For Mum, Dad and Richard,

I love you more than words can ever express.

And to the memory of Tom Smith,

A dear friend sorely missed.

Abstract

This thesis uses a multidisciplinary approach to investigate several aspects of host-parasite relationships, using the European common shrew (*Sorex araneus*) as a model host. Common shrews are known to harbour a variety of micro and macroparasites, but few studies have investigated their blood parasite fauna. To this end, bacteriological and molecular methods were used here to detect haemoparasites in *S. araneus*, and to compare recovered parasite gene sequences with those previously reported from shrews and other species. The results suggest that common shrews harbour a previously undescribed trypanosome, and share other blood parasites with rodents inhabiting their environment.

Given the resource-limited physiology of common shrews and the species richness of their parasite fauna, infection may be more detrimental to *S. araneus* than to other mammalian species. A thorough histological examination conducted here revealed that shrews suffer diverse parasite-mediated pathology, but are capable of mounting immune reactions against infectious agents. Through application of immunohistology, adult *S. araneus* were found to exhibit some signs of exhaustion in their lymphatic tissues, which may affect their ability to mount immune responses. The results from immunohistology also strongly suggested that the 'pancreas of Aselli', a lymphatic organ unique to shrews, functions as a large lymph node draining the gut.

Although helminth parasites are often associated with decreases in host food uptake, few studies of wild vertebrates have found effects of these parasites on host life-history traits. Here, experimental manipulation of helminth burdens provided no evidence for an impact of these parasites on common shrew foraging activity, while treatment with anthelmintics in the field resulted in greater male body mass during the breeding season, and possibly an initial increase in host survival.

Several previous studies have found relationships between host life-history traits and species richness of metazoan parasite assemblages. Here, comparative analysis across the family Soricidae found that larger host species harbour more coccidian parasites, perhaps as a result of increased rates of colonization associated with greater food intake.

Through integration of several specialist techniques, the research presented here provides a broader understanding of the relationships between shrews and their parasites. It is hoped that this work will promote the further application of a multidisciplinary approach to the study of host-parasite systems.

Declaration of work performed

All work described in this thesis was carried out by the author, with the following exceptions. Sectioning of tissue samples and EM photography for **Chapter 3** and **Chapter 4** was carried out by technical staff in the Department of Veterinary Pathology, University of Liverpool. Histological staining was carried out with assistance from Dr. Anja Kipar and technical staff in the Department of Veterinary Pathology, University of Liverpool, and examination of sections was undertaken with Dr. Kipar. Sperm counts and weighing of testes described in **Chapter 6** were conducted by Mr. S.A. Ramm.

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Chapter 1. Introduction

1.1 Parasitism - an overview

Parasitism is characterized by the requirement of one organism (the parasite) to live in or on another (the host) for at least part of its life cycle, benefiting from the association at the host's expense (Matthews, 1998). Parasitic lifestyles have evolved independently in almost every major taxon (Cheng, 1986), such that parasites cannot be placed into any single monophyletic group. On the basis of their size and life-history strategies, parasites can be usefully divided into two broad categories: the 'microparasites' and 'macroparasites' (Anderson & May, 1979). The microparasites include viruses, bacteria and protozoans, and are characterized by their small size, short generation times and high rates of reproduction in the host. The macroparasites comprise the ectoparasitic arthropods (including ticks, fleas and mites) that live upon the host's surface, and the parasitic helminths. In addition to being larger than the microparasites, these metazoan organisms also have longer generation times and direct reproduction within the host is usually absent (Anderson & May, 1979; Anderson, 1982).

The parasitic helminths are contained within three phyla of 'worm-like' creatures: the Platyhelminthes (including the Monogenea, Digenea and Cestoda), Nematoda and the Acanthocephala. Monogeneans are typically ectoparasites of fish, adhering to the skin or gills via a posterior attachment organ known as the haptor or opisthaptor. Adults are hermaphrodites, with cross-fertilization resulting in eggs which develop into oncomiracidia: juvenile stages which swim to new hosts on which to attach and develop (Matthews, 1998). Digeneans are endoparasites of vertebrates, attaching to the host via suckers (usually one at the mouth and one on the ventral surface; Matthews, 1998). Unlike monogeneans, the life cycle of digeneans is indirect, requiring development in at least one intermediate host (usually a snail) before developing as adults in vertebrate hosts (Whitfield, 1982; Matthews, 1998).

Cestodes (or 'tapeworms') typically inhabit the guts of vertebrates, attaching to the intestine via suckers and hooks on the anterior scolex (Whitfield, 1982; Matthews, 1998). Nutrients are absorbed through the tegument of the stobila; a long, flattened section connected to the scolex, comprising many individual sections (proglottids) each

of which contains a hermaphrodite reproductive system (Dunn, 1978; Whitfield, 1982). Eggs are produced through fertilization between worms, proglottids, and possibly within proglottids, and leave the host through the faeces (Whitfield, 1982). Those produced by members of the order Pseudophyllidea develop into free-swimming larva and require two intermediate hosts (typically an arthropod which is then predated upon by a fish), prior to the development of stages infectious to the definitive host. Cyclophyllidian cestodes require just one vertebrate or invertebrate intermediate host, infecting the definitive host through predation (Matthews, 1998).

Nematodes are pseudocoelomic 'roundworms', of which there are over half a million species, most of them free-living or parasites of plants, although some are important parasites of vertebrates and man (Dunn, 1978; Whitfield, 1982). Most species have separate sexes, with eggs hatching and undergoing four larval stages before becoming adults. Transmission can be direct or require intermediate hosts (Whitfield, 1982). Acanthocephalans are pseuocoelomate and also have separate sexes, but are exclusively parasitic. They are also distinguished by the presence of an anterior spiny proboscis used to attach to the intestine of their definitive vertebrate hosts. Life cycles of acanthocephlans are indirect, with arthropod intermediate hosts. If the intermediate host is consumed by a vertebrate in which full development cannot take place, this animal becomes a paratenic host, the parasite being transferred to its definitive host via predation (Matthews, 1998).

1.2 Parasite species richness

There exists considerable variation in the number of parasite species reported from different host species (Price & Clancy, 1983). In part, this may be a consequence of sampling effort (Walther *et al.*, 1995): since many parasites occur at low prevalence (Poulin, 1998a), as more hosts are examined, more rare species will be discovered (Poulin & Morand, 2000). However, important ecological processes may also influence the species richness of parasite faunas (the total number of parasite species reported from one host species; Poulin, 1997). In particular, as hosts originally inherit their parasite faunas from their ancestors, parasite species richness will be partially determined by host phylogeny (Poulin, 1997; Vickery & Poulin, 1998). Over time,

parasite faunas can lose parasite species through extinction, and gain them through speciation and colonization from other host species (Poulin, 1997). Factors influencing rates of colonization, extinction and speciation may therefore be important processes in shaping parasite species richness (Poulin & Morand, 2000). Parasite species richness is predicted to increase with host population density, as parasites which require high transmission rates to persist may only occur in hosts living at high densities (May & Anderson, 1979; Poulin & Morand, 2000; Arneberg, 2002). If larger hosts are colonized by higher numbers of ingested transmission stages as a result of greater food intake, then parasite species richness should also increase with host body mass (Arneberg, 2002). Larger hosts may also more provide more space and a greater array of niches for parasites to occupy (Poulin, 1999).

Comparative analyses of helminth parasite faunas, which control for both sampling effort and host phylogeny, have not always found evidence of proposed relationships with host traits. In a study of directly transmitted nematode parasites of mammals, Arneberg (2002) found parasite species richness to be positively related to both host population density and body mass. A similar investigation in terrestrial mammals which included cestodes, digeneans and nematodes found an effect of host population density, but not body mass, on helminth species richness (Morand & Poulin, 1998), while a study restricted to Iberian rodents also found no relationship between helminth species richness and host body mass (Feliu *et al.*, 1997). Simulation studies suggest parasite extinction or colonization must be frequent events and/or strongly linked to host ecology for relationships to be detectable statistically (Vickery & Poulin, 1998).

Characteristics of parasites themselves may also affect species richness, through rates of speciation, colonization and extinction. Those which remain in or on their hosts throughout their lives have little opportunity to colonize new host species. Phylogenies of such parasites might therefore be expected to mirror more closely those of their hosts. Cospeciation of hosts and parasites appears to be common in lice of seabirds (Paterson & Banks, 2001), and pocket gophers of the rodent family Geomyidae (Hafner & Page, 1995), but not rock-wallabies of the superfamily Macropodidea (Barker, 1991 but see Page *et al.*, 1996). In contrast, parasites which rely on free-living stages for

transmission, such as helminths with indirect life-cycles, may be more likely to colonize new host species through accidental ingestion (Barker, 1991). Parasites with higher rates of transmission may also be more likely to encounter and adapt to new host species (Arneberg, 2002). These hypotheses need to be further developed, as understanding the processes which constrain parasites to their hosts, and those which allow colonization and switching between host species, may be particularly important in predicting and controlling the spread of zoonotic diseases (Ambroise-Thomas, 2000).

1.3 Parasite-mediated pathology and the host immune response

Parasite-mediated pathology occurs when the activities of the parasite, host, or both result in tissue damage and disease (Slauson & Cooper, 1982). Pathological effects of microparasites include direct damage to cellular metabolism through viral replication (Slauson & Cooper, 1982; Lichtenburg, 1984) and production of bacterial toxins which damage cells and interfere with their vital functions (Lichtenburg, 1984; Taussig, 1984). Due to their comparatively large size, helminths can cause physical and mechanical damage by attaching, feeding and migrating through host tissues (Lichtenburg, 1984; Castro, 1990; McLaren, 1990; Wilson, 1990; Matthews, 1998). The host's main defence in fighting and controlling parasitic infections is the immune system, which in jawed vertebrates comprises interactions between many different cell types and mediators (Kasahara et al., 2004). The first line of defence against successfully invading pathogens is innate immunity. Briefly, in order to mount an immune response, the host must first be able to recognise the parasite as being 'foreign' (Medzhitov & Janeway, 2002). Microbes can then be internalized by phagocytic cells and destroyed (Aderem & Underhill, 1999), and virus-infected cells lysed by natural killer cells (Biron *et al.*, 1999). Both host cell types also secrete cytokines, which stimulate the recruitment and activation of leukocytes at the site of infection (Biron *et al.*, 1999; Underhill & Ozinsky, 2002).

As well as forming the most rapid response against pathogens, innate immunity also activates and influences the nature of pathogen-specific adaptive immunity (Fearson & Locksley, 1996). This process begins by macrophages presenting microbe-derived peptides to host T lymphocytes (Germain, 1994), which then in turn activate B cells

within follicles of the spleen and lymph nodes to mature into antibody-producing plasma cells (Parker, 1993). Once antibodies have been produced, they can initiate the effector mechanisms of humoral immunity against extracellular microbes. These include neutralizing microbes by binding to microbial cell receptors, enhancing phagocytosis of antibody-coated microbes, destruction of microbes by antibody-dependent cell-mediated cytotoxicity and activation of the complement pathway, resulting in the formation of the membrane attack complex and cell lysis (Abbas *et al.*, 2000).

Reactions to intracellular microbes are effected via cell-mediated immunity. In delayed-type hypersensitivity, CD4+ Th1 cells and CD8+ T cells activate phagocytes to kill ingested microbes (Oxenius *et al.*, 1998). Other nucleated cells containing foreign antigen in their cystol are destroyed by CD8+ cytotoxic T cells (Kägi *et al.*, 1996).

Helminth infection presents a unique set of problems for the immune system, as these parasites are generally too large to be engulfed by phagocytic cells (Meeusen, 1999). Immune responses against helminths are characterized by activation of Th2 cells, resulting in IgE antibody production and eosinophil activation (Abbas *et al.*, 2000). The role of eosinophils in helminth immunity is not completely understood, but is believed to involve antibody or complement-induced release of toxic proteins and reactive oxygen intermediates (Klion & Nutman, 2004). Eosinophils and mast cells also release mediators causing physiological changes, including vasodilation, mucus secretion and smooth muscle contraction, (Meeusen, 1999) all of which may aid in the ejection of the parasite from the host (Matthews, 1998).

Although immune responses may be effective in controlling parasitic infections, they are not without cost. The mechanisms employed by the immune system to destroy parasites may also incur damage upon host tissues, resulting in immunopathology (Abbas *et al.*, 2000). This may be especially true for helminths, which as a consequence of their large size are only affected by particularly toxic mechanisms (Meeusen, 1999). In addition to damaging host tissues directly, metabolic and nutritional costs are incurred in order to support the up-regulation of the immune system (Lochmiller & Deerenberg, 2000). Immunologic challenge with keyhole limpet haemocyanin was found to induce a 30% increase in resting metabolic rate of laboratory mice (Demas *et al.*, 1997), while similar increases are observed in humans during sepsis (Kreymann *et al.*, 1993; Carlson

et al., 1997), young lambs injected with bacterial pyrogen (Fewell *et al.*, 1991) and in sparrows (*Passer domesticus*) injected with phytohaemagglutin (Martin *et al.*, 2003).

Costs associated with immune activation may impact upon both survival and reproduction in the field: bumblebees (*Bombus terrestis*) challenged under starvation suffered reduction in survival compared to controls (Moret & Schmid-Hempel, 2000), while breeding female sparrows (*P. domesticus*) injected with lipopolysaccaride suffered higher morality of nestlings when brood sizes were artificially increased (Bonneaud *et al.*, 2003). Similarly, pied flycatchers (*Ficedula hypoleuca*) injected with diptheria-tetnus vaccine had lower reproductive output (in terms of both fledgling quality and number) than controls injected with saline (Ilmonen *et al.*, 2000). In humans, results of a meta-analysis of male infertility patients treated with antibiotics suggest immune responses mounted against infections may damage sperm and result in lower ejaculate quality (Skau & Folstad, 2003).

The mounting of immune responses may also reduce attractiveness to potential mates through changes in expression of secondary sex traits (the immunocompetence handicap; Hamilton & Zuk, 1982; Folstad & Karter, 1992). For example carotenoids, yellow-red pigments required for expression of ornamental traits such as bill colouration which must be obtained from food, also function as antioxidants and immunostimulants (Bendich, 1989; Mortensen *et al.*, 1997; Olson & Owens, 1998). Recent experiments have demonstrated that blackbirds (*Turdus merula*) challenged with sheep erythrocytes exhibited less brightly coloured beaks (Faivre *et al.*, 2003), while zebra finches (*Taeniopygia guttata*) fed carotenoids in drinking water were more attractive to females (Blount *et al.*, 2003). These results suggest a trade-off may exist between using carotenoids as part of an immune response, and advertising to potential mates.

Given that parasite pathology and immune reactions may be costly, the host must therefore balance the detrimental effects of infection with those of mounting an immune response (Behnke *et al.*, 1992; Raberg *et al.*, 1998). This may result in less virulent parasites being tolerated by the host rather than being removed completely. As a consequence of the various costs associated with immunity, hosts may be less able to defend themselves against parasites at energetically stressful times in their life history, particularly during reproduction. For example, male *Drosophilia melanogaster* exposed

to higher numbers of females were found to be less able to respond to bacterial infection (McKean & Nunney, 2001), while artificial increases in brood size led to decreases in antibody production in zebra finches (*Taeniopygia guttata*; Deerenberg *et al.*, 1997). Such decreases in immunocompetence associated with reproduction may increase chances of host mortality through a rise in infection intensity. For example, increasing reproductive effort of female collared flycatchers (*Ficedula albicollis*) has been found to result in increased intensity of *Haemoproteus* sp. infections, which in turn were associated with higher host mortality (Nordling *et al.*, 1998). More dramatically, stress related to intense breeding in male dasyurid marsupials *Antechinus swainsonii* and *Antechinus stuartii* has been found to result in involution of lymphatic tissues, presumably mediated through increased levels of glucocorticoids (Poskitt *et al.*, 1984). Males die shortly after their first breeding season, probably as a consequence of observed exacerbation of latent *Listeria* sp. and *Babesia* sp. infections (Barker *et al.*, 1978; Poskitt *et al.*, 1984).

Animals may also be less able to respond to infections later in life as a consequence of age-related changes in lymphatic tissues, a process described as 'immunosenescence' (Franceschi *et al.*, 2000). Although immunosenescence, and its implications for determining health status in old age, has attracted considerable attention in humans (Franceschi *et al.*, 2000), few authors have attempted to study this process in wild animals. Cichon (2003) found older female collared flycatchers (*F. albicollis*) produced lower humoral responses when challenged with sheep erythrocytes, while Saino (2003) reported an age-related decline in antibody responses in barn swallows (*Hirundo rustica*). Both of these studies measured only one aspect of the immune response, namely levels of serum antibodies. Clearly, more work is needed to understand the processes of immunosenescence and it implications for wild animals.

1.4 Anorexia, helminth parasites and vertebrate life-histories

In addition to damage associated with direct pathology, and the costs associated with mounting immune responses, one of the most important detrimental effects of infection is the associated decrease in host food intake (Lochmiller & Deerenberg, 2000). Loss of appetite and temporary anorexia are characteristic of many bacterial, viral

and parasitic diseases, but the reasons for this response are not well understood. Kyriazakis *et al.* (1998) suggests reduction in food intake might increase the effectiveness of the immune response, or allow hosts to become more selective in their diet to avoid further infection. Anorexia appears particularly paradoxical in response to infection by helminth parasites that impose an increased metabolic and nutritional demand on the host (Kyriazakis *et al.,* 1998), either through their own feeding or reductions in digestive efficiency. For example, in white-footed mice (*Peromyscus leucopus*), infection with *Hymenolepis citelli* led to a 2% reduction in digestive efficiency, possibly as a consequence of nutrient loss to parasitism or damage to the gut wall (Munger & Karasov, 1989), while Bailey (1975) reported 1% of host energy appropriated by cestodes infecting rats. However, Sykes (1994) suggests the 1 or 2% decrease in dry matter digestibility caused by endoparasites may be much less detrimental than the associated 30-60% decrease in host food intake (Poppi *et al.*, 1990; Kyriazakis *et al.*, 1998).

By affecting host nutritional status, helminth parasites may impact upon host traits requiring investment of resources. In particular, several studies have demonstrated reductions in body mass and growth rates associated with helminth infection. Laboratory rats *Rattus norvegicus* infected with *Nippostrongylus brasiliensis* were found to lose weight more quickly than uninfected rats (Crompton *et al.*, 1981), most likely as a consequence of reduced food uptake (Crompton *et al.*, 1981; Ovington, 1985). Captive red grouse hens (*Lagopus scoticus*) infected with the nematode *Trichostrongylus tenuis* have been found to gain less weight than uninfected controls prior to laying, again most like as a result of reduced food intake (Shaw & Moss, 1990). A similar effect has been found in cattle: reduction of trichostrongylid infection in calves was found to result in a 22% increase in weight gain in the first season, and a 50-60% increase in the second (Somers *et al.*, 1987). In humans, increases in appetite and growth rate have been found as a result of treatment against hookworm, *Ascaris lumbricoides* and *Trichuris trichiura* in Indonesian school boys (Hadju *et al.*, 1996), and against treatment for hookworm, *A. lumbricoides* and *T. trichiura* in Kenyan school children (Stephenson *et al.*, 1989; Stephenson *et al.*, 1993a; Stephenson *et al.*, 1993b; Adams *et al.*, 1994).

Helminth parasites may also impact upon vertebrate reproduction. Studies using kestrels (*Falco sparverius*) found infection with *Trichinella pseudospiralis* infection delayed egg laying and number of hatchlings produced (Saumier *et al.*, 1986). Similarly, captive red grouse (*L. scoticus*) infected with *T. tenuis* started to lay later in spring and produced fewer eggs at a slower rate than uninfected hens (Shaw & Moss, 1990). Experiments on lab mice (*Mus musculus*) found mothers infected with *Heligmosomoides polygyrus* produced smaller female pups, although there was no similar effect on male offspring (Kristan, 2002). However, wild mice (*M. musculus*) infected with the same parasite produced more pups, and had slightly larger offspring after control for effects of litter size (Kristan, 2004). This may represent a shift towards current reproduction in infected wild mice, perhaps to allow the host to produce as many offspring as possible before parasitism prevents further reproduction (Kristan, 2004).

Effects of helminth infection on host traits can be most clearly demonstrated by experimental manipulation of parasite burdens. Hosts can be treated with anthelmintics, or artificially infected, and changes in host traits compared between treatment groups. Unfortunately, it is much more difficult to perform such studies in the wild than in captivity: anthelmintic drugs are developed primarily for domesticated animals, and there may be no way of knowing whether a certain treatment protocol will prove effective prior to a study. As a consequence, most studies of effects of helminths on wild hosts are observational or correlational rather than relying on direct manipulation of parasite burdens (Grenfell & Gulland, 1995; Gulland, 1995). Observational studies of parasite pathology are generally carried out through necropsy of recovered carcasses (Gulland, 1995), often when parasite epidemics are implicated in depletion of wild populations (for example, wading birds and waterfowl, Wiese *et al.*, 1977; Spalding *et al.*, 1993; Spalding & Forrester, 1993; Work *et al.*, 2004). While integral to understanding how parasites may kill hosts, and thereby impact upon threatened populations, such studies cannot reveal the more subtle costs of parasitism to infected but apparently 'healthy' hosts.

Correlational studies may go further in investigating relationships between host characteristics and parasite burdens, and reveal interesting epidemiological patterns in the process. By dissecting animals of different ages (as assessed by dry lens weight)

Gregory *et al.* (1992) found a convex relationship between age of wood mice (*Apodemus sylvaticus*) and intensity of *H. polygyrus* infection. The decline in parasite numbers with age in older animals may have several explanations, including parasite-induced host mortality, such that the animals with the most intense infections die and are removed from the sampled population (Anderson & Gordon, 1982). Studies of captive populations of wood mice suggest infection with *H. polygyrus* can reduce host survival (Gregory, 1991). However, convex host age - parasite intensity curves can also be generated by acquired immunity reducing intensities in older animals (Gregory *et al.*, 1990).

Although correlational studies may demonstrate associations between parasite burdens and host characteristics, only manipulative studies can ascertain the causation of such relationships (Lehmann, 1993). The small number of studies which have used a manipulative approach in the field have thus far produced conflicting results. The two key findings so far have been the effect of experimental reduction of nematodes on breeding success and winter survival of red grouse (*L. scoticus*; Hudson, 1986; Hudson *et al.*, 1992), and increased daily survival rate in ewes and male lambs (*O. aries*; Gulland, 1992). However, no effect of anthelmintic treatment was found on number of brushtail possums (*Trichosurus caninus*) giving birth, or survival of young to emergence (Viggers *et al.*, 1998). Similarly, treatment against *Hymenolepis citelli* had no effect on survival or the length of time white-footed mice (*P. leucopus*) remained reproductively active (Munger & Karasov, 1991), despite the 2% drop in dry matter digestibility associated with infection in the laboratory (Munger & Karasov, 1989). No effect of treatment was found on body mass or reproduction of showshoe hares (*Lepus americanus*) in Canada (Murray *et al.*, 1998) or Wisconsin (Bloomer *et al.*, 1995), while release of the nematode *Capillaria hepatica* had a minimal effect on survival and reproduction of wild mouse (*Mus domesticus*) populations in Australia (Singleton & Chambers, 1996).

There may be several reasons why not all studies have found effects of helminth parasites on host traits. Firstly, abundance and pathogenicity of helminths will undoubtedly vary between study systems. However, few studies of wild vertebrates have attempted to investigate parasite-mediated pathology in parallel with experiments

exploring wider effects of infection in the field. Gulland (1992) examined pathological lesions associated with nematode infection (*Ostertagia circumcincta*) at the same time as conducting treatment experiments on Soay sheep (*Ovis aries*). The presence of pathological lesions indicated nematodes contributed to mortality in malnourished hosts, which might explain the increase in daily survival associated with anthelmintic treatment. Effects of parasitism may also be disguised if wild hosts increase food acquisition or use bodily reserves to compensate for reductions in their nutritional status (Murray *et al.*, 1998). Experiments that investigate effects of helminths on both host nutritional status and life-history traits, such as those conducted on white-footed mice outlined above (Munger & Karasov, 1989; Munger & Karasov, 1991), may be particularly useful in understanding the full effects of parasites on wild animals.

1.5 Aim of thesis

The aim of this thesis is to explore several aspects of host-parasite interactions using a variety of specialist techniques. It is hoped that this approach will provide a broader understanding of the relationships between parasite and host than studies confined to any one particular discipline. The majority of the research is conducted using one host organism, the European common shrew *Sorex araneus*.

1.6 Energetic strategies of the Soricidae

All shrews belong to the family Soricidae (order: Insectivora) comprising two sub-families: the Soricinae ('red-toothed' shrews, so called because of reddish-brown iron deposits on their teeth, Churchfield, 1990) and the Crocidurinae (or 'white-toothed shrews'). Shrews occur over a range of habitats world wide (Innes, 1994), and feed mainly on small invertebrates (Aitchison, 1987). They are characterized by their long snouts and short legs, otherwise appearing superficially similar to small mice (Churchfield, 1990). Their small size has important consequences for their physiology: small animals have fast metabolic rates (French *et al.*, 1976; Genoud, 1988), yet store less energy in the form of bodily reserves (Calder, 1984; Genoud, 1988). Shrews therefore have a low endurance to starvation (Lindstedt & Boyce, 1985). The two sub-families differ in the relationships between their body sizes and metabolic rates: the

Crocidurinae exhibit basal metabolic rates similar to or slightly higher than predicted from their body weights, while those of the Soricinae are much higher (Genoud, 1988). The fast metabolic rate of Soricinae shrews may be an adaptation to the cold or seasonal climates in which they live, permitting homeostasis during exposure to low temperatures, while the Crocidurinae are generally found in warmer climates, where faster metabolic rates and higher body temperatures might result in overheating in hot weather (Genoud, 1988). The faster metabolisms of the Sorcinae appears to permit a higher rate of biosynthesis: for example, shrews of the genus *Crocidura* (members of the Crocidurinae) usually have less than five offspring per litter, compared to litter sizes greater than five produced by *Sorex* shrews (Innes, 1994). A faster metabolic rate also requires a higher energy budget, which may be difficult to balance during winter when temperatures are lowest and invertebrate prey is scarce. However, if these seasonal variations are predictable, Soricinae shrews can reduce energy expenditure through a reduction in body mass (Dehnel's phenomenon, Pucek, 1970).

1.7 The European common shrew, *Sorex araneus*

The three species of shrew found in mainland Britain, *Sorex araneus*, or common shrew, *Sorex minutus* (the 'pygmy shrew') and *Neomys fodiens* ('water shrew') are all members of the Soricinae. *N. fodiens* is most often encountered in aquatic habitats, while the common and pygmy shrews often occur sympatrically in more terrestrial environments (Churchfield, 1984), with the common shrew generally the more abundant (Michielsen, 1966; Butterfield *et al.*, 1981) and arguably the most well studied. The energetic physiology of *S. araneus* is typical of that of the Soricinae, with oxygen consumption almost 270% higher than would be predicted from body mass (8.3 ± 1.6ml $O_2 \times g^{-1} \times h^{-1}$ at 20°C; Nagel, 1994). Common shrews also store very little fat, less than 10% of their body weight (Churchfield, 1981). As a consequence of their metabolic rate and limited fat stores, common shrews must forage constantly, being neither nocturnal nor diurnal but alternating between roughly hour-long periods of foraging and sleeping (Saarikko & Hanski, 1990). Foraging takes place either on the soil surface or just beneath it (Churchfield, 1980b) with earthworms, beetles, insect larvae and gastropods comprising the majority of the common shrew's invertebrate diet (Pernetta, 1976;

Churchfield, 1994). Studies in captivity suggest *S. araneus* employs random search patterns, rather than olfactory and tactile senses, to locate prey beneath the soil surface (Churchfield, 1980b; Pierce, 1987).

The life cycle of *S. araneus* takes fourteen to sixteen months to complete with only one breeding season in the second year of life (Shillito, 1963; Michielsen, 1966; Pernetta, 1977). The first young are born in mid-May, weighing around 0.4g (Middleton, 1931; Brambell, 1935; Michielsen, 1966; Innes, 1994). Shrews are weaned at around 23d when they disperse from the nest weighing approximately 7.5g (Innes, 1994). Capture-mark-recapture studies suggest the period of dispersal is one of high mortality. Michielsen (1966) reported 42% morality of individuals born in May and June by September, while Churchfield (1980a) recorded 50% mortality within two months of birth. Survival appears to be dependent on the establishment of territories after leaving the nest (Michielsen, 1966; Pernetta, 1977), with a minimum size of approximately 90 m^2 calculated from field experiments (Michielsen, 1991). Once territories have been established, mortality rates level off: by reserving an area over which to forage, shrews may be guaranteeing enough food to survive the winter as sub-adults (Michielsen, 1966).

Common shrews do not hibernate, instead conserving energy over winter by reducing the energy required for maintenance via a decrease in body mass of around 10% (Shillito, 1963; Michielsen, 1966; Pucek, 1970). Both sexes increase in weight with the onset of sexual maturation, occurring earlier in males than females, usually during March or April (Brambell, 1935; Shillito, 1963; Skarén, 1973; Pernetta, 1977). Mature males also exhibit fully developed flank glands and a visible increase in testis mass (Middleton, 1931; Michielsen, 1966; Searle, 1985). Females initially increase in weight at a slower rate than males, but become larger upon reaching maturity at the end of April (Skarén, 1973).

The territorial system of *S. araneus* changes with the onset of breeding. Most females maintain their original territories, the area of which may alter over the breeding season (Shillito, 1963; Michielsen, 1966; Buckner, 1969). Mature males adapt two distinct strategies (Shillito, 1963; Michielsen, 1966; Buckner, 1969; Stockley *et al.*, 1994). Those which mature relatively early establish large ranges, overlapping those of

several females with which they mate. Males maturing later retain the ranges occupied as sub-adults, making long distance visits to more widely dispersed females (Stockley *et al.*, 1994). Genetic analysis reveals a high level of multiple paternity in common shrew litters (Searle, 1990; Tegelström *et al.*, 1991; Stockley *et al.*, 1993) consistent with females mating with several males during their short (two hour) period of oestrus (Dehnel, 1952; Stockley *et al.*, 1993; Stockley *et al.*, 1996). Paternity analysis also suggests the 'overlapping' male strategy to be the more successful, as judged by mean number of offspring fathered (Stockley *et al.*, 1994).

Gestation lasts 20d in common shrews (Innes, 1994), with females having up to three litters and males playing no part in raising offspring (Middleton, 1931; Brambell, 1935). Average litter size is 6.7 offspring at birth (Innes, 1994), although captive studies suggest fewer survive to weaning (mean number 5.5 offspring; Mercer & Searle, 1994). Although a short gestation period allows females to avoid carrying a large litter into late gestation, an early parturition leads to an increase in energy requirements, as lactation is more energetically expensive than pregnancy in common shrews (Genoud & Vogel, 1990; Stockley & Macdonald, 1998). Almost all adults die prior to winter, such that there is little overlap between generations (Adams, 1910; Michielsen, 1966; Buckner, 1969; Pernetta, 1977; Churchfield, 1980a)

1.8 Parasites of common shrews

Common shrews are host to a wide variety of both micro and macroparasites, of which helminths have received the most attention. In the UK, common shrews have been found to harbour more species of helminth than rodents sharing the same habitat (Sharpe, 1964; Lewis, 1968; Roots *et al.*, 1994). This is undoubtedly due to their insectivorous nature (Sharpe, 1964; Lewis, 1968): intermediate stages of several of these parasites have been found in invertebrates preyed upon by *S. araneus* (Kisielewska, 1963; Roots *et al.*, 1994). In the most recent comprehensive survey (Roots, 1992), ten nematode species, ten cestode species, three digenean species and one acanthocephalan species were reported from common shrews in the UK. The cestodes comprised two members of the family Dilepididae, one of which is found in the gut (*Choanotaenia crassiscolex*), the other in the bile duct (*Choanotaenia hepatica*), and eight members of

the Hymenolepididae, all *Hymenolepis* species inhabiting the gut (Roots, 1992; Roots *et al.*, 1994; **Table 1.1**).

Table 1.1 Recent reports of acanthocephalan and cestode parasites in UK *Sorex araneus*

Parasite species	**Location within *S. araneus***
Acanthocephalans	
Centrorhynchus sp.[1,2]	Encysted in body cavity
Cestodes	
Choanotaenia crassicolex[2,3]	Small intestine
Choanotaenia hepatica[2,3]	Bile duct
Hymenolepis diaphana[2,3]	Intestine
Hymenolepis furcata[2,3]	Intestine
Hymenolepis infirma[2,3]	Intestine
Hymenolepis jacutensis[2,3]	Intestine
Hymenolepis prolifer[2,3]	Intestine
Hymenolepis schaldybini[2,3]	Intestine
Hymenolepis scutigera[2,3]	Intestine
Hymenolepis singularis[2,3]	Intestine

1. Possibly *Centrorhynchus* (= *Gordiorhynchus*) *aluconis* (Ewald *et al.*, 1991; Roots *et al.*, 1994)

2. Roots, 1992; 3. Roots *et al.*, 1994

Adult nematodes were recovered from several organs, including the oesophagus, stomach, gut, bladder and liver (Roots, 1992, **Table 1.2**). L4 larvae of the nematode *Porrocaecum* sp. were found encapsulated under the skin and within the body cavity (Roots, 1992, **Table 1.2**). This parasite may be *Porrocaecum spirale,* which occurs as an adult in tawny owls (*Strix aluco*, Roots, 1992). If this is the case, the parasite presumably uses *S. araneus* as a paratenic host. Juvenile stages of the acanthocephalan *Centrorhynchus* sp. also occur encysted in the body cavity of *Sorex araneus* (Ewald *et al.*, 1991, **Table 1.1**). Lewis (1968) listed *Centrorhynchus aluconis* as a parasite from shrews in Wales, without evidence as to how the identification was made. Adult *C.*

aluconis are found in tawny owls (*S. aluco*) across the UK, and it has been suggested that shrews serve as paratenic hosts for this parasite, being transmitted to the final host via predation (Ewald *et al.*, 1991). How the parasite infects *S. araneus* is unknown, but presumably it is carried within an intermediate invertebrate host.

Table 1.2 Recent reports of digenean and nematode parasites in UK *Sorex araneus* (Roots, 1992)

Parasite species	**Location within *S. araneus***
Digeneans	
Brachylaemus fulvus	Oesophagus and stomach
Dicrocoelium soricis	Gall bladder and bile duct
Opisthoglyphe sobelevi	Intestine
Nematodes	
Eucoleus kutori	Stomach lumen
Eucoleus oesophagicola	Mucous membrane of oesophagus
Liniscus incrassatus	Urinary bladder
Longistriata didas	Intestine
Longistriata depressa	Intestine
Longistriata thomasi	Intestine
Longistriata trus	Intestine
Parastrongyloides winchesi	Intestinal mucosa
Porrocaecum sp.[1]	Encapulated in intrascapular adipose tissue and body cavity
Stammerinema soricis	Stomach wall

1. Possibly *Porracaecum spirale* (Roots, 1992)

Several species of flea are found on UK shrews. *Doratopsylla dasycnema* and *Palaeopsylla soricis* are the most common species found on *S. araneus*, but *Ctenophthalmus nobilis*, usually associated with mice and voles, and the mole flea *Hystrichopsylla talpae* have also been reported (Smit, 1957; Churchfield, 1990). Two species of tick occur on *S. araneus* in the UK; *Ixodes trianguliceps* and *Ixodes ricinus*

(Randolph, 1975; Churchfield, 1990), along with three reported species of mite: *Euryparasitus emarginatus*, *Haemogamasus horridus* and *Labidophorus soricis* (Churchfield, 1990).

Several authors have reported finding microparasites in common shrews. Studies from Finland have found a high prevalence of *Pneumocystis carinii* in *S. araneus* (70% infected; Laakkonen *et al.*, 1993), and have also recovered unidentified protozoan cysts from the kidneys (Soveri *et al.*, 1994). Coccidia have also been reported from *S. araneus* in Europe, normally through examination of oocysts in faeces (Levine & Ivens, 1979; Hertel & Duszynski, 1987). As a consequence, almost nothing is known about the life cycles of these parasites within the host.

Blood parasites of *S. araneus* have received less attention, probably because of difficulties in capturing live animals from which to take samples. *Anaplasma phagocytophilum* has been reported from one *S. araneus* from Switzerland (Liz *et al.*, 2000), and *Grahamella* spp. (since reclassified and included in the *Bartonella*, Birtles *et al.*, 1995) has been described from *S. araneus* in several European countries (Hoyte, 1956; Laakkonen *et al.*, 1998; Holmberg *et al.*, 2003). Early reports also suggest shrews may carry *Babesia* sp. and tick-borne encephalitis (Stunkard *et al.*, 1975).

Despite the large number of parasite species associated with *S. araneus*, very little is known about the effects these parasites have upon their host. Given the high metabolic rate and limited energy reserves of common shrews, parasites invoking anorexia or reductions in digestive efficiency or nutrient absorption may be particularly detrimental to this host species (Haukisalmi *et al.*, 1994). Parasitism has even been suggested as a possible cause of the annual mortality of common shrews after the breeding season (Michielsen, 1966; Buckner, 1969; Pernetta, 1977). In addition, very little work has been done to examine the extent of parasite mediated-pathology in common shrews. Vaucher (1971) provided pictures of cestodes attached to the gut wall, and suggested they did little damage to the surface of the intestine. Soveri *et al.* (1994) described histopathological changes in the liver, intestines, lungs, heart and brain, but did not provide photographs.

1.9 Thesis overview

This thesis examines several aspects of host-parasite interactions in the common shrew. The first data chapter (**Chapter 2**) uses bacteriological and molecular techniques to determine the presence or absence of blood parasites in wild-caught common shrews. DNA sequencing is then employed to compare recovered parasite gene sequences with those previously reported from shrews and other species, including rodents sharing the same habitat. This may reveal the extent to which common shrew haemoparasites have been constrained by the phylogeny of their host, or can be transmitted to distantly related species sharing the same environment.

Chapter 3 investigates the extent of parasite-mediated pathology in shrews using histological techniques. This is followed by an immunohistological study of the lymphatic tissues of shrews of different ages in **Chapter 4**. Together, these chapters explore the degree of tissue damage caused by parasites and associated host immune reactions, and the extent to which shrews undergo changes in their capacity to mount immune responses with age. The following two chapters consider the effects of helminth parasitism on shrew behaviour and life-history traits. While most species will undergo temporary anorexia whilst fighting parasitic infections, shrews may have to increase foraging effort in order to replace resources lost as a consequence of infection or face starvation. This hypothesis is tested by treating sub-adult shrews with anthelmintic drugs and comparing their foraging activity to those receiving a water control (**Chapter 5**). A similar protocol is used to investigate possible effects of helminth parasitism on shrew life-history traits in the field (**Chapter 6**). Given the resource limited nature of their physiology, costs of helminth parasitism may be more apparent in common shrews than in other host species.

The final data chapter (**Chapter 7**) extends the study of host-parasite relationships across the family Soricidae. A comparative analysis is used to investigate whether species richness of coccidian parasite faunas is related to host body mass and other life-history traits. A general discussion of the preceding six chapters is presented in **Chapter 8.**

Chapter 2. Haemoparasites of *Sorex araneus*

2.1 Introduction

Infection by haemoparasites can impact upon several important aspects of host biology. Even in the absence of obvious pathology, blood parasites can influence host behaviour (Moore, 2002), reproductive success (Feore *et al.*, 1997; Merino *et al.*, 2000), and population dynamics, the latter a product of decreased host survivorship and altered patterns of fecundity (Grenfell & Gulland, 1995). In the UK, rodent populations have received particular attention as model hosts for the study of wildlife diseases. Rodents harbour a number of important pathogens, many of which can be transmitted to humans or livestock (Webster & Macdonald, 1995; Ogden *et al.*, 1998; Kerkhoff *et al.*, 1999; Homer *et al.*, 2000; Wolfs *et al.*, 2002). Several studies have examined the diversity of UK rodent haemoparasites (Healing, 1981; Turner, 1986; Birtles *et al.*, 1994; Noyes *et al.*, 2002) and are continuing to investigate their ecology, transmission and effects upon host populations (Feore *et al.*, 1997; Begon *et al.*, 1999; Hazel *et al.*, 2000; Birtles *et al.*, 2001; Bown *et al.*, 2003). In contrast, blood-borne pathogens of shrews have been studied only sporadically and in much lesser detail (but see Hoyte, 1956; Stunkard *et al.*, 1975; Birtles *et al.*, 1994; Laakkonen *et al.*, 1998; Holmberg *et al.*, 2003). In part, this may be due to the difficulty in catching and adequately provisioning for live shrews in the field in order to obtain fresh blood samples for analysis.

Common shrews share the same microhabitat as woodland rodents and are believed to come into contact with several rodent species by travelling through their tunnels (Churchfield, 1980b). In addition, shrews and rodents share ectoparasites which have been implicated as vectors for blood-borne pathogens, in particular ticks (Randolph, 1975) and fleas (Smit, 1957). Transmission of haemoparasites might therefore occur between common shrews and rodents, such that *Sorex araneus* may represent an important but largely neglected element of rodent - disease ecology. A detailed study of common shrew haemoparasites could aid in our understanding of the rodent host-pathogen model, and highlight the extent to which haemoparasites are constrained by the phylogeny of their host (in which case, shrews and rodents will have

evolved distinct blood parasite faunas) or can be transmitted and maintained by distantly related species.

This preliminary investigation focuses on three potential shrew parasites more commonly associated with rodents, namely *Anaplasma phagocytophilum,* trypanosomes and *Bartonella* species. *A. phagocytophilum* (formerly *Ehrlichia phagocytophilum, Ehrlichia equi* and the agent of human granulocytic ehrlichiosis [HGE]; Dumler *et al.*, 2001) is a tick-transmitted, obligate intracellular bacterium associated with both human and veterinary diseases, including tick-borne fever in ruminants (Massung *et al.*, 1998; Ogden *et al.*, 1998). *A. phagocytophilum* has been reported from a wide range of vertebrate hosts, including rodents (Ogden *et al.*, 1998) and one *S. araneus* from Switzerland (Liz *et al.*, 2000). The catholic feeding behaviour of its tick vectors, *Ixodes ricinus*, and to a lesser extent *Ixodes trianguliceps*, allows *A. phagocytophilum* to be spread between host species, with the potential for zoonotic transmission from wild animal reservoirs to domesticated animals and humans (Ogden *et al.*, 1998).

The genus *Trypanosoma* comprises unicellular flagellated protozoa parasitizing all classes of vertebrates worldwide (Haag *et al.*, 1998). Host-to-host transmission is carried out by blood-feeding arthropod or leech vectors, entering the vertebrate's blood stream via the vector's saliva during feeding (salivarian transmission) or by ingestion of the vector or its faeces (stercorarian transmission; Stevens *et al.*, 1999a). Trypanosomes have been reported from shrews in Canada and North America (*Sorex vagrans, Sorex cinereus, Sorex fumeus* and *Blarina brevicauda*; Laakkonen *et al.*, 1998) and from former Czechoslovakia (*Sorex alpinus* and *Crocidura suaveolens*; Laakkonen *et al.*, 1998). Trypanosomes from *S. vagrans*, *S. alpinus* and *B. brevicauda* have all been named as *Trypanosoma soricis* (Laakkonen *et al.*, 1998), although no phylogenetic studies have been conducted to investigate whether these parasites are indeed the same species, whether they are unique to shrews, or if shrews are simply hosts to trypanosomes reported from other small mammals.

Members of the genus *Bartonella* are Gram-negative cocco-bacillary proteobacteria, obligate parasites inhabiting the erythrocytes of a wide range of mammalian hosts (Birtles *et al.*, 2001). Several species are emerging as agents of human disease (Daly *et al.*, 1993; Ellis *et al.*, 1999; Kerkhoff *et al.*, 1999; Welch *et al.*, 1999;

Roux *et al.*, 2000; Kosoy *et al.*, 2003; Fabbi *et al.*, 2004). *Grahamella* (since reclassified and included in the *Bartonella*, Birtles *et al.*, 1995) has been described from *S. araneus* in several European countries (Hoyte, 1956; Laakkonen *et al.*, 1998; Holmberg *et al.*, 2003) and from *S. vagrans, S. cinereus*, *S. fumeus* and *B. brevicauda* from North America (Laakkonen *et al.*, 1998). To my knowledge, no study has isolated *Bartonella* from *S. araneus* in the UK, although it has been cultured from the water shrew *Neomys fodiens* (Birtles *et al.*, 1994).

Here, molecular and bacterial culturing techniques are used to determine the presence or absence of these haemoparasites in a sample of wild common shrews. To aid our understanding of the evolutionary relationships between haemoparasites of *S. araneus* and those of other host species, recovered parasite gene sequences are compared with those reported from other hosts and phylogenetic trees are constructed. In addition, effects of host sex and age on parasite prevalence are explored as a first step to investigating the transmission and ecology of haemoparasites in *S. araneus*.

2.2 Materials and Methods

2.2.1 Host sampling

Wild shrews were captured from areas of grassy wasteland in the vicinity of Leahurst field station, Cheshire, England (OS grid reference 331558, 377486) between February 2001 and July 2003. Shrews were caught using Longworth traps baited with fly pupae, previously frozen to prevent eclosion. Traps in which shrews (or other small mammals) had been caught were washed thoroughly prior to being reused.

At culling, cardiac blood samples were taken from 76 animals using a sterile syringe and transferred to an Eppendorf tube. Samples were spun in a centrifuge for 10min at 10000g to separate serum from pellet and the serum removed using a micropipette. Serum and pellet were frozen separately at -18°C until use.

2.2.2 Extraction of DNA for PCR.

DNA was extracted from pellets by alkaline digest prior to detection of trypanosomes and *A. phagocytophilum* by PCR (Bown *et al.*, 2003). Each pellet was dissolved in 500µl 1.25% NH_3 solution, transferred to a sure-lock Eppendorf and spun

briefly at high speed in a centrifuge. Eppendorfs were heated with lids closed at 100°C for 20min in a heating block, centrifuged again and returned to the heating block with lids open for approximately 25min, or until half the liquid in each tube had evaporated. The remaining solution was diluted 1:5 and used as template in subsequent PCR reactions.

2.2.3 Detection and characterization of *A. phagocytophilum* by molecular methods

Infection with *A. phagocytophilum* was detected using a semi-nested PCR targeting an 16S rRNA encoding gene fragment (Massung *et al.*, 1998). First round reactions comprised 2µl 2x PCR master mix (Abgene, Surrey) containing 1.5mmol $MgCl_2$, 20pmol primer ge3a (5' CACATGCAAGTCGAACGGATTATTC), 20pmol primer ge10r (5' TTCCGTTAAGAAGGATCTAATCTCC) and 5µl of DNA template made up to 50µl in total with sterile water. Second stage reactions comprised 4µl PCR Reddymix (Abgene, Surrey) containing 1.5mmol $MgCl_2$, 20pmol primer ge2 (5' GGCAGTATTAAAAGCAGCTCCAGG), 20pmol primer ge9f (5' AACGGATTATTCTTTATAGCTTGCT) and 1µl of first round product made up to 50µl in total with sterile water. Tubes containing distilled water in place of extracted DNA were used as negative controls throughout to warn of potential contamination between samples. Known positive controls were also included in each run as confirmation that reactions had been performed correctly. Cycling conditions for both rounds were as follows:

1. Initial denaturation at 95°C (2min)
2. Thirty cycles of denaturation at 94°C (30s), annealing at 55°C (30s) and extension at 72°C (1min)
3. Extension at 72°C (5min)

Second stage products were visualized on 1% agarose gels containing ethidium bromide and photographed under UV light. Amplicons from PCR positive second stage reactions were cleaned using a Nucleospin kit (Abgene, Surrey) and sent for automated sequencing using the same primers as for the second round of amplification.

2.2.4 Detection and characterization of trypanosomes by molecular methods

Determination of presence/absence of trypanosome DNA was carried out using a semi-nested PCR targeting an 18S rRNA encoding gene fragment (Noyes *et al.*, 2002). First and second stage reactions were prepared as for *A. phagocytophilum* (**Section 2.2.3**) but for using the following trypanosome-specific primers. First round: TRY927F (5'GAAACAAGAAACACGGGAG) and TRY927R
(5' CTACTGGGCAGCTTGGA), second round: SSU561F
(5' TGGGATAACAAAGGAGCA) and SSU561R
(5' CTGAGACTGTAACCTCAAAGC). PCR was carried out under cycling conditions outlined in **Section 2.2.3** with appropriate positive and negative controls and second round products visualized on agarose gels and photographed as described above. PCR products from three positive samples were cleaned using the Nucleospin kit and sent for automated sequencing using the same primers as for the second round of amplification.

2.2.5 Growth of *Bartonella* spp. and characterization of isolates by molecular methods

Five microlitres of each blood pellet was streaked onto 10% horse blood agar plates and incubated at 35°C in an aerobic atmosphere containing 5% carbon dioxide. Plates were grown for up to one month and colonies passaged onto new plates three to four times before harvesting. Crude DNA extracts from colonies were used as templates for two separate PCR reactions. The first targeted a citrate synthase gene (*gltA*) fragment using primers CS443f (5' GCTATGTCTGCATTCTATCA) and CS1137r (5' AATGCAAAAAGAACAGTAAACA) (Birtles & Raoult, 1996). Each reaction contained 15µl 2x master mix, 20pmol of each primer and 1µl colony solution made up to 50µl with sterile water. Appropriate positive and negative samples were included for quality control, with cycling conditions and visualization of products as described for *A. phagocytophilum* in **Section 2.2.3.** DNA from five positive samples was cleaned using the Nucleospin kit and sent for automated sequencing using the same primers used for amplification.

The second PCR targeted the 16S-23S rRNA intergenic spacer region (IGSR; Birtles *et al.*, 2000). This region is too variable to infer relationships between *Baronella* species, but is useful in assessing diversity within species (Birtles *et al.*, 2000). Primers

used were big-F (5' TTGATAAGCGTGAGGTC) and big-R (5' TTGATAAGCGTGAGGTC), with reactions carried out as described for *gltA* with appropriate positive and negative controls. Amplicons were resolved on 3% agarose gels under UV light. Five positive samples were cleaned and sent for sequencing using the primers described above.

2.2.6 Data analysis

2.2.6.1 Parasite prevalence

Differences in parasite prevalence between males and females or sub-adult and adult hosts may reveal possible biases in the mechanics of transmission (Holmberg *et al.*, 2003). Here, generalized linear models with binomial errors and logit link functions (Crawley, 1993) were used to investigate potential effects of host sex, age class and year of collection on the prevalence of shrew haemoparasites. A model was constructed for each parasite genus investigated, with parasite prevalence entered as the dependent variable. Each model contained the following independent variables: host sex (two-level categorical variable, male or female), host age (two-level categorical variable, sub-adult or adult) and year of collection (three-level categorical variable, 2001, 2002 or 2003). Year of collection was included to assess (and control for) potential differences in parasite prevalence between years. To arrive at a minimal model, terms were removed from the maximal model by stepwise deletion, and their significance assessed by comparing the residual deviance of models with and without those terms by χ^2 tests (Wilson & Grenfell, 1997a and erratum Wilson & Grenfell, 1997b) in Genstat 5 (Genstat 5 Committee, 1987). As a final check, terms not included in the minimal model were added and deleted from it individually, and changes in deviance again assessed by χ^2 tests.

2.2.6.2 Analysis of A. phagocytophilum sequence data

A complete 16S rRNA encoding gene fragment sequence recovered from the one shrew found to be PCR positive was assembled by first aligning sequences generated by both primers using Align Plus version 4 (Sci Ed Software, North Carolina, USA), and then deleting primer sequences and ambiguous base pairs. The complete sequence was then compared with other published *Anaplasma* species 16S rRNA encoding gene fragment sequences using version 1.81 of the Clustal X multiple alignment program (Thompson *et al.*, 1997).

2.2.6.3 Analysis of trypanosome sequence data and construction of phylogenetic trees

Complete trypanosome 18S rRNA gene encoding fragment sequences recovered from each shrew were assembled using the same methodology as described above (**Section 2.2.6.2**). Recovered sequences were compared with each other, and with trypanosome 18S rRNA encoding gene fragment sequences published on Genbank (**Table 2.1**) using Clustal X. Where necessary, gaps in the alignment were removed, and 100 bootstrap samples created from the alignment using Seqboot in Phylip version 3.5c (Felsenstein, 1989). Phylogenetic trees were then created by neighbour joining, maximum parsimony and maximum likelihood methods, all using the Phylip suite of programs (DNAdist and neighbor, DNApars and DNAml respectively). Consensus trees for each phylogeny were produced using Consense in Phylip and displayed using Treeview (Page, 1996).

2.2.6.4 Analysis of Bartonella sp. sequence data and construction of phylogenetic trees

Complete *Bartonella* sp. 16S-23S rRNA IGSRs recovered from each shrew were assembled and compared using the same methodology as described above (**Section 2.2.6.3**) The same protocol was used to compare complete sequences of recovered *gltA* fragments, and to create phylogenies based on an alignment of these sequences and those from other recognized *Bartonella* species published on Genbank (**Table 2.2**).

Table 2.1 Species used to construct phylogenetic trees for *Trypanosoma* species with hosts and Genbank accession numbers

Protozoan species	Host species	Accession No.
Bodo caudatus[†]	Free living[1]	X53910[1]
Trypanoplasma borreli[†]	Leech[2]	L14840[2]
Trypanosoma avium	*Aquila pomarina*[3]	AF416559[3]
Trypanosoma bennetti	*Falco sparverius*[4]	AJ223562[4]
Trypanosoma brucei rhodesiense	*Homo sapiens*[5]	AJ009142[5]
Trypanosoma cruzi	*Homo sapiens*[5]	AJ009147[5]
Trypanosoma dionisii	*Pipistrellus pipistrellus*[5]	AJ009151[5]
Trypanosoma evotomys	*Clethrionomys glareolus*[6]	AY043356[6]
Trypanosoma grayi	*Glossina gambiensis*[4]	AJ223565[4]
Trypanosoma grosi	*Apodemus sylvaticus*[6]	AY043355[6]
Trypanosoma lewisi	*Rattus norvegicus*[4]	AJ223566[4]
Trypanosoma mega	*Bufo regularis*[5]	AJ009157[5]
Trypanosoma microti	*Microtus agrestis*[5]	AJ009158[5]
Trypanosoma musculi	*Mus musculus*[4]	AJ223568[4]
Trypanosoma rangeli	*Rhodnius prolixus*[7]	AJ012414[7]
Trypanosoma sp. WM2	*Apodemus sylvaticus*[6]	AY043353[6]
Trypanosoma sp. H25	*Macropus giganteus*[5]	AJ009168[5]
Trypanosoma theileri	*Bos taurus*[5]	AJ009163[5]

1. Fernandes *et al.*, 1993; 2. Maslov *et al.*, 1994; 3. Votypka *et al.*, 2002;

4. Haag *et al.*, 1998; 5. Stevens & Gibson, 1999; 6. Noyes *et al.*, 2002;

7. Stevens *et al.*, 1999b

[†]Included as outgroup for tree rooting

Table 2.2 *Bartonella* species used to construct phylogenetic trees with hosts and Genbank accession numbers

***Bartonella* species**	**Host species**	**Accession No.**
Bartonella alsatica	*Oryctalagus cuniculus*[1]	AF204273[1]
Bartonella bacilliformis	*Homo sapiens*[2]	'KC583'†
Bartonella birtlesii	Woodland rodents[3]	AF204272[3]
Bartonella bovis	*Bos Taurus*[4]	AF293394[4]
Bartonella capreoli	*Capreolus capreolus*[4]	AF293392[4]
Bartonella chomelii	*B. taurus*[5]	AY254308[5]
Bartonella clarridgeiae	*Felis domesticus*[6]	U84386[6]
Bartonella doshiae	Woodland rodents[7]	Z70017[8]
Bartonella elizabethae	*Rattus* spp.[9]/*H. sapiens*[10]	Z70009[8]
Bartonella grahamii	Woodland rodents[7]	Z70016[8]
Bartonella henselae	*Felis domesticus /H.sapiens*[6]	L38987[11]
Bartonella koehlerae	*F. domesticus*[12]	AF176091[12]
Bartonella quintana	*H. sapiens*[13]	Z70014[8]
Bartonella schoenbuchensis	*C. capreolus* [14]	AJ278183[14]
Bartonella taylorii	Woodland rodents[7]	Z70013[8]
Bartonella tribocorum	*Rattus norvegicus*[15]	AJ005494[15]
B. vinsonii arupensis	*rodents/H.sapiens*[16]	AF214557[17]
B. vinsonii berkhoffii	*Canis domesticus*[18]/*H.sapiens*[19]	AF143445[19]
Bartonella vinsonii vinsonii	*Microtus pennsylvanicus* [20]	Z70015[8]
Bartonella sp. SA192UP	*Sorex araneus*[21]	AF391790[21]

1. Heller *et al.*, 1999; 2.Weinman, 1968; 3. Bermond *et al.*, 2000;

4. Bermond *et al.*, 2002; 5. Maillard *et al.*, 2004; 6. Clarridge *et al.*, 1995;

7. Birtles *et al.*, 1995; 8. Birtles & Raoult, 1996; 9. Ellis *et al.*, 1999; 10. Daly *et al.*, 1993; 11. Norman *et al.*, 1995; 12. Droz *et al.*, 1999; 13. Vinson & Fuller, 1961; 14. Dehio *et al.*, 2001; 15. Heller *et al.*, 1998; 16. Welch *et al.*, 1999; 17. Houpikian *et al.*, 2001; 18 Kordick *et al.*, 1996; 19. Roux *et al.*, 2000; 20. Baker, 1946; 21. Holmberg *et al.*, 2003.

† Provided by R. Birtles

2.3 Results

2.3.1 *A. phagocytophilum*

One of 76 shrews was found to be PCR positive for *A. phagocytophilum* (**Table 2.3**). This individual was a sub-adult female caught in scrubland at the Leahurst field station, Cheshire in July 2001, and was carrying eight *I. trianguliceps* tick nymphs at dissection. As only one individual was found to be infected, further analysis of effects of host age and sex on parasite prevalence could not be carried out.

The sequence of the recovered PCR product (550bp, **Appendix A**) was found to be identical to a previously elucidated 16S rRNA gene encoding fragment sequence of *A. phagocytophilum* isolated from a bank vole (*C. glareolus*) in Northwest England (Bown *et al.*, 2003; Genbank accession number, AY082656) which in turn differed by only one base pair to *A. phagocytophilum* sequences isolated worldwide (e.g. AF241532, isolated from a llama (*Lama glama*) in California, Barlough *et al.*, 1997).

Table 2.3 Number of common shrews (both sexes, two age classes) PCR positive for three haemoparasites

Host Sex	Host Age	Number of hosts	Number of hosts PCR positive for: *A. Phagocytophilum*	Trypanosomes	*Bartonella sp.*
Male	Sub-adult	12	0	1	4
	Adult	25	0	3	4
Male total		37	0	4	8
Female	Sub-adult	21	1	1	2
	Adult	18	0	4	1
Female total		39	1	5	3
Combined total		76	1	9	11

2.3.2 Trypanosomes

Trypanosome DNA was found in 9/76 shrew blood samples tested (11.8%, **Table 2.3**). No significant effect of host age or host sex was found on trypanosome prevalence (**Table 2.4**). However, there was a suggestion of an effect of year of collection, although this was not significant at the $P<0.05$ level (**Table 2.4**).

Each of three 18S rRNA products successfully sequenced were identical over 503bp (**Appendix A**), and shared the greatest similarity with *T. lewisi* (Genbank assession number: AJ223566, 95% similarity). Phylogenies constructed based on a multiple alignment of sequences 499bp in length after removal of gaps had variable bootstrap support (10-100%; **Figures 2.1 – 2.3**) and shared several key features. *B. caudatus* and *T. borrelli* grouped together forming an outgroup, as has been shown by previous authors (Maslov *et al.*, 1994; Stevens & Gibson, 1999). *T. brucei* and *T. mega* appeared on two separate branches of the tree, representing the '*T. brucei*' and 'aquatic' trypanosome clades put forward by Stevens and Gibson (1999). *T. cruzi, T. dionisii* and *T. rangeli* grouped together with *T. avium,* forming a '*T. cruzi*' *clade* (Stevens *et al.*, 1999a). In all three trees, trypanosomes from rodents grouped together, and the sequence isolated here from *S. araneus* is closely to, but separate from, this rodent cluster. The branch separating the shrew sequence from the rodent clade had high bootstrap support, between 92% and 100% (**Figures 2.1 – 2.3**).

