino acid sequences of the of the putative mitochondrial ATP synthase D chain proteins from *C. elegans* (*Ce*) and *Drosophila melanogaster* (*Dm*). Amino acid identity is indicated by heavy shading and amino acid similarity by light shading. - denotes a gap inserted to allow sequence alignment.

APPENDIX 3.

Parasite lines derived from isolate ED204.

Dashed lines indicate that infections were initiated with a single iL3 and solid lines indicate that infections were initiated with multiple iL3s. 'direct' and 'indirect' refer to iL3s of direct and indirect development, respectively. Lines that did not produce patent infections are marked as *-ve* . Under the name of each line that produced a patent infection, the *Sr-mvP1* genotype, if known, is shown. *Mixed* denotes that directly developing iL3s of multiple *Sr-mvP1* genotypes were found.

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T2
(1,2)
ED335
direct iL3s
ED335
direct iL3s
ED335
direct iL3s
indirect iL3s
ED132
Homogonic
crossed with
ED335
indirect iL3s
ED383
-ve
ED384
-ve
ED385
-ve
ED386
-ve
ED376
(1,2)
ED377-ED379
all -ve
ED380
(1,2)
ED381
(1,2)
ED382
(1,2)
direct iL3s
ED376
direct iL3s
ED381
ED335
indirect iL3s
ED335(1)
-ve
ED335(2)
mixed
crossed with
ED321
Heterogonic
indirect iL3s
indirect iL3
ED363
(1,2)
ED364-ED367
all -ve
ED335(3)
-ve
ED335(4)
-ve
direct iL3s
ED363

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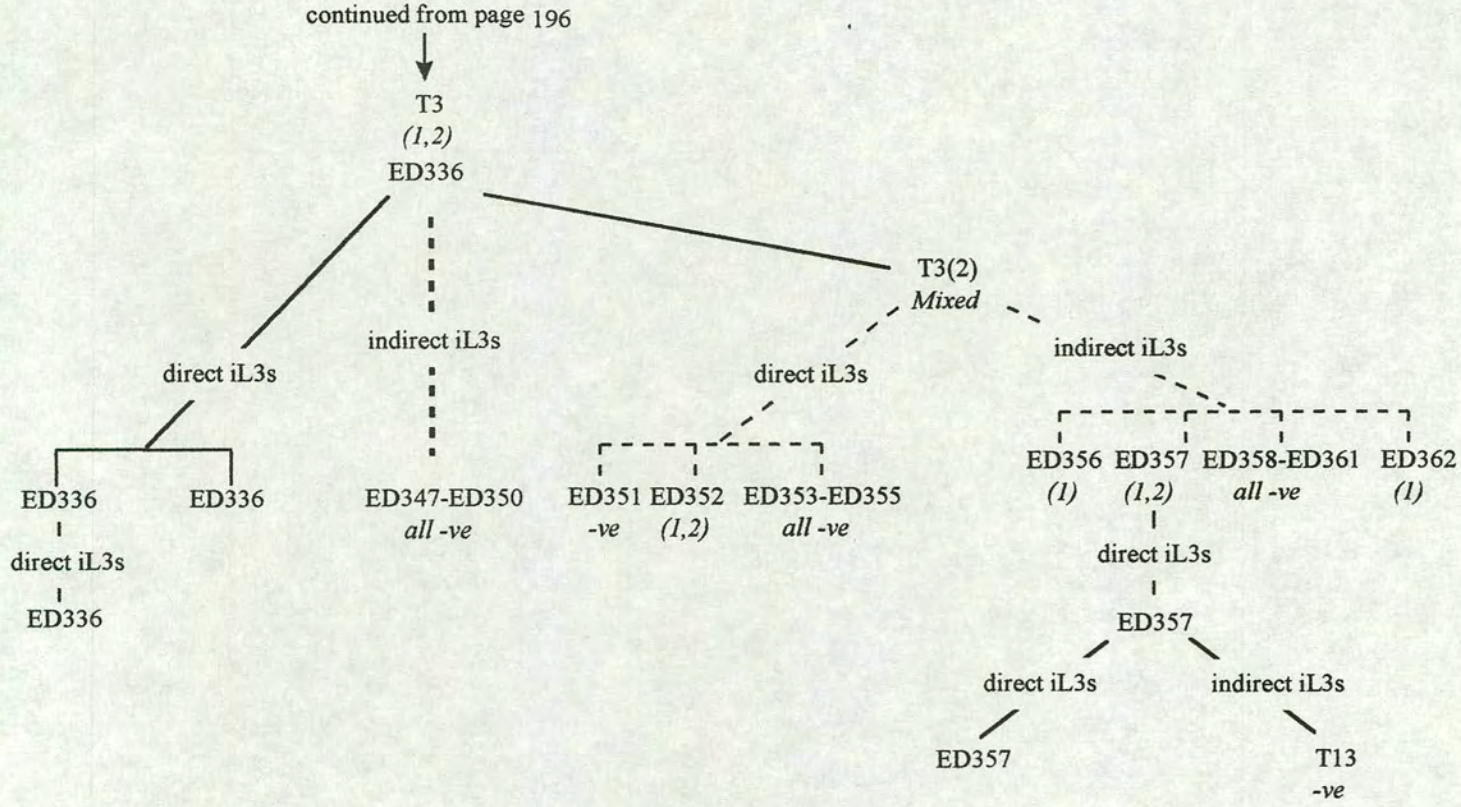

APPENDIX 4.

