# Studies on polyparasitism: helminth infections in Scottish sheep and laboratory rodents

By

Gwenda Hughes

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Department of Zoology

University of Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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## DECLARATION

This thesis describes research carried out by myself unless otherwise cited or acknowledged. It has not, in whole, or in part, been previously presented for any other degree.

Gwenda Hughes

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## SUMMARY

A study of the nature and abundance of parasitic helminth communities of sheep in Scotland (Part 1), and complementary experimental investigations of concurrent helminth infections in rats (Part 2) are described in this dissertation.

In Part 1, a brief discussion on the life histories of common parasites of sheep in temperate climates is presented followed by a review of publications on various aspects of helminth infections of Scottish sheep. The effects of gastrointestinal helminth infections on sheep productivity are also considered, and the rough cost of such infections to the meat industry in Scotland is estimated.

The parasite fauna of Scottish sheep was investigated in an 18-month survey based at the Glasgow abattoir. Most sheep (N = 511) were of Lowland origin, and parasite status was assessed by identifying and counting helminth eggs and protozoan oocysts in the faeces. The diversity and abundance of the helminth community of sheep in Scotland was found to have changed little over the last 70 years. Strongylid nematodes were found to be most common, and *Trichuris ovis* (Nematoda), *Strongyloides papillosus* (Nematoda) and *Capillaria longipes* (Nematoda) were also found to occur frequently. Eggs of *Fasciola hepatica* (Digenea) and of *Moniezia expansa* (Cestoda) were detected in slightly less than 10% of samples. Oocysts of the protozoon, *Eimeria* sp., were discovered in about a fifth of all samples.

Host sex was not found to have any effect on prevalence or egg output of any of the parasite species investigated but, with the exception of sheep infected with *F. hepatica*, young sheep were found to harbour the majority of the infections. The prevalence of most parasite species, and the egg counts of strongylid nematodes and of *T. ovis*, were shown to conform to a distinct seasonal pattern, being high in July and October and low in January and April. Increased exposure to the infective stages of the various helminth species in late spring and summer may have been a consequence of the elevated helminth egg output of the peri-parturient ewe in spring. Additionally, climatic conditions for the development and

transmission of the infective stages of these parasite species may be optimal in spring and summer.

Associations between high egg counts of the strongylids, *T. ovis* and *S. papillosus*, and between the presence of strongylids and high *T. ovis* and *S. papillosus* egg counts, were detected by stepwise multiple regression analysis and by logistic regression analysis, respectively. Seasonal effects were, again, thought to be partly responsible for these observations since temperature and rainfall were found to explain a considerable proportion of the variation in helminth egg counts. Despite this, when season, source and age variables were removed, a positive, though weak, relationship between strongylid and *T. ovis* egg count intensities was discovered in sheep from certain batches. It is suggested that behavioural, physiological and immunological processes may also determine this particular relationship.

In Part 2, a review of the literature on experimental investigations of concurrent parasitic infections in mammalian hosts concentrated specifically on antagonistic and synergistic interactions between parasite species and the consequences of effects on the host. In the experimental studies described, the relationship between *Hymenolepis diminuta* (Cestoda) and *F. hepatica* in rats was examined.

The possibility of interactions between the helminth species was investigated. Both mature and immature *F. hepatica* infections in rats were found to confer no resistance to oral challenge with *H. diminuta* cysticercoids. In fact, *H. diminuta* survival was shown to be slightly enhanced by prior patent infection with *F. hepatica*, which may have resulted from some degree of immunosuppression associated with the concurrent *F. hepatica* infection, or from the increase in bile glucose symptomatic of patent *F. hepatica* infection in rats. The extensive intestinal mast cell response of *F. hepatica*-infected rats around day 35 p.i., may have been responsible for the slightly poorer growth of *H. diminuta* when administered at this time. Rats with mature *H. diminuta* infections offered no resistance to oral challenge with *F. hepatica* metacercariae. Density-dependent effects on *F. hepatica* growth and on *H.*

*diminuta* fecundity were demonstrated. Intraspecific competition for a limiting resource, or increased stimulation of the immune system, both as a consequence of the increased worm burden, may explain these observations.

Host cellular responses to infection with the helminth species were also monitored. The characteristic eosinophilia of *F. hepatica*-infected rats was significantly reduced in concurrent infections with *H. diminuta*. In general, the ability of an *H. diminuta* infection to prevent an *F. hepatica*-induced eosinophilia was more notable than its ability to depress an existing eosinophilia. Eosinophil levels in the bone marrow of rats with the mixed species infections were found to be lower than in the *F. hepatica*-only controls, suggesting that *H. diminuta* effected an inhibitory influence on eosinophil production in *Fasciola*-infected rats. This effect was not, however, consistently observed. Despite the apparent involvement of eosinophils in host resistance to secondary *F. hepatica* infections, rats with combined *F. hepatica* and *H. diminuta* infections were found to offer resistance to secondary challenge with *F. hepatica*, comparable with that of controls. The significance of the latter experiment is not clear, since a pronounced decline in the numbers of circulating eosinophils was not detected in rats with the dual species infections.

# GENERAL INTRODUCTION

In nature, different species of parasitic helminth frequently coexist within an individual host. Human host populations harbour a variety of helminth species, of which 12 are particularly common (Crompton, 1987). These infections occur predominantly in populations living in developing countries and their high prevalence ensures a substantial level of polyparasitism (Crompton, 1987). Concurrent helminth infections in non-human hosts are also widespread and have, for example, been discovered in Scottish sheep (Robertson, 1935), pigs from South Wales (Jenkins & Erasmus, 1963), European tortoises (Schad, 1963), lesser scaup ducks (Bush & Holmes, 1986) and bufflehead ducks (Ewart & McLauchlin, 1990) from Canada, great shearwaters from the central South Atlantic (Hoberg & Ryan, 1989), common gulls from Norway (Kennedy & Bakke, 1989) and in various species of British freshwater fish (Price & Clancy, 1983).

The study of natural parasite communities is of ecological and evolutionary interest. The structure and diversity of helminth infracommunities (i.e. the mean number of species per host) and of helminth component communities (i.e. the total number of helminth species in a given host population) (Kennedy & Bakke, 1989) are dictated by host behaviour, physiology and immunology, as well as the history of interactions between the parasite species (Holmes, 1973). It is thought, for example, that host foraging behaviour and diet is likely to be reflected in the overall species richness of the helminth community; hosts with broad and opportunistic foraging behaviours tend to carry a more diverse range of helminth species (Kennedy & Bakke, 1989). This line of reasoning has been used to explain the rather impoverished helminth community of fish compared with that of birds (Kennedy, Bush & Aho, 1986).

Within a host population, there tends to be a few abundant helminth species, the "core species", and a larger number of rare species, the "satellite species" (Hanski, 1982). Core species frequently occur together and their intensities have also been found to be

highly correlated in lesser scaup ducks (Bush & Holmes, 1986). It is probable that similar patterns of transmission, particularly when a common intermediate host is involved, can at least partially account for associations between parasite species (Bush & Holmes, 1986), although transmission patterns are themselves likely to depend on external phenomena such as climate, topography and geography. Host susceptibility is, however, unlikely to be uniform throughout a population, and may be determined by variations in host behaviour and immune responsiveness, as well as the physiological environment provided by the host. Barger (1984) has suggested that such host-mediated events could be responsible for the significant correlations between the numbers of different trichostrongyle genera in sheep. Hosts which are poor immune responders, in a non-specific sense, may well be expected to harbour high intensity infections overall.

Within an individual host, interactions between helminth species may occur, particularly when they occupy similar sites within the host. Possible outcomes of antagonistic interactions have been examined in a comprehensive article by Holmes (1973). Holmes suggests that antagonistic interactions result from competition between coexisting species, and may lead to either interactive segregation and niche diversification, or to competitive exclusion. Interactive site segregation is probably relatively rare but not unknown. The distribution of the cestode, *Proteocephalus filicollis*, and of the acanthocephalan, *Neoechinorhynchus rutili*, in the alimentary tract of three-spined sticklebacks was found to be significantly different in concurrent infections than in single species infections (Chappell, 1969). The author of this paper suggested that spatial separation of the helminth species during the mixed infections may have resulted from competition, or from the ability of one species to have an adverse influence on the environment of the other. According to Holmes (1973), prolonged coexistence of helminth species in evolutionary time would result in the replacement of interactive segregation with selective segregation, a genetically-controlled process by which helminth species avoid interaction by exploiting different host resources or sites, or by invading the host at different

times. The latter strategy appears to have been adopted by *Cucullanus heterochrous* and *C. minutus*, which mature and occupy the anterior intestine of flounders at different times of year (MacKenzie & Gibson, 1970).

Competitive exclusion is another possible consequence of antagonistic interactions between helminths (Holmes, 1973). This may be evidenced by significantly fewer concurrent infections in a given host population than would be expected, or by the complete absence of one species in the presence of another. In a study on Kenyan cattle, the incidence of concurrent infection with *Fasciola gigantica* and *Echinococcus granulosus* in the livers was extremely low (Froyd, 1960). Regulation of this type of interaction may stem from physical barriers, such as parasite-induced fibrosis of the liver, or from immunological non-specific responses, such as inflammation, which may be detrimental to the survival of a second helminth species (Campbell, Kelly, Townsend & Dineen, 1977; Christensen, Nansen, Fagbemi & Monrad, 1987). Immunological cross-reactivity may also be important between helminth species which are phylogenetically similar (Holmes, 1973; Christensen *et al.*, 1987).

Synergistic interactions, in which the growth and development of one helminth species may be enhanced by the presence of another, have also been observed. Parasite-induced immunotolerance is thought to be the most likely cause of this type of event (Christensen *et al.*, 1987). The survival of *Trichinella spiralis* in mice is extended by 4 weeks in the presence of *Nematospiroides dubius*, for which the immunosuppressive effects of the latter species has been implicated (Behnke, Wakelin & Wilson, 1978). Similarly, the immunological response of mice is found to be impaired by a high-dosage infection with *Echinostomum revolutum*, allowing increased survival of a challenge *Schistosoma mansoni* infection (Christensen, Nydal, Frandsen & Nansen, 1981).

The effects that concurrent helminth infections may have on their host are also numerous. Single species infections are known to elicit a variety of immunological, pathological and physiological responses in the host, and these may be markedly different during multiple species infections. Clearly, the phenomenon termed "competitive exclusion" by Holmes (1973), may be of some benefit to the host if the excluded parasite is of

considerable pathogenicity. Thus protection against infection with *Fasciola hepatica* by prior infection with *Cysticercus tenuicollis* (=*Taenia hydatigena*) is of undoubted advantage to sheep (Campbell *et al.*, 1977). In certain circumstances, however, the ability of concurrent infections to initiate disease can be greater than that predicted by the summation of the effects of individual infections. This has been observed in sheep infected with *Trichostrongylus colubriformis* and *Ostertagia circumcincta*, and was attributed to extensive physiological and metabolic changes during mixed infections (Steel, Jones & Symons, 1982). The pathogenicity of a helminth infection is also likely to be increased in the presence of an infection which has an immunosuppressive influence on the host.

One of the major aims of this thesis was to examine the natural helminth community of a sample of Scottish sheep, specifically parasite diversity and abundance, as well as the possible associations which may exist between different helminth species. Such a study is likely to give a good indication of the distribution of sheep helminth parasites in the field, and also allows valuable, even if somewhat crude, predictions on the extent of chronic, acute and sub-acute disease to be made. Field studies are, nevertheless, not without their disadvantages. Natural helminth communities are frequently diverse and unevenly distributed within the host population (Bush & Holmes, 1986; Kennedy & Bakke, 1989). This very complexity prevents the identification of specific interactions which may occur between helminth species, as well as confounding the influence that individual helminths may have on disease pathology. Experimental studies in the laboratory allow the influence of concurrent infection with known species of helminth on each other, and on the host, to be clarified, since the number of helminth species administered, and the infection protocols, are controlled. To this end, experimental studies using rats and involving helminth species related to those found sheep, were also conducted. The relationship under investigation was that of *Fasciola hepatica* and *Hymenolepis diminuta* in the rat host. This system was chosen for several reasons:

(i) There have been relatively few studies examining concurrent infections involving

digeneans and cestodes.

(ii) As a cestode of the small intestine with a life cycle requiring the ingestion of an invertebrate intermediate host, *H. diminuta* infection in rats has similarities with that of *Moniezia expansa* infections in sheep, even though the latter host is a ruminant.

(iii) *F. hepatica* infections of sheep have been found to be common in Scotland (see, for example, Parnell, Rayski, Dunn & MacKintosh, 1954; present study, Chapter 3), and are likely to be of considerable economic importance (see, for example, Roseby, 1970; Hope Cawdery, 1976).

Part 1 of this thesis deals with natural helminth parasites of sheep. Firstly, the life histories of common parasitic infections of sheep in temperate climates are briefly described, and this is followed by a review of studies to date which have investigated the nature of helminth infections of sheep in Scotland (Chapter 1). The literature examining the importance of such infections, in terms of sheep health and productivity, is then reviewed, and an attempt to assess the possible significance of ovine parasites for the Scottish economy is made (Chapter 2). The final chapter in Part I describes an 18-month epidemiological study investigating natural helminth communities of sheep from various areas of Scotland; this provides a revision of the data on the more common helminth infections of sheep in Scotland, and also studies patterns of association between species of parasite (Chapter 3).

In Part 2, experimental studies of concurrent infections are considered. The section is introduced by a brief chapter reviewing the literature on experimental concurrent parasitic infections, particularly those which are concerned with species of *Fasciola* and *Hymenolepis* (Chapter 4). The remainder of this section deals with experimental studies on concurrent infections of *Hymenolepis diminuta* and *Fasciola hepatica* in rats. A general investigation of growth patterns and fecundity of the individual species, and how these are affected by the presence of a concurrent infection, are considered (Chapter 6). The influence of infection with various combinations of these helminth species on certain immunological responses of the rat host are also discussed (Chapter 7). The experimental methods adopted are

described in Chapter 5.

This dissertation has provided a valuable revision of data on the epidemiology of parasitic infections of sheep in Scotland and an estimation has been made of the current cost of such infections to the meat industry in Scotland. Patterns of association between certain species of helminth found in sheep have also been discovered. In addition, during experimental infections, interactions between helminth species have been identified, together with some interesting effects of these infections on the host. Although these events were observed in the rat host under experimental conditions, they may have significant implications for related infections in sheep and consequently merit further study.

# PART 1

## CHAPTER 1: ASPECTS OF PARASITE INFECTIONS IN SHEEP:

## LIFE HISTORIES AND DISTRIBUTIONS

## 1.1 PARASITES OF SHEEP IN TEMPERATE CLIMATES: HABITATS AND TRANSMISSION

### 1.1.1 Introduction

Domesticated sheep are known to be hosts to a considerable assemblage of parasite species belonging to a variety of classes and phyla. The following section describes those most commonly found in sheep in temperate climates, and summarizes information about their habitats and mechanisms of transmission (see Table 1.1). This section does not include a detailed account of parasite life cycles. Parasites are discussed according to the following categories: Nematoda, Digenea, Cestoda and Protozoa. The texts of Dubey (1977), of Soulsby (1982) and of Urquhart, Armour, Duncan, Dunn & Jennings (1987) are important sources of information, and the classification used is adapted from that of Soulsby (1982).

### 1.1.2 Parasitic Nematoda

1.1.2.1 *Order: Strongylida*

**Superfamily:** Trichostrongyloidea

**Family:** Trichostrongylidae

The trichostrongyles are small, usually hair-like nematodes which parasitize the alimentary tract and have a direct life cycle. Eggs are released in the host's faeces and develop to infective (ensheathed) third stage larvae ($L_3$) on pasture under optimal conditions. Upon ingestion by a susceptible host, larvae undergo a further two moults before maturing in their respective designated sites. The following species are of importance (sites occupied by adult stages within the host are given in parentheses): *Ostertagia circumcincta* (abomasum) and *O. trifurcata* (abomasum); *Haemonchus contortus* (abomasum); *Trichostrongylus axei* (abomasum), *T. colubriformis* (small intestine), *T. vitrinus* (small intestine) and *T. capricola* (small intestine); *Cooperia curticei* (small intestine) and *C. surnabada* (small intestine); *Nematodirus battus* (small intestine), *N. filicollis* (small intestine) and *N. spathiger* (small intestine).

**Table 1.1.** Common parasite species of sheep in temperate climates with a brief description of their habitats and mechanisms of transmission.*

| Parasites | | | site in host | direct/indirect life cycle | mechanism of transmission to ovine host |
|---|---|---|---|---|---|
| Nematoda | | | | | |
| | Strongylida | | | | |
| | | Trichostrongylidae | *Ostertagia circumcincta*<br>*O. trifurcata*<br>*Haemonchus contortus*<br>*Trichostrongylus axei* | abomasum | direct | ingestion of $L_3$ on pasture |
| | | | *T. colubriformis*<br>*T. vitrinus*<br>*T. capricola*<br>*Cooperia curticei*<br>*C. surnabada*<br>*Nematodirus battus*<br>*N. filicollis*<br>*N. spathiger* | small intestine | direct | ingestion of $L_3$ on pasture |
| | | Dictyocaulidae | *Dictyocaulus filaria* | trachea and bronchi | direct | ingestion of $L_3$ on pasture |
| | | Protostrongylidae | *Muellerius capillaris* | alveoli | indirect | ingestion of mollusc harbouring $L_3$ |
| | | | *Protostrongylus* spp. | small bronchioles | indirect | ingestion of mollusc harbouring $L_3$ |
| | | Strongylidae | *Chabertia ovina* | colon | direct | ingestion of $L_3$ on pasture |
| | | | *Oesophagostomum columbianum* | caecum and colon | direct | ingestion of $L_3$ on pasture |
| | | | *O. venulosum* | caecum and colon | direct | ingestion of $L_3$ on pasture |
| | | Ancylostomatidae | *Bunostomum trigonocephalum* | small intestine | direct | ingestion of, or percutaneous infection by, $L_3$ on pasture |
| | Rhabditida | | | | | |
| | | Strongyloididae | *Strongyloides papillosus* | small intestine | direct | ingestion of, or percutaneous infection by, $L_3$ on pasture |
| | Enoplida | | | | | |
| | | Trichuridae | *Trichuris ovis* | large intestine (caecum) | direct | ingestion of eggs containing $L_1$ |
| | | Capillaridae | *Capillaria longipes* | small intestine | direct | ingestion of eggs containing $L_1$ |
| Digenea | | | | | | |
| | | Fasciolidae | *Fasciola hepatica* | bile ducts | indirect | ingestion of metacercariae on vegetation |
| | | Paramphistomatidae | *Paramphistomum* spp. | fore-stomachs | indirect | ingestion of metacercariae on vegetation |
| | | Dicrocoeliidae | *Dicrocoelium dendriticum* | bile ducts | indirect | ingestion of ant harbouring metacercariae |
| Eucestoda | | | | | | |
| | Anoplocephalidea | | | | | |
| | | Anoplocephalidae | *Moniezia expansa* | small intestine | indirect | ingestion of oribatid mite harbouring cysticercoid |
| | Taeniidea | | | | | |
| | | Taeniidae | *Taenia multiceps* | central nervous system | indirect | ingestion of eggs with oncosphere on pasture |
| | | | *T. hydatigena* | on liver in peritoneum | indirect | ingestion of eggs with oncosphere on pasture |
| | | | *T. ovis* | muscle | indirect | ingestion of eggs with oncosphere on pasture |
| | | | *Echinococcus granulosus* | liver and lungs (predominantly) | indirect | ingestion of eggs with oncosphere on pasture |
| Sporozoa | | | | | | |
| | Eucoccidiidae | | | | | |
| | | Eimeriidae | *Eimeria* spp. | intestinal epithelial cells | direct | ingestion of sporulated oocysts on pasture |
| | | | *Isospora* spp. | intestinal epithelial cells | direct | ingestion of sporulated oocysts on pasture |
| | | | *Cryptosporidium* sp. | intestinal lumen | direct | ingestion of sporulated oocysts on pasture |
| | | Sarcocystidae | *Toxoplasma gondii* | extra-intestinal tissues including muscle, liver, lung and brain | indirect | ingestion of sporulated oocysts on pasture |
| | | | *Sarcocystis* spp. | schizonts in endothelial cells of blood vessels; large cysts in muscles | indirect | ingestion of sporocysts on pasture |

* Based on information obtained on consultation of Dubey (1977), Soulsby (1982) and Urquhart, Armour, Duncan, Dunn & Jennings (1987).

**Family:** Dictyocaulidae

*Dictyocaulus filaria* is the major cause of parasitic bronchitis in sheep. The adults reside in the trachea and bronchi and the females produce embryonated eggs which hatch almost immediately, migrate up the trachea, are swallowed and passed out with the faeces. Following larval development on pasture, infective $L_3$ are ingested by grazing sheep. Ingested larvae migrate to the lungs via the lymphatic and blood systems.

**Superfamily:** Metastrongyloidea

**Family:** Protostrongylidae

All protostrongylids known from sheep have been found to inhabit the lungs and have an indirect life cycle involving a molluscan intermediate host. Otherwise, the life cycle is essentially the same as that of the dictyocaulids, with larval development occurring in the mollusc rather than on pasture. Sheep acquire the infection by ingesting the mollusc. Common species in temperate regions are *Muellerius capillaris* (alveoli) and *Protostrongylus* spp. (small bronchioles).

**Superfamily:** Strongyloidea

**Family:** Strongylidae

The strongyles are all intestinal parasites of sheep which have a direct life cycle, closely resembling that of the trichostrongyles. Common species include *Chabertia ovina* (colon); *Oesophagostomum columbianum* (caecum and colon) and *O. venulosum* (caecum and colon).

**Superfamily:** Ancylostomatoidea

**Family:** Ancylostomatidae

The Ancylostomatidae, collectively known as hookworms, are often responsible for considerable morbidity and mortality owing to their blood-sucking activities. The life cycle is

direct and follows that of the trichostrongyles although percutaneous infection with the $L_3$, followed by pulmonary migration, may also occur. The common species is *Bunostomum trigonocephalum* (small intestine).

1.1.2.2 *Order: Rhabditida*

**Superfamily:** Rhabditoidea

**Family:** Strongyloididae

*Strongyloides papillosus* is the only species known to occur in sheep. The life cycle is direct but is unusual in that species of *Strongyloides* alternate between a parasitic and free-living life style. In the parasitic cycle, embryonated eggs are produced by parthenogenesis by the apparently entirely female population residing in the small intestine. The $L_3$ stage may infect the ovine host by skin penetration,or by ingestion, before developing into adult females in the small intestine after a complex tissue migration.

1.1.2.3 *Order: Enoplida*

**Superfamily:** Trichuroidea

**Family:** Trichuridae

*Trichuris ovis* is the prominent trichurid species in sheep from temperate climates. The life cycle is direct and the $L_1$ within the egg is the infective stage. Following ingestion, larval moults occur within the caecal mucosa where the adults are eventually found.

**Family:** Capillariidae

The capillarids are small slender worms of which the only species of possible significance in sheep is *Capillaria longipes*. Adult worms inhabit the small intestine and the life cycle is direct, closely resembling that of *Trichuris ovis*.

### 1.1.3 *Digenea*

**Suborder:** Prosostomata

**Family:** Fasciolidae

*Fasciola hepatica* is the species of economic importance for sheep farmers in temperate regions. The indirect life cycle involves an amphibious snail as intermediate host (*Lymnaea truncatula* in Europe). Adult worms reside in the bile ducts of the sheep and produce eggs which are released with the faeces. Under appropriate conditions, a miracidium hatches and penetrates the snail. After complex and extensive asexual reproduction within the snail, cercariae emerge and encyst to form metacercariae on semi-aquatic vegetation. The metacercariae are infective to susceptible sheep following ingestion.

**Family:** Paramphistomatidae

Adult paramphistomes inhabit the fore-stomachs of ruminants, but otherwise the life cycle is rather similar to that of *F. hepatica*. The important water snail hosts belong to the genera *Planorbis* and *Bulinus*. Sheep become infected when they ingest metacercariae on herbage. There are numerous species, a common one being *Paramphistomum cervi*.

**Family:** Dicrocoelidae

*Dicrocoelium dendriticum* is the only member of the family to be recorded in sheep living in temperate climates. The life cycle parallels that of *F. hepatica* until cercariae are released from the land snail host, *Zebrina detrita* (in Europe). Cercariae emerge in masses called slime-balls which are ingested by ants of the genus, *Formica*. Metacercariae develop within the ant and are infective to sheep when the ant is ingested with vegetation.

### 1.1.4 Cestoda

#### 1.1.4.1 *Order: Anoplocephalidea*

**Family:** Anoplocephalidae

This group comprises, essentially, tapeworms of herbivores. The species most

commonly found in sheep is *Moniezia expansa*, but *M. benedeni* may also occur in the ovine host. Adult worms inhabit the small intestine. Eggs are released with the faeces and are infective to forage mites of the family Oribatidae. Cysticercoids develop within the mite. Sheep acquire the infection by ingestion of infected forage mites during grazing.

1.1.4.2 *Order: Taeniidea*

**Family:** Taeniidae

Sheep are the intermediate hosts for certain species in this family of tapeworms, harbouring either a cysticercus or hydatid cyst, depending on the species. The definitive host, the dog, acquires infection after ingestion of infected sheep flesh. Worms mature in the small intestine and release eggs (with oncospheres) with the faeces. Such eggs contaminate pastures and are infective to sheep upon ingestion. Important species in sheep are (specific name of larval stage and site occupied within the host given in parentheses): *Taenia multiceps* (*Coenurus cerebralis*: central nervous system), *T. hydatigena* (*Cysticercus tenuicollis*: on liver in peritoneum) and *T. ovis* (*Cysticercus ovis*: muscle); *Echinococcus granulosus* (hydatid in liver and lungs).

**1.1.5 Parasitic Protozoa**

**Order:** Eucoccidiidae

**Family:** Eimeriidae

These are mainly intracellular parasites of the intestinal epithelial cells. The life cycle is direct. Oocysts, which are passed with the faeces, develop on pasture and are directly infective to sheep. Following ingestion, sporozoites are released and penetrate intestinal epithelial cells (there is no cellular penetration in the case of *Cryptosporidium* sp.). There then follows a complex process of asexual and sexual reproduction, known as schizogony and gametogony, respectively. There are numerous species and the important genera are *Eimeria*, *Isospora* and *Cryptosporidium*.

**Family:** Sarcocystidae

The life cycles of sarcocystid protozoa are similar to those of *Eimeria* and *Isospora*, with the exception that the asexual and sexual phases occur in the intermediate and definitive hosts, respectively. Sheep are the intermediate hosts. Important species are *Toxoplasma gondii* (extra-intestinal tissues) and *Sarcocystis miescheriana* (blood vessel endothelial cells, muscles). Sheep become infected with *Toxoplasma* following ingestion of sporulated oocysts released in felid faeces, while the ingestion of either sporulated oocysts or of sporocysts of *Sarcocystis* released in canid faeces, may cause infection with this species.

## 1.2 THE DISTRIBUTION AND ABUNDANCE OF HELMINTH INFECTIONS IN SCOTTISH SHEEP: A REVIEW OF THE LITERATURE

There have been few quantitative surveys of the parasitic fauna of sheep in Scotland. Most of the literature regarding parasitic infections of these sheep is concerned with fluctuations in faecal egg counts associated with post-parturient ewes. Although these studies provide important qualitative information on the diversity of helminth infections, they rarely gave an indication of the overall prevalence of individual parasite species in Scottish sheep.

The first recorded survey investigating the occurrence of intestinal parasites of sheep in Scotland was conducted by Cameron between 1921 and 1922. The contents of 700 sheep guts, obtained from the Edinburgh abattoir, were examined for the presence of helminths. This survey was not specifically quantitative since the author supplied no actual figures on prevalences. Nevertheless, an impression of the more important helminths parasitizing Scottish sheep can be gained.

*Nematodirus* was the most common genus of trichostrongyle detected by Cameron, specifically *N. filicollis* and *N. spathiger*, but not *N. battus*. Other trichostrongyles which he observed less frequently were *Ostertagia circumcincta* and *O. trifurcata*, and these were

usually found in association with *Haemonchus contortus* in Lowland sheep (i.e. sheep from the Midland Valley). *Trichostrongylus vitrinus*, *T. extenuatus* (=*T. axei*), *T. instabilis* (=*T. colubriformis*) and *Cooperia curticei* were also occasionally observed. Of the non-trichostrongyle nematodes, *Monodontus trigonocephalus* (=*Bunostomum trigonocephalum*), *Trichuris ovis* and *Oesophagostomum venulosum* were "comparatively common", while *Chabertia ovina*, *Capillaria longipes* and *Strongyloides papillosus* appeared to be of "infrequent occurrence" (Cameron, 1923).

Subsequent papers by other workers suggest these nematodes are typical of Scottish sheep. All were prevalent in 720 sheep viscera collected from 80 farms throughout Scotland (Parnell, Rayski, Dunn & Mackintosh, 1954) and were common helminths of 220 Cheviot sheep from Ettrick in Selkirk (Morgan, Parnell & Rayski, 1951); 148 sheep slaughtered at Dunoon abattoir in Renfrew (Wilson, Morgan, Parnell & Rayski, 1953); 33 sheep originating from farms from all over Scotland (Robertson, 1935); and of 22 fat lambs from East Aberdeenshire (Robertson, 1940).

In these investigations, trichostrongyles were certainly the most important gastro-intestinal nematodes reported, in terms of both prevalence (frequently greater than 90%) and intensity. However, *H. contortus* may not be of particular significance: Fraser & Robertson (1937) describe this trichostrongyle as being "negligible", at least until August, Parnell *et al.* (1954) suggest it is of little consequence to most hill sheep, and Cameron (1923) indicates that it may be confined to certain Lowland sheep only (Cameron, 1923).

There are other prominent nematode infections of Scottish sheep which are not associated with the gastrointestinal tract, the most notable of which are the lung worms. Thirty percent, 48% and 90% of 500 adult sheep slaughtered at an Aberdeen abattoir were infected with *Dictyocaulus filaria*, *Protostrongylus rufescens* and *Muellerius capillaris*, respectively (Robertson, 1935), and 6% of 33 lambs from all over Scotland exhibited symptoms of *D. filaria* infestation (Robertson, 1935). Cases of *D. filaria* and *M. capillaris* infections were also observed in 220 sheep from Ettrick (Morgan *et al.*, 1951) and in 720 Scottish hill sheep (Parnell *et al.*, 1954). Nevertheless, Robertson (1939) suggested that, in

general, Lowland ewes tend to harbour more lung helminths.

The occurrence of digenean infections is perhaps more erratic, but not infrequent. In the original survey conducted by Cameron (1923), *Fasciola hepatica* was the only species of fluke discovered in the 700 sheep investigated. Nevertheless, in a subsequent paper (Cameron, 1931), the same author reports on the "occasional appearance" of the lancet fluke, *Dicrocoelium dendriticum*, in the livers of sheep from the West of Scotland, most notably the Western Isles. Since those sheep encountered in the former survey were slaughtered at the Edinburgh abattoir, they are perhaps unlikely to have originated from the Western Isles. Later studies involving sheep from a variety of regions in Scotland did not report *D. dendriticum* infection.

In contrast, *Fasciola hepatica* infections were frequently recorded. For example, 24% of sheep on an Argyllshire hill farm were infected with *F. hepatica* (Wilson *et al.*, 1953), and the parasite was also well represented in sheep slaughtered at an Aberdeen abattoir (Robertson, 1935), in a mixed group of hill ewes (Robertson, 1939) and in 220 Cheviot sheep from the Southern Uplands (which lie to the south of the Midland Valley) (Morgan *et al.*, 1951). In the study examining the viscera of 720 sheep from hill farming areas throughout Scotland (Parnell *et al.*, 1954), all ewes from some farms in the South West and West of Scotland were reported to harbour *F. hepatica* infections. Perhaps more than any other species, the distribution of *F. hepatica* is associated with the wetter western side of the country (Parnell *et al.*, 1954; Graham & Harris, 1989) where conditions are probably more favourable for the survival of the intermediate snail host.

Cestode infections are not uncommon either. *Moniezia expansa* and *M. benedeni* were both recorded in the original survey by Cameron (1923) and by Parnell *et al.* (1954). Indeed, in three separate surveys conducted by Robertson, involving sheep of different ages and sources (Robertson, 1935, 1939, 1940), approximately one third of the animals were infected with *M. expansa* in each case. Furthermore, the more extensive investigation undertaken by Parnell *et al.* (1954), which is possibly of greater accuracy, suggested that over 40% of sheep

from all over Scotland harboured *M. expansa* infections.

As intermediate hosts of cestodes, sheep in Scotland appear to be most seriously affected by *Taenia hydatigena* (=*Cysticercus tenuicollis*) infection. This parasite has been documented in sheep of mixed ages which originated from various parts of the country (Robertson, 1935, 1939; Morgan *et al.*, 1951; Wilson *et al.*, 1953; Parnell *et al.*, 1954). In those surveys conducted by Robertson, the prevalence of *T. hydatigena* ranged from 15 to 20%. *Taenia multiceps* (=*Coenurus cerebralis*) and *Echinococcus granulosus* were each recorded in only one study conducted by Robertson (1935). In this paper, the author suggested that *T. hydatigena* may be of some significance in Inverness-shire, with cases of the disease occurring in over 16% of farms visited. He further suggested that *E. granulosus* may not be uncommon in Shetland.

There has been a paucity of studies examining the prevalence of protozoan infections of sheep in Scotland. The rather complex and often costly diagnostic procedures which are usually necessary may be partly responsible for this. However, an investigation of coccidia occurring in sheep in the South-West of England (Joyner, Norton, Davies & Watkins, 1966) revealed that almost 95% of 198 sheep exhibited patent infections of *Eimeria arloingi* and that a further nine species of *Eimeria* were common. Such infections may clearly be of some importance in Scottish sheep also.

## 1.3 SUMMARY

Brief details are presented on the habitats and mechanisms of transmission of those parasite species which are most commonly found in sheep in temperate climates. A review of the literature revealed that, until the 1950s, sheep in Scotland harboured a combination of parasite species, of which nematodes, in particular the gastrointestinal trichostrongyles, were especially common. Several species of trichostrongyle nematodes were recorded in sheep from both Highland and Lowland areas of Scotland. The digenean fluke, *Fasciola hepatica*, was frequently detected, although its distribution was associated with the wetter western side of Scotland. Sheep from all over Scotland were commonly found to be both

definitive and intermediate hosts of cestode helminths, but no information regarding the prevalence or distribution of protozoan infections in Scottish sheep was available.

# PART 1

# CHAPTER 2: EFFECT OF GASTROINTESTINAL PARASITIC INFECTIONS ON SHEEP PRODUCTIVITY

## 2.1 INTRODUCTION

Sheep farming in Scotland is of considerable economic importance with over 9 million sheep being farmed in 1989 (D.A.F.S., 1989). In 1987, more than 61 000 tonnes of sheep meat were produced and sold at approximately £3 per kg (D.A.F.S., 1989). Furthermore, about 10 million kg of clipwool was produced and sold at about £1 per kg in 1987 (D.A.F.S., 1989). Any factors which have a detrimental effect on sheep productivity will have significant consequences for the Scottish economy as a whole.

Gastrointestinal helminth parasites are a source of real and threatened economic loss in sheep farming countries throughout the world (Parkins & Holmes, 1989). The extent to which such infections affect productivity varies, and is not always immediately obvious. Production losses associated with fatal infections, and those causing considerable morbidity, are self-evident. The results of more recent work suggest, however, that even in the absence of overt clinical symptoms, parasitic infection may have adverse effects on productivity (Sykes & Coop, 1977a; Parkins & Holmes, 1989).

Prepubescent animals and lactating ewes tend to be the most seriously affected in terms of the diseases caused by gastrointestinal helminth infections. These sheep require a higher energy intake, increased phosphorous and calcium absorption, and a high ratio of amino acid to metabolizable energy absorbed for protein synthesis, relative to animals at other stages of the life cycle (Sykes, 1982). Accordingly, gut-associated helminth infections are likely to be one of the major sources of reduced productivity.

The mechanisms by which gastrointestinal parasitism may influence animal productivity are: (1) depressed voluntary food intake (often referred to as anorexia or inappetence), (2) impaired digestion, (3) decreased nutrient absorption, (4) less efficient nutrient utilization and (5) gastroenteric leakage. These phenomena usually operate together rather than independently, and it is not always clear as to which is of primary importance.

The effects of gastrointestinal parasitism on various aspects of sheep performance are discussed. Since most literature regarding gastrointestinal parasitism and reduced

productivity is concerned with trichostrongyle nematode infections, these parasites predominate in the following review.

## 2.2 BODY WEIGHT

Reduced muscle and fat masses are typical symptoms of gastrointestinal parasite infections in sheep. Such changes are often expressed in terms of weight loss or a decrease in the rate of body weight gain, although analysis of body composition is possibly a more accurate indicator. The latter procedure is destructive, however, and has usually only been included at the end of lengthy experimental studies, when the animals are likely to have experienced at least partial recovery from the acute stages of gastrointestinal parasitic disease (Symons, 1989).

A decrease in growth rate has been observed in sheep infected with *Trichostrongylus colubriformis* (Gardiner, 1966; Steel, Symons & Jones, 1980; Sykes, 1983), *T. vitrinus* (Sykes, 1983), *Ostertagia* spp. (Gardiner, 1966; Symons, Steel & Jones, 1981; Coop, Sykes & Angus, 1982; Leyva, Henderson & Sykes, 1982), *Haemonchus contortus* (Thomas & Ali, 1983; Stevenson, 1989), *Chabertia ovina* (Herd, 1971), the digenean fluke, *Fasciola hepatica* (Sinclair, 1962; Reid, Armour, Urquhart & Jennings, 1970) and the sporozoan parasite, *Sarcocystis* (Munday, 1986).

In the case of trichostrongyle (Herd, 1971; Steel *et al.*, 1980; Symons, *et al.*, 1981) and *Sarcocystis* (Munday, 1986) infections, the severity of weight loss was shown to be positively associated with the number of infectious stages administered, and in *H. contortus* (Abbott, Parkins & Holmes, 1986) and *F. hepatica* (Berry & Dargie, 1976) infections, was found to be additionally influenced by the nutritional status of the host. In the latter two papers, the pathogenicity of disease was aggravated when sheep were fed on poor protein rations. Concurrent trichostrongyle infections were found to exacerbate effects on sheep growth. The resultant decrease in growth rate of sheep with mixed infections was greater than that predicted by the sum of the effects of single species (Steel, Jones & Symons, 1982).

In general, chronic infection with any of these parasite species is likely to cause a depression in host food intake, and this may at least partly account for changes in host body weight (Berry & Dargie, 1976; Sykes & Coop, 1976, 1977b; Steel *et al.*, 1980; Coop, Sykes & Angus, 1981; Symons *et al.*, 1981; Steel *et al.*, 1982). For example, reduced muscle protein synthesis was observed in sheep administered 30 000 larvae of *T. colubriformis* (Steel *et al.*, 1980). This was found to be a consequence of anorexia, and to be a contributory factor in the poor rate of growth observed in these sheep (Steel *et al.*, 1980). However, anorexia need not necessarily have a causative role. A reduction in food consumption would clearly limit the availability of nutrients essential for growth to the host. Nevertheless, skeletal growth is also known to be impaired by parasitic infection (section 2.3); the associated reduction of skeletal musculature may mean that less food is required by the host (Symons, 1989).

Metabolic changes resulting from parasitic infection are well documented, and may also have detrimental consequences for sheep growth. Sykes & Coop (1976) observed a 50% reduction in efficiency of food utilization, specifically nitrogen, in sheep infected with *T. colubriformis*. Similarly, Sykes & Coop (1977b) and Symons *et al.* (1981) observed a decrease in efficiency of utilization of metabolizable energy available for growth in ovine *O. circumcincta* infections, compared with uninfected pair-fed controls. The former authors recorded a reduction in gross efficiency of 30% in the infected animals. Symons *et al.* (1981) and Steel *et al.* (1982) suggested that reduced net nitrogen uptake from the gastrointestinal tract would account for most of the observed poor efficiency of food utilization, and associated decrease in rate of liveweight gain, in trichostrongyle infections. According to (Herd, 1971), weight loss in sheep infected with *Chabertia ovina* was attributable to leakage of plasma protein across the damaged colon wall.

Depressed body weight gain observed in sheep with light *F. hepatica* burdens is likely to be a consequence of impaired feed conversion efficiency, since the level of food intake remains unaffected at low levels of infection (Hawkins & Morris, 1978). Reduced protein, fat and cellulose digestion may be associated with *F. hepatica* infections in sheep (Chubaryan, 1964) and could account for the reduced rate of liveweight gain observed in

these animals (Sinclair, 1967).