Table 2.4 Effects of host age, host sex and year of collection on trypanosome prevalence, as assessed by using a generalized linear model with binomial error distribution and logit link function, N = 76

Independent variable	df	Change in residual deviance[1]	P
Year of collection	2	5.30	<0.08
Host sex	1	0.07	NS
Host age	1	2.00	NS
Host age by sex interaction	1	0.61[2]	NS

1. When added to and deleted from minimal model

2. Added and removed from a model containing host age and host sex

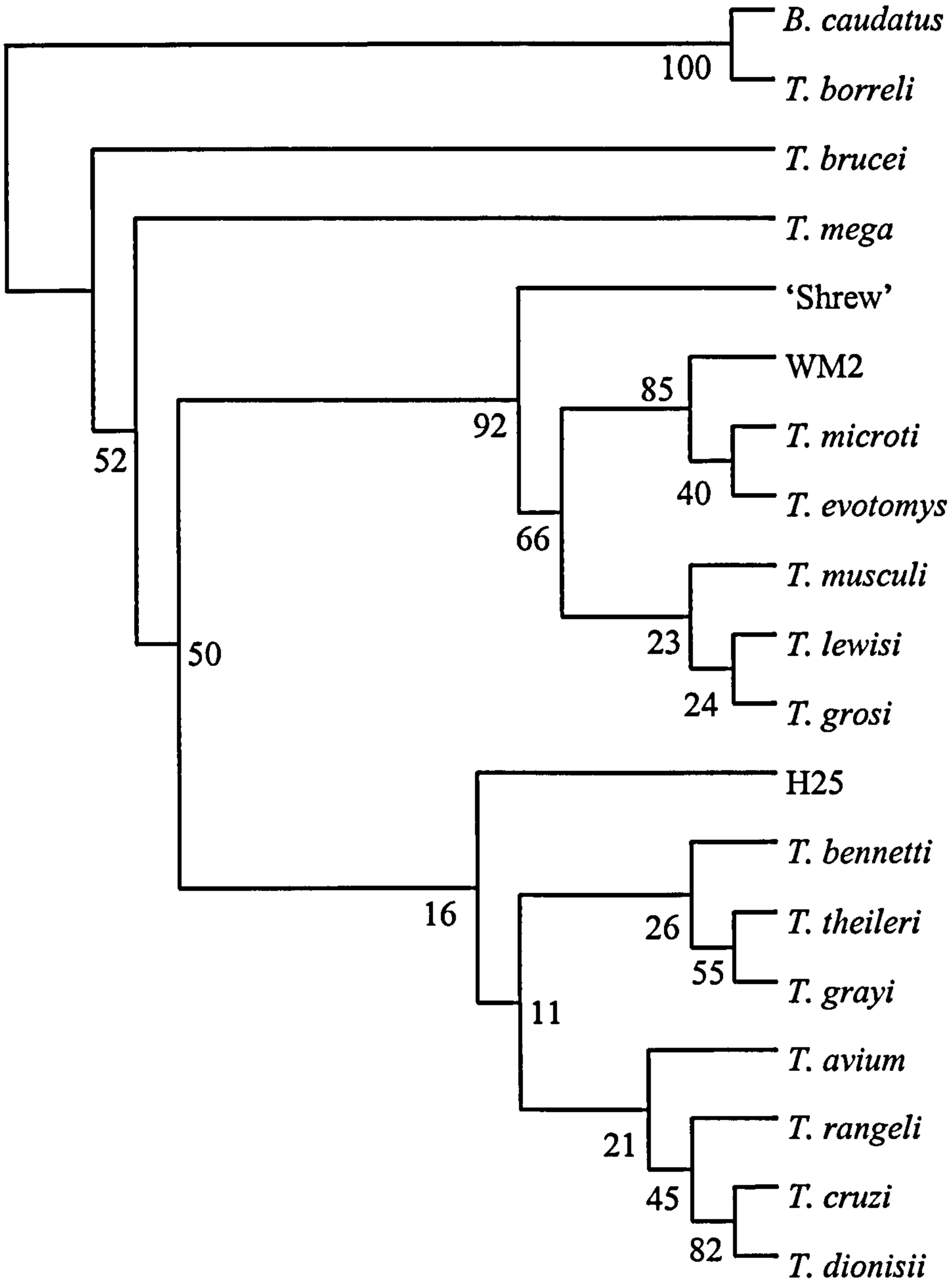


Figure 2.1 Phylogenetic tree constructed using maximum likelihood showing relationships between trypanosome 18S rRNA sequences, including those isolated from *S. araneus* in Cheshire ('Shrew'). Bootstrap values (percentages) are shown at nodes, with Genbank assession numbers and host species for each sequence listed in **Table 2.1**. *B. caudatus* and *T. borreli* are included as an outgroup for tree rooting (Stevens and Gibson, 1999).

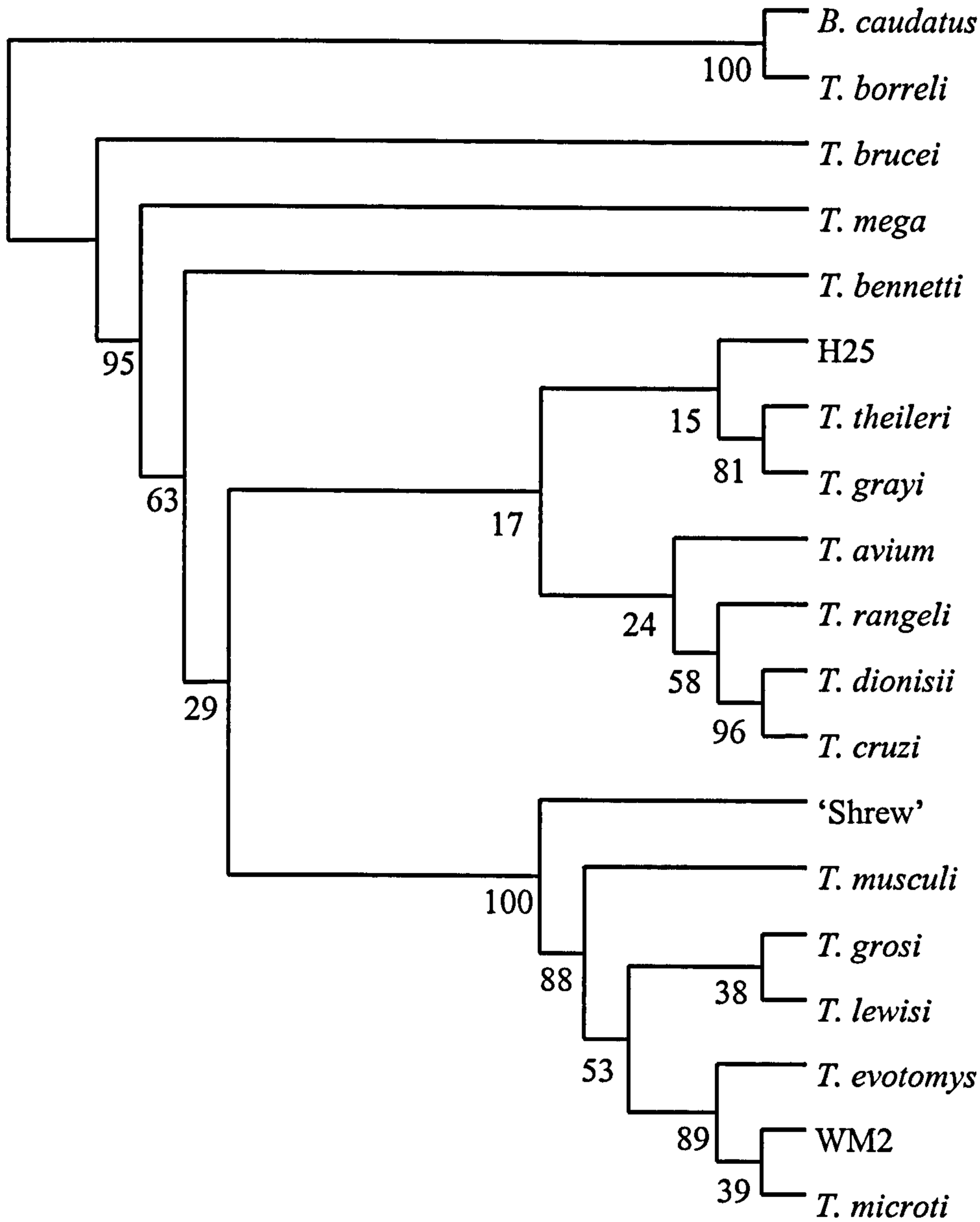


Figure 2.2 Phylogenetic tree constructed using neighbour joining method showing relationships between trypanosome 18s rRNA sequences, including those isolated from *S. araneus* in Cheshire ('Shrew'). Bootstrap values (percentages) are shown at nodes, with Genbank assession numbers and host species for each sequence listed in **Table 2.1.** *B. caudatus* and *T. borreli* are included as an outgroup for tree rooting (Stevens and Gibson, 1999).

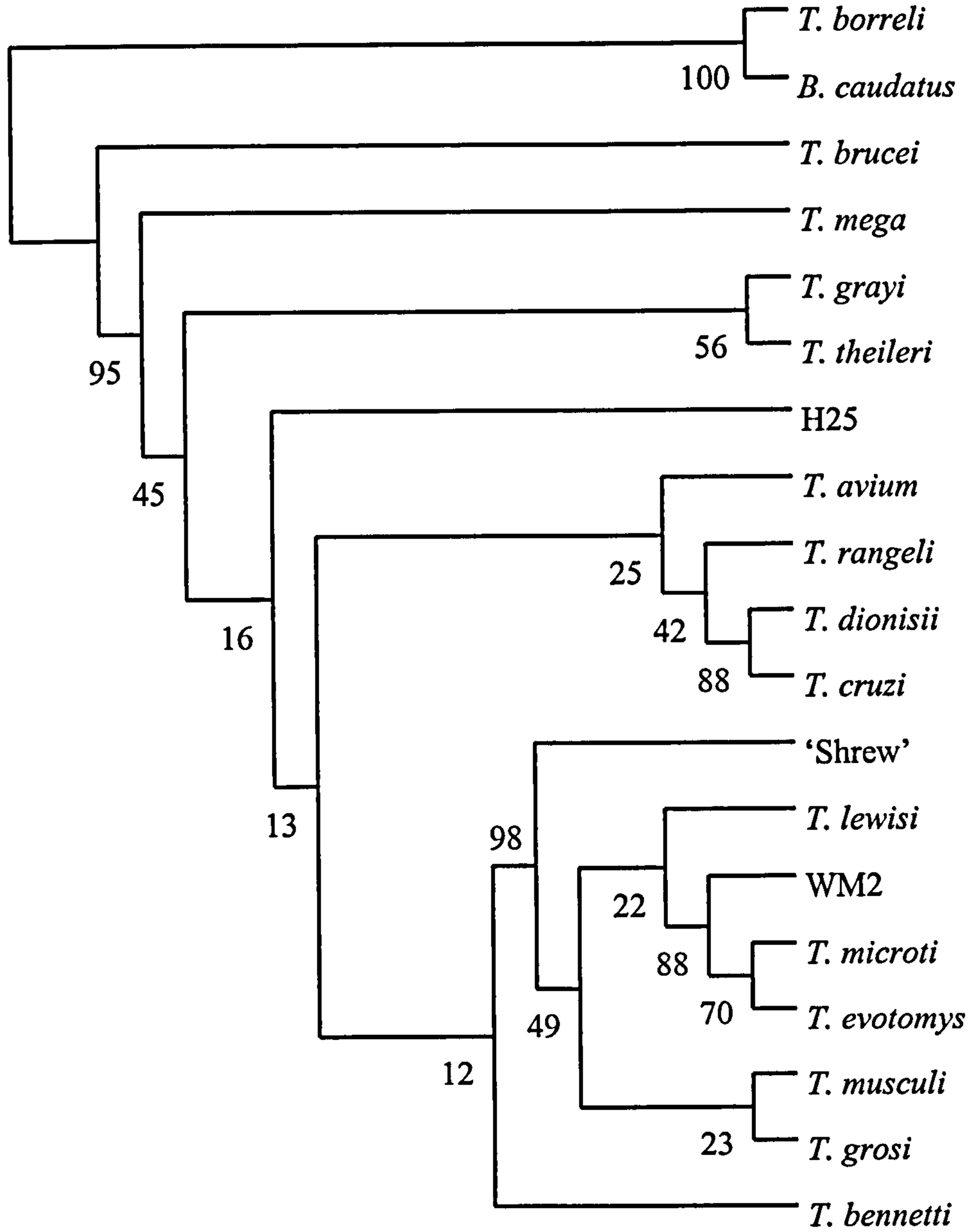


Figure 2.3 Phylogentic tree constructed using maximum parsimony showing relationships between trypanosome 18s rRNA sequences, including those isolated from *S. araneus* in Cheshire ('Shrew'). Bootstrap values (percentages) are shown at nodes, with Genbank assession numbers and host species for each sequence listed in **Table 2.1**. *B. caudatus* and *T. borreli* are included as an outgroup for tree rooting (Stevens and Gibson, 1999).

2.3.3 *Bartonella* sp.

Eleven of 76 shrews (14.5%) were found to be culture positive for *Bartonella* sp., with presence of parasite DNA confirmed by PCR (**Table 2.3**). No effect of host age, host sex or year of collection was found on *Bartonella* sp. prevalence (**Table 2.5**).

Two 16S-23S rRNA intergenic spacer regions were successfully recovered from one shrew each, one 307bp in length, the other 305bp (**Appendix A**). The sequences were 94% similar, differing by 16bp. Citrate synthase gene (*gltA*) fragment sequences were obtained from *Bartonella* sp. recovered from five shrews (**Appendix A**). Comparisons indicated there were two distinct *gltA* sequences, one of which was shared by three isolates (697bp), the other by two isolates (667bp). The two sequences were 99% similar over the 667bp of the shorter sequence, differing by 6bp. The isolates from which different 16S-23S rRNA IGSRS were recovered also differed in their *gltA* sequences.

Comparisons of *gltA* sequences recovered here with those from other *Bartonella* species held in the Genbank database (**Table 2.2**) resulted in an alignment 294bp in length after removal of gaps. All three phylogenetic trees constructed had variable bootstrap support (22% - 100%), but shared several similarities (**Figures 2.4 - 2.6**). *B. bacilliformis* is the most outlying species, a feature shared with previously published phylogenies (Birtles & Raoult, 1996; Holmberg *et al.*, 2003). The three *B. vinsonii* subspecies clustered together, and the human pathogen *B. elizabethae* grouped with two species found in rodents, *B. grahamii* and *B. tribocorum*. Birtles and Roult (1996) found a *gltA* sequence isolated from a rat was identical to that of *B. elizabethae*, and suggested the human disease may be the result of a zoonosis. The trees presented here are in agreement with this hypothesised relationship. Four species isolated from ruminants also grouped together (*B. bovis*, *B. capreoli*, *B. schoenbuchensis* and *B. chomelii*), although in the neighbour joining tree *B. clarridgeiae* isolated from a cat was also included in this cluster (**Figure 2.5**)

In all three trees, the two sequences isolated here from common shrews in Cheshire grouped together with sequence SA192UP, isolated from *S. araneus* in Sweden (Holmberg *et al.*, 2003), and with *B. taylorii* isolated from *Apodemus* sp. (Birtles *et al.*, 1995).

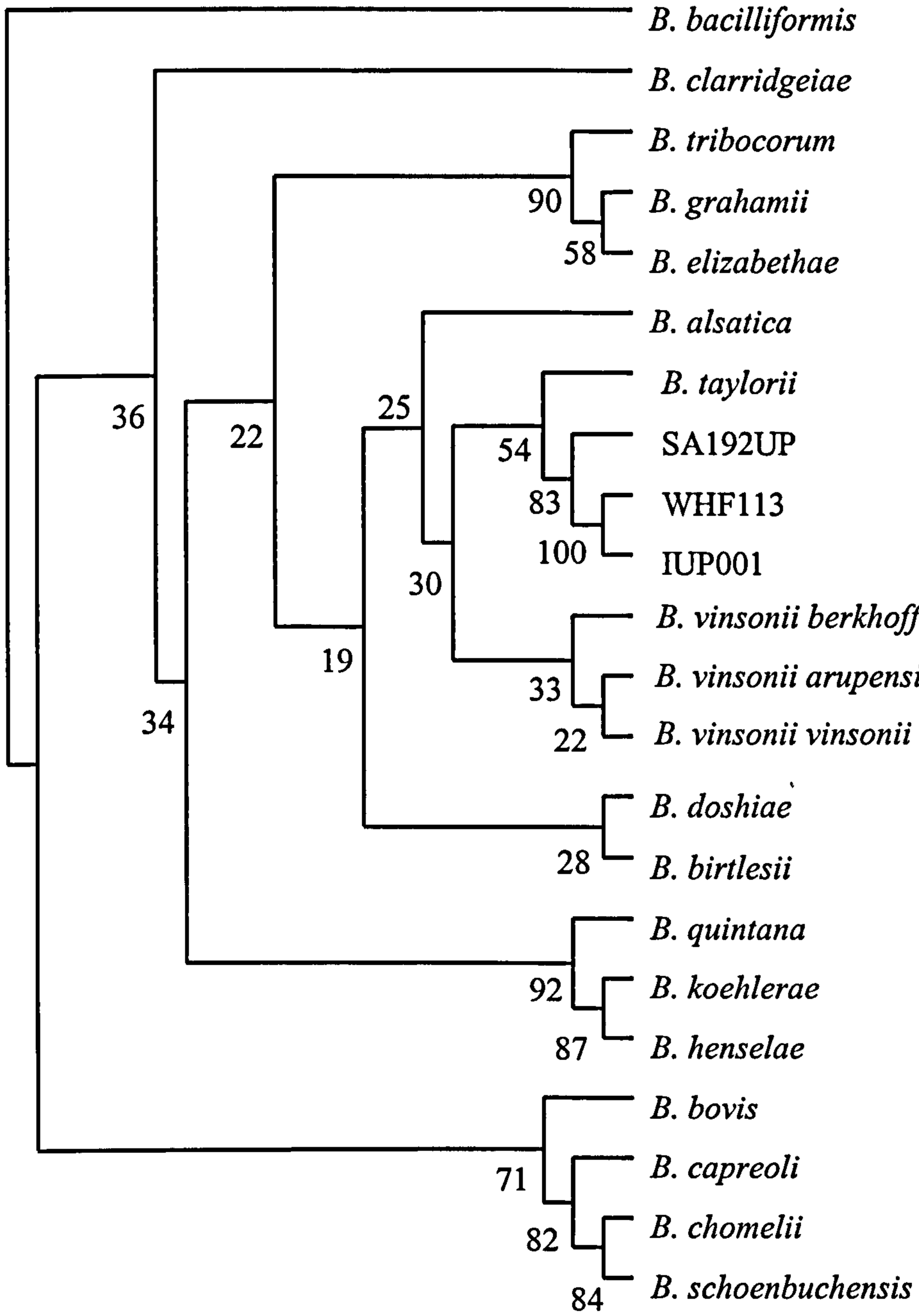


Figure 2.4 Phylogenetic tree constructed using maximum likelihood showing relationships between *Bartonella* species *gltA* fragment sequences, including those isolated from *S. araneus* in Cheshire (WHF001 and IUP001). Bootstrap values (percentages) are shown at nodes, with Genbank assession numbers and host species for each sequence listed in **Table 2.2.**

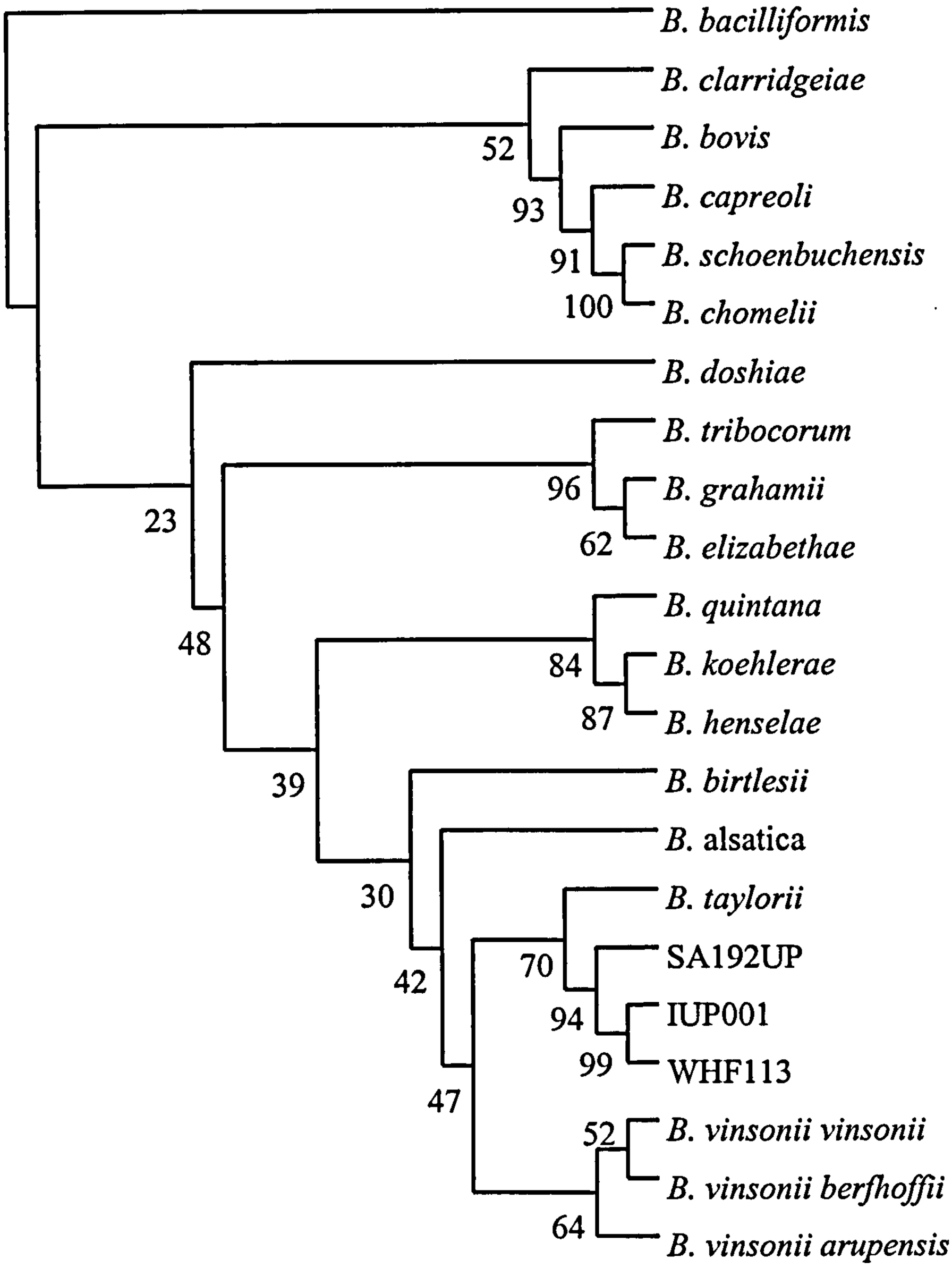


Figure 2.5 Phylogenetic tree constructed using neighbour joining method showing relationships between *Bartonella* species *gltA* fragment 18s rRNA sequences, including those isolated from *S. araneus* in Cheshire (WHF001 and IUP001). Bootstrap values (percentages) are shown at nodes, with Genbank assession numbers and host species for each sequence listed in **Table 2.2**.

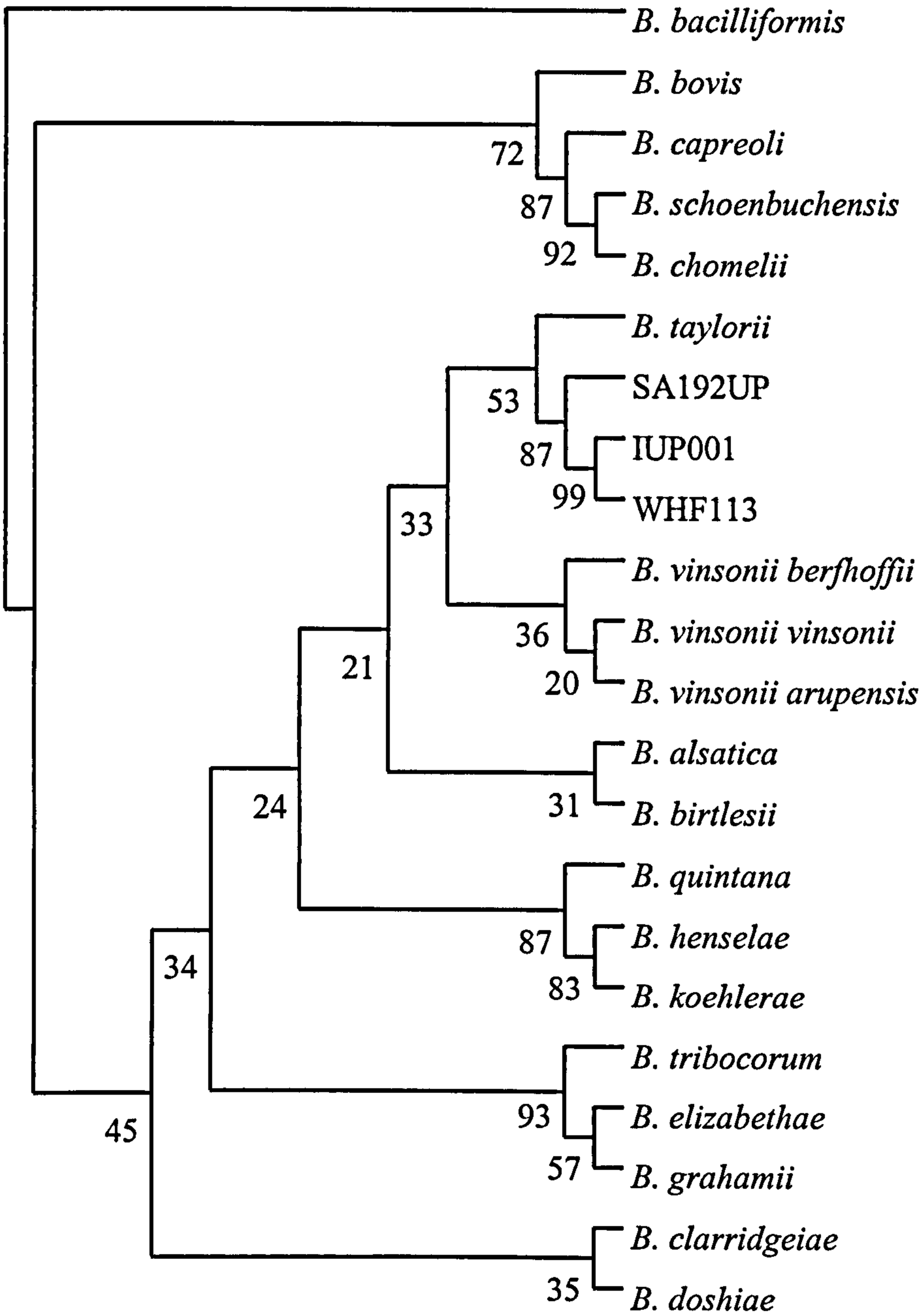


Figure 2.6. Phylogenetic tree constructed using maximum parsimony showing relationships between *Bartonella* species *gltA* sequences, including those isolated from *S. araneus* in Cheshire (WHF001 and IUP001). Bootstrap values (percentages) are shown at nodes, with Genbank assession numbers and host species for each sequence listed in **Table 2.2**.

Table 2.5 Effects of host age, host sex and year of collection on *Bartonella* sp. prevalence, as assessed using a generalized linear model with binomial error distribution and logit link function, N = 76

Independent variable	df	Change in residual deviance[1]	P
Year of collection	2	1.98	NS
Host sex	1	3.06	NS
Host age	1	0.64	NS
Host age by sex interaction	1	0.06[2]	NS

1. When added to and deleted from minimal model

2. Added and removed from a model containing host age and host sex

2.4 Discussion

Of the 76 shrews examined only one was found to be PCR positive for *A. phagocytophilum* (1.3%). This is only the second time *A. phagocytophilum* has been detected from common shrews using PCR, the only previous report being that of Liz *et al.* (2000), who found the parasite in one of five common shrews in Western Switzerland. The results presented here suggest prevalence of *A. phagocytophilum* in common shrews is similar or lower to that found in other small mammals in the UK. Ogden *et al.* (1998) found *A. phagocytophilum* in one of 26 *Clethrionomys glareolus* and zero of 27 *Apodemus sylvaticus*, while Bown *et al.* (2003) found *A. phagocytophilum* in 11% of *C. glareolus* and 1.8% of *A. sylvaticus*. *A. phagocytophilum* is believed to be transmitted between rodents by two species of ixodid tick in the UK, *I. ricinus* and *I. trianguliceps* (Ogden *et al.*, 1998). Both nymph and adult ticks have been shown to be PCR positive for *A. phagocytophilum* (Ogden *et al.*, 1998), although rodents carry very few *I. ricinus* nymphs, and almost no *I. ricinus* adults, despite being major hosts for larvae (Randolph & Craine, 1995). Both species of tick are known to feed on *S. araneus* (Churchfield, 1990; *I. tranguliceps* nymphs were recovered from the one animal infected here), and are likely to be vectors of *A. phagocytophilum* in common shrews.

It has been suggested that the higher prevalence of *A. phagocytophilum* in bank voles may be partially explained by a higher encounter rate of mature males with ticks

coupled with a longer persistence of *A. phagocytophilum* infection than occurs in wood mice (*Apodemus* spp.; Bown *et al.*, 2003). Whether either of these factors could explain the lower prevalence of *A. phagocytophilum* reported here from *S. araneus* is unknown.

The 16S rRNA sequence elucidated from the PCR positive shrew was identical to one previously reported for *A. phagocytophilum* isolated only from *C. glareolus* in Northwest England (Bown *et al.*, 2003). Both the presence of ixodid ticks on common shrews and the sequence data presented here suggest transmission between rodents and shrews may be possible, and that *S. araneus* may be part of the small mammal reservoir of *A. phagocytophilum*. This would seem likely, given the close proximity shared by shrews and wild rodents and the wide host range of *A. phagocytophilum*, but further experiments are needed to demonstrate if transmission between shrews and rodents can be facilitated by ticks. A longitudinal study may reveal whether *A. phagocytophilum* prevalence follows the same seasonal pattern in common shrews as it does in rodents (Bown *et al.*, 2003), and whether the disease is maintained all year round in common shrews, or if infection only occurs as an occasional by-product of the rodent-tick system.

The prevalence of trypanosomes in shrews was higher than that reported for *A. phagocytophilum*, with 11.8% of shrews PCR positive, which is similar to the 13% prevalence of trypanosomes detected in *S. cinereus* from Pennsylvania by inspection of blood smears (Laakkonen *et al.*, 1998). Similar levels of trypanosome prevalence have also been observed in UK bank voles (*C. glareolus*; 11.1%, Healing, 1981; 20%, Turner, 1986 and 19%, Noyes *et al.*, 2002), although prevalence in wood mice (*A. sylvaticus*) appears to be lower (1%, Healing, 1981; 2.2%, Turner, 1986; 8%, Noyes *et al.*, 2002). In the current study, no effect of host gender was found on prevalence, which concurs with investigations of trypanosomes in rodents (Healing, 1981; Turner, 1986). However, previous authors have produced conflicting reports of effects of age on trypanosome prevalence: Healing (1981) found greater prevalence in immature *C. glareolus* and *M. agrestis* than adults, whilst Turner (1986) suggested 'older' *C. glareolus* were more likely to harbour trypanosomes. Here, no significant effect of host age on trypanosome prevalence was found. Transmission of trypanosomes to rodents is believed to occur when the host eats infected fleas or flea faeces during grooming (Hoare, 1972). The lack

of age or sex effects found here suggests sub-adult and adult shrews of both sexes are equally likely to contract trypanosome infections, probably via consuming fleas.

The 519bp 18S rRNA encoding gene fragment sequence isolated using trypanosome specific primers was found to share greatest similarity with a homologous gene from *T. lewisi*. The 5% difference between the sequence isolated from *S. araneus* and any other published on Genbank suggests it may represent the genotype of a previously undescribed *Trypanosoma* species. To my knowledge, this is the first time a trypanosome has been reported from a common shrew. Although similar organisms have been identified from both Old World and New World shrews, often named as *T. soricis* or described as '*T. lewisi*-like' (Laakkonen *et al.*, 1998), no DNA sequence data are available from these parasites which would allow phylogenetic comparisons with the sequence isolated here. In the three phylogenetic trees produced in the current study, the 18S rRNA encoding gene fragment sequence isolated from *S. araneus* groups most closely to, but is separate from, a cluster of rodent trypanosomes. The sequence described here has never previously been isolated from rodents sharing habitat with *S. araneus*, suggesting it may represent a trypanosome species specific to shrews. The flea fauna of common shrews in the UK includes two species rarely found on rodents, *Doratopsylla dasycnema* and *Palaeopsylla soricis* (Smit, 1957). The trypanosome found here might therefore be restricted to shrews by either of these potential flea vectors. However, studies of wild bank voles (*C. glareolus*) and wood mice (*A. sylvaticus*) suggest both rodent species are challenged by each other's associated trypanosomes by a common flea fauna, yet do not seem to become infected (Noyes *et al.*, 2002). Previous authors have suggested trypanosomes may have co-evolved to a certain extent with their hosts (Haag *et al.*, 1998; Noyes *et al.*, 2002 but see Maslov *et al.*, 1996). If so, it would not be surprising to find that trypanosome transmission cannot take place between hosts belonging to different orders (insectivores and rodents). Further work is needed to identify if the trypanosome isolated here is also found in other shrews and whether it represents the genotype of *T. soricis* reported by previous authors.

Bartonella sp. was cultured from 14.5% of shrews examined. This is very close to the 15% prevalence found in Swedish *S. araneus* by PCR (Holmberg *et al.*, 2003) and similar to the 10.4% figure reported from Czechoslovakia (Sebek, 1975), but

considerably lower than the 27% prevalence found in Norway by inspection of blood smears (Hoyte, 1956). No effect of host age or gender was found on *Bartonella* sp. prevalence, which concurs with the earlier observations of Hoyte (1956). It would appear from these results that, as for trypanosomes, shrews of both sexes and age classes are equally likely to become infected by *Bartonella* sp., also thought to be transmitted by arthropod vectors, probably fleas (Birtles *et al.*, 2001; Stevenson *et al.*, 2003).

Two different strains of *Bartonella* sp. were isolated from shrews during this study, exhibiting polymorphisms in both the g*ltA* sequence and 16S-23S rRNA IGSR. Phylogenetic analysis of g*ltA* sequences placed both strains closest to *Bartonella* sp. SA192UP from *S. araneus* in Sweden (Holmberg *et al.*, 2003) and *B. taylorii* isolated from *Apodemus* sp. (Birtles *et al.*, 1995). Previous studies have suggested low host specificity for most *Bartonella* species of rodents, with the same *Bartonella* species able to infect different hosts, and several *Bartonella* species occurring in the same host species (Birtles *et al.*, 1994; Birtles & Raoult, 1996). However, the two *gltA* sequences isolated here have never been reported from rodents, and neither has a similar sequence isolated from common shrews in Sweden (Holmberg *et al.*, 2003). This may suggest a high level of host specificity for *Bartonella* species infecting shrews. As suggested for trypanosomes, *Bartonella* sp. in *S. araneus* may be transmitted by flea species feeding almost exclusively on shrews, such that the strains isolated here and in Sweden may be restricted by their arthropod vector. A similar hypothesis has been suggested to explain the observed divergence between *B. grahamii* associated with bank voles and other species infecting small mammals (Birtles & Raoult, 1996). The clustering of *gltA* sequences from common shrews with *B. taylorii* might suggest a recent host-switching event between two host species sharing the same habitat. Clearly, both proposals need further investigation to fully understanding the evolution of *Bartonella* sp. infections in shrews.

In conclusion, common shrews in the UK are infected with parasite genera previously associated with rodents and other small mammals. In the case of *A. phagocytophilum*, transmission may occur between shrews and other hosts, while the evidence suggests *Bartonella* sp. and trypanosomes of shrews are host specific. Further work is needed to understand the transmission dynamics of shrew haemoparasites, both

within common shrews and between other small mammal species, and the effects these parasites have upon their host.

Chapter 3 Parasite-mediated pathology in *Sorex araneus*

3.1 Introduction

Investigations of wild animal pathology are usually restricted to examination of recovered carcases (Gulland, 1995), often to determine the cause of mortality during wildlife epidemics (Wiese *et al.*, 1977; Spalding *et al.*, 1993; Spalding & Forrester, 1993; Work *et al.*, 2004). In combination with field studies, pathological investigations may also aid our understanding of the physiological mechanisms by which parasites influence host life-history traits. For example, Gulland (1992) examined pathological lesions associated with nematode infection (*Ostertagia circumcincta*) at the same time as conducting treatment experiments on Soay sheep (*Ovis aries*). The presence of pathological lesions indicated nematodes contributed to mortality in malnourished hosts, which might explain the increase in daily survival associated with anthelmintic treatment. Through examination of histological changes in infected tissue, such studies may also provide a broader understanding of host investment in immunity than those which rely solely on measuring antibody titres in serum (Owens & Wilson, 1999). In addition, examination of histological alterations associated with immune reactions provides details of immunopathology, suggested to be one of the major costs to the host of mounting an immune response (Raberg *et al.*, 1998).

It is within this wider framework that parasite-mediated pathology of common shrews is of particular interest. As discussed in **Section 1.8**, shrews are known to withstand infection by a range of micro and macroparasites, and harbour a greater number of helminth species than rodents sharing the same habitat (Sharpe, 1964; Lewis, 1968). Although almost nothing is known about the pathological effects of these parasites, age-related increases in helminth infections have been suggested as a possible factor in the synchronous mortality of adult shrews after the breeding season (Buckner, 1969; Pernetta, 1977). The extent to which *S. araneus* responds immunologically to infection also remains virtually unstudied. Given their resource-limited physiology **(Section 1.7)**, and the energetic and nutrient costs of mounting an immune response **(Section 1.3)**, shrews may have no alternative but to tolerate parasite infestation.

As a first step to exploring the effects of parasitism on *S. araneus,* a thorough pathological investigation of wild common shrews is conducted for the first time, with all major organs examined using standard histological techniques. In addition, quantitative comparisons of helminth abundances are made between host sex and age classes, in order investigate the extent to which shrews suffer increasing levels of parasitism with age. This investigation is complemented by a detailed study of the structure, function and age-related changes in the lymphatic tissues of these animals (**Chapter 4**), which may reveal more about the common shrew's ability to respond to parasitic infection through immune reactions.

3.2 Materials and methods

3.2.1 Animals and tissue processing

Forty-three wild common shrews (19 male, 24 female) were live-caught in the vicinity of Leahurst field station, Cheshire, England (OS grid reference 331558, 377486) between September 2001 and June 2003. Animals were caught either for the purpose of dissection and examination (five animals), or for use as controls in behavioural trials (17 animals, **Chapter 5**) or field experiments (21 animals, **Chapter 6**). Shrews were classified into three age categories: group one consisted of 18 sub-adult animals (eleven female, seven male), group two comprised three pubescent individuals undergoing sexual maturation (two female, one male) and group three was represented by 22 sexually mature animals (eleven female and eleven male) caught during or after the breeding season. All animals appeared healthy when captured, and were killed using an overdose of Fluothane^{tm} (Schering-Plough Animal Health, Hertfordshire, UK) anaesthetic vapour. Full necropsy was performed, and oesophagus, stomach, gut, bladder and carcass were examined for endoparasite infestation. Parasites were identified as nematodes, cestodes, digeneans or acanthocephalans using appropriate keys (Roots, 1992) and counted.

Cardiac blood samples were taken for the detection of haemoparasites (*Bartonella sp.*, trypanosomes and *Anaplasma phagocytophilum*) by bacterial culture and PCR (Birtles & Raoult, 1996; Birtles *et al.*, 2000; Liz *et al.*, 2002; Noyes *et al.*, 2002), as described in **Chapter 2.**

Major organs from all animals were fixed in 4% buffered paraformaldehyde for 24 - 48h, with tissue samples routinely embedded in paraffin wax. 5µm-thick sections were stained with haematoxylin-eosin prior to light microscopy. To facilitate identification of cell populations in liver inflammation, sections were stained using immunohistochemical methods described fully in **Chapter 4**.

3.2.2 Statistical analysis

Mann-Whitney U-tests were employed to investigate if abundances of gut helminths (comprising digeneans, nematodes and cestodes), bladder nematodes, and *Porrocaecum* sp. larvae varied according to shrew sex or age.

The degree of inflammatory infiltration observed in the liver of each animal was assessed as mild, moderate or severe, based on the number of cells and cell layers in portal areas or between hepatic cords. Ordinal logistic regression was then used to test whether severity of inflammation varied with shrew age class or sex.

3.3 Results

3.3.1 Parasitology

All animals harboured helminths. In those examined in detail (N = 36) both nematodes and cestodes were recovered from the digestive tract, while 30/36 (83%) of animals were infected with the digenean *Brachylaemus fulvus* (Roots, 1992), located in the oesophagus, stomach or gut. Additionally, digeneans (identified by egg morphology as *Dicrocoelium soricis*; Roots, 1992) were recovered from the gall bladder lumen of one sub-adult and one adult common shrew. The nematode *Liniscus incrassatus* was found in the urinary bladder of 29 of 36 animals (81%) and larvae of the nematode *Porrocaecum* sp. were present in the intrascapular adipose tissue of 10 of 19 (53%) examined adult animals, reaching a maximum intensity of 68 larvae per shrew, but only two of 12 sub-adults (17%; maximum intensity, two) and none of three pubescent shrews. Acanthocephalans (most likely *Centrorhynchus aluconis*; Ewald *et al.*, 1991) were recovered from the liver of one sub-adult, and the body cavity of another.

Trypanosomes were detected by PCR in blood samples from 6 of 21 (29%) adults and one of three (33%) pubescent shrews tested, while all 14 sub-adult animals

tested were negative. *Bartonella* sp. was cultured from blood of 3/14 (21%) sub-adults, 1/3 (33%) pubescent common shrews and 4/21 (19%) adult individuals. *A. phagocytophilum* was not detected in any of the animals examined.

3.3.2 Effects of shrew age and sex on helminth abundance

Adults were found to harbour more bladder nematodes, *Porrocaecum* sp. larvae and gut helminths than sub-adults (**Table 3.1; Figures 3.1 - 3.3**). In addition, males were found to harbour more gut helminths than females, but no difference in abundances of bladder nematodes or *Porrocaecum* sp. larvae was found between sexes (**Table 3.2**). Whether an interaction exists between effects of host sex and age on parasite abundances could not be formally tested using non-parametric ANOVAs, due to unequal sample sizes between host categories.

3.3.3 Pathohistology

Complete histological details of each animal examined are presented in **Appendix B**. Here, pathological findings are grouped by organ/tissue and presented in alphabetical order.

3.3.3.1 Adipose tissue

Porrocaecum sp. larvae were found encapsulated within cell-rich fibrous connective tissue in adipose tissue of 12 animals. In the three animals examined histologically, infection was not associated with any inflammatory infiltration (**Figure 3.4**). In addition, moderate focal granulomatous steatitis was observed in adipose tissue of one animal.

3.3.3.2 Adrenal glands

Adrenal glands were examined in 36 animals, none of which showed signs of histopathological alteration.

Table 3.1 Parasite abundances of sub-adult and adult common shrews

	Sub-adults				Adults					
	N[1]	**Median**	**25% Quartile**	**75% Quartile**	**N[1]**	**Median**	**25% Quartile**	**75% Quartile**	**U[2]**	**P[3]**
Bladder nematodes[4]	14	1.0	0.0	2.0	21	7.0	1.0	12.0	71.0	<0.05
Porrocaecum sp. larvae[5]	14	0.0	0.0	0.0	19	2.0	0.0	11.0	72.5	<0.05
Gut helminths[6]	13	14.0	9.5	40.0	19	50.0	36.0	79.0	42.5	<0.05

1. Number of shrews
2. Mann-Whitney U-tests comparing parasite abundances of sub-adults and adults
3. Probability of there being no difference in parasite abundances of sub-adults and adults, as judged by Mann-Whitney U-tests
4. *Liniscus incrassatus* (Roots, 1992)
5. Encapsulated in intrascapular adipose tissue and body cavity
6. Comprising nematodes, cestodes and digeneans

Table 3.2 Parasite abundances of female and male common shrews

	Females				Males					
	N[1]	**Median**	**25% Quartile**	**75% Quartile**	**N[1]**	**Median**	**25% Quartile**	**75% Quartile**	**U[2]**	**P[3]**
Bladder nematodes[4]	17	1.0	0.0	16.0	18	2.0	1.0	7.3	133.0	NS
Porrocaecum sp. larvae[5]	15	0.0	0.0	2.0	18	0.0	0.0	6.3	123.5	NS
Gut helminths[6]	14	28.5	13.8	45.3	18	50.0	26.0	89.0	74.0	<0.05

1. Number of shrews
2. Mann-Whitney U-tests comparing parasite abundances of females and males
3. Probability of there being no difference in parasite abundances of females and males, as judged by Mann-Whitney U-tests
4. *Liniscus incrassatus* (Roots, 1992)
5. Encapsulated in intrascapular adipose tissue and body cavity
6. Comprising nematodes, cestodes and digeneans

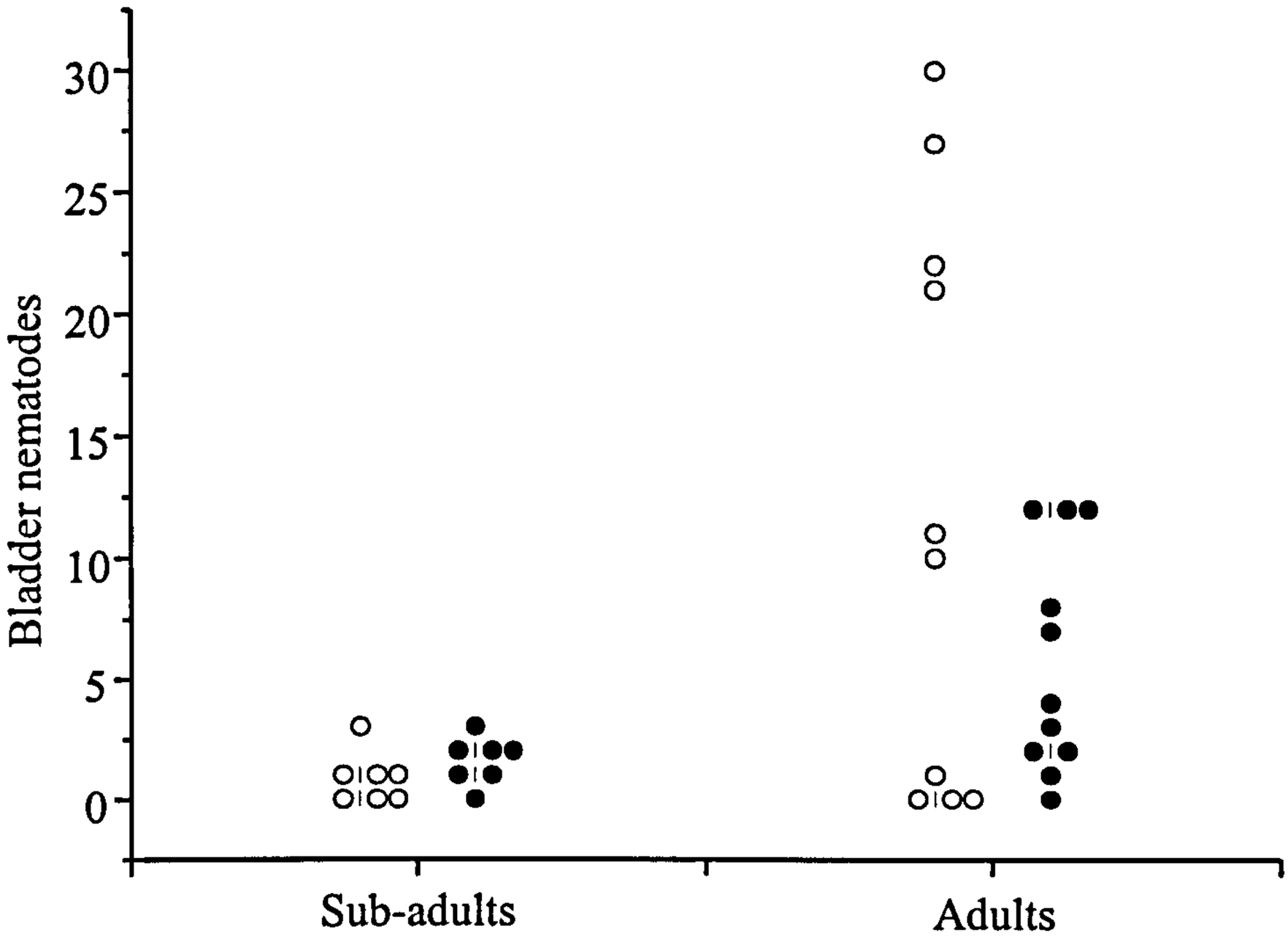


Figure 3.1 Abundance of bladder nematodes (*Liniscus incrassatus*) in female (open circles) and male (filled circles) common shrews of different ages.

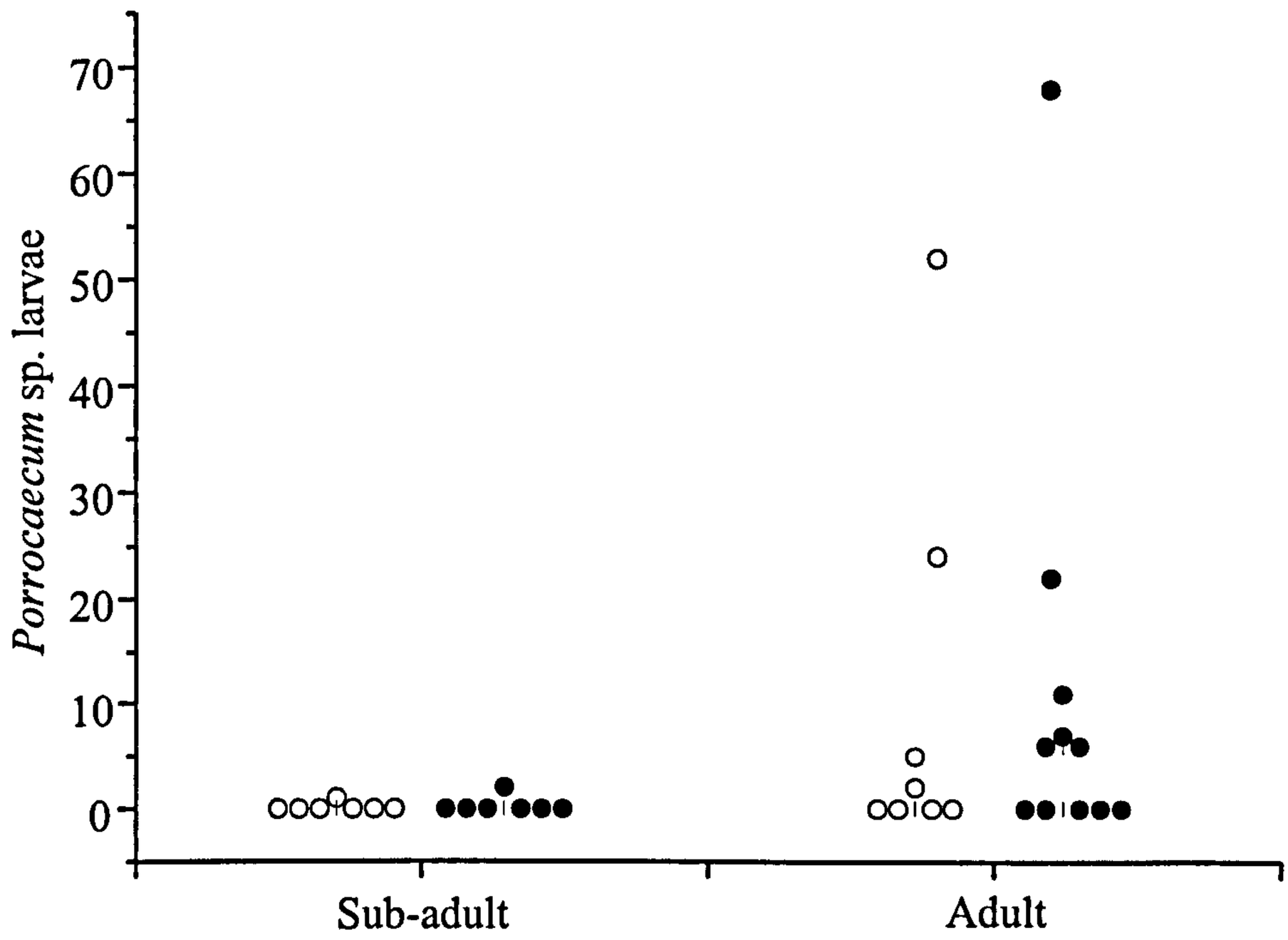


Figure 3.2 Abundance of *Porrocaecum* sp. larvae in female (open circles) and male (filled circles) common shrews of different ages.

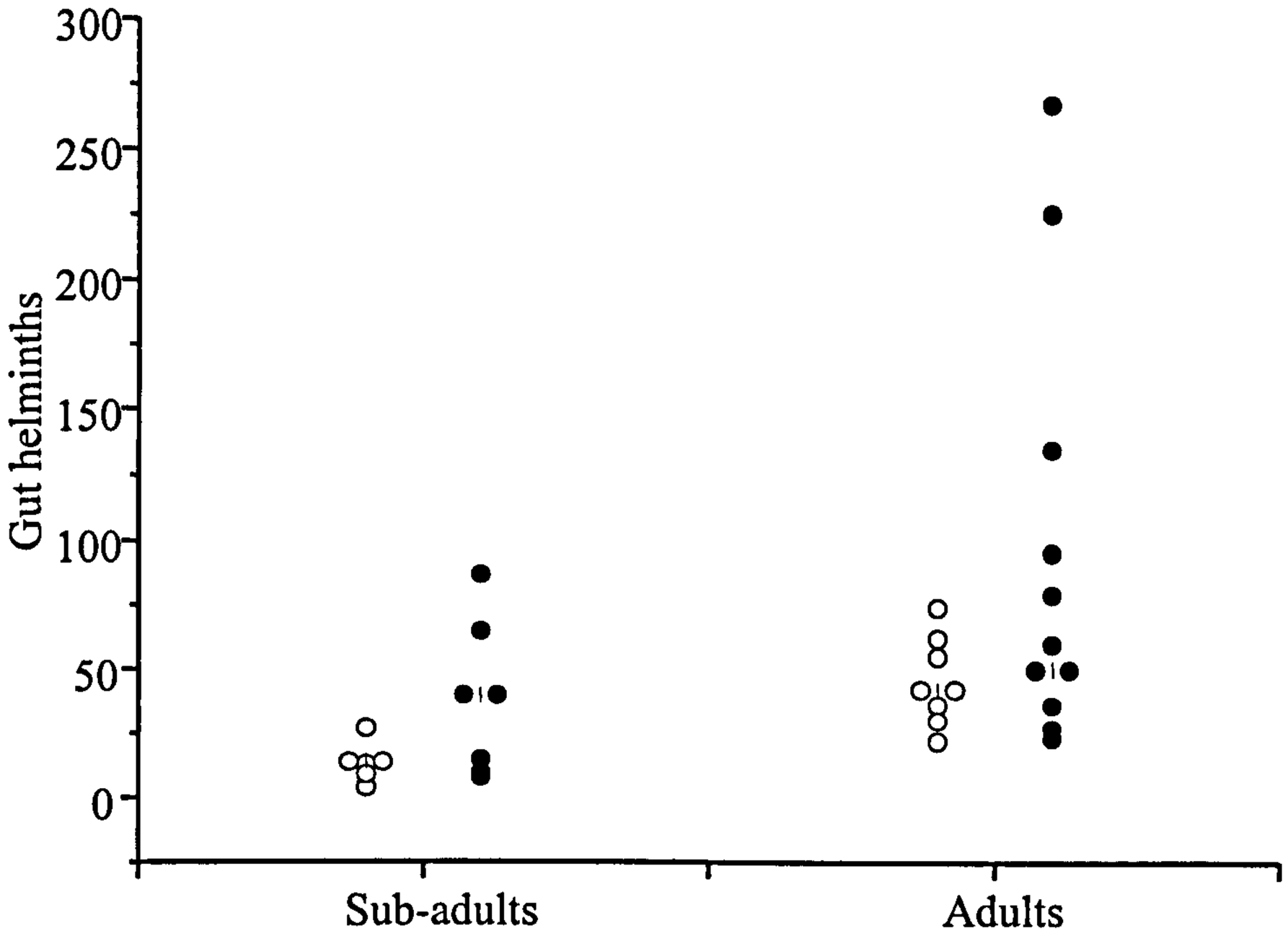
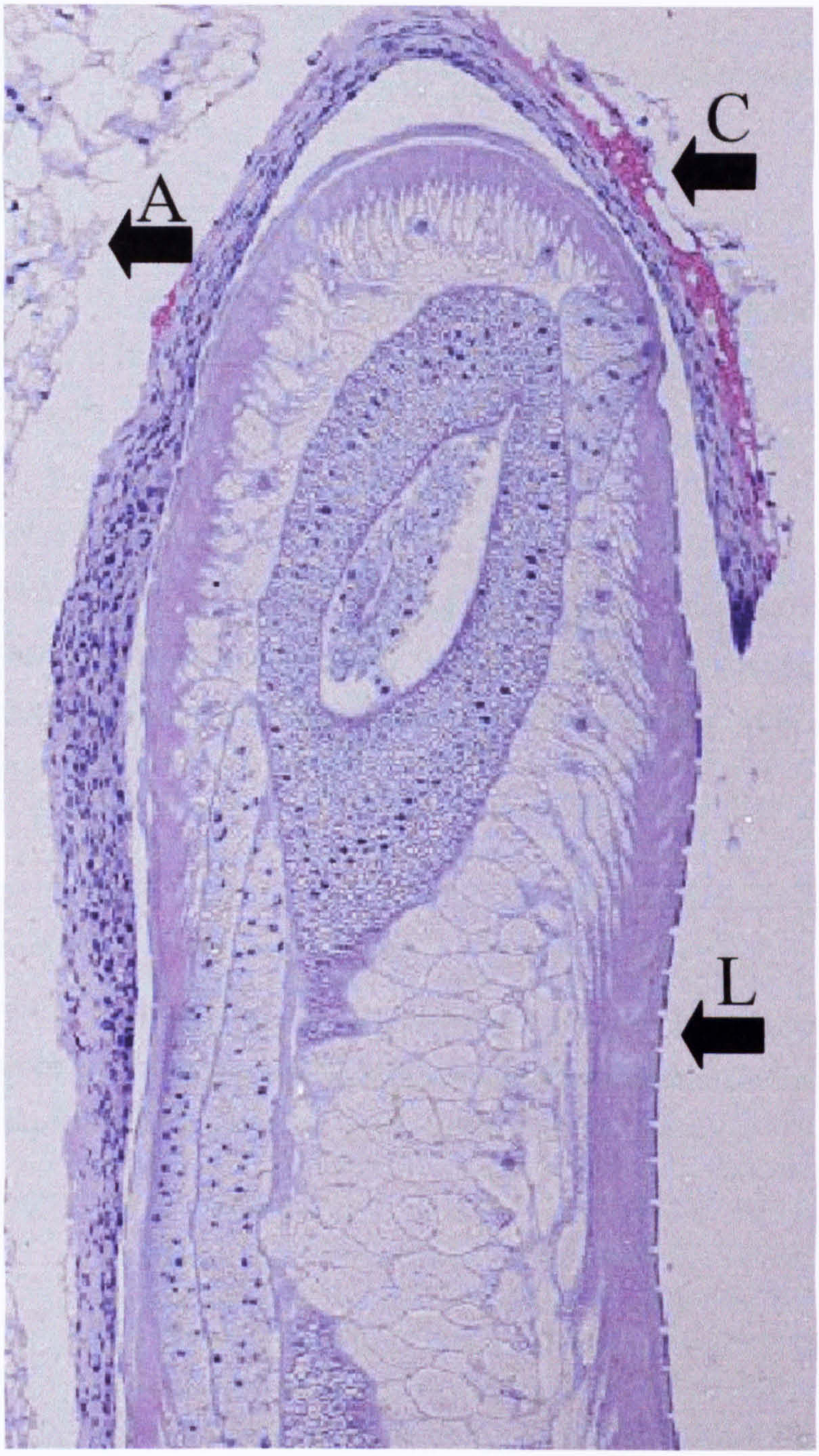


Figure 3.3 Abundance of gut helminths (comprising nematodes, cestodes and digeneans) in female (open circles) and male (filled circles) common shrews of different ages.



x200

Figure 3.4 *Porrocaecum* sp. larva (L) surrounded by fibrous capsule (C) recovered from intrascapular adipose tissue (A) of *S. araneus.*

3.3.3.3 Brain

Brain tissue was examined in 10 animals, none of which showed signs of histopathological alteration.

3.3.3.4 Gall bladder

Infestation of the gall bladder by the digenean *Dicrocoelium soricis* (as identified by egg morphology; Roots, 1992) observed in two individuals resulted in only a mild lymphocytic submucosal infiltration in one animal.