Publications arising from this thesis

The work described in Chapter 2 has been published as:

Harvey, S.C. Paterson, S. & Viney, M.E. (1999). Heterogeneity in the distribution of *Strongyloides ratti* infective stages among the faecal pellets of rats. *Parasitology* **119,** 227-235.

Heterogeneity in the distribution of *Strongyloides ratti* infective stages among the faecal pellets of rats

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SUMMARY

The distribution of helminth parasites within their host population is usually overdispersed and can be described by the negative binomial distribution. The causes of this overdispersion are poorly understood, but heterogeneity in the distribution of infective stages within the environment has been implicated as a possible factor. Here we describe the distribution of infective stages of the rat intestinal nematode parasite *Strongyloides ratti* among the faecal pellets of its host. The distribution of infective stages between faecal pellets is overdispersed and well described by the negative binomial distribution. This overdispersion increases during the course of infection and occurs over a range of infection intensities. Overdispersion of nematode infective stages among faecal pellets may result in increased spatial heterogeneity of the infective stages in the environment and thus may contribute to the generation of overdispersion of adult parasitic stages. In addition, these findings raise important issues regarding the accurate quantification of helminth egg counts from faecal samples.

Key words: overdispersion, *Strongyloides ratti*, spatial heterogeneity, egg counts.

INTRODUCTION

A common observation of natural helminth infections is that the distribution of parasites within a host population is overdispersed, i.e. a minority of hosts harbour the majority of the parasites (Hudson & Dobson, 1995). This overdispersion can be described by the negative binomial distribution (Hunter & Quenouille, 1952; Bliss & Fisher, 1953) and is characterized by 2 parameters, the mean and an inverse measure of the degree of overdispersion, k. Values of k approaching 20 indicate a random or Poisson distribution; lower values of k indicate increasing levels of overdispersion. Generally, helminths have values of k between 0·1 and 1 (Hudson & Dobson, 1995). The common nematode parasite of rats, *Strongyloides ratti*, is no exception. A recent study found it to be overdispersed within the host population, with a mean parasite burden of 36·95 and k of 0·77 (Fisher, 1997). It has been suggested that important factors in the generation of helminth overdispersion are differences in host susceptibility (Gregory, Keymer & Clarke, 1990; Wakelin, 1996) and variable rates of encounter between host and parasite (Crofton, 1971; Anderson & Gordon, 1982).

Differences in host immune function have been implicated in the generation of helminth overdispersion from observations that found increased variability in *Heligmosomoides polygyrus* burdens following repeated exposure compared with a single infection (Keymer & Hiorns, 1989; Gregory *et al.* 1990). However, differences in host susceptibility to helminth infection *per se* are not observed since helminth species are not normally found to covary within hosts (Poulin, 1996). This may be due to the fact that different immune mechanisms mediate responses to different helminth species (Maizels & Holland, 1998). The rate of encounter between host and parasite will depend on the spatial distribution of parasite infective stages in the environment and on the behaviour of the host (Shaw & Dobson, 1998). An even distribution of the infective stages of *Hymenolepis diminuta* has been shown to produce an overdispersed distribution in *Tribolium confusum*. However, increasing the heterogeneity of the distribution of the infective stages within the environment increased the overdispersion, but did not affect the mean intensity of infection (Keymer & Anderson, 1979). Furthermore, the overdispersed distribution of *Howardula aoronymphium* among species of *Drosophila* was found to be due to variation in the exposure to infective stages (Jaenike, 1994). Analysis of the degree of *H. polygyrus* overdispersion in a wild population of *Apodemus sylvaticus* in relation to host age, as measured by weight, found an overdispersed parasite distribution in all weight classes of mice, with the greatest degree of overdispersion in the lowest weight class (Gregory, 1992). One interpretation of these observations is that in young animals, parasite overdispersion is dependent on the rate of encounter between parasites