## 2.3 SKELETAL GROWTH

Trichostrongyle nematode infections may also be accompanied by adverse effects on skeletal development. Sykes, Coop & Angus (1975) reported shorter tibiae, and a general decrease in the volume of the tibiae, lumbar vertebrae and ribs and their costochondral junctions in sheep infected with *Trichostrongylus colubriformis*. Investigations using histological and chemical pathology techniques revealed a combined osteoporosis and osteomalacia (Sykes *et al.*, 1975). Similarly, ovine *T. vitrinus* infections were associated with reductions in bone size, matrix deposition and in the extent of mineralization of bone matrix in the rib (Sykes, Coop & Angus, 1979). Histological changes in the costochondral junction, including rarefaction of cancellous bone, irregularity of the matrix, cross-trabeculation of metaphyseal bone spicules and depletion of osteoblasts, were also observed (Sykes *et al.*, 1979). Furthermore, bone size, as well as the density of bone matrix and its degree of mineralization, were extensively curtailed in sheep with *Ostertagia circumcincta* infections (Sykes, Coop & Angus, 1977). Mineral deposition (calcium or phosphorous) may be reduced by as much as 50% (Sykes & Coop, 1977b).

Poor utilization of energy and protein may account for impaired ovine skeletal growth, at least in the case of the abomasal *O. circumcincta* infections, although a reduction in voluntary food consumption is also involved (Sykes & Coop, 1977b; Sykes *et al.*, 1977). The reduction in bone length and volume growth rate observed in intestinal trichostrongyle infections, such as *T. colubriformis* (Sykes & Coop, 1976) and *T. vitrinus* (Sykes *et al.*, 1979), may result from inadequate phosphorous and calcium absorption (Sykes & Coop, 1976). This impairment of absorption could be due to intestinal oedema, inflammation and flattening of the villi (Gardiner, 1966; Wilson & Field, 1983).

## 2.4 WOOL PRODUCTION

Sheep infected with gastrointestinal parasites are known to be less efficient wool producers than their uninfected counterparts. Young sheep harbouring infections of mixed nematode species exhibited a drop in wool production of up to almost 50% (Brundson, 1964; Fudalewicz-Niemczyk, Malczewski, Nowasad & Petryszak, 1972). In the latter study, shearing yield, and fibre length and diameter were affected. Concurrent infections, at least of *O. circumcincta* and *T. colubriformis*, probably inhibit wool growth to a greater extent than would be expected from the sum of single infections (Steel *et al.*, 1982).

The depression of wool growth related to individual parasite species, however, may not be inconsequential. Significant reductions in wool growth rate and wool fibre diameter were observed over a four-week period in lambs harbouring *T. colubriformis* infections (Steel *et al.*, 1980), and a 40% decrease in wool production was recorded in year-old sheep receiving *T. colubriformis* larvae weekly for six weeks (Barger, Southcott & Williams, 1973). Similarly, dosing regimes with high numbers of *O. circumcincta* larvae in weaner lambs effected an apparent reduction in the rate of wool growth of between 25 and 32% (Symons *et al.*, 1981). Wool growth and its tensile strength were similarly depleted in lactating sheep harbouring *O. circumcincta* infections (Leyva *et al.*, 1982).

Apparently, infections with the digenean fluke, *Fasciola hepatica*, may also influence sheep-wool production. Roseby (1970) recorded a decrease in wool weight of between 20 and 39% from six weeks post infection with 400 to 500 metacercariae, although fibre strength, and suint and wax content of the greasy wool, were not found to be affected. On the other hand, Edwards, al-Saigh, Williams & Chamberlain (1976) observed poor wool quality, in terms of fibre lengths and diameters as well as staple lengths, in sheep given as few as 100 metacercariae. The rate of wool growth was also shown to be significantly depressed in association with *F. hepatica* infection (Hawkins & Morris, 1978).

Similar observations have been reported in sheep with coccidial infections. Sheep harbouring *Eimeria faurei* were found to exhibit a mange-like disorder of the fleece involving a brittleness of the wool fibres (Spindler, 1965).

Reduced voluntary food consumption is believed to be a principal cause of impaired wool production in infections with *T. colubriformis* (Steel *et al.*, 1980), *O. circumcincta* (Symons *et al.*, 1981) and both of these species combined (Steel *et al.*, 1982). It may also contribute to a lesser extent to the decrease in wool growth observed in sheep harbouring *F. hepatica* (Roseby, 1970). However, poor efficiency of net uptake of nitrogen from the gastrointestinal tract in the case of *T. colubriformis* (Steel *et al.*, 1980), *O. circumcincta* (Symons *et al.*, 1981) and mixed *O. circumcincta/T. colubriformis* (Steel *et al.*, 1982) infections, is likely to compound the effects of inappetence on wool growth. Reduced net uptake of nitrogen may be a consequence of impaired digestion and absorption, gastroenteric leakage, or a combination of these factors (Steel *et al.*, 1980; Symons *et al.*, 1981; Steel *et al.*, 1982). Reduced feed conversion efficiency was found to be the probable cause of the depressed rate of wool growth detected in weaner wethers infected with *F. hepatica* (Hawkins & Morris, 1978).

However, there could also be an hormonal explanation for changes in wool quality. Wool may become more tender with increased adrenocortical activity (Lindner & Ferguson, 1956; Paneretto, Chapman, Downes, Reis & Wallace, 1975), a phenomenon which has been associated with *T. colubriformis* infection (Pritchard, Hennessey & Griffiths, 1974), and which may result from hepatic dysfunction in sheep infected with *F. hepatica* (Roseby, 1970).

## 2.5 REPRODUCTION AND LACTATION

Ewe fertility and lactation may be negatively influenced by parasitic infection. Hope Cawdery (1976) demonstrated an inverse relationship between pregnancy rate (defined as the ratio of the number of pregnant ewes to the number of ewes surviving two months following mating) and mortality rate caused by *F. hepatica* infection. The liver damage and blood loss associated with fascioliasis in sheep may exert physiological and nutritional stress, which in turn may impede conception and/or establishment of the foetus (Hope Cawdery, 1976).

Infection with *O. circumcincta* (Leyva *et al.*, 1982) and *H. contortus* (Thomas & Ali, 1983) may interfere with milk production of ewes; infected sheep produced 17% and 23% less milk than uninfected control animals, respectively. Symons (1985) has suggested that the combination of inappetence, gastroenteric protein loss and enhanced gastrointestinal tissue protein metabolism serve to reduce the available amino acid-N for milk production.

## 2.6 PREDICTIONS OF THE ECONOMIC COST OF GUT-ASSOCIATED HELMINTHS OF SHEEP IN SCOTLAND

Gastrointestinal parasitism may clearly have a significant detrimental impact on sheep weight gain, skeletal growth, wool output, fertility and/or lactation. However, no figures are available to allow estimation of the crude economic significance of these infections in Scotland. It may be of interest to extrapolate data on reduced productivity from experimental studies to the natural situation, and to then estimate the possible cost of gastroenteric parasitism in Scottish sheep. It should be recognised that such a calculation will inevitably be based on several major assumptions and, at best, will only produce a rough estimate.

Body weight gain is possibly the simplest measurement of sheep economic performance to use. Since chronic, as opposed to acute, infections are more likely to reflect the typical situation in nature, a percentage change in body weight is best calculated from experimental studies involving daily doses of small numbers of larvae. The calculation can be further simplified by estimating the influence of a single parasite species on body weight gain. Assessing the influence of mixed species infections on sheep productivity would be problematic, especially since concurrent infections are not thought to have strictly additive effects (Steel, 1978; Leng, 1981; Steel *et al.*, 1982).

Data collected during an 18-month epidemiological survey of parasite infections in sheep from all around Scotland, carried out between 1988 and 1990, were analysed and the results are presented in detail in Chapter 3. Evidence for strongylid (predominantly trichostrongyle) nematode infections (excluding *Nematodirus* spp.) was detected in 51.7% of

the 511 sheep investigated. Since these were by far the most common gastrointestinal parasites found, and since the detrimental effects of trichostrongyle infections on sheep productivity are well established, the possible consequences of infection with a single trichostrongyle species will be examined. In the survey, infections with individual strongylid species could not be quantified owing to difficulty with the discrimination of strongylid ova. However, larval cultures revealed that *Trichostrongylus* sp. was one of the most frequently occurring strongylid nematodes. In the following calculation, *T. colubriformis* has been assumed to represent any strongylid infection.

Sykes & Coop (1976) have shown that chronic infections with *T. colubriformis* (2500 larvae/day) may substantially depress body weight gain. At the end of the 13-week experiment, the body weight of the *ad libitum*-fed infected sheep was 79.1% of the *ad libitum*-fed controls. In order to express this as an annual figure, it must be assumed that, outside the experimental period, weight gain in the infected sheep is similar to that in the control sheep. Therefore, in one year, the weight of sheep infected with *T. colubriformis* may be expected to be reduced by

$$(100 - 79.1) \times \frac{13}{52} \%$$

$$= 5.2 \%.$$

The amount of clean sheep meat produced in Scotland in 1989 was reckoned as 63 800 tonnes (1 tonne = 1000kg), and was valued at 282.89p/kg (D.A.F.S., 1989). The percentage of this output which can be taken as having been affected by strongylid infections will be assumed to be equivalent to the estimated percentage of all Scottish sheep which were infected with strongylid nematodes in 1989. The survey described in Chapter 3 predicts an overall prevalence value of 51.7%, so that,

$$\frac{51.7}{100} \times 63\,800$$

= 32 985 tonnes of clean sheep meat produced in 1989 were influenced by strongylid infections, whereas 30 815 (63 800 - 32 985) tonnes were not affected by these

infections.

If chronic infection with *T. colubriformis* reduces body weight by 5.2% per annum (i.e. the weight of infected sheep is 94.8% of that of uninfected controls), and assuming that body weight is a reasonable predictor of meat production, the potential meat output in 1989 can be estimated as 30 185 + (32 985/0.948) = 64 979 tonnes. This suggests that, in 1989, perhaps as much as 1179 (64 979 - 63 800) tonnes of clean meat, with a calculated value of £3,335,273, may have been 'lost' as a consequence of strongylid infections. This is equivalent to an overall reduction in meat production of about 1.8%.

This calculation is intended to give an indication of the possible magnitude of production losses in sheep associated with gastrointestinal helminth infections; it must be noted:

(1) Data on *T. colubriformis* infections were taken to represent all strongylid infections.

(2) In nature, the number of worms per host in a population is not uniform (Anderson & Gordon, 1982). In this calculation, data from an experimental study in which sheep received equal doses of larvae (Sykes & Coop, 1976) were used.

(3) Weight loss per annum was calculated from a 13-week experiment (Sykes & Coop, 1976), and was assumed not to have occurred outside the 13-week period.

(4) Live body weight was assumed to be related to the amount of meat produced.

(5) The influence of strongylid infections on sheep wool output, skeletal growth, reproduction and lactation (Symons, 1989) were not included.

(6) The effects of other gastrointestinal infections which are known to occur in Scottish sheep (see Chapter 3), and which are also likely to have some influence on sheep productivity, were not assessed. This is especially pertinent since concurrent infections are believed to exacerbate the effects of single infections (Steel *et al.*, 1982).

Detailed studies, which specifically investigate the effects of gastrointestinal infections on sheep productivity in Scotland, will be necessary before any accurate predictions can be made. However, despite the problems, the crude calculation of the loss of sheep meat production associated with strongylid nematode infections is clearly useful, as

it helps evaluate the real and potential economic significance of gastrointestinal parasitism of sheep in Scotland.

## 2.7 SUMMARY

A review of the literature revealed that parasites of the gastrointestinal tract, in particular the trichostrongyle nematodes and the digenean fluke, *Fasciola hepatica*, may be responsible for substantial loss of productivity in sheep. Infections with the trichostrongyles have been shown to be associated with a decrease in the rate of body weight gain, reduced skeletal growth, poor wool quality and production, and impaired lactation. A reduction in live-weight gain, wool growth and fertility in sheep has also been detected following infection with *F. hepatica*. Such losses in animal performance have been linked to a combination of factors: depressed voluntary food intake, impaired digestion, decreased nutrient absorption, less efficient nutrient utilization and gastroenteric leakage. The effects of concurrent infections have been found to be greater than expected from the sum of single species infections.

Data on reduced productivity from experimental infections, together with information on natural helminth infections of sheep in Scotland and annual figures from the Scottish sheep meat industry, were used to obtain a rough estimate of the economic significance of gastrointestinal parasitism of sheep in Scotland. Although several assumptions were involved in the calculation, an overall annual reduction in meat production of 1.8%, at a value in 1989 of over £3.3 million, was estimated.

# PART 1

# CHAPTER 3: AN EPIDEMIOLOGICAL SURVEY OF HELMINTH INFECTIONS IN SCOTTISH SHEEP 1988-1990

## 3.1 INTRODUCTION

The nature and distribution of helminth infections of sheep, and their economic significance, were discussed in Chapters 1 and 2. The present study was designed (i) to update and revise the work of earlier surveys regarding the parasitic status of sheep in Scotland, and (ii) to investigate the data collected for possible relationships between the different helminths identified. Specifically, data were examined for patterns in the structure of the helminth communities associated with season, sheep age and sex, as well as the combination of coexisting species. The study was principally concerned with helminth infections associated with the gastrointestinal tract.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Survey design

The survey was based at the Glasgow abattoir where large numbers of sheep from all over Scotland are regularly concentrated. Use of the abattoir allowed sheep from a variety of sources to be investigated whilst simplifying the sampling procedure.

Visits to the abattoir were conducted every three months from October 1988 until April 1990 and a maximum of 100 sheep were sampled at each visit (N = 511). The approximate age, sex and origin of each sheep were recorded. Sheep origin was defined as the catchment area of the market at which sheep were sold (Fig. 3.1). In this study, the majority of sheep were Scottish Blackface and were clearly of Lowland origin although on one occasion, samples were collected from Highland sheep to which no market could be assigned. Total monthly rainfall (mm) and mean monthly temperature (°C) for each market catchment area over the appropriate period were obtained from the Meteorological Office in Edinburgh. Latitude and longitude coordinates were also recorded for each market.

### 3.2.2 Diagnostic procedures

Parasite status was assessed by examining sheep faeces for the presence of cysts, eggs and larvae, and by inspecting livers within the carcass for further evidence of parasitic

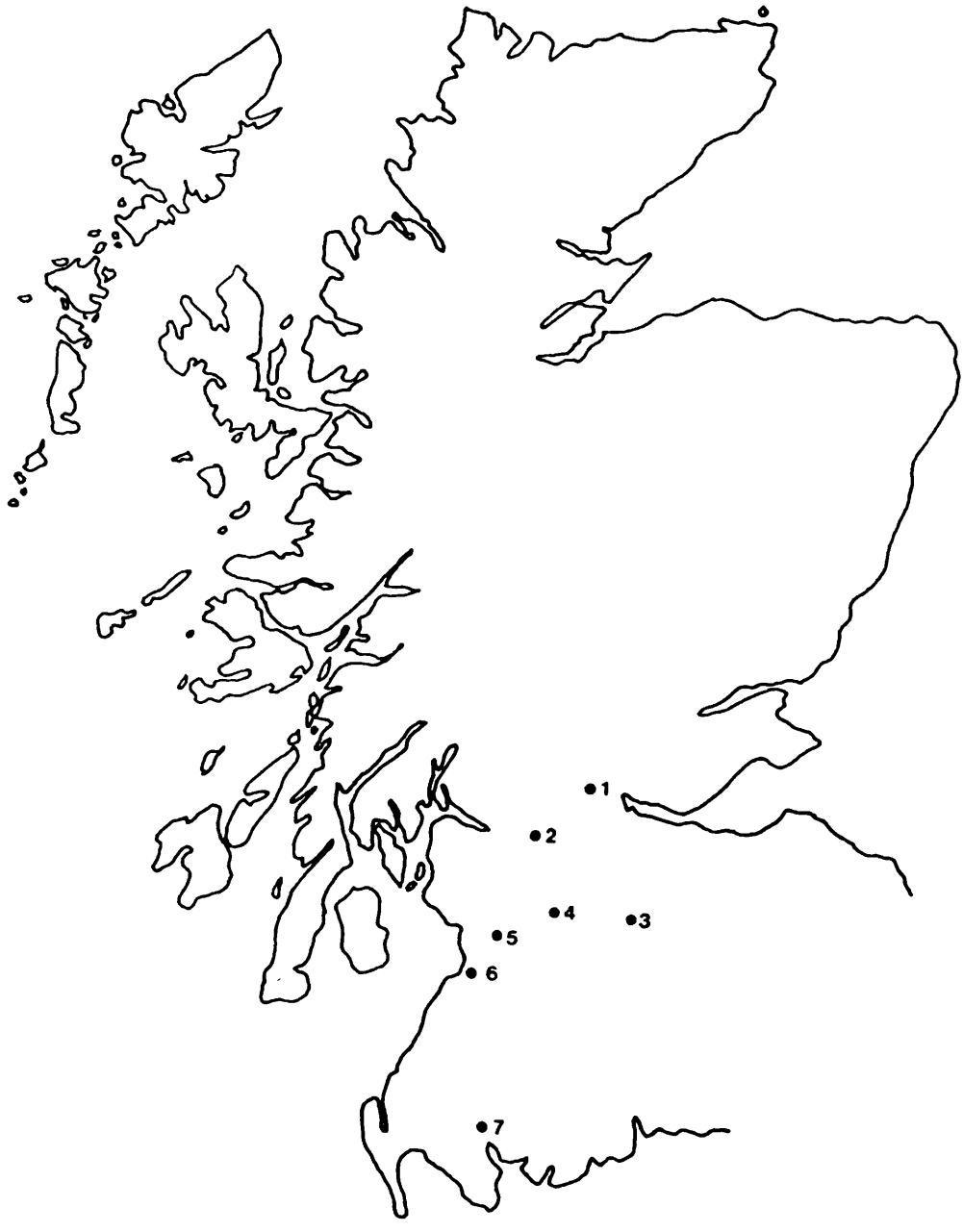



**Fig. 3.1.** Auction markets supplying the Glasgow abattoir which were involved in the present study. 1. Stirling 2. Lennoxtown 3. Lanark 4. Strathaven 5. Kilmarnock 6. Ayr 7. Newton Stewart

infection. Faecal samples of between 5 and 10 g were collected directly from the rectum of the slaughtered animals into 75 ml plastic pots, and were then fixed in 10 % formaldehyde solution until required.

Stool samples were processed in the laboratory by a version of the ether concentration technique (Allen & Ridley, 1970). Each fixed sample was diluted with tap water then macerated with a hand blender until a uniform, aqueous consistency was obtained. The mixture was filtered through two layers of double thickness cotton gauze, and the filtrate transferred to a pre-weighed 10 ml glass centrifuge tube and centrifuged at 2000 r.p.m. for 2 min. The supernatant fluid was discarded and the process repeated twice, although it was centrifuged at 3500 r.p.m. for 5 min on the final occasion. The tube and pellet were inverted for 1 min to drain excess fluid, and weighed. The sediment was resuspended in 6 ml of tap water and thoroughly mixed. Three ml of diethyl ether were added, and the tube stoppered and shaken for about 30 seconds. The stopper was removed and the tube immediately centrifuged at 2000 r.p.m. for approximately 2 min, following which four distinct layers were formed: an upper ether layer containing ether soluble material, a fatty detritus layer, an aqueous layer, and a pellet at the bottom. The detritus layer was dislodged using a wooden spill and the tube quickly inverted to discard the ether, detritus and aqueous layers. The pellet was resuspended in a few drops of water, and one drop of the mixture was pipetted onto a glass slide and examined at x10 magnification. The total number of drops in the suspension was determined and the approximate number of helminth eggs or protozoan oocysts per gram of (sieved) faeces (e.p.g.) calculated, following Hall (1981).

Difficulty in the discrimination of certain strongylid eggs was inevitable, so all strongylid eggs detected were treated as one category for the analyses unless otherwise stated. Eggs in several faecal samples, however, were cultured at 27°C to larval stages (Bairden, pers. comm.) which enabled identification to genus and so gave an indication of commonly occurring strongylids. Oocysts of the protozoon, *Eimeria* sp., were also counted,

but samples were not analysed for the presence of other protozoa as this would have required the use of specific techniques.

### 3.2.3 Data analysis

For certain analyses, parasite data were divided into batches which related to sheep of the same age, source and breed, and which had been slaughtered on the same day. The total number of batches was 16 and the number of sheep per batch ranged from 12 to 80 (mean = 31.9 ± 4.4). (In the text, a sheep batch is to be distinguished from a sheep lot: The latter refers to a group of sheep of mixed ages, but which were auctioned together at the same market). For the investigation of seasonal effects on parasitism, data from all sheep were divided according to the month of slaughter only, i.e. January, April, July and October.

Fisher's exact probability test was used to investigate age and sex effects on the numbers of sheep infected with each parasite species, and to identify possible associations between pairs of parasite species. Prevalence of each parasite species over the different seasons was compared using a Chi-squared test. For investigations concerning parasite fecundity, all egg and oocyst counts were log transformed ($\log_{10}$ [e.p.g. + 1]) in order to minimise overdispersion effects, and thus to allow the use of parametric statistics. A one-way analysis of variance (ANOVA) was used to compare mean egg counts between sheep from different seasons and, when appropriate, significance located using a Tukey test. Egg counts were compared between sheep of different age and sex with the use of a student's t-test. Data from infected sheep only were used. Associations between egg count intensities of the different parasite species, and the way in which these events may be influenced by season and geography, were examined by a stepwise multiple regression procedure on the complete data set. In all analyses, the significance level was taken as $p < 0.05$. Further details of the data analysis are described in the relevant results sections.

## 3.3 RESULTS AND STATISTICAL ANALYSES

### 3.3.1 Parasite Prevalence

#### 3.3.1.1 *Overall prevalence of parasite species in sheep*

Overall prevalences of the species of parasite detected in the sheep are illustrated in Fig. 3.2. Strongylid nematodes were most notable with almost 55% of sheep harbouring patent infections. These include *Nematodirus battus* (17%) and one or both of *N. filicollis* and *N. spathiger* (4%). Larval cultures revealed that *Trichostrongylus* spp., *Ostertagia* spp. and *Oesophagostomum venulosum* were also prominent. Eggs of the enoplid nematodes, *Trichuris ovis* and *Capillaria longipes*, were discovered in 34% and 14% of samples, respectively. Fifteen percent of samples exhibited eggs of the rhabditid nematode, *Strongyloides papillosus*. Other helminths of importance were *Fasciola hepatica* (Digenea) and *Moniezia expansa* (Cestoda). In only one sheep, however, were eggs of the digenean, *Dicrocoelium dendriticum*, present. Cysticerci of *Taenia hydatigena* (Cestoda) were evident in less than one per cent of livers examined. Oocysts of the protozoon, *Eimeria* sp., were present in about 20% of samples.

The occurrence of the species of parasite discovered in batches of sheep is illustrated in Fig. 3.3. Strongylid nematodes were, again, most notable. Although most species could not be distinguished quantitatively, *N. battus* infections were identified in 12 of the 16 batches and *N. filicollis/N. spathiger* in eight of them. All batches of sheep were parasitized by other species of strongylid nematodes. Most batches also included *S. papillosus*, *T. ovis*, *C. longipes*, *F. hepatica*, *M. expansa* and *Eimeria* spp. infections. *Dicrocoelium dendriticum* and *T. hydatigena* infections were detected in only one and two batches, respectively. These data have been summarized in Table 3.1.

#### 3.3.1.2 *Effect of season on parasite prevalence*

The percentages of sheep infected with the different parasites in January, April, July and October are illustrated in Fig. 3.4. Seasonal differences were remarkably similar for most parasites, being lowest in January and April, rising considerably in July, then dropping

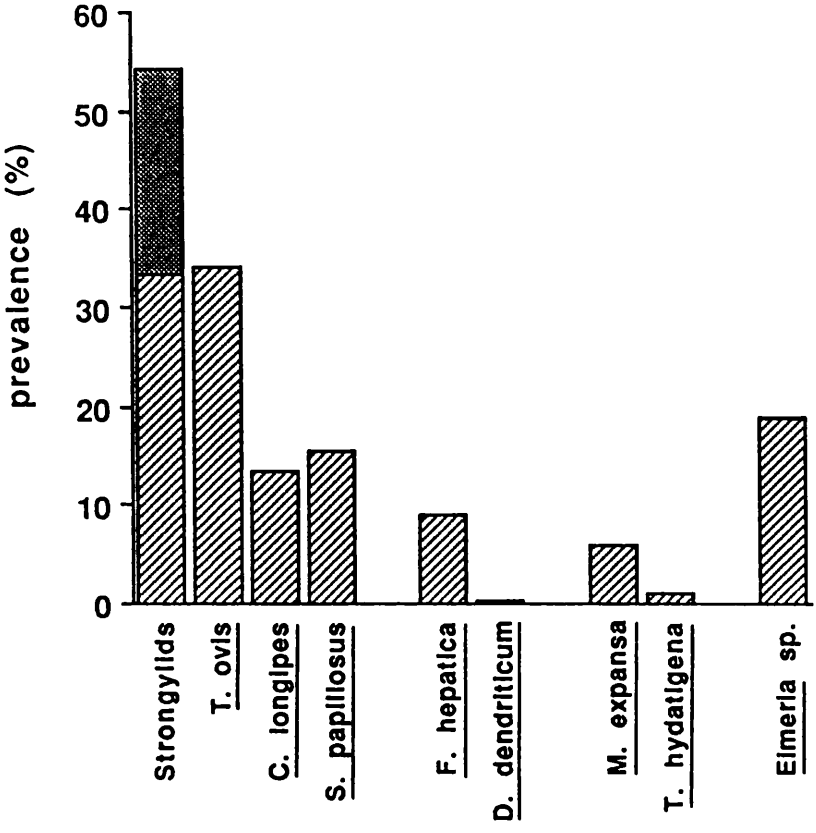



**Fig. 3.2.** Overall prevalences of the species of parasite detected in sheep from around Scotland. Prevalence was determined by the detection of parasite eggs and oocysts in the faeces. Dark shading represents those sheep releasing strongylid eggs of the genus *Nematodirus*. N = 511.

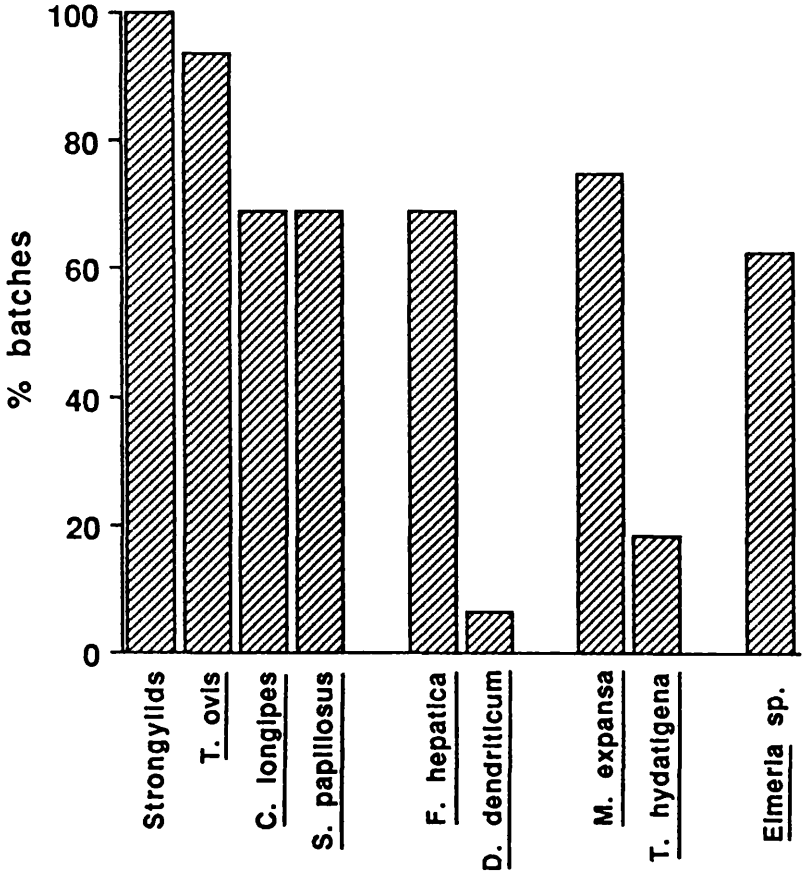



Fig. 3.3. The occurrence of the species of parasite discovered in batches of sheep from around Scotland. Each batch relates to sheep of the same age, source and breed, and which were slaughtered on the same day. N = 16.

**Table 3.1.** Overall prevalence, and occurrence in batches, of parasite species in sheep from around Scotland[a]

| | Overall prevalence (%)[b] | Occurrence in batches (%)[c] | |
|---|---|---|---|
| Strongylids (excl. *Nematodirus* spp.)[d] | 51.7 | 100.0 | Nematoda |
| *Nematodirus battus* | 17.0 | 75.0 | |
| *N. filicollis/spathiger* | 4.1 | 50.0 | |
| *Trichuris ovis* | 34.1 | 93.8 | |
| *Capillaria longipes* | 13.5 | 68.8 | |
| *Strongyloides papillosus* | 15.5 | 68.8 | |
| *Fasciola hepatica* | 9.0 | 68.8 | Digenea |
| *Dicrocoelium dendriticum* | 0.2 | 6.3 | |
| *Moniezia expansa* | 6.1 | 75.0 | Eucestoda |
| *Taenia hydatigena* | 0.9 | 18.2 | |
| *Eimeria* sp. | 18.8 | 62.5 | Sporozoa |

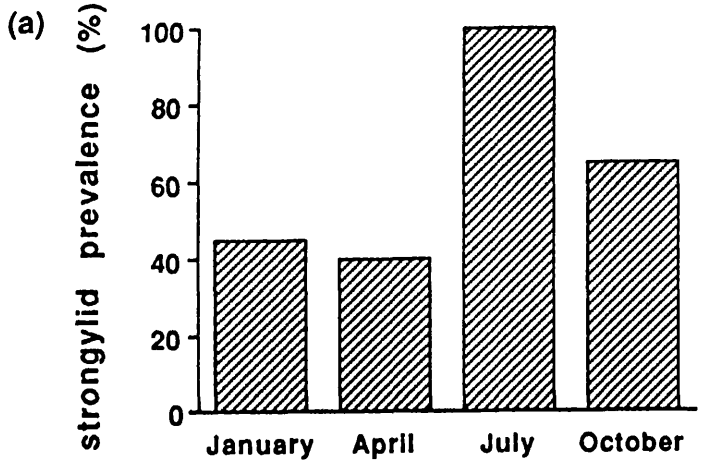
[a] Prevalence was determined by the detection of parasite eggs and cysts in the faeces except *Taenia hydatigena* which was detected by liver examination.

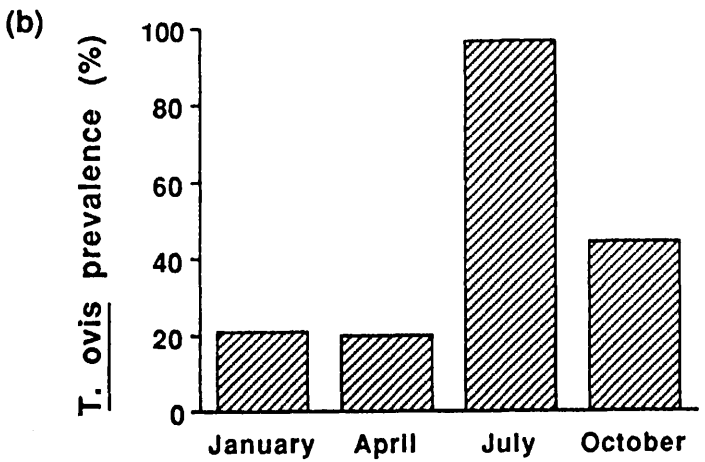
[b] N=511 except for *Taenia hydatigena* where N=321.

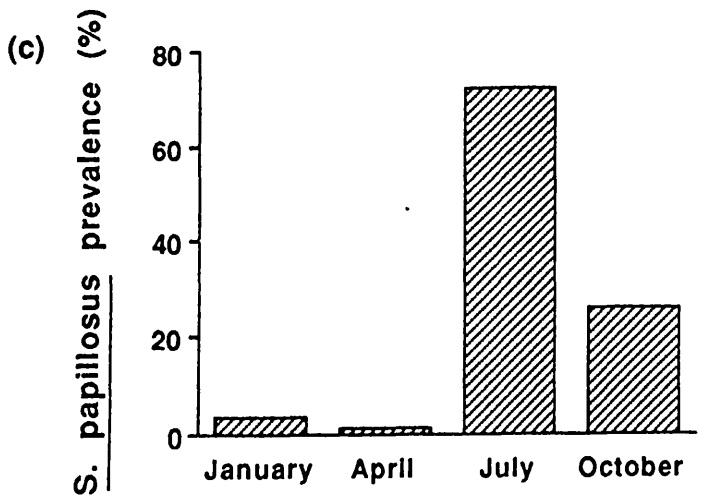
[c] N=16 except for *Taenia hydatigena* where N=11.

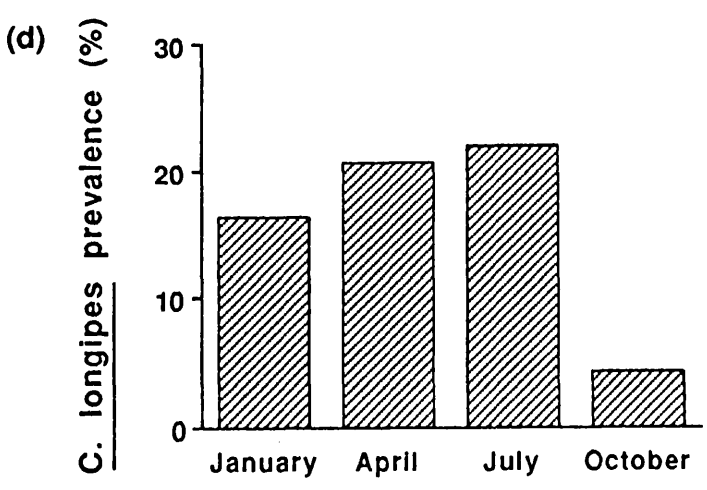
[d] Of the strongylid nematodes, only *Nematodirus* spp. ova could be distinguished. *Nematodirus* spp. ova could be further subdivided into two categories of i) *N. battus*, and ii) *N. filicollis* or *N. spathiger*. Larval cultures revealed that *Trichostrongylus* spp., *Ostertagia* spp. and *Oesophagostomum venulosum* were most prominent amongst remaining strongylids.

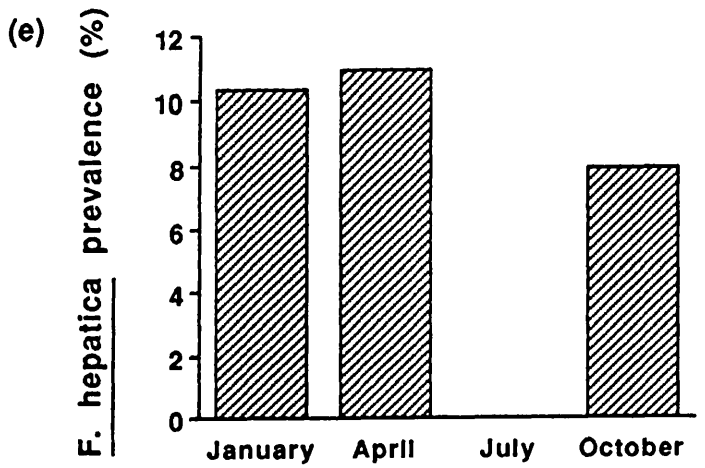
**Fig. 3.4.** The influence of season on parasite prevalence. Prevalence was determined by the detection of parasite eggs and oocysts in the faeces. Data from all sheep were divided according to the month of slaughter only i.e. January (N = 135), April (N = 155), July (N = 32) and October (N = 189) and are presented as percentage values. Actual numbers were compared between months using a Chi-squared analysis. (a) strongylid nematodes, $X^2$ = 53.09, d.f.= 3, $p < 0.00005$; (b) *T. ovis*, $X^2$ = 89.61, d.f.= 3, $p < 0.00005$; (c) *S. papillosus*, $X^2$ = 131.85, d.f.= 3, $p < 0.00005$; (d) *C. longipes*, $X^2$ = 23.50, d.f.= 3, $p < 0.00005$; (e) *F. hepatica*, $X^2$ = 4.47, d.f.= 3, $p > 0.05$; (f) *M. expansa*, $X^2$ = 18.71, d.f. = 3, $p < 0.0005$; (g) *Eimeria* sp., $X^2$ 96.28, d.f.= 3, $p < 0.00005$.

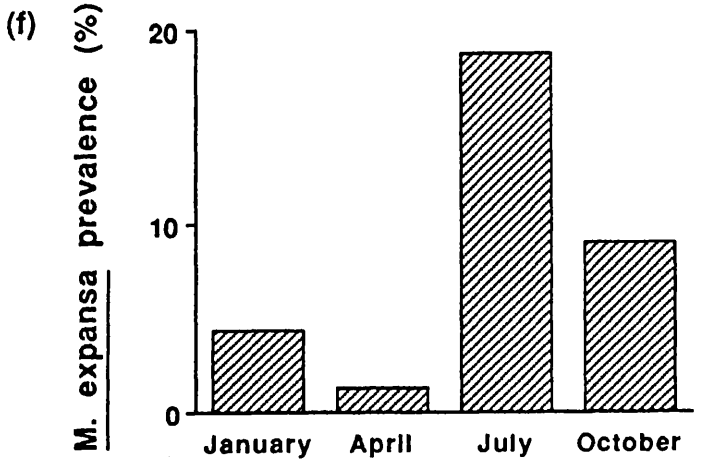

(a)
strongylid prevalence (%)
100
80
60
40
20
0
January
April
July
October

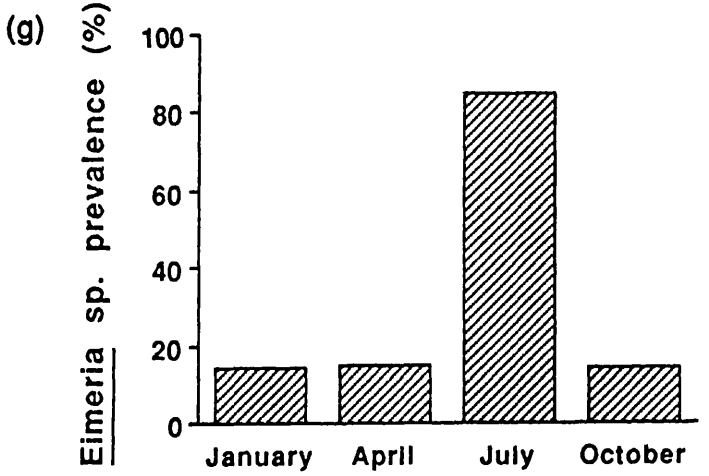

(b)
T. ovis prevalence (%)
100
80
60
40
20
0
January
April
July
October


(c)
S. papillosus prevalence (%)
80
60
40
20
0
January
April
July
October


(d)
C. longipes prevalence (%)
30
20
10
0
January
April
July
October


(e)
F. hepatica prevalence (%)
12
10
8
6
4
2
0
January
April
July
October


(f)
M. expansa prevalence (%)
20
10
0
January
April
July
October


(g)
Eimeria sp. prevalence (%)
100
80
60
40
20
0
January
April
July
October

slightly in October. This pattern was observed for the pooled strongylid species, and for *T. ovis*, *S. papillosus* and *M. expansa*, but in the case of *Eimeria* sp., prevalence in October was as low as in January and April. Chi-squared analyses using the actual numbers of infected and uninfected sheep revealed these differences to be highly significant ($X^2$ = 53.09, d.f.= 3, $p < 0.00005$; $X^2$ = 89.61, d.f.= 3, $p < 0.00005$; $X^2$ = 131.85, d.f.= 3, $p < 0.00005$; $X^2$ = 18.71, d.f.= 3, $p < 0.0005$; $X^2$ = 96.28, d.f.= 3, $p < 0.00005$; respectively). *Capillaria longipes* exhibited a quite different pattern of seasonal prevalence. In January, and in April and July particularly, prevalence of this helminth species was relatively high, but had decreased significantly by October ($X^2$ = 23.50, d.f.= 3, $p < 0.00005$). The prevalence of *F. hepatica* was not found to be significantly different between seasons despite being absent from those sheep slaughtered in July ($X^2$ = 4.47, d.f.= 3, $p > 0.05$).

3.3.1.3 *Effect of sheep sex on parasite prevalence*

The numbers of male and female sheep infected by each parasite species were compared for each batch of sheep. In no batch was sex found to influence the prevalence of any parasite species (Fisher's exact probability > 0.05 in each case).

3.3.1.4 *Effect of sheep age on parasite prevalence*

The effect of sheep age on the prevalence of parasite species was examined. Data from sheep which had been slaughtered on the same day and were of the same breed and market origin, were divided into a group of those < 1 year old and a group of those ≥ 1 year old. This was possible for only two lots of sheep, which were from Ayr and Lennoxtown markets.