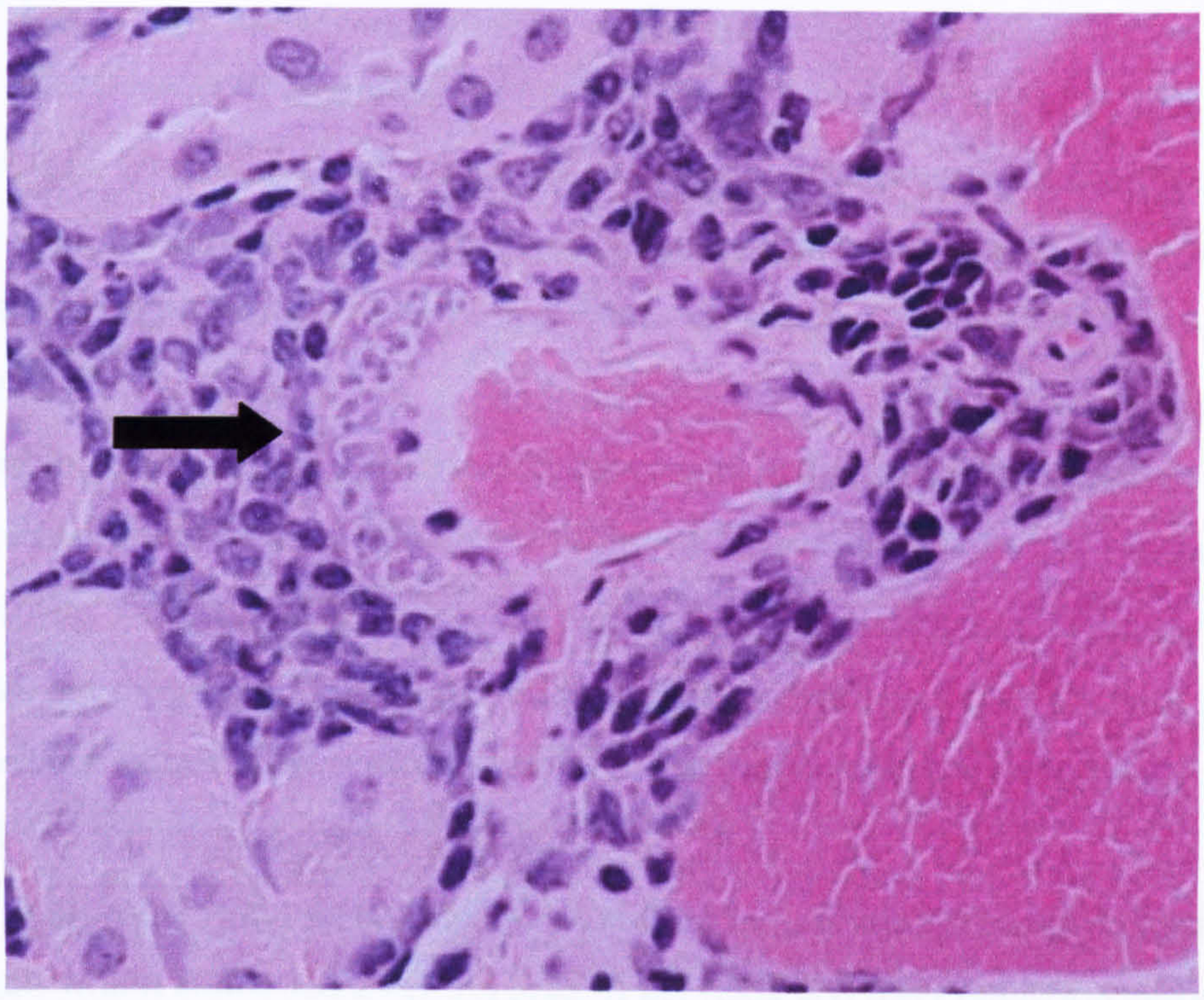
3.3.3.5 Gastrointestinal tract

Intestines of 10 animals were examined histologically, none of which exhibited histological change or inflammatory reaction directly associated with helminths. However, five animals exhibited mild to moderate mixed cellular infiltrations in the mucosa or submucosa.

3.3.3.6 Kidneys

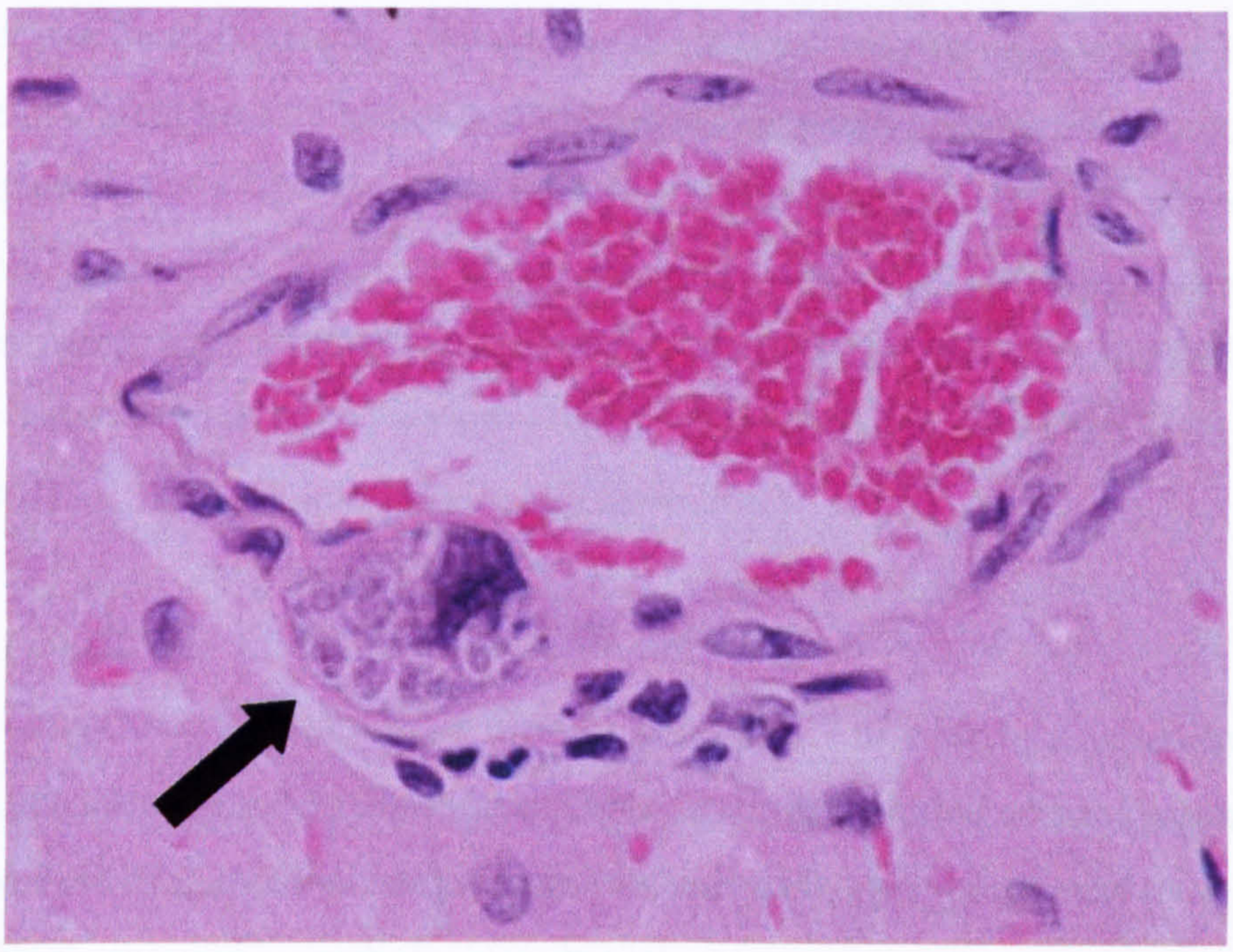
Protozoan cysts were found in kidneys of 21 of 43 (49%) animals from which tissue was examined. These were not associated with reaction in most cases (13 animals) and most commonly mild granulomatous inflammatory infiltration or occasional slight thickening of the affected vessel wall in the remainder (**Figure 3.5a**).

a.



x420

b.



x840

Figure 3.5 Protozoan cysts (arrows) within blood vessel walls in kidney (a.) and myocardium (b.) of *S. araneus*.

3.3.3.7 Liver

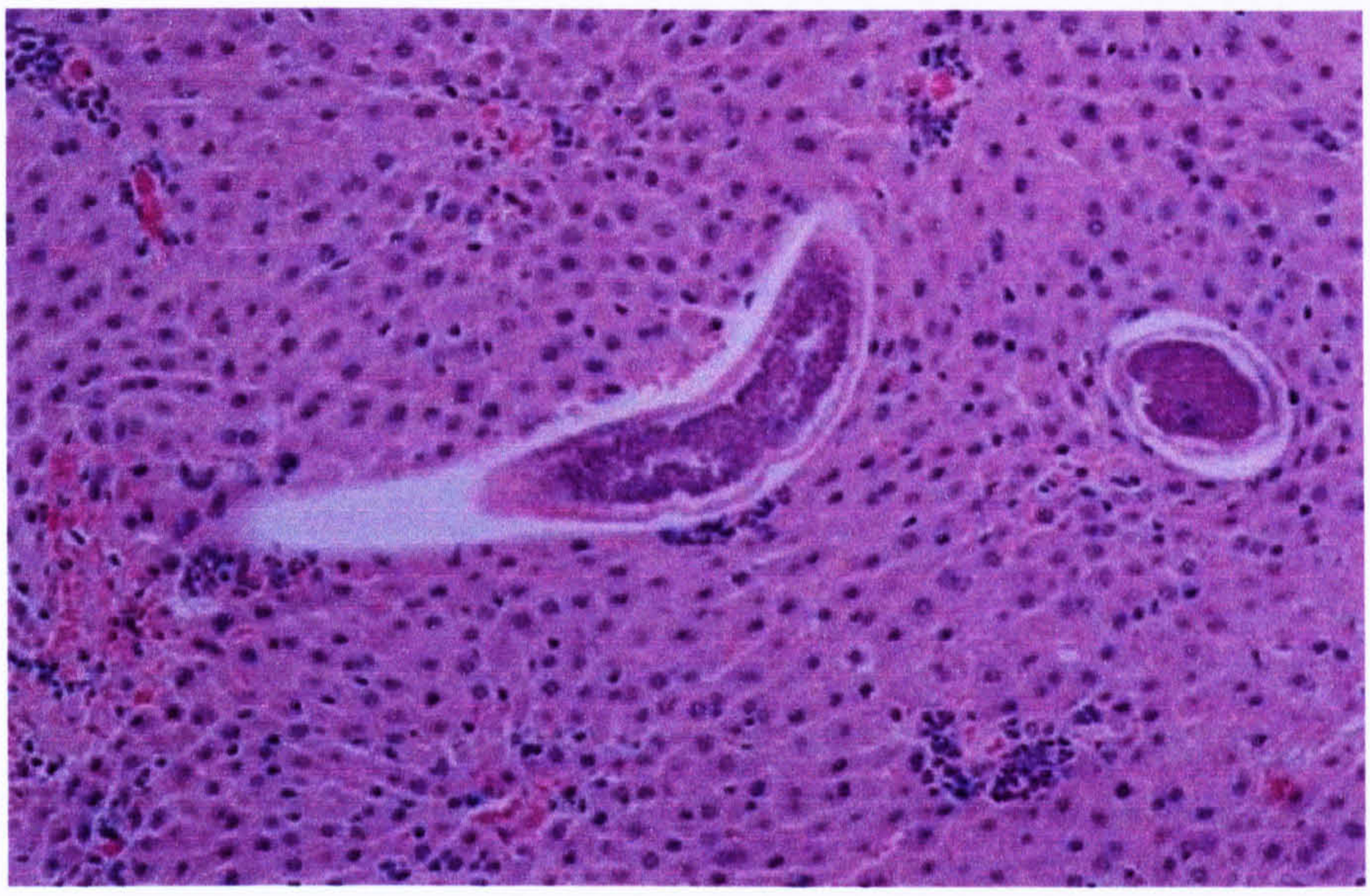
The liver of all examined animals exhibited a variable degree of mixed cellular (neutrophils, lymphocytes and macrophages) portal inflammatory infiltration (**Table 3.3**). No difference in severity of inflammation was found between sub-adult (N = 18) and adult (N = 22) common shrews (Wald = 0.03, df = 1, NS), or between males (N = 22) and females (N = 18; Wald <0.01, df = 1, NS). Inflammation often occurred together with follicle-like accumulations of lymphocytes, which occasionally exhibited germinal centres. Where 'follicles' did not possess germinal centres, lymphocytes were identified as B cells, with strong expression of CD79a and mild to moderate staining for CD45R (see **Section 4.2.2** for explanation of immunohistology). The presence of germinal centres, however, was confirmed by a strong expression of CD45R and a faint reaction for CD79a.

Additional findings included a focal chronic granulomatous inflammation in one sub-adult, which contained a central area of necrosis and mineralisation surrounded by macrophages, epithelioid and multinucleate giant cells and an outer layer of lymphocytes and fibroblasts. Two adults also had a focal necrosuppurative hepatitis, another two focal hepatic necrosis with haemorrhage and pyogranulomatous inflammation, and a fifth extensive multifocal hepatic necrosis with haemorrhage and neutrophil infiltration. The liver of one pubescent shrew also contained helminth parasites which were seen within and outside multifocal suppurative hepatitis and haemorrhage (**Figure 3.6**). Two animals had subendothelial protozoan cysts within portal arteries, which did not induce any alteration.

3.3.3.8 Lungs

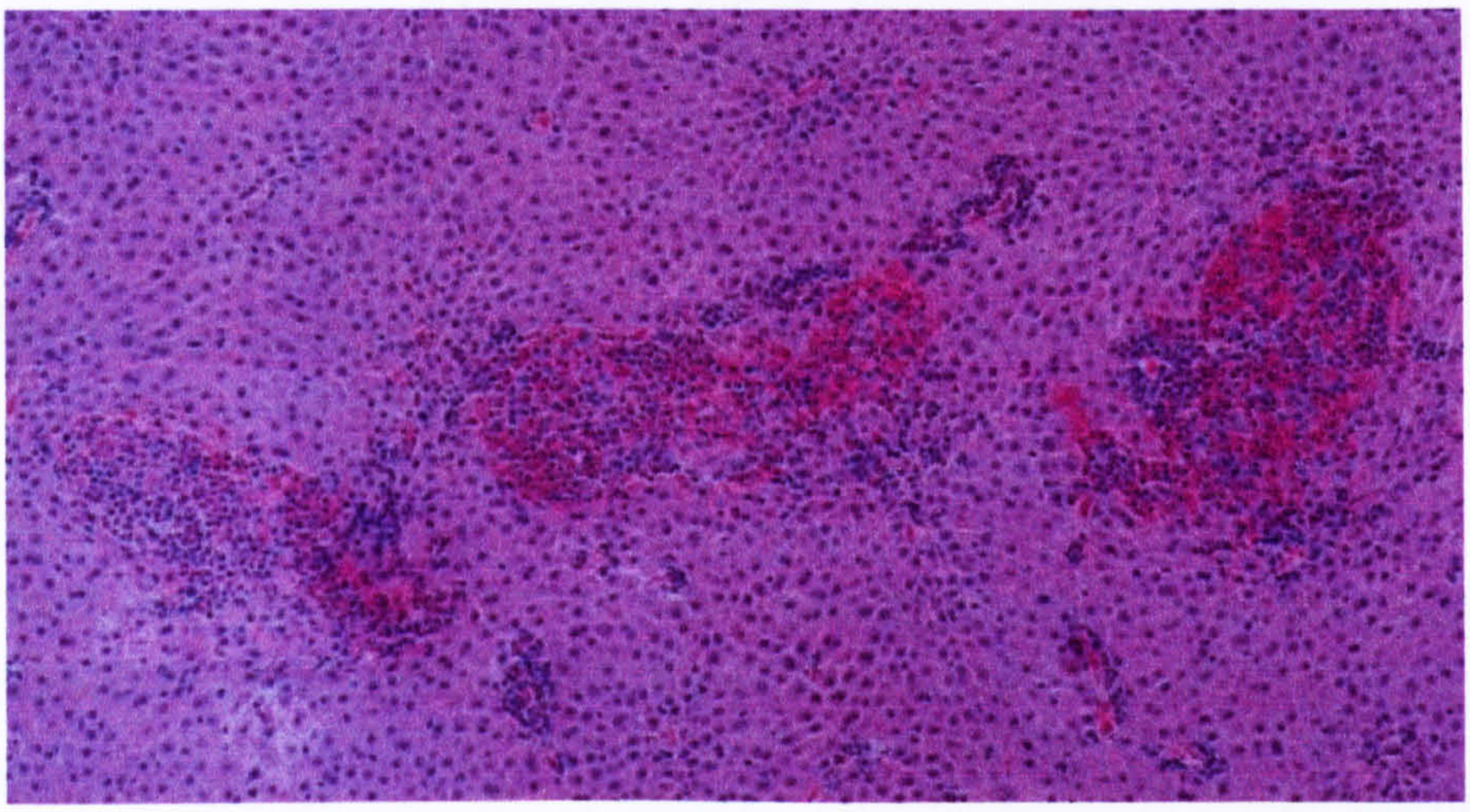
Lung tissue was examined from 41 animals, 27 of which exhibited moderate hyperaemia surrounded by fibroblasts. Other findings included accumulations of lymphocytes (13 animals), haemorrhage (six animals), and granulomas (two animals). Four animals showed no pathological alterations.

a.



x210

b.



x105

Figure 3.6 One or more helminth parasites in hepatic tissue (a.) of *S. araneus*, associated with multifocal necrosuppurative inflammation (b.).

Table 3.3 Severity of liver inflammation in common shrews

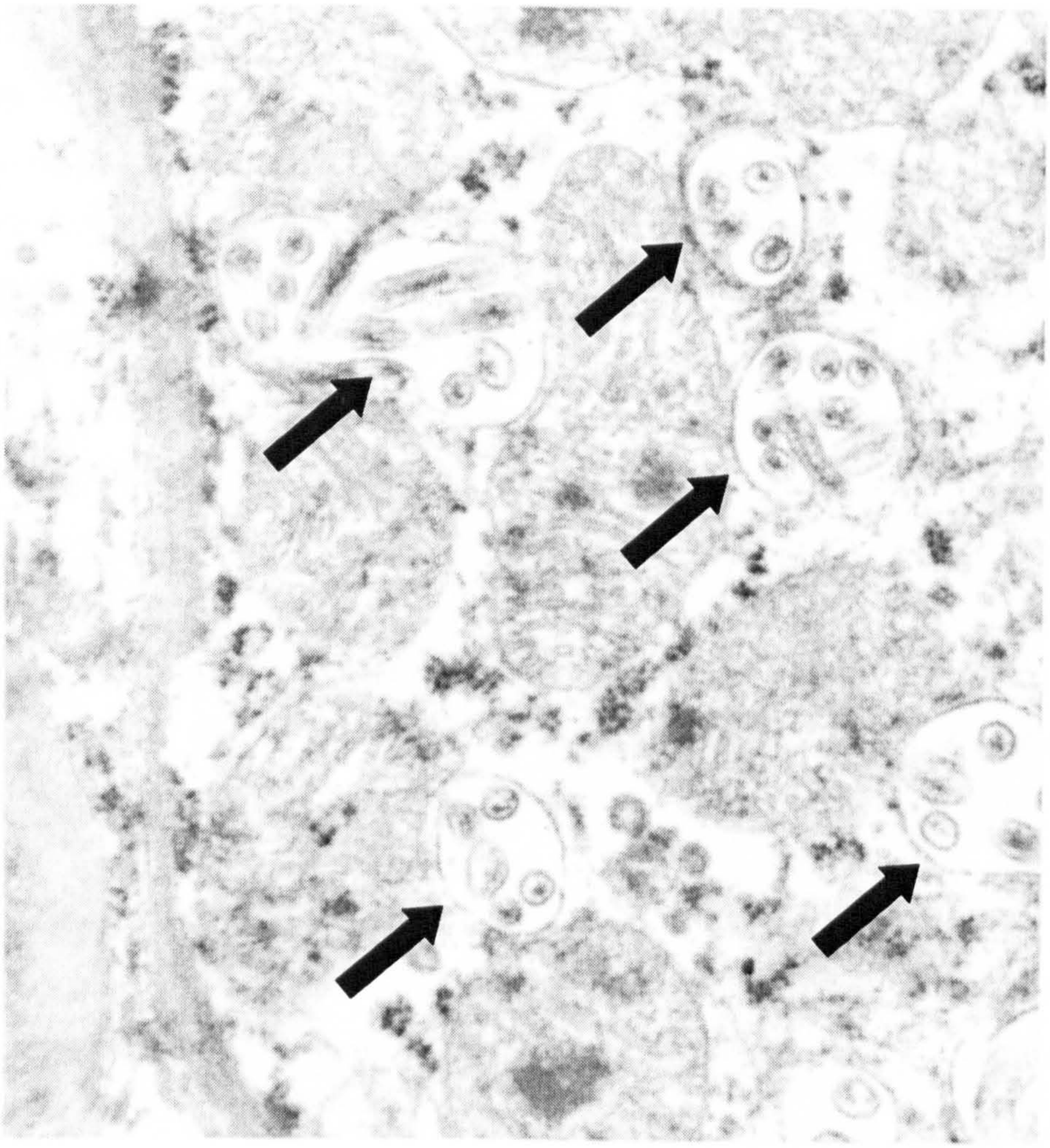
	Mild	Mild to Moderate	Moderate	Moderate to severe	Severe
Sub-adults					
Females	2	4	4	1	0
Males	2	1	4	0	0
Pubesecents					
Females	1	1	0	0	0
Males	0	1	0	0	0
Adults					
Females	4	2	3	1	1
Males	3	2	6	0	0

3.3.3.9 Myocardium

Of 39 examined animals, 14 harboured protozoan cysts in vessel walls within the myocardium (**Figure 3.5b**), associated with mild granulomatous inflammatory infiltration in five animals, and vessel wall thickening in three cases. One shrew exhibited mild focal perivascular infiltration without evidence of cysts. The remaining animals should no evidence of pathological alteration.

3.3.3.10 Oesophagus

Five of seven animals exhibited pathological alterations in the oesophagus. One animal exhibited focal hydropic degeneration of epithelial cells, associated with attachment of the digenean *Brachylaemus fulvus* (Roots, 1992). Two animals exhibited apparent intracytoplasmic structures within degenerating epithelial cells (**Figure 3.7**) associated with focal epithelial proliferation and hydropic degeneration, and two other possessed nematodes between epithelial layers (identified by egg morphology as *Eucoleus oesophagicola*, Roots, 1992), associated with mixed cellar infiltration of the mucosa, and in one animal focal hydropic degeneration of the superficial epithelium.



x28400

Figure 3.7 Apparent intracellular parasites within epithelial cells of the oesophagus of *S. araneus*.

3.3.3.11 Pancreas

Pancreatic tissue was examined in 14 animals, none of which showed signs of pathological alteration.

3.3.3.12 Pancreas of Aselli

The structure and function of this unique organ is described in detail in **Chapter 4 (Section 4.3.2.2)**: only pathological alterations are outlined here. Five of the 41 animals from which tissue were examined had granulomas within the pancreas of Aselli, in one case surrounding a nematode. Two adult males exhibited focal neucrosuppurative inflammation, again in one case surrounding a nematode, while one adult male and one adult female exhibited pyogranulomatous inflammation. Six animals (2 sub-adult females, 3 adult females and one adult male) showed signs of fibrosis, and one adult female had protozoan cysts without inflammatory reaction.

3.3.3.13 Skeletal muscle

In one adult female shrew protozoan cysts with features of *Sarcocystis* sp. were found within skeletal muscle myocytes not causing any reaction. Tissue from the remaining 33 examined animals showed no alteration.

3.3.3.14 Spleen

Protozoan cysts were found in the red pulp of one animal without reaction. Full details of the structure and function of the shrew spleen are presented in **Chapter 4 (Section 4.3.2.1)**.

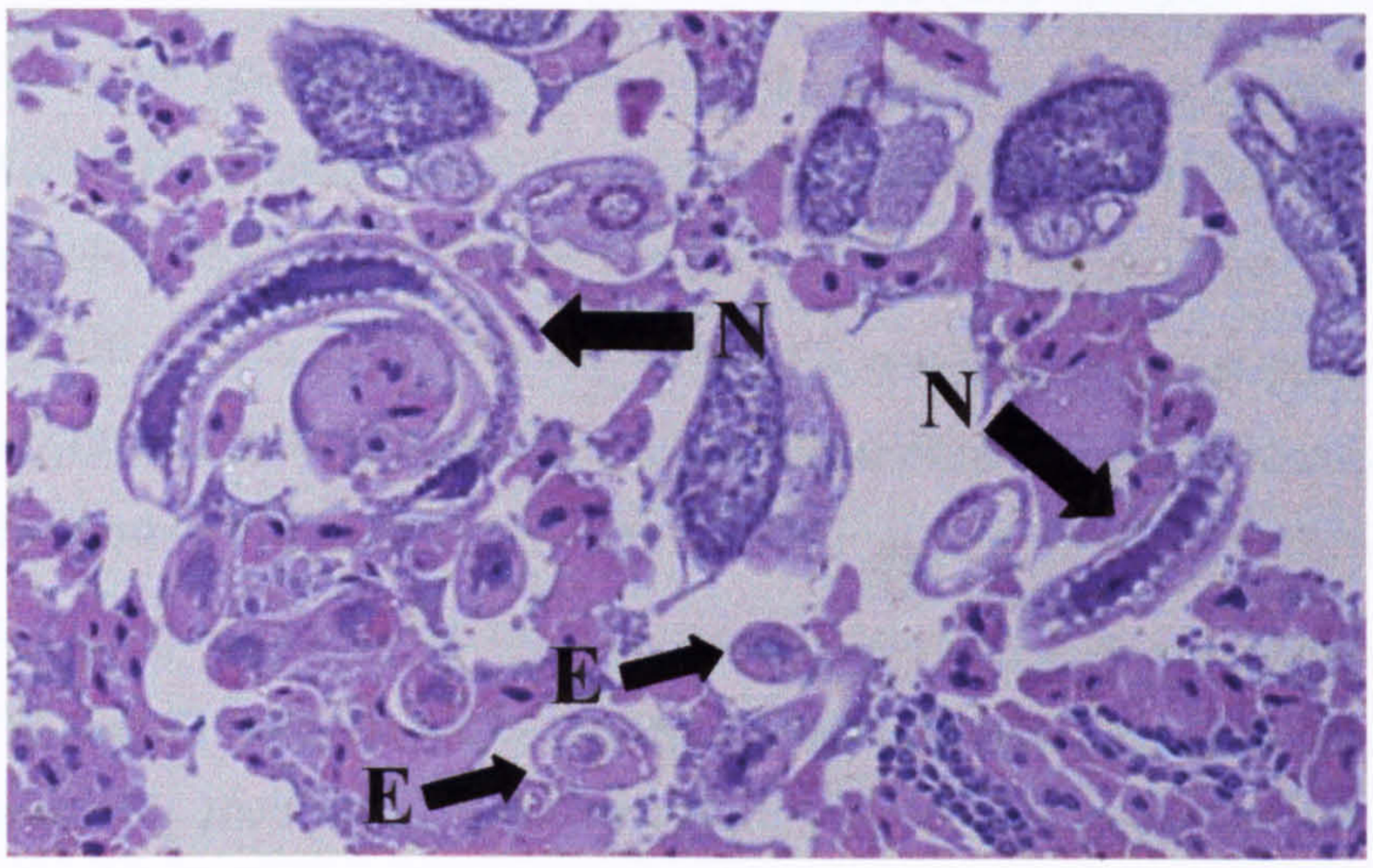
3.3.3.15 Stomach

Stomach tissue from 11 of 14 (79%) animals examined showed no alteration. One common shrew exhibited mild focal accumulations of lymphocytes in the mucosa, while another had a mild submucosal focal neutrophilic leukocyte infiltration. The remaining animal possessed a protozoan cyst within a submucosal vessel wall (without reaction) and exhibited mild mononuclear and moderate mixed cellular infiltrations.

3.3.3.16 Urinary bladder

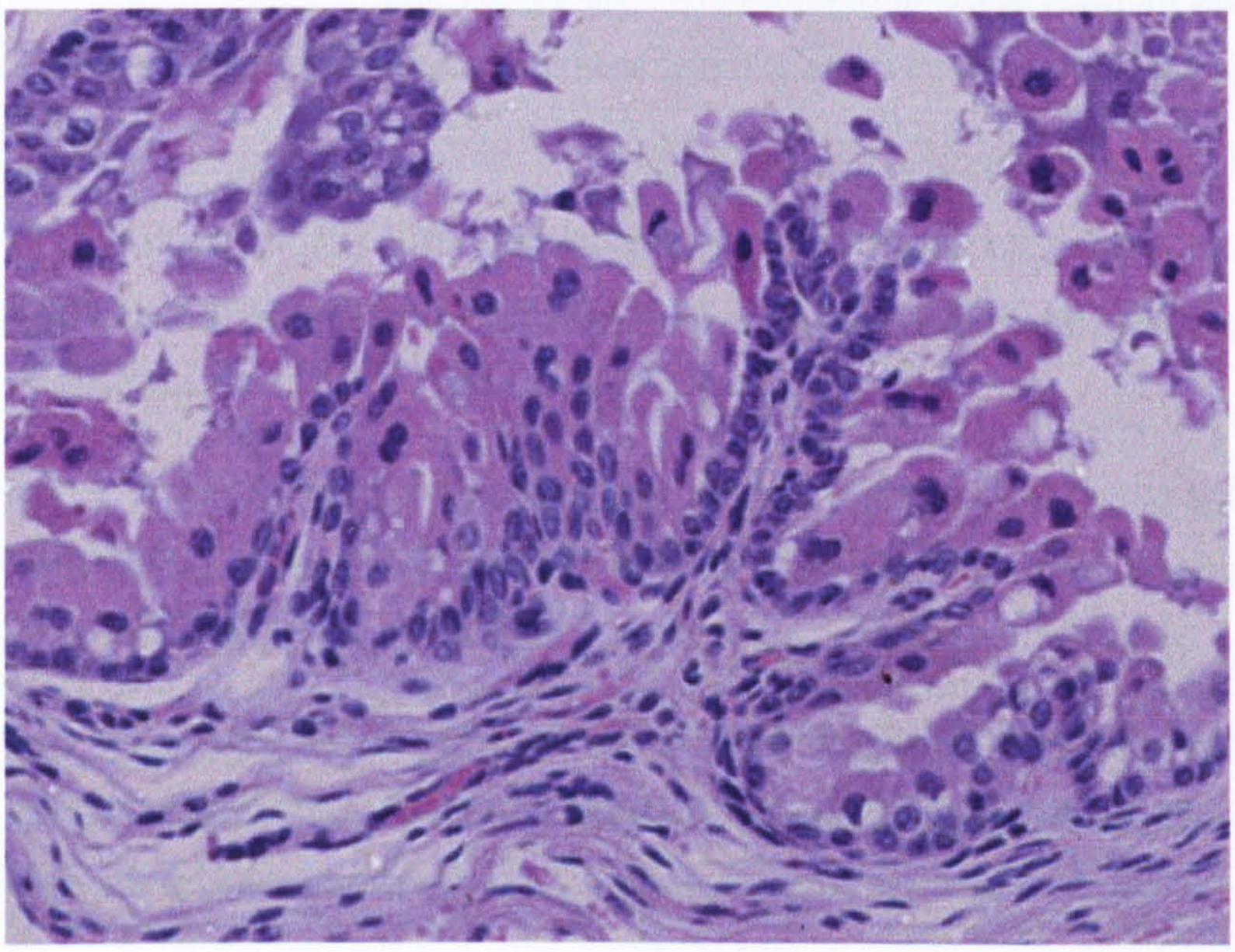
Nematodes in the urinary bladder were found in the lumen and between epithelial cells in ten of 28 (36%) animals (**Figure 3.8a**). The structure of the eggs revealed these nematodes to be *Liniscus incrassatus*, known to inhabit the bladder of shrews (Roots, 1992). In two cases, infection was associated with mild to moderate diffuse mixed cellular mucosal infiltration, while another animal exhibited moderate focal neutrophil infiltration. A further infected individual showed moderate chronic lymphocyte-dominated cystitis, and another exhibited degeneration and sloughing of epithelial cells (**Figure 3.8b**). One animal possessed a protozoan cyst within a vessel wall, without reaction.

a.



x400

b.



x310

Figure 3.8 Nematodes (*Liniscus incrassatus*; N) and nematode eggs (E) within lumen of urinary bladder of *S. araneus*, associated with sheathing of epithelial cells (b.).

3.4 Discussion

Previous studies of parasitism in *S. araneus* have focussed mainly on the identification and quantification of helminths (**Section 1.8**). The results found here concur with those of previous investigations which have found helminth parasitism to be highly prevalent in shrews (Sharpe, 1964; Lewis, 1968; Haukisalmi *et al.*, 1994; Shimalov, 2001). This would seem to suggest *S. araneus* cannot avoid becoming infected by helminths carried in their invertebrate prey (Sharpe, 1964; Lewis, 1968). Intuitively, shrews might be expected to accumulate helminth parasites with age. However, parasite abundances in older animals may decrease as a result of development of host immunity (Gregory *et al.*, 1990), or a positive relationship with host mortality (hosts harbouring high parasite burdens die before reaching old age; Anderson & Gordon, 1982; Gregory *et al.*, 1992). Here, abundances of bladder nematodes, *Porrocaecum* sp. larvae and gut helminths were found to be greater in adults than sub-adults. A study of gut helminths in shrews from Lapland (which similarly partitioned shrews into juvenile and adult classes) found evidence of greater helminth abundance in adult males compared to juvenile males, but no similar trend for females (Haukisalmi *et al.*, 1994). A more detailed temporal study showed prevalence of individual helminth species to vary over the course of the year, with some of the most common species peaking in sub-adults in spring and in adults during the summer of the following year (Kisielewska, 1963). This is most likely a consequence of the increased number of invertebrate intermediate hosts available for consumption during spring and summer (Kisielewska, 1963). It appears therefore that the pattern of helminth abundance in shrews is more complicated than simple linear accumulation with age, but that adults still harbour more helminths than sub-adults.

Here, no evidence was found for a sex difference in abundance of bladder nematodes, or *Porrocaecum* sp. larvae between shrews. However, males were found to harbour more gut helminths then females. Previous studies have found little evidence of sex differences in patterns of parasite infestation in *S. araneus* (Lewis, 1968; Haukisalmi *et al.*, 1994). Differences between sexes in infection are usually attributed to hormonal effects on the immune system (Zuk & McKean, 1996) or differences in behaviour (Tinsley, 1989). No study has yet investigated the effects of hormones on parasite

burdens in common shrews, but the behaviour of males and females is thought to remain similar until the onset of sexual maturity (Barnard *et al.*, 1983; **Section 1.7**). By exhibiting similar patterns of foraging behaviour and food consumption, male and female shrews may have equal exposure to parasites as sub-adults, outweighing any differences in hormonal status that may create discrepancies in patterns of infection. However, differences in adults might be expected as a consequence of hormonal maturation and/or sexual dimorphism in behaviour or food consumption (Haukisalmi *et al.*, 1994). Further study is required to investigate whether the greater abundance of gut helminths in males is related to numbers of one or more individual parasite species (perhaps infecting more males than females through a sex-specific preference for a particular prey item containing intermediate parasite stages), or general relationships between host food intake, immunological status and host sex. In addition, more detailed statistical analysis is needed to ascertain whether the observed difference occurs equally in both sub-adult and adult shrews.

The present study attempted to go beyond quantifying parasite burdens of shrews to examine pathological changes associated with infection. The results suggest shrews suffer a diverse array of parasite-mediated pathology, particularly in association with helminths. Prior to this study, only two attempts had been made to examine pathological changes in common shrews. Vaucher (1971) provided photographs of the attachment of cestodes to the gut wall, and concluded that even the heaviest infection would result in negligible damage to the intestine. Here, no evidence was found for pathological changes associated with gut helminths, although several animals showed signs of gut enteritis which could not be related to a causal organism. Given the food requirements and high metabolic rates of *S. araneus*, any pathological change in the gut which hinders nutrient absorption might be more detrimental to common shrews than to other host species (Haukisalmi *et al.*, 1994). Similarly, helminth parasites occurring in the gall bladder may interfere with bile production, hampering lipid absorption (Soveri *et al.*, 1994) and depleting further the restricted fat reserves of *S. araneus* (Churchfield, 1981).

In common with Soveri *et al.* (1994), who provided brief descriptions of pathology in *S. araneus* from Finland, a high proportion of animals examined here had

protozoan cysts, occurring within vessel walls in the kidneys and myocardium, and occasionally in the liver and other organs. In most instances, these cysts did not induce any alterations apart from an occasional slight thickening of the affected vessel wall or a mild granulomatous inflammatory infiltration. Given the demands placed on the heart by the fast metabolic rate of *S. araneus* (Nagel, 1994), even slight alterations of myocardial tissue may be detrimental to common shrews (Soveri *et al.*, 1994).

The parasite *Porrocaecum* sp. is believed to use *S. araneus* as a paratenic host, remaining encapsulated until consumed by the final host, possibly the tawny owl (*Strix aluco*; Roots, 1992). Previously, it has been suggested that in high numbers this parasite may irritate its shrew host to such an extent as to make its behaviour erratic, increasing the chances of predation (Buckner, 1969). Interestingly, no evidence was found here for an inflammatory reaction associated with encapsulated *Porrocaecum* sp. larvae. This may suggest that, at least at this life-history stage, this parasite is relatively benign, causing little irritation to its host.

The results of this thorough histological examination suggest common shrews are able to withstand both prevalent and diverse parasite-mediated pathology, and are capable of mounting immune reactions against infectious agents, despite their resource-limited physiology. The following chapter considers the lymphatic tissues of common shrews in more detail, in the hope of further understanding their ability to mount immune responses, and any changes in this capacity with age.

Chapter 4. Haemolymphatic tissues in common shrews of different ages

4.1 Introduction

The results presented in **Chapter 2** and **Chapter 3** supplement the findings of previous studies which have reported *Sorex araneus* to be host to a variety of both micro and macroparasites (**Section 1.8**). In addition, **Chapter 3** demonstrated that shrews are able to mount immune responses against many of these parasites despite energetic restrictions placed upon them by their resource-limited physiology (Genoud, 1988). This ability poses interesting questions as to the structure and function of their lymphatic tissues. Until now, studies of the haemolymphatic system in *Sorex araneus* have been fragmentary at best. Most have focussed on the "pancreas of Aselli", a large lymphoid organ, seemingly unique to shrews, which drains the small intestine (Holmes, 1965; Twigg & Hughes, 1970; Tsiperson, 1997). Studies of spleen and bone marrow morphology and function have been restricted to a related species, the musk shrew (*Suncus murinus*; Fukuta *et al.*, 1982; Ishizeki & Tokio, 1989). With the exception of studies examining changes in blood leukocyte counts (Wolk, 1981) and the size of the pancreas of Aselli over time (Twigg & Hughes, 1970), no attempt has been made to examine age-related changes in structure and function of haemolymphatic tissues in common shrews. Senescence of lymphatic tissues ('immunosenescence') could reduce the capacity of *S. araneus* to respond immunologically to infection in later life. If parasites are able to develop unchallenged by the immune system in older individuals, then immunosenescence could be a factor in the age-related increases in parasite abundances reported from common shrews (Haukisalmi *et al.*, 1994; **Chapter 3**). Depletion of lymphatic tissues may also result in previously latent infections becoming uncontrollable and eventually fatal, as has been described in male dasyurid marsupials (Barker *et al.*, 1978; Poskitt *et al.*, 1984, see **Section 1.3**). If this is the case in *S. araneus*, parasitism may contribute to the synchronous mortality of adult common shrews after the breeding season (Buckner, 1969; Pernetta, 1977).

Here, morphological and functional features of common shrew haemolymphatic tissues are defined and age-related changes assessed. To this purpose, both light and electron microscopy are employed, using immunohistological methods to identify

leukocytes (T and B lymphocytes, macrophages/monocytes) and evaluate cellular turnover. Relationships between immunosenescence, parasite burden, and host age class are investigated statistically, as are differences in relative weights of lymphatic tissues between age classes.

4.2 Materials and methods

4.2.1 Animals and tissue processing

Lymphatic tissues were removed at dissection from the 43 wild shrews (19 male, 24 female) used for pathological examination (**Chapter 3**). In most cases weights of spleen, pancreas of Aselli and body mass (minus gut) were recorded at dissection. Prior to light microscopy, major organs from all animals were fixed in 4% buffered paraformaldehyde for 24 - 48h, with tissue samples routinely embedded in paraffin wax. 5µm-thick sections were stained with haematoxylin-eosin, or in the case of haemolymphatic tissues (spleen, pancreas of Aselli, bone marrow (sternum) and in selected cases mesenteric or mediastinal lymph nodes and thymus) used for immunohistological examination. For electron microscopy, tissue samples from the pancreas of Aselli of one sub-adult common shrew were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer (pH 7.4) and routinely embedded in epoxy resin. Both semi-thin sections (1µm) and thin sections were prepared and the latter examined using transmission electron microscopy.

4.2.2 Immunohistology and TUNEL method

Immunohistology and TUNEL method were performed on tissues from 21 common shrews across all age groups. Cross-reactivity of antibodies (the extent to which antibodies developed to label cell populations in other species are applicable for use in shrews) was evaluated using splenic morphology as a guide, the observed morphology being very similar to that from other species. Accordingly, this allowed for identification of lymphatic follicles, T cell zones and red pulp. Both monoclonal and polyclonal antibodies applied in other species (Milner *et al.*, 1996; Kipar *et al.*, 1998a; Kipar *et al.*, 1998b; Köhler *et al.*, 2000) were used to identify leukocytes and proliferating cells in shrew tissues, employing both peroxidase anti-peroxidase and

avidin biotin peroxidase complex methods. Antibodies, their sources and detection methods are listed in **Table 4.1**. T lymphocytes were identified by demonstrating presence of CD3 (a pan T cell marker in many other mammalian species (Milner *et al.*, 1996; Kipar *et al.*, 1998a) whilst antibodies against CD45R of B cells (Butcher *et al.*, 1982; Kipar *et al.*, 1998b) and CD79a (Chu and Arber, 2001) were used in an attempt to stain B cells. Presence of neutrophils and monocytes/macrophages was demonstrated using antibodies against human myeloid/histiocyte antigen (calprotectin; Kipar *et al.*, 1998b) and lysozyme (Kipar *et al.*, 1998a).

Cellular turnover of lymphatic tissues was evaluated according to the number of detectable proliferating and apoptotic cells. Proliferating cells were identified via their expression of the proliferating cell nuclear antigen (PCNA; McCormick & Hall, 1992; Köhler *et al.*, 2000), whilst apoptotic cells were demonstrated *in situ* by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling of DNA fragmentation sites) method (Gavrieli *et al.*, 1992; Köhler *et al.*, 2000) using a commercially available kit according to the manufacturer's instructions (ApopTag™ In Situ Apoptosis Detection Kit; Chemicon, California, USA).

Consecutive tissue sections, incubated with normal rabbit or rat serum or a non-reacting mouse monoclonal antibody, were used as negative controls for polyclonal and monoclonal antibodies respectively. For the TUNEL method, TdT was replaced by distilled water on negative control slides.

4.2.3 Assessment of bone marrow and lymphatic follicle activity

Lymphatic follicles in the spleen and lymph nodes of all animals were classified as relatively large or small, and the presence and degree of depletion (none, mild, moderate or severe) assessed on the basis of the cellularity of the germinal centre. Similarly, bone marrow activity was classified as low, moderate or high based on the ratio of haematopoietic cells to adipose tissue in a cross section of the marrow in the xyphoid bone.

Table 4.1 Antibodies and detection methods used to identify leukocytes and proliferating cells in *S. araneus*

Cells identified	Antibody	Detection method; pre-treatment
B cells (strongest expression in undifferentiated B cells)	Rat anti-mouse CD45R (clone B220, Ly5)[1]	ABC[2]; citrate buffer pre-treatment
B cells (predominantly differentiated B cells)	Mouse anti-human CD79a (clone HM57)[3]	ABC[2]; citrate buffer/EDTA pre-treatment
T cells	Rabbit anti-human CD3[3]	PAP[4]; protease pre-treatment
Monocytes/macrophages, neutrophils, myelomonocytic precursors (not 100% of cells)	Mouse anti-human myeloid/histiocyte antigen (clone MAC387)[3]	PAP[4]; protease pre-treatment
Monocytes/macrophages, neutrophils, myelomonocytic precursors (not 100% of cells)	Rabbit anti-human lysozyme[3]	PAP[4]; protease pre-treatment
Proliferating cells	Mouse anti-human PCNA (clone PC10)[3]	PAP[4]; citrate buffer pre-treatment

1. Cedarlane, Hornby, Canada
2. ABC - avidin biotin peroxidase complex method
3. Dako Cytomation, Ely, Cambridgeshire, UK
4. PAP - peroxidase anti-peroxidase method

4.2.4 Statistical analysis

As in **Chapter 3**, statistical analysis was restricted to sub-adult and adult animals, as sample size for pubescent shrews (N = 3) was too small. General linear models (GLM) were employed to investigate whether relative masses of lymphatic organs (pancreas of Aselli and spleen) differed between common shrews of different age categories and sex. To assess and control for effects of body mass, organ weight (either spleen or pancreas of Aselli) was entered as the dependent variable in a GLM and regressed against the shrew's total weight at culling, minus the weight of the gut (in order to remove variability associated with recently consumed food). Host age (sub-adult or adult) and host sex (male or female) were then added to the model, first individually and then simultaneously, and significance of increases in r^2 associated with adding each term (or both terms together) assessed using F-tests.

Ordinal logistic regression was used to examine whether bone marrow and lymphatic follicle activity (primary/secondary follicles, presence and degree of follicular depletion) in the spleen and pancreas of Aselli varied with common shrew sex and age class. Sex and age category were entered simultaneously as independent variables into each of five models (one per dependent variable described above) and the significance of each term assessed by Wald tests.

In order to investigate potential associations between helminth abundances (as calculated in **Chapter 3**) and host follicle activity, categorical principal component analysis was first used to create a one-dimensional component which combined scores for follicle size and depletion in both the pancreas of Aselli and spleen. Spearman correlations then investigated relationships between this measure and number of macroparasites in the alimentary tract (oesophagus, stomach and gut), number of nematodes in the bladder, and number of *Porrocaecum* sp. larvae encapsulated in the intrascapular region and body cavity.

4.3 Results

4.3.1 Identification of leukocytes, proliferating and apoptotic cells

Immunohistological staining of the spleen showed that all antibodies used cross-reacted with shrew leukocytes. CD45R and CD79a were both expressed by B cells, as confirmed by staining of lymphocytes in follicles, but with different distribution patterns for each antigen. CD45R was strongly expressed in follicular germinal centres, but relatively faintly in well-differentiated B cells within follicular mantle zones. In contrast, staining for CD79a was weaker in follicular germinal centres and stronger in the periphery. Primary follicles, however, were entirely composed of CD79a-positive lymphocytes. Plasma cells were negative or exhibited only a faint staining for both antigens. Staining for CD3 identified lymphocytes in T cell zones and thereby appeared to be a pan T cell marker. Both the myeloid/histiocyte antigen and lysozyme were expressed by monocytes/macrophages and at least a subset of neutrophils. They were both also expressed by a high percentage of cells in the bone marrow.

PCNA expression was observed in follicle germinal centres, T cell zones, bone marrow and splenic red pulp, the sites in the haemolymphatic tissue expected to contain proliferating cells. The majority of megakaryocytes were also positive for PCNA.

TUNEL method identified cells with the morphology of apoptotic cells (Gavrieli *et al.*, 1992; Köhler *et al.*, 2000) as well as apoptotic bodies (both free and within tingible body macrophages), located predominantly in follicular germinal centres.

4.3.2 Relative weights, morphology and composition of lymphatic tissues and bone marrow

4.3.2.1 Spleen

A positive relationship with body mass (minus gut weight) explained 48% of the variability in spleen weight at culling (**Table 4.2, Figure 4.1**). Addition of both host age and sex to the model, either individually or simultaneously, led to significant increases in r^2 (**Table 4.2**). In the model containing all three terms, spleen weight remained positively related to body mass, and adult animals and females had relatively larger spleens than sub-adults and males respectively (**Table 4.3, Figure 4.1**).

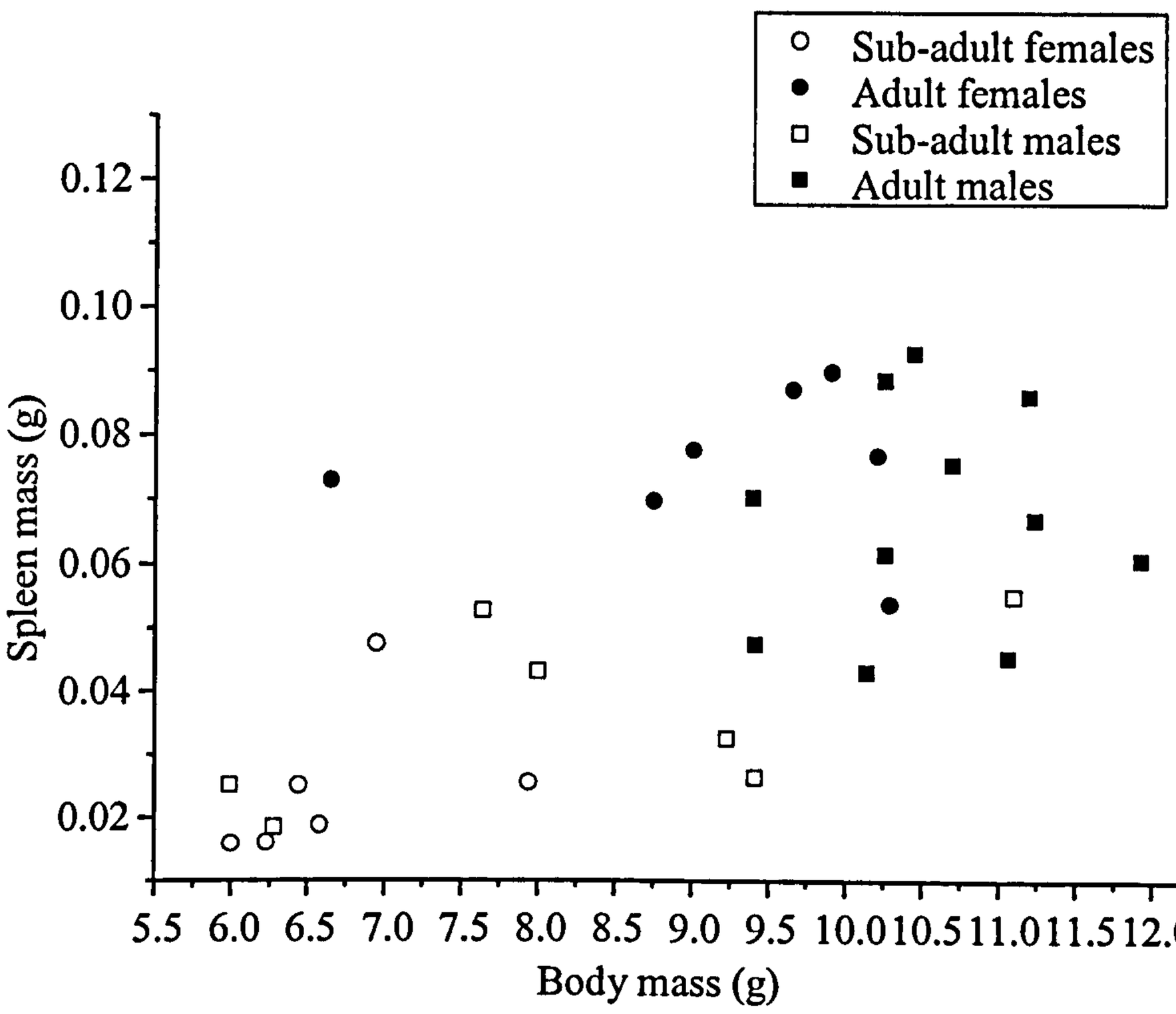


Figure 4.1 Relationship between body mass (minus gut weight) and spleen mass for male and female common shrews of different age classes.

Table 4.2 Changes in r^2 associated with adding terms to a general linear model with spleen weight as the dependent variable. Body mass (minus gut weight) was retained in all models

	R^2 change	**F**	**df**	**P**
Body mass[1]	0.48	28.9	1, 31	<0.001
+ Age	0.15	12.1	1, 30	<0.01
+ Sex	0.11	8.1	1, 30	<0.01
+ Age + Sex	0.20	9.2	2, 29	<0.0001

1. Minus weight of gut

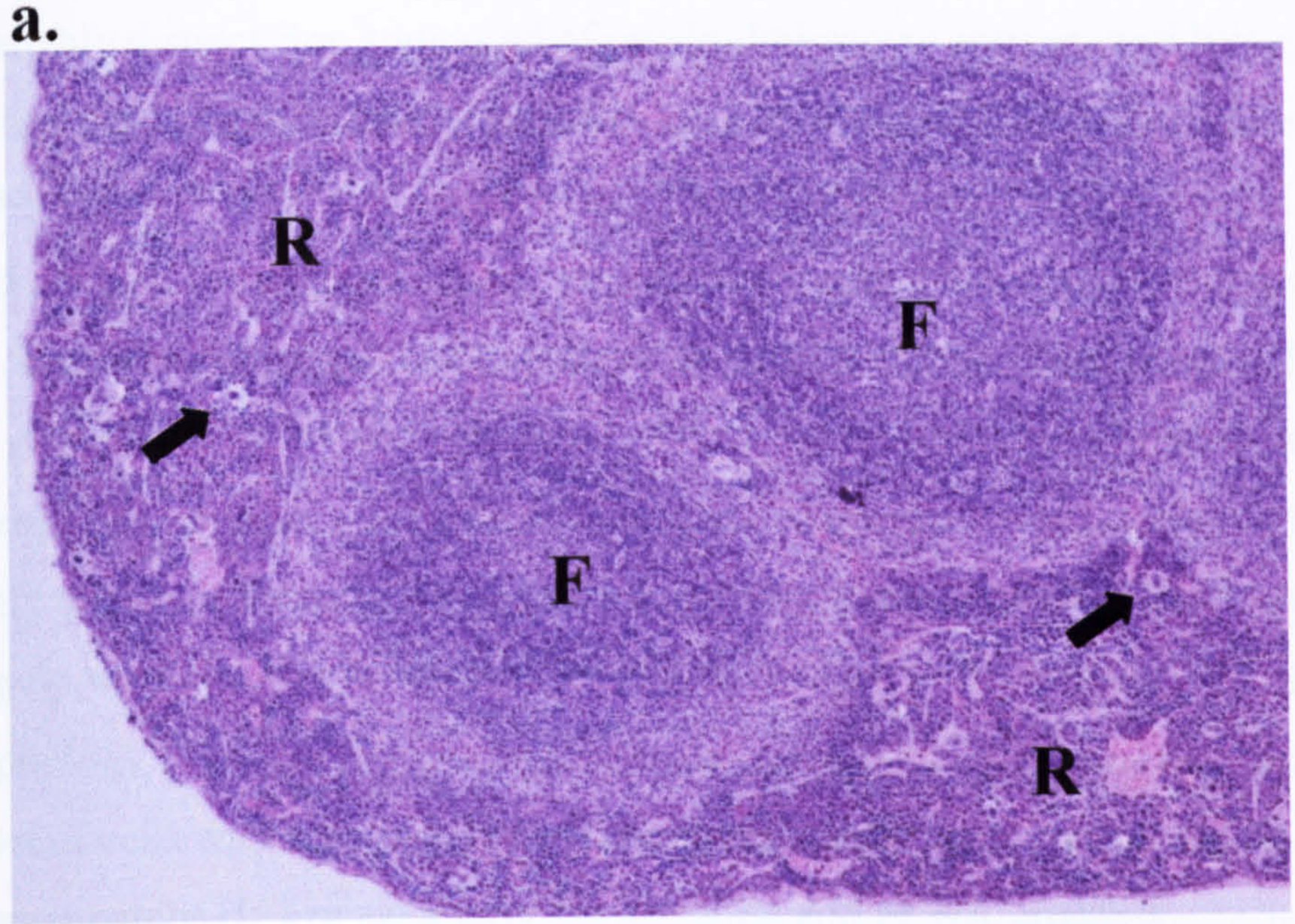
Table 4.3 Partial coefficients of independent variables in a GLM with spleen weight as the dependent variable and host body mass, age category and sex as independent variables

	Unstandardized Coefficient	**SE**	**t**	**df**	**P**
Constant	-0.026	0.016	-1.63	29	NS
Body mass[1]	0.007	0.002	2.88	29	<0.01
Age	0.026 [2]	0.009	2.90	29	<0.05
Sex	-0.014 [3]	0.007	-2.19	29	<0.05

1. Minus weight of gut
2. Indicates larger relative spleen mass in adults than sub-adults
3. Indicates larger relative spleen mass in females than males

The white pulp (lymphatic follicles and T cell zones) was generally confined to the organ's centre, where follicles were arranged singly or in groups (**Figure 4.2**). Follicle centres contained up to 20% CD3-positive T cells. Up to 50% of the cells within germinal centres were PCNA-positive. Follicles and follicle groups were surrounded by

a.



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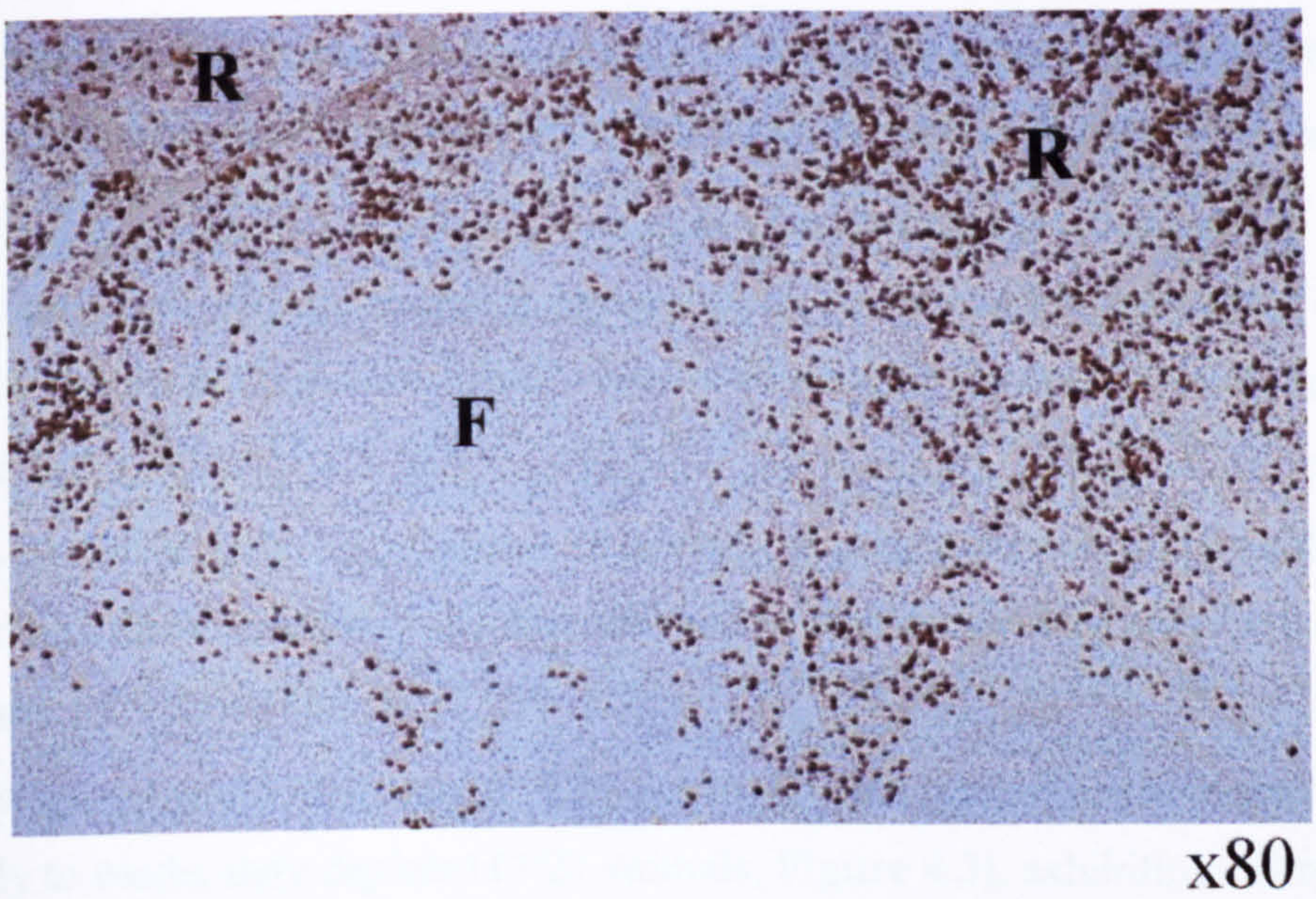

Figure 4.2 Composition of the spleen in *S. araneus* visualized using haematoxylin-eosin staining (a.) and MAC 387 antibodies (b.). Follicle centres (F) are arranged centrally either singularly or in groups, surrounded by red pulp (R) containing erythrocytes and cells positive for myeloid/histiocyte antigen (brown stain, b.), with megakaryocytes dispersed throughout (arrows, a.).

a variably distinct rim, composed of myeloid/histocyte antigen- and lysozyme-positive macrophages and neutrophils, variable numbers of strongly CD79a-positive B cells and CD3-positive T cells, and erythrocytes. T cell zones were arranged around medium-sized arteries, forming periarterial lymphatic sheaths. They appeared similar in size and cell density in all animals and contained up to 10% PCNA-positive cells. The red pulp was generally cell-rich and composed of neutrophils and fewer lymphocytes and macrophages, intermingled with erythrocytes. Numerous megakaryocytes were found evenly distributed throughout (**Figure 4.2a**). In general, 30-50% of cells in the splenic red pulp were positive for myeloid/histiocyte antigen and lysozyme, with the morphology of monocytes/macrophages and neutrophils (**Figure 4.2b**). However, a subset of cells with neutrophil morphology was negative for both antigens. Approximately 10% of cells were proliferating and PCNA-positive.

Differences between age groups were observed in both the composition and functional state of the red and white pulp of the spleen. The amount of both neutrophils and megakaryocytes in the red pulp often appeared higher in adult animals than in sub-adults. The white pulp of sub-adult animals was exclusively comprised of secondary follicles, which often appeared interconnected and formed large groups. Follicles were for the most part large and without signs of depletion (**Figure 4.3**), and included numerous apoptotic cells and tingible body macrophages, as well as several mitotic cells. The three pubescent animals exhibited primary and/or secondary follicles, the latter with features similar to sub-adult shrews. The majority of adult animals exhibited a mixture of primary and secondary follicles (**Figure 4.3**), which were small and only partially connected with clearly visible perifollicular rims. Follicle centres were often mildly to moderately depleted (9/21 animals, **Figure 4.3**), exhibiting collagen deposition in four animals. Where present germinal centres exhibited mitotic as well as apoptotic cells.

Sub-adult shrews were found to have significantly larger follicles than adults (Wald = 17.17, df = 1, $P<0.05$, **Figure 4.3**) but no difference in follice size was found between sexes (Wald = 0.67, d.f. = 1, NS). No significant difference in the presence and degree of follicular depletion was found between males and females (Wald = 0.13, df = 1, NS) or between adult and sub-adult shrews (Wald = 2.10, df = 1, NS, **Figure 4.3**).