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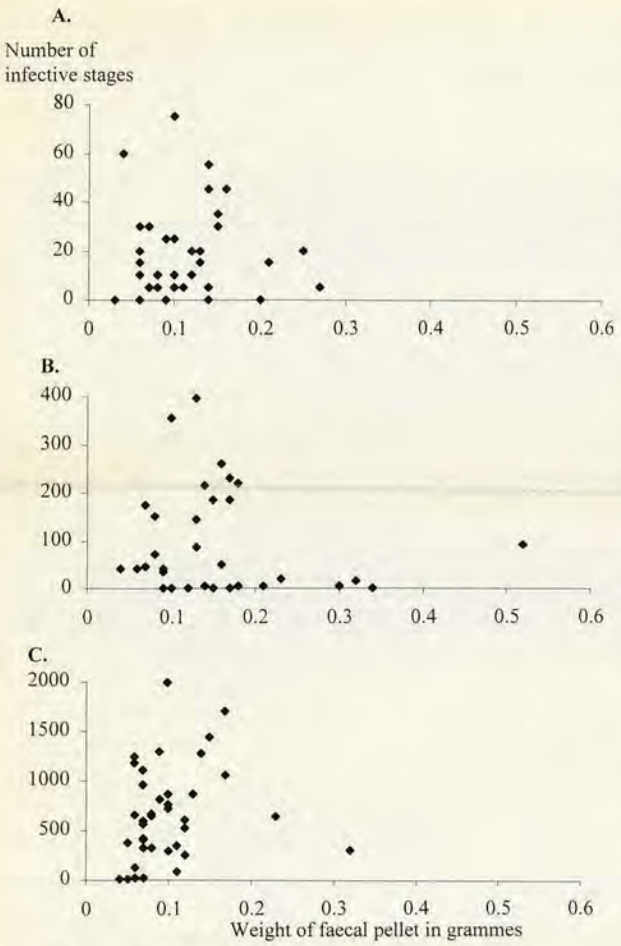

Fig. 1. Scatterplots of the weight of a faecal pellet against the number of infective stages from that faecal pellet for (A) 10 iL3, (B) 100 iL3 and (C) 500 iL3 infections.

and hosts. In older animals overdispersion may be maintained by the development of host immune responses against parasite infection.

Despite the potential importance of a heterogeneous distribution of parasite infective stages in the environment in the generation of an overdispersed distribution of adult stages, there has been little investigation of the way in which such heterogeneity may be generated. The overdispersion of the adult stages of a parasite will, in itself, generate a heterogeneous distribution of infective stages passed by the host population even if infective stages are evenly distributed within the faecal material produced by each host. In contrast, if infective stages are heterogeneously distributed within the faecal material of individual hosts then a heterogeneous distribution of infective stages within the environment would result from the faeces of an individual. The overdispersion of adult parasites within the host population would act to increase the heterogeneity of infective stages within the environment.

Heterogeneity of infective stages between faecal pellets will also have important diagnostic implications. Diagnosis and quantification of the intensity of infection is typically performed by determining egg counts from faecal samples. This sample is often only a small proportion of the total faecal output. The combination of small faecal samples and a heterogeneous distribution of eggs in the faeces may lead to very inaccurate results. Most studies of human helminth infections analyse single stool specimens on consecutive days, weeks or months (Hall, 1981; Sinniah, 1982) and thus detailed comparisons of the distribution of eggs among human stools are rare. However, it has been found that eggs of *Schistosoma japonicum* were overdispersed both within (Yu *et al.* 1998) and between (Ye *et al.* 1998) single human stools. In contrast, only slight overdispersion ($k = 2{\cdot}59$) of *S. japonicum* eggs was observed between stools by Ross *et al.* (1998). The distribution of eggs of *Trichuris trichiura* and *Ascaris lumbricoides* within subsamples of individual stools was also found to be overdispersed (Hall, 1981: Ye *et al.* 1997). However, the relevance of these findings to the distribution of helminth infective stages in rodent faeces is unclear. The nematode parasite *S. ratti* is a good laboratory system for investigating this. Here, we analyse the distribution of infective stages of *S. ratti* between faecal pellets as a function of infection intensity; throughout the course of infection and through a 24 h period. These results are discussed in relation to the generation of spatial heterogeneity of helminth infective stages in the environment and in relation to the accuracy of diagnosis and quantification of helminth infections.