Although the actual numbers of sheep were compared in the analyses, percentage values have been illustrated owing to differing sample sizes (Fig. 3.5). Sheep of less than one year old appeared to harbour the majority of the infections, with the older age class often without any infections at all. These differences were, however, only found to be significant for certain helminth species in sheep from Ayr market (Fisher's exact probability test;

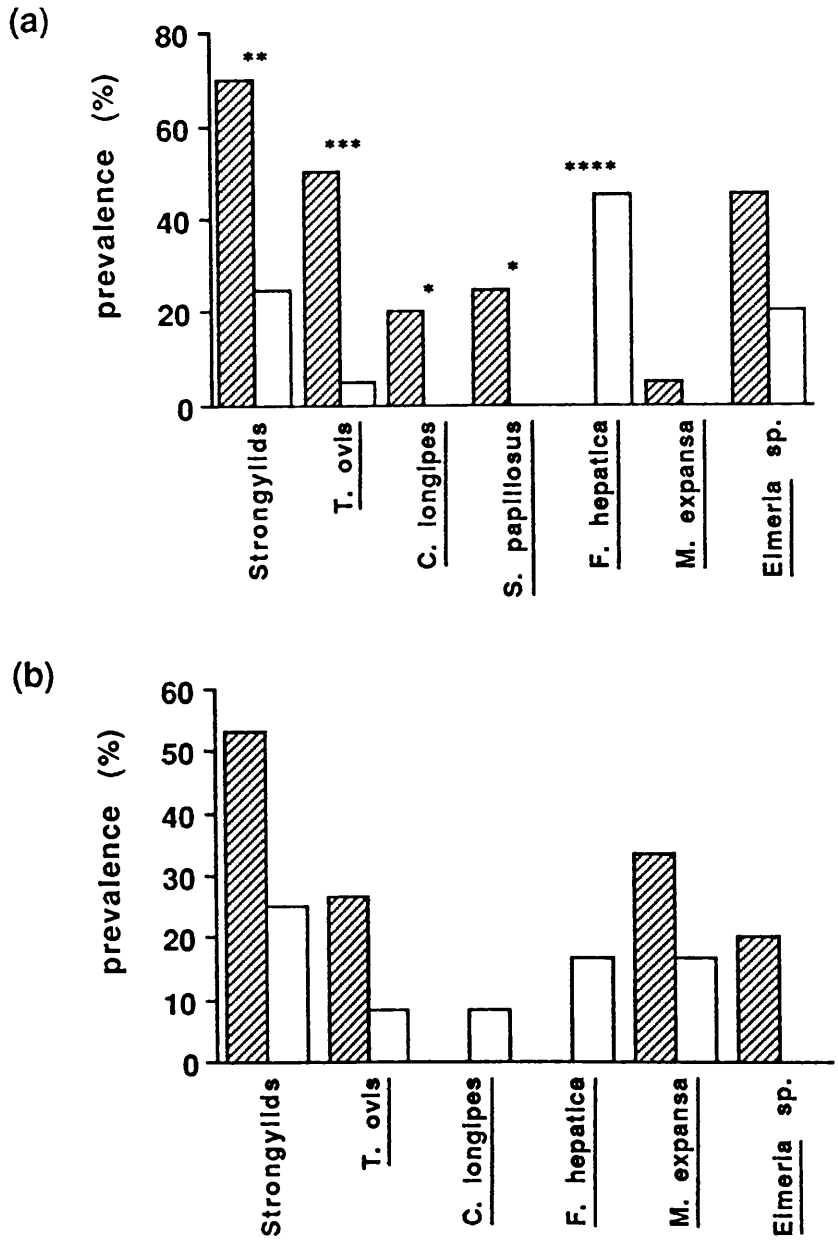



Fig. 3.5. Effect of sheep age on parasite prevalence in sheep from (a) Ayr market, and (b) Lennoxtown market. (▨) < 1 year old; (□) ≥ 1 year old. Percentage values illustrated. Actual numbers compared using Fisher's exact probability test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

pooled strongylid species, p = 0.005; *T. ovis*, p = 0.002; *S. papillosus*, p = 0.024). In all other cases the exact probability was > 0.05 (Fisher's exact test). One noticeable exception to this pattern concerned the prevalence of *Fasciola hepatica* for the two age classes. *Fasciola hepatica* ova were detected in the older age classes of sheep only, although, again, this discrepancy was only found to be significant for sheep from Ayr market (Fisher's exact probability; p = 0.001).

### 3.3.2 Parasite egg/oocyst intensity

#### 3.3.2.1 *Effect of season on parasite egg/oocyst counts*

Mean egg/oocyst counts per sheep of the different parasite species in January, April, July and October are illustrated with standard error bars in Fig. 3.6. Egg counts did not differ significantly by season for most helminth species (One-way ANOVA; *S. papillosus*, $F_{3,75}= 1.89$; *C. longipes*, $F_{3,65}= 1.35$; *F. hepatica*, $F_{2,43}= 0.42$; *M. expansa*, $F_{3,27}= 0.79$; $p > 0.05$ in each case). Strongylid egg counts from sheep killed in July were found to be significantly greater than those from sheep killed in October, which were, in turn, significantly greater than those from sheep killed in January and April (One-way ANOVA followed by Tukey test; $F_{3,252}= 35.94$, $p < 0.0005$). A similar trend was detected for *T. ovis* egg output, although in this case, egg counts did not differ significantly between sheep slaughtered in January, April and October, but were significantly higher in July (One-way ANOVA followed by Tukey test; $F_{3,170}= 15.72$, $p < 0.0005$). *Eimeria* sp. oocyst intensity was significantly higher in sheep killed in July compared with that of those killed in April and October (One-way ANOVA followed by Tukey test; $F_{3,101}= 4.28$, $p < 0.01$).

#### 3.3.2.2 *Effect of sheep sex on parasite egg/oocyst counts*

Sheep sex effects on the egg counts of the parasitic infections ($\log_{10}$ [e.p.g. + 1]) were examined. Mean counts from infected male and female sheep were compared for each batch and were not found to be significantly different for any species of parasite in any batch

**Fig. 3.6.** The influence of season on parasite egg and oocyst counts. Mean egg/oocyst counts ± S.E. of the parasite species in January, April, July and October are presented. Mean counts were calculated from log transformed egg counts ($\log_{10}$ (epg + 1)) of infected sheep only, and were compared using a one-way ANOVA followed by a Tukey test. (a) strongylid nematodes, egg counts in July > egg counts in October > egg counts in January and April, $F_{3,252} = 35.94$, $p < 0.0005$; (b) *T. ovis*, egg counts in July > egg counts in January, April and October, $F_{3,170} = 15.72$, $p < 0.0005$; (c) *S. papillosus*, $F_{3,75} = 1.89$, $p > 0.05$; (d) *C. longipes*, $F_{3,65} = 1.35$, $p > 0.05$; (e) *F. hepatica*, $F_{2,43} = 0.42$, $p > 0.05$; (f) *M. expansa*, $F_{3,27} = 0.79$, $p > 0.05$; (g) *Eimeria* sp., egg counts in July > egg counts in April and October, $F_{3,101} = 4.28$, $p < 0.01$.

(a)
mean strongylid egg count
($\log_{10}$(epg + 1)) ± S.E.
0
1
2
3
4
January
April
July
October
(b)
mean T. ovis egg count
($\log_{10}$(epg + 1)) ± S.E.
0
1
2
3
January
April
July
October
(c)
mean S. papillosus egg count
($\log_{10}$(epg + 1)) ± S.E.
0
1
2
3
January
April
July
October
(d)
mean C. longipes egg count
($\log_{10}$(epg + 1)) ± S.E.
0
1
2
January
April
July
October

(e)
mean F. hepatica egg count
(log10(epg + 1)) ± S.E.
3
2
1
0
January
April
July
October
(f)
mean M. expansa egg count
(log10(epg + 1)) ± S.E.
4
3
2
1
0
January
April
July
October
(g)
mean Eimeria sp. oocyst count
(log10(epg + 1)) ± S.E.
3
2
1
0
January
April
July
October

of sheep (Student's t-test; $p > 0.05$ in each case).

3.3.2.3 *Effect of sheep age on parasite egg/oocyst counts*

Sheep age effects on the egg and oocyst counts of the parasitic infections were examined. Using data from those sheep from Ayr and Lennoxtown markets (see section 3.3.1.3), where the effect of sheep age on parasite prevalence was investigated, mean log egg and oocyst counts of each parasite species in sheep < 1 year old and those ≥ 1 year old, were compared. This investigation was clearly limited, as many of the older sheep from these particular lots were found not to be infected with many of the parasite species. When the sample size was large enough to permit statistical analysis, however, no significant difference between the two age classes was discovered (Student's t-test; $p > 0.05$ in each case).

**3.3.3 Polyparasitism**

3.3.3.1 *Frequency distribution*

The detection of the high prevalences of such a variety of parasite species provided evidence for the common occurrence of mixed species infections. The frequency distribution of parasite infections is illustrated in Fig. 3.7. Almost half of the sheep investigated harboured more than one species of parasite; this is an underestimate of the number of species infecting each sheep as the species of strongylid nematodes were combined.

3.3.3.2 *Prevalence associations*

To investigate whether parasite species were aggregated according to sheep batch, the batches were examined for pairs of parasites which regularly occurred together by constructing a contingency table of parasite presence-absence data as follows:

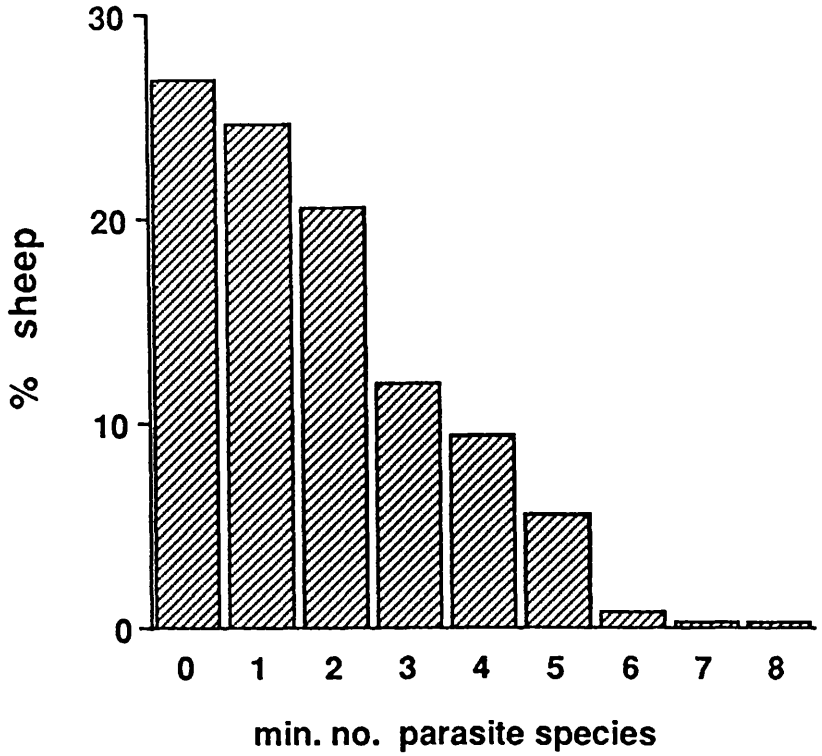



Fig. 3.7. Frequency distribution of parasite infections in 511 sheep from around Scotland. Occurrence was determined by the detection of parasite eggs and oocysts in the faeces. The graph presents an underestimate of the number of parasite species infecting each sheep as the species of strongylid nematodes were combined.

| | | Occurrence of species A in batches (counts) | |
|---|---|---|---|
| | | absent | present |
| Occurrence of species B in batches (counts) | absent | | |
| | present | | |

No pair of parasite species was found to be significantly associated according to sheep batch (Fisher's exact probability test; $p > 0.05$ in each case).

This procedure was repeated for data from sheep of one particular batch, to examine whether certain parasite species were found to coexist within a given sheep more or less frequently than would be expected. Data from sheep belonging to a batch which were greater than one year old, auctioned at Stirling market and slaughtered in April, were chosen because the sample size was relatively large ($n = 80$), and because the overall prevalence of each parasite species was not unduly high (high prevalence of several species would have obscured any associations). The presence of the pooled species of strongylid nematodes was found to be significantly associated with the presence of *Eimeria* sp. (Fisher's exact probability test; $p = 0.018$). No association was found between any other pair of parasite species (Fisher's exact probability test; $p > 0.05$ in each case). The analysis was not carried out on all batches owing to the difficulty inherent in testing such a large number of pairwise combinations, that is, the possibility of incorrectly rejecting the null hypothesis. Considerable parasite diversity and the large number of batches involved generated this problem.

3.3.3.3 *Identifying parasite egg/oocyst intensity associations using stepwise multiple regression analysis*

As strongylids were the most common parasites, it was pertinent to determine the factors which might influence strongylid egg count intensity. Stepwise multiple regression analysis of strongylid egg count intensity per sheep against 10 predictors (*T. ovis*, *C. longipes*, *S. papillosus*, *F. hepatica*, *M. expansa* and *Eimeria* spp. per sheep egg/oocyst counts ($\log_{10}$ [e.p.g. + 1]), mean monthly temperature, total monthly rainfall, latitude and longitude) was carried out using a Minitab statistical package. The latter four predictors were included to help evaluate the influence of season and geography on strongylid egg counts. Age and sex were not included as predictors as this would have rendered the sample size rather small for the given numbers of predictors (N should be $\geq$ [20 x no. of predictors]; see Tabachnick & Fidell, 1989), and since age- and sex-related differences in egg counts had not been detected in this data set. Only strongylid-infected sheep were included in the analysis in order to limit the bias resulting from aggregations of uninfected sheep.

Overall, three of the variables used in the multiple regression analysis had a significant effect on strongylid egg count intensity. These were *T. ovis* and *S. papillosus* egg counts and total monthly rainfall, and together these explained 31.2% of the variation in strongylid egg counts ($F_{3,198} = 31.4$; $R^2 = 0.312$; $p < 0.0005$). However, most of this explained variation was attributable to *T. ovis* egg count intensity ($R^2 = 0.249$ (24.9%); $p < 0.0005$), with *S. papillosus* and rainfall together explaining about 6% ($p < 0.001$ and $p < 0.05$, respectively). These relationships were all positive. No other predictors were found to explain the remaining variation.

It is important to recognize that if certain predictors entered into a stepwise multiple regression analysis are highly correlated with each other, it is likely that only one of these correlated variables would be found to explain variations in the dependent variable. In this way, parameters which had an important influence on strongylid egg count (the dependent variable in the present analysis) may have been obscured. In order to identify relationships

between predictors, a correlation matrix involving all predictors and the dependent variable was constructed using a Minitab statistical package (Table 3.2). Significant positive correlations were in evidence between mean monthly temperature and strongylid, *T. ovis* and *S. papillosus* egg counts, and between longitude and strongylid, *T. ovis* and *S. papillosus* egg counts (Product-moment correlation; see Table 3.2). Since neither temperature nor longitude were found to predict strongylid egg count in the regression analysis, these variables probably explain the same heterogeneity in strongylid egg count intensity as was explained by *T. ovis* and *S. papillosus* egg counts.

3.3.3.4 *Egg count intensity associations between strongylids and T. ovis*

The extent to which the relationship between strongylid and *T. ovis* egg count intensities (see section 3.3.3.3) held in individual batches, was examined. This allowed the effects of age, breed, season and source to be excluded. Single linear regressions of strongylid $\log_{10}$ (e.p.g. + 1) against *T. ovis* $\log_{10}$ (e.p.g. + 1) were carried out for each batch using infected sheep only and where $N \geq 10$ (5 batches). The regression lines have been plotted in Fig. 3.8. In only two of five batches was there a significant relationship detected ($F_{1,17} = 5.67$, $R^2 = 0.21$, $p < 0.05$; $F_{1,9} = 6.43$, $R^2 = 0.35$, $p < 0.05$) with *T. ovis* egg output intensity predicting 21% and 35% of the variation in strongylid egg output, respectively. Both batches comprised Highland lambs of less than one year old which had been slaughtered in October. All five regression equations, however, had positive slopes.

3.3.3.5 *Further investigation of helminth associations using logistic regression analysis*

Since there was such a strong relationship between strongylid, *T. ovis* and *S. papillosus* egg count intensities, an investigation of the effect of strongylid presence on *T. ovis* and *S. papillosus* intensities was undertaken. Although both types of analyses may appear superficially similar, they differed considerably owing to the inclusion of sheep uninfected with strongylids in the latter analysis. Stepwise logistic regression analysis was carried out on strongylid presence-absence data (assigned values of 1 and 0, respectively)

**Table 3.2.** Matrix of product-moment correlation coefficents between all predictors, and between all predictors and the dependent variable, used in the stepwise multiple regression analysis.[a]

| | strongylid egg count (dependent variable) | *T. ovis* egg count | *S. papillosus* egg count | *C. longipes* egg count | *F. hepatica* egg count | *M. expansa* egg count | *Eimeria* sp. egg count | temperature (b) | rainfall (b) | latitude (b) |
|---|---|---|---|---|---|---|---|---|---|---|
| *T. ovis* egg count | 0.459* | | | | | | | | | |
| *S. papillosus* egg count | 0.382* | 0.324* | | | | | | | | |
| *C. longipes* egg count | -0.012 | 0.010 | -0.008 | | | | | | | |
| *F. hepatica* egg count | -0.061 | -0.166 | -0.044 | 0.049 | | | | | | |
| *M. expansa* egg count | 0.190 | 0.088 | 0.105 | 0.115 | -0.002 | | | | | |
| *Eimeria* sp. egg count | 0.157* | 0.286* | 0.147* | 0.261 | -0.073 | -0.071 | | | | |
| temperature (b) | 0.419* | 0.543* | 0.527* | 0.158 | -0.142 | 0.105 | 0.323* | | | |
| rainfall (b) | 0.138 | -0.084 | 0.102 | -0.242 | -0.001 | 0.147 | -0.408* | 0.070 | | |
| latitude (b) | -0.130* | -0.050 | -0.128* | 0.122 | -0.061 | 0.025 | 0.153 | -0.308* | -0.332* | |
| longitude (b) | 0.328* | 0.266 | 0.366* | -0.265 | -0.104 | 0.067 | -0.052 | 0.671* | 0.449* | -0.616* |

[a] N = 257 except (b) where N = 202.

* $p < 0.001$

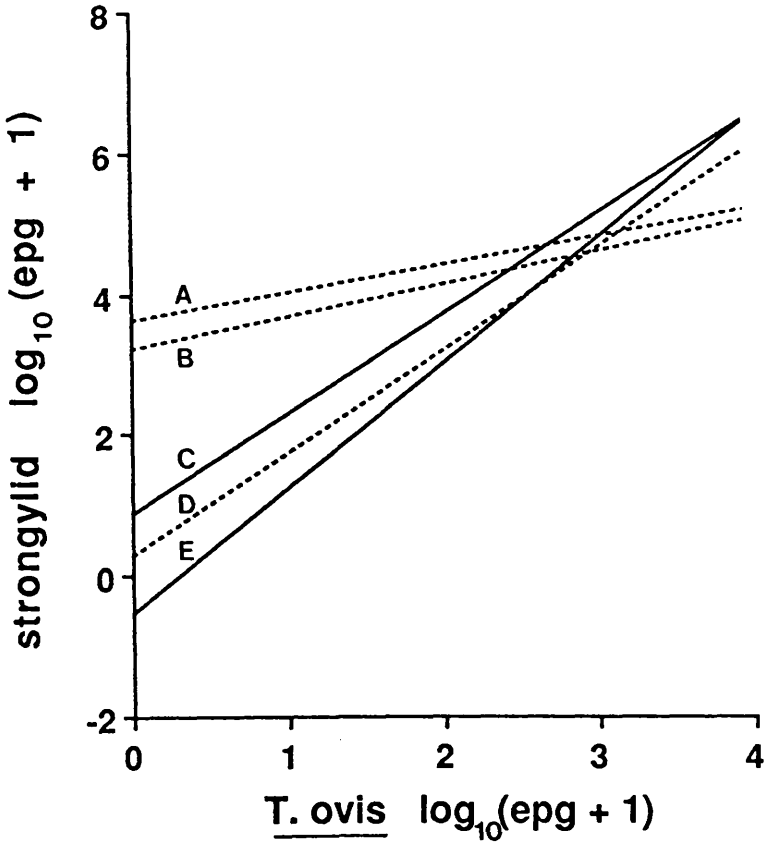



Fig. 3.8. Linear regression lines of strongylid nematode $\log_{10}$ (epg + 1) against *Trichuris ovis* $\log_{10}$ (epg + 1) for 5 separate batches of sheep. Each batch was controlled for age, breed, source and season. A. ≥ 1 year old, Newton Stewart market, October; n = 31, $F_{1,29} = 0.77$, $R^2 = 0.00$, y = 0.39x + 3.62; B. ≥1 year old, Stirling market, July; n = 31, $F_{1,29} = 0.59$, $R^2 = 0.00$, y = 0.45x + 3.21; C. < 1 year old, Highland origin, October; n = 19, $F_{1,17} = 5.67$, $R^2 = 0.21$, y = 1.41x + 0.88; D. ≥ 1 year old, Lanark market, January; n = 10, $F_{1,8} = 1.92$, $R^2 = 0.09$, y = 1.45x + 0.25; E. < 1 year old, Highland origin, October; n = 11, $F_{1,19} = 6.43$, $R^2 = 0.35$, y = 1.76x - 0.54. (——) $p < 0.05$; (----) $p > 0.05$.

against *T. ovis* and *S. papillosus* egg count intensities using a BMDP computer package. The results of this analysis indicated that *T. ovis* egg output was an important predictor of strongylid occurrence ($F_{1,508} = 92.00$, $p < 0.00005$), but that *S. papillosus* egg counts also had a significant independent additional influence ($F_{1,507} = 31.56$, $p < 0.00005$; improvement $X^2 = 36.10$, d.f.= 1, $p < 0.0005$). Both relationships were positive.

### 3.4 DISCUSSION

With respect to the overall prevalence of parasite species in sheep, comparisons of this survey with those conducted previously must be considered with caution, as regional differences may nullify the assumption that any of these surveys are representative of all Scottish sheep. Furthermore, diagnostic procedures varied between surveys. Despite these inconsistencies, some general patterns are apparent and these have been summarized in Table 3.3.

Strongylid nematodes are still common and were detected in all 16 batches of sheep, suggesting that sheep were infected irrespective of their age, origin, and the season of slaughter. Prior to the present work, the most recent study of the gastrointestinal nematodes of Scottish hill sheep found *Ostertagia* and *Trichostrongylus* to be the most commonly occurring strongylid genera (Reid & Armour, 1975). Strongylids which were detected less frequently included *Haemonchus contortus*, *Nematodirus battus*, *N. filicollis*, *Cooperia curticei* and *Oesophagostomum venulosum* (Reid & Armour, 1975). Although the investigative techniques employed in the present study did not allow many strongylid species to be identified and quantified separately, *Nematodirus battus* was shown to persist in three-quarters of the batches examined. The importance of this particular ovine helminth in Scotland may well have increased, as it was not recorded by Cameron during his examination of 700 sheep slaughtered at the Edinburgh abattoir between September 1921 and September 1922 (Cameron, 1923). This is of particular interest as *N. battus* infection can cause considerable morbidity and mortality in lambs (Urquhart, Armour, Duncan, Dunn & Jennings, 1987). Other strongylid infections which are as typical now as when first

**Table 3.3.** A summary of the occurrence of parasite species in sheep from around Scotland (1921-1990)[a]

| Year[b] | 1921-1922 | 1933-1934 | 1935-1939 | 1937-1938 | 1946-1954 | 1972-1973 | 1988-1990 |
|---|---|---|---|---|---|---|---|
| Number sampled | 700 | 33 | 31 | 22 | 591 | 80 | 511 |
| Host status | mixed ages | < 9 months old | mixed ages | fat lambs | mixed ages | ewes > 5 years old | mixed ages |
| Sheep origin | Edinburgh abattoir | Lowland & Highland | Lowland & Highland | E. Aberdeen--shire | Hill sheep throughout Scotland | Dumbarton--shire | Mainly Lowland |
| Strongylids (excl. *Nematodirus* spp.) | +++ | 100.0 | 100.0 | 100.0 | +++ | +++ | 51.7 |
| *Nematodirus battus* | A | 90.9 | 41.9 | 72.7 | +++ | + | 17.0 |
| *N. filicollis/spathiger* | +++ | | | | | | 4.1 |
| *Trichuris ovis* | ++ | 75.8 | 45.2 | 72.7 | 63.5[c] | ++ | 34.1 |
| *Capillaria longipes* | + | A | 45.2 | 90.0 | ≤ 6.0 | — | 13.5 |
| *Strongyloides papillosus* | + | 9.1 | A | A | ++ | A | 15.5 |
| *Fasciola hepatica* | ++ | A | 3.2 | A | ≤ 38.6[d] | — | 9.0 |
| *Dicrocoelium dendriticum* | A | A | A | A | A | — | 0.2 |
| *Moniezia expansa* | ++ | 33.3 | 32.3 | 36.4 | 42.0 | — | 6.1 |
| *Taenia hydatigena* | — | 15.2 | 19.4 | A | ++ | — | 0.9 |
| *Eimeria* sp. | — | — | — | — | — | — | 18.8 |
| Source | Cameron (1923) | Robertson (1935) | Robertson (1939) | Robertson (1940) | Parnell *et al.* (1954) | Reid & Armour (1975) | Present study |

[a] Where possible the number of sheep infected is given as a % prevalence value. +++ is used to denote common throughout the year; ++ found throughout the year; + infrequent; A absent; — no information available.

[b] Period during which survey was conducted.

[c] Number sampled = 262.

[d] Number sampled = 720.

investigated by Cameron (1923), are *Trichostrongylus* spp., *Ostertagia* spp. and *Oesophagostomum venulosum*, although this list is almost certainly incomplete.

*Trichuris ovis* prevalence appears to have declined slightly since surveyed by Parnell *et al.* (1954), but must still be considered significant with 34% of sheep and 94% of batches being affected by this ovine helminth. Similarly, a decrease in the numbers of sheep harbouring patent *Fasciola hepatica* and *Moniezia expansa* infections is apparent. It is of interest, however, that in a substantial number of sheep exhibiting extensive liver damage resulting from *F. hepatica* infestation, no *F. hepatica* eggs were recovered from their faeces. It is possible that sheep are being treated specifically against *F. hepatica* prior to market sale, in an attempt to minimize ostensible disease symptoms (McPhee, pers. comm.). *Dicrocoelium dendriticum* has probably always been rare and more typical of the Western Isles (Cameron, 1931; Parnell *et al.*, 1954) and was observed in only one sheep from Lennoxtown market in the present survey. Since this survey was completed, however, several lots of sheep from the north of Scotland, exhibiting considerable liver damage due to *D. dendriticum* infection, have been slaughtered at the Glasgow abattoir (McPhee, pers. comm.). Quantitative data on *T. hydatigena* prevalence is lacking, but reports by Robertson (1935, 1939), Wilson *et al.* (1953) and Parnell *et al.* (1954) suggest it was previously of greater concern in Scotland.

There is an indication that *Capillaria longipes* and *Strongyloides papillosus* infections are now more frequent than when first reported by Cameron in 1923, and later by Parnell *et al.* in 1954, with the former author rarely detecting either species in a sample of 700 sheep from all over Scotland. In a study based at a Dumbartonshire hill farm between April 1972 and November 1973, *S. papillosus* was not found in any of the sheep investigated (Reid & Armour, 1975). In the current survey, almost 70% of batches contained sheep infected with these helminth species and, overall, about 16 % and 14% of sheep were found to be passing *S. papillosus* and *C. longipes* eggs, respectively.

In general, it appears that, although infection frequencies have fluctuated with a tendency to decline, species diversity of helminths associated with the gastrointestinal tract

of sheep in Scotland has remained largely unchanged for about 70 years. This outcome would, perhaps, not have been anticipated, owing to the strategic administration of anthelmintic treatments, improved animal husbandry and management, and an awareness of the importance of gastrointestinal diseases in domestic livestock, all of which are associated with modern farming.

Sheep age was found to have considerable influence on the prevalence of parasite species in the sheep from Ayr and Lennoxtown markets which were investigated, with the younger age class harbouring the majority of the infections. This result is not altogether surprising as, in the case of the trichostrongyles, young lambs acquire the infections in late summer but are thereafter relatively resistant to reinfection (Soulsby, 1982; Urquhart *et al.*, 1987). Similarly, older sheep are likely to be immune to most other gastrointestinal nematode infections (Parkins & Holmes, 1989) and to *M. expansa* and *Eimeria* spp. (Soulsby, 1982). Consequently, when an infection is eventually lost, for example following anthelmintic treatment, it is not replaced. This results in older sheep exhibiting lower prevalence rates.

In the light of these observations, the failure to detect any discrepancy in egg output between the two age classes may appear incongruous. Certainly, the development of resistance to reinfection with age implies that infection intensities and egg counts will be lighter, as well as prevalence rates being lower, in older sheep. However, the results were inconclusive since the older sheep were frequently found not to be infected with many of the parasite species present.

The exception to the age-prevalence pattern described above concerned the prevalence of *Fasciola hepatica* which was only observed in the older age class of sheep. This is, again, a predictable result. The prepatent period of an *F. hepatica* infection is relatively long, and sheep show little or no resistance to repeated challenge with *F. hepatica* (Boray, 1967). Sheep are thus more likely to show evidence of infection as the duration of exposure increases.

The possible influence of sheep sex on parasite prevalence and egg counts was of interest, but within the limits of this survey was found to have no ostensible effect upon either of these parameters for any of the parasites discovered, at any time of year. This may initially appear rather surprising in some respects, owing to the well-documented rise of parasite egg output in ewes, first observed by Taylor (1935). This increase is confined to lactating ewes (for example, see Connan, 1967; Gibbs, 1967; Jansen, 1967; Arundel & Ford, 1969; O'Sullivan & Donald, 1970) which have been found to harbour larger adult parasite populations (O'Sullivan & Donald, 1970). Impairment of the immunological capacity of the ewe associated with hormonal changes during lactation, may culminate in the resumption of the development of arrested 4th-stage trichostrongyle larvae, as well as the maturation of a higher proportion of newly-ingested larvae, and increased individual worm fecundity (O'Sullivan & Donald, 1970). These events explain the pronounced rise in populations of *Ostertagia* spp. and *Trichostrongylus* spp., mirrored by a comparable increase in the faecal egg counts of these nematodes, which was discovered in a sample of Scottish hill ewes during the month of April (Reid & Armour, 1975). In the current study, the failure to identify sex differences between nematode egg counts of sheep killed in April may reflect the rather low numbers of sheep present in some of the samples being compared or, what is more likely, the negligible proportion of sheep slaughtered commercially which comprise lactating ewes.

Although strongylid egg counts in female sheep were not significantly higher in April, seasonal effects on strongylid egg counts of males and females combined, were apparent. Specifically, strongylid faecal egg output in July and October was considerably greater than in January and April. These observations conform broadly with those of other workers investigating seasonal variations in nematode egg output in Scottish hill lambs (for example, see Morgan, Parnell & Rayski, 1950; Reid & Armour, 1975). Possibly the most important determinant of strongylid worm burdens, and by implication faecal egg counts, is the availability of infective larvae on pasture. The ingestion of overwintered $L_3$ larvae of *Ostertagia* spp. and *Trichostrongylus* spp. by young lambs in spring may become manifest by

a substantial rise in the numbers of strongylid eggs passed during June and July (Urquhart *et al.*, 1987). A second annual peak in the faecal egg output of these strongylid species in lambs may be expected in September and October, following the intake of infective larvae resulting from the contamination of pasture with nematode ova by peri-parturient ewes in April and May (Urquhart *et al.*, 1987). These phenomena could also explain why overall prevalence of strongylid nematodes was highest in July and October, as a greater proportion of lambs would be exposed to infection directly prior to these months.

The availability of infective $L_3$ larvae to sheep may not be the only factor which determines faecal egg output. It now seems probable that the level of arrested larval development, and hence the magnitude of the adult worm population, may also be dictated by certain environmental parameters (Reid & Armour, 1972). Although the mechanisms by which such changes operate are subject to considerable speculation, Reid & Armour (1972) postulate that seasonal effects on endocrine or metabolic functions of the $L_4$, possibly mediated by the host, may account for the increased development of the $L_4$, which starts in autumn and proceeds until January. Alternatively, environmental stimulation of the free-living larval stages prior to ingestion may influence the degree of arrested larval development (Urquhart *et al.*, 1987).

Similar events may have helped orchestrate the pronounced seasonal effects on the faecal egg count of strongylid nematodes detected in the present survey. In a study of sheep infected with species of *Ostertagia*, *Trichostrongylus* and *Nematodirus* carried out by Reid & Armour (1972), the ratio of inhibited 4th stage larvae to adults was found to increase considerably throughout late autumn and winter. One could predict that maturation of the arrested larvae would result in an increase in faecal egg counts in spring and summer. This process may have been of minimal import in the current study, however, since although strongylid egg counts were high in July, they were comparatively low in April.

Seasonal variability in worm prevalence and faecal egg output was not confined to the strongylid nematodes. In fact, with the exception of *F. hepatica*, the prevalence rates of all

other parasite species varied significantly with season. As with the strongylids, prevalence tended to be lowest in January and April, highest in July, and somewhere between the two in October. Faecal egg counts of *T. ovis* were also found to follow a seasonal pattern comparable to those of the strongylid nematodes. It would seem plausible that availability of the infective stages of these parasites had, again, a dominant influence on both the prevalence and intensities of these infections, and that this was a function of certain climatic and host-related factors. Most of these species are likely to exhibit elevated egg or oocyst output in response to the relaxed immunity of the breeding ewe in spring, and the development of the infective stages is likely to be optimal in warm and humid conditions typical of April and May. Transmission to the susceptible host may well be maximal at this time of year, producing a peak of patent infections in summer and autumn.

This is a very generalized interpretation of the results, and does not hold equally well for the individual parasite species. The life cycle of *Moniezia expansa* is also dependent on the prevalence and distribution of the intermediate host, an oribatid mite. Nevertheless, in a study of several pastures in South East Scotland carried out by Rayski (1945), these mites were found to occur all year round, although there was a tendency for their numbers to increase in summer at certain localities. *Capillaria longipes* displayed a quite different seasonal prevalence distribution from that of most other parasite species, being relatively uniform in January, April and July, then falling off rather dramatically in October. Clearly, factors which determine the seasonal distribution of this helminth are more complex. Finally, the prevalence and egg counts of *F. hepatica* were not found to vary significantly with season, despite the noticeable absence of this parasite from sheep culled in July. This latter observation may be an anomaly connected with the relatively small data set collected for the month of July. Seasonal influences on the prevalence and intensity of *F. hepatica* infection were expected. Ollerenshaw (1974) suggests that, in Britain, acute fascioliasis in sheep may be expected to occur from October until the beginning of April following summer infection, or during August, September and October following infection in winter. The timing of these events is dictated by successful development of the eggs on pasture and of

the larval stages in the intermediate snail host, *Lymnaea truncatula*, both of which are temperature-regulated (Ollerenshaw, 1974). It is interesting to note that the observed seasonal prevalence rates of *F. hepatica* resemble those predicted for the incidence of acute disease.

With such a prevalent and diverse assortment of gastrointestinal parasite species, it was hardly surprising to find that mixed species infections were common. What was of particular interest, however, was the discovery that high strongylid egg counts were associated with high *T. ovis* and *S. papillosus* egg counts, and that the presence of strongylids was related to high *T. ovis* and *S. papillosus* egg output. Furthermore, in one particular batch of sheep, concurrent infections with strongylid nematodes and *Eimeria* sp. were found to occur more frequently than would be predicted by chance alone.

Such associations are not unprecedented. Annan, Crompton, Walters & Arnold (1986) reported significant co-occurrences of hookworm, *Trichuris trichiura* and *Ascaris lumbricoides* infections in rural pre-school children in Ghana and similarly, Robertson, Crompton, Walters, Nesheim, Sanjur & Walsh (1989) detected a pronounced association between the occurrence of *A. lumbricoides* and *T. trichiura* in Panamanian school children. Robertson *et al.* (1989) also demonstrated strong correlations between the egg output of these two helminths from the same hosts.

Whether positive associations between the egg output of two or more species of helminth within a host reflect a tendency for high intensity infections of different helminths to occur together, is worthy of consideration. The crux of the argument centres on the relative merits of the faecal egg count as an estimator of worm burden. Certain recent publications concerned with a variety of human, ovine and bovine nematodes have observed strong positive correlations between faecal egg count and intensity of infection (Roberts & Swan, 1981; Bryan & Kerr, 1989; Murrell, Leighton, Boswell & Gasbarre, 1989; Forrester & Scott, 1990), suggesting that egg counts are an adequate estimate of infection intensity when more direct forms of measurement are impracticable. Other workers have detected more

convincing relationships between faecal egg count and total worm weight (Coadwell & Ward, 1982). However, density-dependent constraints on parasite fecundity are considered to be of critical importance by Anderson & Schad (1985), who suggest that egg counts may be used to discriminate between individuals with high and low hookworm burdens, but that more detailed predictions of infection intensity would be unjustified.

Regardless of whether faecal egg counts are worthy estimators of worm burden or not, it is interesting that, as with their egg counts (Robertson *et al.*, 1989), the numbers of *A. lumbricoides* and *T. trichiura* have been shown to be correlated in the human host (Bundy, Cooper, Thompson, Didier & Simmons, 1987). Positive associations between the intensities of certain helminth infections have also been observed in the ovine host. Whitlock, Crofton & Georgi (1972) described positive correlations in the numbers of trichstrongylids, notably *Haemonchus contortus cayugensis* with *Ostertagia* spp., *Cooperia* sp., *Trichostrongylus* spp. and *Nematodirus* spp., and Barger (1984) found comparable responses involving species of *Haemonchus*, *Ostertagia*, *Trichostrongylus* and *Nematodirus* in sheep of similar age and grazing history.

The mechanisms responsible for these types of event are likely to be complex and remain poorly understood. In this study, sheep batch differences, which may reflect variations pertaining to animal husbandry, may explain how high egg counts of strongylids, *T. ovis* and *S. papillosus* occurred together. Flocks of sheep which rarely receive anthelmintic treatment, for example, may be inclined to exhibit higher parasite egg counts overall. Since almost all batches were affected by strongylid and *T. ovis* infections, however, there must be other significant considerations.

Variations in host immunity associated with sheep breed may be reflected in faecal egg counts (Boyce, Courtney & Loggins, 1987). Ovine breed variation in response to infection with *Haemonchus contortus* (Yazwinski, Good, Moncol, Morgan & Linnerud, 1980; Courtney, Parker, McClure & Herd, 1984; Courtney *et al.*, 1985) and *Fasciola hepatica* (Boyce *et al.*, 1987) has been recorded. If all species of helminth in a more 'susceptible' breed of sheep produce relatively more eggs, high egg counts could become aggregated by

sheep breed. Sheep breed differences were, however, unlikely to have had much influence in the present study, as most sheep were Scottish Blackface.

Barger (1984) recognized that seasonal variability may generate such correlations, in that, high intensity infections of all parasite species, which have infective stages with similar developmental requirements, are likely to occur together. The pronounced seasonal distribution of parasite prevalence and egg counts in this study suggests the possible importance of this variable in determining associations between helminth egg counts. This is particularly pertinent when one considers that seasonal egg count patterns were remarkably similar for the pooled strongylid species, *T. ovis* and *S. papillosus*.

The regression analysis revealed that a small proportion of the variation in strongylid egg output could be attributed to fluctuations in total monthly rainfall. Rainfall is an important predictor of the availability of goat trichostrongyle larvae on pasture (Rahman & Collins, 1990), and of the larval development of *Trichostrongylus colubriformis* (Barnes, Dobson, Donald & Waller, 1988) and *Ostertagia circumcincta* (Waller & Thomas, 1978) on pasture. It would, however, be rash to regard the variation in strongylid egg production which was attributable to total monthly rainfall, as being the sum involvement of climatic factors. Data from the correlation matrix (Table 3.2) indicate that strongylid, *T. ovis* and *S. papillosus* egg counts were all highly correlated with mean monthly temperature. Overall, these results suggest that in warmer and wetter seasons of the year, or in warmer and wetter regions of Scotland, *T. ovis*, *S. papillosus* and strongylid nematode infections of sheep exhibit higher faecal egg counts, which may reflect higher intensity infections. If so, this effect is likely to be related to the developmental properties and transmission of the infective stages of these species, which will be favoured in a warm and moist environment, as discussed earlier. It should be remembered, however, that the meteorological and geographical data included in the analysis were clearly only crude estimates of parameters affecting specific batches of sheep.