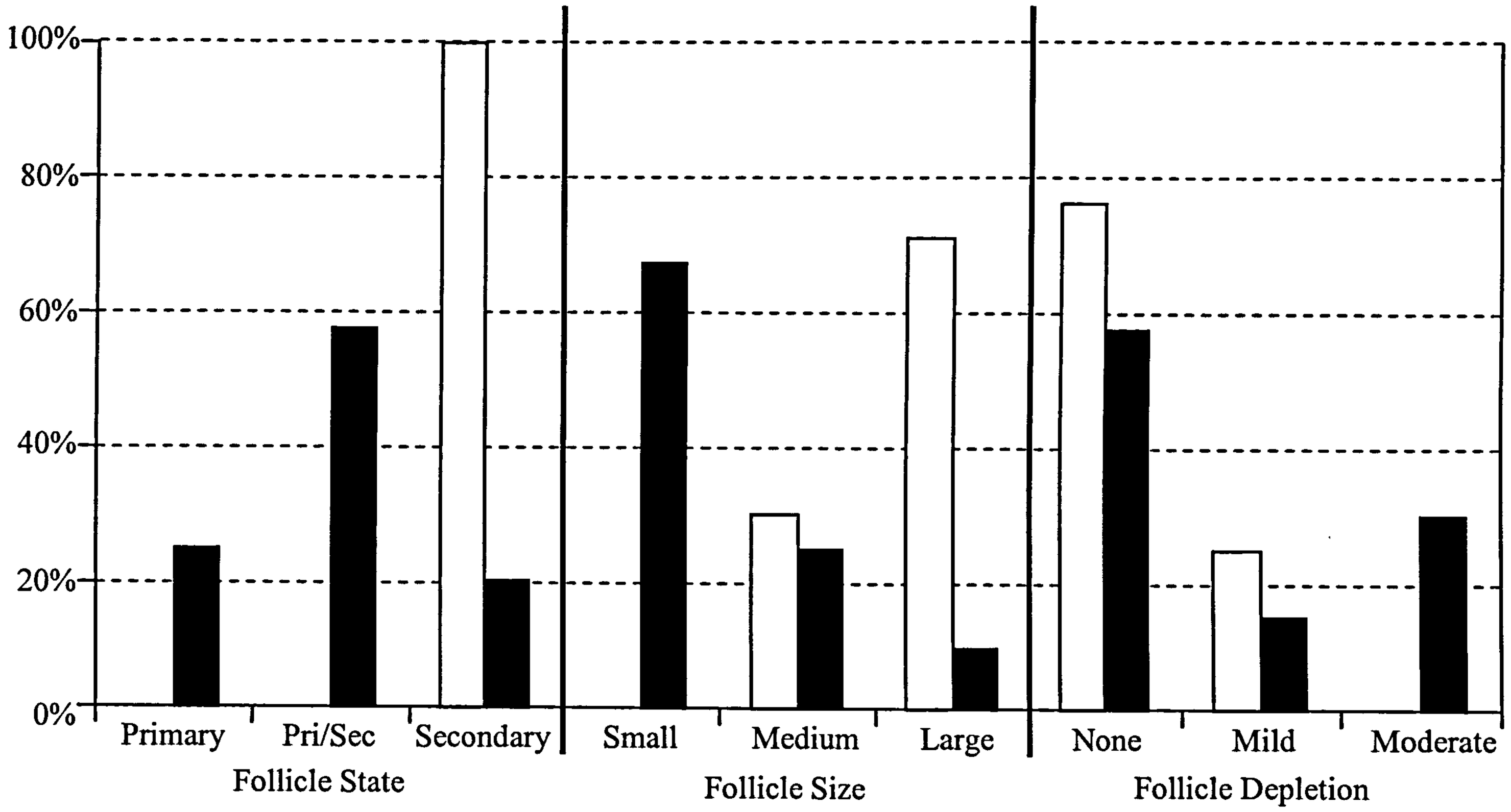

Figure 4.3 Comparison of follicle activity and functional state expressed in the spleen of sub-adult (open bars) and adult (filled bars) common shrews.

4.3.2.2 Pancreas of Aselli

After the removal of one extreme outlier (standardized residual = 4.6, **Figure 4.4**), a positive relationship with body weight explained 32% of variation in pancreas of Aselli weight (**Table 4.4, Figure 4.4**). However, neither host age nor sex significantly increased r^2 when added to the model (**Table 4.4, Figure 4.4**).

Table 4.4 Changes in r^2 associated with adding terms to a GLM exploring variability in pancreas of Aselli weight with body mass (minus gut weight) as an independent variable

	r^2	F	df	P
Body weight[1]	0.32	14.7	1, 31	<0.001
+ Age	<0.01	0.09	1, 30	NS
+ Sex	<0.01	0.06	1, 30	NS
+ Age + Sex	<0.01	0.06	2, 29	NS

1. Minus weight of gut

In general, the pancreas of Aselli exhibited a composition very similar to that of a lymph node (**Figure 4.5**). Beneath the capsule were marginal sinuses of variable width, containing vacuolated cells (generally negative for both myeloid/histiocyte antigen and lysozyme), disseminated lymphocytes and few myeloid/histiocyte antigen- and lysozyme-positive macrophages. Also present were variable numbers of neutrophils, either disseminated throughout or as small accumulations. In four adult shrews, the marginal sinuses exhibited focal to extensive fibrosis.

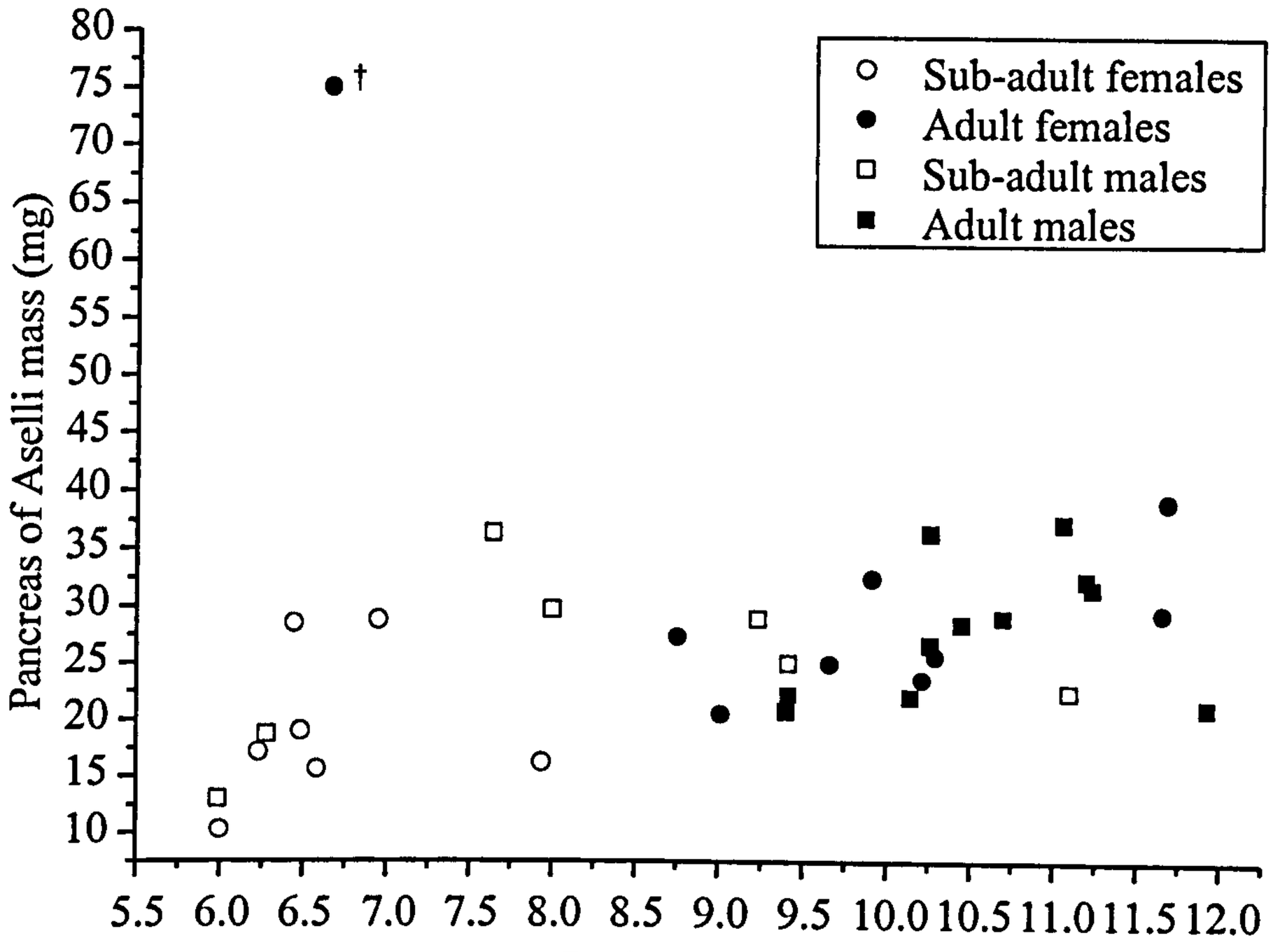

Figure 4.4 Relationship between body mass (minus gut weight) and pancreas of Aselli mass for male and female shrews of different age classes.

† Outlier removed prior to analysis (see text for details).

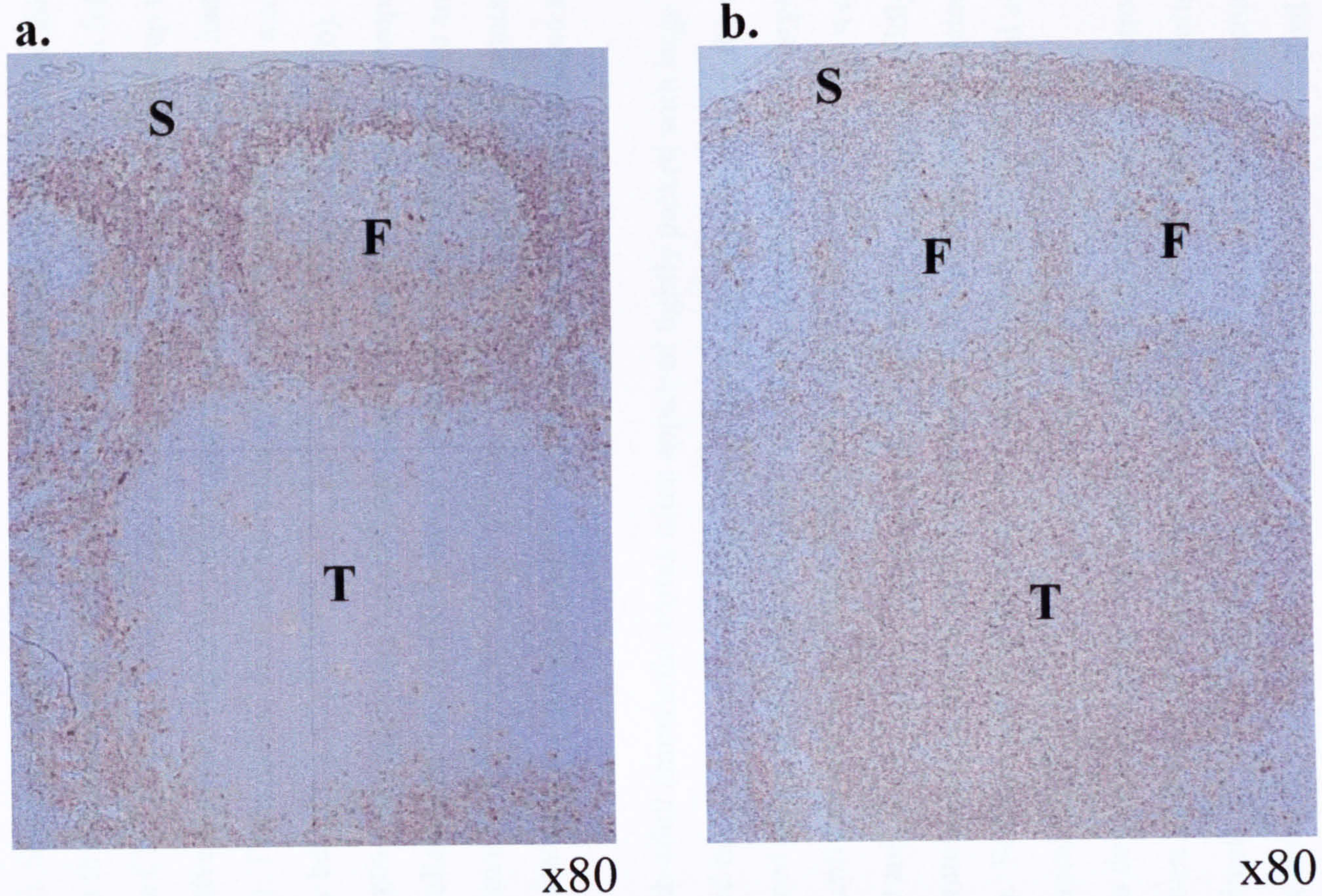

Figure 4.5 Composition of the pancreas of Aselli in *S. araneus* as visualized using immunohistological staining for B cells (a.) and T cells (b). Beneath the marginal sinus (S) are follicles (F) and a sub-cortical T cell zone (T).

Beneath the sinuses lay a cortex composed of follicles (**Figure 4.5**). Bar one adult male, which exhibited a mixture of primary and secondary follicles, the pancreas of Aselli of all animals contained only secondary follicles, regardless of host age or sex (**Figure 4.6**). Staining of follicle B cells for CD45R and CD79a was comparable to that described in the spleen (**Figure 4.5a, Section 4.3.2.1**). Up to 20% of cells in follicle centres were CD3-positive T cells (**Figure 4.5b**). Follicle centres exhibited up to 50% PCNA-positive, proliferating cells, and apoptosis was also frequent in most instances (**Figure 4.7**). T cell zones, almost entirely composed of CD3-positive cells, were located immediately beneath the follicles, thereby forming a paracortex (**Figure 4.5b**). T cell zones were generally similar in size and cell density in animals of all age groups. The centre (medulla) contained relatively loosely arranged, mostly empty sinuses and was otherwise almost entirely composed of plasma cells (**Figure 4.8**). Sinuses contained low numbers of scattered macrophages which expressed myeloid/histocyte antigen and/or lysozyme.

In sub-adult animals the cortex often appeared tightly packed with large follicles that exhibited no, mild or moderate depletion (**Figure 4.6**) In adult shrews, the cortex often contained only a small number of follicles (9/20 animals; 45%), with large areas of cortex frequently devoid of follicles (5/20; 25%). For the most part, follicles were either small (13/20 animals; 65%) or of moderate size (6/20 animals; 30%), and mildly (2/20; 10%), moderately (13/20; animals 65%) or severely (1/20 animals; 5%) depleted (**Figure 4.6**). Follicles occasionally seemed to extend outwards into marginal sinuses and in two animals exhibited central collagen deposition.

Areas devoid of follicles were also devoid of T cell zones. As a consequence, overall numbers of T cells in the pancreas of Aselli often seemed lower in adults than sub-adult animals. Where the cortex was devoid of follicles, plasma cells extended to the marginal sinuses or beyond to the capsule, such that the medulla often occupied most of the organ in adult animals. Accordingly, the whole organ frequently appeared as an accumulation of plasma cells, surrounded by a fragmentary cortex and paracortex.

Extramedullary haematopoiesis was observed in 9/21 adult animals, as represented by scattered megakaryocytes within the outer medulla.

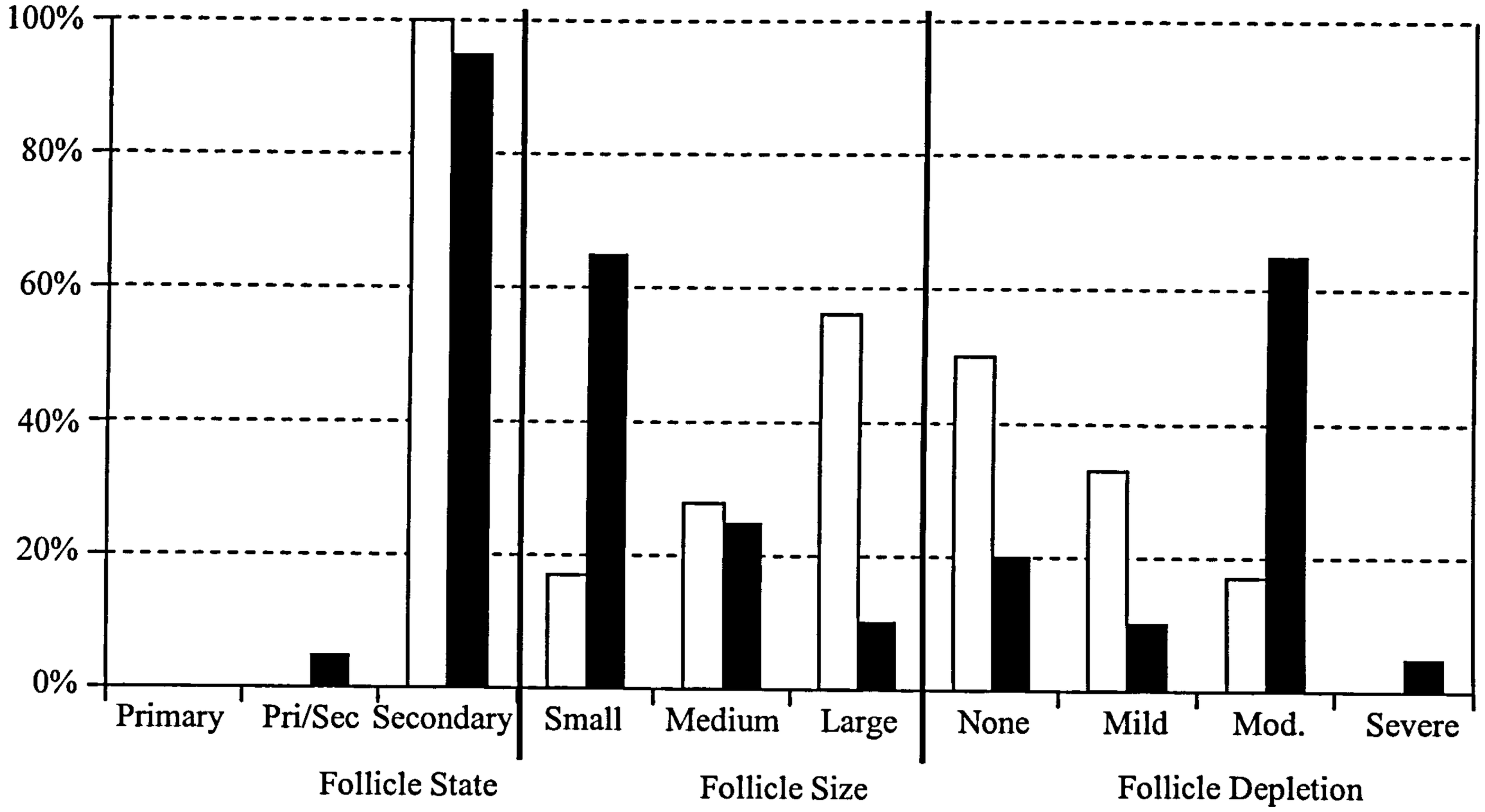

Figure 4.6 Comparison of follicle activity and functional state expressed in the pancreas of Aselli of sub-adult (open bars) and adult (filled bars) common shrews.

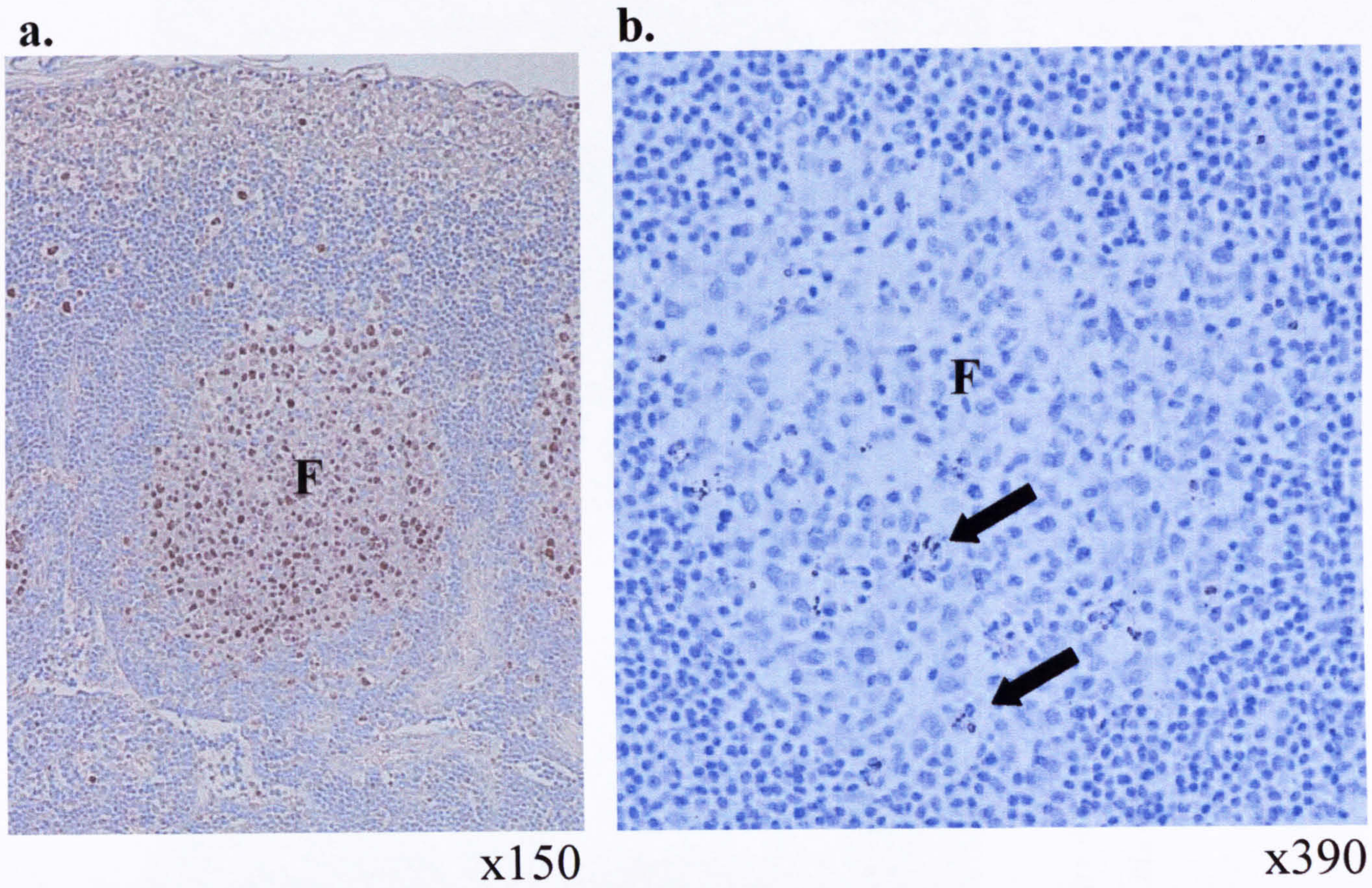

Figure 4.7 Cells undergoing proliferation (brown stain, a.) and apoptosis (arrows, b.) in follicles (F) within the pancreas of Aselli of *S. araneus*.

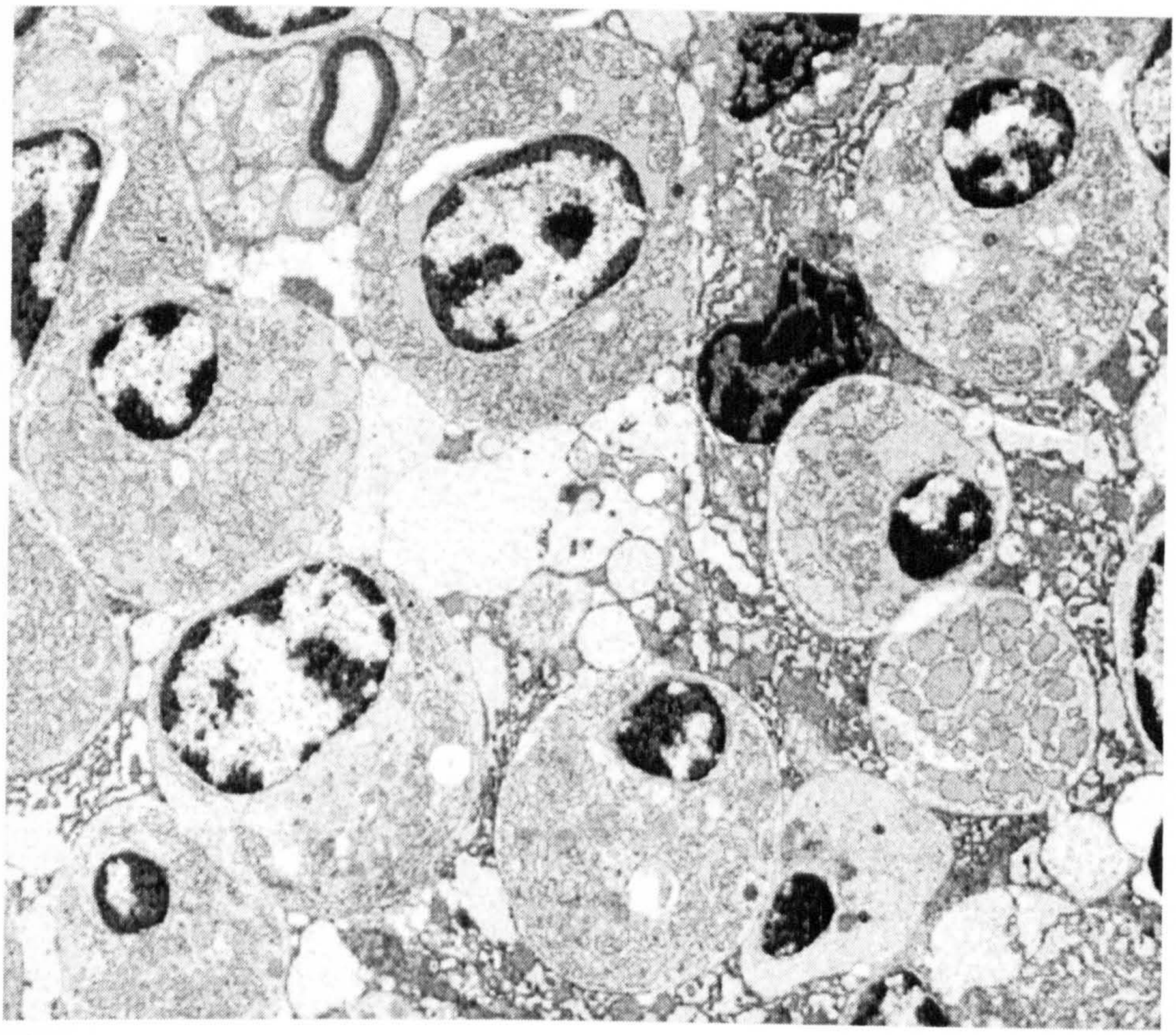

Figure 4.8 Scanning EM photograph of plasma cells within the medulla of the pancreas of Aselli of *S. araneus*.

Sub-adult shrews had larger follicles than adults (Wald = 11.06, df = 1, P<0.05, **Figure 4.6**), but there was no difference between sexes (Wald = 0.30, df = 1, NS). In adult shrews, follicular depletion was more severe than in sub-adults (Wald = 13.28, df = 1, P<0.05 **Figure 4.6**), but there was again no difference between sexes (Wald = 0.42, df = 1, NS).

4.3.2.3 Lymph nodes

All features normally associated with mammalian lymph nodes were represented in *S. araneus*: a cortex containing primary and secondary follicles, paracortex, lymphatic cords, medulla and both marginal and medullary sinuses. Compared to lymph nodes in other mammalian species (Köhler *et al.*, 2000; Wünschmann *et al.*, 2000), the medulla often appeared to contain a high number of plasma cells, particularly in adults.

4.3.2.4 Thymus

Thymic tissue was recovered from seven animals spread across all age groups. It was generally represented by a variable number of lymphocyte layers surrounding blood vessels, encased by a thin capsule of fibrous connective tissue. The thymus seemed to exhibit variable degrees of involution in all animals, regardless of age.

4.3.2.5 Bone marrow

The bone marrow generally exhibited moderate to high activity with all cell lines known from other mammalian species present. Approximately 10% of cells were identified as T cells, 10% as B cells (CD79a-positive, CD45R-negative) and 30% to 50% myeloid/histiocyte antigen- and/or lysozyme-positive cells. Megakaryocytes were also visible (**Figure 4.9**). At least 30% to 40% of all cells were PCNA-positive, representing relatively high levels of proliferation. Using ordinal logistic regression, no difference in bone marrow activity was found between sub-adult and adult shrews (Wald = 1.91, d.f. = 1, NS), or between sexes (Wald = 0.74, df = 1, NS).

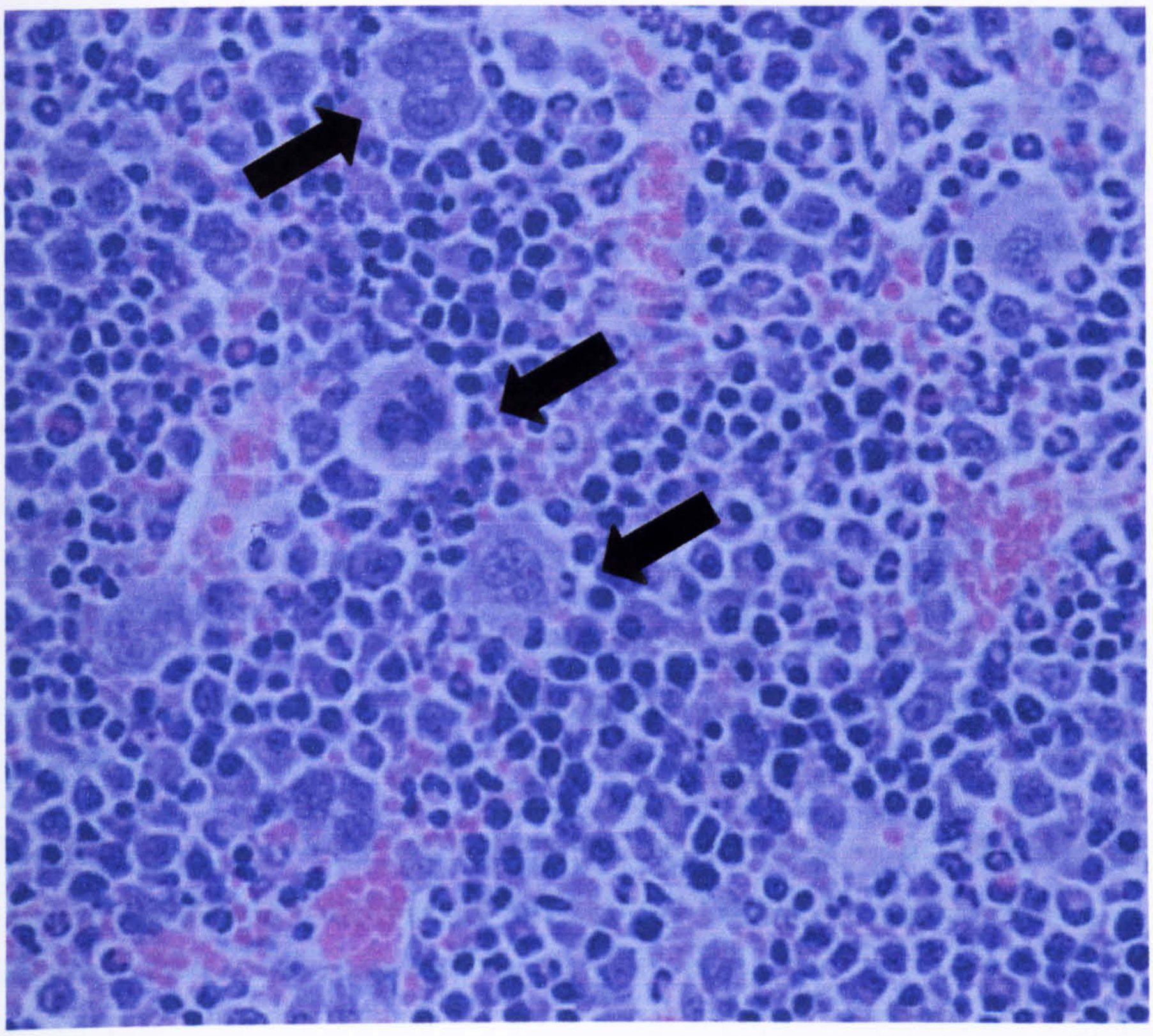

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Figure 4.9 Megakaryocytes (arrows) within bone marrow tissue of *S. araneus*.

4.3.4 Association of follicle activity and helminth abundance

The categorical principal component derived from measures of follicle size and depletion carried an Eigenvalue of 2.66, explaining 69% of the variance in pancreas of Aselli follicle size, 76% of the variance in pancreas of Aselli follicle depletion, 77% of the variance in spleen follicle size but only 44% of the variance in spleen follicle depletion. The component contrasted large follicle size (component loadings, spleen: 0.88, pancreas of Aselli: 0.83) with follicle depletion (component loadings, spleen: -0.67, pancreas of Aselli: -0.87). Thus a high score represented larger follicle size and a lower degree of follicle depletion. The mean rank of component scores was higher in sub-adult shrews (N = 18) than adults (N = 22; U = 49.0, $P<0.01$), confirming that sub-adult animals generally had larger, less depleted follicles than adults, with no evidence of a difference between males (N = 18) and females (N = 22; U = 190.0, NS). In sub-adult shrews, there was no significant relationship between component scores and number of gut helminths (N = 13, r_s = 0.31, NS), nematodes recovered from the bladder (N = 14, r_s = 0.28, NS) or *Porrocaecum* sp. larvae (N = 14, r_s = 0.08, NS). Similarly, in adult animals, no relationship was found between component scores and number of gut helminths (N = 19, r_s = 0.10, NS), bladder nematodes (N = 21, r_s = -0.19, NS) or encysted *Porrocaecum* sp. larvae (N = 19, r_s = -0.11, NS).

4.4 Discussion

The current investigation has addressed several previously unknown aspects of the common shrew's haemolymphatic tissues. By first assessing the morphology and function of the pancreas of Aselli, and developing methods for the *in situ* identification of shrew leukocytes, proliferating and apoptotic cells, this study has evaluated the functional state of haemolymphatic tissues in sub-adult, pubescent and adult shrews. In general, it was found that leukocyte markers which are used in other mammalian species can also be applied in *S. araneus*. Thereby CD3, part of the T cell receptor complex that is expressed by the T cell lineage from the pre-T cell stage onwards (Abbas *et al.*, 2000), can be regarded as a pan-T cell marker in common shrews. However, in contrast to other species, none of the B cell markers tested appeared to be a "pan B cell marker" in *S. araneus* (Monteith *et al.*, 1996). CD45R of rodent and feline B cells is clearly expressed most intensely in shrew B cells prior to final differentiation, whereas staining for CD79a intensifies with differentiation of B cells. The latter finding has also been observed in the dog and cat (A. Kipar, pers comm.), rendering CD79a a marker for well-differentiated B cells. At least in some mammalian species, CD79a (Igα, part of the B cell antigen receptor) is also only expressed at detectable levels after the pre-B cell stage (Abbas *et al.*, 2000; Chu & Arber, 2001).

As in other species, myeloid/histiocyte antigen and lysozyme can be regarded as markers for mature monocytes/macrophages and neutrophils and their precursors, myelomonocytic cells (Horny *et al.*, 1990; Kipar *et al.*, 1998a) in common shrews. However, in both the splenic red pulp and in sinuses of the pancreas of Aselli, a proportion of cells with the morphology of neutrophils expressed neither antigen. Accordingly, the question arises as to whether these cells might actually represent a morphologically indistinguishable heterogeneous population of granulocytes which cannot be further identified by the methods applied. Furthermore, the vacuolated cells within the marginal sinuses of the pancreas of Aselli and granulomas were also negative for both antigens. It remains to be seen whether these nevertheless represent a subset of perhaps specialized macrophages. Finally, both staining of proliferating cells using an antibody against human PCNA, and the application of the TUNEL

method for the detection of nucleosome-sized DNA fragments typical of apoptosis, proved effective in the common shrew.

The composition of the spleen in *S. araneus* was found to be very similar to that of the musk shrew, containing a central white pulp with red pulp predominantly located in the periphery (Fukuta *et al.*, 1982). The variably distinct rim of macrophages, lymphocytes, neutrophils and erythrocytes surrounding follicles and follicle groups seems to be homologous to the "marginal" or "intermediate zone", previously described in other species (including the musk shrew) and considered the site of most intensive blood filtration in the spleen (Snook, 1950; Fukuta *et al.*, 1982). To my knowledge, no previously published study has compared spleen weights of sub-adult and adult common shrews. Here, relative spleen weight was found to be greater in adults than sub-adults, and greater in females than males. Infection with the nematode *Heligmosomoides polygyrus* has been shown to lead to increases in relative spleen size in house mice (Kristan, 2002), while cliff swallows fumigated against ectoparasites have been found to contain smaller spleens than untreated birds (Brown & Brown, 2002). What changes in spleen size represent in terms of immunocompetence is highly debatable (Smith & Hunt, 2004), but if an increase in spleen size is a reaction to exposure to parasites, older shrews might be expected to harbour more parasites than sub-adults, and females more so than males. As described in **Chapter 3**, abundances of bladder nematodes, *Porrocaecum* sp. larvae and gut helminths were higher in adults than sub-adults. However, abundances of gut helminths (but not bladder nematodes or *Porrocaecum* sp. larvae) were higher in males than females.

The results presented here corroborate findings of a previous morphological study which classified the pancreas of Aselli as an "encapsulated lymphoid organ, exhibiting cortex with follicular lymphoid structures, and medulla with numerous plasma cells" (Holmes, 1965). The immunohistological examination undertaken here strongly suggests that the pancreas of Aselli is a specialised lymph node. It was found to contain a high number of plasma cells, but - as it also contained a T cell producing "paracortex" – it cannot be regarded exclusively as a specific site of B cell production and/or a functional analogue of the bursa of Fabricius in birds, as has been previously

suggested (Tsiperson, 1997). However, the pancreas of Aselli exhibited some relatively striking and specific features in so far as the centre (medulla) is almost entirely composed of plasma cells. The results of the current study suggest that the pancreas of Aselli is used for the "storage" of plasma cells, the number of which increases with age. Such a feature has not been described previously in any other species. Considering the diversity of micro and macroparasites harboured by common shrews, it seems plausible that this accumulation of plasma cells represents an effective host response to a multitude of infectious agents/antigens. This finding is supported by the relatively high number of plasma cells found in other shrew lymph nodes examined here.

No difference in relative mass of the pancreas of Aselli was found between sub-adults and adults or between males and females. Results of a previous longitudinal study (Twigg & Hughes, 1970) suggested the relative mass of the pancreas of Aselli declines steadily over the lifetime of the common shrew, although no statistical analysis was conducted. This may still be the case, if the test used here was not sensitive enough to detect a difference between sub-adult and adult shrews.

The predominance of large, active, secondary follicles found both in the spleen and pancreas of Aselli of young animals represents an activated immune system, responding effectively to a diverse array of infectious agents. Old animals, however, exhibited characteristics indicative of immune system exhaustion: follicles were generally smaller and were often depleted, with a smaller proportion of primary follicles, many of which were "non-active". This seems to indicate decreased follicular activity in adult animals, as represented by impaired germinal centre reactions resulting in a reduction in lymphocyte production (Herrera *et al.*, 2000). Impairment of germinal centre reactions is a known feature of immunosenescence in vertebrates and has been studied extensively: in man it has been shown to be a product of defective T cell-dependent B cell activation (Fernandez-Gutierrez *et al.*, 1999; Herrera *et al.*, 2000). Reduced lymphocyte production as a consequence of follicular and T cell impairment could explain why significantly lower numbers of white blood cells, and specifically lymphocytes, have been reported in older common

shrews (Wolk, 1981). Old adult *S. araneus* have also been found to have higher numbers of neutrophils in their peripheral blood (Wolk, 1981). This may, perhaps, be a reflection of the greater number of neutrophils within the splenic red pulp of adult common shrews, often observed in the current study.

No relationship was found here between lymphatic follicle activity and helminth abundances. However, experimental manipulation of parasite burdens would be required to separate fully the effects of parasite abundances on follicle activity, given the changes in both found to occur with age.

Haematopoietic activity seems not to change with age in the common shrew, as both bone marrow and splenic red pulp exhibited similarly intense activity in animals of all age groups. The results of the current investigation on *S. araneus* concur with those previously obtained from the musk shrew (*S. murinus*; Fukuta *et al.*, 1982), identifying both the splenic red pulp and bone marrow as physiological sites of erythropoiesis, leukocytopoiesis and platelet production over the animal's lifespan. In this aspect, shrews are similar to some reptiles, whereas in other mammals the capacity for haematopoiesis in the spleen seems to cease after birth (Fukuta *et al.*, 1982). In old shrews, there was also evidence for haematopoiesis in the pancreas of Aselli, as megakaryocytes were observed in the medulla of some individuals.

Previously, it has been suggested that for short-lived species facing intense selection pressure, surviving infection is dependent on ability to mount an immediate, innate immune response, as detrimental effects of infection would quickly result in starvation or predation (Lochmiller & Deerenberg, 2000). As demonstrated in **Chapter 3**, shrews of every age group exhibited a variable degree of mixed cellular portal inflammation in the liver, and often some degree of granulomatous inflammation, usually in response to helminths. This indicates sub-adult and adult shrews are able to mount both phagocyte-dominated and cell-mediated immune responses, even under the constraints placed upon them by their limited internal resources. The presence of numerous active secondary follicles in the spleen and pancreas of Aselli, the development of small lymphatic follicles in portal areas in the liver and the generally high number of plasma cells in the pancreas of Aselli all

demonstrate that shrews remain consistently able to mount systemic, specific immune responses. The increasing number of plasma cells in the medulla of the pancreas of Aselli with advancing age might even suggest a 'refocusing' of the immune system, from reacting to novel antigens in follicles as a young animal, to combating previously experienced parasites or pathogens with appropriate antibody responses as an adult.

Chapter 5. Effects of helminth parasitism on common shrew foraging behaviour

5.1 Introduction

Several vertebrate species have been shown to suffer reductions in growth rate, survival and breeding success as a result of helminth parasitism, even in the absence of serious pathology (reviewed in **Section 1.4**). Studies on captive animals and school children strongly suggest these detrimental effects may primarily be a consequence of reduced food intake in infected hosts (**Section 1.4**). The mechanisms by which such reductions in appetite occur are not well understood, but may be linked to increased levels of cytokines associated with infection (Pearson *et al.*, 1990; Hadju *et al.*, 1996). Whether anorexia is an adaptive host response to parasitism is also unknown. It perhaps reflects hosts becoming more selective in food intake, in order to reduce chances of further infection and select foods high in antiparasitic compounds (Kyriazakis *et al.*, 1998). Such a response appears particularly paradoxical when associated with those helminth infections which impose increased nutritional demands upon the host through direct feeding, impairment of digestion, and/or energetic and nutrient costs associated with immune reactions (Kyriazakis *et al.*, 1998; Lochmiller & Deerenberg, 2000).

As previously discussed (**Section 1.7**), *Sorex araneus* has a high metabolic rate even for its small size (Genoud, 1988; Nagel, 1994), stores very little energy in the form of bodily reserves (Churchfield, 1981), and as a consequence must forage constantly or risk starvation (Genoud, 1988). These characteristics have made common shrews useful models for investigations of foraging activity. Competition, time constraints and prey availability have all been shown to alter foraging activity of common shrews (Barnard & Brown, 1981; Barnard *et al.*, 1983; Barnard & Brown, 1985; Barnard & Hurst, 1987), but no study so far has considered the effects of parasitism. As almost all common shrews harbour helminth parasites (Shimalov, 2001), becoming infected through ingestion of infective stages contained in their invertebrate prey (Kisielewska, 1963; Sharpe, 1964; Lewis, 1968), parasitism may be an important determinant of foraging activity in this species. Given their limited energy reserves, parasitized shrews may have no choice but to forgo anorexia (assuming this is a functional response against parasites), and forage at a faster rate than unparasitized individuals in order to replace

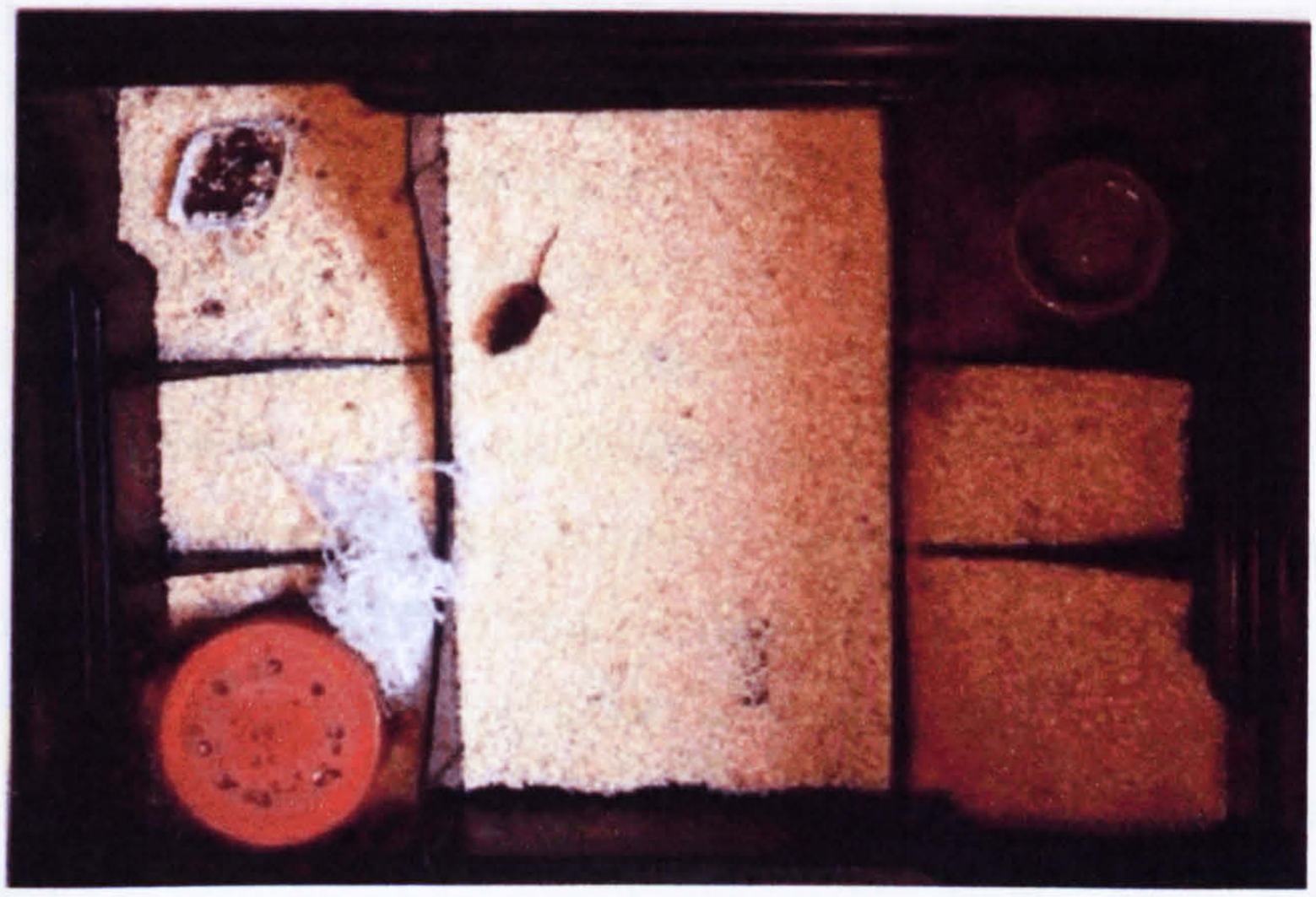
resources lost as a consequence of infection. To investigate this hypothesis, foraging activity of wild sub-adult common shrews carrying their natural parasite burdens is compared with those treated with anthelmintic drugs. This is the first time anthelmintic treatment has been attempted in *S. araneus*, and this experiment serves as a precursor to a field study which investigates effects of helminths on common shrew life-history traits using a similar technique (**Chapter 6**).

5.2 Materials and Methods

5.2.1 Capture and housing of experimental subjects

Sub-adult common shrews were live captured in the vicinity of Leahurst Field Station, Cheshire, England (OS grid reference 331558, 377486), between June 2002 and February 2003. Shrews were initially sexed according to the method of Searle (1985), and confirmed as sub-adults by weight at capture and fur condition, and by absence of testes and flank gland development in males and absence of nape scars (indicating they had not been mated; Crowcroft, 1957), lactation, and pregnancy in females. Shrews were kept in individual enclosures with a floor space measuring 60cm x 42cm (**Figure 5.1**). Each enclosure was furnished with sawdust to a depth of 5mm and a circular nest box (diameter 116mm, height 73mm) containing paper wool bedding. Water was provided *ad libitum* in a water bowl and shrews were fed 20g of minced ox heart per day, supplemented with 5% Nafag additive for carnivores no. 8340 (Nafag Ag, Labortierfutter, CH-9201 Gossau, Switzerland).

a.



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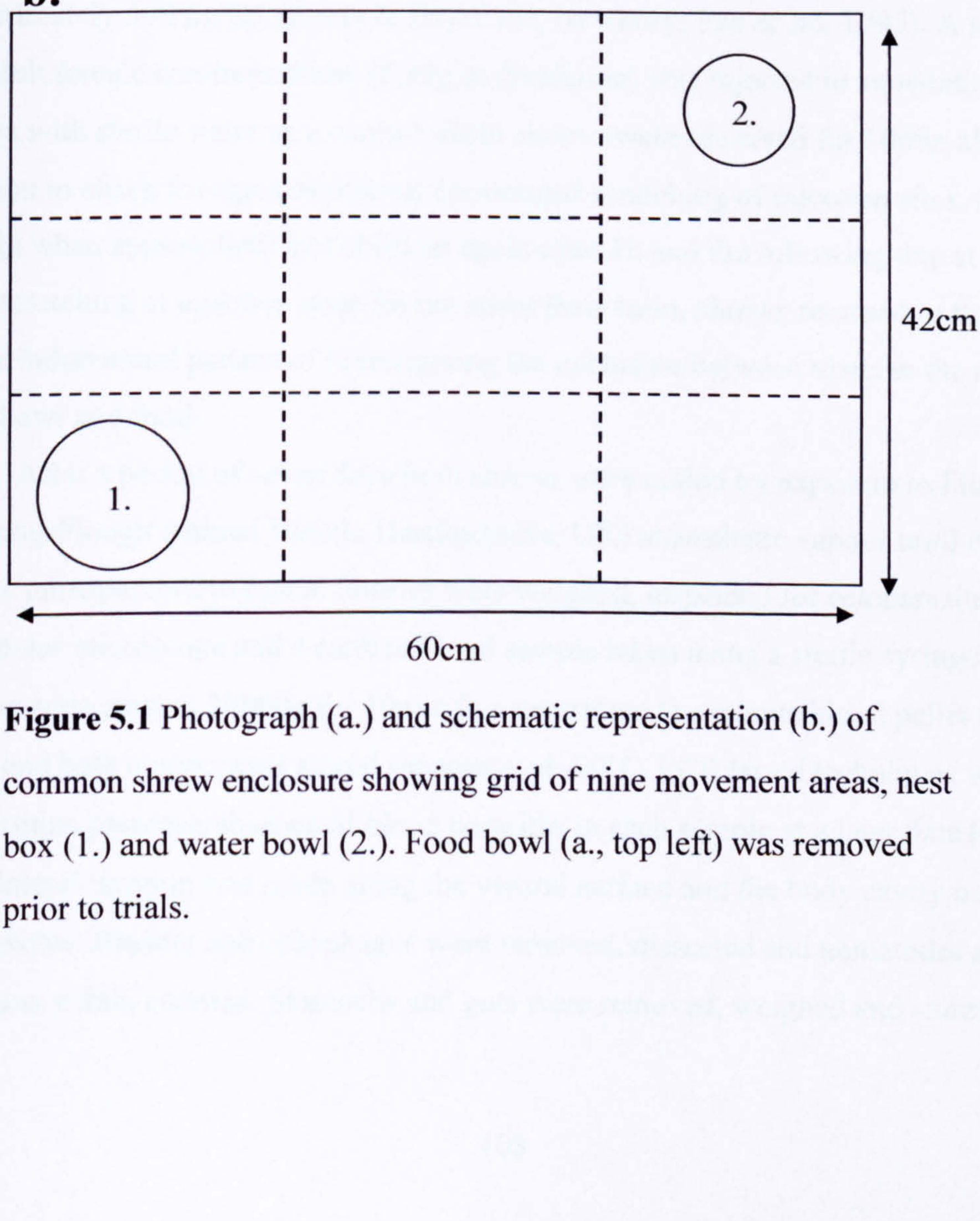

Figure 5.1 Photograph (a.) and schematic representation (b.) of common shrew enclosure showing grid of nine movement areas, nest box (1.) and water bowl (2.). Food bowl (a., top left) was removed prior to trials.

5.2.2 Pilot study to determine appropriate dose rates of anthelmintics

No record of treating common shrews with anthelmintics had been published prior to this study. Given the common shrew's faster metabolic rate compared to laboratory animals of a similar size (Genoud, 1988; Nagel, 1994), dose rates per unit body mass were likely be higher than those required to treat laboratory rodents (Bishop, 1998). To ascertain safe but effective dose rates for experimental use, one wild sub-adult female common shrew (body weight 7.54g at dissection) brought into captivity was injected subcutaneously with praziquantel, effective against cestodes and digeneans (Thomas & Andrews, 1977; Thomas & Gönnert, 1977; Butcher *et al.*, 1996; Adnyana *et al.*, 1997) and ivermectin, effective against nematodes (Campbell *et al.*, 1983; Klement *et al.*, 1996). Dose rates per unit body mass were those suitable for rats and mice (20µg ivermectin in solution, approximately 2000 µg/kg (Ivomec, Merial Animal Health Limited, Essex; Ostlind, 1985; Huerkamp, 1993), 5µg praziquantel in solution, approximately 500 µg/kg (Droncit, Bayer plc, Newbury; Fan & Ito, 1995). A second sub-adult female common shrew (8.93g at dissection) was injected in an identical fashion with sterile water as a control. Both shrews were observed for 10min after injection to check for signs of distress (prolonged scratching of injection sites, not moving when approached) and checked again after 1h and the following day at feeding. After scratching at injection sites for not more than 1min, shrews returned to their normal behavioural pattern of investigating the enclosure between visits to the nest box, water bowl and food.

After a period of seven days both shrews were culled by exposure to Fluothane^{tm} (Schering-Plough Animal Health, Hertfordshire, UK) anaesthetic vapour until they became unresponsive to touch. Shrews were weighed, inspected for ectoparasites under a binocular microscope and a cardiac blood sample taken using a sterile syringe. Blood samples were spun at 10000g for 10min in a centrifuge to separate blood pellet from serum and both components stored separately at -20°C. PCR based techniques were used to determine presence/absence of blood parasites in each sample at a later date (**Chapter 2**). A lateral incision was made along the ventral surface and the body cavity inspected for parasites. Bladder and oesophagus were removed, dissected and nematodes and digeneans within counted. Stomachs and guts were removed, weighed and stored in

formalin at dissection. At a later date, each gut was soaked in Hanks saline and dissected under x40 magnification. Parasites found were identified as nematodes, cestodes or digeneans using keys (Roots, 1992) and the numbers of each per shrew recorded. Dissections were carried out 'blind' with respect to the treatment category of each subject.

Abundances of cestodes and nematodes were found to be lower in the treated shrew, although neither was eliminated completely (Table 5.1). In addition, more digeneans were recovered from the treated shrew than the mock-treated shrew (Table 5.1). In an attempt to ascertain dose rates which would completely remove helminths from common shrews, a further group of four sub-adults (two males and two females, body weights 6.98g to 8.72g at dissection) was treated with an increased dose rate of 80µg ivermectin in 0.04ml solution and 23µg praziquantel in 0.04ml, with two further sub-adults (one male, 9.10g, one female, 7.67g) receiving two injections of 0.04ml sterile water as controls. Shrews were checked for signs of side effects as described above, and were found to scratch at sites of injection for no more than 1min. Shrews were dissected after seven days, and their parasite burdens compared as described above, again with dissections carried out 'blind' with respect to treatment category. There was some evidence to suggest a lowering of cestode abundance by anthelmintic treatment, although evidence for a decrease in abundances of nematodes and digeneans remained ambiguous (Table 5.1). Despite subjects showing no sign of ill effects associated with drug administration, dose rates were not increased further to avoid risking toxic side effects. Only small numbers of shrews were used at both stages of this preliminary investigation, both for welfare reasons and because of the time involved in capturing large numbers of shrews from the wild.

Table 5.1 Helminth abundance in treated and mock-treated shrews during pilot study

Treatment category	Dose rate	Sex	Cestodes	Nematodes	Digeneans
Treated	Initial[1]	Female	7	1	4
Mock-treated†	Initial[2]	Female	60	5	2
Treated†	Increased[3]	Male	3	1	1
Treated†	Increased[3]	Male	9	5	4
Treated†	Increased[3]	Male	8	3	0
Treated†	Increased[3]	Female	0	2	1
Mock-treated†	Increased[2]	Male	32	9	2
Mock-treated†	Increased[2]	Female	14	8	6

1. Initial dose rate: 20µg ivermectin in 0.04ml sterile water, 5µg praziquantel in 0.04ml sterile water

2. 2 x 0.04ml sterile water control

3. Increased dose rate: 80µg ivermectin in 0.04ml sterile water, 23µg praziquantel in 0.04ml sterile water

†. Animals included in main behavioural analysis

5.2.3 Effects of anthelmintic treatment on host behaviour

Twelve wild caught shrews were assigned to either a treated or mock-treated category (six individuals per category) attempting to balance for numbers of males and females in each group. This included seven animals from the pilot study (Table 5.1) All individuals were classed as 'sub-adults' weighing between 6.98 and 10.29g at dissection and lacking signs of sexual maturation as described in Section 5.2.1. All shrews were housed as described in Section 5.2.1. To reduce helminth abundances in the treatment group (four male, two female) ivermectin (80µg ivermectin in 0.04ml in sterile water) and praziquantel (23µg praziquantel in 0.04ml in sterile water) were administered by subcutaneous injection, as described in Section 5.2.2. Two injections of 0.04ml sterile water were similarly administered to the six individuals in the mock-treated category (three male, three female) the day after capture, again on opposite flanks.

Seven days after treatment/mock-treatment shrews were temporarily removed from their enclosures and the sawdust and bedding material replaced in order to ensure all cached food had been removed. Fifty fly casters (previously frozen to prevent eclosion) were mixed in the sawdust as prey items before shrews were returned to their enclosures. Subjects were videoed from above the enclosure using a tripod-mounted camera for 130min which included a 10min acclimatization period prior to the main 2h trial.

5.2.4 Analysis of behavioural trials

Videos of each trial were analyzed after the experiment, and shrew behaviour classified using the following parameters. Foraging activity was summarized by two measures: number of times a shrew approached and sniffed a prey item over the 2h observation period (pupae were visible on the video tape as black objects contrasted against the light colour of the sawdust), and the number of prey items consumed or cached during the trial. The number of food items eaten or cached during the 10min acclimatization period was also recorded, as this could potentially affect foraging rate during the main trial. A general measure of activity was calculated by visually dividing the enclosure into a grid of nine areas (**Figure 5.1.b**) and recording the number of times the subject moved from one to another. Time spent in the nest box, number of nest box entries and water bowl visits were recorded separately, with the latter defined as the number of times the shrew made contact with the water. Escape attempts were recorded as bouts of attempting to climb the walls of the enclosure, usually starting from the roof of the nest box. Analysis was carried out 'blind' with respect to shrew treatment category.

5.2.5 Assessment of post-treatment parasite abundances

Shrews were culled and dissected within 24h after the end of each trial, as described in **Section 5.2.2**, with cestode, nematode and digenean abundances determined as for the pilot study. Again, all dissections were carried out without knowing the shrew's treatment category.

5.3 Results

5.3.1 Efficacy of treatment

Anthelmintic drugs were administered to the treatment category in the expectation that the abundances of cestodes, nematodes and digeneans per host would be lowered below that of the mock-treated controls. Differences in the behaviour between the two groups might then be attributed to differences in parasite burden. Administration of anthelmintic drugs was found to significantly lower median cestode abundance in the treated group below that of the mock-treated group, **(Table 5.2, Figure 5.2)**. Median nematode abundance was also lower in the treated group than in the controls, although this difference was not found to be significant **(Table 5.2, Figure 5.3)**. Inspection of **Figure 5.3** indicates one treated individual carried a much greater number of nematodes than any other member of either category (28 nematodes), while the remaining treated animals had slightly lower nematode abundances than the mock-treated category. No significant difference in median abundance of digeneans was found between treated and mock-treated shrews **(Table 5.2, Figure 5.4)**. Median number of fleas per host also did not differ between the two groups **(Table 5.2)**, although only three fleas were recovered in total.

Differences in the number of parasites between males and females may indicate a sex difference in susceptibility to infection or host immune response (Bundy, 1988; Tinsley, 1989; Holmberg *et al.*, 2003). Inspection of **Figure 5.2** might suggest higher cestode abundances in males than females in the mock-treated category, although firm conclusions cannot be drawn from such small numbers ($N_{male} = N_{female} = 3$). With the exception of one female carrying 28 nematodes **(Figure 5.3)**, numbers of each helminth per shrew otherwise appear broadly similar within treatment categories **(Figures 5.2 - 5.4)**.