MATERIALS AND METHODS

Parasites and maintenance

S. ratti was maintained by serial passage in randomly bred, size-matched, 6-week-old female Wistar rats (Bantin & Kingman, UK) which were used in all experiments. Food and water were provided *ad libitum* and animals were held in a 12 h light/dark cycle, with the dark cycle commencing at 18.30. All infections were by subcutaneous injection. Inocula of 100 and 500 infective 3rd-stage larvae (iL3s) were prepared by dilution and inocula of 10 iL3s by counting individual larvae under a dissecting microscope. Faeces from infected animals were collected as described elsewhere (Viney, Matthews & Walliker, 1993). Faecal pellets were cultured by placing individual pellets in the wells of a 24-well culture dish (Nunc) with a small quantity of distilled water and maintained at 19 °C. An animal infected with *S. ratti* passes eggs and larvae in faeces. After faecal culture, these grow into iL3s, free-living males or free-living females. The total number of these stages present in cultured faeces was used as a direct measure of the number of eggs and larvae passed in fresh faeces. This was determined by repeatedly rinsing each culture well with distilled water and

Table 1. Analysis of the goodness of fit of the number of infective stages per faecal pellet relative to the Poisson and negative binomial distributions for the (A) 10, (B) 100 and (C) 500 iL3 infections in Exp. 1.

	Day p.i.	Number of		Number of infective stages per pellet			Poisson		Negative binomial	
		Pellets	Infective stages	Mean	Variance	k	Deviance	P value	Deviance	P value
(A)	5	43	725	16·9	308	1·35	141·57	< 0·01	34·75	0·74
	10	43	180	4·2	100	0·24	112·00	< 0·01	8·74	> 0·99
	15	39	485	12·4	3995	0·05	559·66	< 0·01	5·24	> 0·99
(B)	5	33	3070	93·0	12053	0·47	812·55	< 0·01	30·62	0·49
	10	48	750	15·6	956	0·25	375·67	< 0·01	20·56	> 0·99
	15	32	410	12·8	1814	0·12	331·33	< 0·01	8·21	> 0·99
(C)	5	38	25405	668·6	234170	1·22	2791·56	< 0·01	42·97	0·20
	10	48	13015	271·1	113281	0·43	3592·07	< 0·01	47·79	0·40
	15	44	210	4·8	259	0·09	183·56	< 0·01	5·63	> 0·99

determining the number of worms present by dilution counting. Here, we refer to the number of worms that developed in faecal cultures as the number of infective stages passed by a host.

Experiment 1 : distribution of infective stages among faecal pellets through an infection

Three rats were infected with 10, 100 or 500 iL3s of isofemale line ED335. Faeces were collected overnight between 18.00 and 09.00, on days 5, 10, 15 and 20 p.i., cultured and the number of infective stages present in 20% of the total volume determined as described above. For the pellets collected on day 5 p.i. each pellet was weighed.

Experiment 2 : distribution of infective stages among faecal pellets through a 24 h period

Four rats were infected with 500 iL3s of isofemale line ED248. Faeces were collected on day 5 p.i. in 4 consecutive 6 h collection periods, i.e. period 1, 18.00–00.00; period 2, 00.00–06.00; period 3, 06.00–12.00 and period 4, 12.00–18.00. Faecal pellets were cultured and the number of infective stages present in 10% of the total volume determined, as described above.

Statistical analysis

The distribution of infective stages among faecal pellets was fitted to Poisson and negative binomial distributions. For the negative binomial distribution, k was estimated by iterative scoring and maximum likelihood using moment estimates as initial values (Bliss & Fisher, 1953). The standard error of k was estimated as the square root of the inverse of the Fisher information at the maximum likelihood estimate of k (Bliss & Fisher, 1953; McCullagh & Nelder, 1989). Goodness of fit of observed data to both the Poisson and negative binomial distributions was investigated by fitting generalized linear models (GLMs) with a log link function to the data and examining the relative deviances from the models using Pearson's χ^2 statistic (McCullagh & Nelder, 1989). GLM fitting was performed in Matlab using glmlab, a publicly available routine (http://mathworks.com), modified to accommodate the negative binomial distribution.

GLMs were fitted to a negative binomial error distribution by estimating k within each round of the iteratively reweighted least squares algorithm. This method allows the log likelihood of the model to be maximized over both k and the regression coefficients (β) (Lawless, 1982). Log likelihoods for groups of independent models were calculated by summing log likelihoods. To test whether the addition of extra parameters (β or k) significantly improves the goodness of fit, the likelihood ratios of different

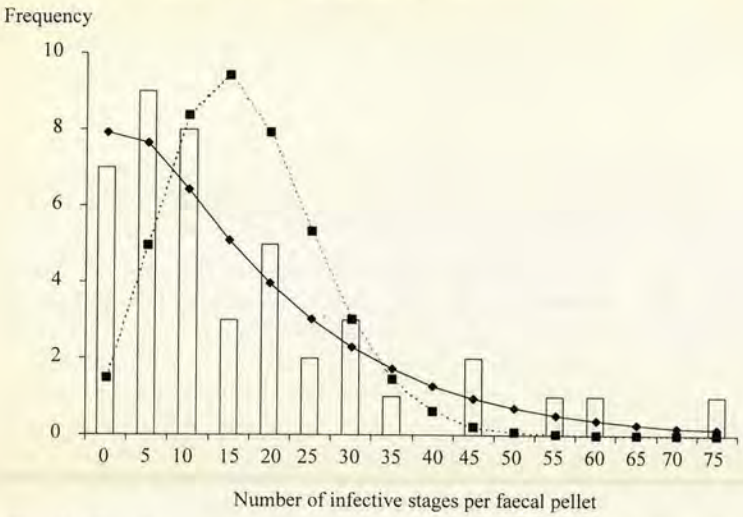

Fig. 2. The frequency distribution of the number of infective stages per faecal pellet from the 10 iL3 infection on day 5 p.i. and the fitted Poisson (◆) and negative binomial (■) distributions.