Nevertheless, within-batch regression equations between strongylid and *T. ovis* egg

production, plotted on the same axes (Fig. 3.8), revealed a tendency for one to increase with the other even when age, breed, season and source variables were excluded. Although it should be emphasized that only two of the five regression equations were significant, it is possible that behavioural, physiological or immunological mechanisms have a part to play in the relationship between strongylid and *T. ovis* egg counts. Similar events may explain why concurrent infections with strongylid nematodes and *Eimeria* sp. were found to occur more frequently than would be expected in the one batch of sheep for which such associations were investigated. A given sheep host, for example, may be predisposed to certain behaviour patterns which render it more vulnerable to acquiring infections. It is known that the grazing behaviour of an individual sheep may vary considerably from the average of the whole flock (Fraser & Broom, 1990). This may be important since strongylid and *T. ovis* infections are transmitted to sheep by the ingestion of infective stages on pasture. However, this does not really explain why the strongylids and *T.ovis* specifically were associated, since all species of parasite detected in the present study are transmitted to sheep in this manner. Heterogeneity in host physiology or resistance to infection may account for certain hosts carrying heavier worm burdens overall (Anderson & May, 1985). This phenomenon is exquisitely demonstrated by the lactating ewe infected with strongylid nematodes, in which the temporary relaxation of immunity, possibly associated with increased levels of prolactin (Connan, 1974), allows the maturation of arrested larvae, the survival of a greater proportion of newly-ingested larvae, and probably induces increased egg production per worm (O'Sullivan & Donald, 1970). The combined effect of these processes is to cause an increase in the burden and faecal egg count of concurrent species of strongylid nematodes. It should be recognized, however, that an association between the strongylids and *T. ovis* may have been easier to detect since they were the most common, or indeed, may have been a function of their high prevalence.

Whatever the causes, animals which consistently pass large numbers of helminth eggs will be important foci for disease transmission and, where this is a reasonable indication of worm burden, will also be the most seriously affected in terms of disease. Furthermore, such

hosts will provide a basis for the regulation of parasite populations by means of density-dependent processes (Anderson & Gordon, 1982).

## 3.5 CONCLUSIONS AND SIGNIFICANCE OF STUDIES

Gastrointestinal helminth infections are an intrinsic feature of sheep farming in Scotland. The abundance and diversity of these parasites appears to have declined only slightly since first investigated by Cameron (1923). Perhaps of particular concern are the extent and character of multiple species infections detected in the present study, which may have serious consequences in terms of sheep productivity, particularly since sheep farming is of considerable economic importance in Scotland. The abundance and nature of helminth infections must surely warrant some concern and the need for a thorough assessment of their influence on sheep productivity in Scotland is evident.

## 3.6 SUMMARY

An 18-month study, based at the Glasgow abattoir, investigated the helminth community associated with the gastrointestinal tract of 511 sheep from around Scotland. Faecal samples were examined for the presence of parasite eggs and oocysts, and livers were monitored for evidence of parasitic infestation. Comparisons with previous studies suggested that parasite diversity has changed little over the last 70 years, although infection frequencies appear to have declined. Sheep sex was not found to have any effect upon prevalence or egg output of any of the parasites discovered, but young sheep (< 1 year old) were shown to harbour the majority of infections in two lots of sheep investigated. The prevalence and egg counts of most species of parasite were found to peak in July, and in October to a lesser extent.

Mixed species infections were common, and stepwise multiple regression analysis was used to study relationships between egg count data of different parasite species, and the effect of season and geography on such associations. The intensity of *Trichuris ovis* faecal egg counts was found to explain about 25% of the variation in strongylid nematode egg counts. *Strongyloides papillosus* egg count intensities were also weakly related to those of the strongylids. In addition, stepwise logistic regression analysis indicated that the presence of strongylids was associated with high *T. ovis* and *S. papillosus* egg counts. These relationships were positive. A further small proportion of the variation in strongylid egg output was attributed to fluctuations in total monthly rainfall, and it is suggested that increased moisture content on pasture may enhance strongylid larval development and thus favour successful transmission to the ovine host. The discovery that *T. ovis*, *S. papillosus* and strongylid egg counts were highly correlated with mean monthly temperature, suggests that development and transmission of the infective stages is also enhanced in a warmer environment. No other predictors could be found to explain the remaining variation. When sheep batches were considered separately, with the exclusion of effects of age, breed, season and source, linear regression analysis revealed a tendency for strongylid nematode egg production to increase with that of *T. ovis*. It is suggested that host-mediated behavioural,

physiological or immunological mechanisms may also, therefore, help to explain this association.

As sheep farming is of considerable economic importance in Scotland, a detailed evaluation of the effects of multiple species infections on sheep productivity, particularly high intensity infections, is desirable.

# PART 2

# CHAPTER 4: EXPERIMENTAL INVESTIGATIONS OF CONCURRENT PARASITIC INFECTIONS IN MAMMALIAN HOSTS

## 4.1 INTRODUCTION

There is a wealth of literature on concurrent infections involving various combinations of parasite species in mammalian hosts. Most studies investigated parasitic infections of laboratory rodents, and often concentrated on possible interactions between the parasites, as well as their influence on the host. The diversity of parasite life histories lends itself to a considerable array of possible interactions between species, since they will depend on migration patterns, sites occupied and pathogenicity of one or more of the infecting agents. For the same reasons, the effect of these infections on the host would be expected to vary with the combination of species harboured. In this chapter, various types of interaction, which have been observed between parasite species in experimental concurrent infections will be reviewed. In addition, certain effects of these infections on the host will be discussed. Throughout this discussion, preference will be given to examples involving infection with either *Fasciola* spp. or *Hymenolepis* spp., whenever possible, since concurrent infections with *F. hepatica* and *H. diminuta* were the subject of the present study.

## 4.2 ANTAGONISTIC INTERACTIONS BETWEEN PARASITE SPECIES

Whereas some antagonistic interactions may result from competition for a limiting resource, or from direct mechanical interference (see Holmes, 1973), most are probably induced by immunological non-specific factors (see Christensen, Nansen, Fagbemi & Monrad, 1987). Antagonistic interactions resulting from immunological cross-reactivity are, on the other hand, most commonly in evidence between closely related species (Christensen *et al.*, 1987), and are usually manifest by the impaired ability of one parasite species to establish within the host in the presence of another. Baboons harbouring mature *Schistosoma haematobium* infections may develop considerable resistance to challenge with *S. mansoni*, expressed in terms of impaired faecal egg counts and reduced worm establishment (Webbe, James, Nelson, Ismail & Shaw, 1979). Evidence for the involvement of cross-reacting antigens stemmed from the cytotoxic properties of sera from baboons infected with *S. haematobium* to schistosomula of *S. mansoni in vitro* (Webbe *et al.*, 1979).

Reduced establishment of *S. mansoni* has been observed in mice previously infected with *S. bovis*, *S. mattheei* or *S. rodhaini* (Nelson, Amin, Saoud & Teesdale, 1968). The apparent necessity for considerable deposits of eggs produced by the primary infection to elicit this resistance, argues in favour of a specific immune response (Christensen *et al.*, 1987), as it is analogous to observations on homologous *S. mansoni* challenge infections in mice (Dean, 1983).

Nevertheless, according to Campbell, Kelly, Townsend & Dineen (1977), cross-reactivity may be associated with the resistance demonstrated in sheep with 12-week *Cysticercus tenuicollis* (=*Taenia hydatigena*) infections when challenged with *F. hepatica*. Despite this, immunisation regimes involving *T. hydatigena* antigen preparations were found to be ineffective at inducing immunity in rats to infection with *F. hepatica* (Rajasekariah, Rickard, Montague & Mitchell, 1979). The observed resistance of sheep (Monrad, Christensen, Nansen & Frandsen, 1981), calves (Sirag, Christensen, Nansen, Monrad & Frandsen, 1981) and Sudanese cattle (Yagi, Younis, Haroun, Gameel, Bushara & Taylor, 1986) harbouring *Schistosoma bovis*, to subsequent administration of *F. hepatica* and *F. gigantica* (in the case of the Sudanese cattle) metacercariae, has not been linked to functional cross-reacting antigens. This is perhaps more surprising since the phylogenetic relationship between *Fasciola* and *Schistosoma* is closer than between *T. hydatigena* and *F. hepatica*. Despite this, mice and hamsters have been shown to acquire immunity to infection with *Schistosoma mansoni* following exposure to adult *F. hepatica* worm antigen (Hillyer, Del Llano de Diaz & Reyes, 1977), and a *Fasciola/Schistosoma* cross-reactive defined immunity antigen was considered to be responsible for the development of immunity in mice to infection with *F. hepatica* (Hillyer, 1985). The role of possible cross-reacting antigens during concurrent infections involving *Fasciola* spp. requires further clarification.

Evidence for antagonistic interactions between helminths mediated by non-specific immune responses is more convincing. The early expulsion of *Hymenolepis diminuta* from mice infected with 70-day-old *S. mansoni* was attributed to a pronounced intestinal

inflammation associated with the release of eggs by the blood fluke (Andreassen, Odaibo & Christensen, 1990a). During such an inflammatory response, an increase in intestinal permeability may result in net movement of complement into the gut lumen, which might account for the destrobilation and premature expulsion of *H. diminuta* (Andreassen *et al.*, 1990b). Inflammation of the gut was thought to be responsible for the poor survival of *H. diminuta* in mice with *Echinostoma caproni* infections of at least 7 days old (Andreassen *et al.*, 1990b).

Similar events have been demonstrated between *Hymenolepis* species and nematodes. Overall *H. diminuta* survival and size was impaired in mice harbouring 7-day old *Ascaris suum* infections at the time of challenge (Bindseil & Andreassen, 1981). The authors suggest that this effect may have been attributable to cellular changes in the alimentary tract following the return of the nematode larvae to the gut from day 4 p.i. onwards. Comparable changes in the cellular composition of the intestine accompanying the rejection of *Trichinella spiralis* from mice, have been shown to have adverse effects on the growth of *Hymenolepis microstoma* (Howard, Christie, Wakelin, Wilson & Behnke, 1978). The non-specific inflammatory response associated with the rejection of *T. spiralis* was implicated, since stunting of *H. microstoma* growth was not recorded if *T. spiralis* expulsion occurred after the scolex of *H. microstoma* had attached to the bile duct (i.e. the post-intestinal stage) (Howard *et al.*, 1978). Nevertheless, gut inflammation during *E. caproni* infection of mice was found to have negligible influence on growth and development of challenge *H. microstoma* infections (Andreassen *et al.*, 1990b).

The establishment of *Fasciola hepatica* in rats may also be influenced by non-specific host immune responses. The intestinal eosinophilia elicited in rats infected with *Nippostrongylus brasiliensis* 4 weeks previously, appeared to render the animals refractory to challenge with *F. hepatica* metacercariae (Doy, Hughes & Harness, 1981a). *Nippostrongylus*-induced damage of the intestine was not believed to be involved in this resistance, since rats with 14-day-old *N. brasiliensis* infections, in which gut damage is likely to be considerable, were susceptible to challenge with *F. hepatica* (Doy *et al.*, 1981a).

Other indirect mechanisms may generate antagonistic interactions between parasite species. Changes in the environment of the host may also have a part to play. In the presence of *N. brasiliensis* and *Moniliformis moniliformis*, an *H. diminuta* infection of rats was shown to exhibit reduced dry weight and length, and to occupy a more posterior position in the small intestine, compared with single *H. diminuta* infections (Holland, 1987). The author suggests that these observations may be related to the gross pathological changes of the jejunum which have been found to accompany *N. brasiliensis* infections in rats, such as a thickened and fluid-filled jejunum, oedematous and irregularly shaped villi, and damage to the integrity of the intestinal wall (see Ogilvie & Jones, 1971). Presumably, the non-specific inflammatory response associated with both *N. brasiliensis* (see Ogilvie & Jones, 1971) and with *M. moniliformis* (Holland, 1987) could also have had some influence with respect to these interactions.

In certain cases, however, more direct forms of interference between helminth species have been implicated. Holmes (1961) has suggested that in concurrent infection with *M. moniliformis* and *H. diminuta* in rats, competition for carbohydrate may at least partly account for detrimental effects on *H. diminuta* growth, resulting in lighter and shorter worms with a lower mean weight:length ratio. The restricted range of intestine occupied by the latter helminth was, particularly, regarded as evidence for competitive inhibition, since distributional limitations of free-living organisms have often been associated with competitive interactions (Holmes, 1961). Other workers suggested that the production of ethanol by *M. moniliformis* might have effected the posteriad shift of *H. diminuta* (Ward & Crompton, 1969), and Holland (1987) has also speculated that *Nippostrongylus* may produce a substance which could account for impaired *H. diminuta* growth observed when both species inhabit the same rat. Similar interactions have been demonstrated between mixed species infections comprising *F. hepatica* and *Hymenolepis microstoma* in mice (Lang, 1967). Results from this experiment suggested that *H. microstoma* migrated from the proximal region of the common bile duct to the distal end of the bile duct, to the junction of the bile

duct and the duodenum, or even to the duodenum itself, following oral challenge with *F. hepatica*. Attachment at this less favourable site appeared to reduce the survival rate of *H. microstoma*. The release of toxic substances by *F. hepatica*, or superior competition by *F. hepatica* for a limiting resource, were suggested mechanisms through which the antagonistic effects directed against *H. microstoma* may have operated (Lang, 1967).

### 4.3 SYNERGISTIC INTERACTIONS BETWEEN PARASITE SPECIES

Synergistic interactions between parasite species infecting the same individual host are less well documented, and are generally believed to be a consequence of parasite-induced immunosuppression (Christensen *et al.*, 1987). Hopkins (1980) has demonstrated that the intensity and dry weight of *H. diminuta* infections of mice are considerably greater when they are concurrent with *Nematospiroides dubius* infections. A depression in the host's immune response was possibly responsible for this effect (Hopkins, 1980). The presence of both larval and adult stages of *N. dubius* in mice have been shown to inhibit expulsion of a concurrent infection with *Trichinella spiralis*, an event which is usually completed by day 15 (Behnke, Wakelin & Wilson, 1978).

Concurrent infection with certain protozoa may also exert a positive influence on the survival and growth of *H. diminuta*. Machnicka & Chromanski (1980) studied infections of *H. diminuta* in mice concurrently infected with *Trypanosoma cruzi* and found the tapeworm's development was enhanced to the extent that sexual maturity was attained. *Hymenolepis diminuta* does not become sexually mature in immunocompetent mice (Hopkins, Subramanian & Stallard, 1972). Similar results were obtained from mice concurrently infected with *T. brucei*, although the overall effect was less pronounced (Fagbemi & Christensen, 1984). In both examples, a trypanosome-induced immunosuppression was thought to account for the enhanced *H. diminuta* survival and growth; immunosuppression is well-documented during trypanosomiasis (see Mansfield, 1978). Machickna & Chromanski (1980) suggested that both humoral and cell-mediated responses to *H. diminuta* antigens were inhibited. Immunotolerance towards *Trichuris muris* in mice, exhibited by

delayed worm rejection, was also observed following infection with the rodent piroplasms, *Babesia hylomysci* and *B. microti* (Phillips & Wakelin, 1976).

In addition to enhanced growth and survival, parasite-induced immunosuppression may also exert a synergistic influence on the fecundity of a concomitant helminth infection. Elevated egg production per worm has been observed in *Haemonchus contortus* infections of sheep which had been simultaneously dosed with *F. hepatica* metacercariae (Presidente, Knapp & Nicol, 1973). Evidence for the involvement of immunosuppression in this example, was provided by the considerable impairment of the circulating eosinophilia demonstrated in sheep with the dual infections (Presidente *et al.*, 1973). The fecundity of *Ornithobilharzia turkestanicum* in mice has been found to increase during combined infection with *S. bovis* or *S. haematobium*, although the authors do not comment on whether helminth-induced immunotolerance was involved (Massoud & Nelson, 1972).

### 4.4 EFFECT OF CONCURRENT PARASITIC INFECTIONS ON THE HOST

The ability of certain parasite species to induce immunosuppression may render a host harbouring a combination of parasite species susceptible to serious disease. Impaired immune responses in mice infected with *Babesia* spp. (Phillips & Wakelin, 1976), *Plasmodium berghei* and *Tryp. brucei* (Phillips, Selby & Wakelin, 1974) have been correlated with the delayed expulsion of *Trichuris muris* from the same host. Phillips & Wakelin (1974) speculated on possible implications for the epidemiology for human helminthiasis, and suggest that acute protozoan infections may exacerbate many of the disease symptoms associated with helminth infections.

Helminth-induced immunotolerance is likely to elicit similar events. A greater proportion of a given dose of *S.mansoni* develop in mice harbouring more than 15 *Echinostomum revolutum* than in fluke-free mice (Christensen, Nydal, Frandsen & Nansen, 1981). Immunosuppression was also implicated in the enhanced pathogenicity and mortality demonstrated in sheep with concurrent *F. hepatica* and *H. contortus* infections, and the

observed depression of the numbers of circulating eosinophils tended to support this idea (Presidente *et al.*, 1973). The validity of this interpretation is debatable when it is noted that calves, harbouring *S. bovis* and superimposed *F. hepatica*, exhibited an impaired eosinophilic response, but experienced less extensive liver damage, compared with *F. hepatica*-only infected controls (Sirag *et al.*, 1981). In fact, in the latter example considerable resistance to the challenge with *F. hepatica* ran in parallel with the reduced eosinophilia, which suggests that the latter does not represent a generalised immunosuppression.

Besides immunosuppression, other phenomena linked with mixed species infections may amplify the symptoms of disease. During experimental concurrent infections of *Trichostrongylus colubriformis* and *Ostertagia circumcincta* in sheep, Steel, Jones & Symons (1982) recorded more pronounced reductions in live weight gain and wool growth when compared with sheep given *O. circumcincta* larvae only. Sheep given only *T. colubriformis* larvae did not exhibit impaired productivity. Sustained gastro-intestinal damage and inflammatory responses in sheep with the mixed infections resulted in greater gastro-enteric plasma loss, which certainly contributed to the impairment of the animals' health. A more substantial and prolonged level of inappetence in these sheep was also involved (Steel, Jones & Symons, 1982).

Concurrent parasitic infections do not always exacerbate the detrimental effects of single species infections on the host. Certain helminths may, in fact, offer a protective role, and render the host totally or partially refractory to infection with another. Thus, prior infection of sheep (Monrad *et al.*, 1981) and calves (Sirag *et al.*, 1981) with *S. bovis* caused a decrease in the survival rate of challenge *F. hepatica* infections, and consequently reduced the liver damage, such as fibrosis, bile-duct enlargement and calcification, normally associated with *F. hepatica* infection. Similar results were observed in sheep infected with *Taenia hydatigena* when later challenged with *F. hepatica* (Campbell *et al.*, 1977). The timing of the primary *T. hydatigena* infection was important; sheep with 3-week infections expressed no immunity to challenge with *F. hepatica*, whereas those with 12-week infections exhibited pronounced resistance. Time taken for the development of the immunological effector

mechanisms was believed to be a possible cause of the apparent discrepancy. The significance of this particular result cannot be over-emphasised, since infection of sheep with *T. hydatigena* is of extremely low pathogenicity (Campbell *et al.*, 1977).

## 4.5 SUMMARY AND AIMS OF EXPERIMENTAL STUDIES

Clearly, interactions between 2 or more species of parasite infecting an individual host are diverse. Antagonistic interactions are most common and are often expressed in terms of reduced survival, more rapid expulsion, migration to a less favourable site, and impaired growth and fecundity of one species in response to another. Synergistic interactions usually involve delayed rejection, or enhanced growth and fecundity of one species. These events may be mediated by the parasites themselves, perhaps following competition for a restricted resource, or by direct mechanical interference, but most appear to be governed by the host, particularly its immune response.

The host responds to concurrent infections in a variety of ways. Where one infecting species is a recognised immunosuppressive agent, the pathogenicity of a concurrent infection may be considerably increased. Certain combinations of helminth species have, however, been shown to impair certain aspects of the host's reaction (notably the eosinophilic response) even though the individual species do not elicit these events when in single species infections. Certain species of helminth may sensitise the host, and render it refractory to challenge with another.

For the remainder of Part 2, experimental concurrent infections involving *Fasciola hepatica* and *Hymenolepis diminuta* in rats are discussed. The course of these investigations can be broadly divided into 2 categories: (i) a study of the influence of each species on the survival, growth and fecundity of the other (Chapter 6), and (ii) the effect of concurrent infections with these species on the rat, specifically their ability to influence eosinophil levels in the host (Chapter 7). Details of the experimental methods adopted are described in Chapter 5.

# PART 2

# CHAPTER 5: MATERIALS AND METHODS

## 5.1 ANIMAL PROCEDURES

### 5.1.1 Experimental animals

All experiments were conducted under Home Office supervision (PPL 60/00370) using outbred male Wistar rats older than 7 weeks, by which time rats are considered to be fully immunocompetent (see Ogilvie & Jones, 1971). Rats were housed in cages of approximately 50 x 32 x 18 cm lined with wood shavings. The rats were kept at an ambient temperature of about 20°C in a 12 h light/dark cycle and at a density of 7 per cage unless otherwise specified. Food (CRM diet) and water were freely available.

### 5.1.2 Maintenance of *Hymenolepis diminuta* life cycle

Rats harbouring adult *Hymenolepis diminuta* were deeply anaesthetized with diethyl ether before being killed by cervical dislocation. Each rat usually harboured between 7 and 10 worms, and this was sufficient for the infection of 100 flour beetles (*Tribolium confusum*). Worms were dissected from the rats' small intestines, and mature proglottids were removed and macerated in 0.9% aqueous NaCl solution (hereafter referred to as saline solution).

Approximately 100 flour beetles, housed in 2 plastic 'sandwich' boxes (170 x 115 x 60 mm) with small air holes in the lid, were fasted for four days prior to infection. Drops of the macerated proglottids in saline solution were applied liberally to filter paper lining the boxes, and the beetles were left to feed on these for 1 h. Following this stage, quantities of whole wheat flour were added to the boxes, and the beetles were housed in a room maintained at 25°C until mature cysticercoids had developed in the haemocoel (approximately 4 weeks). Cysticercoids were dissected from beetles in 0.9% saline solution under a binocular microscope at x6 magnification, and were administered to lightly anaesthetized rats (usually 10 cysticercoids/rat). The infections took about 3 weeks to develop to patency in rats.

### 5.1.3 *Fasciola hepatica* metacercariae

A prolonged attempt to establish the life cycle of *Fasciola hepatica* in the laboratory had to be abandoned owing to numerous difficulties experienced in culturing the algae (*Oscillatoria* spp.) on which the intermediate snail host, *Lymnaea truncatula*, was to feed. Algae grow well on wet clay nourished with plant food which is constantly illuminated with high intensity fluorescent lighting and maintained at 20°C (Graham, pers. comm.). It was, however, difficult to obtain clay soil locally, and a loamy soil with agar added as a binding agent was used instead. This technique had only limited success as algal cultures often only survived for a few days. Consequently, snail stocks could not be fed daily and mortality was high.

Thereafter, metacercariae of *F. hepatica* were ordered from the Parasitology Department, Central Veterinary Laboratory in Weybridge, Surrey, several weeks prior to each experiment. Metacercariae were stored in tap water at 4°C until required.

### 5.1.4 Experimental infections

Rats were lightly anaesthetized with diethyl ether before infection with *H. diminuta* cysticercoids and *F. hepatica* metacercariae. Infective stages were administered orally in 0.5 ml of either 0.9% saline solution or 0.5 ml tap water using a siliconized Pasteur pipette. When both species were given concurrently, they were administered in 0.9% saline. The infection doses were always 10 *H. diminuta* cysticercoids and 10 *F. hepatica* metacercariae. At the end of each experiment, animals were killed by deep ether anaesthesia followed by cervical dislocation.

## 5.2 HELMINTHOLOGICAL MEASUREMENTS

### 5.2.1 Recovery of helminths

#### 5.2.1.1 *F. hepatica*

The following procedure was adapted from that described by Harness, Doy & Hughes (1977) for extracting flukes from mouse liver. The liver and bile duct were removed from

each freshly killed rat, weighed and incubated in 0.9% saline at 37°C for 1 h. If they were not examined immediately thereafter, livers were stored at 4°C for no longer than 48 h before examination. After incubation, most adult and juvenile flukes migrated out from the bile ducts and liver tissue, respectively. Juvenile flukes were identified using a binocular microscope at x6 magnification. Each liver was then gently macerated in saline, using the blunt end of curved forceps, in order to detect any remaining juvenile flukes. All flukes were counted and stored in a deep-freeze at -20°C until required for dry weight measurements.

5.2.1.2 *H. diminuta*

The small intestine from the pylorus to the ileo-caeco-colic junction was removed from each rat. To recover adult or almost adult worms (≥ 17 days post infection (p.i.)), the nozzle of a 10 ml syringe containing 0.9% saline solution was inserted into the duodenal opening and, while the intestine was positioned vertically over a crystallizing dish, worms were flushed out. In those experiments where small, juvenile worms were to be recovered (< 8 days p.i.), the entire intestine was immersed in 0.9% saline, dissected and pinned open on a waxed dissecting dish. The opened gut was examined at x20 magnification under the binocular microscope and any worms removed with fine forceps. In both cases, the number of scolices retrieved was counted and worms were stored at -20°C until required for dry weight measurements.

**5.2.2 Length and weight measurements**

5.2.2.1 *F. hepatica*

In certain experiments, fluke length was measured before freezing. In these cases, worms were relaxed by chilling for at least 1 h at 4°C, then measured from anterior to posterior tips on a glass petri-dish positioned over graph paper graduated in millimetres.

For weight measurements, flukes were defrosted and excess moisture removed with absorbent tissue. They were placed on pre-weighed aluminium foil and weighed in mg to 2

decimal places on a Sartorius precision balance. Each fluke plus foil was then transferred to an oven set at 100°C and thereafter weighed every 6-9 h until the weight was observed to have stabilized to within ± 0.05 mg.

5.2.2.2 *H. diminuta*

In experiment 5, individual *H. diminuta* length was measured. Worms were relaxed as for *F. hepatica* (section 5.2.2.1), gently straightened and juxtaposed along the measuring side of a ruler, then measured to the nearest 0.5 of a mm.

Weight measurements were made as described for *F. hepatica* (section 5.2.2.1), with the exception that individual worms were not weighed separately. Extensive fragmentation of worms occurred, particularly in regions of mature proglottids, so that entire worms were seldom recovered. Since there was always more than 1 worm per rat, individual worm weights would have been highly inaccurate.

**5.2.3 Egg production**

5.2.3.1 *Kato Katz technique (experiments 1, 2 and 3)*

In experiments 1, 2 and 3, faecal pellets were collected from individual rats between 0900 and 1000 h at specific intervals (depending on the experiment) and stored at 4°C until required. Samples were processed using a version of the Kato Katz technique (WHO, 1985) to determine helminth egg counts per rat. Each pellet was allowed to soften for several hours in vials containing about 5 ml tap water, and was then macerated with a wooden spill until completely disintegrated. The mixture was transferred to a centrifuge tube, made up to 10 ml with tap water, and centrifuged for 5 min at 2000 r.p.m. The supernatant fluid was discarded and the pellet was pressed through stainless steel mesh onto a broad glass slide. The sieved material was passed through a stainless steel template which apportioned approximately 26 mg onto a glass slide (several test runs yielded a value of 25.67 ± 1.50). Three to four drops of 3% malachite green in 50% glycerol were added to the slide and mixed well before examination at x40 magnification. The number of eggs per gram of

(sieved) faeces (e.p.g.) was then calculated.

5.2.3.2 *Modified McMaster technique for H. diminuta egg counts (experiment 4)*

Several of the results obtained using the Kato Katz method were found to be highly variable (section 6.4.1.2), so the modified McMaster method (H.M.S.O., 1971) was used to determine *H. diminuta* egg counts in experiment 4. (This technique could not have been used in experiments 1, 2 and 3 since insufficient faecal material was collected in those experiments).

For individual faecal collections, rats were housed in cages (33.5 x 20.5 x 19.0 cm) and the entire quantity of faeces produced by each rat over a 24-h period was collected on the same day of each week of the experiment. Each faecal sample was thoroughly broken up and mixed with a hand blender. Three grams were removed and added to 42 ml of water before being mixed with a hand blender until of a uniform, aqueous consistency. The mixture was filtered through two layers of double thickness cotton gauze, and 10 ml of the filtrate were transferred to a centrifuge tube and centrifuged at 2000 r.p.m. for 2 min. The remaining mixture was retained. The supernatant fluid was discarded and the sediment loosened by gentle agitation. The tube was filled with saturated aqueous NaCl solution up to the 10 ml mark and inverted several times (covering open end with thumb) until the sediment was evenly suspended. Approximately 0.5 ml were withdrawn with a Pasteur pipette and run into one chamber of a McMaster counting slide. The counting chamber (one $cm^2$) was systematically searched under low power (x6) and all eggs counted. This process was repeated for the other counting chamber. The two values were added so that each egg represented 50 per g of faeces (the volume examined under both chambers was 0.30 ml, which is 2/300 of the original 45 ml which contained 3 g of faeces).

5.2.3.3 *Zinc sulphate flotation technique for F. hepatica egg counts (experiment 4)*

Relatively dense fluke eggs do not float in saturated NaCl solution, so the zinc sulphate flotation technique was adopted for the determination of *F. hepatica* egg counts in experiment 4. Fifteen ml of the retained filtrate (section 5.2.3.2) were transferred to a centrifuge tube, and centrifuged at 2000 r.p.m. for 2 min. The supernatant fluid was discarded and replaced with saturated zinc sulphate solution after loosening the sediment. The tube was inverted several times with the open end covered, before being returned to the centrifuge. Saturated zinc sulphate solution was added to the tube drop by drop until a positive meniscus had been formed. An 18 $mm^2$ glass coverslip was carefully positioned on top and the tube centrifuged at 1000 r.p.m. for 2 min. Following this, the coverslip was removed, held vertically and adhering eggs were washed into a conical centrifuge tube using $\leq$ 10 ml of water. This tube was centrifuged at 2000 r.p.m. for 2 min. The supernatant fluid was siphoned off leaving $\leq$ 0.1 ml in the tube, which was then pipetted onto a glass slide. The tube was then rinsed with a further 0.1 ml which was added to the same slide. An 18 $mm^2$ coverslip was placed over the drops and all eggs counted at x40 magnification. Each egg represented one per gram of faeces (15 ml of the original 45 ml, which contained 3 g of faeces, were used).

5.2.4 **Estimation of food intake by measuring faecal output (experiment 4)**

To measure individual faecal output, rats were housed in cages (33.5 x 20.5 x 19.0 cm) and the entire quantity of faeces produced by each rat over a 24-h period was collected on the same day of each week of the experiment. Each sample was transferred to a pre-weighed large glass petri dish and weighed in g to 2 decimal places on a Sartorius balance. Samples with petri dishes were transferred to an oven set at 100°C and thereafter weighed every 6-9 h until the weight was observed to have stabilized to within $\pm$ 0.05 g. It was assumed that the quantity of faeces produced over a fixed period could be taken as a rough estimate of food consumption since faecal output must be related to food intake.

## 5.3 HAEMATOLOGICAL AND HISTOLOGICAL TECHNIQUES

### 5.3.1 Blood cells

#### 5.3.1.1 *Differential counts for eosinophils*

The tip of a rat's tail was swabbed with 70% ethanol and a small incision was made using clean, sharp dissecting scissors. One drop of blood was applied to a glass slide and the end of another glass slide was drawn across this to produce a blood film. The incision was again swabbed with 70% ethanol to minimize the possibility of infection before the rat was returned to its cage. No evidence was obtained to suggest that this procedure had any ill effects on the rats. Blood smears were allowed to dry for at least 30 min, then stained with Leishman's stain (Gurr, BDH Ltd.) for 3 min and differentiated in distilled water for 7 min. When dry, blood films were examined under the light microscope at x40 magnification. Cells were counted by moving the slide with its film systematically under the objective lens, starting at that point where the smear first became one cell thick. Care was taken to avoid counting cells situated in the periphery of the smear where cells are often ruptured and where the larger cells tend to be over-represented. The number of eosinophils per 100 cells was counted; eosinophils can be easily identified by the presence of granules which stain bright pink with Leishman's stain.

#### 5.3.1.2 *Absolute white blood cell counts*

For the collection of larger samples of blood (not greater than 0.3 ml) it was necessary to restrain each rat in a modified perspex chamber with a vertical slit at one end through which the animal's tail was brought and held firmly before sampling.

In experiment 1, approximately 0.3 ml of blood per rat was periodically sampled by superficial venesection (as described in section 5.3.1.1) into a 0.3 ml vial containing 0.5 mg EDTA $K_2$ (Microvette). Each sample was analysed on a Technicon H1 Hematology System in the Department of Haematology, Western Infirmary, to determine absolute eosinophil counts.

In experiment 6, approximately 50 $\mu$l of blood were collected in a 1 ml Eppendorf

containing 50 $\mu$l of white cell diluting fluid (WCDF: 0.01 % gentian violet in 3 % acetic acid (McMenamin, 1986)). Each sample plus WCDF was gently mixed using a vortex mixer. As it was clearly not possible to collect exactly 50 $\mu$l of blood, the mixed samples were compared to known volumes of water ranging from 70 to 130 $\mu$l in 1 ml Eppendorfs, and the total volume estimated to within 10 $\mu$l. This allowed the actual volume of blood collected to be estimated. Known volumes of WCDF and water were dispensed using Gilson pipettes. A further 900 $\mu$l of WCDF were added and the entire sample was thoroughly mixed by vortex mixer. Approximately 0.5 ml of the diluted sample were run into both chambers of an improved Neubauer haemocytometer and examined with a light microscope at x10 magnification. White cell diluting fluid lyses all red cells and stains the nuclei of all white cells a dark purplish-blue. The total number of white cells within the grid of each chamber could, therefore, be easily counted. Since the volume covering each grid is $10^{-4}$ ml ($2 \times 10^{-4}$ ml covering two grids), absolute white cell counts per ml of blood were calculated as follows:

$$\frac{\text{total number of cells in 2 grids} \times \dfrac{\text{total volume of diluted sample of WCDF + blood (ml)}}{2 \times 10^{-4}}}{\text{estimated blood volume (ml)}}$$

**5.3.2 Peritoneal exudate cells**

The following method for the investigation of peritoneal exudate cells was adapted from that of McMenamin (1986). Rats were deeply anaesthetized with diethyl ether and killed by cervical dislocation. Ten ml of warm Hanks's Balanced Salt Solution (HBSS) (Sigma Chemical Company Ltd.), at a pH of approximately 6.8, were injected into the peritoneal cavity at the flap of skin directly anterior to the hip, using a 21 gauge needle. The abdomen was gently massaged for about 5 min before the peritoneal cavity was exposed. The medium containing peritoneal exudate cells was collected using a 10 ml syringe and filtered through 2 layers of lens tissue to obtain a single cells suspension and to remove debris. To

wash the cells, the suspension was transferred to a 10 ml glass centrifuge tube, made up to 10 ml with HBSS and centrifuged at 1100 r.p.m. for 7 min. The sediment was resuspended in 10 ml HBSS.

In order to make differential counts, 2-5 drops of the suspension were added to the cuvettes of a cytocentrifuge and centrifuged at 600 r.p.m. for 5 min. The slides with discs of cells were allowed to dry for at least 30 min and were stained with Leishman's stain (Gurr, BDH Ltd.) for 5 min, then differentiated in distilled water for a further 20 min. Following this, slides were rinsed in tap water, allowed to dry and mounted in Histomount (National Diagnostics).

Various cell types may be unevenly distributed throughout smears. In order to limit any bias for or against a particular cell type, cells were counted along the diameter and systematically across one semi-circle of each smear, under the x40 objective. Only those regions which were no greater than one cell thick were examined. Cells on the extreme periphery of the smear were not counted as they were often ruptured. The number of eosinophils per 500 cells was counted.

#### 5.3.3 Bone marrow cells

The following method for investigating bone marrow cells was adapted from Spry (1971). Rats were deeply anaesthetized with diethyl ether and killed by cervical dislocation. Following removal of the medium from the opened peritoneal cavity (section 5.3.2), the left hind leg of each rat was liberally swabbed with 70 % ethanol. This helped prevent loose hair becoming airborne and sticking to exposed tissue. The skin around the leg was cut and peeled back to the foot, and the intact femur separated from the hip and femoro-tibial joints using bone scissors. Tissue around the femur was removed using scissors and a scalpel, and the femur then severed with bone scissors near the hip joint. Following this, the tip of the femur at the knee joint was snipped with bone scissors until the cavity was just exposed. Ten ml of HBSS were then forced through the bone cavity using a 19 gauge needle on a syringe, expelling a plug of bone marrow. In order to obtain a single suspension, the plug was gently

aspirated several times through the 19 gauge needle and filtered through two layers of clean lens tissue. The filtrate was transferred to a glass centrifuge tube, made up to 10 ml with HBSS and spun at 1100 r.p.m. for 7 min. Cells were resuspended in 10 ml of fresh HBSS.

Differential cell counts were obtained by essentially the same procedure described in section 5.3.2 for peritoneal cells. Since the bone marrow cell suspension was slightly more concentrated, however, only 1-4 drops were added to the cuvettes of the cytocentrifuge. Counts were made of the number of immature and mature eosinophils per 500 nucleated cells only. A haematology atlas (McDonald, Dodds & Cruickshank, 1978) was consulted to assist with the identification of certain cell types.

**5.3.4 Intestinal cells**

The following method for the investigation of intestinal cells was adapted from that described by Harness, Doy & Hughes (1977). Rats were deeply anaesthetized with diethyl ether and killed by cervical dislocation. When appropriate, mature *H. diminuta* worms were recovered (section 5.2.1.2) prior to the histological procedures. The posterior two-thirds of the small intestine were cut into two pieces, each of which was flushed through with 10 % neutral buffered formaldehyde solution (NBF) using a 10 ml syringe. Each piece was coiled, with the anterior end first, round a glass rod between 2 corks set at about 5 mm apart, and transferred to a beaker containing NBF. Beakers were covered with pieces of weighted perspex to keep the intestines immersed and to prevent evaporation of the fixative. The intestines were fixed in this way in NBF for at least 24 h. Thereafter, corks and glass rods were removed and the coiled 'discs' of tissue transferred to individual, covered petri dishes containing NBF, and were fixed for a further 6 days. All samples to be processed were transferred to individual stainless steel baskets in a large beaker and thoroughly rinsed by running tap water continuously through the beaker for at least 12 h. To ensure that they were small enough for histological sectioning, intestinal 'discs' were cut diagonally and one half discarded. Each semi-circle of tissue was dehydrated in serially graded alcohols followed

by Histoclear (National Diagnostics), then embedded in paraffin wax. Intestines were sectioned longitudinally at 7 $\mu$m and 2-4 semi-circles of tissue transferred to a glass slide. Only those sections which were cut at greater than 1500 $\mu$m into the tissue were used.

Tissues were stained with haematoxylin and eosin (H & E). Sections were cleared in Histoclear then rehydrated in serially graded alcohols to distilled water. Following this, they were stained for 5-10 min with Mayer's haematoxylin (Mayer, 1903) and rinsed in Scott's tap water before being brought to 90 % alcohol through serial concentrations. Sections were then stained in 1% aqueous eosin Y for 1 minute, fully dehydrated in absolute alcohol for 1 min, and transferred to Histoclear before mounting in Histomount (National Diagnostics).

Sections were examined under the light microscope using the x40 objective lens. The total number of eosinophils encountered crossing between 8 and 14 longitudinal sections of gut wall was counted. Counts were expressed as the mean number of eosinophils per gut wall traverse. Eosinophils were identified by virtue of their cytoplasmic granules which stain a bright orange-pink with eosin, and also by the characteristic shape of their nuclei.

# PART 2

## CHAPTER 6: CONCURRENT INFECTIONS INVOLVING *FASCIOLA HEPATICA* AND *HYMENOLEPIS DIMINUTA* IN RATS: INTERSPECIFIC EFFECTS

## 6.1 INTRODUCTION

It is clear that during experimental concurrent infections both heterologous synergistic and antagonistic interactions may be observed between helminths (Chapter 4). Such interactions may be expressed in terms of enhanced or reduced parasite establishment, survival, growth, and fecundity. The results of experiments carried out to investigate possible interspecific effects between the digenean, *Fasciola hepatica*, and the cestode, *Hymenolepis diminuta*, in the rat host are discussed in this chapter.

## 6.2 OBJECTIVES

The objectives of the experiments carried out were as follows:

(1) To investigate whether the survival, growth and fecundity of *H. diminuta* infection in rats was affected by a prior, patent infection with *F. hepatica*, and also whether any of the above parameters in an established *F. hepatica* infection, were in any way influenced following challenge with *H. diminuta* cysticercoids (experiments 1 and 4).

(2) To study the survival, growth and fecundity of an *F. hepatica* infection in rats previously infected with *H. diminuta*. Any influence of the *F. hepatica* challenge on the established *H. diminuta* was also examined (experiment 2).

(3) To investigate the influence of *F. hepatica* and *H. diminuta* on each other's establishment and/or survival, growth and fecundity, when the infective stages of both species were administered simultaneously (experiment 3).

(4) To test whether the extensive mucosal mast cell proliferation believed to peak on day 35 of *F. hepatica* infections in rats, affects *H. diminuta* establishment, survival or growth (experiment 5).

(5) A general investigation of growth patterns of the individual species, especially in relation to worm fecundity, in conjunction with the study on interspecific interactions.