Table 5.2 Comparison of parasite abundances in mock-treated and treated shrews by Mann-Whitney U-tests

	Mock-treated (N = 6)			Treated (N = 6)				
	Median	25% quartile	75% quartile	Median	25% quartile	75% quartile	U	P
Cestodes	43.5	11.8	64.5	2.0	0.8	8.3	2.0	<0.01
Nematodes	9.0	7.0	9.5	2.5	1.0	10.5	7	NS
Digeneans	2.0	0.0	3.0	1.0	0.0	4.0	16	NS
Fleas	0.0	0.0	1.0	0.0	0.0	0.3	15	NS

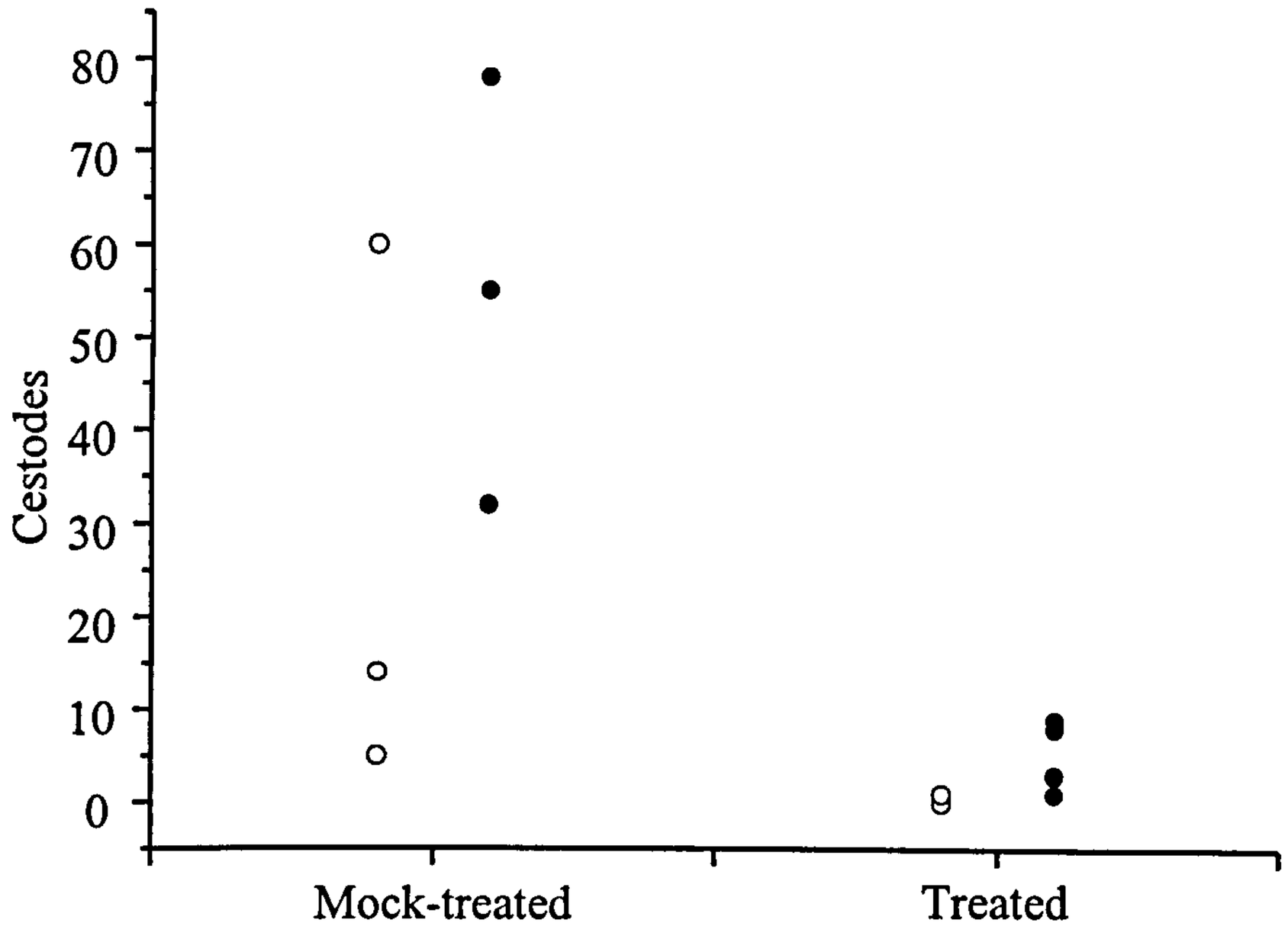

Figure 5.2 Cestode abundances of female (open circles) and male (filled circles) treated and mock-treated common shrews.

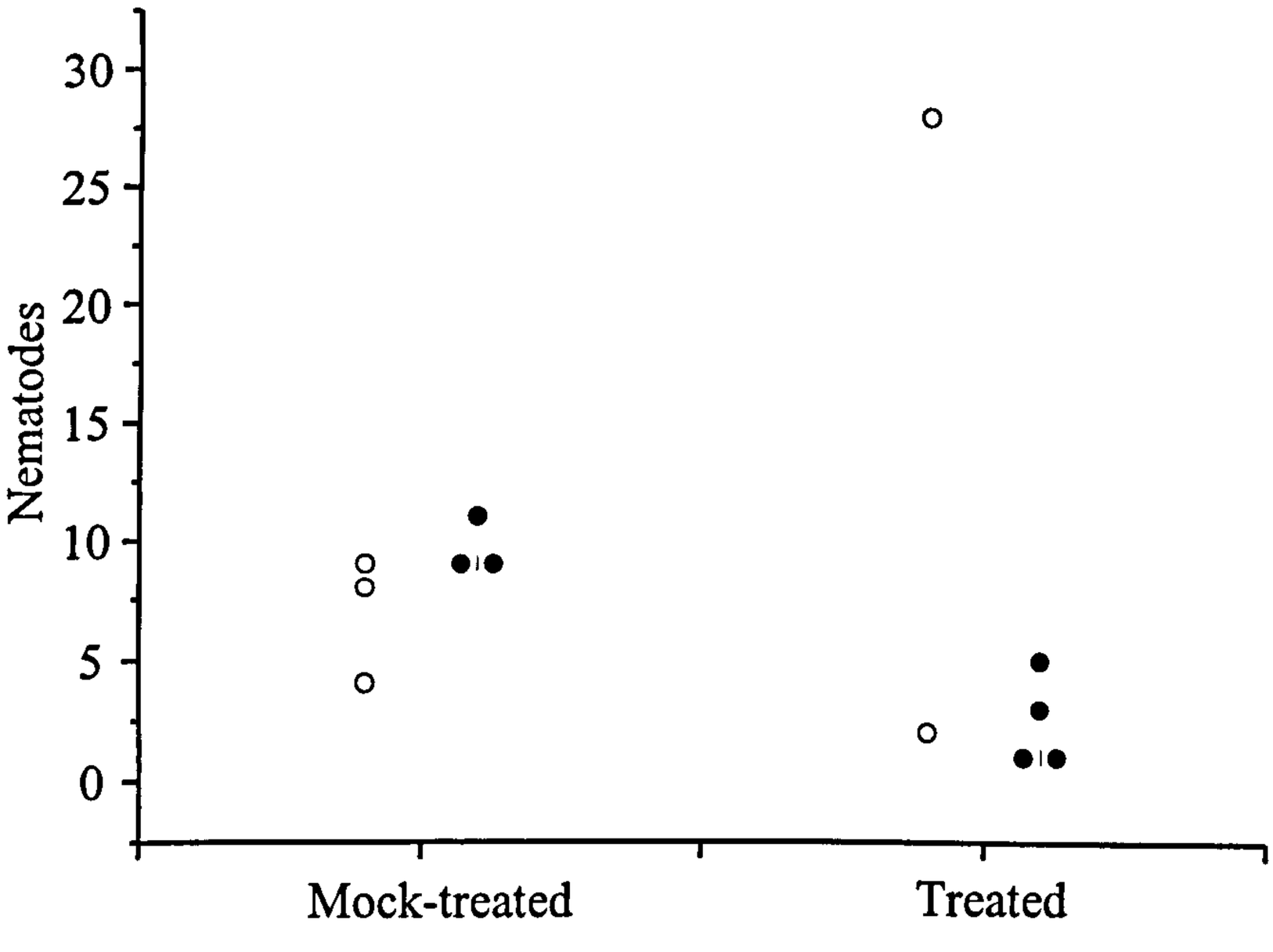

Figure 5.3 Nematode abundances of female (open circles) and male (filled circles) treated and mock-treated common shrews.

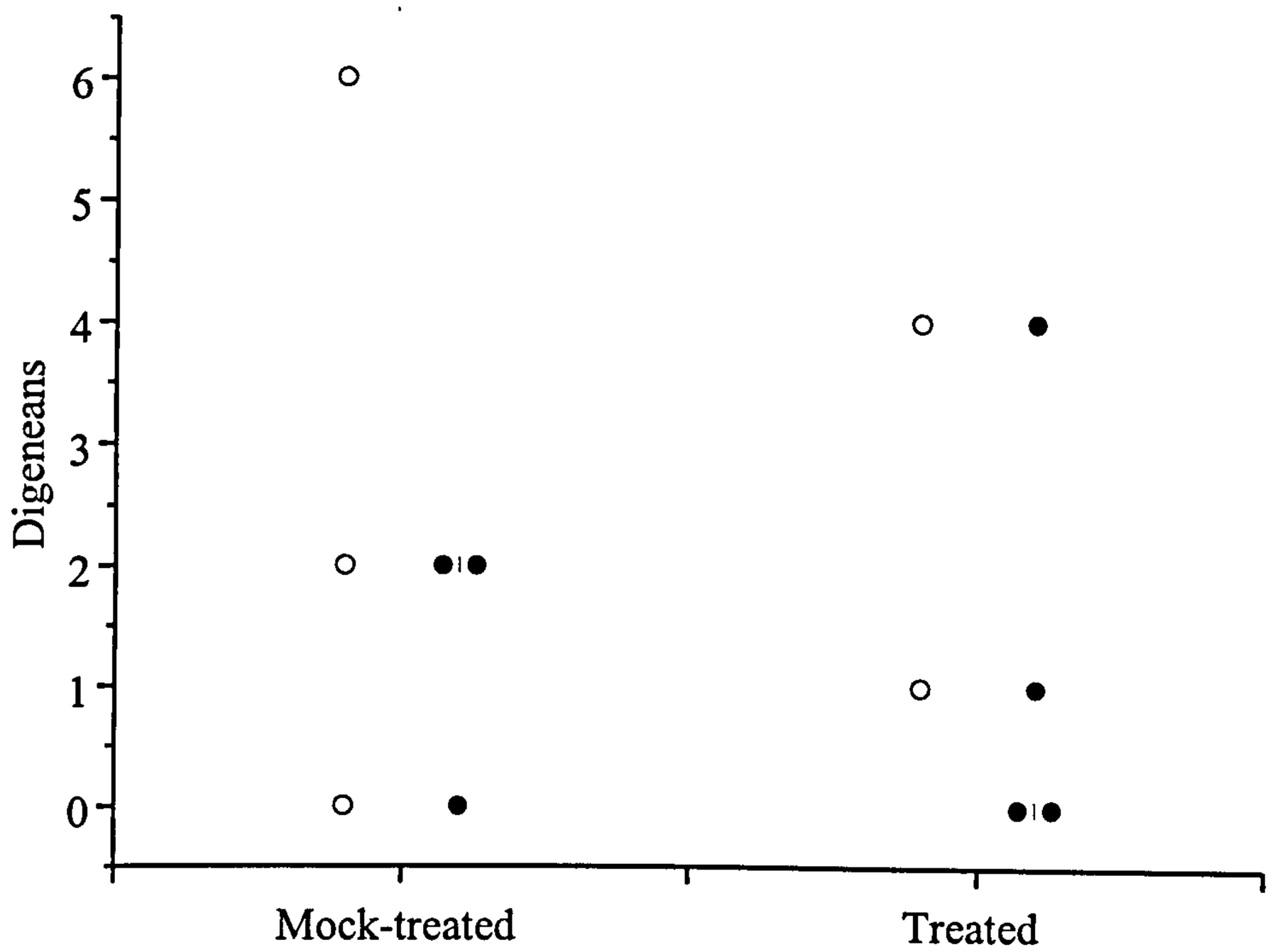

Figure 5.4 Digenean abundances of female (open circles) and male (filled circles) treated and mock-treated common shrews.

5.3.2 Correlations between behavioural measures

Considerable between-subject variation was observed in several of the behavioural measures recorded (**Figures 5.5 - 5.8**). Variation in some of these measures may be correlated, and simply reflect differences in general movement between shrews. Total movement count (the number of times a shrew moved from one movement area to another) was positively correlated with the number of water bowl visits and the number of prey items eaten or cached during the trial (**Table 5.3**). A higher movement count may also reflect a subject spending more time in the open area of the enclosure, and less time under cover in the nest box. The results showed that movement count was negatively correlated with both time spent in the nest box and the average duration of nest box visits (**Table 5.3**). Therefore, total movement count seems to provide a good indicator of general movement for use in subsequent analysis. Not all measures were correlated with movement count however: neither number of prey investigations nor bouts of escape attempts were significantly correlated with movement count (**Table 5.3**).

Interest in prey items during the main trial might be negatively correlated to the number of food items consumed during acclimatization, if shrews eat enough in the initial 10min period to become satiated. However, no such relationship was found (**Table 5.3**).

Interestingly, the number of bouts of escape attempts was positively correlated with the number of prey investigations during the main trial (**Table 5.3**). Perhaps this suggests a general level of interest in investigating the home enclosure, independent of general movement

Table 5.3 Spearman correlations between behavioural measures of common shrews, N = 12

	Correlation coefficient	Water bowl visits	Time in nest box	Average time in nest box	Prey eaten or cached during trial	Food eaten or cached pre-trial	Prey investigations during trial	Escape bouts
Total movement	r_s	0.71	-0.58	-0.77	0.57	-0.22	0.40	0.05
count	P	<0.01	<0.05	<0.01	<0.05	NS	NS	NS
Water bowl	r_s		-0.54	-0.76	0.24	-0.09	0.44	0.21
visits	P		0.07	<0.01	NS	NS	NS	NS
Time	r_s			0.86	-0.43	0.35	-0.49	-0.25
in nest box	P			<0.01	NS	NS	NS	NS
Average time in	r_s				-0.59	0.12	-0.66	-0.41
nest box	P				<0.05	NS	0.02	NS
Prey eaten or	r_s					-0.08	0.20	-0.14
cached during trial	P					NS	NS	NS
Food eaten or	r_s						0.05	0.14
cached pre-trial	P						NS	NS
Prey investigated	r_s							0.63
during trial	P							<0.05

Figure 5.5 Movement count of female (open circles) and male (filled circles) treated and mock-treated common shrews.

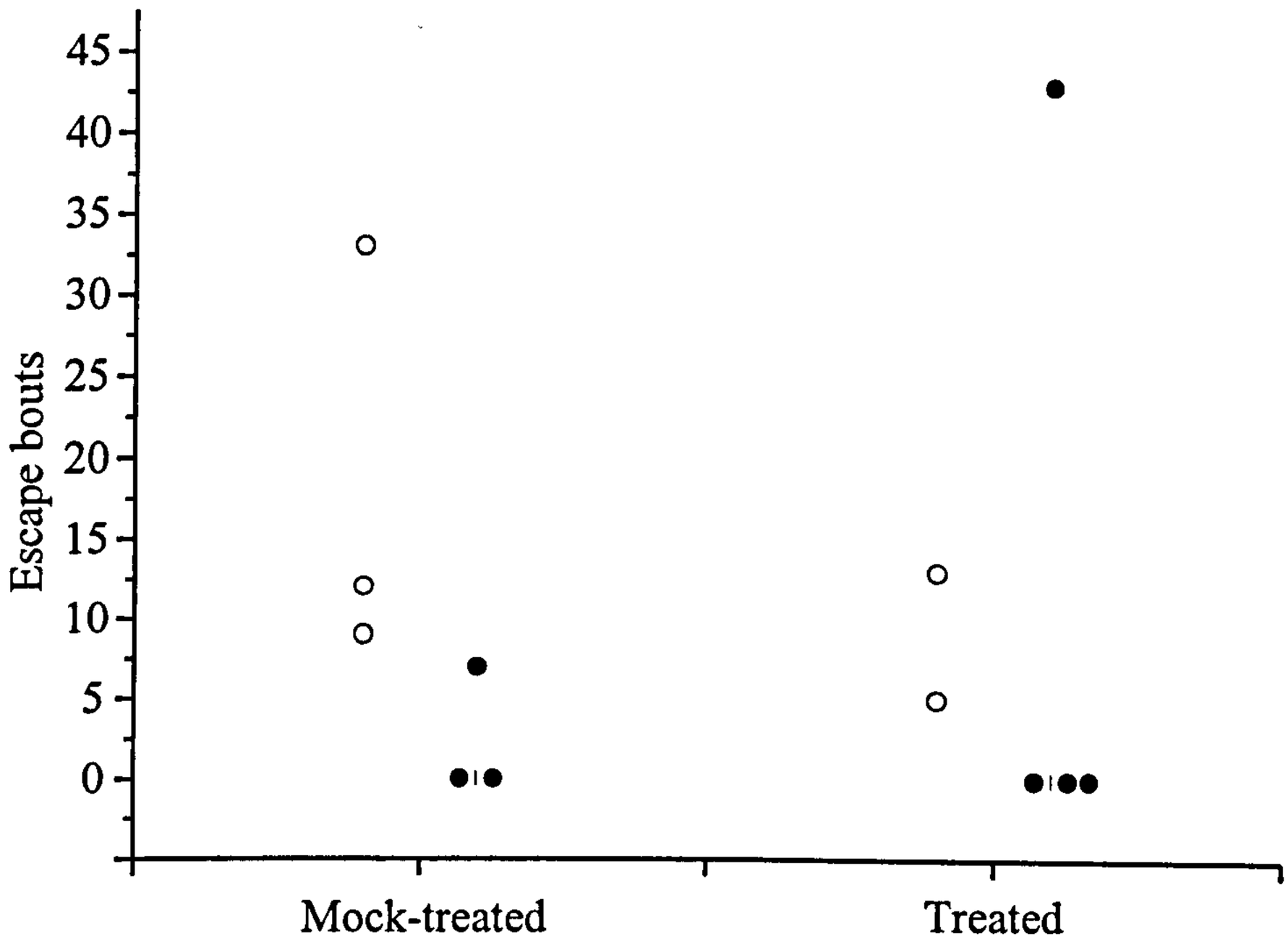

Figure 5.6 Number of escape bouts of female (open circles) and male (filled circles) treated and mock-treated common shrews.

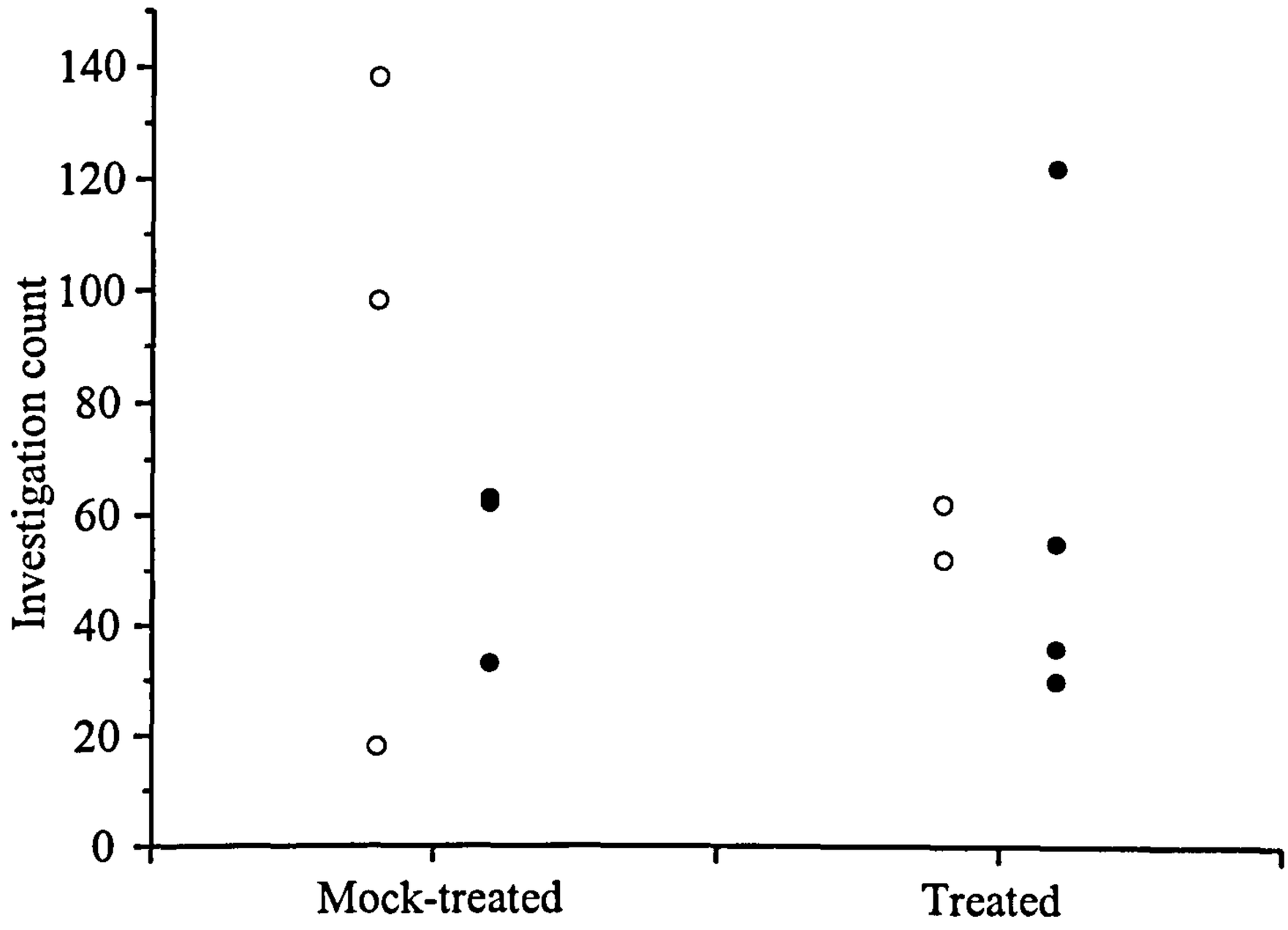

Figure 5.7 Investigation count of female (open circles) and male (filled circles) treated and mock-treated common shrews.

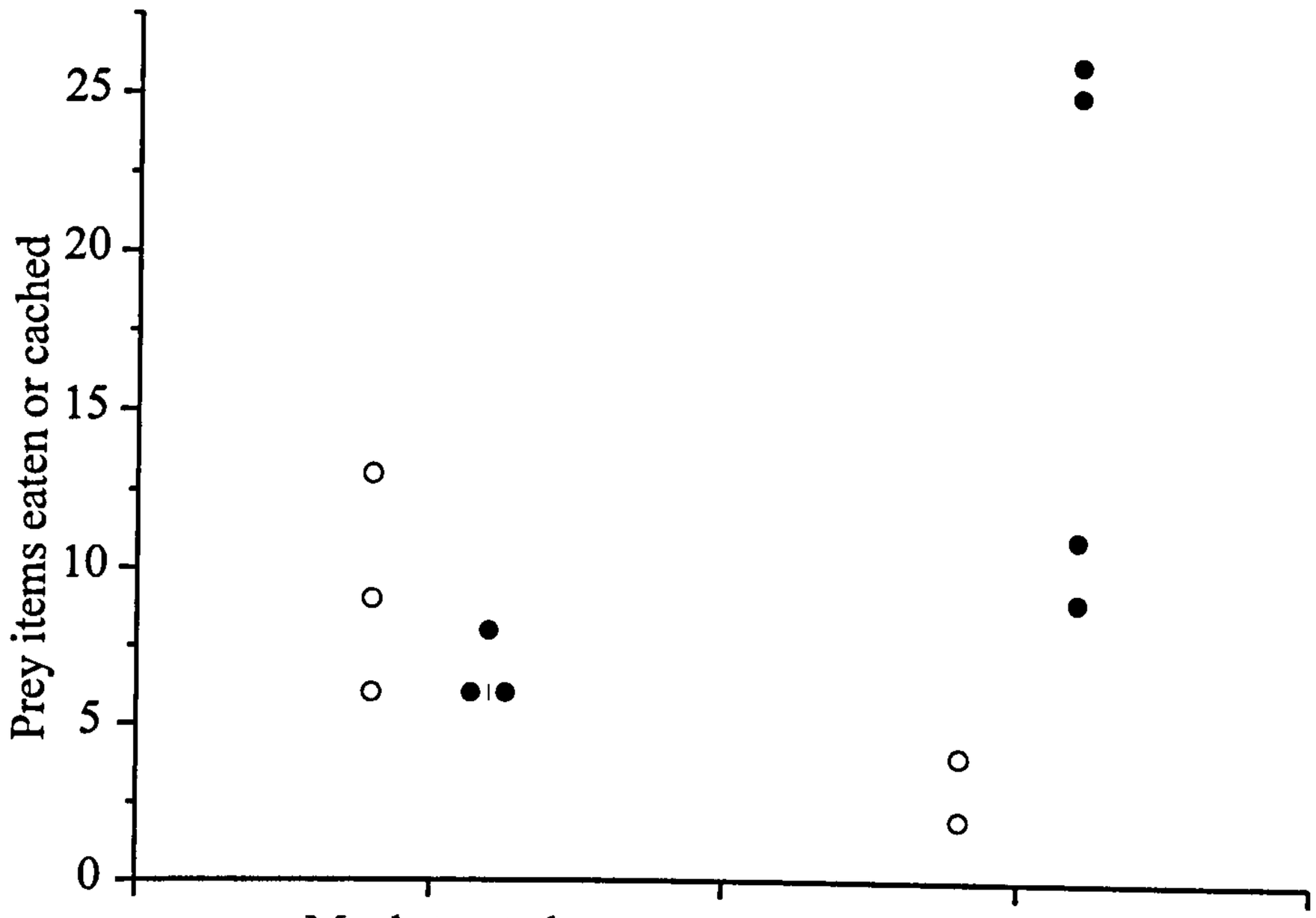

Figure 5.8 Number of prey items eaten or cached by female (open circles) and male (filled circles) treated and mock-treated common shrews.

5.3.3 Differences in host behaviour between treatment categories.

If foraging activity in common shrews is related to parasite abundances, successful anthelmintic treatment would be expected to result in differences in behaviour between treated and mock-treated categories. However, no difference between groups was found with respect to measures of movement, escape attempts, or interaction with prey (**Table 5.4, Figures 5.5 - 5.8**). As treatment was not totally effective in removing parasites, relationships might still exist between residual parasite abundances and behavioural measures. However, no evidence of such relationships was found (**Table 5.5**). Shrew body mass might also affect behaviour; for example if larger shrews spend more time foraging in order to maintain their greater body mass, or if common shrew behaviour changes with age, as reflected by body mass. Here, no behavioural measure was significantly correlated with body mass (minus gut weight, to remove variability in mass associated with recently consumed food; **Table 5.5**).

Table 5.4 Differences in behavioural measures between treatment categories, $N_{treated} = N_{mock\text{-}treated} = 6$

	Mock-treated			Treated				
Behavioural measure	**Median**	**25% quartile**	**75% quartile**	**Median**	**25% quartile**	**75% quartile**	**U**	**P**
Total movement count	220.5	131.8	467.0	269.0	176.3	331.5	16.0	NS
Prey eaten or cached	7.0	6.0	10.0	10.0	3.5	25.3	14.5	NS
Prey investigations	62.5	29.3	108.0	53.5	34.5	77.0	14.5	NS
Escape bouts	8.0	0.0	17.3	2.5	0.0	20.5	16.0	NS

Table 5.5 Spearman correlations between behavioural measures and parasite abundances and host body mass, N =12

Behavioural measure	Correlation coefficient	Parasites per host				Shrew body mass (minus gut)
		Cestodes	Nematodes	Digeneans	Fleas	
Total movement count	r_s	0.09	-0.18	-0.03	0.03	-0.41
	P	NS	NS	NS	NS	NS
Prey eaten or cached	r_s	0.16	-0.47	-0.20	-0.20	-0.26
	P	NS	NS	NS	NS	NS
Prey investigated	r_s	0.11	0.02	-0.30	0.50	0.21
	P	NS	NS	NS	NS	NS
Escape bouts	r_s	-0.32	-0.03	0.00	0.38	0.10
	P	NS	NS	NS	NS	NS

5.4 Discussion

Comparisons of parasite abundances between treated and mock-treated shrews suggest the protocol developed may be at least partially effective in reducing cestode burdens in *S. araneus*. The results suggest the dose used against nematodes may have some effect, although it did not result in a statistically significant difference between treated and mock-treated groups. In addition, there appeared to be no effect of treatment on abundance of digeneans. However, firm conclusions cannot be drawn from these limited sample sizes. Attempting to reduce parasite burdens of common shrews is an ambitious objective. Compared to rodents of a similar size, shrews are rarely kept in laboratories (the exception being *Suncus murinus* in the US) or as domestic pets. As such, there is little demand to develop treatment regimes against common infectious agents. Adapting protocols from mammals of similar size is difficult, as the faster metabolic rate of shrews may mean any injected compound will be metabolized before it can become effective (Genoud, 1988; Bishop, 1998). Much larger doses may therefore be required. In addition, wild common shrews are host to several helminth species (Vaucher, 1971; Vaucher & Durette-Desset, 1973; Roots, 1992; Roots *et al.*, 1994) which may vary in their sensitivity to anthelmintics. As such, the effectiveness of any drug may differ between individuals harbouring different parasite communities. Despite these difficulties, development of an effective treatment protocol could provide a powerful tool for investigating effects of parasitism in common shrews. Comparisons between treated and mock-treated shrews may allow experimenters to investigate casual relationships between parasitism and host traits that cannot be inferred from correlational studies. Reducing natural parasite burdens of *S. araneus* may also result in more naturalistic experiments than artificial infection of helminth-free shrews with a limited number of parasite species. Given our limited knowledge of the life cycles of these parasites, artificial infection is likely to be a less practical option than anthelmintic treatment.

Several experiments have demonstrated effects of helminth parasites on food intake of vertebrate hosts. Grouse (*Lagopus scoticus*; Shaw & Moss, 1990), rats (*Rattus norvegicus*; Crompton *et al.*, 1981; Ovington, 1985), reindeer (*Rangifer tarandus*; Arneberg *et al.*, 1996) and humans (Hadju *et al.*, 1996) have all been found to either

decrease food intake as a consequence of infection, or increase food intake with anthelmintic treatment. Despite a significantly lower median abundance of cestodes in the treated group, and the suggestion of a lower median nematode abundance, no obvious pattern of behavioural differences was found between the two host categories. In addition, no strong relationships were found between number of parasites post-treatment and host behaviour. However, considerable variation in behaviour was recorded between shrews, suggesting the methods used were sensitive enough to detect differences between individuals, but that these could not be directly attributed to variation in parasite abundances. Future comparisons of common shrew foraging activity before and after treatment may allow for control of individual variation in behaviour.

The results presented here cannot prove or disprove whether common shrews alter their activity in response to parasitic infection in the wild. However, they perhaps suggest that parasitism at the level recorded here does not have a dramatic effect on foraging and general activity of sub-adults. Given their fast metabolism and high energy demands (Genoud, 1988), if common shrews were unable to forage for any reason, including potential effects of parasitism, they would be expected to be removed quickly from natural populations. As such, sufficiently heavily parasitized animals may not be readily available for study. However, common shrews can contain upwards of 100 helminths and survive to adulthood (Haukisalmi *et al.*, 1994). If parasitized shrews do increase their foraging effort to replace nutrients lost to parasites and associated immune responses, these differences could not be detected here with the small sample sizes involved. Although shrews store little fat in the wild, they can quickly become obese in captivity (Churchfield, 1981; Churchfield, 1990); this may explain the greater body weights for sub-adult shrews reported here than would be expected in the wild (Shillito, 1963; Michielsen, 1966). These additional energy reserves may also disguise any effect of parasitism on shrew foraging activity.

In conclusion, the treatment protocol used appears at least partially effective in reducing helminth burdens of sub-adult common shrews, although this in itself could not explain differences in host behaviour. Although treatment was not totally successful, it did not appear to have any lasting detrimental effects on shrews during the period of

investigation. In the following chapter, a similar treatment regime is used to explore effects of parasitism life-history traits of common shrews under field conditions.

Chapter 6. Effects of helminth parasitism on wild common shrews

6.1 Introduction

Of the small number of experiments that have examined effects of helminth parasitism on life-history traits of vertebrates in the wild, few have reported significant results associated with anthelmintic treatment (**Section 1.4**). If detrimental effects of helminth parasites are the result of an impact on host nutritional status as studies of captive animals suggest (**Section 1.4**), they may only be detectable when hosts are unable to compensate for losses in energy intake through increased food acquisition or use of internal reserves (Tripet & Richner, 1997; Murray *et al.*, 1998), or when energetic costs are exacerbated by demands of reproductive activity, parental care, and lactation (Festa-Bianchet, 1989; Johnson & Albrecht, 1993; Kristan, 2002). As described in previous chapters, the strictly resource-limited physiology (Churchfield, 1981; Genoud, 1988) and high investment in reproduction (Genoud & Vogel, 1990) of *S. araneus* may make any detrimental effects of helminth parasitism easier to detect than in other mammalian species. Here, wild common shrews are treated in the field with either anthelmintic drugs or a water control, using a similar protocol to that described in **Chapter 5**. Treatment categories are then compared to investigate whether administration of anthelmintics results in lower abundances of cestodes, nematodes and digeneans, and changes in several key host traits.

Previous studies have shown common shrews begin to gain weight rapidly prior to the breeding season, with males starting to grow earlier than females (Shillito, 1963; Skarén, 1973; Pernetta, 1977). Mature males adopt one of two mating strategies: some expand their winter territories to overlap with those of several females, while the remainder make repeated long-distance visits to potential mates (Buckner, 1969; Michielsen, 1966, Shillito 1963; Stockley *et al.*, 1994). In general, it is males that mature earlier which adopt the overlapping strategy, and father the most offspring (Stockley *et al.*, 1994). Here, effects of helminth parasitism on time of maturation and growth rate are investigated through comparison of body weights of treated and mock-treated groups. Comparisons are made in the field prior to the breeding season, when shrews which began to grow earlier would be expected to be heavier, and more accurately in the

lab at dissection. In addition, an indicator of shrew activity is used to compare movements of animals prior to and during the breeding season, which may reveal if parasitism influences choice of male mating strategy and general movement of females.

Number of offspring fathered by common shrews has also been found to be positively related to testes mass and number of females overlapped by 'overlapping' males, and sperm counts in 'non-overlapping' males (Stockley *et al.*, 1996). To investigate effects of parasitism on sperm counts and testes mass, comparisons of these traits are made between treated and mock-treated groups at dissection.

Finally, as several authors have suggested parasitism to be a factor in mortality of common shrews (Michielsen, 1966; Buckner, 1969; Pernetta, 1977), effects of helminths on shrew survival are investigated experimentally through comparison of treated and mock-treated groups.

6.2 Materials and Methods

6.2.1 Trapping grid

Trapping took place between 7th August 2002 to 24th June 2003 on undisturbed grassland owned by Wetherstone's House, Neston, Cheshire (OS coordinates 331430, 377535). The field is approximately rectangular and bordered along its edges by a stone wall (southwest edge), a wire mesh fence (southeast), a planted tree border (northeast) and an area of bracken (northwest). When trapping commenced in August 2002, the field contained intermittent patches of long grass, nettles and ragwort between larger areas of shorter grass. Patches of long grass receded over winter, but grew back from March - July to give a more uniform cover over the entire field.

Initially, 45 Longworth traps (Chitty & Kempson, 1949) were placed over a five by nine grid, with on average approximately 10m between each trap (**Figure 6.1**). In order to maximise trapping success, priority was given to placing traps in areas of cover likely to be inhabited by common shrews, rather than adhering to a regularly spaced grid. From 23rd August 2002 an additional 15 traps (three rows of five traps) were added to extend the grid across the northwest side of the field (**Figure 6.1**). Each trap contained hay bedding and fly pupae (frozen first to prevent eclosion), the latter acting as both bait and sustenance for captured animals.

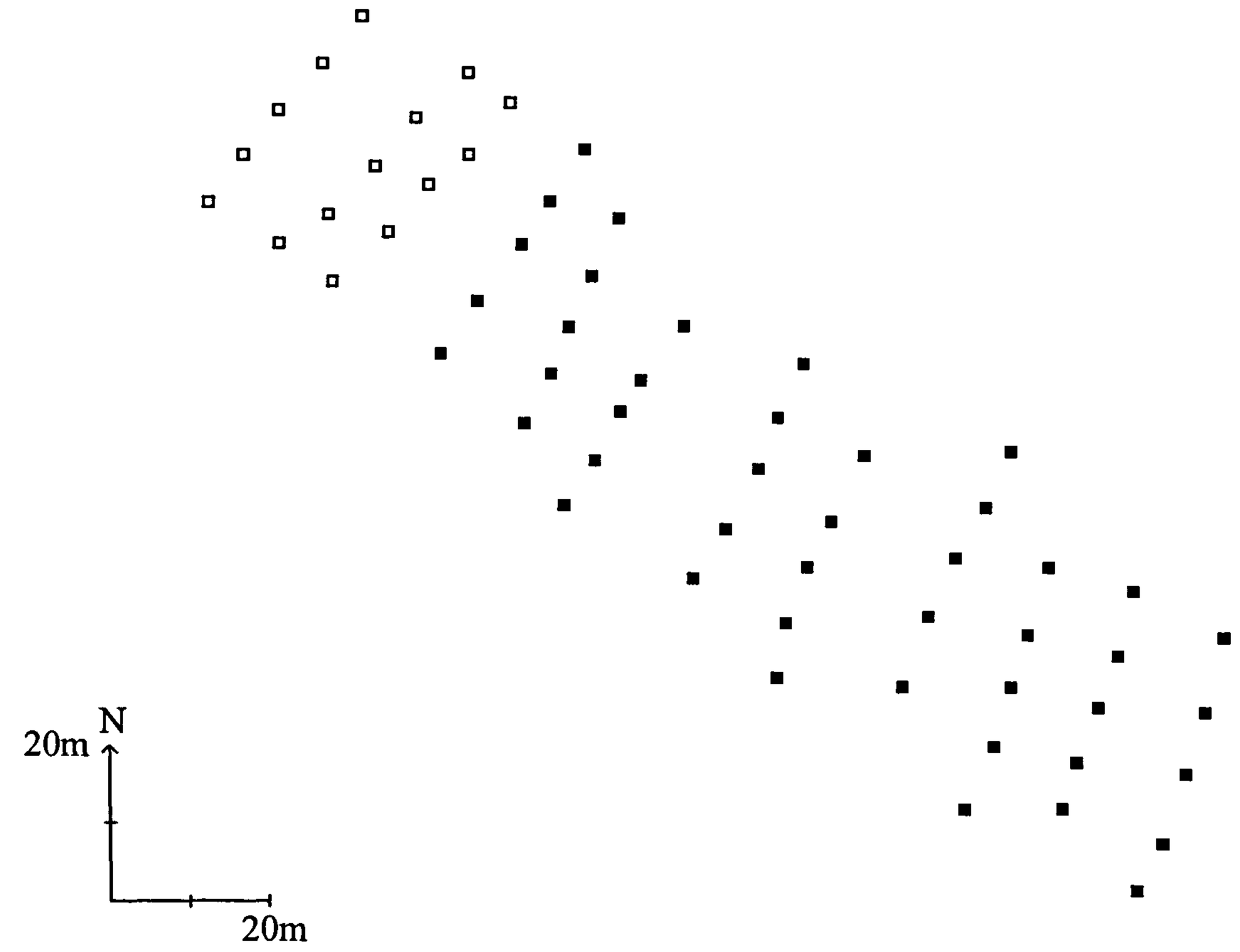

Figure 6.1 Scale representation of trapping grid for field experiment. ■: traps set from 07.08.02. □ : additional traps from 23.08.02.

Traps were set on 'pre-bait' (allowing animals to enter and leave traps without capture) overnight in order to allow shrews to become familiar with entering traps and therefore increase capture rate. On trapping days, traps were set on 'capture' from 9am onwards, ensuring each contained ample bedding and fly pupae. Traps were checked every 2 - 3h, with two rounds of trapping per day between 7th August 2002 and 13th March 2003, increased to a maximum of four rounds after 14th March 2003.

6.2.2 Trapping schedule

Trapping began with two trial periods in August 2002 (trapping on 7th - 8th August, 26th - 29th August) and December 2002 (10th – 13th December, 17th – 19th December), to ensure that shrews could be pit-tagged, injected and successfully recaptured. From 14th January 2003 to 14th March 2003, trapping took place on Mondays to Fridays for two week periods, with a fortnight between each session (to allow for trapping at other sites in order to capture sub-adult shrews used in experiments described in **Chapter 5**). From 17th March 2003, the trapping schedule was altered such that trapping took place on Mondays, Wednesdays and Fridays every week.

Trapping did not take place during heavy rain, as it proved too difficult to tag and treat animals under these conditions, nor during the middle of excessively hot days, to avoid shrews over-heating in traps.

6.2.3 Tagging and anthelmintic treatment of shrews

On first capture, a 12mm Avid Friendchip™ electronic identification chip (Avid plc., East Sussex, United Kingdom), was injected into the scruff of each common shrew using a single-use disposable syringe. Each chip is associated with a unique number that can be ascertained using a 'pit-tag reader', such that each shrew could be identified on subsequent captures. Tagged shrews were then alternately assigned to either the treatment or mock-treatment group, in which they remained for the duration of the experiment. On subsequent captures, shrews in the treatment category were injected subcutaneously with anthelmintic drugs, using the same protocol and dose rates as for the behavioural experiment (80µg ivermectin in 0.04ml sterile water and 23µg praziquantel in 0.04ml sterile water, **Section 5.2.3**). Shrews in the mock-treatment

category were similarly injected with two doses of 0.04ml sterile water. Animals in both categories were retreated throughout the experiment, in order to reduce infection by newly ingested parasites in the treatment category. As a precaution against retreating shrews too often (potentially leading to toxic side effects), a minimum period of 14d was left between treatments. Initially, shrews were treated or mock-treated on their first capture. However, this was later modified such that shrews were first treated/mock-treated on their second capture, to avoid stress associated with being pit-tagged and treated in one handling session.

6.2.4 Collection of morphometric data.

Shrew identification number and trap position were recorded at each recapture. Animals were weighed in a plastic bag using a spring balance, and sexed by the presence or absence of nipple patches in sub-adults and testis bulge in adults (Searle, 1985).

Whether the shrew was currently moulting was also recorded, along with development of flank glands. Where flank glands were visible, their size was rated from one to three using the following criteria: one, a small, dark mark on either side, less than 3mm long. Two, dark patches larger than 3mm long. Three, large dark patches with a waxy appearance. All shrews were checked for ectoparasites by back-combing the fur, paying particular attention to the ears and fur on the back of the neck and base of the tail. Testes development was recorded for males, and where visible the size of the testes bulge rated from one to three (one: small testes beginning to develop. two: testes in mid development. three: large testes, completely developed).

Females were checked for the presence of a 'nape-scar' indicating they had recently been mated (Crowcroft, 1957), lactating nipples and pregnancy, as assessed by the presence of a 'pear'shaped abdomen.

6.2.5 Collection and dissection of shrews

Female shrews showing signs of pregnancy (first pregnant shrew captured 8th May 2003) were brought into captivity for dissection. Similarly, mature males (as assessed by weight, flank gland and testes development) were brought in from 28th May 2003 onwards. Shrews were housed individually as described for sub-adults in **Section**

5.2.1, and dissections carried out as per **Section 5.2.2**, with all organs weighed and parasites quantified as before. In addition, the uterus of females was weighed, and the number of embryos present recorded. For males, individual weights of reproductive organs were recorded and an assessment of sperm count performed as described below. All dissections and sperm counts were carried out 'blind' with respect to the treatment history of the subject.

6.2.6 Sperm counts and weighing of male sex organs

To ensure maximum viability of sperm, weighing of male reproductive organs and sperm counts were carried out while the remainder of the shrew was being dissected, using the following protocol (Searle & Beechey, 1974). The left testis was removed and weighed, and the caput of the right epididymis placed in a watchglass containing 0.1ml of 1% citrate solution. This was then macerated using a scalpel blade and scissors before a further 0.9ml of 1% citrate solution was added to the watchglass. Contents were mixed using a pipette, and a small amount of the suspension pipetted under the coverslip of a double-chambered Neubauer haemocytometer. The hameocytometer was then placed in a box containing moist cotton wool for 15min, to allow sperm to disperse evenly beneath the coverslip. The haemocytometer was then placed under a microscope and the number of sperm heads in the four corner squares and one centre square (each square representing 0.1µl of solution) of each of the two counting chambers counted. In borderline cases (where sperm heads lay across a border between two squares), only heads located on the upper or left limiting lines of a given square were included in the count. The mean number of sperm per 0.1µl square for both chambers were summed and averaged, to give number of sperm per 0.1µl solution.

6.2.7 Data analysis

6.2.7.1 Effects of anthelmintic treatment and host sex on helminth abundance

Distributions of macroparasites between hosts are often aggregated, such that a small proportion of hosts harbour relatively high numbers of parasites compared to the rest of the population, resulting in right skew and long tails when plotted as histograms (Shaw & Dobson, 1995). Such distributions often fail to meet the entry requirements for

parametric tests (Wilson & Grenfell, 1997a), and thus may best be analyzed using non-parametric techniques. Alternatively, in some cases, parasite data can be normalized by log-transformation (adding one first to remove zeros), although this may not be successful if the original distribution is highly skewed (Wilson & Grenfell, 1997a). In addition, aggregated data can be analyzed using a generalized linear model (GLM) with an appropriate error structure (either the negative binomial or overdispersed Poisson distribution) and link function (usually a log link; Wilson & Grenfell, 1997a).

To investigate whether number of cestodes, nematodes and digeneans per mature shrew captured at the end of the field study differed between treatment categories, data were analyzed using a non-parametric Mann-Whitney U-test, a general linear model on log-transformed data (adding one first to remove zeros) and a generalized linear model with overdispersed Poisson errors and a log link. In addition, the general and generalized models were employed to investigate whether host sex or an interaction between sex and treatment category had an effect on abundance of each helminth class. Significance of terms in each model was assessed by backwards deletion using F-tests (Crawley, 1993). To check the minimal model had been reached, terms found to be non-significant by stepwise deletion were individually added to and deleted from the model, and change in deviance assessed by F-tests as before. For models with overdispersed Poisson errors, the dispersion parameter was estimated empirically as the ratio between the calculated Pearson's χ^2 and residual degrees of freedom (McCullagh & Nelder, 1983; Crawley, 1993; Wilson & Grenfell, 1997a). Residual analysis was undertaken for each model to ascertain which gave the best fit for the given data set, and the results of models compared both to each other and the non-parametric U-test.

6.2.7.2 Comparison of shrew body weights between treatment categories at dissection

To remove variability associated with weight of recently consumed food, the weight of the gut was subtracted from total body mass prior to group comparisons. In females, the situation was complicated by the presence of developing embryos, which may make up a considerable (but highly variable) proportion of their body mass. As a consequence, mass of developing embryos was also subtracted from females, with remaining body mass of males and females considered separately.

6.2.7.3 Analysis of movement data

Potentially, anthelmintic treatment could create differences in movement patterns of shrews compared to controls, for example if treated animals are able to defend larger territories, or if treated males adopt a different mating strategy (Shillito, 1963; Michielsen, 1966; Buckner, 1969; Stockley *et al.*, 1994). Here, number of different traps visited by an individual over half a calendar month is used as an approximate measure of movement. This measure is calculated for three periods for which most data are available (last half of March, first and second half of April). Number of traps visited per period is then compared between treatment groups using a generalized linear mixed model with Poisson errors (Elston *et al.*, 2001; Paterson & Lello, 2003). Shrew identity is included as a random factor, to take into account taking repeated measurements from the same set of animals. Previous studies have shown mature males and female shrews exhibit different patterns of activity during the breeding season (Shillito, 1963; Michielsen, 1966; Buckner, 1969; Stockley *et al.*, 1994). As a consequence, each sex is analyzed separately for an effect of treatment.

6.2.7.4 Comparison of testis mass and sperm counts between treatment categories

Sperm counts were log-transformed (after adding 1) prior to analysis to meet the parametric entry requirements of t-tests and linear regression. No such transformation was required for testis mass. T-tests were used to compare both measures between treatment categories. To compare relative testis mass between groups, body weight (minus gut) and treatment category were entered simultaneously in a general linear model, with significance of terms assessed by t-tests of parameter estimates.

6.2.7.5 Comparison of survival between treatment categories

To investigate whether treatment groups differed in their survival, a Kaplan-Meier analysis was performed to compare the number of days an animal 'survived' from the date on which it was first treated (Kaplan & Meier, 1958). Unfortunately, whether an animal actually died cannot be ascertained from trapping data. Time survived for each animal is therefore assumed to be the date at which it was last caught (animals caught only once were assumed to live only one day after capture); this is clearly a limited

approach as animals may simply leave the trapping site, or may remain but never be recaptured.

The advantage of the Kaplan-Meier technique is that it is non-parametric, and therefore does not require any assumptions regarding the shape of the survival function. In addition, it allows for entry of 'right-censored' cases: individuals for whom time of death (as defined by the crude measure used) cannot be calculated. In this case, this represents the subset of animals collected in for dissection at the end of the experiment. Tests for a difference between the survival curves of the two treatment groups were carried out using both the Breslow (1970) and more robust Tarone-Ware (Tarone & Ware, 1977) statistic.

6.3 Results

6.3.1 Effects of treatment and host sex on number of parasites per shrew

6.3.1.1 Cestodes

The distribution of cestodes between shrews at dissection (N = 38) showed marked positive skewness (2.23, SE = 0.38), a long right hand tail, and a high level of aggregation (k = 0.69, calculated from the moment estimate equation given in Wilson & Grenfell (1997a; 1997b); **Figure 6.2**). Log_e-transformation of the data (adding 1 first to remove zeros) appeared to reduce and change direction of skewness (-0.47, SE = 0.38, **Figure 6.3**), and the Kolmogorov-Smirnov test found no evidence for a difference between the transformed data and the normal distribution (Z = 0.66, N = 38, NS).

A Mann-Whitney U-test suggested a difference in median cestode abundance between the treated and mock-treated categories which bordered on significance, with abundances higher in the mock-treated category (**Table 6.1, Figure 6.4**). Analysis of log_e-transformed data by general linear model also found a difference between the treated groups, with mock-treated shrews again harbouring more cestodes than treated shrews ($t = 2.24$, $df = 36$, $P<0.05$, **Figure 6.5**). However, no evidence was found for an effect of host sex on number of cestodes per shrew ($F = 1.44$, $df = 1, 35$, NS), nor for an interaction between treatment category and host sex ($F = 0.03$, $df = 1, 34$, NS). Analysis of untransformed data using overdispersed Poisson errors found no effect of treatment

Table 6.1 Median parasite abundances in mock-treated and treated shrews with Mann-Whitney U-test

	Mock-treated				Treated					
	N	Median	25% quartile	75% quartile	N	Median	25% quartile	75% quartile	U	P
Cestodes	17	39.0	15.5	52.0	21	17.0	8.0	47.0	112.0	= 0.05
Nematodes	17	36.0.	17.5	66.5	20	19.0	13.0	44.3	127.5	NS
Digeneans	17	3.0	2.0	6.0	21	2.0	1.0	4.0	108.0	<0.05

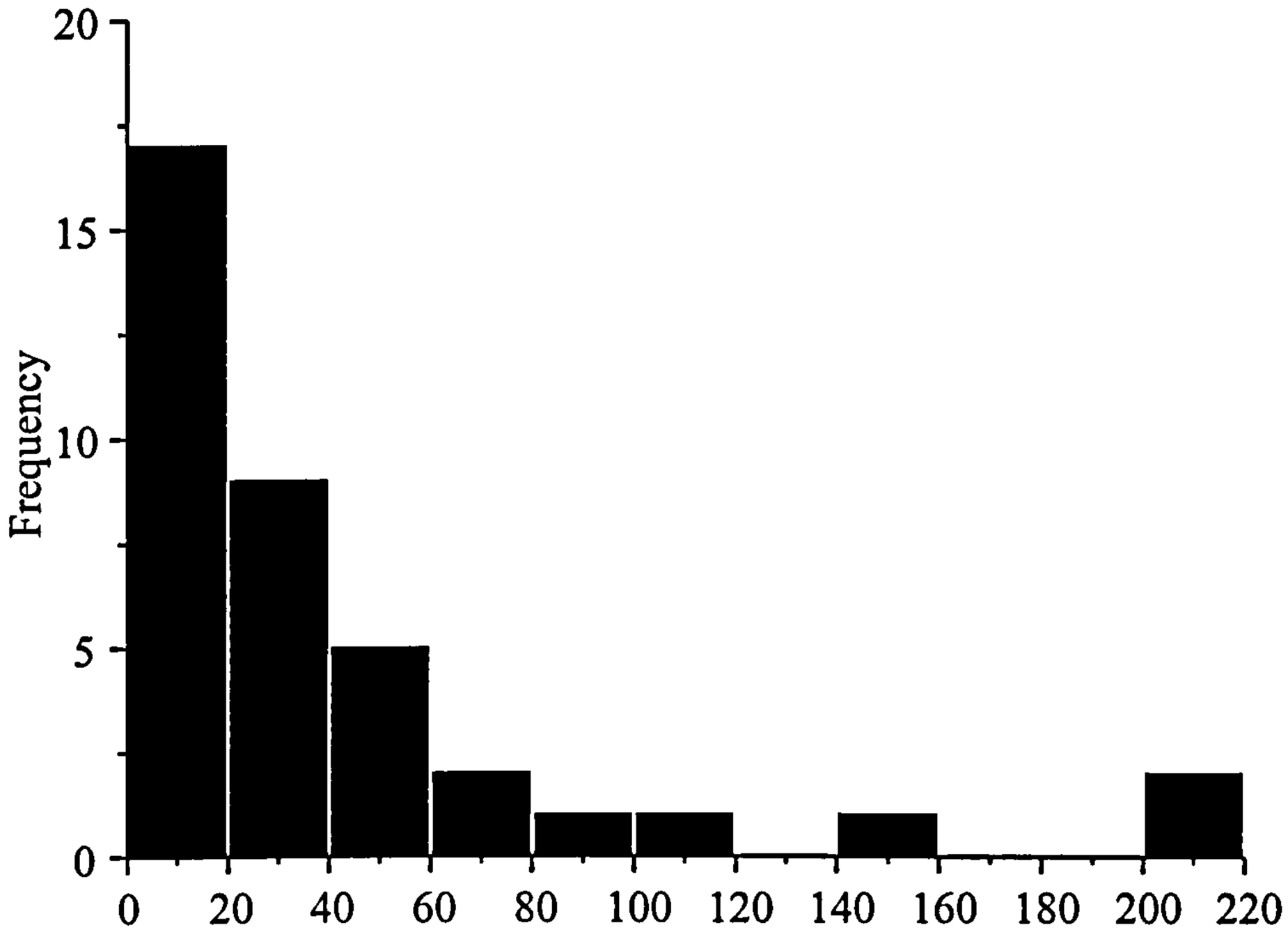

Figure 6.2 Histogram of cestode abundance per shrew at dissection.

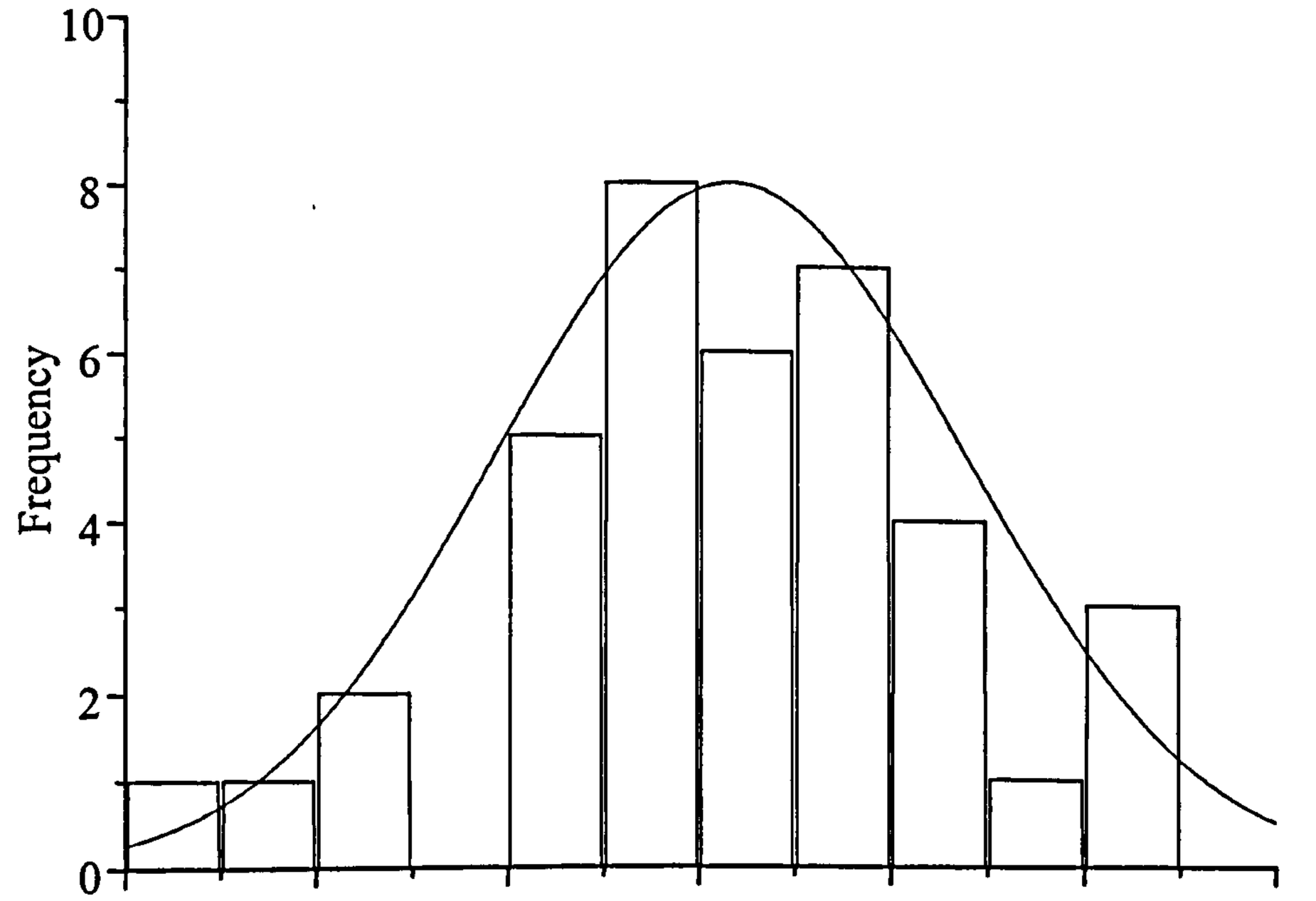

Figure 6.3 Histogram of log-transformed cestode abundance per shrew at dissection.

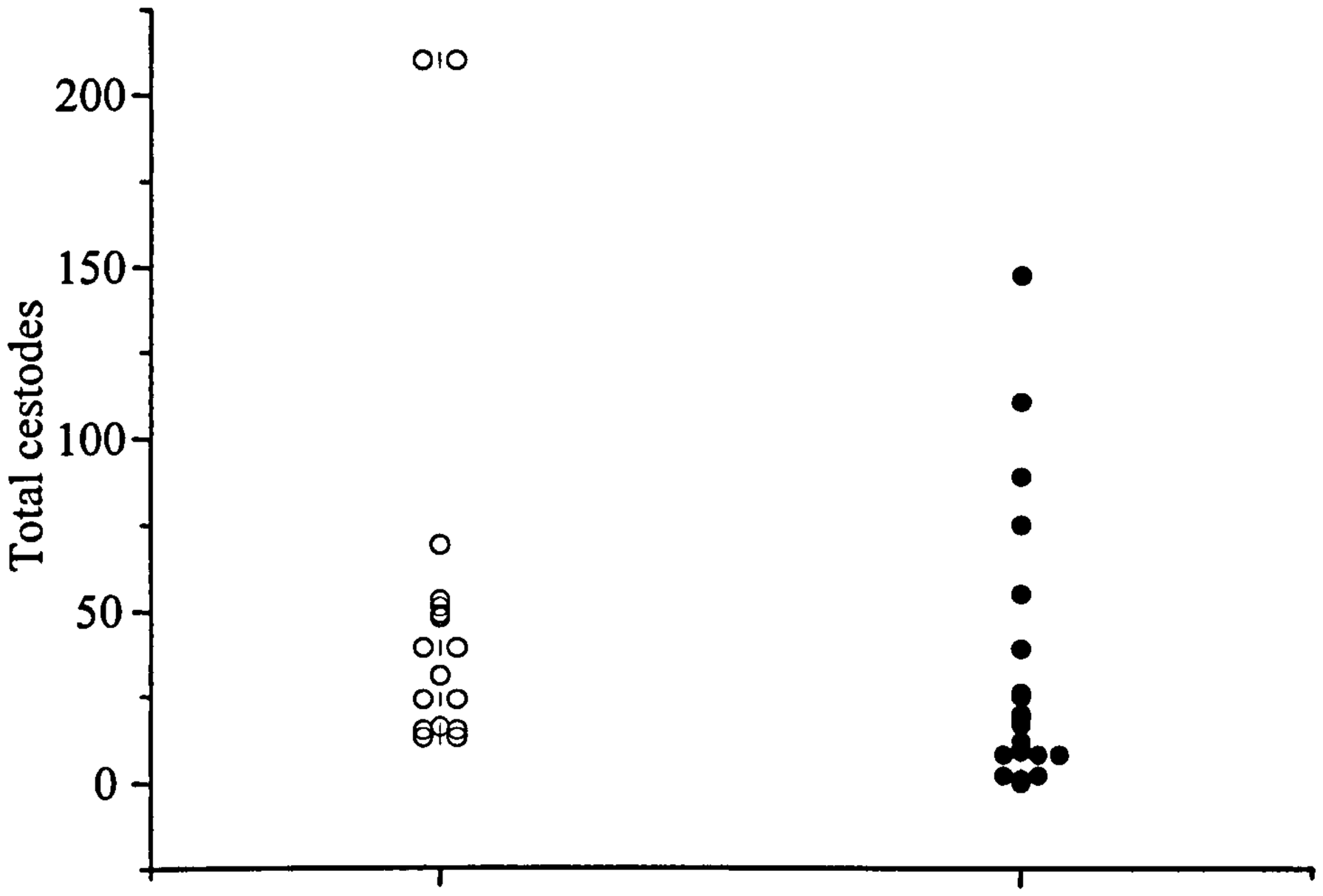

Figure 6.4 Cestode abundances of mock-treated and treated common shrews at dissection.