models were compared to each other (Venables & Ripley, 1997). Calculations were performed in Matlab v5.2 (Mathworks Inc., Natick, MA) using code converted from S-plus (Mathsoft, Cambridge, MA) (Venables, available at: ftp://lib.stat.cmu.edu).

RESULTS

Experiment 1 : distribution of infective stages among faecal pellets through an infection

The number of infective stages per faecal pellet plotted against faecal pellet weight is shown in Fig. 1. For each infection dose a GLM, using a negative binomial error distribution, of the number of infective stages per faecal pellet on day 5 p.i. and including the weight of each faecal pellet was constructed. For all infection doses there was no association between the number of infective stages per faecal pellet and the weight of the faecal pellet (null deviance = 34·75, Δdeviance = 0·15, $P = 0{\cdot}70$, null deviance = 30·62, Δdeviance = 0·53, $P = 0{\cdot}47$ and null deviance = 42·97, Δdeviance = 1·14, $P = 0{\cdot}29$ for the 10, 100 and 500 iL3 infections, respectively).

The distribution of the number of infective stages per faecal pellet was significantly overdispersed compared with the Poisson distribution but was well described by the negative binomial distribution (Table 1). On day 20 p.i., for all infection doses, there were insufficient faecal pellets containing infective stages to derive estimates of k or to fit the Poisson and negative binomial distributions. The fit of the Poisson and negative binomial distributions to the data from the 10 iL3 infection on day 5 p.i. is shown in Fig. 2. To investigate whether the degree of overdispersion differed between infection doses or between days p.i. a single GLM with a common k was constructed containing all explanatory variables and interactions and compared with individual GLMs that contain the same number of data points and regression coefficients, but that were split by *Day p.i.* or *Infection Dose* and fitted separately. This allowed k to be estimated separately for each day p.i. and each infection dose and the significance of separate ks to be determined by examining the log likelihood ratios (Shaw, Grenfell & Dobson, 1998) (Table 2). These estimated values of k for the different infection doses and days p.i. are shown in Fig. 3. This shows that k differs between different days p.i. (Table 2 and Fig. 3 A) and between different infection doses (Table 2 and Fig. 3 B).

Experiment 2 : distribution of infective stages among faecal pellets through a 24 h period

The number of faecal pellets produced by each rat during each 6 h collection period was analysed by fitting a GLM to these data, assuming Poisson errors. *Rat* and *Collection Period* were fitted as main effects and significance tested by examining deviance residuals. This identified a significant effect of *Collection Period* on the number of faecal pellets produced (Δdeviance = 47·73, $\Delta_{\text{D.F.}} = 3$, $P < 0{\cdot}001$) and showed that there was no significant effect of *Rat* on the number of faecal pellets produced (Δdeviance = 2·50, $\Delta_{\text{D.F.}} = 3$, $P = 0{\cdot}475$, null model: deviance = 75·45, residual D.F. = 15) Comparison of parameter estimates showed that the number of faecal pellets produced in collection period 2 (00·00–06·00) was significantly greater than in other collection periods (Δdeviance = 28·07, $\Delta_{\text{D.F.}} = 1$, $P < 0{\cdot}001$).

The distribution of infective stages between faecal pellets, for these data combined for all rats and all collection periods, was significantly overdispersed relative to the Poisson distribution (deviance = 2359, D.F. = 207, $P < 0{\cdot}001$), but was well described by the negative binomial distribution (deviance = 184·2, D.F. = 206, $P = 0{\cdot}86$) with an overall mean

Table 2. Analysis of k between infection doses and days p.i. by comparison of a GLM with a common k for all groups with separate GLMs split by *Infection Dose* or by *Day p.i.*

Model	D.F.	2 × log likelihood	χ^2	ΔD.F.	P value
Common k for all treatments. Terms: *Intercept* + *Infection Dose* + *Day p.i.* + *Infection Dose* · *Day p.i.*	358	65192	—	—	—
Separate ks for days p.i. Terms: *Intercept* and *Infection Dose*	356	65275	83·68	2	< 0·001
Separate ks for infection doses. Terms: *Intercept* and *Day p.i.*	356	65201	9·20	2	0·01