## 6.3 EXPERIMENTAL PROTOCOLS

### 6.3.1 Experiment 1

Four groups (A,B,C and D), each having 7 rats per group, were used in the experiment. The infection procedure is described in section 5.4.1. On day 0, each rat from two of the groups (A and B) received 10 *F. hepatica* metacercariae. On day 74, each rat from group B, with mature *F. hepatica* infections, together with each rat from group C, which were uninfected, were given 10 *H. diminuta* cysticercoids. Group D rats represented the uninfected control group. The experiment was terminated on day 124 when *post-mortem* examinations were carried out.

In a primary infection in rats, *F. hepatica* is observed to begin the production of eggs by about 10 weeks p.i. (personal observation). Faecal samples were collected regularly at known intervals from day 60 until the end of the experiment (day 124), and examined for the presence of eggs (section 5.2.3.1). When the rats were killed, mature *F. hepatica* and *H. diminuta* were removed as detailed in section 5.2.1., and length and wet and dry weight measurements taken (section 5.2.2). *Fasciola* length was the only measure of fluke size in this experiment, but it appears to be a reliable indicator since length and dry weight are highly correlated (section 6.4.3.4).

The experimental protocol is summarised in Table 6.1.

**Table 6.1.** Experimental protocol for experiment 1.

| Rat group (n) | day 0 | day 74 | day 124 |
|---|---|---|---|
| A (7) | 10 *F. hepatica* metacercariae/rat | --- | experiment end |
| B (7) | 10 *F.hepatica* metacercariae/rat | 10 *H. diminuta* cysticercoids/rat | experiment end |
| C (7) | --- | 10 *H. diminuta* cysticercoids/rat | experiment end |
| D (7) | --- | --- | experiment end |

### 6.3.2 Experiments 2 and 3

Four groups (A,B,C & D), each having 7 rats per group, were used in experiment 2. The infection procedure used is described in section 5.4.1. On day 0, 10 *H. diminuta* cysticercoids were administered to each rat in groups B and C. Fifty-six days later, each rat from group B, together with each rat from group A, received 10 *F. hepatica* metacercariae. Group D rats remained uninfected throughout the duration of the experiment.

Rats of groups A, C and D from experiment 2 were also used in experiment 3. (Experiments 2 and 3 ran concurrently in order to limit the number of experimental animals used). In addition, each rat from a further group of seven (group E) received 10 *H. diminuta* cysticercoids and 10 *F. hepatica* metacercariae simultaneously on day 0 of the experiment.

The experiments were terminated over a two-day period (days 161 and 162) owing to the large number of animals to be examined at *post-mortem*. Care was taken to divide each rat group for this purpose, to minimize any group bias associated with unequal infection courses.

In a primary infection of *Hymenolepis diminuta* in rats, worm egg production is observed to begin 16 to 17 days p.i. (Roberts, 1961). Faecal samples were collected at regular intervals from day 14 onwards, and examined for the presence and intensity of helminth eggs (section 5.2.3.1). When the experiment finished, mature worms were removed from rats as described in section 5.2.1, and length and wet and dry weight measurements were taken (section 5.2.2).

Since experiment 3 ran concurrently with experiment 2, for certain measurements it was necessary to compare 3, as opposed to 2, group medians in order to avoid multiple pairwise testing. When this was the case, a Kruskal-Wallis analysis of variance was used, and any significance between the groups located using the method of multiple comparisons between treatments detailed in Siegel & Castellan (1988).

The experimental protocols are summarised in Tables 6.2 and 6.3.

**Table 6.2.** Experimental protocol for experiment 2.

| Rat group (n) | day 0 | day 56 | day 161-2 |
|---|---|---|---|
| A (7) | --- | 10 *F. hepatica* metacercariae/rat | experiment end |
| B (7) | 10 *H. diminuta* cysticercoids/rat | 10 *F. hepatica* metacercariae/rat | experiment end |
| C (7) | 10 *H. diminuta* cysticercoids/rat | --- | experiment end |
| D (7) | --- | --- | experiment end |

**Table 6.3.** Experimental protocol for experiment 3.

| Rat group (n) | day 0 | day 105/6 | day 161-2 |
|---|---|---|---|
| A* (7) | 10 *F. hepatica* metacercariae/rat | experiment end | |
| C (7) | 10 *H. diminuta* cysticercoids/rat | --- | experiment end |
| D (7) | --- | --- | experiment end |
| E (7) | 10 *H. diminuta* cysticercoids + 10 *F. hepatica* metacercariae/rat | --- | experiment end |

* *F. hepatica* infection did not run concurrently with that of group E

### 6.3.3 Experiment 4

Two groups of 10 rats were used in an experimental procedure which followed that for rats of groups B and C in experiment 1. On day 0, each of 10 rats (group A) were administered 10 *F. hepatica* metacercariae. Seventy-four days later, these rats, together with another 10 uninfected rats (group B), each received 10 *H. diminuta* cysticercoids. The

experiment ended on day 124 when all rats underwent *post-mortem* examination.

Rats were housed individually in cages (section 5.2.3.2) to facilitate the collection of relatively large amounts of faecal samples per rat. The entire quantity of faeces produced in one 24 hour period was collected from each rat at the same time twice per week. One of these weekly samples was examined for helminth eggs (sections 5.2.3.2 and 5.2.3.3), and the other was dry weighted to give an estimate of food intake (section 5.2.3.4).

The doses of *H. diminuta* cysticercoids and of *F. hepatica* metacercariae administered to rats were later found to be inaccurate. Consequently, the original aims of the experiment could not be fulfilled, although some useful information was still procured from the data collected.

### 6.3.4. Experiment 5

Each rat from a group of 7 (A) was given 10 *F. hepatica* metacercariae on day 0 of the experiment. Thirty-five days later, each of these rats, together with each rat from a further group of 7 (B), received 10 *H. diminuta* cysticercoids. The experiment was terminated on day 42 of the experiment when *post-mortem* examinations were carried out.

When the rats were killed, *F. hepatica* and *H. diminuta* were removed as detailed in section 5.2.1., and length and wet and dry weight measurements taken (section 5.2.2).

The experimental protocol is summarised in Table 6.4.

**Table 6.4.** Experimental protocol for experiment 5.

| Rat group (n) | day 0 | day 35 | day 42 |
|---|---|---|---|
| A (7) | 10 *F. hepatica* metacercariae/rat | 10 *H. diminuta* cysticercoids/rat | experiment end |
| B (7) | --- | 10 *H. diminuta* cysticercoids/rat | experiment end |

## 6.4 STATISTICAL ANALYSIS

Since most of the morphometric data recorded were not found to be normally distributed, all data collected were analysed using non-parametric statistics. Consequently, where appropriate, data were presented in tables as group medians with inter-quartile ranges. The exception concerns the presentation of helminth egg counts over the course of the experiment, which were illustrated graphically as group means with standard error bars for clarity. (Egg count data were, nevertheless, compared between groups using non-parametric tests.)

For the comparison of two or more group medians, the Wilcoxon-Mann-Whitney test or the Kruskal-Wallis analysis of variance were used. When more than two groups were being compared, significance was located using the method for multiple comparisons between treatments detailed in Siegel & Castellan (1988).

Associations between sets of data were examined using Spearman's rank correlation technique. Comparison of these relationships between treatment groups, however, had to be conducted using analysis of covariance (ANCOVA), which is a parametric test.

The experimental level of significance was taken to be $p < 0.05$. Two-tailed probabilities were used for all statistical analyses. In all tables presented in the text, 'S' denotes a significant result and 'NS' a non-significant result.

## 6.5 RESULTS

### 6.5.1 Helminth egg counts

#### 6.5.1.1 *Experiment 1*

In experiment 1, egg production of *F. hepatica* and *H. diminuta* in rats which harboured patent *F. hepatica* infections at the time of infection with *H. diminuta*, was investigated. Mean values for *Hymenolepis diminuta* faecal egg counts in rats from groups B and C throughout the duration of the experiment are illustrated with standard error bars in Figure 6.1. Until day 31 of the infection (day 105 of the experiment), *H. diminuta* egg counts

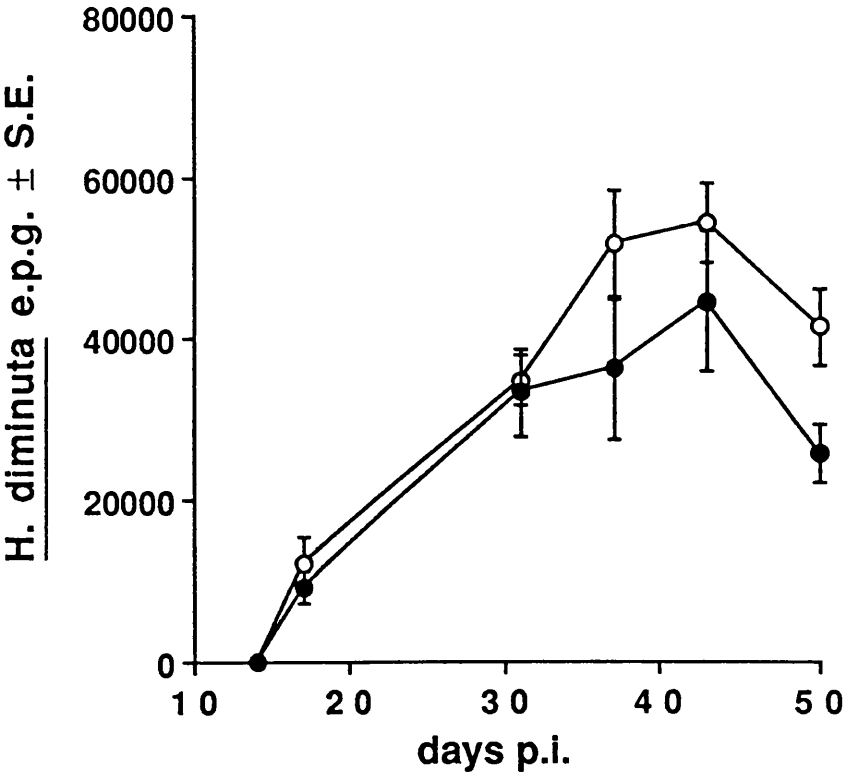



**Fig. 6.1.** Mean *Hymenolepis diminuta* faecal egg counts ± S.E. of the rats of groups B and C in experiment 1. Group B rats harboured 74-day old *F. hepatica* infections (10 metacercariae/rat) at the time of infection with *H. diminuta* (10 cysticercoids/rat); group C rats were infected with *H. diminuta* only (10 cysticercoids/rat). ( o ) group B; ( ● ) group C.

from B and C rats were found to be rather variable, but appeared to increase at comparable rates. Thereafter, *H. diminuta* faecal egg counts from B rats, concurrently infected with *F. hepatica*, were consistently greater than those from C rats, which harboured *H. diminuta* only. A significant difference between the median egg counts of the groups was detected only on day 50 of the infection (day 124 of the experiment) (Wilcoxon-Mann-Whitney test; $W = 71.0$, $n_1 = 7$, $n_2 = 7$; $p < 0.05$).

The mean faecal egg counts of *F. hepatica* in rats from groups A and B for the duration of the experiment are shown with standard error bars in Figure 6.2. Although egg counts were again variable, the *F. hepatica* egg output from those rats with concomitant *H. diminuta* infections (group B) was consistently lower than that of those harbouring *F. hepatica* only (group A), from before the *H. diminuta* cysticercoids were administered until at least day 90 of the experiment. This difference was found to be significant on day 77 of the experiment, 3 days after *H. diminuta* cysticercoids had been given to group B rats, (Wilcoxon-Mann-Whitney test; $W = 68.5$, $n_1 = 7$, $n_2 = 7$; $p < 0.05$), although a drop in *F. hepatica* egg output was observed in both rat groups. Egg counts remained low in the mixed infection group for a further 2 weeks, but recovered fairly rapidly in the *Fasciola*-only control rats.

6.5.1.2 *Experiments 2 and 3*

Experiments 2 and 3 were designed to study *F. hepatica* and *H. diminuta* egg production in rats which harboured mature *H. diminuta* infections at the time of infection with *F. hepatica*, and in rats which received *F. hepatica* and *H. diminuta* infections simultaneously, respectively. Preliminary examination of faecal samples, however, revealed that the apparent variability in egg counts for *H. diminuta*, and especially for *F. hepatica*, observed in experiment 1, were evident in a more exaggerated form in experiments 2 and 3. The rather small sample size, as well as the diagnostic procedure used (section 5.2.3.1), was thought to be responsible for this. The former problem could have been countered by

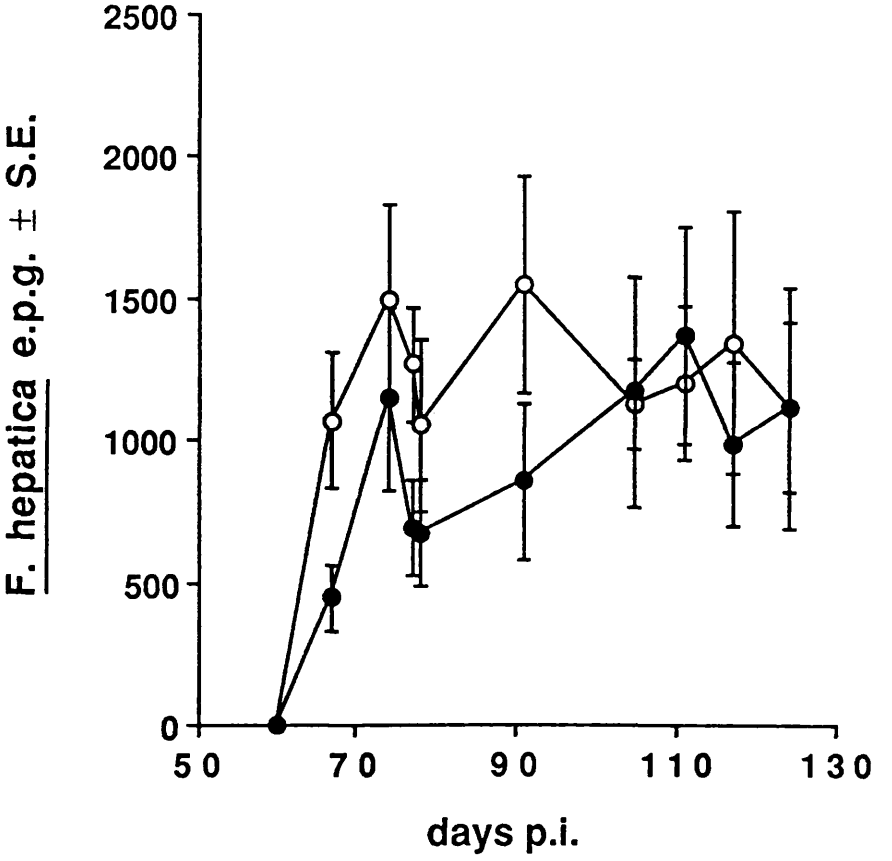



**Fig. 6.2.** Mean *Fasciola hepatica* faecal egg counts ± S.E. of the rats of groups A and B in experiment 1. Group A rats harboured *F. hepatica* only (10 metacercariae/rat); group B rats were infected with *H. diminuta* (10 cysticercoids/rat) on day 74 post infection with *F. hepatica* (10 metacercariae/rat). ( ○ ) group A; ( ● ) group B.

housing rats in individual cages, but this was impracticable owing to the scale of the experiment (experiments 2 and 3 combined involved 35 rats). It was therefore decided that examination of faecal samples should not be completed.

### 6.5.2 Helminth intensity

#### 6.5.2.1 *Experiment 1*

In experiment 1, the survival of *H. diminuta* and *F. hepatica* in rats which harboured mature *F. hepatica* infections at the time of infection with *H. diminuta*, was investigated. The median number of *H. diminuta* scolices, which is the best measure of the number of worms, recovered from the rats of groups B and C is shown with inter-quartile range in Table 6.5. Rats with the concurrent *F. hepatica* infections (group B) were found to have significantly more *H. diminuta* at the end of the experiment than those with *H. diminuta* only (Wilcoxon-Mann-Whitney test; $W = 69$, $n_1 = 7$, $n_2 = 7$; $p < 0.05$).

**Table 6.5.** Median number of *H. diminuta* scolices recovered from the rats in groups B and C of experiment 1, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median number of *H. diminuta* scolices | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| B (7) | 9.00 | 6.00, 10.00 | 0.039 (S) |
| C (7) | 5.00 | 5.00, 7.00 | |

The median number of *F. hepatica* recovered from the rats of groups A and B is shown with inter-quartile range in Table 6.6. No significant difference between those rats harbouring concurrent *F. hepatica/H. diminuta* infections (group B), and those with *F. hepatica* infections only (group A) was detected (Wilcoxon-Mann-Whitney test; $W = 58$, $n_1 = 7$, $n_2 = 7$; $p > 0.05$).

**Table 6.6.** Median number of *F. hepatica* recovered from the rats in groups A and B of experiment 1, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median number of *F. hepatica* | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (7) | 3.00 | 2.00, 4.00 | 0.516 (NS) |
| B (7) | 2.00 | 1.00, 4.00 | |

6.5.2.2 *Experiment 2*

In experiment 2, the survival of *H. diminuta* and *F. hepatica* in rats which harboured mature *H. diminuta* infections at the time of infection with *F. hepatica*, was investigated. The median number of *H. diminuta* scolices recovered from the rats of groups B and C is shown with inter-quartile range in Table 6.7. The number of *H. diminuta* recovered did not appear to differ significantly between those rats which were later challenged with *F. hepatica* metacercariae (group B), and those which were not (group C) (Kruskal-Wallis ANOVA; $H = 0.22$, d.f.$= 2$, $p > 0.05$).

**Table 6.7.** Median number of *H. diminuta* scolices recovered from the rats in groups B, C and E of experiments 2 and 3, compared using Kruskal-Wallis ANOVA.

| Experimental group (n) | Median number of *H. diminuta* scolices | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| B (6) | 7.50 | 5.50, 9.25 | |
| C (7) | 7.00 | 5.00, 9.00 | 0.895 (NS) |
| E (6) | 7.00 | 6.00, 9.25 | |

Median *F. hepatica* worm burdens recovered from the rats of groups A and B are displayed with their inter-quartile ranges in Table 6.8. There was no apparent difference between the *F. hepatica* worm burden of those rats previously infected with *H. diminuta* (group B), and that of those harbouring *F. hepatica* only (group A) (Kruskal-Wallis ANOVA, $H = 2.02$, d.f.$= 2$, $p > 0.05$).

**Table 6.8.** Median number of *F. hepatica* recovered from the rats in groups A, B and E of experiments 2 and 3, compared using Kruskal-Wallis ANOVA.

| Experimental group (n) | Median number of *F. hepatica* | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (5) | 4.00 | 3.00, 5.00 | |
| B (6) | 2.50 | 0.75, 4.25 | 0.365 (NS) |
| E (6) | 3.00 | 1.00, 4.00 | |

6.5.2.3 *Experiment 3*

In experiment 3, the survival of *H. diminuta* and *F. hepatica* in rats infected with both species simultaneously, was investigated. The median number of *H. diminuta* recovered from the rats of groups C and E is shown with interquartile range in Table 6.7. No significant difference was discovered between the number of *H. diminuta* recovered from the *H. diminuta*-only control rats and those which received a simultaneous infection with *F. hepatica* metacercariae (Kruskal-Wallis ANOVA, $H = 0.22$, d.f.$= 2$, $p > 0.05$).

Similarly, the number of *F. hepatica* recovered from those rats which received a simultaneous dose of *H. diminuta* cysticercoids (group E), was not observed to vary significantly from that of those which received *F. hepatica* metacercariae only (group

A)(Table 6.8: Kruskal-Wallis ANOVA, H = 2.02, d.f.= 2, $p > 0.05$).

6.5.2.4 *Experiment 5*

In experiment 5, the survival of an *H. diminuta* infection given to rats harbouring 35-day old *F. hepatica* infections which are known to be accompanied by an extensive intestinal mast cell response, was investigated. The median number of *H. diminuta* recovered from the rats of groups A and B is displayed with interquartile range in Table 6.9. No significant difference between the number of *H. diminuta* recovered from rats with 35-day old *F. hepatica* infections (A), and those with *H. diminuta* only (B), was found (Wilcoxon-Mann-Whitney, W = 56.5, $n_1 = 7$, $n_2 = 7$, $p > 0.05$).

**Table 6.9.** Median number of *H. diminuta* recovered from the rats in groups A and B of experiment 5, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median number of *H. diminuta* scolices | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (7) | 8.00 | 2.00, 10.00 | 0.651 (NS) |
| B (7) | 7.00 | 5.00, 8.00 | |

**6.5.3 Helminth morphometrics**

6.5.3.1 *Experiment 1*

The growth of *H. diminuta* and *F. hepatica* in rats which harboured mature *F. hepatica* infections at the time of infection with *H. diminuta*, was investigated in experiment 1. The total dry weight of *H. diminuta* recovered from each rat was compared in the rats of groups B and C, and median values with inter-quartile ranges are shown in Table 6.10. Although greater in the rat group with the mixed infection (group B), total *H. diminuta* dry weight per rat was not found to differ significantly between the rat groups (Wilcoxon-Mann-Whitney

test; W = 60, $n_1$ = 7, $n_2$ = 7; $p > 0.05$).

**Table 6.10.** Median total dry weight of *H. diminuta* recovered from the rats in groups B and C of experiment 1, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median *H. diminuta* dry weight (mg) | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| B (7) | 1430 | 690, 1450 | 0.368 (NS) |
| C (7) | 760 | 440, 1190 | |

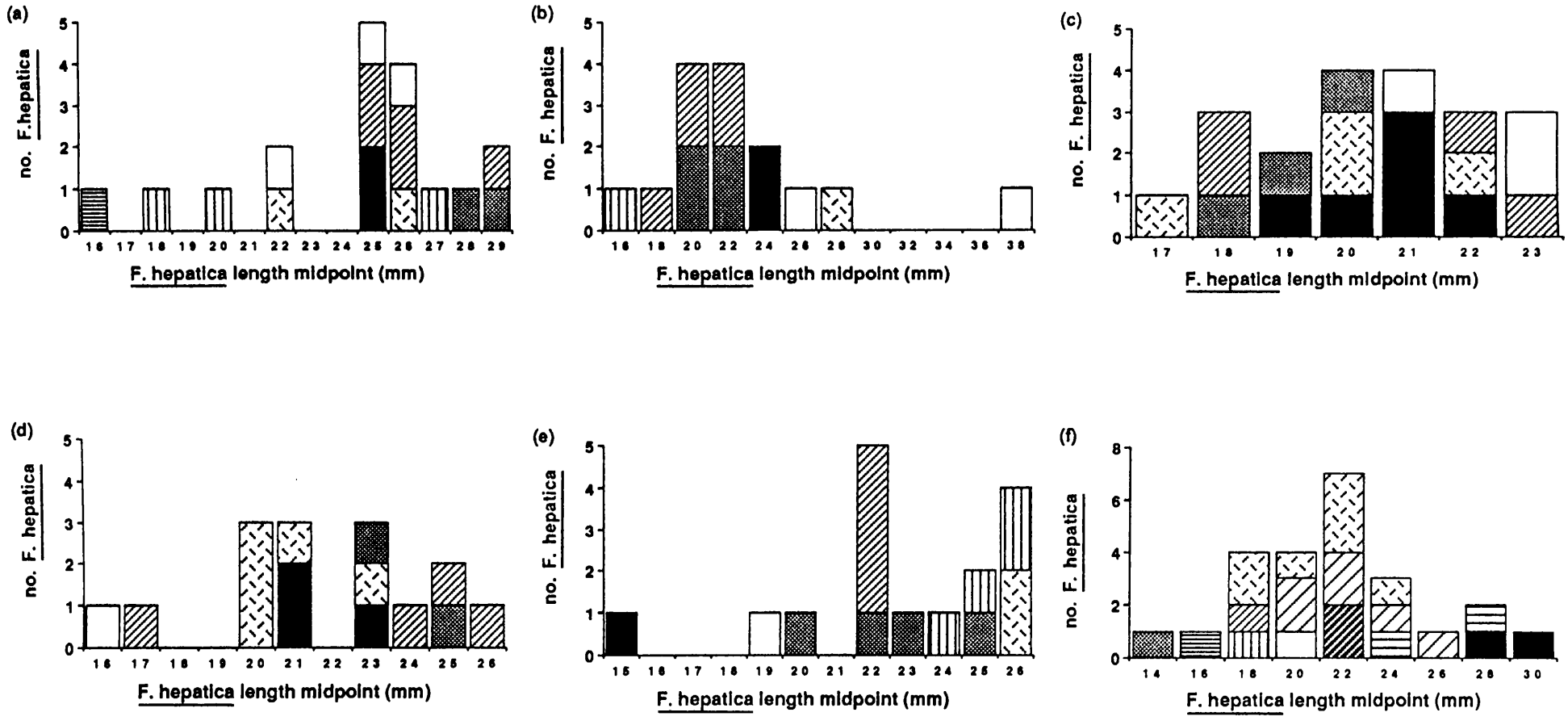
Histograms of the length of *F. hepatica* recovered from the rats of groups A and B are illustrated in Fig. 6.3 (a) and (b), respectively. The distribution of worm length in the rats of group A did not appear to be normal and appeared to be fragmenting into different size classes. Worm length within an individual rat often varied considerably (Fig. 6.3 (a) and (b)).

Median *F. hepatica* lengths for both groups are illustrated with inter-quartile ranges in Table 6.11. No significant difference between the length of *F. hepatica* in those rats with the single species infections (group A), and that of those which had been challenged with *H. diminuta* (group B), was discovered (Wilcoxon-Mann-Whitney test; W = 359.5, $n_1$ = 18, $n_2$ = 15; $p > 0.05$).

**Table 6.11.** Median length of *F. hepatica* recovered from the rats in groups A and B of experiment 1, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median *F. hepatica* length (mm) | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (18) | 25.00 | 22.00, 26.25 | 0.055 (NS) |
| B (15) | 21.50 | 19.50, 23.00 | |

**Fig. 6.3.** The length distributions of *F. hepatica* recovered from each group of rats in experiments 1, 2, 3 and 4. Each distinct hatch pattern represents *F. hepatica* recovered from an individual rat. (a) experiment 1, group A rats; (b) experiment 1, group B rats; (c) experiment 2, group A rats; (d) experiment 2, group B rats; (e) experiment 3, group E rats; (f) experiment 4, group A rats.


(a)
no. F.hepatica
F. hepatica length midpoint (mm)
(b)
no. F. hepatica
F. hepatica length midpoint (mm)
(c)
no. F. hepatica
F. hepatica length midpoint (mm)
(d)
no. F. hepatica
F. hepatica length midpoint (mm)
(e)
no. F. hepatica
F. hepatica length midpoint (mm)
(f)
no. F. hepatica
F. hepatica length midpoint (mm)

6.5.3.2 *Experiment 2*

The growth of *H. diminuta* and *F. hepatica* in rats carrying patent *H. diminuta* infections at the time of infection with *F. hepatica*, was studied in experiment 2. The median total dry weight of *H. diminuta* per rat is illustrated with inter-quartile range for the rats from groups B and C in Table 6.12. No significant difference in worm weight was observed between rats with the concurrent *F. hepatica* infection (group B), and those harbouring *H. diminuta* only (group C) (Kruskal-Wallis ANOVA, H = 1.46, d.f.= 2, $p > 0.05$).

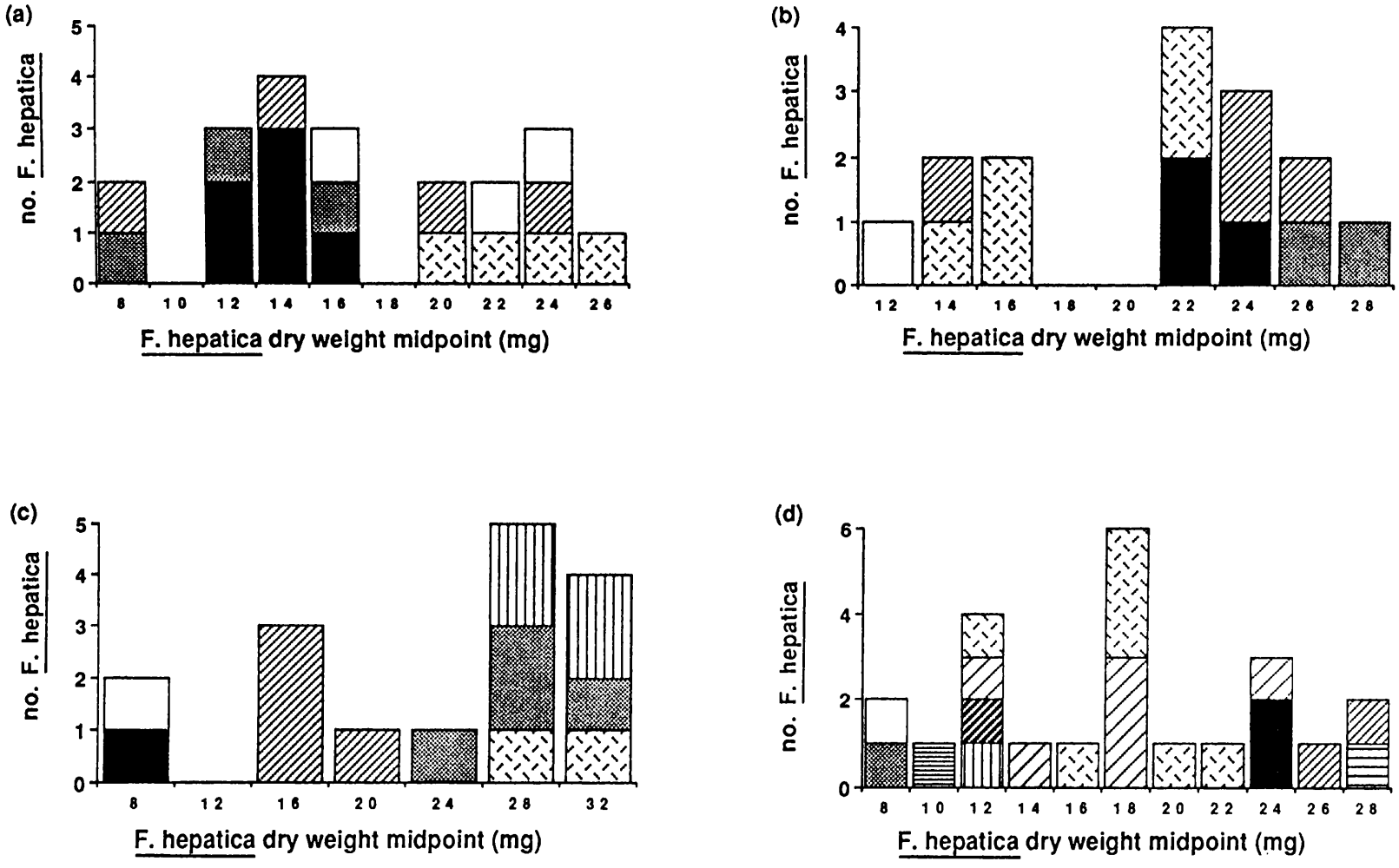
**Table 6.12.** Median total dry weight of *H. diminuta* recovered from the rats in groups B, C and E of experiments 2 and 3, compared using Kruskal-Wallis ANOVA.

| Experimental group (n) | Median *H. diminuta* dry weight (mg) | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| B (6) | 773.8 | 590.5, 880.8 | |
| C (7) | 694.7 | 493.4, 749.6 | 0.483 (NS) |
| E (6) | 690.0 | 404.0, 864.0 | |

As in section 6.5.3.1, histograms of *F. hepatica* length did not appear to be normally distributed, particularly in the case of those recovered from rats of group B (Fig. 6.3 (c) and (d)). The distributions of worm weights were bimodal (Fig. 6.4 (a) and (b)), and different weight categories were often present within the same rat (6.4 (a) and (b)).

No significant difference between the median lengths of *F. hepatica* recovered from rats with concomitant *H. diminuta* infections (group B), and that of those infected with *F. hepatica* only (group A) was found (Table 6.13: Wilcoxon-Mann-Whitney test, W = 321.0, $n_1 = 15$, $n_2 = 20$, $p > 0.05$). Comparison of *F. hepatica* dry weights between rat groups A and B of experiment 2, however, revealed a significant difference in median worm weights, the

**Fig. 6.4.** The dry weight distributions of *F. hepatica* recovered from each group of rats in experiments 2, 3 and 4. Each distinct hatch pattern represents flukes recovered from an individual rat. (a) experiment 2, group A rats; (b) experiment 2, group B rats; (c) experiment 3, group E rats; (d) experiment 4, group A rats.


(a)
no. F. hepatica
0
1
2
3
4
5
8
10
12
14
16
18
20
22
24
26
F. hepatica dry weight midpoint (mg)
(b)
no. F. hepatica
0
1
2
3
4
12
14
16
18
20
22
24
26
28
F. hepatica dry weight midpoint (mg)
(c)
no. F. hepatica
0
1
2
3
4
5
8
12
16
20
24
28
32
F. hepatica dry weight midpoint (mg)
(d)
no. F. hepatica
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
F. hepatica dry weight midpoint (mg)

group with the concomitant *H. diminuta* infection harbouring heavier worms (Table 6.14: Wilcoxon-Mann-Whitney test, $W = 335.0$, $n_1 = 15$, $n_2 = 20$, $p < 0.05$). (Morphometric data on *F. hepatica* worms recovered from rat group E of experiment 3 were not included in a combined analysis, since these worms were 56 days younger than those in groups A and B of experiment 2).

**Table 6.13.** Median length of *F. hepatica* recovered from the rats in groups A and B of experiment 2, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median *F. hepatica* length (mm) | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (20) | 20.35 | 19.23, 21.50 | 0.092 (NS) |
| B (15) | 21.30 | 20.00, 23.60 | |

**Table 6.14.** Median dry weight of *F. hepatica* recovered from the rats in groups A and B of experiment 2, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median *F. hepatica* dry weight (mg) | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (20) | 16.33 | 12.58, 22.05 | 0.032 (S) |
| B (15) | 22.42 | 16.10, 24.82 | |

6.5.3.3 *Experiment 3*

In experiment 3, the growth of *H. diminuta* and *F. hepatica* in rats which were given infections with both species simultaneously, was examined. Statistical analysis detected no significant disparity in total dry weight of *H. diminuta* per rat between the *H. diminuta*-only

control rats (group C), and those which received *F. hepatica* metacercariae simultaneously (group E) (Table 6.12: Kruskal-Wallis ANOVA, $H = 1.46$, d.f.$= 2$, $p > 0.05$).

Morphometric data on *F. hepatica* recovered from the rats of group E were not compared to those from rats of group A, since those worms in the former group were 56 days younger than those in the latter group. Nevertheless, the distributions of *F. hepatica* length and of *F. hepatica* dry weight were skewed (Figs. 6.3 (e) and 6.4 (c), respectively), and the weight histogram resembled a bimodal distribution more than any other (Fig. 6.4 (c)). Unlike the *F. hepatica* length and weight histograms in experiments 1 and 2, however, different size classes of worm rarely occurred within an individual host.

6.5.3.4 *Experiment 4*

In experiment 4, the experimental procedure followed that for rats of groups B and C in experiment 1, and was designed to examine the growth of *H. diminuta* infections in rats harbouring mature *F. hepatica* infections, in greater detail. As this was not possible (see section 6.3.3), certain aspects of *F. hepatica* growth were studied. *Fasciola* length was normally distributed (Fig. 6.3 (f)) but worm weights appeared to fall into different size categories, which often resided within an individual rat (Fig. 6.4 (d)).

The relationship between *F. hepatica* length and dry weight was investigated. Individual *F. hepatica* dry weights were plotted against their corresponding lengths (Fig. 6.5) and, although some scatter of points is evident, there is, nevertheless, a highly significant correlation between the two variables ($R_S = 0.610$, $n = 24$, $p = 0.002$).

6.5.3.5 *Experiment 5*

The growth of *H. diminuta* in rats harbouring 35-day old *F. hepatica* infections, which are believed to be accompanied by an intestinal mastocytosis, was studied in experiment 5. The median length of *H. diminuta* recovered from the rats of groups A and B are shown with inter-quartile ranges in Table 6.15. Although the *H. diminuta* were shorter in those rats which harboured 42-day old *F. hepatica* infections than in those rats which did not, worm

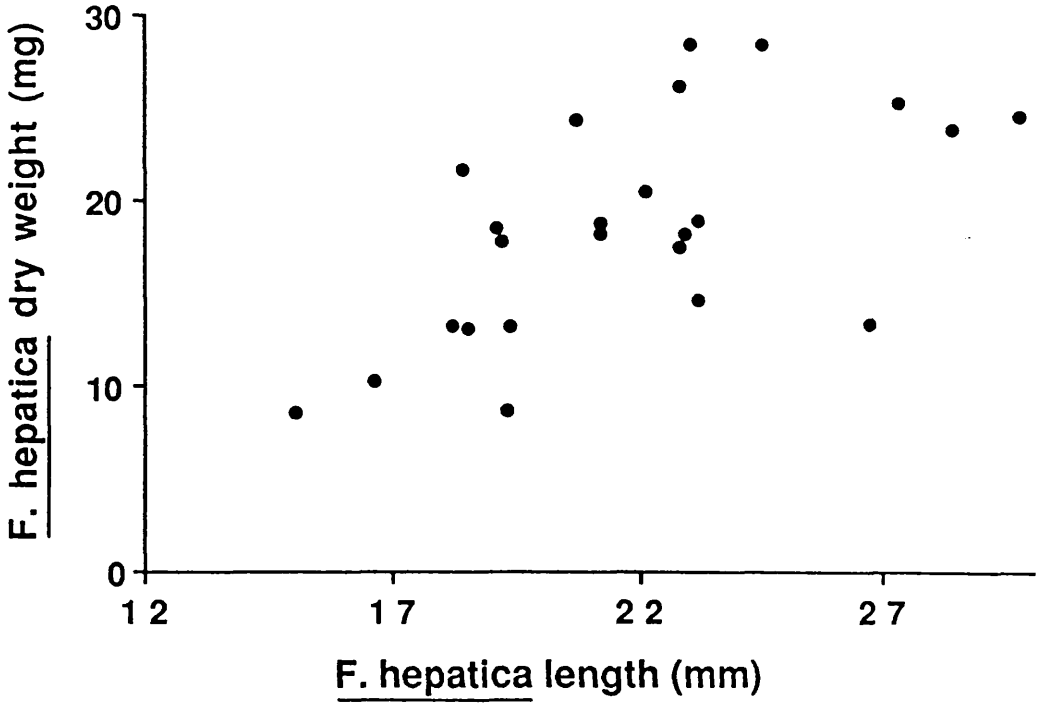



**Fig. 6.5.** Relationship between *F. hepatica* length and *F. hepatica* dry weight in flukes recovered from the rats of group A in experiment 4. Spearman's rank correlation coefficient ($R_S$) = 0.610, n = 24, p = 0.002.

length was not found to be significantly different between the groups (Wilcoxon-Mann-Whitney test, W = 30.0, $n_1$ = 6, $n_2$ = 7, p > 0.05). No significant difference was detected between median total dry weight of *H. diminuta* recovered from the same groups of rats (Table 6.16: Wilcoxon-Mann-Whitney test, W = 38.0, $n_1$ = 6, $n_2$ = 7, p > 0.05).

**Table 6.15.** Median length of *H. diminuta* recovered from the rats in groups A and B of experiment 5, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median length of *H. diminuta* (mm) | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (6) | 33.00 | 17.25, 43.25 | 0.100 (NS) |
| B (7) | 46.00 | 38.50, 48.00 | |

**Table 6.16.** Median dry weight of *H. diminuta* recovered from the rats in groups A and B of experiment 5, compared using Wilcoxon-Mann-Whitney test.

| Experimental group (n) | Median dry weight of *H. diminuta* (mg) | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (6) | 8.01 | 1.04, 10.65 | 0.617 (NS) |
| B (7) | 7.25 | 4.63, 11.91 | |

### 6.5.4 Eggs per gram faeces (e.p.g.) as a measurement of worm burden

#### 6.5.4.1 *Experiment 1*

Egg counts obtained from faecal samples collected at the end of the experiment (day 124) were used to investigate the relationship between e.p.g. and worm burden for the 2 species of helminth. The number of *H. diminuta* recovered did not appear to be related to egg output for those in rats of group B or for those in rats of group C (see Table 6.17). Furthermore, the total dry weight of *H. diminuta* worms recovered from each rat was not

found to be associated with e.p.g. for either group of rats (see Table 6.18).

**Table 6.17.** Spearman's rank correlation coefficients for *H. diminuta* e.p.g. against *H. diminuta* numbers, for the rats in each group of experiments 1 and 4.

| Experiment | Experimental group (n) | $R_S$ | Significance (p value) |
|---|---|---|---|
| 1 | B (7) | 0.000 | > 0.50 (NS) |
| | C (7) | -0.449 | > 0.20 (NS) |
| 4 | A (9) | 0.192 | > 0.50 (NS) |
| | B (6) | -0.435 | > 0.20 (NS) |

**Table 6.18.** Spearman's rank correlation coefficients for *H. diminuta* e.p.g. against *H. diminuta* dry weight, for the rats in each group of experiments 1 and 4.

| Experiment | Experimental group (n) | $R_S$ | Significance (p value) |
|---|---|---|---|
| 1 | B (7) | 0.382 | > 0.20 (NS) |
| | C (7) | 0.179 | > 0.50 (NS) |
| 4 | A (9) | 0.603 | > 0.05 (NS) |
| | B (6) | 0.486 | > 0.20 (NS) |

No significant correlation was observed between the number of *F. hepatica* recovered and *F. hepatica* e.p.g., in the rats from groups A and B (see Table 6.19). For group A rats, however, the associated probability between mean *F. hepatica* length and *F. hepatica* egg output was less than 0.05 (see Table 6.20). Furthermore, all correlation coefficients associated with *F. hepatica* e.p.g. and worm number or size were positive and of considerable magnitude. (Mean *F. hepatica* length was used since total *F. hepatica* length of all worms per rat combined, is a rather meaningless concept).