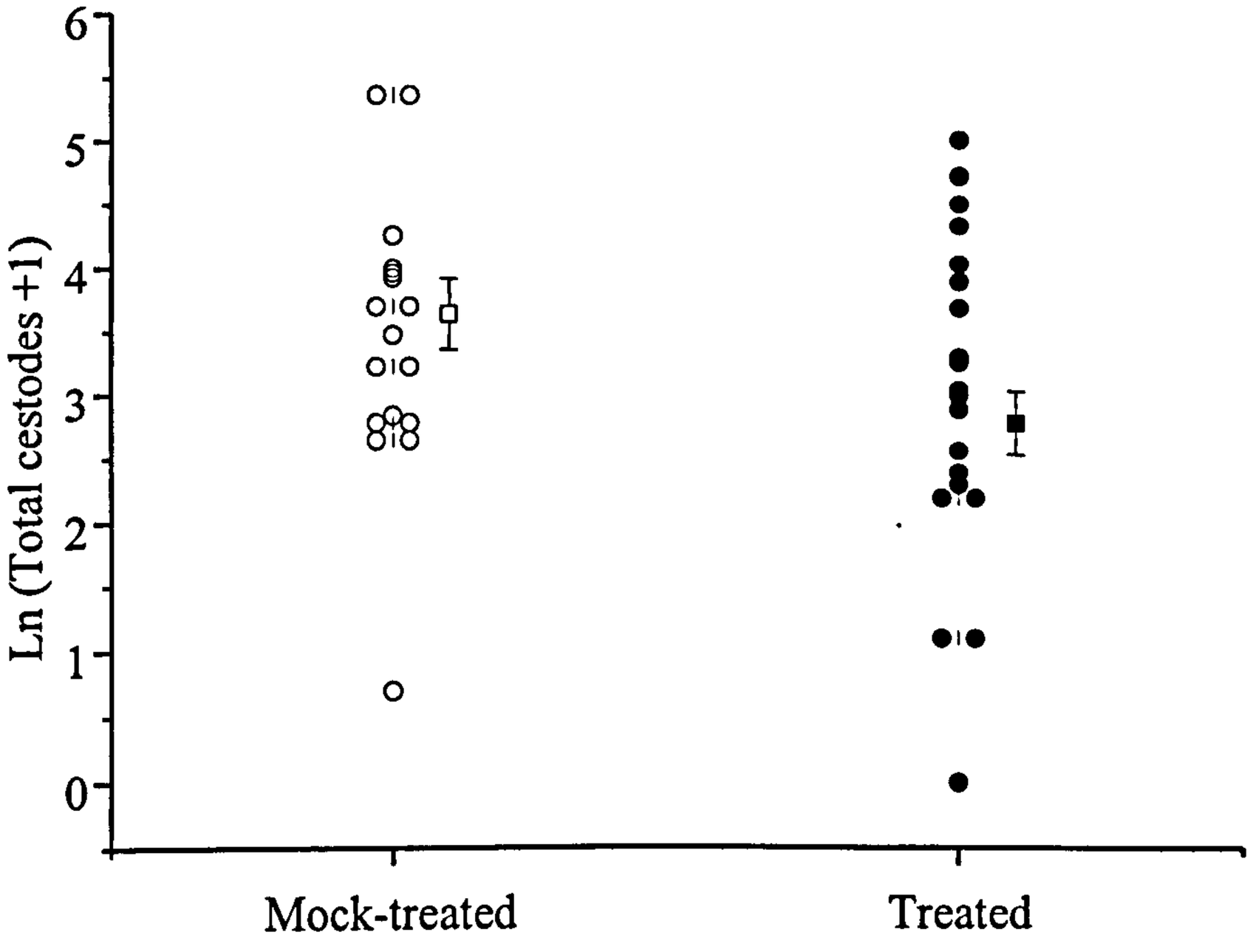

Figure 6.5 Log-transformed (+1) cestode abundances of mock-treated and treated shrews at dissection. Squares represent group means predicted by general linear model (see text for details).

category ($F = 2.25$, $df = 1, 36$, NS), sex ($F = 2.07$, $df = 1, 36$, NS), or an interaction between the two ($F = 0.36$, $df = 1, 34$, NS) on number of cestodes per shrew. There is therefore a discrepancy between the results of the two models as to whether there is a significant effect of treatment on cestode abundance. **Figure 6.6** and **Figure 6.7** show q-q plots of standardized deviance residuals from the analysis of log-transformed data and overdispersed Poisson model respectively. In both cases, treatment category is entered as the only independent variable. Whilst neither show a perfect straight line (which would represent normally distributed residuals), the distinct curve in **Figure 6.7** seems to suggest the Poisson model does not fit the data as well as the normal model applied to the $\log_e$-transformed data. In addition, as the non-parametric test (which makes no distributional assumptions) also found a difference between the treated and mock-treated categories, it would appear that there is at least some evidence for a lowering of cestode abundances by anthelmintic treatment.

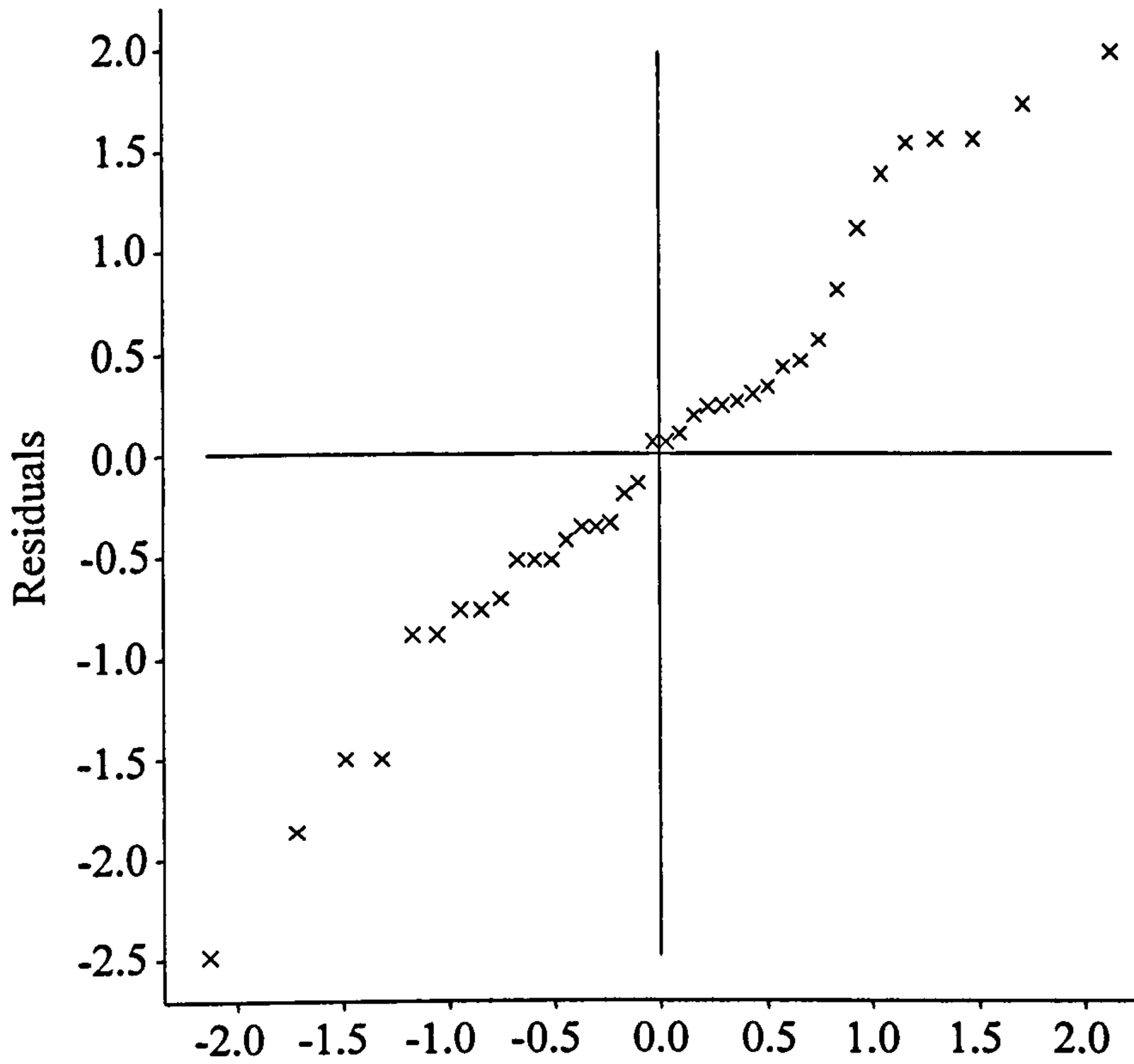

Figure 6.6 q-q plot of standardized residuals from analysis of log-transformed cestode data, with treatment category as independent variable and normal errors.

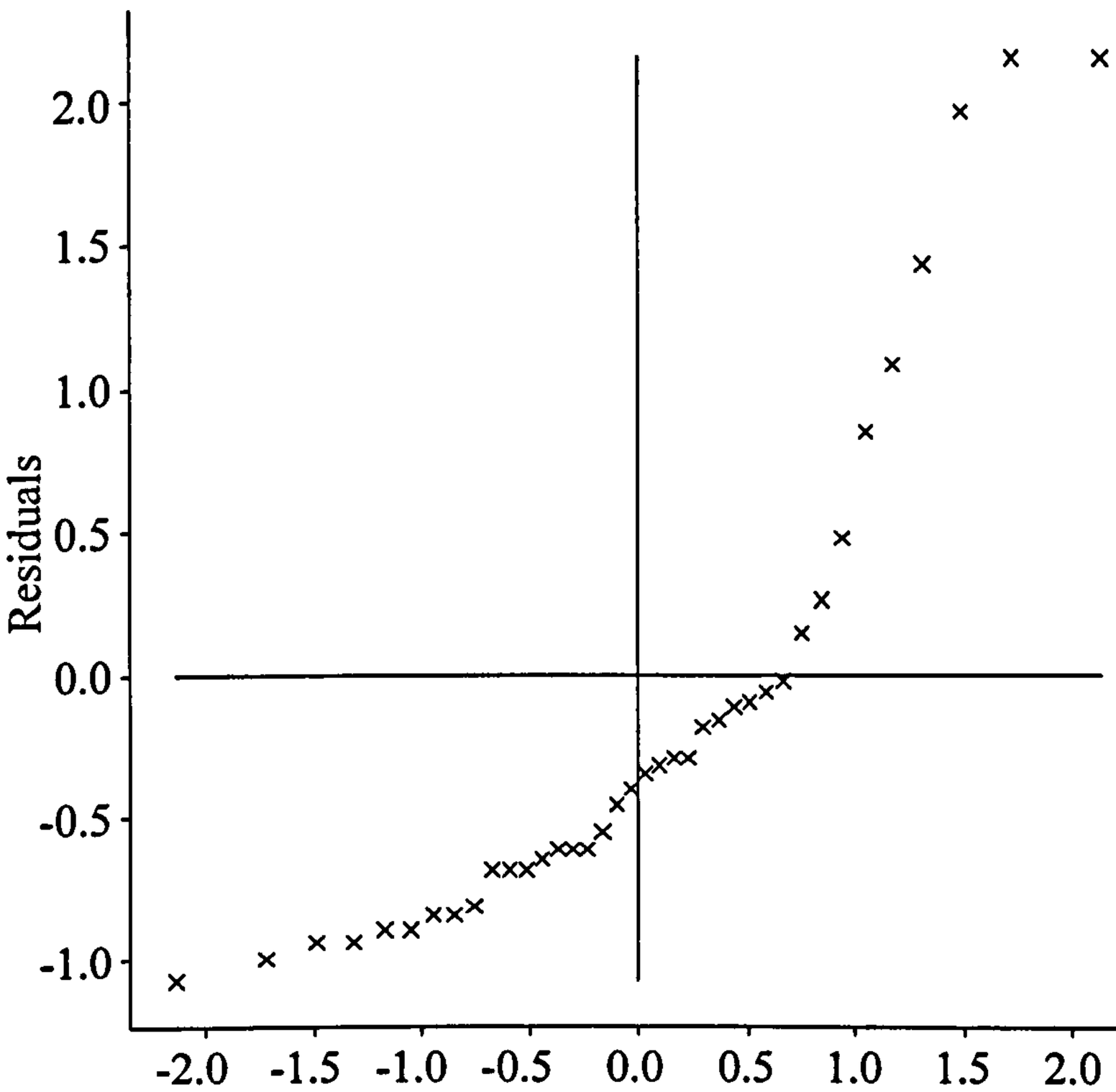

Figure 6.7 q-q plot of standardized residuals from analysis of cestode data using overdispersed Poisson errors, with treatment category as independent variable.

6.3.1.2 Nematodes

The distribution of nematodes between shrews (N = 37, one shrew was excluded as not all nematodes could be recovered at dissection) also exhibited right hand skew (0.76, SE = 0.39), although this did not appear as pronounced as for the cestode data (**Figure 6.8**). Aggregation, while still evident (k estimate = 2.39), was also less marked than found for cestodes. Log_e-transformation of the data (adding 1 first to remove zeros) of the nematode data reduced skewness (0.01, SE = 0.39), and the Kolmogorov-Smirnov test found no evidence for a difference between the transformed data and the normal distribution (Z = 0.729, N = 37, NS). However inspection of **Figure 6.9** does not suggestion a close match to the Gaussian distribution.

A Mann-Whitney U-test found no difference between treatment categories in the mean ranks of number of nematodes per shrew (**Table 6.1, Figure 6.10**). Analysis of log_e-transformed data found no difference between treatment groups (F = 1.51, df = 1, 35, NS, **Figure 6.11**). However, there was some suggestion of a difference between host sexes with a trend towards more nematodes per male than female shrew (F = 3.13, df = 1, 35, P<0.1), but no evidence of a treatment by sex interaction (F = 0.52, df = 1, 33, NS). An overdispersed Poisson mode applied to untransformed data found no effect of treatment category (F = 1.67, df = 1, 35, NS), host sex (F = 1.75, df = 1, 35, NS), or an interaction between the two (F = 0.21, df = 1, 33, NS) on nematode abundances.

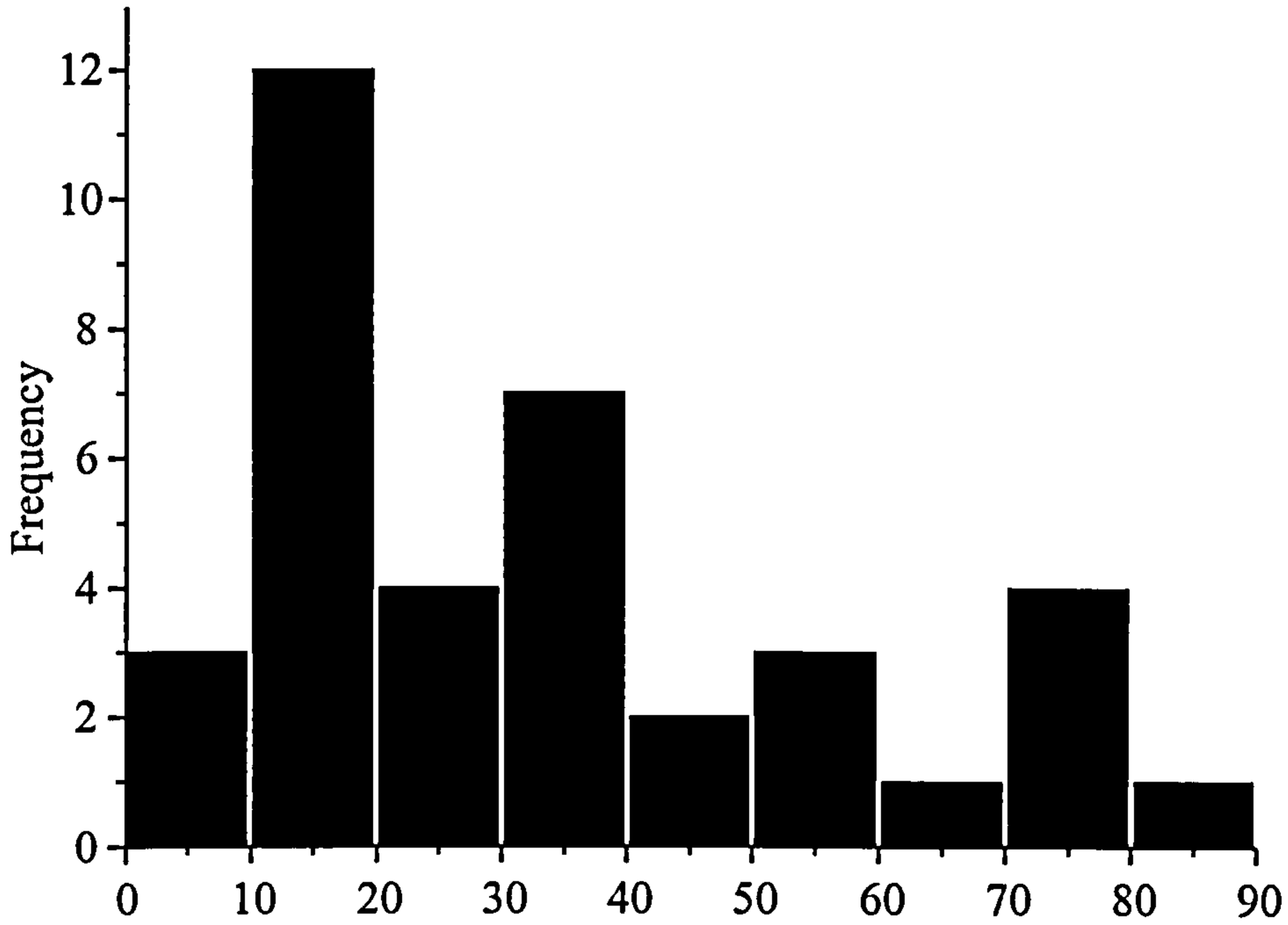

Figure 6.8 Histogram of nematode abundance per shrew at dissection.

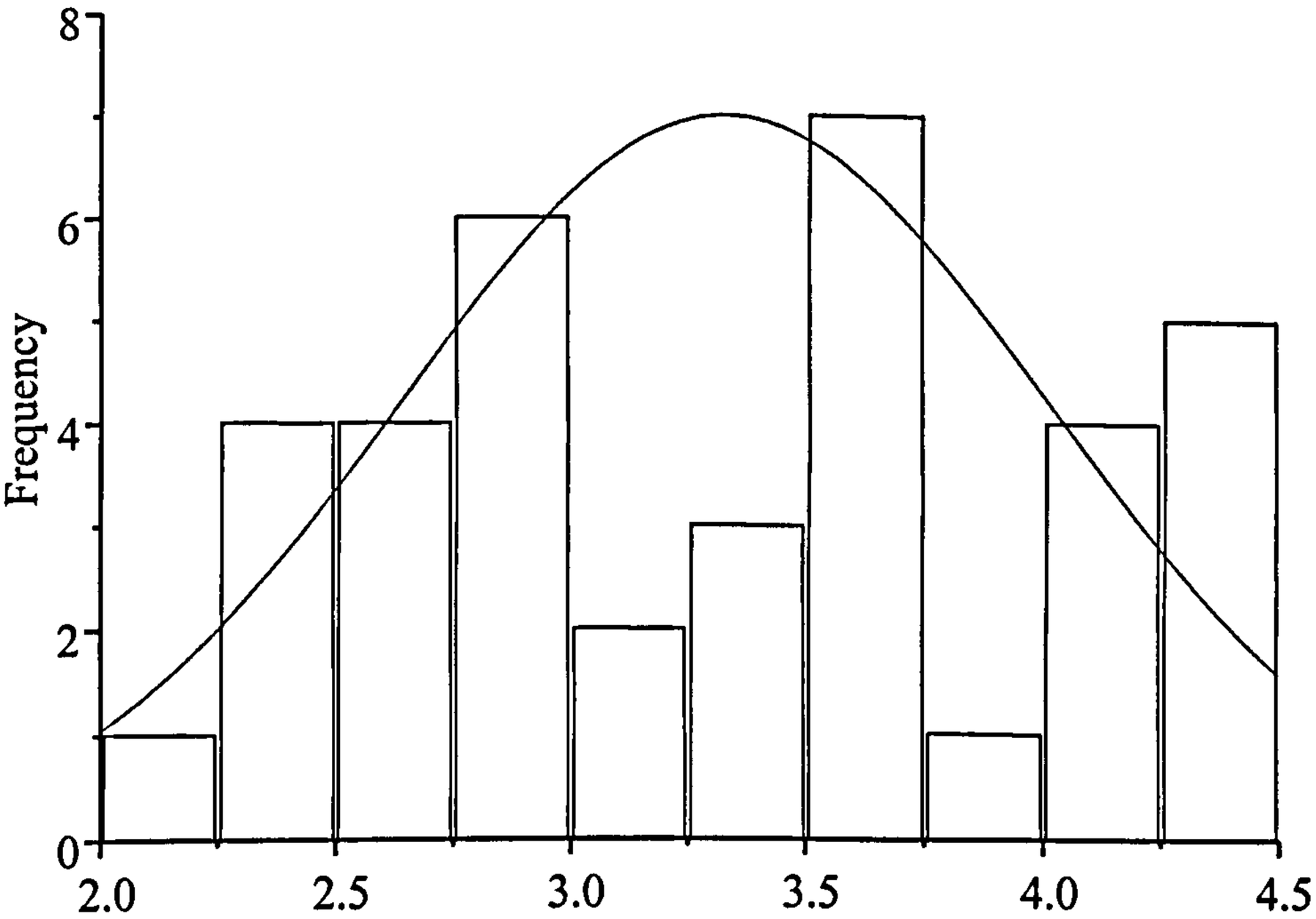

Figure 6.9 Histogram of log-transformed nematode abundance per shrew at dissection.

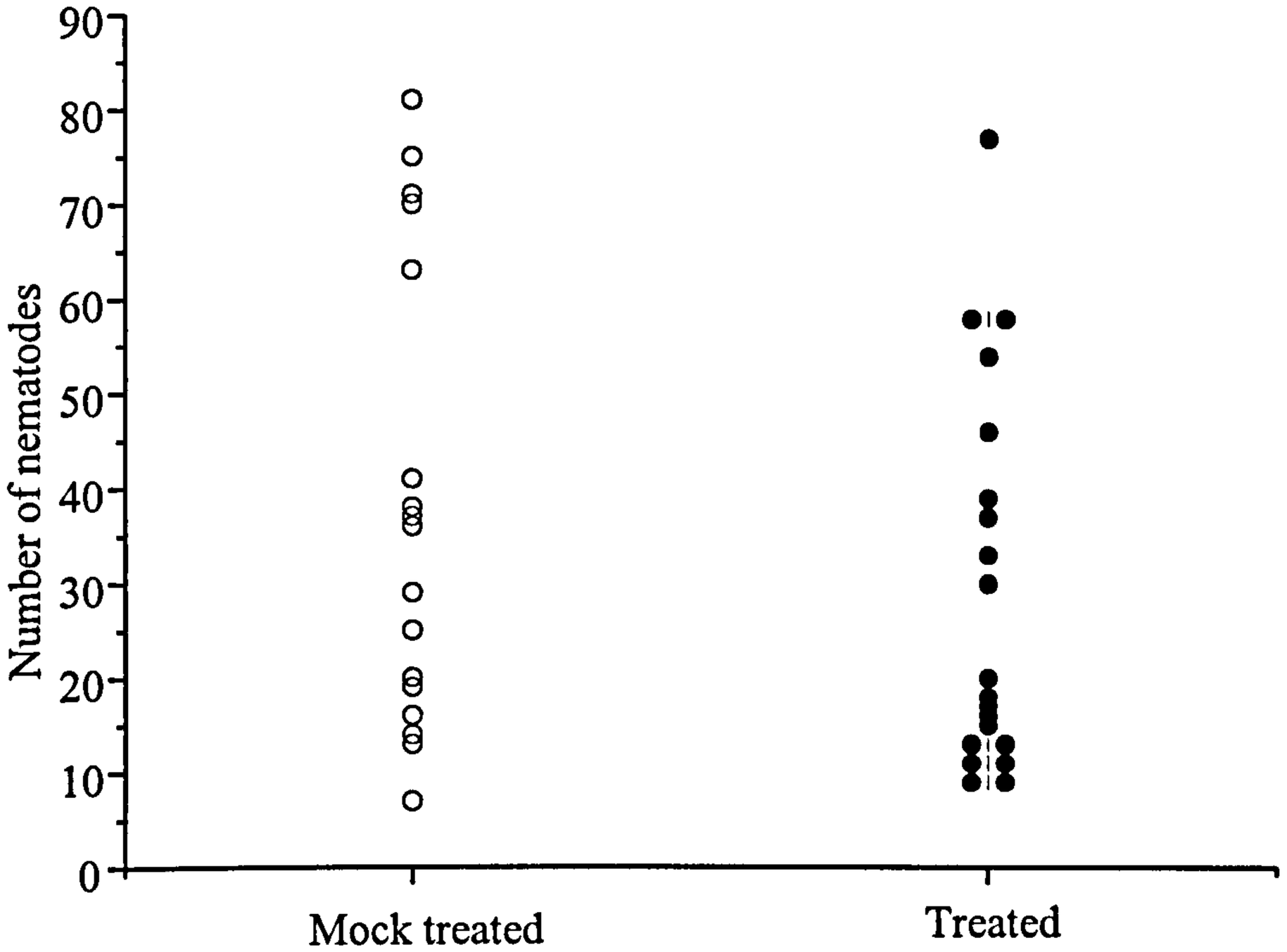

Figure 6.10 Nematode abundances of mock-treated and treated shrews at dissection.

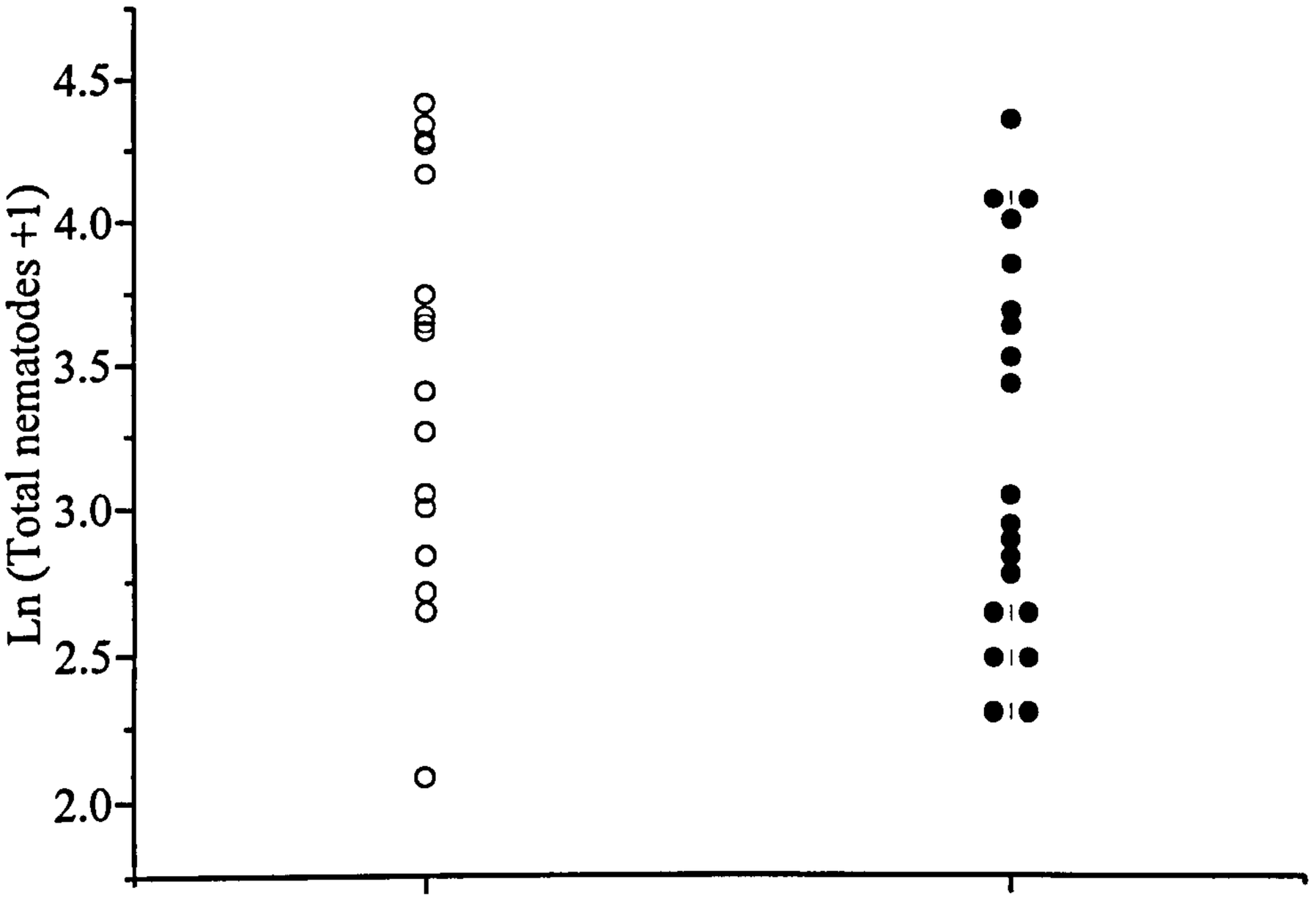

Figure 6.11 Log-transformed (+1) nematode abundances of mock-treated and treated shrews at dissection.

6.3.1.3 Digeneans

The distribution of digeneans between shrews appeared aggregated and positively skewed (k estimate = 3.70, skew = 0.76, skew SE = 0.38, **Figure 6.12**). Log_e transformation of the data reduced and changed direction of skew (-0.30, SE =0.38), resulting in an approximate fit to the Gaussian distribution (N = 38, Z = 0.83, NS; **Figure 6.13**).

A Mann-Whitney U-test found lower median abundance of digeneans in the treated group (**Table 6.1, Figure 6.14**), and analysis of log-transformed data gave a similar result (t = 2.34, df = 36, P<0.05, **Figure 6.15**), but found no evidence for a sex difference (F = 1.75, df = 1, 35, NS) or a treatment by sex interaction (F = 0.21, df = 1, 33, NS). In addition, analysis of untransformed data by generalized linear model also found a significant difference between treatment categories, with fewer digeneans per shrew in the treatment category (t = 2.17, df = 36, P<0.05, **Figure 6.14**), but also found no difference between sexes (F = 0.71, df = 1, 35, NS), or any evidence for an interaction between treatment and host sex (F = 0.95, df = 1, 34, NS).

6.3.2 Comparison of body weights of treated and mock-treated common shrews

Body weight (minus gut weight) of males approximated a Gaussian distribution (N = 24, Kolmogorov Smirnov Z = 0.50, NS) at post mortem. Body weights of males in the treated group (N = 13, mean = 11.3g, SE = 0.28) were found to be heavier than those in the mock-treated category (N = 11, mean = 10.6g, SE = 0.23; t = 2.08, df = 22, P<0.05; **Figure 6.16**).

Female body weights (minus gut and embryos) were too sparsely distributed to appear normal, although a Kolmogorov-Smirnov test found no evidence for a difference between the data and the expected distribution (N = 14, Z = 1.19, NS). Neither a t-test (t = 0.84, df = 12, NS) nor a Mann-Whitney U-test ($N_{mock\text{-}treated} = 6$, $N_{treated} = 8$, U = 22, NS) found a difference between the weights of the two groups (**Figure 6.17**).

Figure 6.18 and **Figure 6.19** show weights of male and female shrews recorded throughout the experiment. Both graphs show a general decrease in body mass over the winter period, then a steady increase over the spring associated with sexual maturation.

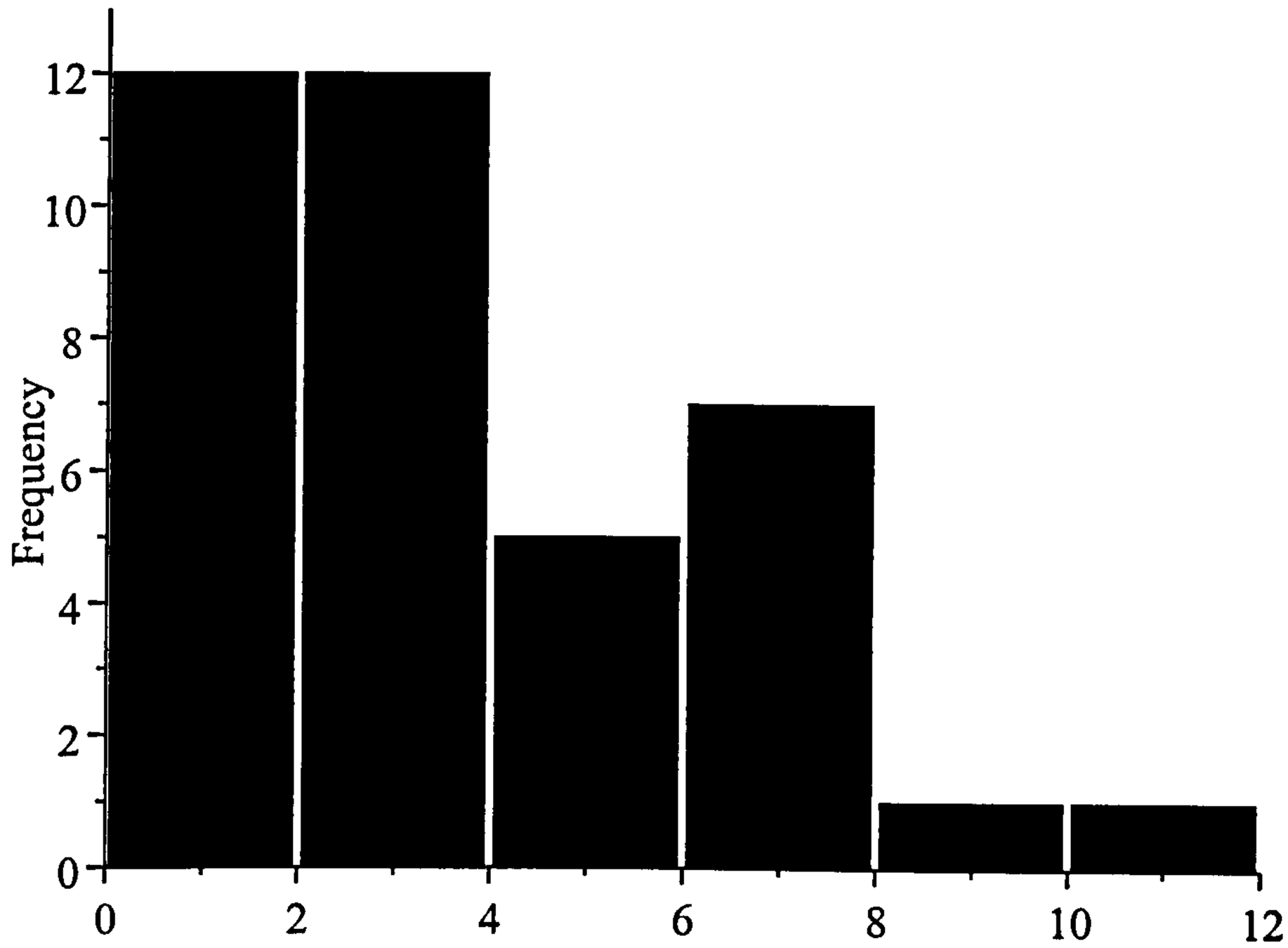

Figure 6.12 Histogram of digenean abundance per shrew at dissection.

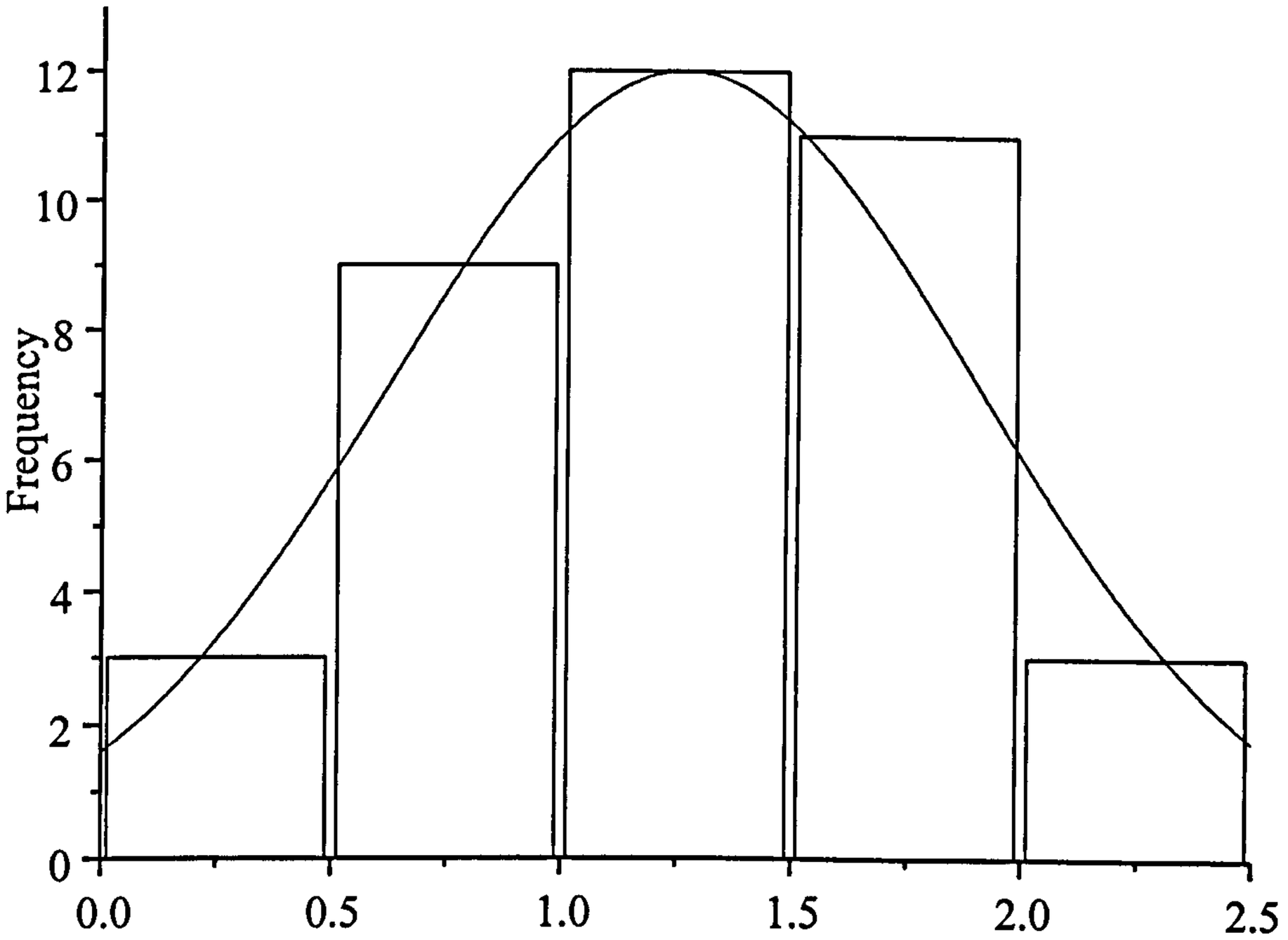

Figure 6.13 Histogram of log-transformed digenean abundance per shrew at dissection.

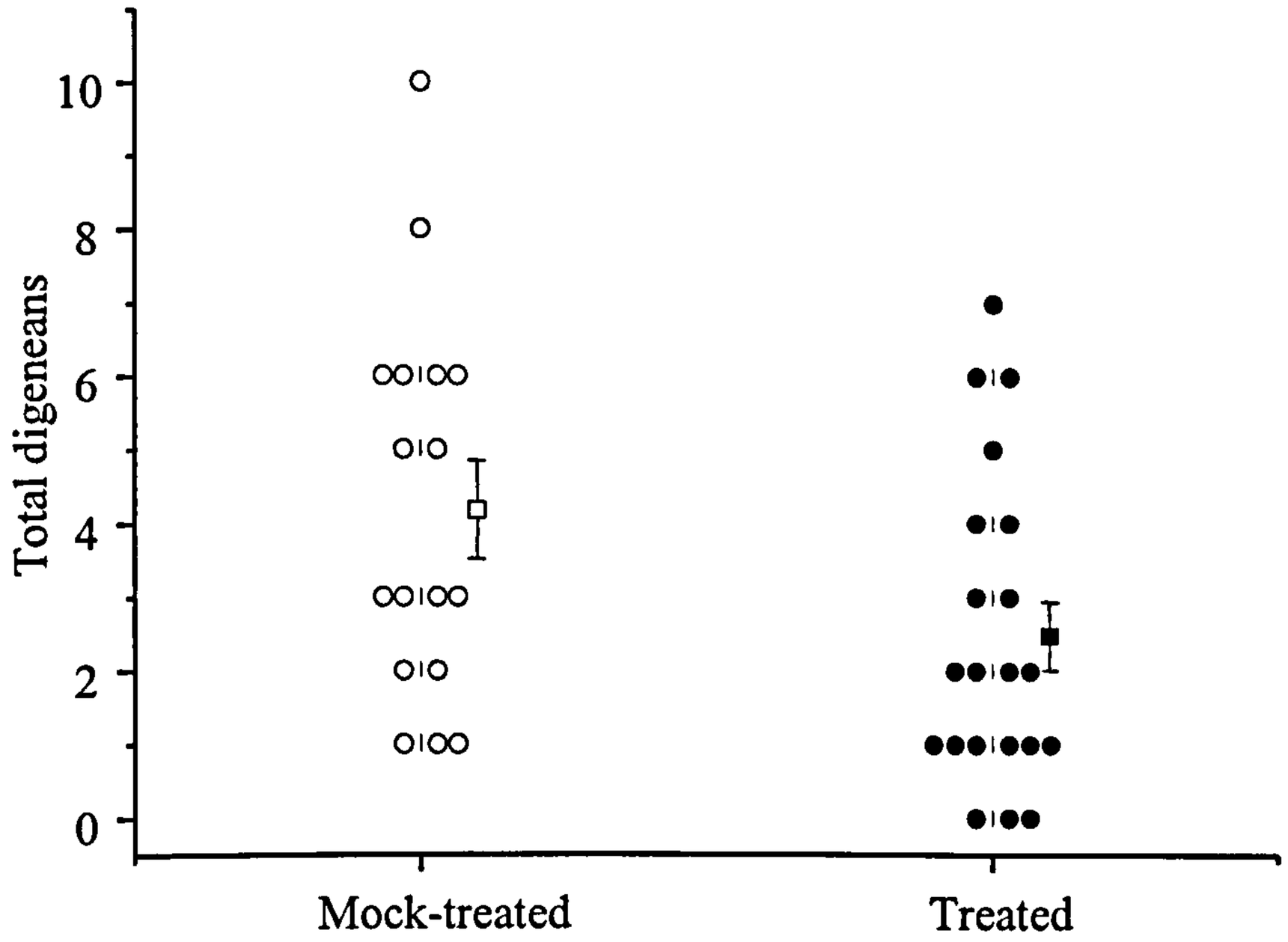

Figure 6.14 Digenean abundances of mock-treated and treated shrews at dissection. Squares represent group means predicted by generalized linear model (see text for details).

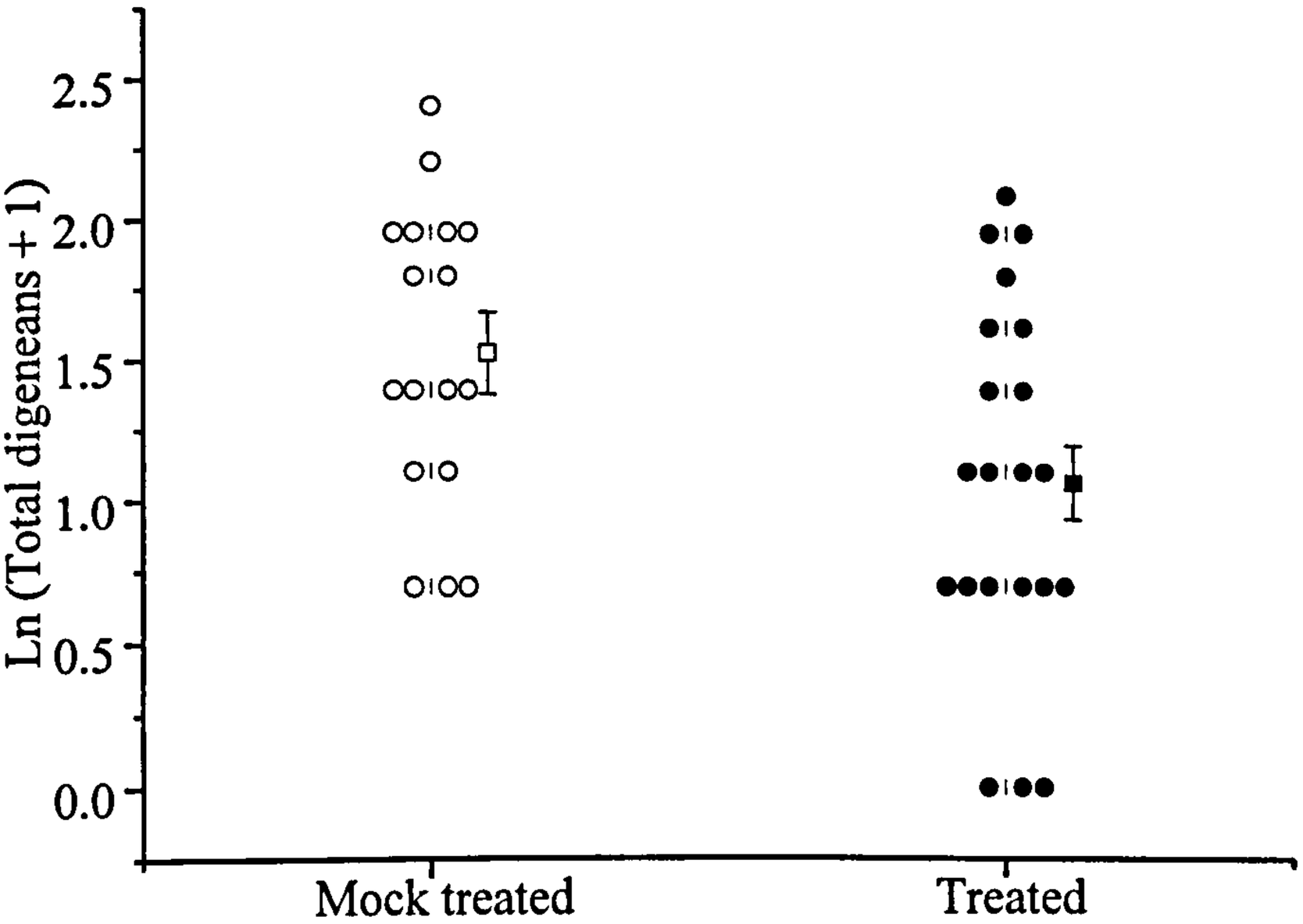

Figure 6.15 Log transformed (+1) digenean abundances of mock-treated and treated shrews at dissection. Squares represent group means predicted by general linear model (see text for details).

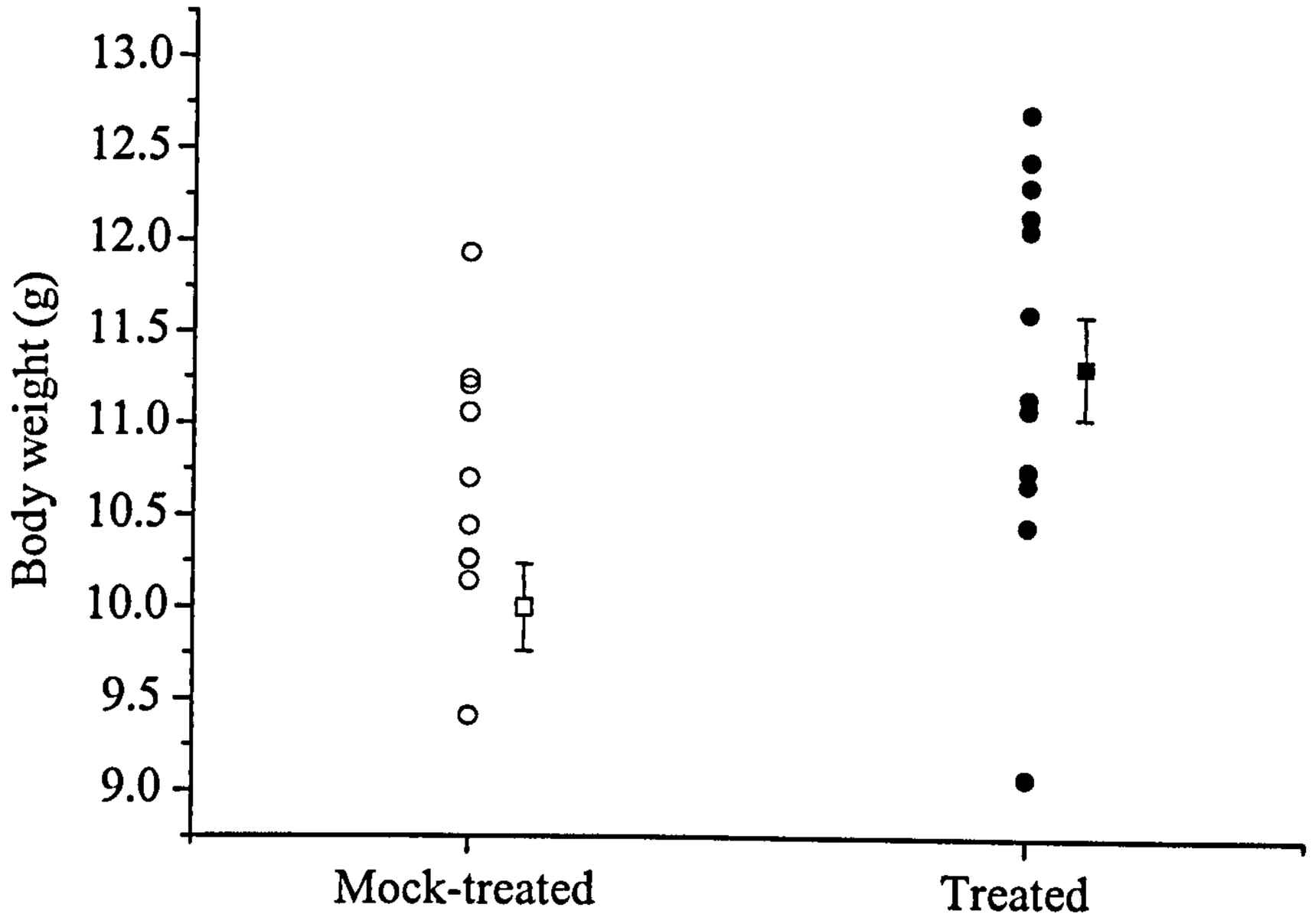

Figure 6.16 Body weights (minus weight of gut) of male shrews at dissection. Squares represent group means with standard errors.

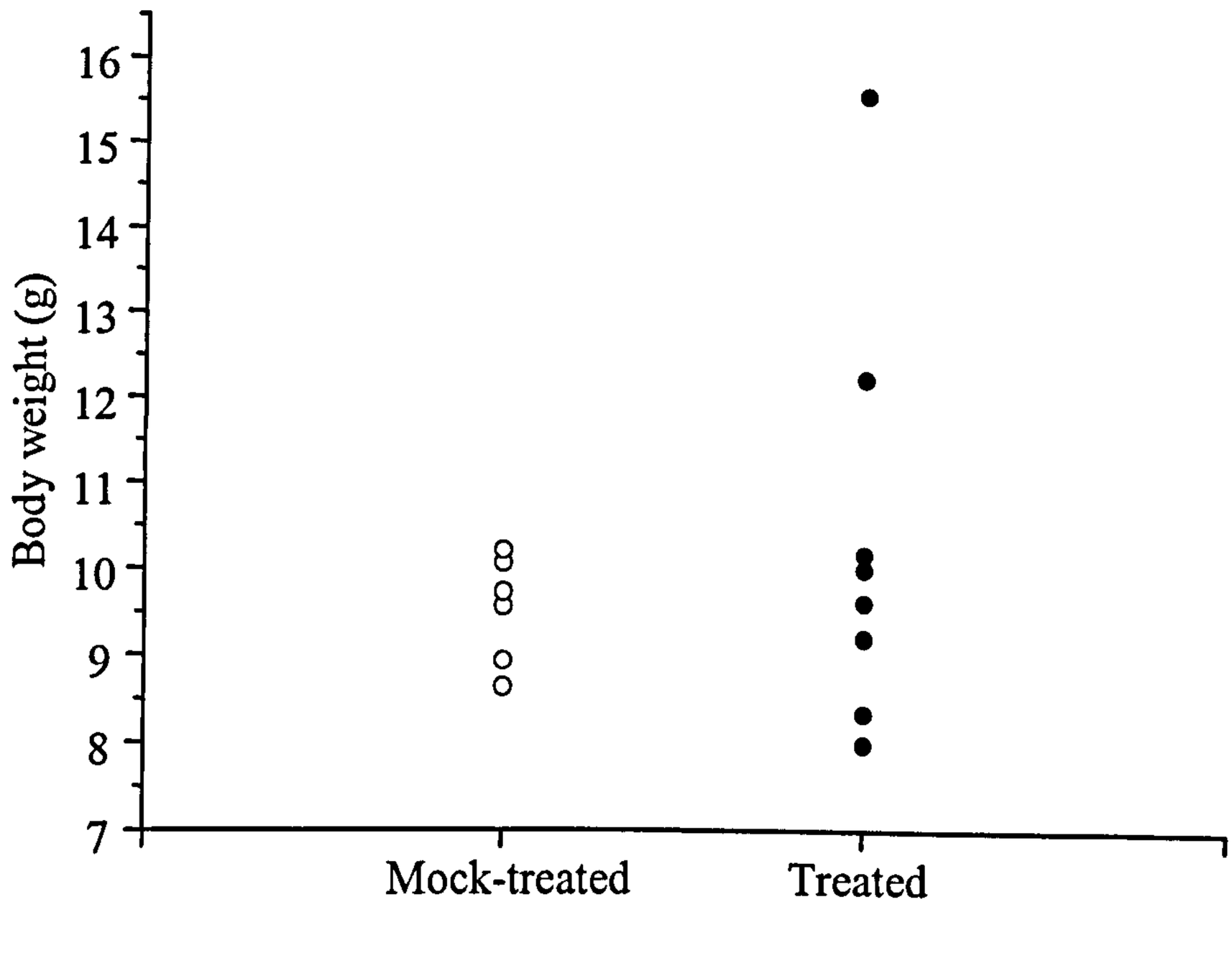

Figure 6.17 Body weights of female shrews at dissection (minus weight of gut and embryos).

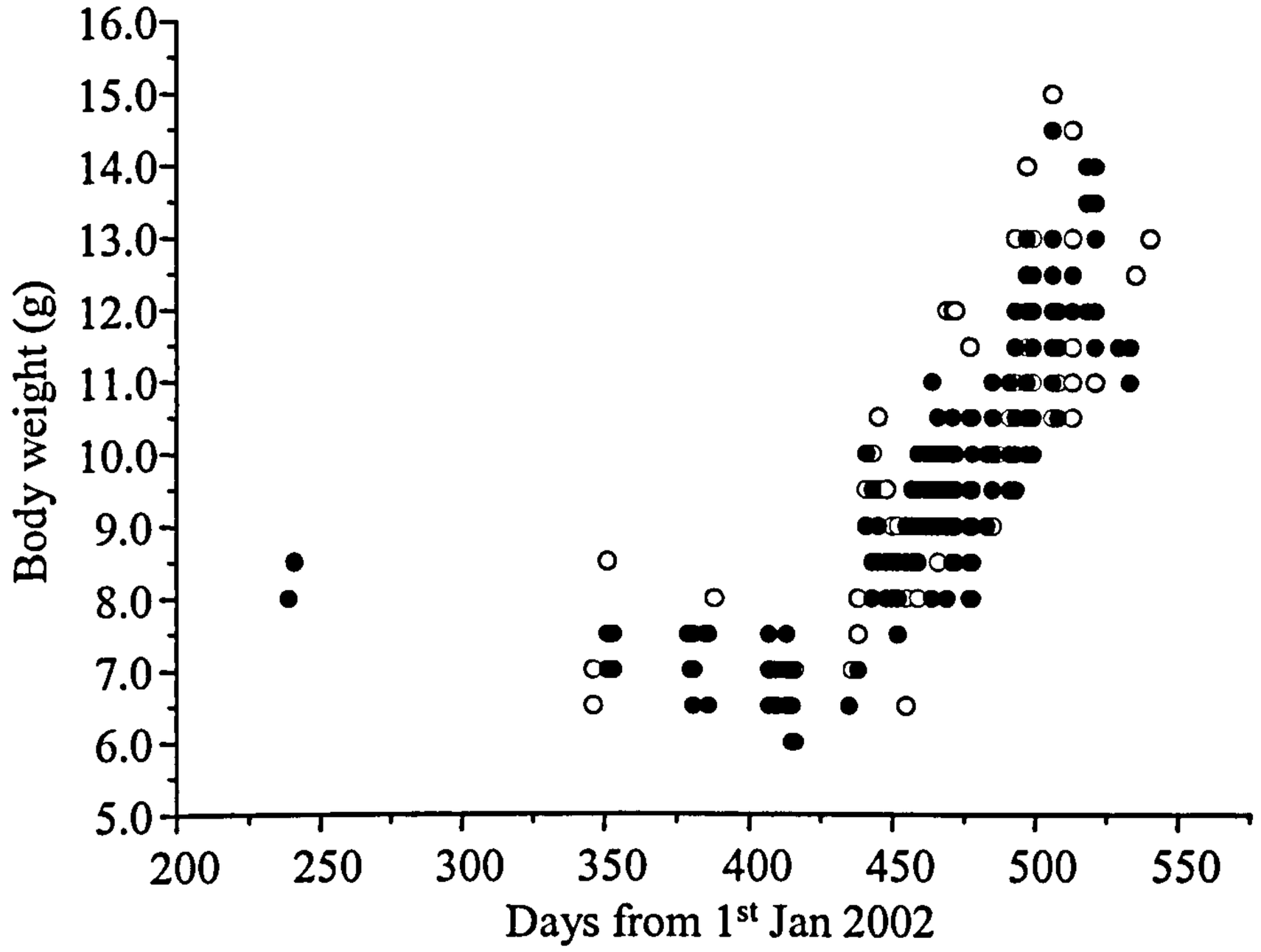

Figure 6.18 Body weights of treated (filled circles) and mock-treated (open circles) male shrews.