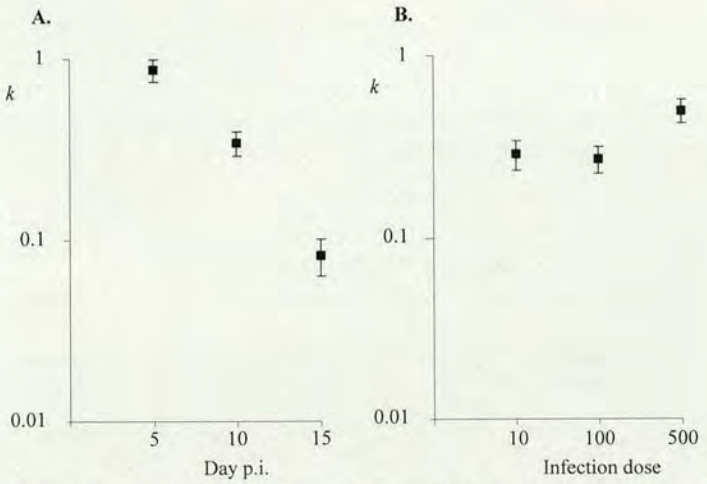

Fig. 3. The estimated values of k from GLMs (Table 2) (A) on days 5, 10 and 15 days p.i. for all infection doses combined and (B) for the 10, 100 and 500 iL3 infection doses for days 5, 10 and 15 p.i. combined. Error bars are ±1 S.E.

Table 3. Analysis of k between rats and collection periods by comparison of a GLM with a common k for all groups with separate GLMs split by *Rat* or by *Collection Period*

Model	D.F.	2 × log likelihood	χ^2	ΔD.F.	P value
Common k for all treatments. Terms: *Intercept* + *Rat* + *Collection Period* + *Rat* · *Collection Period*	191	8527	—	—	—
Separate ks for collection period. Terms: *Intercept* and *Rat*	188	8531	3·63	3	0·30
Separate ks for rat. Terms: *Intercept* and *Collection Period*	188	8531	3·67	3	0·30

and k of 114 and 0·744, respectively. For the faecal collection period comparable to Exp. 1 (collection periods 1 and 2 combined), for all 4 rats combined, the distribution of infective stages was overdispersed with a mean and k of 128 and 0·836, respectively. To investigate whether the degree of overdispersion differed between rats and collection periods, a GLM with a common k for all groups was compared with individual GLMs to which k values were fitted separately for *Collection Period* and for *Rat* (Table 3). This showed that there was no significant difference in k between rats or between collection periods.

A GLM with a normal error distribution of the natural log transformed number of infective stages passed by each rat showed that there was no significant variation between rats in the number of infective stages passed by each rat over the entire 24 h period, but that the mean number of infective stages passed in each 6 h collection period did vary ($\chi^2 = 7·91$, D.F. = 3, $P = 0·048$). The parameter estimates of the mean number of infective stages

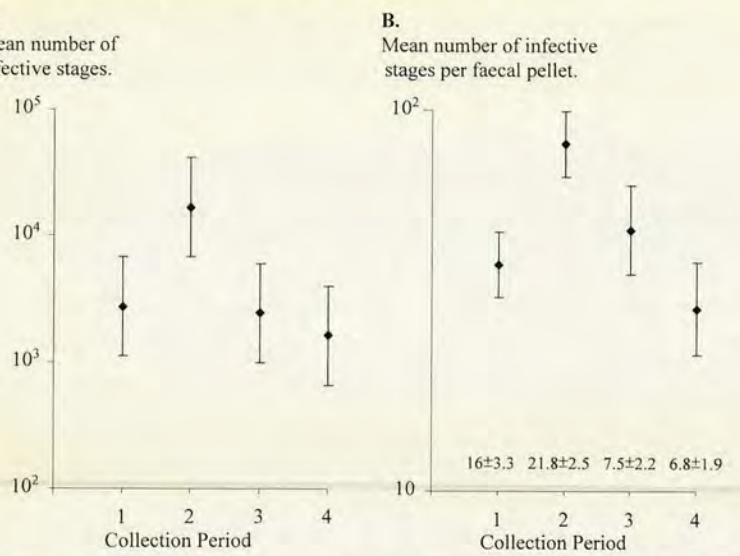

Fig. 4. (A) The mean number of infective stages over the 4 six h collection periods. Error bars are ±1 S.E. (B) The mean number of infective stages per faecal pellet over the 4 six h collection periods. Error bars are ±1 S.E. Also shown are the mean number of faecal pellets per collection period ±1 S.E.