**Table 6.19.** Spearman's rank correlation coefficients for *F. hepatica* e.p.g. against *F. hepatica* numbers, for the rats in groups A and B of experiment 1.

| Experimental group (n) | $R_S$ | Significance (p value) |
|---|---|---|
| A (7) | 0.400 | > 0.20 (NS) |
| B (7) | 0.505 | > 0.20 (NS) |

**Table 6.20.** Spearman's rank correlation coefficients for *F. hepatica* e.p.g. against mean *F. hepatica* length, for the rats in groups A and B of experiment 1.

| Experimental group (n) | $R_S$ | Significance (p value) |
|---|---|---|
| A (7) | 0.857 | < 0.05 (S) |
| B (7) | 0.714 | = 0.10 (NS) |

6.5.4.2 *Experiment 4*

The relationship between *H. diminuta* worm burden and egg production on the last day of the experiment was investigated. No significant relationship between *H. diminuta* e.p.g. and worm number could be detected statistically for infections in the rats of groups A and B (see Table 6.17). In addition, there was no detectable relationship between *H. diminuta* e.p.g. and total worm dry weight, again for either group (see Table 6.18).

Since 5 of the 10 rats in group A harboured only 1 *F. hepatica* worm, the data were not considered useful for any analysis of the relationship between egg count and worm burden.

### 6.5.5 Density-dependent effects on worm burden

In each experiment, the influence of worm burden on individual worm size was examined. No significant correlation between mean *H. diminuta* dry weight and the number of

worms recovered was observed in any group of rats of any experiment, with the exception of the rats from group B of experiment 4, for which the associated correlation coefficient had a probability of less than 0.05 (see Table 6.21). It is perhaps worth noting, however, that 4 of the correlation coefficients were negative and rather high in value.

**Table 6.21.** Spearman's rank correlation coefficients for mean *H. diminuta* dry weight against the total number of *H. diminuta* recovered, for the rats in each group of experiments 1, 2, 3 and 4.

| Experiment | Experimental group (n) | $R_s$ | Significance (p value) |
|---|---|---|---|
| 1 | B (7) | 0.315 | > 0.20 (NS) |
| | C (7) | -0.655 | > 0.10 (NS) |
| 2 | B (6) | -0.771 | > 0.10 (NS) |
| | C (7) | -0.071 | > 0.50 (NS) |
| 3 | E (6) | 0.555 | > 0.20 (NS) |
| 4 | A (9) | -0.570 | > 0.10 (NS) |
| | B (6) | -0.928 | < 0.05 (S) |

The above procedure was repeated for *F. hepatica*, using mean *F. hepatica* length per worm versus the number of worms recovered for experiment 1 (since weight data were not available), and mean *F. hepatica* weight per worm against the number of worms harvested for the remaining experiments. There was clearly no evidence for density-dependent effects operating for *F. hepatica* since no significant relationship was observed between mean worm size and the number of worms recovered in almost all cases (see Table 6.22). Experiment 4 provided the exception, with a positive correlation occurring between the number of worms recovered and mean weight per worm.

**Table 6.22.** Spearman's rank correlation coefficients for mean *F. hepatica* length (experiment 1) or mean *F. hepatica* weight (experiments 2, 3 and 4) against the total number of *F. hepatica* recovered, for the rats in each group of experiments 1, 2, 3 and 4.

| Experiment | Experimental group (n) | $R_s$ | Significance (p value) |
|---|---|---|---|
| 1 | A (7) | 0.309 | > 0.50 (NS) |
| | B (7) | 0.204 | > 0.50 (NS) |
| 2 | A (5) | 0.053 | > 0.50 (NS) |
| | B (5) | 0.000 | > 0.50 (NS) |
| 3 | E (6) | 0.555 | > 0.20 (NS) |
| 4 | A (10) | 0.728 | < 0.05 (S) |

### 6.5.6 Density-dependent effects on worm egg output

#### 6.5.6.1 *Experiment 1*

Egg count data from day 124 of the experiment were used to examine egg production per unit quantity of worm tissue at the various infection intensities observed. No significant correlation was detected between the number of *H. diminuta* recovered and the number of eggs produced per worm at the end of the experiment for rats in groups B or C (see Table 6.23). A significant relationship between the total dry weight of *H. diminuta* per rat and egg production per gram of *H. diminuta* tissue was, however, observed between *H. diminuta* egg output per gram of tissue and total *H. diminuta* dry weight for rat group B (see Table 6.24). In all cases the correlation coefficients were negative and of considerable magnitude.

**Table 6.23.** Spearman's rank correlation coefficients for the number of eggs produced per worm against the numbers of *H. diminuta* recovered, for the rats in each group of experiments 1 and 4.

| Experiment | Experimental group (n) | $R_S$ | Significance (p value) |
|---|---|---|---|
| 1 | B (7) | -0.606 | > 0.10 (NS) |
| | C (7) | -0.767 | > 0.05 (NS) |
| 4 | A (9) | -0.843 | < 0.01 (S) |
| | B (6) | -0.986 | < 0.02 (S) |

**Table 6.24.** Spearman's rank correlation coefficients for *H. diminuta* egg production per gram of tissue against total *H. diminuta* dry weight, for the rats in each group of experiments 1 and 4.

| Experiment | Experimental group (n) | $R_S$ | Significance (p value) |
|---|---|---|---|
| 1 | B (7) | -0.837 | < 0.05 (S) |
| | C (7) | -0.679 | > 0.10 (NS) |
| 4 | A (9) | -0.833 | = 0.01 (S) |
| | B (6) | -0.771 | < 0.05 (S) |

This tendency for *H. diminuta* egg production to be inversely related to total worm burden was examined further using linear regression analysis. Specifically, the elevations and slopes of the regression lines were compared between infection in rats of treatment groups B and C, in order to investigate whether the elevated *H. diminuta* egg production associated with those rats with concurrent *F. hepatica* infections (section 6.4.1.1) had any influence on the observed density dependence.

Regression lines for the scatter plots of *H. diminuta* egg production per worm against the number of worms recovered, are illustrated for rat groups B and C in Fig. 6.6 (a). The regression line for those rats which harboured concomitant mature *F. hepatica* infections

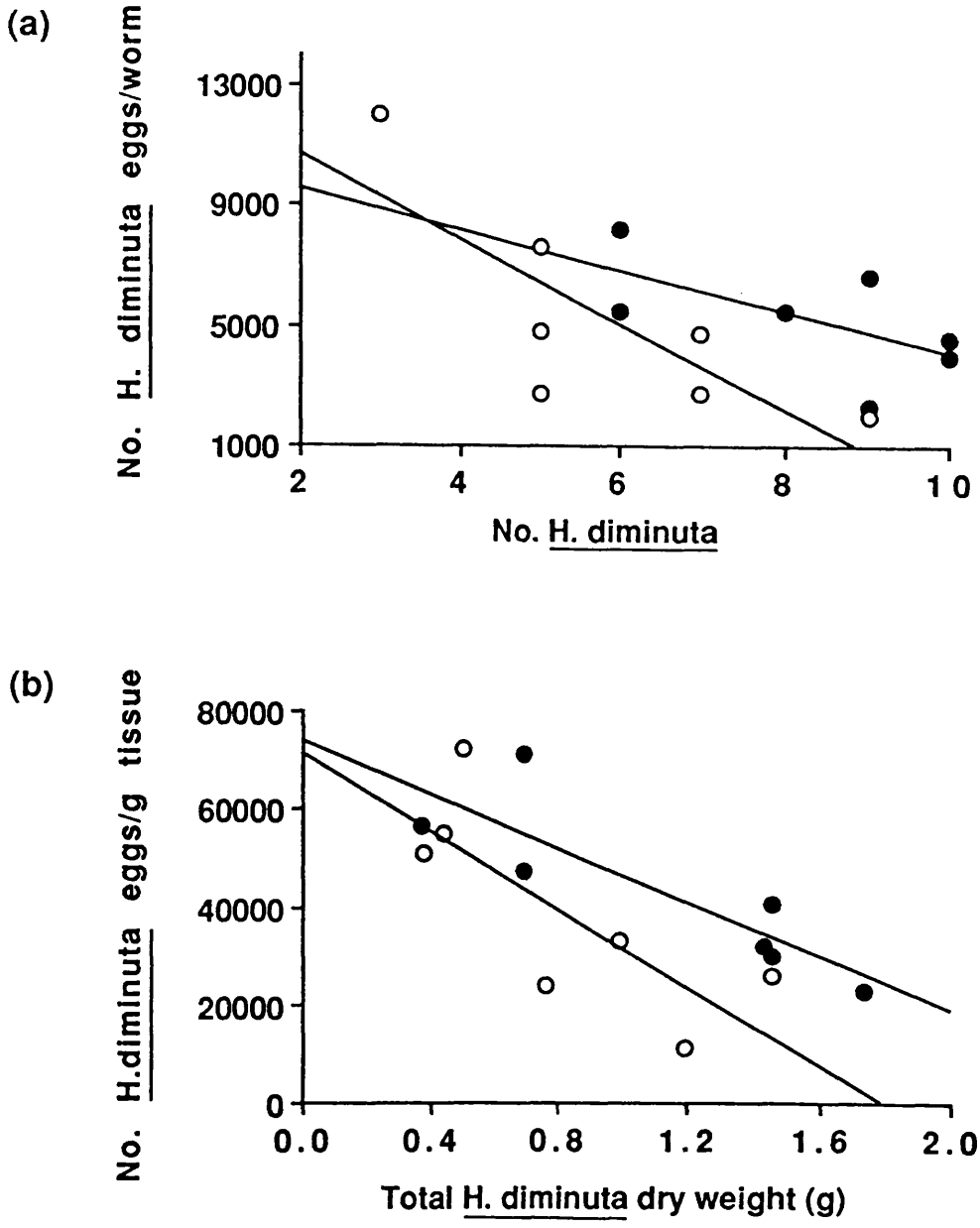



Fig. 6.6. Comparison of density-dependent effects on *H. diminuta* fecundity in the rats of groups B and C in experiment 1. Group B rats harboured 74-day old *F. hepatica* infections (10 metacercariae/rat) at the time of infection with *H. diminuta* (10 cysticercoids/rat); group C rats were infected with *H. diminuta* only (10 cysticercoids/rat). ( ● ) group B rats; ( ○ ) group C rats. (a) Regression lines for the scatter plots of *H. diminuta* egg production per worm versus the number of worms recovered; ANCOVA, $F_{slope} = 1.31$, d.f.= 1, 10, $p > 0.05$; $F_{height} = 3.82$, d.f.= 1, 10, $p > 0.05$. (b) Regression lines for the scatter plots of *H. diminuta* egg production per gram of worm tissue versus total *H. diminuta* dry weight; ANCOVA, $F_{slope} = 0.61$, d.f. = 1, 10, $p > 0.05$; $F_{height} = 4.05$, d.f.= 1, 10, $p > 0.05$.

(group B) is more elevated than that of those harbouring *H. diminuta* only (group C). However, no significant difference between the slopes, or between the elevations, of the regression lines was found (Analysis of covariance; $F_{slope} = 1.31$, d.f.=1,10, $p > 0.05$; $F_{height} = 3.82$, d.f.= 1,10, $p > 0.05$).

Plots of *H. diminuta* egg output per gram of tissue versus total *H. diminuta* weight for groups B and C, are shown with corresponding regression lines in Fig. 6.6 (b). These regression lines exhibit a similar pattern to that illustrated in Fig. 6.6 (a), the height of the line being greater in the rats harbouring mixed species infections (group B). Yet again, however, the regression lines did not differ significantly from each other in terms of either slope or elevation (Analysis of covariance; $F_{slope} = 0.61$, d.f.= 1, 10, $p > 0.05$; $F_{height} = 4.05$, d.f.= 1, 10, $p > 0.05$).

In the case of *F. hepatica*, the correlation coefficients suggest no obvious relationship between the number of eggs produced per worm and the number of worms recovered, for rat group A or for rat group B (see Table 6.25). There was, also, no clear association between eggs produced per mm length of worm tissue and mean *F. hepatica* length per rat for either group (see Table 6.26), although a significant and positive correlation coefficient was observed in group A rats.

**Table 6.25.** Spearman's rank correlation coefficients for the number of eggs produced per *F. hepatica* against the number of *F. hepatica* recovered, for the rats in groups A and B of experiment 1.

| Experimental group (n) | $R_s$ | Significance (p value) |
|---|---|---|
| A (7) | 0.200 | > 0.50 (NS) |
| B (7) | -0.348 | > 0.20 (NS) |

**Table 6.26.** Spearman's rank correlation coefficients for the number of eggs produced per mm length of *F. hepatica* tissue against mean *F. hepatica* length, for the rats in groups A and B of experiment 1.

| Experimental group (n) | $R_S$ | Significance (p value) |
|---|---|---|
| A (7) | 0.857 | < 0.05 (S) |
| B (7) | -0.371 | > 0.20 (NS) |

6.5.6.2 *Experiment 4*

Egg count data from the last day of the experiment were used to study possible density-dependent effects on *H. diminuta* egg production. Egg production per *H. diminuta* was negatively correlated with the number of worms recovered for the rats in groups A and B (see Table 6.23). It should be noted, however, that the relationship detected for the infections in the rats of group B was curved and not linear.

A similar pattern was observed between *H. diminuta* egg output per gram of tissue and total worm weight. In rats of both experimental groups, the calculated correlation coefficients were negative and significant (see Table 6.24). Density-dependent effects involving *F. hepatica* were not examined because of the very low diversity in helminth intensities.

## 6.6 DISCUSSION

Under the experimental conditions used and doses administered, a mature primary infection of *Fasciola hepatica* (Digenea) in outbred Wistar rats does not appear to be associated with any resistance to oral challenge with *Hymenolepis diminuta* (Eucestoda). No evidence was found to suggest that *Hymenolepis* intensity, total dry weight or fecundity were impaired when the cysticercoids were administered to rats with patent *Fasciola* infections. This conclusion applies when the *F. hepatica* infection is given after the *H. diminuta*

infection, or if both species are administered simultaneously.

This finding may seem rather surprising, since infection with *F. hepatica* has been associated with an apparent resistance of mice to challenge with *Schistosoma mansoni* (Christensen, Nansen, Frandsen, Bjorneboe & Monrad, 1978), and of rats to challenge with *Nippostrongylus brasiliensis* (Goose, 1977) and the metacestode stage of *Taenia taeniaeformis* (Campbell, Kelly & Martin, 1979). Furthermore, partial expulsion of mature *Hymenolepis microstoma* infections was detected in mice following challenge with *F. hepatica* (Lang, 1967).

Indeed, in experiment 1, significantly more *H. diminuta* worms were recovered from those rats with patent *F. hepatica* infections, and this was probably responsible for the elevated *H. diminuta* egg production observed in these rats. In these experiments, it is impossible to determine whether enhanced worm establishment, improved worm survival or a combination of these accounts for the increased *H. diminuta* recovery, but it clearly may signify some type of synergistic interaction with the *F. hepatica* infection.

There are numerous reports of synergistic interactions between helminth species (see Christensen, Nansen, Fagbemi & Monrad, 1987), which are manifested by increased establishment, delayed expulsion, impaired natural resistance, as well as improved growth and fecundity. The survival of *H. diminuta* in mice is considerably enhanced by prior infection with *Nematospiroides dubius* (= *Heligmosmoides polygyrus*) (see Hopkins, 1980) and inhibition of host (sheep) resistance to *Haemonchus contortus* coinciding with the concurrent administration of an *F. hepatica* infection has been demonstrated by Presidente, Knapp & Nikol (1973). In the latter study, faecal egg counts and the duration of patency of *H. contortus* was greater in the dual infections. It is not possible to determine whether the increase in *H. contortus* egg output resulted from greater egg production per worm, as faecal collections were discontinued before the experiment's completion. The authors did assert, however, that establishment of both parasites in the concurrent infections was comparable to that of the respective single infection controls, although the complete data set was not disclosed. An increase in individual worm egg production is likely to explain the higher

faecal egg counts reported. This is in contrast to results obtained from experiment 1 of the present study, where the significantly greater median *H. diminuta* egg count in rats harbouring a concurrent *F. hepatica* infection, appeared to be at least partly attributable to a greater overall worm burden.

The explanation of the observed improved establishment or survival of *Hymenolepis* infections in the presence of *Fasciola* merits further consideration and study. The immunosuppression observed to accompany *Hel. polygyrus* infections in mice, and *T. spiralis* infections in rats and mice (see Shimp, Crandall & Crandall, 1975; Terry & Hudson, 1982; Ali & Behnke, 1984), may help to elucidate the synergistic effects these species have on the survival of certain other helminths. Hopkins (1980) noted that the survival of *H. diminuta* in mice was substantially improved in the presence of *Hel. polygyrus*, perhaps as a result of a depression in the host's immune response following oral administration of antigens (Shimp *et al.*, 1975). Delayed rejection of *N. brasiliensis* (Wescott & Colwell, 1980) and of *T. spiralis* (Behnke, Wakelin & Wilson, 1978) in mice, also occurred in the presence of *Hel. polygyrus* infections. In addition, concurrent *T. spiralis* infections have been associated with the delay in expulsion of *Hymenolepis nana* in mice (Ferretti, Gabriele, Palmas & Wakelin, 1984) and *Strongyloides ratti* in rats (Moqbel & Wakelin, 1979). An increase in *Schistosoma mansoni* establishment in mice with heavy infections of *Echinostoma revolutum* has been reported (Christensen, Nydal, Frandsen & Nansen, 1981). In this example, suppression of innate resistance mechanisms was thought to accompany heavy *E. revolutum* infections in mice, and to have mediated the synergystic effects on the challenge *S. mansoni* infection observed. Furthermore, mice with late pre-patent *S. mansoni* infections were found to allow greater establishment of *Ascaris suum*, which was explained in terms of high-intensity unspecific stimuli (Bindseil, 1970).

Increased immunotolerance resulting from the concomitant *Fasciola* infection could explain the increase in *H. diminuta* establishment and/or survival, as well as the enhanced *H. diminuta* egg production, in rats. The increase in *H. diminuta* egg production itself need not

be solely attributable to the heavier *Hymenolepis* worm burden. Immunosuppression of the host may directly improve egg production per worm, as observed in trichostrongyle infections of the immunosuppressed lactating ewe (see Connan, 1967; Gibbs, 1967; Jansen, 1967; Arundel & Ford, 1969; O'Sullivan & Donald, 1970). However, unlike the infections described above, *H. diminuta* does not appear to stimulate an immune response detrimental to worm establishment or survival in the rat host at dosing regimes of up to 10 cysticercoids per rat (Hesselberg & Andreassen, 1975). It is difficult to imagine possible improvements to a highly immunotolerant environment. Nevertheless, concomitant infection with *Fasciola hepatica* was believed to interfere with the development of host resistance to *Haemonchus contortus* in sheep (Presidente *et al.*, 1973), suggesting that the concept of *F. hepatica*-induced immunosuppression should not be entirely disregarded.

Enhancement of *H. diminuta* establishment or survival could also operate through certain physiological mechanisms. A study of the energy sources and metabolism of *Fasciola hepatica* in outbred Wistar rats obtained evidence for a greater concentration of glucose in the bile of infected rats compared with uninfected control rats (mean values = 1.9 and 5.2 mg/100 ml, respectively) (Coles, Simkin & Barrett, 1980). Chandler (1942) reported that restriction of dietary carbohydrate resulted in reduced establishment and size of *H. diminuta* worms. By implication, therefore, an increase in dietary carbohydrate, in the form of glucose entering the duodenum via the bile duct, may enhance *Hymenolepis* establishment and growth. This is especially pertinent since *Hymenolepis diminuta* has been known for a long time to be dependent on glucose (or starch) as its principal energy source (Read, 1956; Laurie, 1957).

*Hymenolepis diminuta* was, however, found to be slightly, though not significantly, shorter one week after the administration of the cysticercoids to rats harbouring 35-day old *F. hepatica* infections. In rats, intestinal mast cells (and consequently eosinophils) are known to increase considerably following a primary infection with *F. hepatica* (Doy, Hughes & Harness, 1978; Pfister & Meierhofer, 1986). This increase is believed to peak around day 35 (Doy, Hughes & Harness, 1981; Pfister & Meierhofer, 1986) and is associated with the local

anaphylactic response triggered following challenge *F. hepatica* infections (Doy *et al.*, 1978; Doy *et al.*, 1981). Such a reaction could effect a negative influence on the growth of *H. diminuta*. The administration of *H. diminuta* cysts to *Trichinella spiralis*-infected mice (Behnke, Bland & Wakelin, 1977) or to *N. brasiliensis*-infected rats (Morcock & Roberts, 1976), was accompanied by stunted *H. diminuta* worms, for which the non-specific inflammatory response associated with the expulsion of the nematodes appeared to be responsible. According to Hopkins (1980), there are no studies on interspecific interactions involving *H. diminuta* and nematodes to which specific cross immunity can be attributed. In addition, intestinal inflammation induced by the eggs of patent *S. mansoni* infections in mice enhanced the expulsion of a superimposed *H. diminuta* infection (Andreassen, Odaibo & Christensen, 1990). To determine whether the intestinal mast cell response in immature *F. hepatica* infections in rats actually does restrict *H. diminuta* growth at its initial stages, it would be necessary to repeat the experiment using much larger sample sizes.

*Fasciola hepatica* infections in rats do not appear to be adversely influenced by concomitant infections with *H. diminuta*, whether given before, after, or simultaneously with the latter helminth. Nevertheless, the notable drop in *Fasciola* egg production detected in both rat groups 3 days after the administration of *Hymenolepis* cysticercoids to one of the groups, persisted for 2 weeks in those rats with the dual infections, but recovered quickly in the *F. hepatica*-only controls. This may suggest some form of *H. diminuta*-induced suppression of *Fasciola* egg production, although the problems associated with identifying patterns in such highly variable data cannot be over-emphasised.

Egg production by *S. mansoni* may be suppressed in the presence of concomitant *T. spiralis* infections (Aboul Atta & El-Sheikh, 1981) and *F. hepatica* challenge of sheep harbouring 12-week infections of *Cysticercus tenuicollis* were found to exhibit reduced *Fasciola* establishment and egg excretion (Campbell, Kelly, Townsend & Dineen, 1977). The authors of the latter paper believed this interference to be immunologically mediated, rather than the result of cestode-induced pathological changes in the liver, as fibrosis was less

severe in the immunologically responsive hosts. The reduction in *F. hepatica* egg output, however, was almost certainly a function of reduced worm establishment and size. This differs from the study by Aboul Atta & El-Sheikh (1981) in which the capacity of egg production per pair of *S. mansoni* worms was depressed. *Fasciola* survival and length were apparently unaffected by the challenge *Hymenolepis* infection in the present study, and a reduction in individual *Fasciola* fecundity is most likely to explain lower egg counts overall. Processes through which an impairment of *F. hepatica* egg production may operate are difficult to determine, but *H. diminuta* is known to stimulate a humoral immune response and immunoglobulins have been observed to coat *H. diminuta* in the rat (Befus, 1975). It should be recognised, however, that although no statistically significant difference was detected, slightly fewer and smaller *F. hepatica* were recovered from those rats which were later challenged with *H. diminuta*, and this could be at least partly responsible for the lower egg counts.

The significantly heavier *Fasciola* recovered from those rats which received *H. diminuta* cysticercoids 56 days prior to the *F. hepatica* infection could also signify some form of *H. diminuta*-mediated influence. Although this result appears analogous to that of experiment 1 where a greater number of *Hymenolepis* were discovered in rats which had previously been infected with *Fasciola*, the resemblance is probably superficial. The enhanced *Hymenolepis* establishment or survival was accompanied by an overall greater (though not significant) worm weight. There were, however, fewer *Fasciola* worms in those rats which harboured the heavier worms, which reciprocates with the idea that as the number of worms harboured by a host increases, the mean weight per worm decreases.

Density-dependent effects on individual worm weight and fecundity have been documented for several cestode, nematode and one acanthocephalan species (Keymer, 1982). High population densities have been found to reduce individual worm size or survival of *H. diminuta* in rats (Chandler, 1939; Roberts, 1961; Hesselberg & Andreassen, 1975), *H. microstoma* in mice (Jones & Tan, 1971), *H. nana* in mice (Ghazal & Avery, 1974), *Moniliformis moniliformis* in rats (Holmes, 1961), *Necator americanus* in humans (Hill,

1926), *Ancylostoma duodenale* and *A. caninum* in humans and dogs (Sarles, 1929; Krupp, 1961; Schad, Soulsby, Chowdhury & Gilles, 1975) and *Ascaris lumbricoides* and *A. suum* in humans and pigs (Jorgensen, Nansen, Nielsen, Eriksen & Andersen, 1975; Beaver, 1980; Leikina, Poletaeva & Martsinovsky, 1980). Egg production per unit of worm tissue may also be inversely related to overall worm burden, for example in *Hymenolepis diminuta* in rats (Hesselberg & Andreassen, 1975; Keymer, Crompton & Singhvi, 1983), *H. microstoma* in mice (Jones & Tan, 1971), *H. nana* in mice (Ghazal & Avery, 1974), *A. lumbricoides* in humans (Croll, Anderson, Gyorkos & Ghadirian, 1982; Thein-Hlaing, Than-Saw, Htay-Htay-Aye, Myint-Lwin & Thein-Maung-Mying, 1984), *Trichuris trichiura* in humans (Bundy, Thompson, Cooper, Golden & Anderson, 1985) and hookworm in humans (Anderson & Schad, 1985). Such examples of density-dependence are believed to operate through at least 2 distinct types of process: intraspecific competition for limited nutrients or space, and increased stimulation of the immune system, both as a consequence of the greater worm burden.

Correlations of mean worm weight or length against worm density for both *H. diminuta* and *F. hepatica* for all experiments in the present study did not provide convincing evidence for a negative relationship between the two variables. Although a density-dependent influence on individual worm weight for *H. diminuta* in rats has been clearly demonstrated (Chandler, 1939; Roberts, 1961; Hesselberg & Andreassen, 1975; Chappell & Pike, 1976; Keymer *et al.*, 1983), it is probable that worm burdens were insufficiently variable in the present investigation to isolate this effect. For this very reason, it would be unwise to abandon the contention that high density *F. hepatica* infections in rats may restrict the size of the individual worms.

Another reason for entertaining the possibility that infection intensity may influence *Fasciola* worm weight, concerns the distribution of worm weights within the experimental rat groups. *Fasciola* dry weights frequently exhibited bimodal distributions within experimental groups of rats. Studies by Keymer *et al.* (1983) and Chappell & Pike (1976)

have reported the bimodality of *H. diminuta* worm weights in rats administered relatively high infection doses (> 15 worms). Furthermore, bimodal length distributions were documented from high dosage (100 000 larvae) *Ostertagia* spp. infections in sheep (Dunsmore, 1960).

These phenomena appeared to be mediated by a density-dependent mechanism. In the investigation of *Ostertagia* spp. in sheep, worm length was normally distributed in those sheep receiving only 1000 infective larvae (Dunsmore, 1960), indicating that worms only separate into two distinct size populations at high dosing regimes. Chappell & Pike (1976) suggested that weight loss resulting from the actions of the host's immune response, or from competition for a limiting resource, may account for the bimodality of weight distributions observed in their study on *H. diminuta* in rats. This could explain why different size classes of *Fasciola* worms appeared to reside within individual hosts which, in itself, implies that an obvious inverse relationship between worm burden and worm size is unlikely to be detected. If intraspecific competition results in different size categories occurring within one host, this argues for the likelihood of parasite-mediated density-dependence. (Since individual *H. diminuta* worm weights were not collected during the present studies, the phenomenon was not investigated further for this species).

The influence of *H. diminuta* burden on worm fecundity was more conspicuous. There was a clear tendency for egg production per worm, or per gram of worm tissue, to decrease with increasing worm number, or total worm weight, respectively, as evidenced by the large and negative correlation coefficients (Tables 6.23 and 6.24). It should be emphasised, however, that these relationships were not always found to be statistically significant; larger sample sizes would be needed to establish the relationship.

Density-dependent effects on *H, diminuta* fecundity have been observed in rats harbouring mean worm burdens of between 0.6 and 96.0 (Keymer *et al.*, 1983) and between 1 and 13 (Hesselberg & Andreassen, 1975). In both studies, however, the question of density dependence was specifically addressed, and rats were administered a considerable range of infection doses. In the present study, each rat received only 10 cysticercoids, and all apparent density-dependent effects occurred over the range of infection intensities generated by

differences in worm establishment or survival. This, perhaps, serves to emphasise the sensitivity of *H. diminuta* to relatively small changes in worm density.

There is no evidence from the present experiments, however, to suggest that the fecundity of *Fasciola hepatica* is a function of infection intensity or total worm mass. The variability in infection intensity may have been too low to produce density-dependent effects on worm fecundity. Unlike those for *H. diminuta*, *Fasciola* egg counts appeared to have a positive relationship with worm size (Table 6.20). In any case, the apparent bimodal nature of *F. hepatica* size distributions suggest that density-dependent effects do operate, but these may only be manifest by reduced fecundity per unit worm size at much higher infection intensities.

The very low variability in *Hymenolepis* intensity, together with the acuteness of its density-dependent reaction, help to explain why egg counts were poor predictors of worm burden. Certain recent literature has suggested that e.p.g. counts provide a rough estimate of infection intensity (Bryan & Kerr, 1989; Murrell, Leighton, Boswell & Gasbarre, 1989; Forrester & Scott, 1990). In each of these studies, however, natural infections, which provided a considerable spectrum of infection intensities, were studied. It is probable, therefore, that most faecal egg count procedures are not sensitive enough to detect relatively small differences in worm burden, but are adequate to discriminate gross discrepancies in helminth intensities. Nevertheless, the phenomenon of density-dependence on individual worm fecundity does serve to confound the relationship between e.p.g. and worm burden (Anderson & Schad, 1985), so that predictions of infection intensity from egg count data alone are unlikely to be profitable.

## 6.7 SUMMARY

An investigation into possible interspecific effects between concurrent infections with the digenean, *Fasciola hepatica*, and the eucestode, *Hymenolepis diminuta*, in the rat host was carried out. In the process, population dynamics of the individual species were

examined. The results and conclusions can be summarised as follows:

(1) Patent infection with neither species conferred resistance to oral challenge with the other.

(2) Significantly more *H. diminuta* were recovered from rats with patent *F. hepatica* infections (compared to *H. diminuta*-only control rats), which may signify some type of synergistic interaction. The increased *H. diminuta* establishment or survival was probably responsible for the elevated egg counts and greater total worm weights recorded in these rats, and could have resulted from (as yet unestablished) immunosuppression associated with the concurrent *F. hepatica* infection, or from the increase in bile glucose symptomatic of patent *F. hepatica* infections in rats, although there are other possible explanations.

(3) Rather stunted week-old *H. diminuta* were, however, recovered when the cysticercoids were administered to rats with 35-day old *F. hepatica* infections. The extensive intestinal mast cell response believed to accompany *F. hepatica* infections in rats at that stage, may have been responsible for this example of poor growth in *H. diminuta*.

(4) A slight reduction in *F. hepatica* egg output was recorded in rats which were challenged with *H. diminuta*, and may have been a consequence of *H. diminuta*-induced depression in *Fasciola* fecundity, or a result of the slightly, but not significantly, lower number of *F. hepatica* recovered from these rats compared to the single infection controls.

(5) Heavier *Fasciola* were recovered from rats which received *H. diminuta* prior to the *F. hepatica* infection. This was not, however, believed to be analogous with the result obtained for *H. diminuta* (2), but was thought to be a consequence of certain density-dependent mechanisms. The observation that *Fasciola* weight distributions were bimodal in character, a phenomenon which often typifies density-dependence, reinforced this contention.

(6) *Hymenolepis* burden was not found to impose constraints on worm size within the limited range of infection intensities of these experiments.

(7) *H. diminuta* egg production was, however, found to be very sensitive to relatively small increases in overall worm quantity.

(8) Density-dependent depression of *H. diminuta* fecundity, in conjunction with low

variability of infection intensities, was thought to account for the lack of any relationship detected between *H. diminuta* burden and e.p.g.

(9) Egg output by individual *Fasciola* did not appear to be impaired by the small increases in intensity which occurred in these experiments, and mean *Fasciola* length was positively, though weakly, related to total e.p.g.

# PART 2

## CHAPTER 7: CONCURRENT INFECTIONS INVOLVING *FASCIOLA HEPATICA* AND *HYMENOLEPIS DIMINUTA* IN RATS: EFFECTS ON THE HOST

## 7.1 INTRODUCTION

A characteristic feature of helminth infections involving a tissue-invasive stage in mammalian hosts, is the disproportionate increase in the blood and tissues of the host of the polymorphonuclear leucocyte, the eosinophil (Sanderson, Campbell & Young, 1988). Eosinophils, along with neutrophils, basophils and mast cells, are granulocytic cells, all of which contain granules with histological staining qualities peculiar to each cell type (Roitt, Brostoff & Male, 1985). The granules of the eosinophil comprise an arginine-rich major basic protein (MBP) (Gleich, Loegering & Maldonado, 1973; Gleich, Loegering, Keuppers, Bajaj & Mann, 1974) which may represent as much as 50% of the granule protein (Butterworth, 1984), the eosinophil cationic protein (ECP), also rich in arginine (Olsson & Venge, 1974; Olsson, Venge, Spitznagel & Lehrer, 1977), and a variety of more minor proteins (Butterworth, 1984). The basic nature of these granules is responsible for their strong affinity with acid dyes such as eosin.

The biological role of the eosinophil was rather obscure until relatively recently (Sanderson *et al.*, 1988). However, one important function of the cell concerns its ability to neutralize many mast cell products, such as histamine (Butterworth, 1984), platelet activating factor (Benveniste, 1974) and heparin (Butterworth, 1984), released during IgE-dependent degranulation in immediate hypersensitivity reactions (Butterworth, 1984). Upon degranulation, mast cells release certain mediators which actively recruit eosinophils to the site of the reaction where degradation or neutralization of these products occurs (Butterworth, 1984). Mediators responsible for this recruitment include eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay & Austen, 1971; Kay, Stechschulte & Austen, 1971), histamine (Clark, Gallin & Kaplan, 1975) and its oxidative metabolite, imidazole acetic acid (Turnbull & Kay, 1976), certain lypoxygenase and cyclooxygenase derivatives of arachidonic acid (Goetzl & Gorman, 1978) as well as some uncharacterized polypeptides of intermediate molecular weight (Boswell, Austen & Goetzl, 1978). This series of events may have evolved as part of the host's defence against relatively large, metazoan, tissue-invasive parasites (Butterworth, 1977; McLaren, 1980), with the eosinophil

employed principally as an effector cell in antibody-dependent, cell-mediated immunity to reinfection (Glauert, Butterworth, Sturrock & Houba, 1978). This hypothesis contends that the invading helminth stimulates an increase in the number of circulating eosinophils along with a local antigen-induced IgE-dependent degranulation of mast cells. The release of certain mast cell mediators serves to localize the eosinophils at the site of the infection, and also to enhance the ability of eosinophils, in the presence of antibody or complement, to initiate destruction of the helminth. Furthermore, eosinophils may then inactivate mast cell mediators and inhibit further mast cell degranulation.

Evidence for the involvement of the eosinophil in immune damage to helminths is well-documented in the case of schistosomula of *Schistosoma mansoni* (see, for example, Butterworth, Sturrock, Houba, Mahmoud, Sher & Rees, 1975; Butterworth, David, Franks, Mahmoud, David, Sturrock & Houba, 1977; McLaren, Mackenzie & Ramalho-Pinto, 1977; Glauert *et al.*, 1978, Capron, Rosseaux, Mazingue, Bazin & Capron, 1978; Kazura, Fanning, Blumer & Mahmoud, 1981; Veith, Pestel, Loiseau, Capron & Capron, 1985), and has also been presented in studies on *Trichinella spiralis* larvae (Grover, Butterworth, Sturrock & Bass, 1983), microfilariae of *Onchocerca volvulus* (Greene, Taylor & Aikawa, 1981) and infective larvae of *Dictyocaulus viviparus* (Knapp & Oakley, 1981).

In the *S. mansoni* schistosomulum system, eosinophils have been found to adhere to the antibody- (IgG (Anwar & Kay, 1977) and IgE (Capron, Bazin, Joseph & Capron, 1981)) or complement- (C3b and C3d (Anwar & Kay, 1977)) coated helminth, and degranulate on its surface (McLaren *et al.*, 1977; Glauert *et al.*, 1978; Caulfield, Korman, Butterworth, Hogan & David, 1980). This results in the gradual destruction of the syncytial membrane which is then phagocytosed by eosinophils which have not degranulated (Glauert *et al.*, 1978). Such damage may be attributable to the toxic nature of the granule contents, particularly MBP (Butterworth, Wassom, Gleich, Loegering & David, 1979) and ECP (Mclaren, McKean, Olsson, Venge & Kay, 1981).

Despite its ability to stimulate an eosinophilia in the rat *in vivo*, however, studies on

*Mesocestoides corti in vitro* did not show that eosinophils either adhered to or damaged the metacestodes in the presence of specific anti-sera, complement or granulocyte stimulating agents (Cook, Ashworth & Chernin, 1988).

In the case of *Fasciola hepatica*, rat eosinophils have been demonstrated to adhere to juvenile flukes *in vitro* (Goose, 1978; Doy, Hughes & Harness, 1980; Duffus & Franks, 1980). A similar study by Glauert, Lammas & Duffus (1985), however, found bovine eosinophils to be comparatively ineffective at causing damage to juvenile flukes, for which the presence of a protective antigen/antibody layer on the parasite's surface appeared to be responsible. This precipitate is believed to be generated by the rapid turnover of the highly antigenic outer glycocalyx (Hanna, 1980; Duffus & Franks, 1981), a feature which is absent from *S. mansoni* schistosomula (Glauert *et al.*, 1985).

Nevertheless, both rats (Hayes, Bailer & Mitrovic, 1973; Hughes Harness & Doy, 1976) and cattle (Ross, 1967) are known to develop a resistance to secondary infection with *F. hepatica*. Elevated intestinal eosinophil and mast cell numbers (Doy, Hughes & Harness, 1978; Doy, Hughes & Harness, 1981; Pfister & Meierhofer, 1986), as well as slightly higher goblet cell counts (Pfister & Meierhofer, 1986), may be associated with resistance in rats, although it seems more likely that the majority of challenge flukes are destroyed in the peritoneal cavity following penetration of the gut wall (Davies & Goose, 1981; Hughes, Harness & Doy, 1981; Burden, Bland, Hammet & Hughes, 1983). Circumstantial evidence for the involvement of peritoneal eosinophils in rat immunity to secondary infection arose from the studies by Davies & Goose (1981) and Burden *et al.* (1983), in which transmission and scanning electron microscopy was used to investigate the host's response to juvenile *F. hepatica* in secondary infections of rats. Burden *et al.* (1983) studied the early migration of the flukes and discovered that challenge flukes were found to be rapidly coated with antibody whilst still in the gut lumen. A proportion of these appeared unable to penetrate the intestinal mucosa, possibly trapped in antibody-rich mucus. The majority of challenge flukes, however, penetrated the mucosa, shed their antigen/antibody precipitate, perhaps as a consequence of mechanical disturbance or of tegumental surface turnover, but were

rapidly re-coated with antibody upon entering the peritoneal cavity. Flukes were thereafter seen to be encapsulated by host cells, predominantly eosinophils, in the initial stages. Evidence for eosinophil-mediated damage to the flukes included the appearance of flask-shaped pits in the tegument directly beneath recently degranulated eosinophils, followed by the eventual erosion of the entire syncytium.

It may be that the failure of bovine eosinophils to cause significant damage to juvenile *F. hepatica in vitro*, attributed to the presence of a protective layer of antigen/antibody complexes, could be reversed with the addition of certain factors, such as degranulating mast cells, which may be present *in vivo* (Duffus & Franks, 1980; Glauert *et al.*, 1985). Indeed, this may explain why the major basic protein purified from bovine eosinophils was able to elicit damage, and even death, in juvenile *F. hepatica* at relatively low concentrations (Duffus, Thorne & Oliver, 1980). It has also been suggested that the inability of mice to develop substantial protection against secondary *F. hepatica* infections, in marked contrast to rats, may be a function of the relatively poor eosinophil response observed in the former rodent compared to that detected in the latter (Milbourne & Howell, 1990).