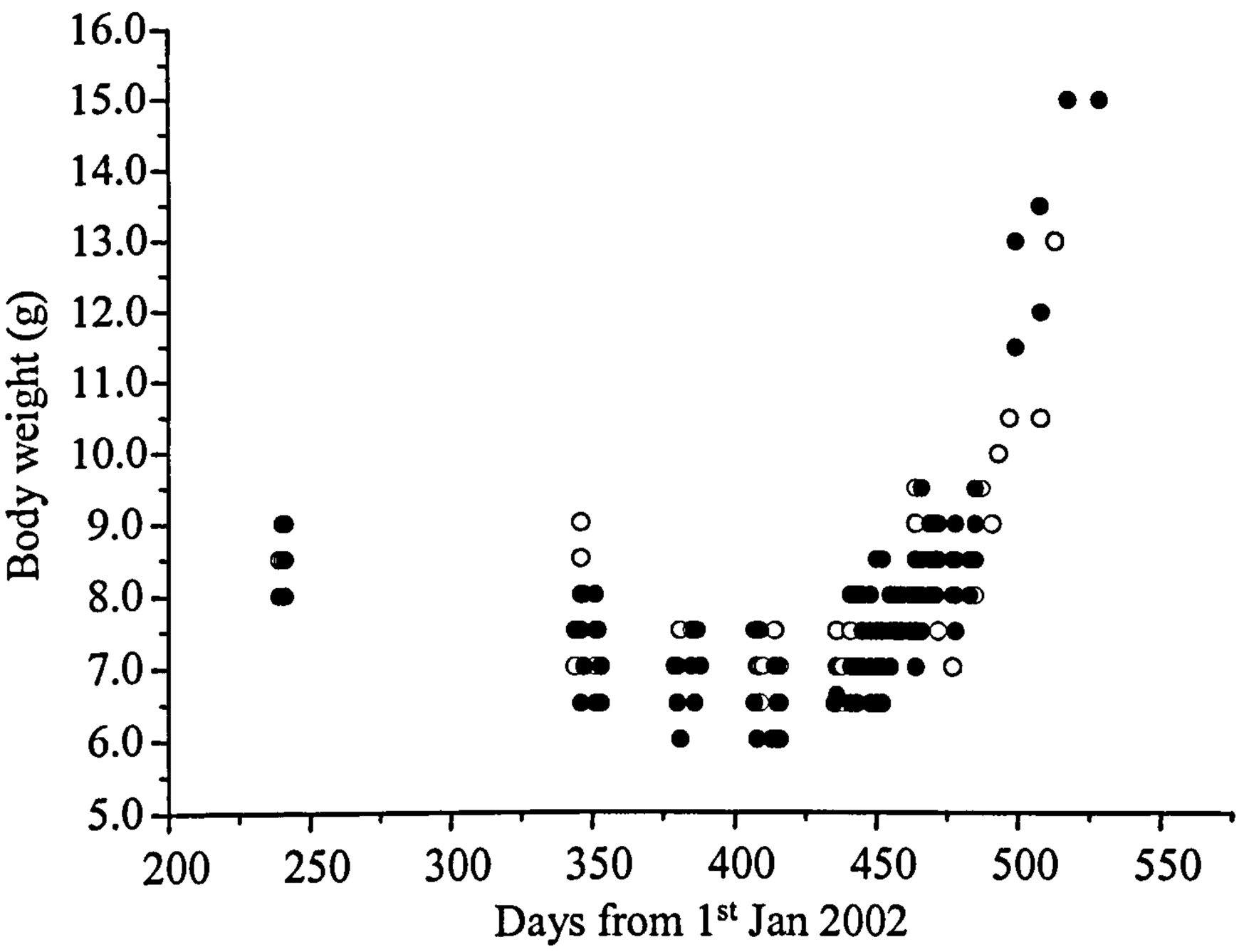

Figure 6.19 Body weights of treated (filled circles) and mock-treated (open circles) female shrews.

Neither graph shows an obvious difference between weights of treated and mock-treated shrews, although such a difference could be disguised by individual differences between animals. Comparing average weights of males between days 435 and 455 (corresponding to the last two weeks in March and first two weeks in April, approximate time of sexual maturation), no difference was found between treatment categories ($N_{mock\text{-}treated} = 4$, $N_{treated} = 5$, $U = 6.0$, NS). Similarly, a comparison of average weights of females at approximate time of sexual maturation (between days 441 and 469, corresponding to the whole of April) also found no difference between treatment categories ($N = 16$, $t = 0.7$, NS).

6.3.3 Comparison of movement between treated and mock-treated common shrews

Number of different traps visited by males and females over three subsequent half-month periods is shown in **Figure 6.20** and **Figure 6.21** respectively. Controlling for differences between individuals using an error term, no significant effect of treatment, time period (1 - 3) or an interaction between the two was found for males or females (**Table 6.2**). Inspection of the figures suggests a possible difference between the two sexes, with male shrews visiting more traps than females. Combining data from both treatment categories, such a difference was found to be statistically significant (**Table 6.3**).

Table 6.2 Effects of treatment and sampling period on number of different traps visited by male and female shrews

Sex	Factor	Wald	df	P
Males	Treatment category	0.5	1	NS
	Time period	0.1	2	NS
	Treatment by period interaction	0.7	2	NS
Females	Treatment category	0.0	1	NS
	Time period	2.0	1	NS
	Treatment by period interaction	0.4	2	NS

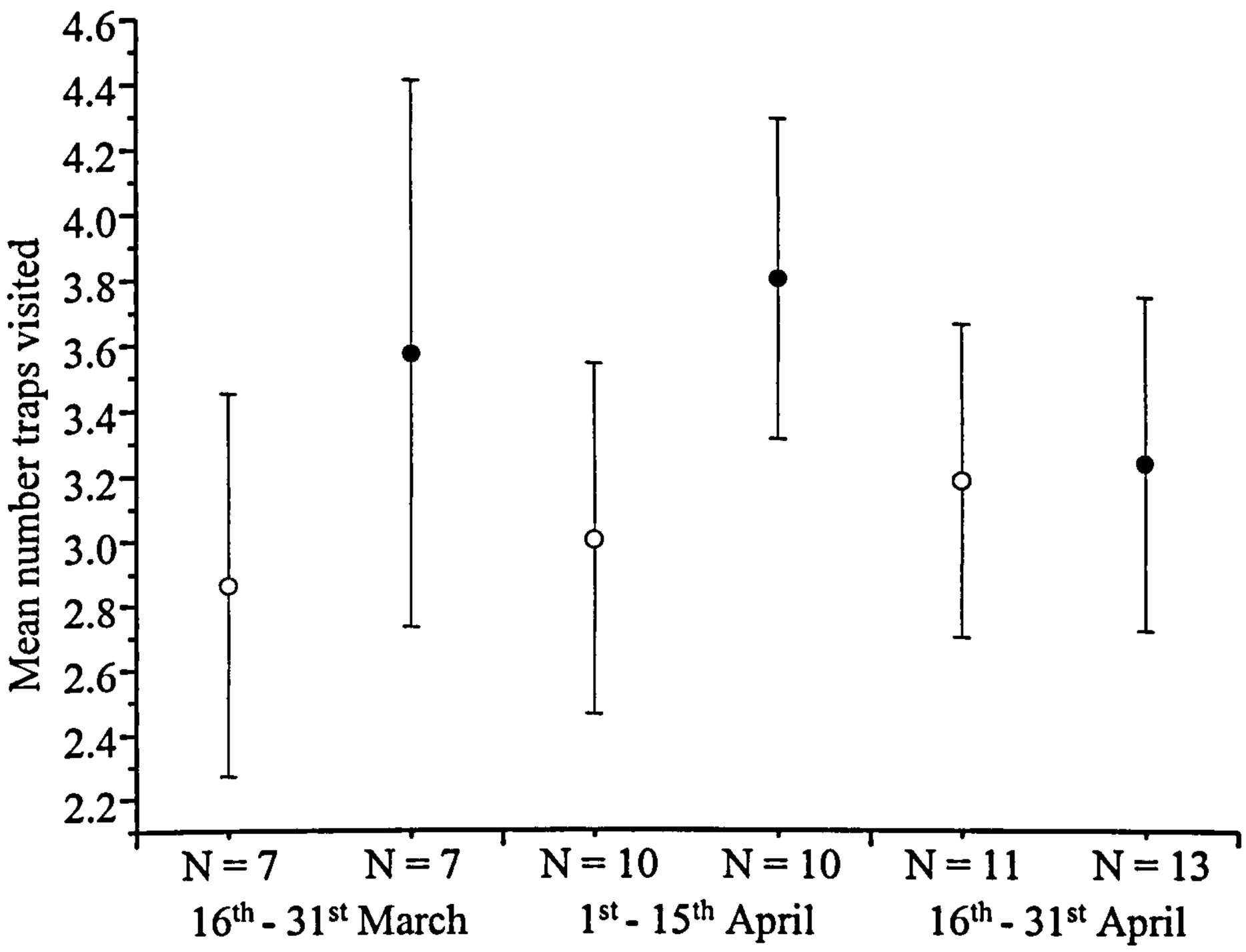

Figure 6.20 Average number of different traps visited by mock-treated (open circles) and treated (filled circles) male shrews. Bars represent standard errors.

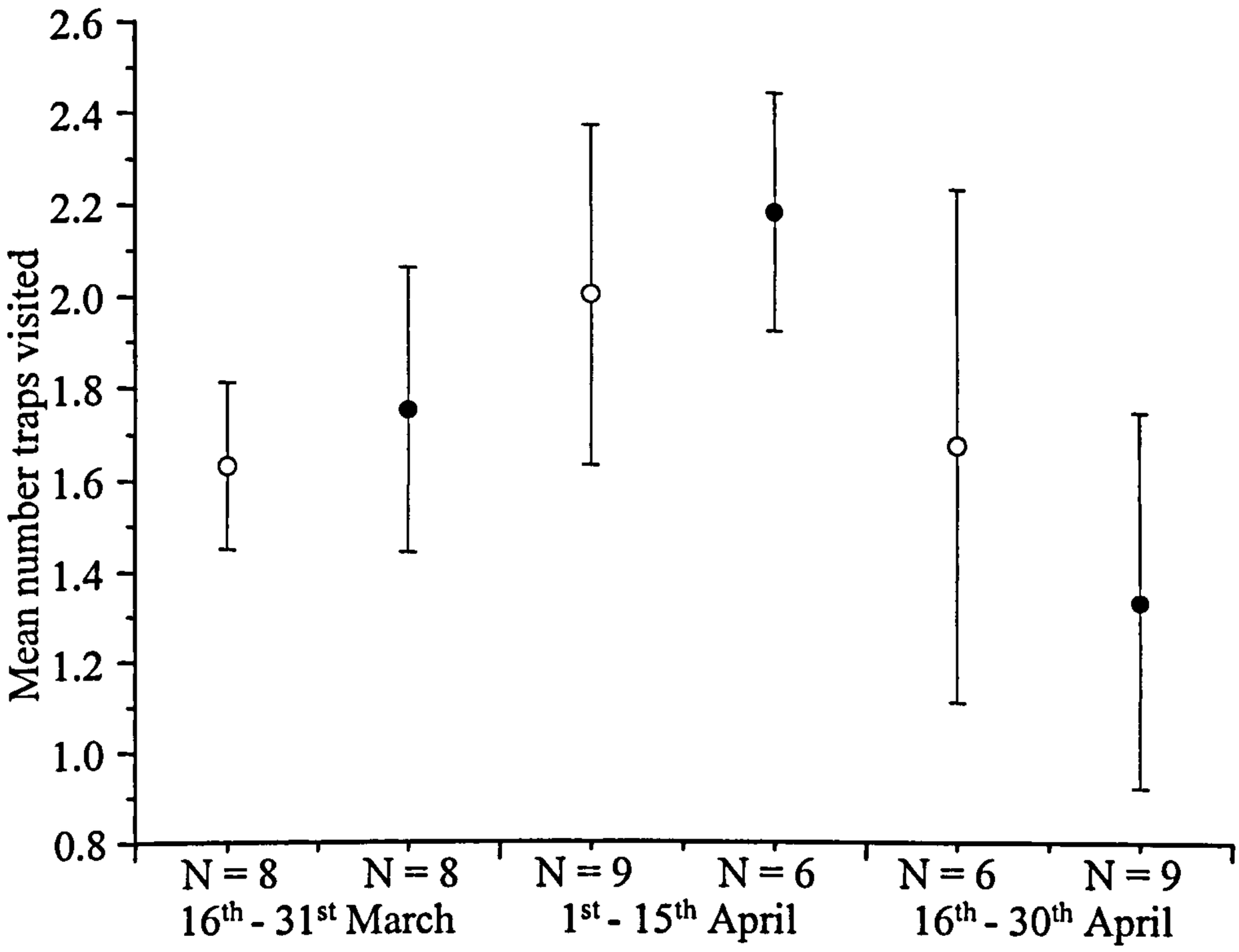

Figure 6.21 Average number of different traps visited by mock-treated (open circles) and treated (filled circles) female shrews. Bars represent standard errors.

Table 6.3 Effects of host sex and sampling period on number of different traps visited.

Factor	Wald	df	P
Sex[1]	18.1	1	<0.05
Time period	0.5	2	NS
Sex by period interaction	0.7	2	NS

1. Predicted (back-transformed) means: females 1.73, males 3.22.

6.3.4 Comparison of sperm counts and testis mass of treated and mock-treated shrews

No difference in $\log_e$-transformed sperm count or absolute testis mass was found between treatment categories (sperm count: N = 23, t = 0.52, NS; **Figure 6.22**, testis mass: N =24, t = 0.62, NS; **Figure 6.23**). In a general linear model with testis mass as the dependent variable, and treatment category and body mass (minus gut weight) entered simultaneously as independent variables, neither term was found to be related to testis mass (body mass: df = 21, t = 0.66, NS; treatment category: df = 21, t = 0.29, NS). This suggests there is no difference in relative testis mass between treatment groups.

6.3.5 Survival of treated and mock-treated shrews

Cumulative survival from first injection for both treated and mock-treated shrews is shown in **Figure 6.24.** It appears from the graph that, initially, a higher proportion of treated shrews survived than mock-treated shrews. However, further from the time of first treatment (around 230d), the two survival curves cross, suggesting mock-treated shrews outlived treated shrews. The associated Breslow and Tarone-Ware statistic (the latter considered more robust for comparisons of survival curves with different shapes, Tarone & Ware, 1977) both give some indication there may be a difference in survival between treated (N = 30) and mock-treated (N = 30) shrews (Breslow 3.89, df = 1, P<0.05; Tarone-Ware 3.44, df = 1, P<0.07).

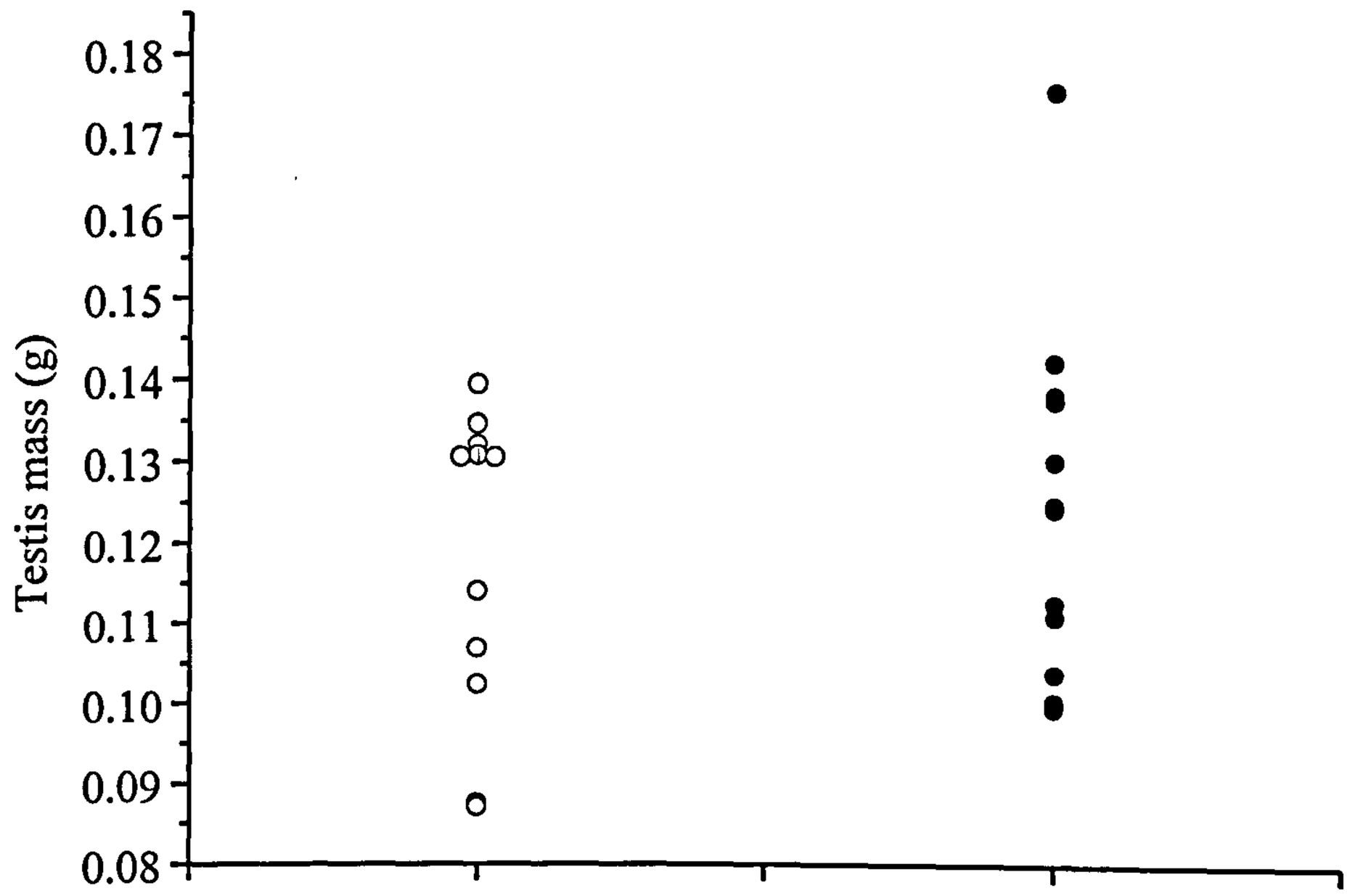

Figure 6.22 Testis mass of treated (filled circles) and mock- treated (open circles) shrews.

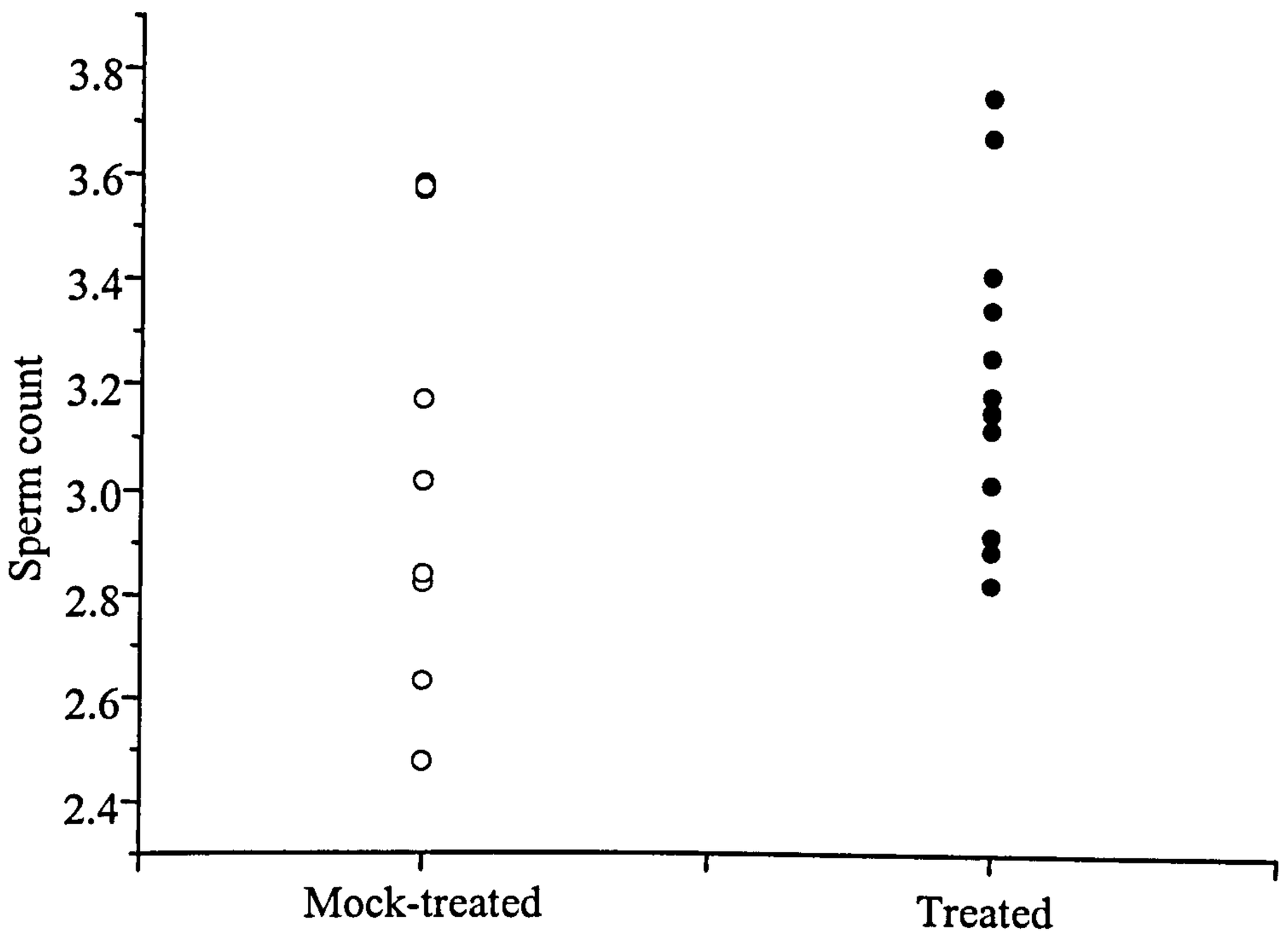

Figure 6.23 Log-transformed sperm counts of treated (filled circles) and mock-treated (open circles) shrews.

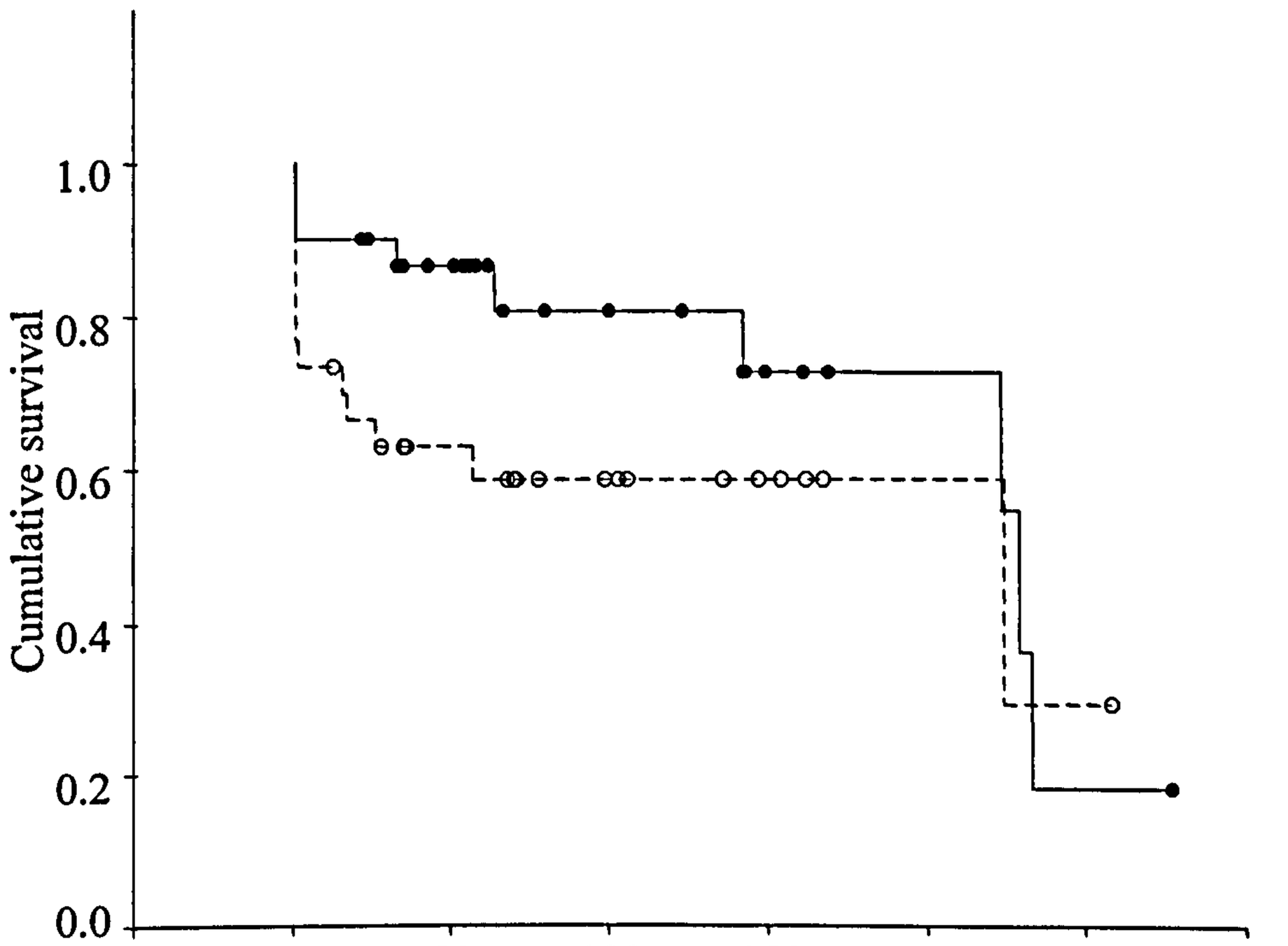

Figure 6.24 Cumulative survival plot for treated (full line) and mock-treated (dotted line) shrews. Circles indicated censored cases (shrews brought in for dissection).

6.4 Discussion

Prior to the experiments presented in this thesis, no attempt had ever been made to reduce helminth burdens of common shrews. From data collected at dissection, the greatest effect of treatment in the field appeared to be on digenean abundance, with all three statistical tests used finding significantly fewer parasites per shrew in the treatment group. Comparing cestode abundances between groups, treatment appeared to significantly lower number of cestodes, as judged by Mann-Whitney U-tests and analysis of $\log_e$-transformed data, but not by application of the generalized linear model with overdispersed Poisson errors. Analysis of residuals suggested the general linear model applied to $\log_e$-transformed data gave a better fit than that of the Poisson generalized linear model. As the non-parametric test found a difference between the two groups (although it bordered on significance), which makes no assumption regarding the data distribution, it appears anthelmintic treatment -while not totally effective in eradicating cestodes- did significantly lower their abundance in the treatment category.

No difference was found in nematode abundance between the two groups by any of the tests used. A breakdown of number of nematodes at each site in the body (intestinal tract, oesophagus, bladder and encapsulated in adipose tissue) would be required to determine if treatment was totally ineffective, only effective at certain sites, or only against certain nematode species.

No significant differences in abundances of nematodes, cestode or digeneans was found between sexes. As discussed in **Section 3.4**, previous studies have also found little evidence of sex differences in patterns of parasite infestation in shrews (Lewis, 1968; Haukisalmi *et al.*, 1994).

Treated males were found to be heavier at dissection than those in the mock-treated category, once variability of recently consumed food was removed by subtracting gut weight. This suggests that reductions in the abundance of digeneans and/or cestodes allowed male shrews to reach a larger body mass by the breeding season. Helminth infection has previously been linked to reduced growth rates in humans (Hadju *et al.*, 1996) and captive animals (Crompton *et al.*, 1981; Shaw & Moss, 1990; Sykes, 1994). Whether larger body size is advantageous to shrews is unknown: behavioural studies suggest an individual's competitive ability may be determined more by the biting

strength of the jaw than body mass (Hanski *et al.*, 1991). However, shrews grow considerably prior to the breeding season, with males gaining weight faster than females (Shillito, 1963; Skarén, 1973; Pernetta, 1977). This would seem to suggest a selective advantage in body size related to breeding success. Greater body mass at dissection might also be related to the time at which shrews began to increase in weight prior to the breeding season. As males which mature earlier are more likely to adopt the more successful 'overlapping' mating strategy (Stockley *et al.*, 1994) this might indicate a potentially important effect of parasitism on shrew breeding success. However, no difference in male body mass was found between treatment categories at approximate time of maturation. This could suggest male shrews only begin to lose weight as a consequence of helminth parasitism during the breeding season. It is perhaps more likely that fluctuations in body weight associated with recently consumed food, coupled with difficulties in weighing live animals in the field with balances only accurate to 0.5g, may disguise differences detectable when weighing culled animals (with guts removed) in the laboratory. In addition, no difference in weights between females was found at dissection or in the field. At face value, this might indicate a sex-specific response to parasitism in shrews, with females compensating for effects of parasitism in other ways, perhaps by reducing reproductive effort. However, analysis of female body weights was complicated by the presence of embryos at different stages of development, the weights of which were subtracted prior to analysis. Some females were also found to be lactating, suggesting they were already rearing litters at time of dissection. Differences in female investment in current reproduction might therefore disguise any detectable effect of parasitism on body mass.

Analysis of animal movements based on trapping data has always presented problems for ecologists. Here, numbers of different traps visited by an individual over three half-month periods was used as a crude indicator of shrew activity prior to and during the breeding season. Despite this simplification, a significant difference between the sexes was found, with males visiting more traps than females. This would seem to concur with more detailed studies of shrew movements, which have shown that females maintain their relatively small winter territories during the breeding season, while males employ one of the two mating tactics described above (Shillito, 1963; Michielsen, 1966;

Buckner, 1969). A difference between treatment categories (within each sex) may have indicated some effect of helminth burden on shrew activity. Given that patterns of movement are related to mating strategy and reproductive success in male shrews (Stockley *et al.*, 1994), a difference between treatment groups might indicate an effect of helminth parasitism on breeding success. However, no such effect was found here.

Both sperm count and testis mass have been related to reproductive success in male shrews; number of offspring fathered has been found to be positively related to testes mass and number of females overlapped by 'overlapping' males, and sperm counts in 'non-overlapping' males (Stockley *et al.*, 1996). Here, no difference was found in testis size or sperm counts between treatment categories, suggesting treatment did not allow male shrews to invest directly in these aspects of reproduction. Clearly, more work is needed to clarify the relationships between male body size, mating strategy, testis size, sperm count and reproductive success.

Of the small number of experiments which have investigated effects of helminth parasites on wild vertebrates, few have found an effect on host survival (**Section 1.4**). Given their limited energy reserves (Churchfield, 1981), common shrews may be more susceptible than most host species to starvation through effects of helminth infection on nutritional status (Lindstedt & Boyce, 1985; Genoud, 1988; Haukisalmi *et al.*, 1994). However, results from field studies suggest that once juvenile common shrews have established territories, the mortality rate remains low until after the breeding season (Michielsen, 1966; Pernetta, 1977; Churchfield, 1980a; Churchfield, 1984). The results presented here suggest a trend towards a difference in survival curves between treatment categories, with initial increased survivorship in shrews receiving anthelmintics, although the lines appear to cross after 230d from first injection. This may perhaps represent a long term toxicity effect of one or both of the drugs delivered. Larger sample size and a greater understanding of shrew survivorship curves would allow for more detailed analysis, controlling for effects of time of year and host characteristics (sex and weight, for example) on survival (Tabachnick & Fidell, 2001). However, even with the basic statistical technique, small sample sizes and only partially successful treatment regime applied, it would appear helminths (in particular cestodes and/or digeneans) may

have some effect on survival rates of common shrews prior to and during the breeding season.

Chapter 7. Coccidian parasite faunas and shrew life-history traits: a comparative analysis

7.1 Introduction

Understanding the processes that govern species richness remains a central topic in community ecology (Simberloff & Moore, 1997). Recently, parasite communities have received particular interest. In part, this is due to the relative ease with which their habitat (the host individual) can be defined, and available resources quantified (space within the host, for example; Holmes & Price, 1986). Parasite communities can also be studied at different hierarchical levels, from the species found within one individual (a parasite infracommunity), through to those occurring in a host population (the parasite component community), to the entire range of parasites infecting a host species (the host's parasite fauna; Poulin, 1997).

Parasite faunas develop over evolutionary time, as new parasites colonize component communities, and become extinct from all host populations (Poulin, 1998b). Several factors have been suggested to influence these processes, and create the observed differences in parasite fauna species richness between hosts. Kennedy (1976; 1990) proposed chance events and parasite dispersal as significant contributors, whilst Brooks (1980) emphasized the effects of host-parasite cospeciation. Host population density (Morand & Poulin, 1998) and geographical range (Gregory, 1990) are also predicted to be important contributors. Host body size has been proposed as a key determinant of species richness, as larger hosts may supply a greater array of niches for occupation by parasites (Kennedy *et al.*, 1986; Price, 1987; Poulin, 1995). Larger hosts may also acquire more species as a consequence of greater food intake, increasing opportunities for colonization by accidentally ingested parasites (Poulin, 1995; Arneberg *et al.*, 1998a; Arneberg, 2002).

Comparative evidence of relationships between host body size and species richness of parasite faunas and communities has been reported in several studies (Price & Clancy, 1983; Bell & Burt, 1991; Guégan *et al.*, 1992; Parker, 1993; Poulin, 1995; Arneberg, 2002; but see Gregory, 1990; Feliu *et al.*, 1997). However, these relationships are often weakened when sampling effort (the number of hosts examined) or host geographical range is taken into account (Gregory, 1990; Walther *et al.*, 1995; Guégan

& Kennedy, 1996; Poulin, 1997). Poulin (1995; 1997; 1998b) has also emphasised the need to control for phylogenetic relationships between host species, as closely related hosts are more likely to carry similar parasite faunas, and share traits which may affect rates of parasite colonization and extinction (Poulin, 1998b). When host phylogeny is controlled for, relationships between host size and parasite species richness are again often (but not always) weakened (Poulin, 1995; Poulin, 1997; Morand & Poulin, 1998).

To date, the majority of studies which have found significant relationships between host traits and parasite species richness have focused on helminths (but see Poulin, 1995 for ectoparasites of fish). Here, comparative analysis is used to investigate whether body mass and related host life-history traits might influence the species richness of coccidian faunas. In contrast to helminths, coccidia are protozoan parasites which rely on replication within host cells (usually in the intestine) to complete their life cycle. The end result of replication is the formation of oocysts, which are passed out into the faeces, and will infect the next host if ingested (Cox, 1982). Whilst helminths often form long-lasting endemic infections, coccidian infections usually last less than a fortnight, and may be accompanied by host immunity (Seville *et al.*, 1996).

Shrews are an interesting focus group for an analysis of coccidian fauna species richness, as their semi-fossorial lifestyle may make them particularly susceptible to infection by coccidia (Hertel & Duszynski, 1987). This is partly because the shrew's environment provides conditions conducive for oocyst survival, including adequate moisture and protection from UV light. Foraging in underground tunnels could also bring shrews into contact with oocysts deposited by other small mammals, increasing possibilities for colonization. Shrews also exhibit considerable interspecific variation in their life-history traits, especially between the two sub-families, the Soricinae and Crocidurinae (Innes, 1994; Symonds, 1999b), making them ideal candidates for the comparative approach undertaken here.

In addition to body mass, several related host traits may also influence rates of parasite colonization and extinction, and thereby influence coccidian species richness. Metabolic rate and growth rate are likely to reflect energy requirement and food intake, which could influence rates of parasite colonization as described above. Host longevity may also influence parasite species richness, as host lifespan may limit the time

available for parasitic infection and colonization to take place (Poulin, 1997; Nunn, 2002). Here, potential associations between these traits, host body mass and coccidian species richness are investigated through comparative analysis.

7.2 Materials and methods

7.2.1 Dataset

Data on coccidian parasites of shrews were collected from primary and secondary literature (Levine & Ivens, 1979; Hertel & Duszynski, 1987; McAllister & Upton, 1989, **Table 7.1**). Forty-six species of coccidia were found reported from 32 species of shrew. The majority of the dataset consists of named species of parasite, most of which were described from examination of oocysts in the faeces. In addition, 19 partially named or reported but unidentified coccidia species are also included. Twenty-nine species of coccidia (including seven partially unnamed or unnamed species) were reported from the 13 species of shrew for which phylogenetic data were also available. The number of coccidia species reported from each host species was counted to give a measure of species richness. The total number of hosts examined in each study was also recorded, in order to control for sampling effort of each shrew species.

Life-history data for shrews were derived from published reviews (Innes, 1994; Symonds, 1999b). The traits included in the dataset were adult body mass, growth rate prior to weaning, life span, and metabolic rate (**Table 7.1**).

Table 7.1 Life history-traits[†] and coccidian species richness for 32 species of shrew

Host	Number of hosts examined	Coccidia species	Adult mass (g)	Lifespan (months)	Metabolic rate (ml O_2 h^{-1} g^{-1})	Growth rate (g/day)	Coccidia References
Blarina brevicauda	22	3	17.83	23.6	3.90		1, 2
Crocidura leucodon	7	2	9.82		2.55	0.30	2
Crocidura russula		1	10.81	37.5	2.23	0.37	2
Crocidura schweitzeri	6	2					2
Crocidura suaveolens		1	6.96	48.0		0.32	2
Cryptotis parva	14	1	5.13	21.0	5.09	0.15	3
Neomys anomalus	8	2	12.48		4.98	0.31	2
Neomys fodiens	3	2	16.51	16.3	4.42	0.50	2
Sorex araneus	109	9	8.92	15.7	8.17	0.31	2
Sorex arcticus	2	0		16.5			1
Sorex asper		2					1
Sorex bendirii	1	0					1
Sorex caecutiens	7	1	5.90	16.0			1
Sorex californicus		1					1
Sorex cinereus	63	2	3.90	20.5	11.90	0.16	1
Sorex fontinalis	7	2					1

Host	Number of hosts examined	Coccidia species	Adult mass (g)	Lifespan (months)	Metabolic rate (ml O_2 h^{-1} g^{-1})	Growth rate (g/day)	Coccidia references
Sorex fumeus	16	4	7.60	17.0			1
Sorex haydeni	4	1					1
Sorex hosoni	1	0					1
Sorex hoyi	3	0	3.39		16.70		1
Sorex longirostris	8	2	3.10				1
Sorex minutus		2	4.02	16.2	11.02	0.10	1
Sorex nanus	1	0	2.60				2
Sorex ornatus	2	1	5.10	16.0			1
Sorex pacificus	12	6					1
Sorex palustris	50	5	12.70				1, 2
Sorex preblei	2	0					1
Sorex tenellus	2	2					1
Sorex trowbridgii	105	5	7.40	18.0			1
Sorex unguiculatus	48	3	13.20				1
Sorex vagrans	112	5	5.94	17.5	5.52	0.15	1
Suncus murinus		2	46.70	30.0	1.68	1.39	1

† Shrew life-history data from Innes, 1994 and Symonds, 1999b. 1. Hertel & Duszynski, 1987; 2. Levine & Ivens, 1979; 3. McAllister & Upton, 1989.

7.2.2 Comparative analyses

Initial analysis of potential relationships between host life-history traits and coccidia species richness were carried out without controlling for host phylogeny. As parasite species richness is likely to be correlated with sampling effort (Walther *et al.*, 1995) this was controlled for by taking residual species richness from a regression of species richness on host sample size. Residuals were also used to control for effects of body mass where necessary when investigating relationships between parasite faunas and host life-history traits such as growth rate and longevity. All data were log-transformed, and relationships were investigated using standard linear regression analysis.

Analyses based on direct comparisons between species, such as those described above, can give misleading results due to the non-independence of traits shared by closely related taxa (Harvey & Pagel, 1991). These regressions have been included to allow for comparisons with similar analyses which do not control for phylogeny (Kennedy *et al.*, 1986; Poulin, 1995; Arneberg *et al.*, 1998a; Morand & Poulin, 1998). In addition, relationships that hold both with and without controlling for phylogeny are more likely to remain intact as the shrew phylogeny is further refined (Nunn, 2002). In the second part of the analysis, host phylogeny was controlled for using the CAIC 2.0 software package (Purvis & Rambaut, 1995), which is based on Felsenstein's (1985) comparative method. This method creates independent contrasts by calculating the differences in trait values between pairs of sub-taxa within the inputed phylogeny. Here, the shrew phylogeny of Symonds (1999a; based on George, 1988; Dannelid, 1991; Ducommun *et al.*, 1994; George & Sarich, 1994; Maddalena & Ruedi, 1994) was used with branches set to equal lengths. Relationships between contrasts were then explored using linear regression forced through the origin. Again, all data were log-transformed, and contrasts for each host trait regressed against corrected contrasts for species richness. Species richness was corrected for sampling effort by taking residuals from a regression of species richness against host sample size after control for phylogeny.

7.3 Results

When species were analysed without control for phylogeny, significant positive relationships were found between body mass and coccidian species richness (**Figure 7.1, Table 7.2**), and between growth rate and coccidian species richness (**Table 7.2**). However, body mass and growth rate were also significantly related ($F = 108.9$, $df = 1, 9$, $r^2 = 0.92$, $P<0.01$), and the relationship between growth rate and species richness was no longer significant after controlling for this relationship (**Table 7.2**). Relationships between body mass, growth rate and coccidian species richness could not be further investigated through multiple regression due to small sample size ($N = 7$), and high collinearity (variance inflation factor = 8.60) between body mass and growth rate. No evidence was found for a significant relationship between life span and coccidian species richness (**Table 7.2**), nor for a significant relationship between life span and body mass ($F = 1.53$, $df = 1, 13$, $r^2 = 0.11$, NS). Metabolic rate was not significantly related to coccidian species richness either before (**Table 7.2**) or after ($F = 0.06$, $df = 1, 8$, $r^2 = 0.01$ NS) control for a significant negative relationship with body mass ($F = 19.9$, $df = 1, 11$, $r^2 = 0.66$, $P<0.05$).

Using CAIC to control for host phylogeny, coccidian species richness again increased significantly with host adult body mass (**Figure 7.2, Table 7.3**). A significant positive relationship was also found between contrasts in growth rate and coccidian species richness (**Table 7.3**). Again, there was a significant positive relationship between contrasts in body mass and growth rate ($F = 51.6$, $df = 1, 10$, $r^2 = 0.85$, $P<0.001$), and the relationship between growth rate and species richness was no longer significant after controlling for this relationship (**Table 7.3**). Sample size ($N = 6$) was too small to separate the relationships between these three variables by multiple regression (body mass and growth rate as predictors of coccidian species richness). After control for phylogeny, no significant relationship was found between contrasts in life span and coccidian species richness (**Table 7.3**), or between contrasts in body mass and life span ($F = 0.001$, $df = 1, 9$, $r^2 < 0.001$, NS). Contrasts in coccidian species richness were not significantly related to contrasts in metabolic rate either before ($F = 3.08$ $df = 1, 9$, $r^2 = 0.31$, NS, **Table 7.3**), or after ($F = 0.001$, $df = 1, 8$, $r^2 < 0.001$, NS) control for a

significant negative relationship between contrasts in body mass and metabolic rate ($F = 11.04$, $df = 1, 11$, $r^2 = 0.53$, $P<0.01$, **Table 7.3**).

Table 7.2 Associations between host traits and coccidian species richness with control for sampling effort but not host phylogeny

Host trait	F	df	r^2	P
Body mass	5.3	1, 16	0.26	<0.05
Growth rate	25.5	1, 6	0.84	<0.01 [1]
Life span	2.2	1, 10	0.20	NS
Metabolic rate	4.3	1, 8	0.38	<0.08

1. Not significant after control for body mass; $F = 0.18$, $df = 1, 6$, $r^2 = 0.04$, NS

Table 7.3 Associations between host traits and coccidian species richness with control for sampling effort and host phylogeny

Host trait	F	df	r^2	P
Body mass	18.9	1, 8	0.73	<0.01
Growth rate	8.3	1, 7	0.62	<0.05 [1]
Life span	1.1	1, 6	0.21	NS
Metabolic rate	3.1	1, 9	0.31	NS

1. Not significant after control for body mass; $F = 0.02$, $df = 1, 6$, $r^2 = 0.004$, NS

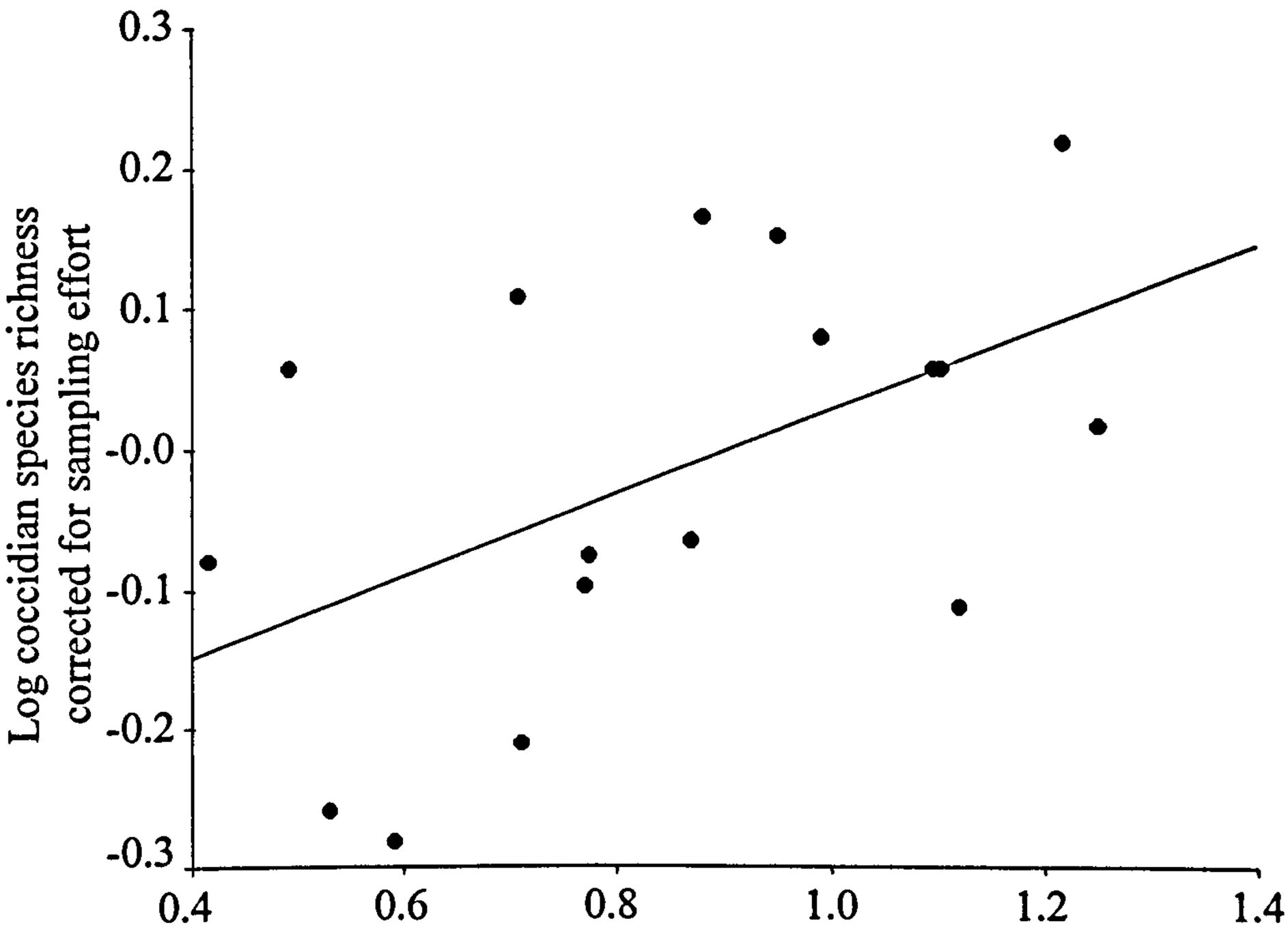

Figure 7.1 Linear regression analysis of Log_{10} host body mass and Log_{10} coccidian species richness for 17 species of shrew. Coccidian species richness is corrected for sampling effort.

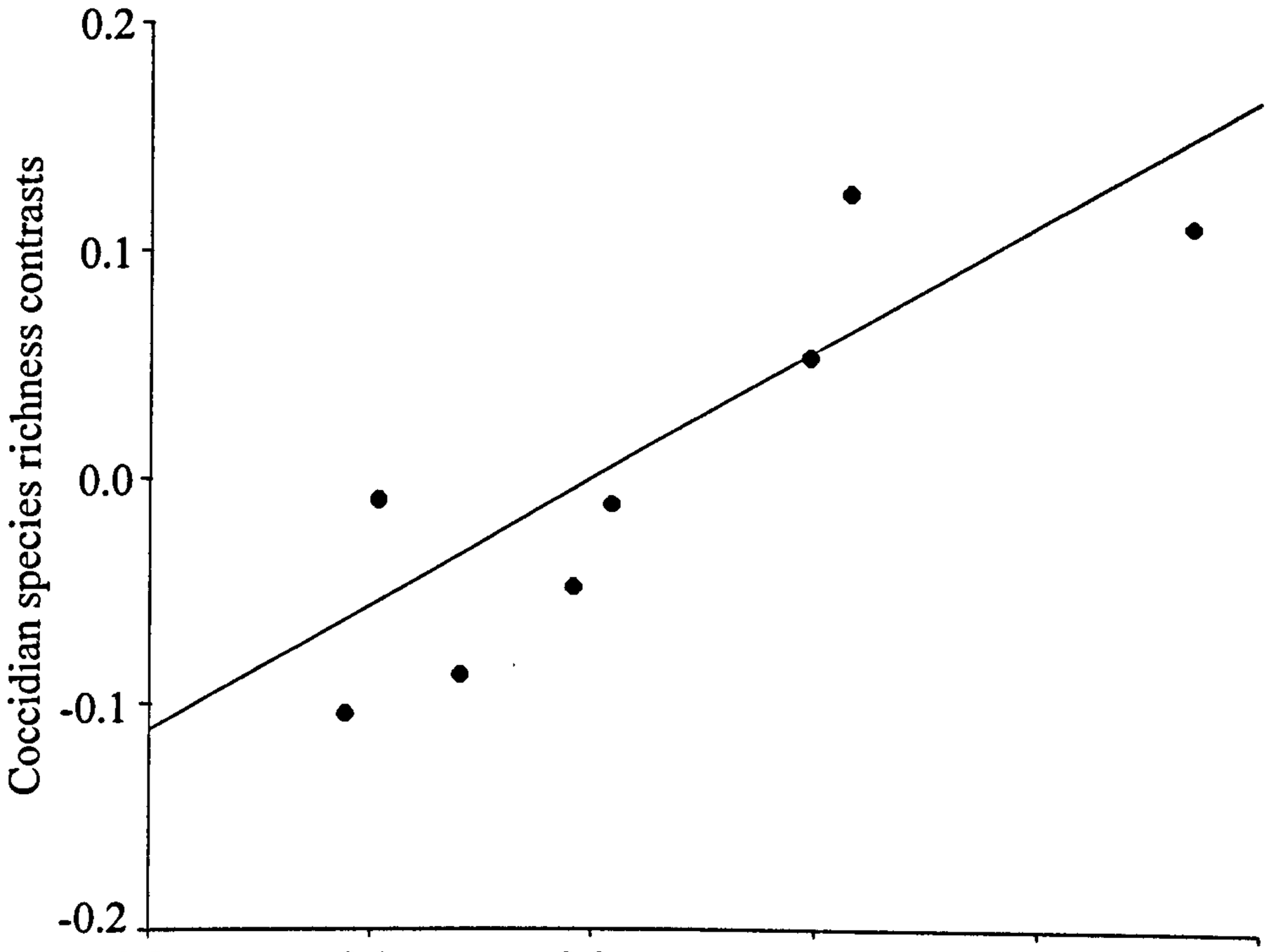

Figure 7.2 Linear regression analysis of host body mass and coccidian species richness, controlling for host phylogeny. Coccidian species richness is corrected for sampling effort.

7.4 Discussion

Results of the analyses presented indicate that host species body mass is correlated with coccidian fauna species richness across the Soricidae. Significant positive relationships between host body mass and coccidian species richness were found both before and after control for host phylogeny. Similar relationships between host body mass and parasite species richness have been reported previously in studies of mammals (Poulin, 1995; Arneberg, 2002) and fish (Poulin, 1995), although not all similar studies have found evidence of such relationships (mammals, Morand & Poulin, 1998; birds, Poulin, 1995; rodents, Feliu *et al.*, 1997).

One explanation for the relationship between host mass and parasite species richness reported here is that space or energy availability within the host might limit parasite numbers, such that larger hosts are able to accommodate more parasite species (Poulin, 1995). However, as Arneberg *et al.* (1998b) point out in their study of nematodes in mammals, there is more energy and space available within the host than is ever exploited by parasites. Alternatively, larger host species may provide a greater diversity of niches for parasites to occupy (Kennedy *et al.*, 1986; Poulin, 1995). This could explain the relationship found here, if different coccidian species use different niches. However, in a study of coccidian communities in rodents, Seville *et al.* (1996) found no evidence of negative interactions between species of coccidia. They suggested that the environment of the intestinal epithelium is non-limiting, given the rate at which new cells are produced and replaced, minimizing interactions between coccidian species. Perhaps the most likely explanation is that larger animals require a greater food intake, or eat a greater variety of prey, which may serve as alternative hosts for some of the coccidian species reported from shrews (Bush *et al.*, 2001). Increased food intake could therefore increase opportunities for colonization by accidentally ingested parasites (Poulin, 1995; Arneberg *et al.*, 1998a; Arneberg, 2002).

Of the other life-history traits considered in the present analysis, host growth rate was also correlated with coccidian species richness, both with and without control for phylogeny. However, host body mass was also correlated with growth rate, and the effects of these two variables on coccidian species richness could not be separated with the sample sizes involved. Although caution is required in interpreting results of

analyses based on relatively small sample sizes, a plausible explanation for the observed relationships might be that shrews which grow faster reach a greater body mass, and need more energy to do so. As such, they are likely to require greater food intake, increasing opportunities for colonization by coccidia as described above. However, following similar reasoning, a correlation between metabolic rate and coccidian fauna species richness might also be expected, although no such relationship was found in the present analysis. Moreover, no evidence was found for a relationship between host life span and coccidian species richness. Again, the sample sizes involved are small but the results presented here suggest that host longevity does not limit colonization by coccidia. This may be due to the relatively short time required for coccidia to successfully invade and replicate within the host (less than two weeks; Seville *et al.*, 1996).

Other factors likely to influence parasite fauna richness include host geographical range (Guégan & Kennedy, 1996), and chance events (Kennedy, 1976). Host density has also been shown to influence parasite species richness in several host taxa (Arneberg *et al.*, 1998a; Morand & Poulin, 1998; Arneberg, 2002), and would be worthy of further investigation in relation to the results presented here. Analysis of broader taxonomic groups could also reveal whether relationships between host body mass and coccidian species richness are confined to shrews, or form part of a more general trend. Finally, although this analysis takes into account host phylogeny, it does not control for relationships between coccidia; a technique that could control for both would increase further the power of comparative analyses (Arneberg *et al.*, 1998b).

Chapter 8. Discussion

This thesis has explored several aspects of host-parasite interactions using a variety of different techniques. Here, an attempt is made to integrate findings from these different investigations, and to draw general conclusions regarding host-parasite relationships in shrews. The results of each individual chapter are reviewed, and opportunities for further research suggested.

Sorex araneus was chosen as a host system partly for its diverse parasite fauna (Vaucher, 1971; Vaucher & Durette-Desset, 1973; Stunkard *et al.*, 1975; Roots, 1992). Most studies of shrew parasites have focussed on the identification of helminths (Lewis, 1968; Vaucher, 1971; Vaucher & Durette-Desset, 1973; Ewald *et al.*, 1991; Roots, 1992; Roots *et al.*, 1994). In comparison, haemoparasites have attracted relatively little attention, with only occasional reports of organisms discovered in blood smears or by PCR, usually as an aside to studies concentrating primarily on rodents (Hoyte, 1956; Laakkonen *et al.*, 1998; Liz *et al.*, 2000; Holmberg *et al.*, 2003). The investigation described in **Chapter 2** found UK common shrews are host to at least three haemoparasites; a trypanosome, *Bartonella* sp., and *Anaplasma phagocytophilum*. To my knowledge, trypanosomes have never been previously identified from *S. araneus*. The 5% difference between the 18S rRNA sequence found here and the closest match on Genbank suggests the isolated genotype may represent a previously unreported species. Although detected by PCR, the parasite has yet to be seen through examination of blood smears. This would confirm the parasite in question is a trypanosome, and would permit comparison of morphometric features with similar parasites in other host species. Such comparisons may aid in deciding whether the genotype found here does indeed represent a previously unreported species. In addition, genetic comparisons with trypanosomes reported from other species of shrew (Laakkonen *et al.*, 1998) may reveal the extent to which trypanosomes have cospeciated with their shrew hosts, or have been acquired from other host species sharing the same habitat.

As discussed in **Section 2.1**, both *A. phagocytophilum* and certain species of *Bartonella* are known to be pathogenic in humans and domestic animals. It is highly unlikely humans would acquire either pathogen directly from shrews, but nevertheless

S. araneus may be an important reservoir host for both diseases. In particular, transfer may take place between common shrews and rodents sharing the same habitat, which may be more likely to come into contact with humans and domesticated animals. It is hoped the research undertaken here will be extended to investigate whether shrews share other diseases with rodents, such as cowpox virus (Feore *et al.*, 1997), and tuberculosis (Cavanagh *et al.*, 2002), and the mechanisms by which these diseases are transmitted within shrew populations, and between host species. In addition, although no evidence was found here of pathological changes associated with these parasites, further work is needed to investigate their effects on the health status and fecundity of shrews, similar to studies carried out on cowpox and tuberculosis in wild rodents (tuberculosis: Cavanagh *et al.*, 2002; cowpox; Bennett *et al.*, 1997; Feore *et al.*, 1997; Telfer *et al.*, 2002) and *Bartonella birtlesii* in BALB/c lab mice (Boulouis *et al.*, 2001).

One of the major aims of this thesis was to investigate effects of parasitism in shrews from a variety of different perspectives. It was hoped this would provide a broader insight than a study constrained to any one particular discipline. While shrews are known to harbour a wide variety of micro and macroparasites (**Section 1.8**), prior to this study virtually nothing was known regarding the effects of these organisms upon their host. The histological investigation of **Chapter 3** was conducted to assess the level of damage associated with these parasites in shrew tissues, and the host's immune response towards them. While many investigations of wild animal pathology are conducted on recovered carcasses or during wildlife epidemics (Gulland, 1995), the survey conducted in **Chapter 3** was performed using a cross-section of apparently healthy animals, in the hope of providing a more accurate picture of the pathology suffered by live shrews. The results suggest common shrews suffer diverse parasite-mediated pathology, mainly as a consequence of helminth infection, yet still survive until breeding. Whether any of these infections are likely to become fatal is unknown, although given the resource-limited physiology of *S. araneus*, infection by parasites which might disrupt digestion (such as digeneans inhabiting the bile duct) or interfere with nutrient transport (by infecting cardiac tissue, for example) may be more serious than in other species (Soveri *et al.*, 1994).

Most metazoan parasites found within common shrew tissue sections could be identified using keys based on morphology and characteristics of eggs (Roots, 1992). However, several animals harboured protozoan cysts, and one animal exhibited what appeared to be an intracellular parasite in the oesophagus, which could not be identified. It is hoped these parasites may be of interest to those with specialist knowledge of similar organisms in other hosts. One organism conspicuously absent from examined animals was the fungal parasite *Pneumocystis carinni* (Laakkonen, 1995). This pulmonary pathogen has been observed at prevalences of 70% in *S. araneus* in Finland (Laakkonen *et al.*, 1993), but was not found in any of the 41 animals from which lung tissue was examined here. Surveys of other shrew populations are required to investigate whether this parasite is indeed absent in British *S. araneus*[1].

In order to limit the number of shrews used for this thesis, the majority of animals dissected for histological examination were those used as controls for behavioural and field experiments (**Chapter 5** and **Chapter 6**). Unfortunately, this meant only a relatively small number of intestinal tracts could be examined histologically, as most were dissected in order to quantify helminth abundances. However, in a previous study Vaucher (1971) found little evidence of histopathological change associated with gut cestodes in shrews, and suggested that even in very high numbers they will damage only a very small proportion of the intestinal mucosa.

The examination of lymphatic tissues conducted in **Chapter 4** required the use of specialist staining techniques to label populations of lymphocytes and assess levels of cellular turnover. The results demonstrate that immunohistological techniques developed for use in humans and laboratory animals can also be applied in *S. araneus*. It is hoped this success will encourage others studying shrews to use these techniques, and attempt to extend their use to other wild mammalian species.

The lymphatic tissues of *S. araneus* warranted investigation for several reasons. Until now, the structure and function the Pancreas of Aselli had not been adequately described. Here, results of immunohistological staining strongly suggest it functions as a very large lymph node draining the intestine. Interestingly, other species of shrew also

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[1] Interestingly, the one pygmy shrew examined in this study was found to exhibit a pulmonary fungal infection.

exhibit modifications to the standard mammalian lymphatic system. Several species (but not *S. araneus*) have been found to possess 'anal tonsils' (Dryden & Conaway, 1970), the function of which remains unknown. The short-tailed shrew *Blarina brevicauda* has an extrapulmonary appendage on the right lung, described as the 'pulmonary appendix' (Parke, 2002). This structure has been suggested to function as an immunologic organ, as it receives aspirated antigens and contains large numbers of IgA-secreting plasma cells (Parke, 2002). The investigation conducted here found the Pancreas of Aselli also contains large number of plasma cells. Perhaps these features suggest something about the physiology or lifestyle of shrews that requires the development of additional lymphatic organs to sample antigens and produce antibodies. It is hoped the immunohistological methods used here will be employed to investigate the lymphatic systems of other species of shrew, in the hope of exploring this possibility more fully. A comparative analysis that could control for effects of phylogeny (similar to the one described in **Chapter 7**) might also be useful in identifying lifestyle characteristics associated with these modifications.

Although immunity may be one of the major physiological mechanisms regulating host survival (Lochmiller & Deerenberg, 2000), and immunosenescence has been recognized as a major factor in determining human health in later life (Franceschi *et al.*, 2000), few studies have investigated this process in wild animals (barn swallows, *Hirundo rustica*, Saino *et al.*, 2003; collared fly catchers, *Ficedula albicollis*, Cichon *et al.*, 2003). Here, shrews were found to be able to respond to both micro and macroparasites with immune reactions both prior to and during the breeding season, despite lymphatic tissues exhibiting some signs of deterioration in adulthood (**Chapter 4**). While adult common shrews did show some signs of immunosenescence, and results from the field study suggest parasites may have a role in shrew mortality (**Chapter 6,** see below), from the evidence presented here it seems unlikely that rapid involution of lymphatic tissues, combined with exacerbation of latent infection, results in the death of adult shrews after breeding, as is believed to occur in *Antechnius* spp. (Barker *et al.*, 1978; Poskitt *et al.*, 1984). However, this hypothesis can only be confirmed by examination of adult shrews after the conclusion of the breeding season. Unfortunately, catching the small number of remaining adults at this time of year is difficult, as most

traps are occupied by the much larger number of dispersing juveniles (Michielsen, 1966; Churchfield, 1980a; Moraleva, 1989; Gliwicz & Jancewicz, 2001).