Table 4. The results of GLM analyses of the mean number of infective stages per faecal pellet

Model	Term	Residual D.F.	2 × log likelihood	χ^2	ΔD.F.	*P* value
Null		206	8469·51	—	—	—
Model 1	*Collection Period* (factor; periods, 1, 2 and (3+4))	204	8489·38	19·88	2	< 0·001
Model 2	*Collection Period* (polynomial)	204	8489·10	19·60	2	< 0·001
Model 3	*Collection Period* (factor; periods, 1, 2 and (3+4))	—	—	20·59	2	< 0·001
	Rat (factor)	—	—	6·13	3	0·105
	Rat × *Collection Period*	195	8523·40	27·89	6	< 0·001

produced in each 6 h collection period is shown in Fig. 4A. This shows that the greatest number of infective stages was passed in collection period 2 (00.00–06.00).

The number of infective stages per faecal pellet was examined by fitting a GLM with a negative binomial error structure to the observed data (Table 4). The error structure required k to be estimated from the data and was assumed to be constant between time-periods and rats which, as shown in Table 3, is a valid assumption. In Models 1 and 3, collection periods 3 and 4 were combined since this did not result in a significant increase in deviance (Δdeviance = 1·86, D.F. = 1, P = 0·173). *Collection Period* is significantly associated with the mean number of infective stages per faecal pellet when considered as a factor (Model 1) or as a quadratic function (Model 2) (Table 4). The highest mean number of infective stages per faecal pellet is in collection period 2 (00.00–06.00) (Fig. 4B). Model 4 shows that there is no significant difference between *Rat* in the mean number of infective stages per faecal pellet when considered as a main effect (Table 4). However, there is a significant interaction between the factors *Rat* and *Collection Period* (Table 4).

DISCUSSION

In both experiments, the distribution of the infective stages of *S. ratti* among host faecal pellets was significantly overdispersed and well described by the negative binomial distribution. Variation in the weight of faecal pellets did not explain the distribution of infective stages among faecal pellets and thus the observed overdispersion is not a trivial function of faecal pellet weight. The distribution of the infective stages among host faecal pellets was overdispersed for the 3 infection doses used. A reduction in the observed degree of overdispersion was found for the 500 iL3 infection dose, which may be a consequence of the very large number of eggs, and hence greater density, in the faeces. For the 2 isofemale lines used, for comparable infection doses (500 iL3s) and during comparable sampling periods (day 5 p.i.), the mean production of infective stages

was different, but the overdispersion among the faecal pellets was similar. Thus, ED335 and ED248 had a mean and k of 669 and 1·22 and 128 and 0·836, respectively. This overdispersion of infective stages among faecal pellets may be due to heterogeneity in the timing of egg production and egg laying or due to the behaviour of the eggs in the intestinal lumen. If the production and laying of eggs by parasitic females varies over time and varies similarly for cohorts of worms, then this may also lead to the generation of an overdispersed distribution of infective stages among faecal pellets. Furthermore, the eggs of *S. ratti* are laid in the intestinal epithelium and thus are not immediately liberated into the intestinal lumen. It is not known how eggs enter the intestinal lumen, but it may well be due to epithelial abrasion. Such a process may lead to the liberation of clumps of eggs into the intestinal lumen which may also generate an overdispersed distribution. In *S. ratti*, it has been reported that some eggs are laid in strings of varying lengths, held together with a mucus secreted by the parasitic female (Sandground, 1925). This would lead to some clumping of the eggs and may also generate an overdispersed distribution of infective stages among faecal pellets. However, this phenomenon has only been reported for *Strongyloides* and as there is evidence that the infective stages of other nematodes are overdispersed among faecal pellets, this cannot be the general basis of the generation of heterogeneity in egg distribution.

There was no significant difference in the degree of overdispersion of the infective stages between the 4 collection periods throughout a 24 h period. In contrast to the consistency of overdispersion across a 24 h period, the degree of overdispersion significantly increased during infection. During the course of an infection with *S. ratti*, rats become increasingly immune and lose intestinal worms (Moqbel & McLaren, 1980). As shown above, an infection dose of 500 iL3s results in a decreased level of overdispersion, but there is no change in the degree of overdispersion between the 10 iL3 and 100 iL3 infection doses. Thus, the increased overdispersion later in an infection is unlikely to be a simple function of the reduced numbers of parasitic females. It is therefore probable that the greater overdispersion later in infection is a result of increasing heterogeneity in the egg production by the parasitic females rather than a reduction in the number of parasitic females *per se*. However, it is unclear if this increased overdispersion is a consequence of the age of the parasitic females or a response to increasing host immunity. It has been shown that increasing host immunity reduces the number of eggs *in utero* and results in changes to the ultrastructural morphology of *S. ratti* parasitic females (Moqbel & McLaren, 1980). It may be that these functional and morphological changes in the parasitic females are the cause of the increased heterogeneity of egg production over infection. In addition, over the course of infection the distribution of parasitic females within the small intestine changes. In the initial stages of infection, the majority of the parasitic females are located in the quarter of the small intestine immediately below the pyloric sphincter, but as the infection progresses they move to the posterior of the small intestine (Moqbel & Denham, 1977). This may reduce the opportunity for the eggs to be dispersed in the lumenal contents, with the result that the overdispersion of infective stages among the faecel pellets is increased.