In the rat host, *Hymenolepis diminuta* was previously thought not to evoke an immune response (Roberts & Mong, 1968). There is, however, increasing evidence to suggest that an immunologically-mediated expulsion of *H. diminuta* occurs both in secondary infections (Featherston & Copeman, 1990) and in relatively heavy (40-100 cysticercoids) primary infections (Hesselberg & Andreassen, 1975; Hindsbo, Andreassen & Ruitenberg, 1982; Featherston & Copeman, 1990). In addition, the growth of secondary *H. diminuta* infections given to rats 3-10 days after the chemotherapeutic expulsion of a primary infection, is substantially reduced (Hopkins & Andreassen, 1991). The mechanisms behind these events have not been clarified, although a significant intestinal eosinophilia was observed to develop in Wistar rats following the administration of 100 *H. diminuta* cysticercoids until at least day 20 p.i., and appeared to be associated with a concomitant increase in mucosal mast

cells (Hindsbo *et al.*, 1982). In a more recent study, mastocytosis was not observed at 20 days p.i. in rats given only 40 *H. diminuta* cysticercoids, but was shown to have developed by day 30 p.i., to persist until at least day 47 p.i., and to have declined by day 62 p.i. (Featherston & Copeman, 1990). It is also of interest that a blood eosinophilia was detected in a human experimentally infected with *H. diminuta* (Turton, Williamson & Harris, 1975).

Changes in the intestinal eosinophil and mast cell populations may well be associated with *H. diminuta* expulsion (Hindsbo *et al.*, 1982; Featherston & Copeman, 1990). This may operate by a process similar to that postulated for the rejection of *N. brasiliensis* and *T. spiralis* in rats, in which the systemic secretion of rat mast cell protease II (RMCP II) by functionally active mucosal mast cells has been implicated (Woodbury, Miller, Huntley, Newlands, Palliser & Wakelin, 1984). Other mast cell secretions which may be of importance in relation to nematode expulsion include histamine, serotonin, and the arachidonic acid metabolites (particularly prostaglandins and leukotrienes) (see review by Rothwell, 1989). It is thought likely that eosinophils play more of an accessory role in this rejection (Rothwell, 1989). Nevertheless, evidence for the active involvement of eosinophils in the expulsion of *H. diminuta* from mice was presented by Van der Vorst, Dhont, Joris, De Rycke, Cesbron & Capron (1990), who correlated eosinophil peroxidase (EPO) concentrations in the intestinal lumen with the rejection process.

Since the eosinophil may be of considerable importance in both *F. hepatica* and *H. diminuta* infections, blood samples were taken at fortnightly intervals to monitor any gross changes in the levels of eosinophils in the rats of experiments 1, 2 and 3 described in chapter 5. These investigations revealed that the characteristic eosinophilia detected in *F. hepatica*-infected rats was either partially or wholly reduced by combined infection with *H. diminuta*. The results from these experiments, together with the results of experiments designed to investigate this phenomenon in greater detail, are described in this chapter.

## 7.2 OBJECTIVES

The objectives of the experiments described in this chapter were as follows:

(1) To monitor levels of circulating eosinophils in rats infected with *F. hepatica*, with *H. diminuta* and with both species combined (experiments 1, 2 and 3).

(2) Following the discovery that eosinophil numbers in rats with concurrent *F. hepatica/H.diminuta* infections were substantially lower than those of rats harbouring *F. hepatica* only, the possible consequences of this in terms of host resistance to reinfection with *F. hepatica* was investigated (experiment 6). (Experiments 4 and 5 have been discussed in Chapter 6).

(3) To study the observed depression of circulating eosinophil levels in rats with the mixed infections in greater detail, specifically to determine whether it was a consequence of *H. diminuta*-induced suppression of eosinophil production, or whether the cells were being sequestered elsewhere (experiments 7 and 1).

## 7.3 EXPERIMENTAL PROTOCOLS

### 7.3.1 Experiments 1, 2 and 3

Details of the infection regimes used in experiments 1, 2 and 3 have already been described (sections 6.3.1 and 6.3.2), and are summarised in Tables 7.1, 7.2 and 7.3. The rationale behind the time-courses of these experiments concerns their foremost objective, to investigate possible interspecific effects between *F. hepatica* and *H. diminuta* in rats (see section 6.2). In each experiment, blood was sampled and examined every two weeks (section 5.3.1). In the case of experiment 1, blood was initially analysed on Technicon H1 Hematology System as this is reputed to provide extremely accurate differential and absolute blood cell counts. Use of this machine had to be abandoned six weeks after the start of the experiment, however, as the rat blood samples were frequently found to clot before the analysis could be conducted. Clotting occurred despite the use of EDTA-coated vials for the collection of blood. Consequently, for the remainder of experiment 1, and for the

duration of experiments 2 and 3, blood smears were taken and eosinophil numbers expressed as a proportion of 100 leucocytes counted.

At the end of experiment 1, the intestines of each rat were removed and, following recovery of the worms, were processed in order to determine the extent of eosinophil infiltration into the lamina propria (section 5.3.4).

**Table 7.1.** Experimental protocol for experiment 1.

| Rat group (n) | day 0 | day 74 | day 124 |
|---|---|---|---|
| A (7) | 10 *F. hepatica* metacercariae/rat | --- | experiment end |
| B (7) | 10 *F.hepatica* metacercariae/rat | 10 *H. diminuta* cysticercoids/rat | experiment end |
| C (7) | --- | 10 *H. diminuta* cysticercoids/rat | experiment end |
| D (7) | --- | --- | experiment end |

**Table 7.2.** Experimental protocol for experiment 2.

| Rat group (n) | day 0 | day 56 | day 161-2 |
|---|---|---|---|
| A (7) | --- | 10 *F. hepatica* metacercariae/rat | experiment end |
| B (7) | 10 *H. diminuta* cysticercoids/rat | 10 *F. hepatica* metacercariae/rat | experiment end |
| C (7) | 10 *H. diminuta* cysticercoids/rat | --- | experiment end |
| D (7) | --- | --- | experiment end |

**Table 7.3.** Experimental protocol for experiment 3.

| Rat group (n) | day 0 | day 105/6 | day 161-2 |
|---|---|---|---|
| A* (7) | 10 *F. hepatica* metacercariae/rat | experiment end | |
| C (7) | 10 *H. diminuta* cysticercoids/rat | --- | experiment end |
| D (7) | --- | --- | experiment end |
| E (7) | 10 *H. diminuta* cysticercoids + 10 *F. hepatica* metacercariae/rat | --- | experiment end |

* *F. hepatica* infection did not run concurrently with that of group E

### 7.3.2 Experiment 6

The infection procedure used is described in section 5.1.4. On day 0 of the experiment, the rats of two groups of 7 (A and B) each received 10 *F. hepatica* metacercariae. Twenty-nine days later, each rat from group A, together with each rat from a further two groups of 7 (C and D), were given 10 *H. diminuta* cysticercoids. The rats from groups A and B each received a challenge *F. hepatica* infection of 10 metacercariae on day 43 of the experiment. On the same day, each rat from group D, along with each rat from another group of 7 (E), were administered 10 *F. hepatica* metacercariae. The experiment was terminated on day 64 and *post-mortem* examinations were carried out.

For the duration of the experiment, blood films were made every 14 days to monitor the proportion of circulating leucocytes which comprised eosinophils (section 5.3.1.1). When the rats were killed, *F. hepatica* and *H. diminuta* were removed and counted as detailed in section 5.2.1, after which wet and dry weight measurements were taken (section 5.2.2).

The experimental protocol is summarised in Table 7.4.

**Table 7.4.** Experimental protocol for experiment 6.

| Rat group (n) | day 0 | day 29 | day 43 | day 64 |
|---|---|---|---|---|
| A (7) | 10 *F. hepatica* metacercariae/rat | 10 *H. diminuta* cysticercoids/rat | 10 *F. hepatica* metacercariae/rat | exp. end |
| B (7) | 10 *F. hepatica* metacercariae/rat | --- | 10 *F. hepatica* metacercariae/rat | exp. end |
| C (7) | --- | 10 *H. diminuta* cysticercoids/rat | --- | exp. end |
| D (7) | --- | 10 *H. diminuta* cysticercoids/rat | 10 *F. hepatica* metacercariae/rat | exp. end |
| E (7) | --- | --- | 10 *F. hepatica* metacercariae/rat | exp. end |

### 7.3.3 Experiment 7

The infection procedures used are described in section 5.1.4. On day 1 of the experiment, each rat from 4 groups of 7 (A, B, C and D) were given 10 *F. hepatica* metacercariae. Thirteen days later (day 14), the rats from groups A and B each received 10 *H. diminuta* cysticercoids. On day 19, the rats from groups A and C, along with 7 uninfected control rats (group E), were killed and *post-mortem* examinations were carried out. The rats from groups B and D, and a further group of 7 uninfected control rats (F), were killed on day 33 when *post-mortem* examinations were undertaken. The timing of these procedures was chosen to maximize possible discrepancies in eosinophil counts occurring between the experimental groups, since circulating eosinophil levels in *F. hepatica*-infected rats were found to be greatest on and between days 16 and 44 p.i. (see section 7.5.1.3).

Blood was sampled from all experimental rats (section 5.3.1) every 3 or 4 days starting from day 0, and both differential eosinophil counts and absolute white blood cell counts were made. When rats were killed, all worms were recovered and counted (section 5.2.1), and wet and dry weight measurements taken (section 5.2.2). Peritoneal exudate cells

and bone marrow cells were collected from each rat and spun onto glass slides using a cytocentrifuge (sections 5.3.2 and 5.3.3, respectively). After staining, the number of mature or immature eosinophils per 500 nucleated cells was counted (sections 5.3.2 and 5.3.3).

The experimental protocol is summarised in Table 7.5.

**Table 7.5.** Experimental protocol for experiment 7.

| Rat group (n) | day 1 | day 14 | day 19 | day 33 |
|---|---|---|---|---|
| A (7) | 10 *F. hepatica* metacercariae/rat | 10 *H. diminuta* cysticercoids/rat | exp. end | |
| B (7) | 10 *F. hepatica* metacercariae/rat | 10 *H. diminuta* cysticercoids/rat | --- | exp. end |
| C (7) | 10 *F. hepatica* metacercariae/rat | --- | exp. end | |
| D (7) | 10 *F. hepatica* metacercariae/rat | --- | --- | exp. end |
| E (7) | --- | --- | exp. end | |
| F (7) | --- | --- | --- | exp. end |

## 7.4 STATISTICAL ANALYSIS

All cell count data were analysed using standard parametric statistics and were expressed graphically as group means with standard errors. Group means were compared using one-way analysis of variance (ANOVA) (as there was always more than two groups) and, when appropriate, significance located using the Tukey test. A paired t-test was employed to compare cell counts of individual rats between time points.

Since most of the worm count and morphometric data recorded from the experiments described in Chapter 6 were not found to be normally distributed, all worm data described in this chapter were analysed using non-parametric statistics. These data were either presented in tables as group medians with inter-quartile ranges, or illustrated graphically as group

means with standard error bars for clarity.

For the comparison of two or more group medians, the Wilcoxon-Mann-Whitney test or the Kruskal-Wallis analysis of variance were used. When more than two groups were being compared, significance was located using the method for multiple comparisons between treatments detailed in Siegel & Castellan (1988).

The level of significance was taken to be $p < 0.05$. Two-tailed probabilities were used for all statistical analyses. In tables presented in the text, a significant result is denoted by an 'S', and a non-significant result by 'NS'.

## 7.5 RESULTS

### 7.5.1 Eosinophil counts in experiments 1, 2 and 3

#### 7.5.1.1 *Experiment 1*

In this experiment, circulating eosinophil levels in rats harbouring primary patent *F. hepatica* infections when challenged with *H. diminuta* cysticercoids (group B), as well as in rats with the respective single-species infections (groups A and C), and in uninfected control rats (group D), were monitored. In addition, the degree of eosinophil infiltration into the intestinal tissues of these rats was investigated.

The mean number of eosinophils per 100 leucocytes counted from the rats of each group are presented from days 67 to 124 in Fig. 7.1. Although data are available for only one time point (day 67) before the *H. diminuta* infections were administered (see section 7.3.1), eosinophil levels in the rats of groups A and B, which harboured 9.5-week old *F. hepatica* infections, were greater than those from the rats of groups C and D, which were uninfected at this time. These differences were, however, only found to be significant between groups A and D (One-way ANOVA followed by Tukey test; $F_{3,24} = 4.33$, $p < 0.05$).

A significant decrease in circulating eosinophil levels was recorded between days 67 and 77 in the rats from group B, coinciding with the administration of *H. diminuta* cysticercoids to these rats (day 74) (Paired t-test; $t = 3.93$, $n = 7$, $p < 0.01$). This drop was

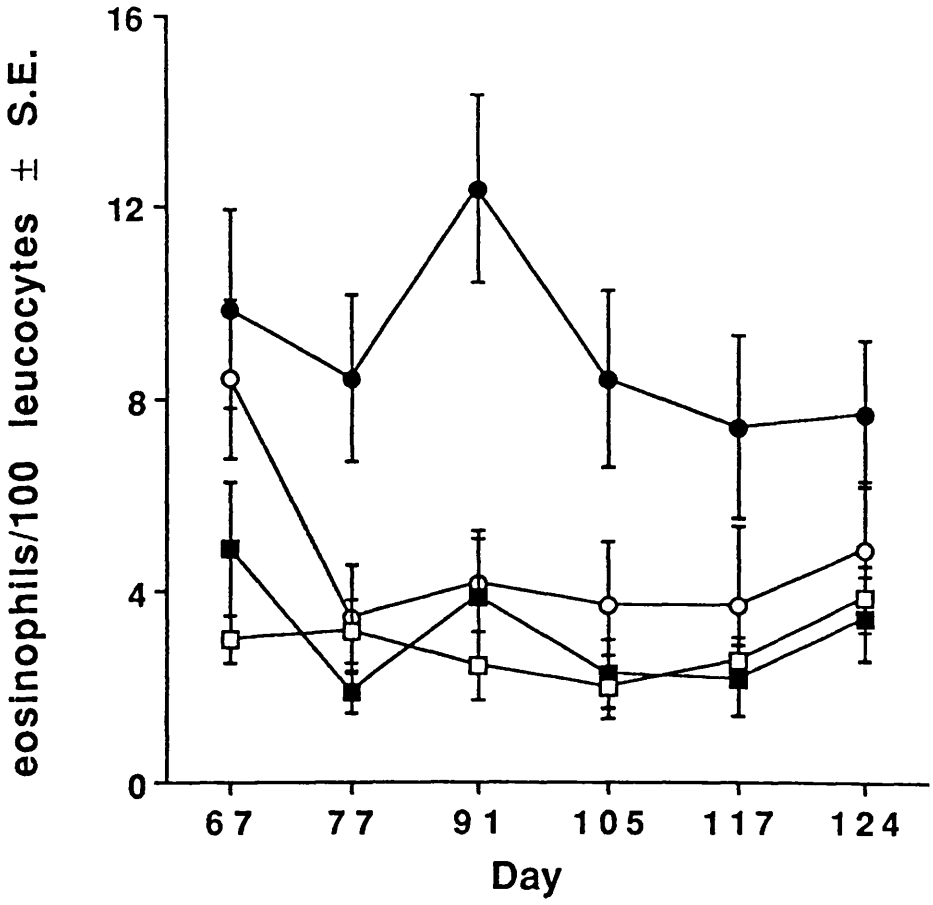



**Fig. 7.1.** Circulating eosinophil levels (mean number/100 leucocytes ± S.E.) in the rats of groups A, B, C and D in experiment 1. Group A rats each received 10 *F. hepatica* metacercariae on day 0; group B rats each received 10 *F. hepatica* metacercariae on day 0 and 10 *H. diminuta* cysticercoids on day 74; group C rats each received 10 *H. diminuta* cysticercoids on day 74; group D rats were kept as uninfected controls. ( ● ) group A rats; ( ○ ) group B rats; ( ■ ) group C rats; ( □ ) group D rats.

not observed in the rats of group A, which did not receive the challenge *H. diminuta* infection (Paired t-test; $t = 0.58$, $n = 7$, $p > 0.05$). Although no significant change in circulating eosinophils was found in the rats of groups C and D over the same time interval (Paired t-test; $t = 2.07$, $n = 7$, $p > 0.05$; $t = -0.31$, $n = 7$, $p > 0.05$; respectively), levels were observed to drop in group C rats following infection with *H. diminuta* (Fig. 7.1).

On days 77 and 91 of the experiment, the rats from group A exhibited a significant eosinophilia compared to all other rat groups, which did not differ significantly from one another (One-way ANOVA followed by Tukey test; $F_{3,24} = 6.78$, $p < 0.003$; $F_{3,24} = 11.34$, $p < 0.0005$; respectively). Thereafter, eosinophil counts in group A rats were observed to decrease gradually as the experiment progressed. By day 105, a significant difference was detected between the rats of groups A and C, and groups A and D, only (One-way ANOVA followed by Tukey test; $F_{3,24} = 5.76$, $p < 0.005$), and on day 117, was observed between group A and C rats only (One-way ANOVA followed by Tukey test; $F_{3,24} = 3.21$, $p < 0.05$). At the end of the experiment, no significant difference between the levels of circulating eosinophils was found between the groups (One-way ANOVA; $F_{3,24} = 2.62$, $p > 0.05$).

The number of eosinophils in the middle and posterior thirds of the small intestines of the rats in groups A, B, C and D, are shown with standard error bars in Fig. 7.2. Although eosinophil counts are slightly higher in the middle third of the intestines of group A rats, no significant difference was detected between the different groups of rats (One-way ANOVA; $F_{3,24} = 1.66$, $p > 0.05$). In the case of the posterior thirds of the intestines, however, eosinophil levels were considerably higher in the rats of group A, which harboured *F. hepatica* infections only, compared with those rats with concurrent *F. hepatica*/*H. diminuta* infections (group B), with *H. diminuta*-only infections (group C) and with uninfected control rats (group D), which did not differ from each other (One-way ANOVA followed by Tukey test; $F_{3,24} = 8.45$, $p = 0.001$).

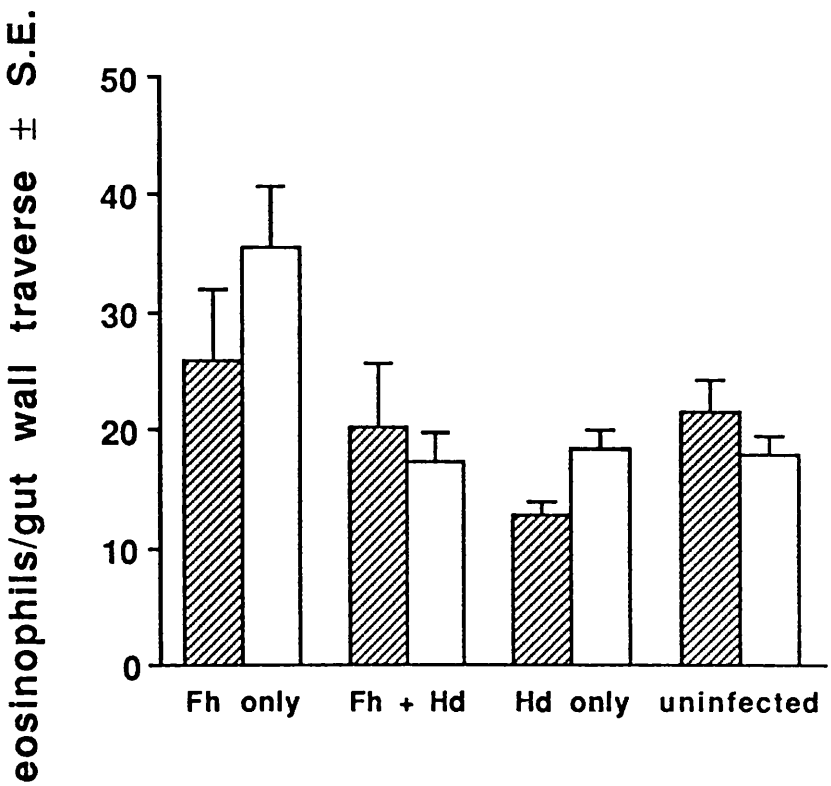



**Fig. 7.2.** Number of eosinophils (mean number/gut wall traverse ± S.E.) in the middle and posterior thirds of the small intestines of rats in the 4 groups of experiment 1. **Fh only** (group A rats): each rat received 10 *F. hepatica* metacercariae on day 0; **Fh + Hd** (group B rats): each rat received 10 *F. hepatica* metacercariae on day 0 and 10 *H. diminuta* cysticercoids on day 74; **Hd only** (group C rats): each rat received 10 *H. diminuta* cysticercoids on day 74; **uninfected** (group D rats): each rat was kept as an uninfected control. (▨) middle third; (□) posterior third.

7.5.1.2 *Experiment 2*

The effect of a prior, patent infection of *H. diminuta* on the development of a circulating eosinophilia following challenge with *F. hepatica*, was investigated in this experiment. The mean number of eosinophils per 100 leucocytes counted are illustrated with standard error bars for the rats of groups A, B, C and D from day 0 to day 127 in Fig. 7.3. From day 0 until day 58, no significant disparity between eosinophil counts in the different groups of rats was discovered (One-way ANOVA; $F_{3,24}$= 1.70, 0.29, 1.54, 0.30, 0.42 and 1.04 on days 0, 2, 16, 30, 44 and 58, respectively; $p > 0.05$ in each case). On day 72, however, 16 days after the administration of *F. hepatica* metacercariae, eosinophil levels in group A rats were significantly higher than those of the other three groups of rats (One-way ANOVA followed by Tukey test; $F_{3,24}$= 23.41, $p < 0.0005$). The rats of group B, which were given *F. hepatica* metacercariae at the same time as the rats of group A, but which harboured 56-day old *H. diminuta* infections at this time, did not exhibit significantly elevated eosinophil levels compared to uninfected (group D), or *H. diminuta*-only infected (group C), rats (One-way ANOVA followed by Tukey test, see above). In group-A rats, thereafter, the proportion of leucocytes which comprised eosinophils gradually began to decline, whereas eosinophil levels in group-B rats were observed to increase slightly on day 86 (Fig 7.3). From day 86 until day 114, significant discrepancies in the levels of circulating eosinophils could only be found between the rats of groups A and C, and groups A and D (One-way ANOVA followed by Tukey test; $F_{3,24}$= 6.30, $p < 0.005$; $F_{3,24}$= 4.62, $p < 0.02$; $F_{3,23}$= 4.58, $p < 0.02$; days 86, 100 and 114, respectively). At the end of the experiment on day 127, eosinophil counts were not detected to be significantly different between the groups of rats (One-way ANOVA; $F_{3,23}$= 1.67, $p > 0.05$).

7.5.1.3 *Experiment 3*

The eosinophil response in rats infected simultaneously with *F. hepatica* and *H. diminuta* was monitored in experiment 3. Mean eosinophil counts with standard error bars

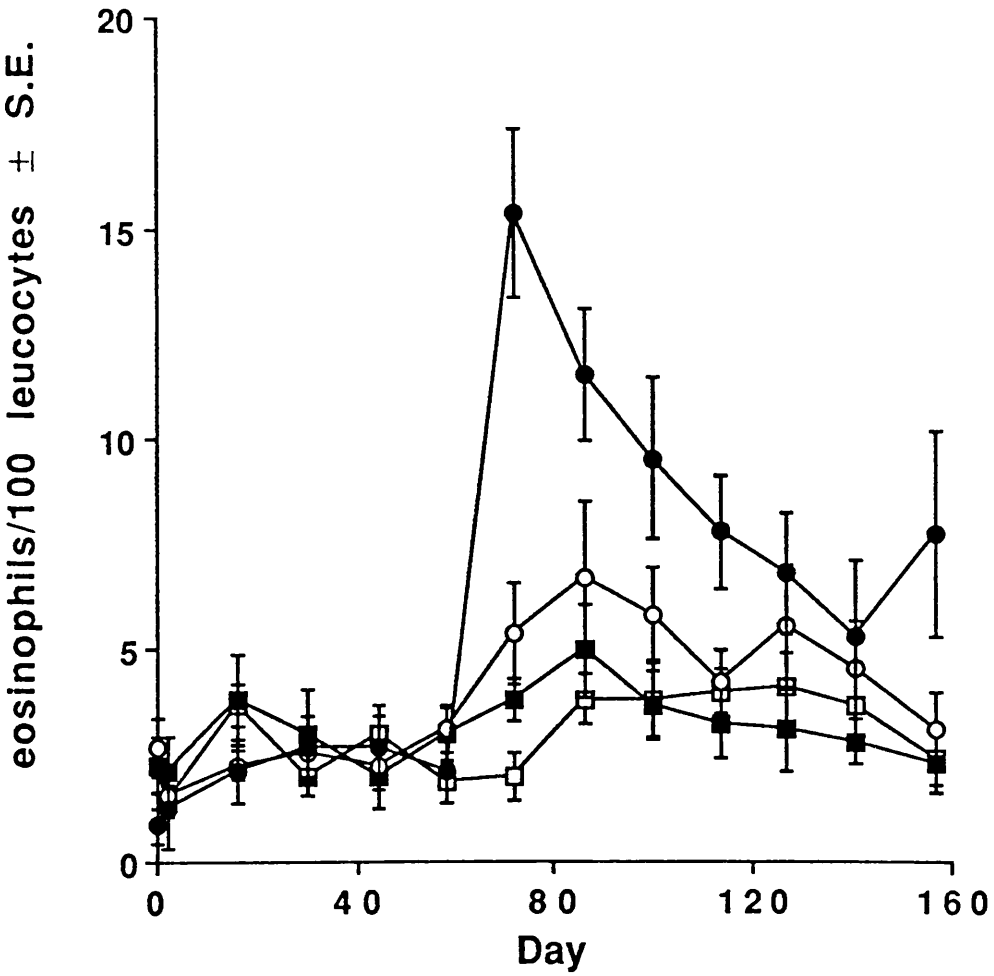



Fig. 7.3. Circulating eosinophil levels (mean number/100 leucocytes ± S.E.) in the rats of groups A, B, C and D in experiment 2. Group A rats each received 10 *F. hepatica* metacercariae on day 56; group B rats each received 10 *H. diminuta* cysticercoids on day 0 and 10 *F. hepatica* metacercariae on day 56; group C rats each received 10 *H. diminuta* cysticercoids on day 0; group D rats were kept as uninfected controls. ( ● ) group A rats; ( ○ ) group B rats; ( ■ ) group C rats; ( □ ) group D rats.

for each group of rats from day 0 to day 127 of the experiment are illustrated in Fig. 7.4. Although the *F. hepatica* infections in the rats of groups A and E did not run concurrently, since group A rats acted as *F. hepatica*-only controls for experiment 2, data from these groups of rats were plotted on the same graph to allow comparison of eosinophil responses.

On day 16, levels of circulating eosinophils were significantly greater in those rats which harboured *F.hepatica* only (group A) compared to all other groups of rats (One-way ANOVA followed by Tukey test; $F_{3,24}= 10.70$, $p < 0.0005$). Despite the apparently higher eosinophil counts of rats administered *F. hepatica* and *H. diminuta* simultaneously (group E), compared to uninfected (group D) and *H. diminuta*-only infected (group C) controls, this difference was not found to be statistically significant (One-way ANOVA followed by Tukey test, see above). Thereafter, the circulating eosinophilia observed in group A rats was observed to decline. On days 30, 44 and 58, significant differences in mean counts were consistently detected between the rats of groups A and C, A and D, E and C and E and D (one-way ANOVA followed by Tukey test; $F_{3,24}= 15.65$, $p < 0.0005$; $F_{3,24}= 10.54$, $p < 0.0005$; $F_{3,22}= 9.63$, $p < 0.0005$; respectively). By day 72, eosinophil counts of the rats of groups A and D only were observed to differ (One-way ANOVA followed by Tukey test; $F_{3,22}= 4.43$, $p < 0.02$). From this point on, data from the rats of groups C, D and E only were available, and no significant difference was discovered between the groups on days 86, 100 and 114 (One-way ANOVA followed by Tukey test; $F_{2,17}= 2.32$, 0.40 and 2.56, respectively; $p > 0.05$ in each case). On the last day of the experiment (127), numbers of circulating eosinophils were again observed to rise in rats with concurrent *F. hepatica/H. diminuta* infections (group E) (One-way ANOVA followed by Tukey test; $F_{2,17}= 5.03$, $p < 0.02$).

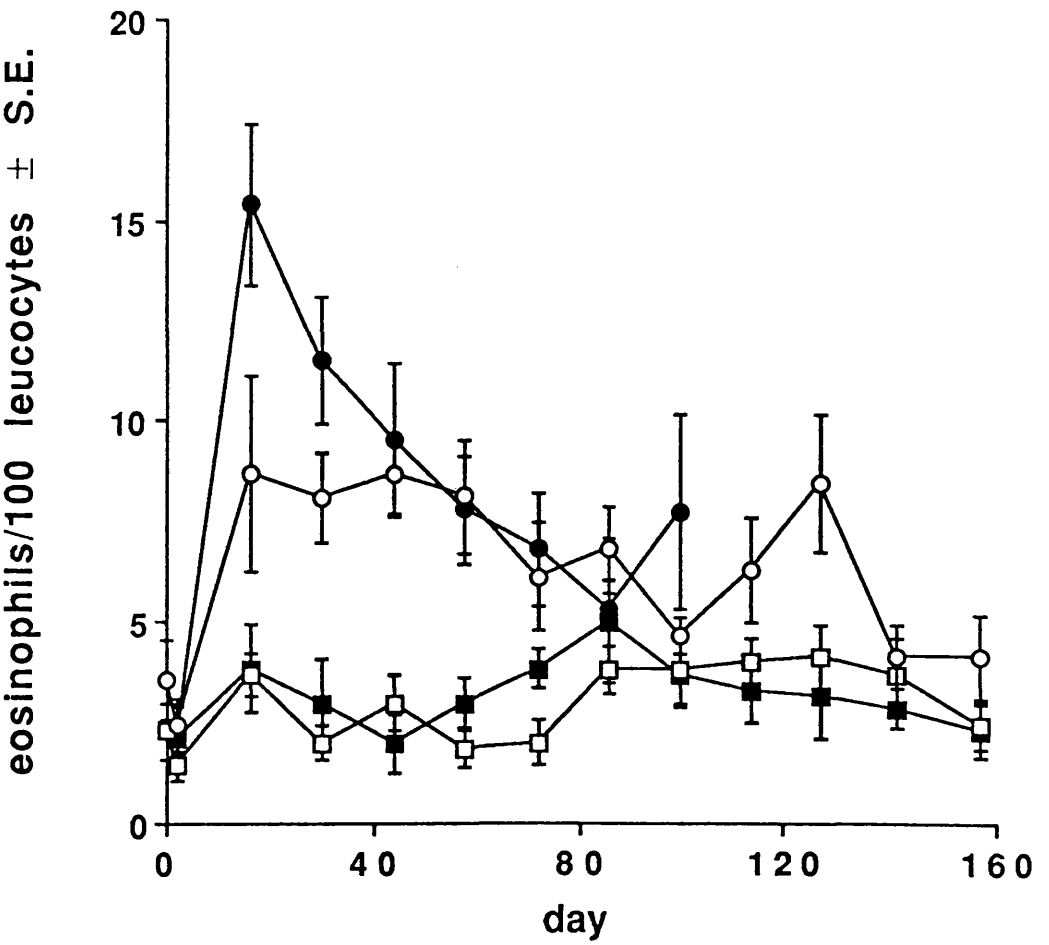



Fig. 7.4. Circulating eosinophil levels (mean number/100 leucocytes ± S.E.) in the rats of groups A, C, E and D in experiment 3. Group A rats each received 10 *F. hepatica* metacercariae on day 0; group C rats each received 10 *H. diminuta* cysticercoids on day 0; group E rats each received 10 *F. hepatica* metacercariae and 10 *H. diminuta* cysticercoids on day 0; group D rats were kept as uninfected controls. ( ● ) group A rats; ( ■ ) group C rats; ( ○ ) group E rats; ( □ ) group D rats.

### 7.5.2 Influence of concurrent infection with *H. diminuta* on the ability of rats to resist reinfection with *F. hepatica* (experiment 6)

#### 7.5.2.1 *Levels of circulating eosinophils*

In this part of experiment 6, the influence of a superimposed *H. diminuta* infection on the eosinophil response of rats harbouring primary and secondary *F. hepatica* infections, was investigated. Mean eosinophil counts with standard error bars are presented for all groups of rats from day 0 until the end of the experiment on day 63 in Fig. 7.5. By day 14 of the experiment, a disparity in the levels of circulating eosinophils was detected between the groups of rats, the rats in groups A and B, with 14-day old *F. hepatica* infections, having significantly higher counts than the other 3 (C, D and E), at this point uninfected, groups of rats (One-way ANOVA followed by Tukey test; $F_{4,30}= 8.09$, $p < 0.0005$). This pattern was maintained until day 47 (One-way ANOVA followed by Tukey test; $F_{4,30}= 16.59$ and 13.01 on days 32 and 47, respectively; $p < 0.0005$ in each case), despite the administration of *H. diminuta* cysticercoids to the rats of groups A, C and D on day 29. It is worth noting that a slight drop in eosinophil number was observed on day 47 in the rats with the concurrent *F. hepatica/H. diminuta* infections (group A), compared to that of those harbouring *F. hepatica* only (group B) (Fig. 7.5). On day 63, 20 days after the administration of *F. hepatica* metacercariae to the rats of groups A, B, D and E, eosinophil counts were higher in group E rats (which harboured a primary *F. hepatica* infection only) than in any other group of rats (Fig. 7.4). The difference was only found to be significant between the rats of groups E and A and of groups E and C (One-way ANOVA followed by Tukey test; $F_{4,28}= 6.86$, $p = 0.001$).

#### 7.5.2.2 *Recovery of F. hepatica*

To determine whether the survival of primary or secondary *F. hepatica* infections was influenced by a superimposed *H. diminuta* infection, the numbers of *F. hepatica* recovered from all primary, and from all 21-day old infections, were compared between the groups of

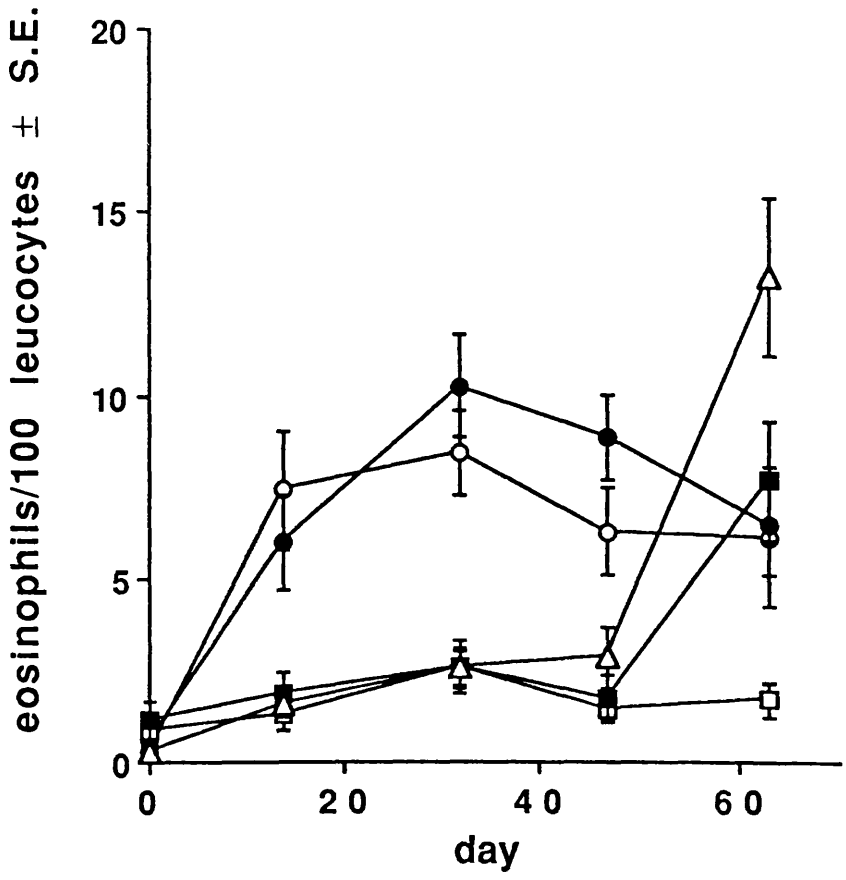



Fig. 7.5. Circulating eosinophil levels (mean number/100 leucocytes ± S.E.) in the rats of groups A, B, C, D and E in experiment 6. Group A rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0, were infected with *H. diminuta* (10 cysticercoids/rat) on day 29, and were given a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group B rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0 and a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group C rats each received an *H. diminuta* infection (10 cysticercoids/rat) on day 29; group D rats each received an *H. diminuta* infection (10 cysticercoids/rat) on day 29 and a primary *F. hepatica* infection (10 metacercariae/rat) on day 43; group E rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 43. ( ○ ) group A rats; ( ● ) group B rats; ( □ ) group C rats; ( ■ ) group D rats; ( △ ) group E rats.

rats. The mean number of *F. hepatica* recovered from all primary infections are illustrated with standard error bars in Fig. 7.6. Despite the difference in ages between the primary infections of rats in groups A and B (64 days) and groups D and E (21 days), all groups of rats were found to harbour similar burdens (Kruskal-Wallis ANOVA; $H = 0.22$, d.f.$= 3$, $p > 0.05$).

The mean numbers of *F. hepatica* recovered from the 21-day old infections in the rats of groups A, B, D and E, are presented with standard error bars in Fig. 7.7. In the rats of groups A and B, for which the 21-day old flukes represented secondary *F. hepatica* infections, significantly fewer worms were recovered than from group D and E rats (Kruskal-Wallis ANOVA followed by multiple comparisons procedure (section 7.4); $H = 19.72$, d.f.$= 3$, $p < 0.0005$).

7.5.2.3 *F. hepatica weights*

The effect of a concurrent *H. diminuta* infection on the growth of both primary and secondary *F. hepatica* infections was also investigated. Mean fluke dry weights in the 64-day old primary infections only (recovered from the rats in groups A and B) are shown with standard error bars in Fig. 7.8. Flukes were found to be significantly heavier in the rats of group A, which also harboured *H. diminuta* and secondary *F. hepatica* infections, compared to those of group B, which harboured a concomitant secondary *F. hepatica* infection only (Wilcoxon-Mann-Whitney test; $W = 52.0$, $N_1 = 6$, $n_2 = 6$, $p < 0.05$).

The mean wet weights of the 21-day old flukes in the same groups of rats are illustrated with standard error bars in Fig. 7.9 (wet weights were used since, when dry, the worms were often found to be of 'zero' weight). Although the worms in the rats of group A appear to be considerably heavier than those in the other 3 groups of rats, no significant difference in fluke weight was detected between the groups (Kruskal-Wallis ANOVA; $H = 3.71$, d.f.$= 3$, $p > 0.05$). The anomaly probably stems from the presence of a minority of atypically large worms found in certain group A rats; this is evidenced by the presence of very large error bars associated with the mean value.

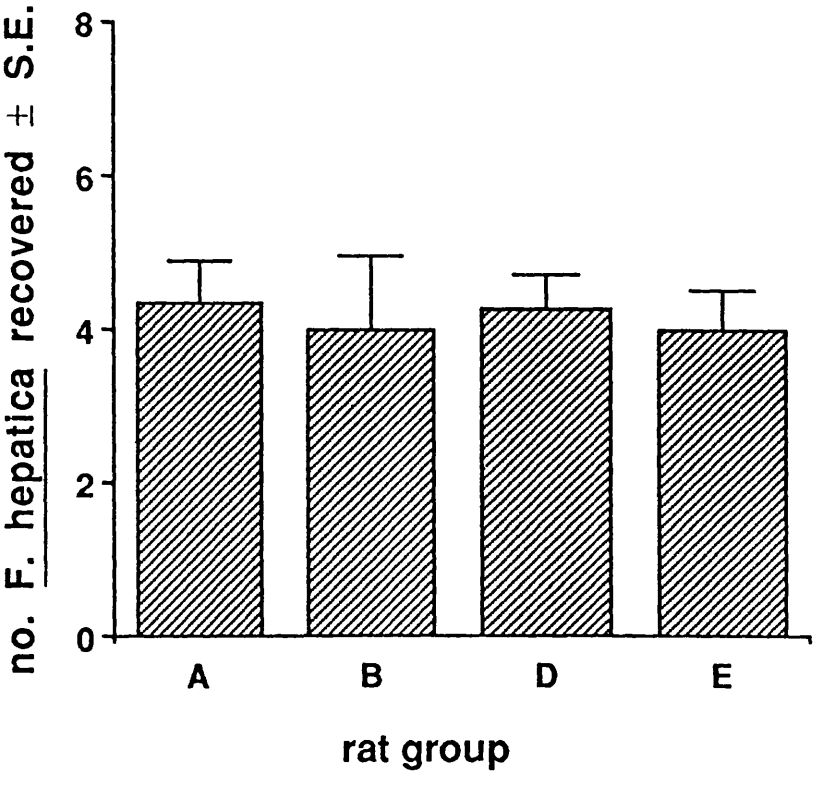



Fig. 7.6. The mean numbers (± S.E.) of *F. hepatica* recovered from the primary infections of the rats in groups A, B, D and E in experiment 6. Group A rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0, were infected with *H. diminuta* (10 cysticercoids/rat) on day 29, and were given a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group B rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0 and a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group D rats each received an *H. diminuta* infection (10 cysticercoids/rat) on day 29 and a primary *F. hepatica* infection (10 metacercariae/rat) on day 43; group E rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 43. Kruskal-Wallis ANOVA; $H = 0.22$, d.f. $= 3$, $p > 0.05$.