Chapter 5 and **Chapter 6** attempted to investigate the effects of helminth parasitism on common shrew behaviour and life-history traits. Both studies relied on a manipulative approach, comparing shrews carrying their natural parasite burdens with those treated with anthelmintics. Treatment was partially successful. In the laboratory, treatment significantly reduced cestode abundances in comparison to controls, and there was some evidence for an effect on abundances of nematodes, but not digeneans. Due to the small sample sizes involved, it is difficult to draw firm conclusions on drug efficacy from the laboratory study, especially as helminth abundances prior to treatment were unknown. Larger numbers of animals were involved in the field experiment, although results cannot be compared directly to the behaviour trials, as animals were both repeatedly treated and continually exposed to infection. Abundances of both cestodes and digeneans were reduced below those of the control group in the field, but no effect was found on nematode abundances. These results would seem to suggest that the dosage of praziquantel administered was sufficient to lower abundances of cestodes and digeneans, but that ivermectin did not significantly affect total nematode abundance.

Despite the limited efficacy of treatment, the field study described in **Chapter 6** found treated males were heavier at dissection than mock-treated controls. To my knowledge, this is the first experimental field study to demonstrate a reduction in body weight associated with helminth parasitism in a wild mammal. As previously discussed, studies of laboratory animals suggest that reduction in host nutritional status may be the major cause of reduced growth associated with helminth infection in vertebrates (**Section 1.4**). If this is the case, detrimental effects of helminth parasites in the wild may only be detectable when hosts are unable to compensate for losses in energy intake through increased food acquisition or use of internal reserves (Tripet & Richner, 1997; Murray *et al.*, 1998). The fast metabolism and limited fat stores of *S. araneus* (Churchfield, 1981; Genoud, 1988; Nagel, 1994) might make any effect of helminths on host nutrition more visible than in other host species, resulting in the detectable effect on body mass found here.

While reduction in food intake has been suggested as the most important mechanism by which helminths impact upon host nutritional status (**Section 1.4**), the behavioural experiment described in **Chapter 5** found no effect of treatment on any measure of foraging activity in common shrews, although sample sizes were too small to draw firm conclusions. As reported in a previous study of foraging in common shrews (Barnard *et al.*, 1983), general activity was found to vary considerably between individuals. A simpler experiment, in which rates of food consumption are compared directly between treatment categories, rather than through behavioural assay of foraging effort, might be more useful in investigating effects of helminth parasitism on food intake in *S. araneus*, and understanding whether common shrews do undergo anorexia as a consequence of infection. In addition, only sub-adult shrews were used in this experiment: if impact on nutritional status is positively related to infection intensity (Hall, 1985), effects of helminth parasites on shrew foraging activity may be more visible in adults carrying heavier parasite burdens.

In addition to anorexia, there are a number of other mechanisms by which helminth parasites may impact upon host nutritional status, resulting in the observed reduction in shrew growth rate. As mentioned above, digeneans blocking the bile duct may prevent the release of bile and proper absorption of lipids (Soveri *et al.*, 1994), while many helminths can reduce digestive efficiency through damage to the intestine, or through their own feeding (Hall, 1985). As previously discussed, only a small number of shrew guts were examined histologically (**Chapter 3**). Given the diversity of helminths which occur in the intestine of *S. araneus* (**Section 1.8**), the effects of these parasites on host digestive efficiency may warrant further study. The results of **Chapter 3** did reveal that common shrews mount immune responses to helminths within host tissues. As immune reactions require investment of host energy and nutrients (Lochmiller & Deerenberg, 2000), this may represent another potentially serious effect of infection on host nutrition.

One aspect of helminth infection not considered here was the impact on host metabolism. Fever is a common response to many microparasite infections, and can result in a 30% increase in metabolic rate (Lochmiller & Deerenberg, 2000). Macroparasite infection does not generally result in high fever (Hall, 1985), but given

the resource-limited physiology of shrews, any increase in metabolism could potentially result in starvation. In addition, extra heat produced as a result of faster metabolism might result in shrews overheating during the summer. Tools for measuring rates of metabolism in shrews have already been developed (Buckner, 1964; Genoud & Vogel, 1990; Poppitt *et al.*, 1993; Nagel, 1994), and by combining these techniques with the treatment methods applied in **Chapter 5** and **Chapter 6**, an investigation into the effects of helminth infection on shrew metabolic rate should be feasible.

There was some evidence to suggest treatment in the field resulted in greater initial survivial of common shrews in comparision to mock-treated controls. Unfortunately, the analysis was complicated by the eventual crossing of the two survival curves, making it difficult to draw firm conclusions. Only a small number of previous experiments have reported an effect of helminth parasitism on survival of wild vertebrates (**Section 1.4**). Given the shrew's limited toleration for starvation (Lindstedt & Boyce, 1985; Genoud, 1988), any impact of helminth parasitism on host nutritional status through the mechanisms discussed above may result in death more quickly than in other host species. As previously discussed, whether mortality can result from any of the histological alterations associated with parasites described in **Chapter 3,** perhaps combined with the gradual deterioration of lymphatic tissues reported in **Chapter 4,** remains unknown.

Though no effect of anthelmintic treatment was found here on traits associated with male reproductive success (sperm count, testis mass, movement), future studies might consider the effects of helminth parasites on reproductive output of female *S. araneus*. Lactation is considered to be energetically costly in this species, raising energy intake by 300% (Genoud & Vogel, 1990), and females also produce more offspring than survive to weaning (Mercer & Searle, 1994). Whether helminth infection can limit the number of offspring a female can raise might feasibly be tested through comparisons of treated and mock-treated shrews in captivity.

The emphasis of investigation was shifted for **Chapter 7,** which explored relationships between shrew life-history traits and species richness of their coccidian parasite faunas. It is now recognized that host phylogeny may be an important factor in determining species richness of parasite faunas (Poulin, 1995; Poulin, 1997; Poulin,

1998b), and this was controlled for through a comparative analysis technique (Felsenstein, 1985; Purvis & Rambaut, 1995). This approach has been previously applied by several authors investigating helminth assemblages (Gregory *et al.*, 1990; Poulin, 1995; Feliu *et al.*, 1997; Poulin, 1997; Arneberg *et al.*, 1998a; Morand & Poulin, 1998; Arneberg, 2002), but never (to my knowledge) to coccidian faunas. Coccidian species richness was found to be positively correlated with host body mass and growth rate, although these two traits are themselves related. Some studies of helminths (but not all) have also found relationships between species richness and host body mass (Poulin, 1995; Arneberg, 2002, but see Feliu *et al.*, 1997; Morand & Poulin, 1998). Greater food consumption of larger hosts, resulting in increased rates of colonization, is perhaps the most likely explanation for this relationship (Poulin, 1995; Arneberg *et al.*, 1998a; Arneberg, 2002), although experiments are needed to test this hypothesis. Studies which examine the processes by which individual hosts and host populations acquire coccidian parasites, and how coccidia become extinct from host populations, would also be useful in understanding how host traits might influence coccidian species richness.

Previous investigations of parasitism in common shrews have, for the most part, been limited to the identification and quantification of helminths. By using modern molecular techniques to identify haemoparasites present in *S. araneus*, this thesis has provided an important contribution to the study of parasitism in this species. The current study has also gone beyond the identification of parasites to explore the effects of these organisms upon their host. The results demonstrate that parasitism clearly has several important implications for common shrews, both in terms of pathology and broader effects upon host life-history traits. The histological examination conducted here reveals that, despite their resource-limited physiology, common shrews are able to mount immune responses against these parasites, and may even have developed a large, specialized lymph node (the Pancreas of Aselli) as an additional defence against gut parasitism.

By drawing on the shrew parasite literature, the comparative analysis conducted here has identified host traits which may be important in determining coccidian species richness in members of the Soricidae. To my knowledge, this represents the first attempt to use such a technique to identify patterns in shrew parasite assemblages. Shrews have

proven an excellent model system for this thesis, and it is hoped research will continue into host-parasites interactions in *S. araneus* and other members of this family. As mentioned in the introduction (**Section 1.5**) and discussed in **Chapter 7,** members of the Crocidurinae have different life-history strategies to the Soricinae shrews, exhibiting lower metabolic rates for their body size and longer life spans but producing smaller litters (Genoud, 1988; Innes, 1994). Comparisons between sub-families may reveal the extent to which the detrimental effects of parasitism described here are a consequence of the 'live fast - die young' strategy of *S. araneus*, or are common across all shrew species.

Above all, this thesis has demonstrated that a multitude of specialist techniques can be successfully integrated into an ecological framework, and provide a broader understanding of host-parasit relationships. Recently, it has been suggested that more studies of this nature are required to further our understanding of the ecology of malaria, in order to develop new strategies to control this important human disease (de Roode & Read, 2003). It is hoped the work conducted here will contribute to promoting an interest in different aspects of host-parasite relationships in evolutionary biology, and encourage those with specialist techniques already working in this field to interpret their results in a wider evolutionary context. Such an interdisciplinary approach would undoubtedly further our understanding of host-parasite relationships in wild animals, and have practical benefits for the control of important human and animal diseases.

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Host-Parasite Interactions in the Common Shrew (*Sorex araneus*)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Daniel Peter Bray

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Appendix A. Sequences isolated from haemoparasites of *Sorex araneus*

Anaplasma phagocytophilum 16S rRNA encoding gene fragment sequence

TTAACGGATTATTCTTTATAGCTTGCTATAGAGAATAATTAGTGGCAGACGG
GTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAAT
GGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGAT
GAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGAT
GATCTATAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGATACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCA
AGCCTGATCCAGCTATGCCGCGTGAGTGAGGAAGGCCTTAGGGTTGTAAAA
CTCTTTCAGTAGGGAAGATAATGACGGTACCTACAGAAGAAGTCCCGGCAA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCAAGCGTTGTTCGGAA
TTATTGGGCGTAAAGGGCATGTAGGCGGTTCGGTAAGTTAAAGGTGAAATG
CCAGGGCTTAACCCTGGAGCTGCTTTTAATACTGCCAA

Trypanosome 18S rRNA encoding gene fragment sequence

GCTTTTGTTGGTTTTACAGTCTATTGGAGATTATGGGGCTGTGCGACAAGCG
CTCGAGTGCACTCCTTTCGGTGTGACACTCGGCGCCTTTGTGGGAAATCTGC
GGTTGTGTTCTCGGGCTTCGGCCTCGCAGGGCACATATCCGTCTTCCCTCAAC
TCGCGGCATCCAGGAATGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGTG
CGTCAGAGGTGAAATTCTTAGACCGCACCAAGACGAACTACAGCGAAGGCA
TTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGGGATCGAAGAT
GATTAGAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGG
GGAGTTTTTGGTCGTAGGCGGGGTCGGGTTCATCTCGCTCCTCGTCTCGCCA
ATGTATATCAATTTACGTGCATATTCTTTCTGGTCCTCGCAAGAGGGTCCATC
TACGGGAATATCCTCAGCACGTTATCTGACTTCTTCACGCGAAAGCTTTGAG

Bartonella sp. 16S-23S rRNA intergenic spacer region sequence WHF018

GGAGGTTCAAGTCCTCCCAGGCCCACCAATTTATCTATCCATTTGCCTTTATC
CGTTAATTTGCTGTTTTATCGGTCCGTTTGTTAAGTGTTGGTAACAGTTTTTG
TAATGATGAAAAACAGTCCTGCTTATAAAAGACCTACTTATAAAAGGCTTGT
TTCTAAGTTATGACGCTTATTCATTTCGTTTAGGTAAGAGAAACTTCAAGCG
GTTCAAACGCAAAACACTTTGAATTTTGCAAAACAATTTGCGTTTTGAAGTG
ATTCAAGTTCGTGATTTCGACTTAAAAGTTTCGAATGC
TTATCTT

Bartonella sp. 16S-23S rRNA intergenic spacer region sequence F

GGAGGTTCAAGTCCTCCCAGGCCCACCAATTTATCTATCCATTTGCCTTTATC
CGTTTATTTGCTGTTTTATCGGTCCGTTTGTTAAGTGTTGGCAACAGTTTTTGT
AATGATGAAAAACAGTCCTGCTTATATAAAGACCTACTTATAAAAGGCTTGT
TTTTAAAATGTGACGCTTATCCATTCCGCTTAGGCAAGAGAAACTTCAGGCG
GTTCAAACGCAGAACGCTTTGAATTTTGTAAAACAATTTGCGTTTTGAAGTG
ATTCAAGTTCGTGATTTCGACTTAAAAGTTTCGAATGCTTTATCCTTTTAG

Bartonella sp. citrate synthase gene fragment sequence IUP001

CGATTCTATTGATATTACAGACTCACAACAAAGAATGATTGCTTCTGTTCGC
CTCATCTCAAAGGTTCCAACTCTTGCTGCGATGGCATATAAATATAGTATCG
GGCAAGCCTTTGTTTATCCACGAAATGATCTTAGTTACGCTGCTAATTTTCTC
CGTATGTGCTTTTGCGTTCCTTGTGAAGAATACAAAATCAATCCCGTTCTTAC
TCGAGCAATGGATCGAATTTTTATTCTTCATGCAGACCATGAACAAAATGCT
TCTACATCCACTGTACGCCTTGCAGGATCATCAGGTGCTAATCCGTTTGCAT
GTATCGCAGCAGGTGTTGCGTGCCTTTGGGGACCAGCGCATGGTGGAGCTAA
TGAAGCATGCTTAAAGATGCTACAAGAAATAGGTTCCGTTGAGAGAATTCCT
GAATTCATTGCACGTGCAAAAGATAAAAATGATCCTTTCCGCCTTATGGGGT
TTGGGCACAGAGTCTATAAAAATTATGATCCGCGCGCAAAAATCATGCAAA
AAACCTGCCATGAAGTTTTAAAAGAGCTTAACATCCAAGATGATCCACTTCT
TGATATAGCTATAGAACTTGAAAAAATCGCCCTGAGCGATGAATATTTTGTT
GAGAAAAAGCTTTATCCAAATGTCGATTTCTATTCTGGAATT

***Bartonella* sp. citrate synthase gene fragment sequence WHF113**

CGATTCTATTGATATTACAGACTCACAACAAAGAATGATTGCTTCTGTTCGC
CTCATCTCAAAGGTTCCAACTCTTGCTGCGATGGCATATAAATATAGTATCG
GGCAAGCCTTTGTTTATCCACGCAATGATCTTAGTTACGCTGCTAATTTCCTC
CGTATGTGCTTTTGCGTTCCTTGTGAAGAATACAAAATCAATCCCGTTCTTAC
TCGAGCAATGGATCGAATTTTTATTCTTCATGCAGACCATGAACAAAATGCT
TCTACATCCACTGTACGCCTTGCAGGATCATCAGGTGCTAATCCGTTTGCAT
GTATTGCAGCAGGTGTTGCATGCCTTTGGGGACCAGCGCATGGTGGAGCTAA
TGAAGCATGCTTAAAGATGCTACAAGAAATAGGTTCCGTTGAGAGAATTCCT
GAATTCATTGCACGTGCAAAAGATAAAAATGATCCTTTCCGCCTTATGGGGT
TTGGGCACAGAGTCTATAAAAATTATGATCCGCGTGCAAAAATCATGCAAA
AAACCTGCCATGAAGTTTTAAAAGAGCTTAACATCCAAGATGATCCACTTCT
TGATATAGCTATAGAACTTGAAAAAATCGCCCTGAACGATGAATATTTTGTT
GAGAAAAAGCTTTATCCAAATGTCGATTTCTATTCTGGAATTACATAAAAGC
TCTAGGATTCCCAACTGAAA

Appendix B. Histopathological findings from *Sorex araneus*

Sub-adult females

Animal H01-1217

Organ	Histopathology
Adipose tissue	Nematode larva (*Porrocaecum* sp.) encapsulated by cell-rich fibrous connective tissue, no inflammatory infiltration.
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Brain	Unaltered.
Kidneys	Very mild tubular protein cast formation, one subendothelial protozoan cyst in cortical vessel.
Liver	Moderate to severe mixed cellular portal infiltration with follicle-like lymphocyte accumulations, indicating germinal centre formation. One portal artery with subendothelial protozoan cyst; bile ducts: few degenerate cells.
Lung	Moderate hyperaemia; very mild perivascular/bronchial mononuclear infiltration.
Lymph node	Moderately-sized secondary follicles, otherwise filled with lymphocytes.

Animal H01-1217 (continued)

Organ	Histopathology
Myocardium	One artery and one vein with subendothelial protozoan cyst, with very mild granulomatous infiltration.
Oesophagus	Parasites (digeneans; *Brachylaemus fulvus*) attached to squamous epithelium, with focal hydropic degeneration of surrounding epithelial cells.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with large, mildly depleted secondary follicles which occasionally formed two layers; beneath are T cell zones; focal areas in cortex without follicles; focally wide cortical sinuses with vacuolated cells, lymphocytes, macrophages and neutrophils (occ. also as small accumulations); centre filled with plasma cells (relatively loosely arranged, with distinct medullary sinuses), focally extending to marginal sinuses/capsule.
Skeletal muscle	Unaltered.
Intestine	Few lymphocytes in mucosa, mild mixed cellular infiltration in upper submucosa; Peyer's patches with secondary follicles, otherwise unaltered.

Animal H01-1217 (continued)

Organ	Histopathology
Spleen	Centre vastly filled with numerous, large, often connected (up to 4) secondary follicles, surrounded by rim of lymphocytes, macrophages, neutrophils and erythrocytes (rim surrounds groups of connected follicles, not every single one). Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered.
Urinary bladder	Intraluminal nematodes (*Liniscus incrassatus*).

Animal H01-1508

Organ	Histopathology
Bone marrow	High activity [+++]
Kidneys	Mild focal mixed (lymphocytes, macrophages, few neutrophils) interstitial infiltration.
Liver	Mild mixed portal and perivascular infiltration.
Lung	Mild perivascular lymphocyte accumulation.
Myocardium	Mild focal perivascular granulomatous infiltration.

Animal H01-1508 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with relatively large secondary follicles (with numerous apoptoses; cortex not completely filled); beneath are T cell zones; relatively narrow marginal sinuses with vacuolated cells, numerous lymphocytes and neutrophils; centre filled with plasma cells, focally extending to marginal sinuses/capsule; in marginal sinus/capsule one granuloma with necrotic centre, surrounded by lymphocytes, neutrophils and fibroblasts as well as thick fibrous connective tissue capsule.
Spleen	Centre filled with numerous, often connected (up to 6) large secondary follicles; groups are surrounded by rims of lymphocytes, neutrophils, macrophages and erythrocytes; in germinal centres numerous apoptotic cells. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.

Animal H01-1510

Organ	Histopathology
Kidneys	Two vessels with subendothelial protozoan cyst, no reaction.
Liver	Mild to moderate portal mixed inflammatory infiltration.
Lung	Mild perivascular lymphocyte accumulations, few disseminated neutrophils.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex almost completely filled with large secondary follicles, beneath are T cell zones; focally wide marginal sinuses with vacuolated cells, lymphocytes and small focal accumulations of neutrophils; loose medullary sinuses (containing few lymphocytes and macrophages); centre filled with plasma cells; relatively few apoptoses.
Skeletal muscle	Unaltered.
Spleen	Centre filled with large secondary follicles with small rim of neutrophils, macrophages and lymphocytes; relatively few apoptoses; one protozoan cyst in vessel wall (no reaction). Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.

Animal H02-0136

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	Moderate activity [++]
Gall bladder	Unaltered.
Kidneys	Focal granulomatous infiltration, especially around arteries with subendothelial protozoan cyst; occasional protozoan cyst without reaction.
Liver	Mild to moderate mixed portal infiltrate with occasional infiltration of bile ducts.
Lung	Mild focal periarteriolar/bronchiolar lymphocyte accumulations, focally few disseminated neutrophils.
Myocardium	Mild focal periarterial granulomatous infiltrate, one protozoan cyst without reaction.
Pancreas of Aselli	Cortex with relatively small, mildly to moderately depleted secondary follicles; beneath are T cell zones; marginal sinuses relatively wide with vacuolated cells and fewer lymphocytes, focally extensive capsular fibrosis and neutrophil accumulations; in one large area neutrophil infiltration also in capsule, with embedded nematode (51.2µm by 4.88µm); centre filled with plasma cells, focally extending to marginal sinuses/capsule.
Skeletal muscle	Unaltered.
Small intestine	Unaltered.

Animal H02-0138

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Very mild focal mononuclear infiltration.
Large intestine	Unaltered.
Liver	Moderate portal mixed cellular infiltration with occasional follicle-like accumulations of lympocytes with germinal centre formation, occasional infiltration of bile ducts.
Lung	Unaltered.
Myocardium	One subendothelial protozoan cyst, no reaction.
Pancreas of Aselli	Cortex with moderately-sized, mildly depleted secondary follicles (cortex not completely filled); beneath are T cell zones; relatively wide marginal sinuses with vacuolated cells, lymphocytes and neutrophils (relatively low numbers), focal fibrosis; granuloma, comprised of vacuolated cells around nematodes (perhaps several cross sections 17μm in diameter), surrounded by capsule of fibroblasts 260μm diameter; focal neutrophil accumulation; centre filled with plasma cells; mild extramedullary haematopoiesis (few megakaryocytes).

Animal H02-0138 (continued)

Organ	Histopathology
Skeletal muscle	Unaltered.
Small intestine	Unaltered.
Spleen	Centre filled by relatively large, often connected secondary follicles with rim of macrophages, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered.

Animal H02-0139

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Mild focal granulomatous perivascular infiltration (with one subendothelial protozoan cyst).
Liver	Moderate mixed cellular portal infiltration with occasional small follicles; occasioanally inflammatory cells and degenerate cells in bile duct lumina.
Lung	Very mild focal lymphocyte accumulations
Myocardium	Unaltered.

Animal H02-0139 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with relatively small, mildly depleted secondary follicles; beneath are T cell zones; relatively wide marginal sinuses with vacuolated cells, lymphocytes and (focally) neutrophils; centre filled with plasma cells (centre is relatively small).
Skeletal muscle	Unaltered
Small intestine	Mild submucosal mixed cellular inflammatory infiltration.
Spleen	Centre filled with large, connected secondary follicles with relatively indistinct rim of macrophages, lymphocytes and neutrophils. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered.

Animal H02-0181

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	Moderate to high activity [++/+++]
Brain	Unaltered.
Kidneys	Very mild mononuclear interstitial infiltration; one protozoan cyst, no reaction.

Animal H02-0181 (continued)

Organ	Histopathology
Liver	Mild to moderate mixed portal infiltration with occasional follicle-like accumulations of lymphocytes and infiltration of bile ducts.
Lung	Unaltered.
Mediastinal lymph node	Relatively small secondary follicles in cortex; distinct T cell zones.
Myocardium	Unaltered.
Ovarium	Unaltered.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with numerous moderately-sized to large secondary follicles; beneath are T cell zones; relatively narrow sinuses with vacuolated cells, lymphocytes and focally extensively relatively many neutrophils; centre filled with plasma cells, focally extending to marginal sinuses/capsule.
Skeletal muscle	Unaltered
Spleen	Centre filled with moderately-sized, often connected, mildly depleted secondary follicles with numerous apoptotic cells; with relatively distinct rims of macrophages, neutrophils and lymphocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.
Uterus	Unaltered.

Animal H02-0182

Organ	Histopathology
Adipose tissue	Nematode, encapsulated by cell-rich capsule, no inflammatory reaction.
Adipose tissue (mediastinum)	Moderate focal granulomatous steatitis.
Adrenal glands	Unaltered.
Bone marrow	Moderate to high activity [++/+++]
Brain	Unaltered.
Kidneys	Unaltered.
Liver	Mild to moderate portal infiltration and occasional cells in bile ducts.
Lung	One small granuloma with central vacuolated cells, surrounded by fibroblasts.
Mediastinal lymph node	Moderately-sized secondary follicles, distinct T cell zones; relatively wide sinuses with vacuolated cells and lymphocytes.
Myocardium	Unaltered.
Pancreas	Unaltered.

Animal H02-0182 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with numerous, relatively small, mildly depleted secondary follicles, standing out into wide marginal sinuses with vacuolated cells, few lymphocytes and scattered neutrophils; beneath are T cell zones; centre filled with plasma cells widely expanding to marginal sinuses/capsule.
Skeletal muscle	Unaltered.
Spleen	Centre filled with connected, moderately-sized secondary follicles, with rim of macrophages, lymphocytes and neutrophils. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Thymus	Moderate involution? Represented by thin layers of lymphocytes around vessels, with fine fibrous connective tissue capsule.
Urinary bladder	Unaltered.

Animal H03-0223

Organ	Histopathology
Bone marrow	High activity [+++]
Gall bladder	Unaltered.
Kidneys	Moderate focal mononuclear interstitial infiltration (lymphocytes, macrophages) around vessels with protozoan cysts in thickened wall.

Animal H03-0223 (continued)

Organ	Histopathology
Liver	Mild portal, predominantly mononuclear infiltration.
Lung	Moderate hyperaemia, moderate focal peribronchial and vascular lymphocyte accumulations.
Myocardium	Protozoan cyst, completely occluding vessel (?), no reaction.
Oesophagus	Unaltered.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with mildly to moderately depleted secondary follicles (cortex not completely filled), beneath are T cell zones, focally wide marginal sinuses with vacuolated cells, macrophages, lymocytes and neutrophils, centre filled with plasma cells, focally extending to marginal sinuses; mild extramedullary haematopoiesis (few megakaryocytes).
Skeletal muscle	Unaltered.
Small intestine	Moderate mixed cellular mucosal infiltration.
Spleen	Centre with partly connected, mildly depleted secondary follicles with numerous apoptotic cells, surrounded by rim of macrophages, lymphocytes and fewer neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered.

Animal H03-0438

Organ	Histopathology
Adrenal glands	Unaltered
Kidneys	Mild focal mixed cellular infiltration in the renal pelvis.
Liver	Moderate portal mixed cellular infiltrations with occasional follicles-like accumulations of lymphocytes.
Lung	Moderate hyperaemia, focal alveolar haemorrhage, mild peribronchial and vascular lymphocyte accumulations.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex with numerous, moderately sized, mildly depleted secondary follicles; beneath are T cell zones; focally wide marginal sinuses with vacuolated cells, macrophages, lymphocytes; centre filled with plasma cells, focally extending to marginal sinuses and capsule.
Spleen	Centre filled with moderately sized, often connected, mildly depleted secondary follicles, surrounded by rim of macrophages, lymphocytes, fewer neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.

Animal H03-0541

Organ	Histopathology
Adipose tissue	Encapsulated parasite (*Porrocaecum* sp.), surrounded by cell-rich fibrous connective tissue capsule.
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Mild tubular protein cast formation, focal mild mononuclear interstitial nephritis.
Liver	Moderate, predominantly mononuclear portal infiltration; focal chronic granulomatous inflammation with central necrosis and mineralisation, surrounded by multinucleate giant cells, macrophages and epithelioid cells; outer layer of lymphocytes and fibroblasts.
Lung	Moderate hyperaemia.
Myocardium	One vessel with small protozoan cyst in wall, no reaction.
Oesophagus	Parasite larvae (nematode in cross section, 73µm diameter) and eggs (65µm by 28µm, with two polar plugs, consistent with *Eucoleus oesophagicola*) between upper epithelial cell layers; focal hydropic degeneration of superficial epithelium, mild diffuse mixed cellular infiltration of mucosa.
Pancreas of Aselli	Cortex almost completely filled with large secondary follicles with numerous apoptotic cells; beneath are T cell zones; relatively narrow marginal sinuses with macrophages, lymphocytes and scattered neutrophils; centre filled with plasma cells, focally extending to marginal sinuses/capsule.
Skeletal muscle	Unaltered.

Animal H03-0541 (continued)

Organ	Histopathology
Spleen	Centre filled with often connected, mildly depleted secondary follicles, surrounded by rim of macrophages, lymphocytes and fewer neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.

Sub-adult males

Animal H01-1509

Organ	Histopathology
Bone marrow	High activity [+++]
Kidneys	Mild tubular protein cast formation; one protozoan cyst in vessel wall, no reaction.
Liver	Mild mixed portal infiltration.
Lung	Moderate periarterial/peribronchial, predominantly mononuclear infiltration.
Myocardium	Mild focal periarterial granulomatous infiltrate, in one affected artery, subendothelial protozoan cyst.
Pancreas of Aselli	Cortex almost completely filled by large secondary follicles, with relatively many apoptotic cells; beneath are T cell zones; relatively narrow marginal sinuses with (focally) vacuolated cells, lymphocytes and neutrophils; centre filled with plasma cells, focally extending to marginal sinuses.

Animal H01-1509 (continued)

Organ	Histopathology
Spleen	Centre filled with numerous large, often connected (up to 3) secondary follicles, with rim of lymphocytes, macrophages, lymphocytes and erythrocytes, germinal centres with numerous apoptotic cells. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.

Animal H02-0135

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Several subendothelial protozoan cysts in arteries, one within mild granulomatous infiltrate, otherwise mild interstitial granulomatous infiltration.
Liver	Moderate portal mixed cellular infiltration with follicle-like lymphocyte accumulations, indicating germinal centre formation with numerous apoptotic cells with occasional infiltration of bile ducts, one portal artery with subendothelial protozoan cyst.
Lung	Unaltered.
Myocardium	One subendothelial protozoan cyst; no reaction.

Animal H02-0135 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex almost completely filled with predominantly large secondary follicles; beneath are T cell zones; occ. wide marginal sinuses with vacuolated cells, lymphocytes and occasional neutrophils; centre filled with plasma cells focally extending to marginal sinuses.
Skeletal muscle	Unaltered.
Small intestine	Unaltered.
Spleen	Centre filled with often connected, large secondary follicles with numerous apoptotic cells, surrounded by rim with macrophages, neutrophils and lymphocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered.

Animal H02-0137

Organ	Histopathology
Adrenal glands	Unaltered.
Kidneys	Very mild mononuclear interstitial infiltration, one protozoan cyst without reaction.
Large intestine	Unaltered.

Animal H02-0137 (continued)

Organ	Histopathology
Liver	Moderate portal mixed cellular infiltration with small follicle-like lymphocyte accumulations.
Lung	Very mild periarterial lymphocyte accumulations.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex with large, mildly depleted secondary follicles; beneath are T cell zones; moderately wide marginal sinuses with vacuolated cells, relatively numerous lymphocytes and neutrophils, centre filled with plasma cells.
Small intestine	Unaltered.
Spleen	Centre filled with relatively large, often connected secondary follicles with numerous apoptotic cells, surrounded by rims of macrophages and lymphocytes with neutrophils. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered.

Animal H02-0140

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	Moderate activity [++]
Kidneys	Mild focal interstitial granulomatous infiltration.
Large intestine	Unaltered.
Liver	Mild portal mixed cellular infiltration with occasional follicle-like accumulations of lymphocytes.
Lung	One small granuloma, composed of vacuolated cells, surrounded by fibroblasts; moderate focal, predominantly mononuclear interstitial infiltration, mild to moderate perivascular and peribronchial lymphocyte accumulations.
Myocardium	Mild focal (periarterial) granulomatous infiltration with subendothelial protozoan cyst.
Pancreas of Aselli	Cortex with moderately-sized mildly depleted secondary follicles; beneath are T cell zones; focally wide sinuses with vacuolated cells, lymphocytes and neutrophils; centre filled with plasma cells.
Skeletal muscle	Unaltered.
Small intestine	Mild submucosal mixed inflammatory infiltration.

Animal H02-0140 (continued)

Organ	Histopathology
Spleen	Centre filled with large, connected (up to eight) secondary follicles with numerous apoptotic cells and occasional mitoses in germinal centres; with distinct rims of macrophages, lymphocytes and neutrophils. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered.

Animal H03-0437

Organ	Histopathology
Bone marrow	High activity [+++]
Gall bladder	Unaltered.
Kidneys	Moderate hyperaemia.
Liver	Moderate mixed cellular portal infiltration.
Lung	Moderate hyperaemic, focal alveolar haemorrhage.
Myocardium	Intravascular protozoan cyst (in vessel wall), no reaction.
Pancreas	Unaltered.

Animal H03-0437 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with moderately sized secondary follicles (cortex in large areas not filled); beneath are T cell zones; focally wide marginal sinuses with vacuolated cells, disseminated macrophages and lymphocytes and extensive focal accumulations of neutrophils, occasionally surrouding areas of necrosis; centre filled with plasma cells, widely extending to marginal sinuses; in medulla several protozoan cysts within vessel walls, no reaction.
Skeletal muscle	Unaltered
Spleen	Centre filled with often connected, relatively large secondary follicles, surrounded by rim of macrophages, lymphocytes, fewer neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.

Animal H03-0539

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Brain	Unaltered.

Animal H03-0539 (continued)

Organ	Histopathology
Gall bladder	Lumen filled with one large parasite (digenean *Dicrocoelium soricis*) with brown eggs (roughly 32µm by 22µm) containing 2-3 pigmented granules, mild focal lymphocytic infiltration.
Kidneys	Focal medullary tubular mineralisation.
Liver	Mild to moderate mixed cellular (lymphocyte, plasma cell-dominated) portal infiltration, moderate hyperaemia.
Lung	Moderate hyperaemia in trachea, otherwise unaltered.
Myocardium	One protozoan cyst in arterial wall, no reaction.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex almost completely filled with relative large secondary follicles; beneath are T cell zones; relatively narrow marginal sinuses with vacuolated cells, lymphocytes, macrophages and neutrophils; centre filled with plasma cells, focally extending to marginal sinuses.
Skeletal muscle	Unaltered.
Spleen	Centre filled with large, often connected secondary follicles, surrounded by rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.

Animal H03-0540

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Unaltered.
Liver	Moderate mixed cellular (lymphocyte, plasma cell dominated) portal infiltration with small follicular accumulations of lymphocytes.
Lung	Moderate hyperaemia in trachea, otherwise unaltered.
Myocardium	Unaltered.
Oesophagus	Unaltered.
Pancreas of Aselli	Cortex with mildly to moderately depleted, large secondary follicles (cortex not completely filled); beneath are T cell zones; focally wide marginal sinuses with vacuolated cells, lymphocytes, macrophages and scattered neutrophils; centre filled with plasma cellss, focally extending to marginal sinuses.
Skeletal muscle	Unaltered.
Spleen	Centre filled with large, often connected secondary follicles with numerous apoptotic cells, surrounded by rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.

Pubescent females

Animal H02-1006

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Brain	Unaltered.
Kidneys	One protozoan cyst, subintimal, in renal medulla, no reaction.
Liver	Mild to moderate portal mixed cellular infiltration with occasional small follicle-like accumulations of lymphocytes and occasional infiltration of bile ducts.
Lung	Moderate hyperaemia.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex with relatively small, mildly depleted secondary follicles (large areas of cortex are free of follicles); beneath are T cell zones; often relatively wide marginal sinuses with vacuolated cells, lymphocytes and few neutrophils; centre filled with plasma cells widely extending to sinuses.
Skeletal muscle	Unaltered.
Spleen	Centre with relatively small primary follicles, with thin rim of macrophages, neutrophils and lymphocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urethra	Unaltered.

Animal H02-1502

Organ	Histopathology
Adrenal glands	Unaltered.
Brain	Unaltered.
Gall bladder	Unaltered.
Kidneys	Unaltered.
Liver	Multifocal necrosuppurative hepatitis with haemorrhage and (intralesional) nematodes (400-800µm by 20-30µm max, unlikely to be *Calodium cholidicola* as too small and no subcuticular papillae observed). Moderate mixed cellular portal infiltration with follicle-like accumulations of lymphocytes and focal infiltration of bile ducts.
Lung	Moderate hyperaemia, focal perivascular and peribronchial lymphocyte accumulations.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex with numerous large secondary follicles with numerous apoptotic cells and tingible body macrophages; beneath are large T cell zones; relatively wide marginal sinuses with vacuolated cells, macrophages, lymphocytes and relatively numerous neutrophils; centre relatively small and filled with plasma cells, intermingled are neutrophils.
Spleen	Centre filled with large, often connected secondary follicles with numerous apoptotic cells and tingible body macrophages in germinal centres, surrounded by rim of macrophages, lymphocytes, fewer neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.

Pubescent males

Animal H02-801

Organ	Histopathology
Bone marrow	High activity [+++]
Brain	Unaltered.
Kidneys	One protozoan cyst (subintimal) in vain, no reaction.
Liver	Very mild to moderate portal mixed cellular infiltration, occasionally with infiltration of bile ducts.
Lung	Moderate hyperaemia.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex with relatively small, mildly depleted secondary follicles; beneath are T cell zones; relatively narrow marginal sinuses with vacuolated cells and occasional lymphocytes and neutrophilic leukocytes; centre filled with plasma cells; in large areas expanding to marginal sinuses/capsule.
Skeletal muscle	Unaltered.
Spleen	Centre filled with mildly to moderately depleted secondary follicles with distinct rim of macrophages, lymphocytes and few neutrophilic leukocytes.
Urinary bladder	Unaltered.

Adult females

Animal H02-158

Organ	Histopathology
Brain	Unaltered.
Gall bladder	Unaltered.
Kidneys	Mild multifocal mononuclear interstitial infiltration, mild tubular protein cast formation.
Liver	Mild to moderate mixed portal infiltration with occasional follicle-like accumulations of lymphocytes.
Lung	Moderate hyperaemia; mild multifocal purulent alveolar pneumonia with few desquamated macrophages and focal peribronchial accumulations of lymphocytes.
Pancreas of Aselli	Cortex almost completely filled with large secondary follicles with numerous apoptotic cells; beneath are T cell zones; moderately wide marginal sinuses with vacuolated cells, lymphocytes, neutrophils and apoptotic cells; centre filled with plasma cells.
Spleen	Centre filled with large secondary follicles with distinct rim of macrophages, lymphocytes and erythrocytes; numerous apoptotic cells in follicle centres. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.

Animal H02-1588

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	Moderate activity [++]
Brain	Unaltered.
Kidneys	Very mild interstitial mononuclear infiltration.
Liver	Mild portal fibrosis and mononuclear or mixed infiltration, occasional leukocytes between hepatic cords, multifocal haemorrhage.
Lung	Moderate hyperaemia, mild alveolar oedema and occasional haemorrhage.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex almost completely filled with large secondary follicles with relatively numerous apoptotic cells; beneath are large T cell zones; relatively narrow marginal sinuses with vacuolated cells and lymphocytes; centre filled with plasma cells.
Skeletal muscle	Unaltered.
Spleen	Centre filled with large secondary follicles, small mantle zones with numerous apoptotic cells, surrounded by rim of erythrocytes, macrophages and lymphocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.

Animal H03-0224

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Brain	Unaltered.
Kidneys	Mild focal chronic interstitial nephritis (plasma cell-dominated).
Liver	Mild to moderate mixed cellular portal infiltration.
Lung	Moderate hyperaemia, mild focal peribronchial and vascular lymphocyte accumulations.
Myocardium	Protozoan cyst in thickened vessel wall, no reaction.
Oesophagus	Moderate mixed cellular mucosal infiltration; focal epithelial hydropic degeneration with neutrophil infiltration and intracytoplasmic structures (protozoan?) within (degenerating) epithelial cells; cestode within lumen most likely a dorsal or ventral slice (722µm by 278µm) of the digenean *brachylaemus fulvus*.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with few, relatively small secondary follicles, occasional T cell zones are beneath; relatively narrow marginal sinuses with vacuolated cells, lymphocytes and neutrophils (accumulations); centre filled with plasma cells, which widely extend to sinuses and capsule; granuloma in capsule, with central dystrophic calcification, surrounded by vacuolated cells, multinucleated giant cells and macrophages/epithelioid cells with scattered neutrophils.

Animal H03-0224 (continued)

Organ	Histopathology
Skeletal muscle	Unaltered
Small intestine	Moderate mixed cellular mucosal infiltration.
Spleen	Centre with relatively small primary/secondary follicles, often connected; with distinct rim of neutrophils, lymphocytes, macrophages and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Mild focal mucosal lymphocyte accumulations.
Urinary bladder	Lumen with nematodes (diameter 102µm cross section, with eggs identified as *Liniscus incrassatus*: 49 x 19.5um), parasites partly encapsulated between/in epithelial cells, moderate chronic lymphocyte-dominated cystitis.

Animal H03-1088

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Moderate hyperaemia, focal lymphoplasmacellular perivascular interstitial infiltration in medulla.
Liver	Moderate portal, mononuclear (lymphocytes, plasma cells, fewer neutrophilic leukocytes) infiltration.

Animal H03-1088 (continued)

Organ	Histopathology
Lung	Moderate hyperaemia, moderate focal peribronchial and vascular lymphocyte accumulations.
Myocardium	Unaltered.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with relatively few, moderately depleted, small secondary follicles; beneath are T cell zones; focally wide marginal sinuseswith vacuolated cells, lymphocytes, macrophages and multiple accumulations of neutrophils and lymphocytes; centre vastly filled with plasma cells, in large areas extending to marginal sinuses.
Skeletal muscle	Unaltered.
Spleen	Centre with solitary, primary/secondary follicles, surrounded by distinct rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Few nematodes in longitudinal section (85µm by 12µm) in lumen and between/in epithelial cells. Most likely *Liniscus incrassatus*.

Animal H03-1164

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Moderate tubular protein cast formation, mild multifocal interstitial mononuclear infiltration.
Liver	Mild mixed cellular (focal) portal infiltration.
Lung	Moderate hyperaemia, focal alveolar haemorrhage.
Myocardium	Unaltered.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with relatively small, moderately depleted secondary follicles, large areas of cortex devoid of follicles; beneath are T cell zones; often wide marginal sinuses with vacuolated cells, lymphocytes, neutrophils and macrophages; mild extramedullary haematopoiesis; centre filled with plasma cells, often extending to marginal sinuses/capsule; focal pyogranulomatous infiltration in capsule.
Skeletal muscle	Unaltered.

Animal H03-1164 (continued)

Organ	Histopathology
Spleen	Centre with few, relatively small, mildly depleted, occasionally connected primary/secondary follicles; with rim of neutrophils, macrophages, lymphocytes and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and very numerous megakaryocytes.
Thymus	Moderate involution. Remnants represented by lymphocyte accumulations around blood vessels, with thin fibrous capsule.
Urinary bladder	Unaltered.

Animal H03-1165

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Moderate hyperaemia, mild to moderate tubular protein cast formation.
Liver	Mild portal mixed cellular, plasma cell-dominated infiltration.
Lung	Moderate hyperaemia.
Myocardium	Unaltered.

Animal H03-1165 (continued)

Organ	Histopathology
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with relatively small, moderately depleted secondary follicles, large areas of cortex devoid of follicles; beneath are T cell zones; often wide marginal sinuses with vacuolated cells and scattered lymphocytes and macrophages and neutrophil accumulations; centre filled with plasma cells, often extending to sinuses/capsule.
Skeletal muscle	Unaltered.
Spleen	Centre with few, relatively small primary/secondary follicles; with distinct rims of macrophages, neutrophils, lymphocytes and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and numerous megakaryocytes.
Urinary bladder	Nematodes in lumen and in thin capsule within superficial epithelial cell layers, mucosa otherwise unaltered. Nematode larva in longitudinal section, 93µm by 10µm. Most likely *Liniscus incrassatus*.

Animal H03-1167

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]

Animal H03-1167 (continued)

Organ	Histopathology
Gall bladder	Unaltered.
Kidneys	Moderate hyperaemia, mild to moderate tubular protein cast formation.
Liver	Moderate portal mixed cellular (dominance of lymphocytes and plasma cells) portal infiltration with occasional follicle-like accumulations of lymphocytes.
Lung	Moderate hyperaemia, focal accumulation of foamy macrophages within alveoli.
Myocardium	Unaltered.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with moderately-sized, moderately depleted secondary follicles (cortex not completely filled); beneath are T cell zones; relatively wide marginal sinuses with vacuolated cells, numerous neutrophils, lymphocytes and macrophages; centre filled with plasma cells, often extending to marginal sinuses; mild extramedullary haematopoiesis.
Skeletal muscle	Unaltered.
Spleen	Few moderately depleted primary/secondary follicles, with distinct rim of neutrophils, macrophages and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and very numerous megakaryocytes.
Urinary bladder	Mild mixed cellular infiltration of the mucosa, few nematodes in lumen.

Animal H03-1188

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Mild tubular protein cast formation, mild focal chronic interstitial nephritis; one protozoan cyst.
Liver	Intense portal mixed cellular inflammatory infiltration; multifocal haemorrhage and neutrophil-dominated inflammatory infiltration of the parenchyma, multifocal hydropic degeneration of hepatocytes.
Lung	Moderate hyperaemia.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex with few, small, moderately depleted secondary follicles; beneath are T cell zones; relatively narrow marginal sinuses with vacuolated cells, lymphocytes and macrophages, focal mild fibrosis; centre filled with plasma cells, in large areas extending to marginal sinuses/capsule.
Skeletal muscle	Unaltered.
Spleen	Centre with relatively few, rarely connected, small primary/secondary follicles, surrounded by distinct rim of macrophages, lymphocytes, neutrophils and erythroytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and numerous megakaryocytes.
Urinary bladder	Nematode larvae in longitudinal section, 98µm by 22µm, most likely *Liniscus incrassatus*, encapsulated between superficial epithelial cells; moderate focal neutrophil infiltration of epithelium and mucosa.

Animal H03-1189

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Mild chronic interstitial nephritis with one central protozoan cyst, one protozoan cyst in thickened vessel wall and one protozoan cyst almost completely occluding vessel with thickened wall.
Liver	Mild portal mixed cellular infiltration.
Lung	Moderate hyperaemia, mild focal peribronchial and vascular lymphocyte accumulations.
Pancreas of Aselli	Cortex with moderately sized, moderately deleted secondary follciles (cortex not completely filled); beneath are T cell zones; focally wide marginal sinuses with focal fibrosis and vacuolated cells, lymphocytes and macrophages, occasional accumulations of neutrophils; in cortex one granuloma with central necrosis and mineralisation, surrounded by neutrophils, macrophages and epithelioid cells with collagen deposition; centre filled with plasma cells, in large areas extending to marginal sinuses/capsule; moderate extramedullary haematopoieses (numerous megakaryocytes).
Skeletal muscle	Numerous variably sized, long protozoan cysts within muscle fibres, no reaction.
Spleen	Centre with relatively small, occasionally connected, primary follicles, surrounded by distinct rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and very numerous megakaryocytes.

Animal H03-1189 (continued)

Organ	Histopathology
Thymus	Moderate involution? Remnants represented by layers of lymphocytes (and macrophages) around blood vessels, with thin fibrous capsule.
Urinary bladder	Nematode larvae in longitudinal section, 102µm by 22µm, most likely *Liniscus incrassatus*, in capsule within superficial epithelium, nematode larvae in lumen.

Animal H03-1192

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Gall bladder	Unaltered.
Kidneys	Multiple vessels with protozoan cysts, mild chronic interstitial nephritis, occasionally in association with cysts; mild tubular protein cast formation.
Liver	Moderate to severe multifocal mixed cellular (neutrophilic leukocytes) parenchymal infiltration; moderate mixed cellular portal infiltration with follicle-like accumulations of lymphocytes.
Lung	Moderate hyperaemia.
Myocardium	Unaltered.

Animal H03-1192 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with relatively few, small, moderately depleted secondary follicles; beneath are T cell zones; wide marginal sinuses with vacuolated cells, lymphocytes, macrophages and focal accumulations of neutrophils; in cortex granuloma with central necorsis and mineralisation; surrounded by macrophages/epithelioid cells, neutrophils, lymphocytes and collagen (outer layer), second small granuloma in marginal sinuses, composed of neutrophils, macrophages and fewer lymphocytes, with small amounts of collagen; centre vastly filled with plasma cells, in large areas extending to marginal sinuses.
Skeletal muscle	Unaltered.
Spleen	Centre with relatively few, moderately sized, mildly depleted primary/secondary follicles, surrounded by rim of macorphages and fewer lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and numerous megakaryocytes; vessels with protozoan cysts in vessel wall, no reaction. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Mild mixed cellular mucosal inflammatory infiltration.

Animal H03-1424

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Gall bladder	Several cestodes with brown/yellow eggs.
Kidneys	Mild tubular protein cast formation, one protozoan cyst without reaction.
Liver	Moderate portal mixed cellular infiltrations.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with scattered small, moderately to severely depleted secondary follicles with central collagen deposition; beneath are T cell zones; moderately wide marginal sinuses with vacuolated cells, disseminatred lymphocytes and macrophages and multiple small neutrophil accumulations, multifocal extensive fibrosis; centre filled with plasma cells, in most areas extending to marginal sinuses; moderate extramedullary haematopoiesis (megakaryocytes).
Skeletal muscle	Unaltered.
Spleen	Centre with numerous, often connected small primary/secondary follicles with focal central collagen deposition, surrounded by rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.

Animal H03-1424 (continued)

Organ	Histopathology
Stomach	Unaltered.
Urinary bladder	Several nematode larvae in lumen and between epithelial cells.

Adult males

Animal H03-1381

Organ	Histopathology
Adrenal glands	Unaltered
Kidneys	One protozoan cyst within glomerulum, no reaction.
Liver	Mild to moderate portal mixed cellular infiltration with occasional smal follicle-like accumulations of lymphocytes.
Lung	Moderate hyperaemia, focal alveolar haemorrhage, moderate multifocal neutrophil accumulations.
Mediastinal lymph node	Composed of lymphocytes (no follicles discernible), intermingled with neutrophils; numerous apoptoses.
Myocardium	Unaltered.
Pancreas	Unaltered.

Animal H03-1381 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with small primary/secondary follicles (large areas of cortex without follicles); beneath are T cell zones; often wide marginal sinuses with vacuolated cells and numerous degenerating cells (apoptotic cells) and lymphocytes, macrophages, neutrophils; centre filled with plasma cells, often extending to sinuses; very numerous tingible body macrophages between plasma cells.
Skeletal muscle	Unaltered.
Spleen	Centre with partly connected, relatively small primary/secondary follicles with several apoptotic cells, surrounded by distinct rim of macrophages, neutrophils, lymphocytes and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Thymus	Mild to moderate involution. Represented by tightly packed layers of lymphocytes around blood vessels, thin fibrous capsule.

Animal H03-1384

Organ	Histopathology
Adrenal glands	Unaltered.
Gall bladder	Unaltered.
Kidneys	Unaltered.
Liver	Moderate, predominantly mononuclear portal infiltration with follicle-like accumulations of lymphocytes.
Lung	Moderate hyperaemia.
Myocardium	Unaltered.
Pancreas	Unaltered.
Pancreas of Aselli	Cortex with relatively small, mildly depleted secondary follicles with relatively numerous apoptotic cells, large areas without follicles; beneath are relatively small T cell zones; wide marginal sinuses with vacuolated cells, neutrophils, macrophages and lymphocytes; centre filled with plasma cells, in large areas extending to sinuses/capsule; mild extramedullary haematopoiesis (few megakaryocytes).
Spleen	Centre with relatively small primary follicles with distinct rims of neutrophils, macrophages, lymphocytes and fewer erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	One protozoan cyst in vessel wall between muscle layers, otherwise unaltered.

Animal H03-1406

Organ	Histopathology
Adrenal gland	Unaltered.
Bone marrow	High activity [+++]
Gall bladder	Unaltered.
Kidneys	Mild tubular hyaline droplet/protein cast formation, mild focal plasmacellular infiltration in renal pelvis.
Liver	Moderate portal mixed cellular infiltration, focal chronic granulomatous infiltration, focal necrosuppurative inflammation.
Lung	Moderate hyperaemia, focal alveolar haemorrhage, focal pyogranulomatous subpleural infiltration with type ii pneumocyte hyperplasia; trachea: unaltered.
Mediastinal lymph node	Relatively small secondary follicles; marginal sinuses with numerous macrophages and scattered lymphocytes.
Myocardium	One protozoan cyst in arterial wall, with mild focal granulomatous infiltration.
Oesophagus	Focal epithelial proliferation and hydropic degeneration with intracytoplasmic structures (protozoan?) within (degenerating) epithelial cells.

Animal H03-1406 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with relatively few, small to moderately sized, moderately depleted secondary follicles; beneath are T cell zones; relatively narrow marginal sinuses with vacuolated cells, scattered lymphocytes and macrophages; centre vastly filled with plasma cells, in large areas extending to marginal sinuses/capsule; mild extramedullary haematopoiesis (megakaryocytes).
Skeletal muscle	Unaltered
Spleen	Centre with few, occasionally connected, moderately depleted, small primary/secondary follicles, surrounded by rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered.
Thymus	Intense involution? Represented by lymphocytes surrounding blood vessels, with thin fibrous capsule.
Urinary bladder	Unaltered.

Animal H03-1409

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Mild focal interstitial plasma cell infiltrations.
Liver	Focal necrosis with haemorrhage and pyogranulomatous infiltration in the periphery; moderate portal mixed cellular infiltrate.
Lung	Moderate hyperaemia.
Myocardium	Unaltered.
Skeletal muscle	Unaltered.
Spleen	Centre with moderately sized, occasioanlly connected primary follicles, surrounded by distinct rim of macrophages, lymphocytes, neutrophils and erythrocytes). Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Unaltered.

Animal H03-1410

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Gall bladder	Unaltered.
Kidneys	Scattered protozoan cysts, no reaction.
Liver	Mild portal mixed cellular infiltration.
Lung	Moderate hyperaemia.
Myocardium	One protozoan cyst in thickened vessel wall (slightly bulging), no reaction.
Pancreas of Aselli	Cortex with moderately sized, mildly to moderately depleted secondary follicles; beneath are T cell zones; relatively wide marginal sinuses with vacuolated cells, scattered macrophages and lymphocytes and few neutrophil accumulations; centre filled by plasma cells, focally extending to marginal sinuses.
Skeletal muscle	Unaltered.
Spleen	Centre with few, occasionally connected, small primary/secondary follicles, surrounded by distinct rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and numerous megakaryocytes.

Animal H03-1410

Organ	Histopathology
Thymus	Moderate involution? Represented by layers of lymphocytes around blood vessels, with thin fibrous connective tissue capsule.
Urinary bladder	Nematode larvae within detritus (free in lumen).

Animal H03-1411

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Gall bladder	Unaltered.
Kidneys	Mild interstitial lymphoplasmacellular infiltration.
Liver	Very mild portal mixed cellular infiltration.
Lung	Unaltered.

Animal H03-1411 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with few, relatively small, moderately depleted secondary follicles with occasional central collagen deposition; beneath are T cell zones; wide marginal sinuses with vacuolated cells, scattered lymphocytes and macrophages and larger accumulations of neutrophils, focal fibrosis; centre vastly filled with plasma cells, in large areas extending to marginal sinuses/capsule.
Skeletal muscle	Unaltered.
Spleen	Centre with small, occasionally connected, moderately depleted secondary follicles with central collagen deposition, surrounded by rim of macrophages, lymphocytes, fewer neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Thymus	Intense involution? Represented by few layers of lymphocytes around blood vessels, with thin fibrous connective tissue capsule.
Urinary bladder	Unaltered

Animal H03-1412

Organ	Histopathology
Adrenal glands	Unaltered.
Kidneys	Protozoan cysts without reaction; mild focal interstitial pyogranulomatous infiltration.
Liver	Multifocal extensive hepatocellular necrosis and haemorrhage, with neutrophil infiltration; moderate portal mixed cellular infiltration.
Lung	Moderate multifocal perivascular lymphocyte infiltration, focal granulomatous infiltrate around hair fragment.
Myocardium	Protozoan cysts in arterial walls.
Pancreas of Aselli	Cortex with moderately sized, moderately depleted secondary follicles, occasionally sticking out into marginal sinuses; beneath are T cell zones; focally wide marginal sinuses with vacuolated cells and scattered macrophages and lymphocytes as well as small neutrophil accumulations; centre vastly filled with plasma cells, in large areas extending to marginal sinuses/capsule.
Spleen	Centre with relatively small, occasionally connected, moderately depleted secondary follicles with central collagen deposition, surrounded by distinct rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Urinary bladder	Numerous nematode larvae (most probably *Liniscus incrassatus* in cross section) within detritus in lumen and between superficial epithelial cells, with degeneration and sloughing of superficial epithelial cells.

Animal H03-1414

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Kidneys	Moderate hyperaemia, protein cast formation, protozoan cysts (one in venous wall) without reaction.
Liver	Moderate mixed portal infiltration with occasional follicle-like accumulations of lymphocytes.
Lung	Moderate hyperaemia, focal predominantly granulomatous peritracheal infiltration.
Myocardium	Unaltered.
Oesophagus	Few nematode larvae (*Eucoleus oesophagicola*) Between upper epithelial cell layers, moderate mixed cellular submucosal infiltration.
Pancreas of Aselli	Cortex with few, relatively small, moderately depleted secondary follicles; beneath are T cell zones; focally wide marginal sinuses with vacuolated cells and disseminated lymphocytes and macrophages; centre filled with plasma cells, focally extending to marginal sinuses; mild extramedullary haematopoiesis (few megakaryocytes); focal pyogranulomatous inflammation in medulla.
Skeletal muscle	Unaltered.

Animal H03-1414 (continued)

Organ	Histopathology
Spleen	Centre with relatively small, often connected, moderately depleted secondary follicles, surrounded by rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Unaltered. Subserosal granuloma with central necrosis, surrounded by macrophages, epithelioid cells and outer layer of neutrophils, lymphocytes and fibroblasts.

Animal H03-1419

Organ	Histopathology
Adrenal glands	Unaltered.
Bone marrow	High activity [+++]
Gall bladder	Unaltered.
Kidneys	Mild tubular protein cast formation, protozoan cyst without reaction.
Liver	Mild to moderate portal mixed cellular infiltration; focal circular fibrosis (old granuloma).
Lung	Moderate hyperaemia; multifocal subpleural pyogranulomatous infiltration with central haemorrhage and multinucleate gianT cells; moderate perivascular mononuclear infiltration.
Mediastinal lymph node	Relatively small follicles, wide marginal sinuses with vacuolated cells and extensive neutrophil infiltration, extending to capsule; numerous plasma cells in medulla.

Animal H03-1419 (continued)

Organ	Histopathology
Myocardium	Unaltered.
Pancreas of Aselli	Cortex with moderately sized, moderately depleted secondary follicles, often sticking out into marginal sinuses; beneath are T cell zones; focally wide marginal sinuses with vacuolated cells, macrophages and scattered lympocytes focal necrosuppurative inflammation, extending into capsule; centre filled with plasma cells, in most areas extending to marginal sinuses; mild extramedullary haematopoiesis (few megakaryocytes).
Skeletal muscle	Unaltered.
Spleen	Centre with partly connected, moderately sized, mildly depleted secondary follicles, surrounded by distinct rim of macrophages, lymphocytes, neutrophils and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, neutrophils and megakaryocytes.
Stomach	Mild mononuclear infiltrates; moderate multifocal mixed cellular infiltrates in wall; one protozoan cyst in submucosal vessel wall, no reaction.
Urinary bladder	Unaltered.

Animal H03-1421

Organ	Histopathology
Adrenal glands	Unaltered.
Gall bladder	Unaltered.
Intestine	Several endoparasites within lumen, perhaps at least two cestodes in cross section, given flattened shape of sections 660µm x 296µm, 481µm x 212µm.
Kidneys	Mild chronic interstitial (lymphocytes, plasma cells) nephritis; one protozoan cyst without reaction.
Liver	Moderate multifocal, predominantly portal mixed cellular infiltration, multifocal (small areas) haemorrhage and necrosis in parenchyma, with neutrophilic leukocyte infiltrations.
Lung	Mild disseminated (alveolar) neutrophilic leukocyte infiltrations; mild perivascular mononuclear (lymphocyte) infiltration.
Myocardium	Unaltered.
Pancreas of Aselli	Cortex with relatively small, moderately depleted secondary follicles (large areas of cortex devoid of collicles), occasionally sticking out into marginal sinuses; beneath are T cell zones; relatively wide marginal sinuses with vacuolated cells, disseminated macrophages and lymphocytes and focal accumulations of neutrophils and plasma cells; centre filled with plasma cells, in most areas extending to marginal sinuses; mild extramedullary haematopoiesis (few megakaryocytes).

Animal H03-1421 (continued)

Organ	Histopathology
Spleen	Centre with moderately sized, occasionally connected, mildly to moderately depleted primary/secondary follicles with occasional central collagen deposition, surrounded by distinct rim of macrophages, lymphocytes, neutrophils and erythrocytes cell-rich red pulp with macrophages, lymphocytes, very numerous neutrophils and megakaryocytes.
Stomach	Mild submucosal focal neutrophilic leukocyte infiltration.

Animal H03-1425

Organ	Histopathology
Adrenal glands	Unaltered.
Kidneys	Mild, predominantly plasmacellular interstitial infiltrates (and in renal pelvis); mild medullary tubular calcification, one small old granuloma.
Liver	Mild portal mixed cellular infiltration; focal necrosis with haemorrhage and neutrophilic leukocytes infiltration in periphery.
Lung	Moderate hyperaemia.
Myocardium	Unaltered.

Animal H03-1425 (continued)

Organ	Histopathology
Pancreas of Aselli	Cortex with very few small follicles, beneath are T cell zones; relatively wide marginal sinuses with vacuolated cells, lymphocytes and multiple neutrophil accumulations; centre filled with plasma cells, in large areas extending to marginal sinuses/capsule; focal necrosuppurative inflammation with parasite in centre (species unknown, measures 12µm by 17µm) in medulla.
Skeletal muscle	Unaltered.
Spleen	Centre with small, occasionally connected primary follicles, surrounded by very distinct rim of macrophages, lymphocytes, macrophages and erythrocytes. Cell-rich red pulp with macrophages, lymphocytes, very numerous neutrophils and megakaryocytes.
Stomach	Unaltered.
Urinary bladder	Unaltered.