To analyse the distribution of infective stages among faecal pellets with greater precision, we sampled faeces in 4 equal 6 h periods. This showed that the production of faecal pellets is similar between rats and is at its greatest between 00.00 and 06.00. Behavioural studies of rats have found that they are most active at night, with peak foraging behaviour occurring 2–3 h after dark (Barnett, Cowan & Prakash, 1975). This increased activity is accompanied by a higher body temperature and increased metabolic activity (Honma & Hirochige, 1978). The dark period of the study animals commenced at 18.30 and hence a peak in faecal output between 00.00 and 06.00 is not unexpected and is likely to result from peak feeding activity a number of hours earlier. In addition to the peak in faecal output between 00.00 and 06.00, the greatest number of infective stages passed also occurred during this period.

There was also a significant difference between collection periods in the mean number of infective stages per faecal pellet. Thus, the observed peak in the number of infective stages passed between 00.00 and 06.00 is due to a combination of the peak in faecal production and the peak in mean number of infective stages per faecal pellet. Parasitic females of *S. ratti* lie buried in the mucosa of the small intestine where eggs are deposited (Dawkins, 1989). Therefore, it is likely that the liberation of eggs from the mucosa is related to, or dependent on, gut activity. Thus, a peak in the number of infective stages passed by a host would be expected to coincide with a peak in faecal production, as was observed. However, a peak in the mean number of infective stages per faecal pellet was also observed in the same time-period. This strongly suggests that the production of eggs by parasitic females varies through time. These data support previous suggestions that such variation in egg production occurs in *S. ratti* (Sandground, 1928). The findings also agree well with a study of *Aspiculuris tetraptera* which found that over a 24 h period egg production peaked at the same time as the peak in faecal output (Phillipson, 1974). Furthermore, this peak in *A. tetraptera* egg production was found to be due to an increase in the mean number of eggs per faecal pellet. For *S. ratti*, there is probably a delay between the time at which eggs

were laid by parasitic females and their liberation into the intestinal lumen. Thus the peak egg production of a parasitic female is likely to occur before the peak faecal production of the host and may coincide with the peak in rat activity that occurs 2–3 h after dark (Barnett *et al.* 1975). The analysis of the mean number of infective stages per faecal pellet also identified a significant interaction between the factors *Rat* and *Collection Period*. Biologically this implies that there is variation between different rat and time-period combinations in the number of infective stages per faecal pellet. The biological significance of this finding is unclear, given the limited number of animals used and the small number of faecal pellets produced in some time-periods.

The overdispersion of the infective stages of *S. ratti* between faecal pellets may contribute to the spatial heterogeneity of the infective stages in the environment. However, spatial environmental heterogeneity will also depend on the defaecation behaviour of rats, about which little is known. Spatial heterogeneity of the infective stages in the environment may in turn be involved in the production of overdispersed distributions of adult parasites. Experimental studies of *H. polygyrus* infection in *A. sylvaticus*, indicated that variation in immunocompetence is important in generating overdispersion of adult parasites within the host population (Gregory *et al.* 1990). However, increased spatial heterogeneity of infective stages within the environment and the resulting change in the rate of encounter between host and parasite is also known to increase overdispersion (Keymer & Anderson, 1979). Our observations demonstrate the basis by which such spatial heterogeneity may be generated. Further study of the distribution of infective stages in the environment should now be a priority.

These observations also have important practical consequences for parasitological studies. For example, when determining the number of infective stages per gram of faeces from an infected animal, the result that is obtained will be heavily dependent on what proportion of faecal output is considered and over what time-period faeces were collected. Thus, for *S. ratti*, to obtain an accurate measure of the number of infective stages in faeces, a substantial proportion of the daily faecal output must be collected and treated as a whole. It is likely that our observations may explain the extreme variation in the daily worm output that have been observed in studies that have extrapolated from single faecal pellets (Sheldon, 1937). The observation of considerable variation in daily egg output seen in other helminth infections (Hall, 1981) suggests that the overdispersion of the infective stages of *S. ratti* reported here may also occur in other helminth systems. These results also suggest that extreme care may have to be taken when relying on faecal egg counts to determine either the existence or intensity of helminth infections. It would be informative to determine if the overdispersion of infective stages among faecal pellets observed here occurs in helminth infections of medical or veterinary importance and to investigate its role in parasite distribution and transmission.

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