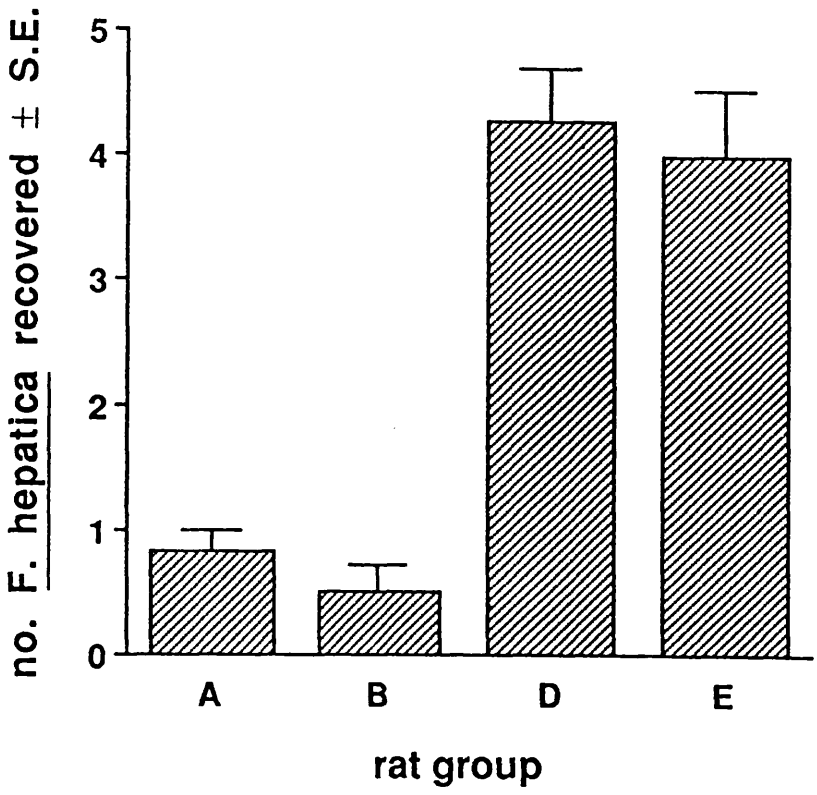



Fig. 7.7. The mean numbers (± S.E.) of *F. hepatica* recovered from all 21-day old infections (those given on day 43) of the rats in groups A, B, D and E in experiment 6. Group A rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0, were infected with *H. diminuta* (10 cysticercoids/rat) on day 29, and were given a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group B rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0 and a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group D rats each received an *H. diminuta* infection (10 cysticercoids/rat) on day 29 and a primary *F. hepatica* infection (10 metacercariae/rat) on day 43; group E rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 43. Significantly more *F. hepatica* were recovered from the rats of groups D and E than from those of groups A and B; Kruskal-Wallis ANOVA followed by multiple comparisons between treatments (Siegel & Castellan, 1988); $H = 19.72$, d.f. = 3, $p < 0.0005$.

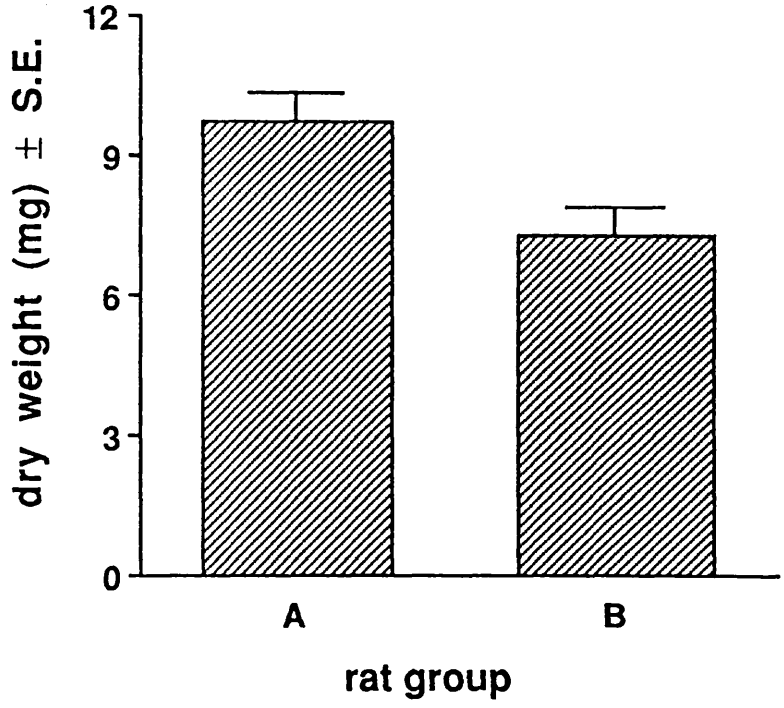



Fig. 7.8. Mean dry weights (± S.E.) of *F. hepatica* recovered from the primary infections of the rats in groups A and B in experiment 6. Group A rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0, were infected with *H. diminuta* (10 cysticercoids/rat) on day 29, and were given a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group B rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0 and a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; Significantly heavier *F. hepatica* were recovered from the rats of group A; Wilcoxon-Mann-Whitney test; $W = 52.0$, $n_1 = 6$, $n_2 = 6$, $p < 0.05$.

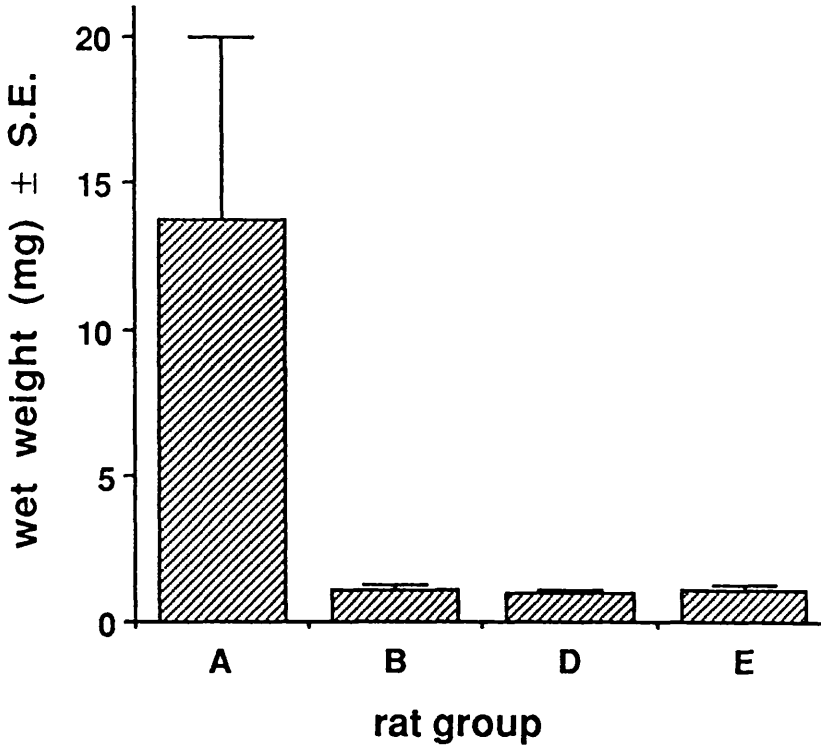



Fig. 7.9. Mean wet weights (± S.E.) of *F. hepatica* recovered from all 21-day old infections (those given on day 43 of the experiment) of the rats in groups A, B, D and E in experiment 6. Group A rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0, were infected with *H. diminuta* (10 cysticercoids/rat) on day 29, and were given a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group B rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 0 and a secondary *F. hepatica* infection (10 metacercariae/rat) on day 43; group D rats each received an *H. diminuta* infection (10 cysticercoids/rat) on day 29 and a primary *F. hepatica* infection (10 metacercariae/rat) on day 43; group E rats each received a primary *F. hepatica* infection (10 metacercariae/rat) on day 43. No significant difference was detected between the groups; Kruskal-Wallis ANOVA; $H = 3.71$, d.f. $= 3$, $p > 0.05$.

### 7.5.3 Detailed investigation of changes in the eosinophil levels of rats in response to concurrent infection with *F. hepatica* and *H. diminuta* (experiment 7)

#### 7.5.3.1 *Absolute white blood cell counts*

The effect of *H. diminuta* challenge on the total leucocyte counts of rats with 14-day old *F. hepatica* infections was investigated. Mean leucocyte counts per ml of blood from day 0 until day 18, in the case of rats from groups A, C and E, and from day 0 until day 32, in the case of rats from groups B, D and F, are illustrated with standard error bars in Fig. 7.10. White blood cell counts appeared to be relatively stable until day 18 when they appeared to rise in the rats of group C, and in the rats of group D to a lesser extent. The difference was only of borderline significance, however (One-way ANOVA; $F_{5,36}= 2.48$, $p = 0.05$). The increase was observed in rats which harboured primary *F. hepatica* infections only, and was not evident in rats with concurrent *F. hepatica/H. diminuta* infections (groups A and B), or in uninfected control rats (groups E and F). On day 22, no significant difference in leucocyte count could be detected between group B, D and F rats, although levels were clearly higher in rats from group B, with concomitant *F. hepatica/H. diminuta* infections, and in those from group D, which harboured *F. hepatica* only, in particular (One-way ANOVA; $F_{2,18}= 2.49$, $p > 0.05$). This trend continued until one day prior to the end of the experiment (32), a significant difference in white cell counts being detected between the rats of D and F on days 26 and 32 (One-way ANOVA followed by Tukey test; $F_{2,18}= 5.38$, $p < 0.02$; $F_{2,18}= 6.97$, $p < 0.01$; respectively).

#### 7.5.3.2 *Levels of circulating eosinophils*

The eosinophil response of rats with 2-week old *F. hepatica* infections to a superimposed *H. diminuta* infection was monitored in some detail. The mean numbers of circulating eosinophils per 100 leucocytes ± the standard error, are presented from day 0 until day 18 for the rats of groups A, C and E, and from day 0 until day 32 for the rats of groups B, D and F, in Fig. 7.11. Eosinophil levels did not vary significantly between the

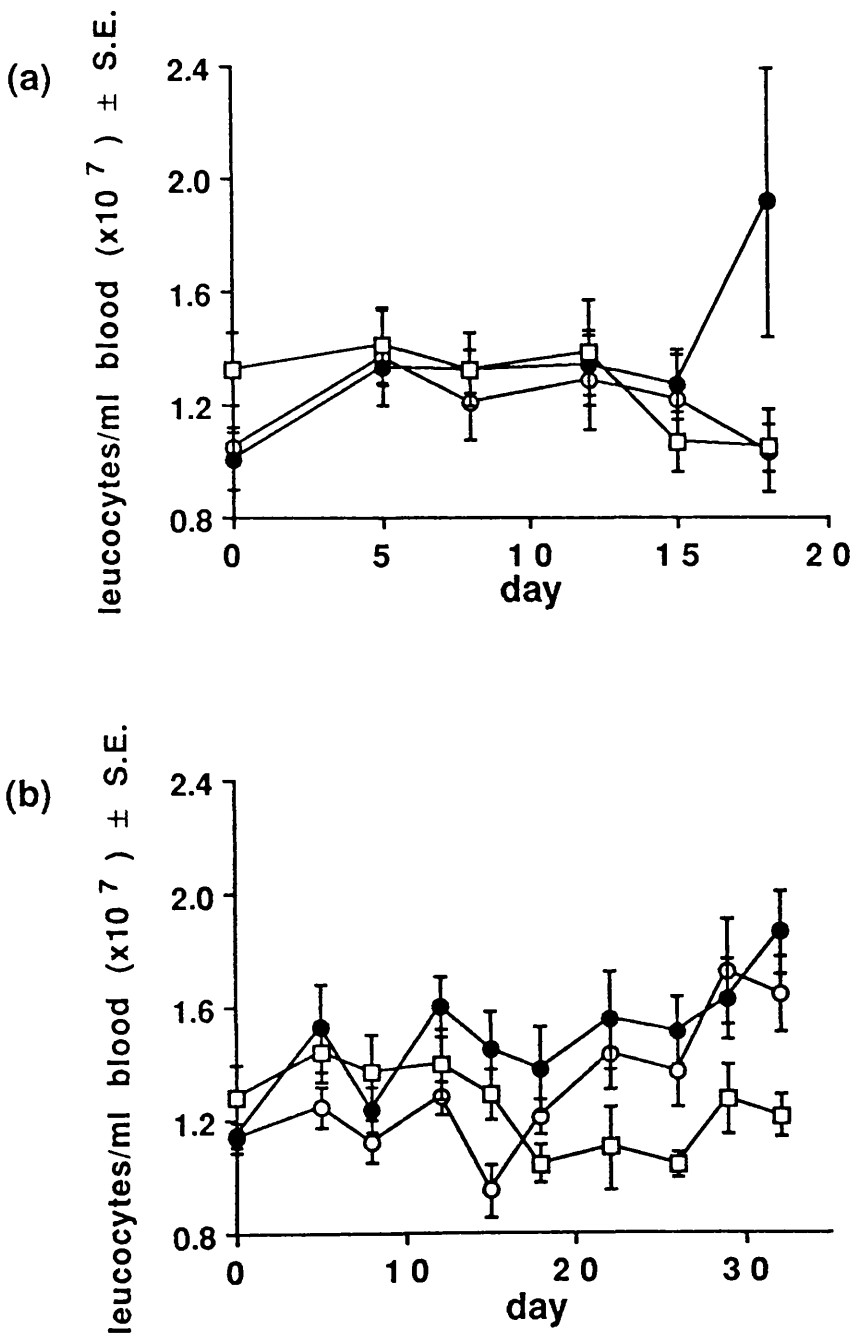



Fig. 7.10. Absolute leucocyte counts (mean number/ml blood ± S.E.) in the rats of groups A, B, C, D, E and F in experiment 7. The rats of groups A and B each received 10 *F. hepatica* metacercariae on day 1 and 10 *H. diminuta* cysticercoids on day 14; the rats of groups C and D each received 10 *F. hepatica* metacercariae on day 1; the rats of groups E and F were kept as uninfected controls. (a) Leucocyte counts in groups A, C and E which were killed on day 19; ( ○ ) group A rats; ( ● ) group C rats; ( □ ) group E rats. (b) Leucocyte counts in groups B, D and F which were killed on day 33; ( ○ ) group B rats; ( ● ) group D rats; ( □ ) group F rats.

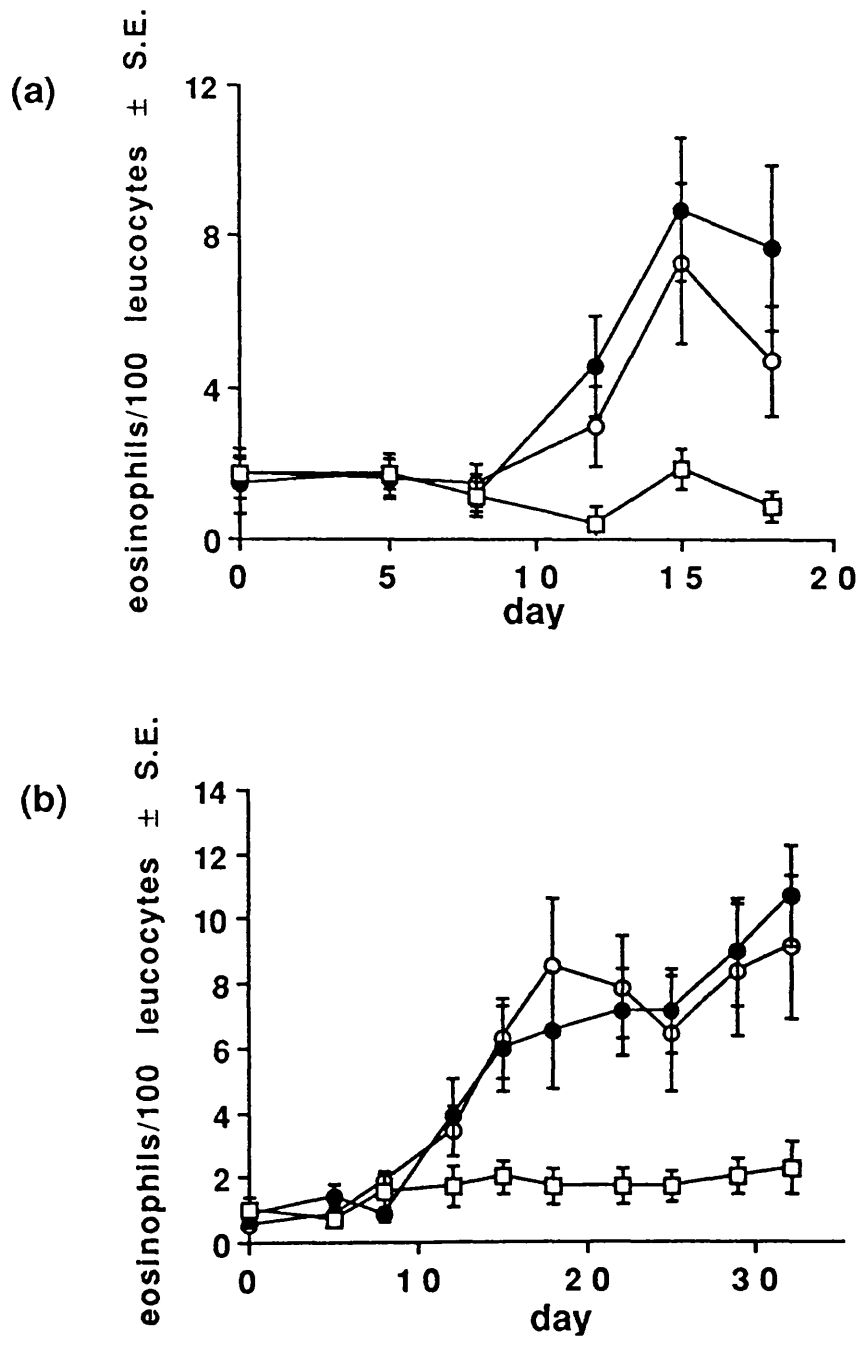



Fig. 7.11. Circulating eosinophil levels (mean number/100 leucocytes ± S.E.) in the rats of groups A, B, C, D, E and F in experiment 7. The rats of groups A and B each received 10 *F. hepatica* metacercariae on day 1 and 10 *H. diminuta* cysticercoids on day 14; the rats of groups C and D each received 10 *F. hepatica* metacercariae on day 1; the rats of groups E and F were kept as uninfected controls. (a) Leucocyte counts in groups A, C and E which were killed on day 19; ( ○ ) group A rats; ( ● ) group C rats; ( □ ) group E rats. (b) Leucocyte counts in groups B, D and F which were killed on day 33; ( ○ ) group B rats; ( ● ) group D rats; ( □ ) group F rats.

groups until day 12 of the experiment, when they were significantly greater in the rats from group C compared with those from group E (One-way ANOVA followed by Tukey test; $F_{5,36}= 2.52$, $p < 0.05$). Numbers were, however, generally greater in the rats of groups A, B, C and D, which harboured primary *F. hepatica* infections only at this time, than in the uninfected control rats (groups E and F) (Fig. 7.11). This trend was found to continue until day 32 (One-way ANOVA followed by Tukey test; day 15, $F_{5,36}= 4.03$, $p = 0.005$, sig. btw. groups C and E, C and F; day 18, $F_{5,36}= 4.03$, $p = 0.005$, sig. btw. groups B and E, B and F, C and F; day 22, $F_{2,18}= 7.21$, $p = 0.005$, sig. btw. groups B and F, D and F; day 25, $F_{2,18}= 5.08$, $p < 0.02$, sig. btw. groups D and F; day 29, $F_{2,18}= 6.11$, $p < 0.01$, sig. btw. groups B and F, D and F; day 32, $F_{2,18}= 7.49$, $p < 0.005$, sig. btw. groups B and F, D and F). It should be noted that on no occasion was there a significant difference in eosinophil counts detected between those rats harbouring *F. hepatica* only (groups C and D), and those with concurrent *F. hepatica/H. diminuta* infections (group A and B).

7.5.3.3 *Levels of eosinophils in peritoneal exudate*

The extent of eosinophil infiltration into the peritoneal cavity of rats infected with *F. hepatica*, was examined at 5 and 19 days post challenge with *H. diminuta*. The mean numbers of eosinophils counted per 500 peritoneal exudate cells in the rats of groups A, C and E on day 19, and in the rats of groups B, D and F on day 33, are presented with standard error bars in Fig. 7.12. The rats of groups A and C, with mixed *F. hepatica/H. diminuta* and *F. hepatica*-only infections, respectively, were found to have significantly elevated eosinophil counts compared to the uninfected control rats (group E) (One-way ANOVA followed by Tukey test; $F_{2,17}= 45.23$, $p < 0.0005$). Although rats with the concurrent infection had slightly lower eosinophil counts than those with *F. hepatica* alone, this difference was not significant (One-way ANOVA followed by Tukey test, see above). The same pattern was observed in rats, with corresponding infections, killed on day 33. Rats which harboured *F. hepatica* only (group D), or which had concurrent *F. hepatica/H. diminuta* infections (group B), had significantly greater numbers of eosinophils than their uninfected counterparts

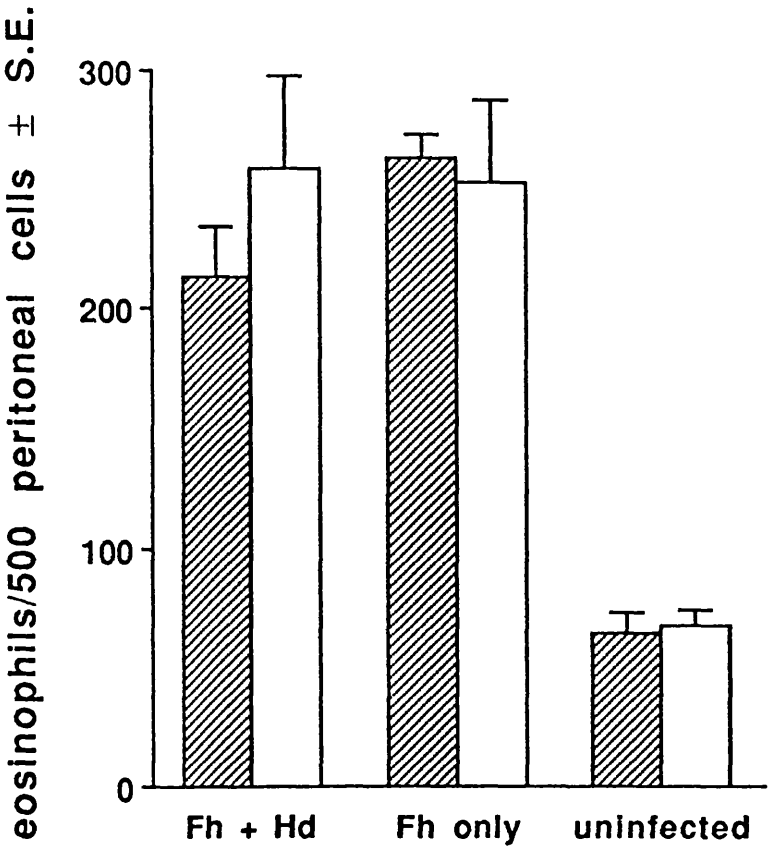



Fig. 7.12. The mean numbers (± S.E.) of eosinophils per 500 peritoneal exudate cells in the six groups of rats in experiment 7. **Fh + Hd** (rat groups A and B): each rat received 10 *F. hepatica* metacercariae on day 1 and 10 *H. diminuta* cysticercoids on day 14; **Fh only** (rat groups C and D): each rat received 10 *F. hepatica* metacercariae on day 1; **uninfected** (rat groups E and F): each rat was kept as an uninfected control. (▨) rats killed on day 19; (□) rats killed on day 33.

(group F) (One-way ANOVA followed by Tukey test; $F_{2,17}$= 11.13, p = 0.001).

7.5.3.4 *Levels of eosinophils in bone marrow*

Eosinophil production in the bone marrow of *F. hepatica*-infected rats was monitored at 5 and 19 days after a challenge *H. diminuta* infection. The mean number of immature and mature eosinophils per 500 nucleated bone marrow cells counted are shown with standard error bars for the rats of groups A, C and E on day 19, and for the rats of groups B, D and F on day 33, in Fig. 7.13. On day 19, uninfected rats (group E) were found to have significantly lower eosinophil counts than rats with concomitant *F. hepatica/H. diminuta* infections (group A) which were, in turn, observed to exhibit significantly lower eosinophil numbers than those rats which were infected with *F. hepatica* only (group C) (One-way ANOVA followed by Tukey test; $F_{2,17}$= 32.95, p < 0.0005). On day 33, however, eosinophil levels in rats with the mixed infections (group B) were not found to differ from those in rats harbouring *F. hepatica* alone (group D), although both groups were found to have significantly elevated counts compared with the uninfected control rats (group F) (One-way ANOVA followed by Tukey test; $F_{2,18}$= 17.43, p < 0.0005).

7.5.3.5 *Worm recovery*

To determine whether the survival of 14-day old *F. hepatica* infections was influenced by a superimposed *H. diminuta* infection, and also whether infection procedures were consistent, the numbers of worms recovered were compared between the different groups of rats for the respective helminth species. The median numbers of *F. hepatica* recovered from the rats of groups A, B, C and D are shown with inter-quartile ranges in Table 7.6. No significant difference in infection intensity was detected between rats killed on day 19 (groups A and C) and those killed on day 33 (groups B and D), or between rats challenged with *H. diminuta* on day 14 (groups A and B) and those which were not (groups C and D) (Kruskal-Wallis ANOVA; H = 5.85, d.f.= 3, p > 0.05). The median number of *H.*

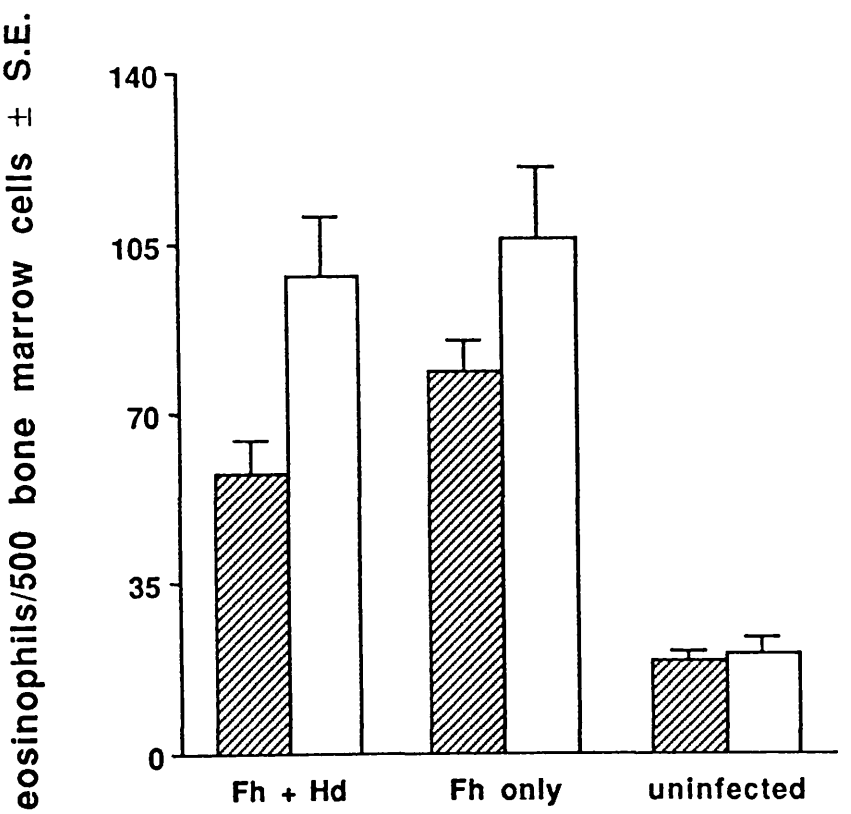



**Fig. 7.13.** The mean numbers (± S.E.) of mature and immature eosinophils per 500 nucleated cells in the bone marrow of rats from the six groups in experiment 7. **Fh + Hd** (rat groups A and B): each rat received 10 *F. hepatica* metacercariae on day 1 and 10 *H. diminuta* cysticercoids on day 14; **Fh only** (rat groups C and D): each rat received 10 *F. hepatica* metacercariae on day 1; **uninfected** (rat groups E and F): each rat was kept as an uninfected control. (▨) rats killed on day 19; (□) rats killed on day 33.

*diminuta* recovered from the rats of groups A and B are shown with inter-quartile ranges in Table 7.7. Significantly more *H. diminuta* were recovered from the 19-day old infections from group B rats than from the 5-day infections in group A rats (Wilcoxon-Mann-Whitney test; $W = 27.5$, $n_1 = 6$, $n_2 = 7$, $p < 0.05$), for which the difficulty in detecting the immature worms from the intestinal mucosa of the latter rats was likely to be a cause. The rats in both these groups were administered *F. hepatica* metacercariae 14 days prior to the *H. diminuta* infection.

**Table 7.6.** Median number of *F. hepatica* recovered from the rats of groups A, B, C and D in experiment 7, compared using Kruskal-Wallis ANOVA.

| Rat group (n) | Median | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (7) | 2.00 | 0.00, 3.00 | |
| B (7) | 1.00 | 0.00, 3.00 | |
| | | | 0.120 (NS) |
| C (7) | 3.00 | 3.00, 5.00 | |
| D (7) | 2.00 | 2.00, 3.00 | |

**Table 7.7.** Median number of *H. diminuta* recovered from the rats of groups A and B in experiment 7, compared using Wilcoxon-Mann-Whitney test.

| Rat group (n) | Median | Inter-quartile range | Significance (p value) |
|---|---|---|---|
| A (7) | 6.50 | 3.50, 7.75 | |
| | | | 0.041 (S) |
| B (7) | 9.00 | 8.00, 10.00 | |

## 7.6 DISCUSSION

Results from these investigations reveal that the characteristic circulating eosinophilia of *F. hepatica*-infected rats (Rajesekariah & Howell, 1981) may be at least partially or wholly reduced by combined infection with *H. diminuta*. Eosinophil numbers were observed to decline when *H. diminuta* was given to rats with mature *F. hepatica* infections, and were found to increase only slightly following administration of *F. hepatica* to rats harbouring mature *H. diminuta* infections. When both species were administered simultaneously, however, the effect was less marked, although levels of circulating eosinophils in rats with the combined infections were still less than those with *F. hepatica* only.

Suppression of an *F. hepatica*-induced eosinophilia of the host by a concurrent helminth infection has rarely been described, but is not altogether unprecedented. In a study by Presidente, Knapp & Nicol (1973), the eosinophilic response of sheep to simultaneous infection with *F. hepatica* and *Haemonchus contortus* was lower than that recorded for sheep harbouring *F. hepatica* only, although levels in the former sheep were still much higher than in uninfected, or *H. contortus*-only infected sheep. In addition, calves with 10-week old *Schistosoma bovis* infections which were challenged with *F. hepatica* exhibited a much reduced eosinophilia compared to those which received the *F. hepatica* infection only (Sirag, Christensen, Nansen, Monrad & Frandsen, 1981). In this experiment, the eosinophilic response in calves infected with *S. bovis* alone was similar to that in calves with the mixed species infections. The authors interpreted this observation as a failure of the challenge dose of *F. hepatica* to accelerate the moderate, *S. bovis*-induced eosinophilia.

The functional significance of these events is not clear since the parasites involved behave in a variety of ways. Concurrent infection with *F. hepatica* appeared to inhibit the development of resistance to *H. contortus* in the experiment conducted by Presidente *et al.* (1973), as determined by the duration of *H. contortus* fecundity. The authors interpreted the reduced eosinophilia as evidence for an immunosuppressive effect following simultaneous

infection with these species. In the study by Sirag *et al.* (1981), *S. bovis* egg production was unaffected by prior challenge with *F. hepatica*, although the liver-fluke burdens were themselves significantly lower than in calves infected with *F. hepatica* alone. In the experiments described here, the depressed eosinophilia was concomitant with slightly better survival of *H. diminuta* given to rats harbouring patent *F. hepatica* infections. Taken together, these phenomena are suggestive of an immunodepressed host. Yet it is important to recognise that an impaired eosinophilic response was also observed during various other dosing regimes involving both species, with which no effect on the survival, size or fecundity of either species was associated (see Chapter 6). Nevertheless, since depression of the typical eosinophilic response was detected in *F. hepatica*-infected rats, sheep and calves following combined infection with *H. diminuta*, *H. contortus* and *S. bovis*, respectively, the observation is not likely to be an artifact.

In the papers described, the authors do not attempt to elucidate mechanisms which may impair a circulating eosinophilia. Eosinopenia has been shown to accompany many acute infections in their initial stages (Weiner & Morkovin, 1952), and it has been suggested that the recruitment of eosinophils to the vicinity of an inflammatory reaction could account for this (Bass, 1975). Infection of rats with *H. diminuta* induces mastocytosis of the alimentary tract (Hindsbo, Andreassen & Ruitenberg, 1982; Featherston & Copeman, 1990), an event which may be responsible for the accumulation of eosinophils in the small intestine of *H. diminuta*-infected rats (Hindsbo *et al.*, 1982) and mice (Van der Vorst, Dhont, Cesbron, Capron, Dessaint & Capron, 1988).

There is no evidence from the present studies to suggest that elevated levels of eosinophils accompanying *F. hepatica* infection of rats, were diminished as a consequence of their increased sequestration to the lamina propria following infection with *H. diminuta*. Numbers of eosinophils were found to be significantly lower in the posterior small intestines of rats which had harboured concurrent *F. hepatica*/*H. diminuta* infections compared with those which had been infected with *F. hepatica* alone. Furthermore, rats which harboured the concurrent infections, together with those infected with *H. diminuta* only, did not exhibit

an intestinal eosinophilia when compared with uninfected controls. Besides, kinetics of the numbers of intestinal eosinophils were found to correspond closely with those of the numbers of blood eosinophils in *Nippostrongylus brasiliensis*-infected rats for the duration of a 40-week experiment (Nawa & Hirashima, 1984). An extensive mastocytosis of the alimentary tract of rats has been associated with this infection (Ogilvie & Jones, 1971).

Since an elevated sequestration of eosinophils to the gut did not appear to accompany the challenge *H. diminuta* infection to *Fasciola*-infected rats, the possibility that their production in, or release from, the bone marrow was being depressed, was considered. Oscillations in the numbers of circulating blood cells are likely to be a consequence of changes in bone marrow production (Tyazhelova, 1987). Results from experiment 7 revealed that reduced eosinophil production in the bone marrow, relative to that of rats infected with *F. hepatica* alone, may be associated with rats harbouring concurrent infections. Although the numbers of mature and immature eosinophils were significantly elevated in the bone marrow of rats infected with both *F. hepatica* and *H. diminuta*, of the first groups of rats to be killed in this experiment, those with the dual species infections exhibited significantly lower eosinophil counts than those which harboured *F. hepatica* alone. The result was not conclusive, since the effect was not repeated in rats which received the same infection regime, but whose infections were allowed to develop for a further 14 days. In any case, in this experiment rats with concurrent infections did not display a marked decline in the numbers of circulating eosinophils when compared with those harbouring only *F. hepatica*, although counts were slightly lower. Furthermore, absolute leucocyte counts did exhibit a tendency to be reduced in rats with the dual infections.

The failure of this detailed experiment to verify previous observations on eosinophil numbers, may concern the timing of the infection procedures. Depression of the *F. hepatica*-induced eosinophilia was found to be most pronounced when *H. diminuta* cysticercoids were introduced to rats with mature *F. hepatica* infections. The experiment investigating bone marrow eosinophilia involved the administration of *H. diminuta* to rats which had received

*F. hepatica* metacercariae only 14 days previously.

Nevertheless, at least in certain situations, the characteristic *F. hepatica*-induced eosinophilia of rats appeared to be depressed by concomitant infection with *H. diminuta*, and this event merits further consideration. While detailed hypotheses are not advisable at this stage, it may be profitable to consider mechanisms by which suppression of eosinophil production in the bone marrow of rats with the mixed species infections could operate.

Immunological reactions, including the development of the eosinophilic response, are modulated by a network of events controlled by lymphokines (Klein, 1990). Eosinophilia is generally believed to be mediated by T-cells (Basten & Beeson, 1970) which produce, amongst other factors, eosinophil differentiation factor (EDF) which is also known as interleukin-5 (IL-5) (Sanderson, Campbell & Young, 1988). Interleukin-5 has been shown to cause proliferation of eosinophils in bone marrow cultures by stimulating pre-committed eosinophil precursors (Sanderson *et al.*, 1988), and is, itself, thought to be produced following T-cell stimulation with interleukin-2 (IL-2) (Enokihara, Furusawa, Nakakubo, Kajitani, Nagashima, Saito, Shishido, Hitoshi, Takatsu, Noma, Shimizu & Honjo, 1989). The generation of cells committed to the eosinophil lineage appears to result from interleukin-3 stimulation of bone marrow stem cells (Klein, 1990). The eosinophil response may be downregulated by feedback mechanisms acting on lymphokine production. Besedovsky, Del Rey, Sorkin & Dinarello (1986) suggest that following infection, or during inflammatory or immune responses, interleukin-1 concentrations in the circulation may reach a level which stimulates the pituitary-adrenal axis and results in increased glucocorticoid levels in the blood. Glucocorticoids inhibit the production and action of several lymphokines, including IL-2 (Besedovsky, Del Rey, Sorkin & Dinarello, 1986).

The existence of such a complex network of interactions invites speculation on the patterns of lymphokine release which follow different combinations of antigenic stimulation. Immunological events during single species infections with *F. hepatica* or *H. diminuta* may interact when both species infect the same host.

Athymic nude rats infected with *F. hepatica* have been shown to exhibit a pronounced

blood eosinophilia (Doy & Hughes, 1982), which suggests that eosinophilia in rats need not be entirely T-cell dependent. The biological activity of the eosinophil may be regulated by substances released by helminths (Silberstein & David, 1987), and it is conceivable that they are capable of eliciting a negative effect on the cell's production. Doy & Hughes (1982) contend that the species of parasite used may determine the mechanism which induces eosinophil leucocytosis in rats. It is possible, therefore, that species-specific modulation of eosinophilia exists, and was in some part responsible for the reduced eosinophilia associated with challenge *H. diminuta* infections to *F. hepatica*-infected rats.

Regardless of the cause, the consequence of an impaired eosinophil response, in terms of the ability of rats to mount an effective immunity to reinfection with *F. hepatica*, was of interest. The eosinophil is believed to be an important effector cell involved in the immune killing of secondary *F. hepatica* infections of rats, operating most effectively on juvenile flukes entering the peritoneal cavity (Davies & Goose, 1981; Burden, Bland, Hammet & Hughes, 1983). It has been suggested that the inferior eosinophilia of mice infected with *F. hepatica*, relative to rats, accounts for the failure of mice to develop resistance to secondary infection (Milbourne & Howell, 1990). Furthermore, sheep which developed strong resistance to reinfection with *Trichostrongylus colubriformis*, were found to exhibit a significantly higher blood eosinophilia than their *T. colubriformis*-infected counterparts, which offered only weak resistance to reinfection (Dawkins, Windon & Eagleson, 1989).

These studies suggest that the magnitude of the eosinophilic response mounted will influence the ability of the host to develop resistance to secondary infection. Results from the present studies, however, are not entirely supportive of this idea. Rats demonstrated effective resistance to secondary *F. hepatica* infections even when *H. diminuta* was administered 14 days prior to the secondary fluke infection (experiment 6). Since the dosing procedures resulted in consistent levels of intensity in primary *F. hepatica* infections of the different groups of rats, intensity data are open to little dispute. One interesting discovery

was that fluke weights in both primary and secondary infections were greater in rats with the superimposed *H. diminuta* infections (A), than in their age-matched controls. This could be explained in terms of an *H. diminuta*-induced synergistic effect on fluke growth, perhaps associated with a reduction in circulating eosinophils. Such an interpretation should be considered with caution, however, since fluke size in those rats given *H. diminuta* before a primary *F. hepatica* infection (D), was not abnormally large.

As in experiment 7, rats with the mixed species infections did not display a depressed eosinophilia to the extent predicted from the earlier experiments, although data from only a few time points were recorded. If experiments 6 and 7 are comparable in this respect, the observation that, in experiment 7, eosinophil numbers in the peritoneal exudate of *F. hepatica*-infected rats was unaffected by challenge with *H. diminuta*, is informative: most effective immunity to secondary *F. hepatica* infections appears to operate in the peritoneal cavity (Davies & Goose, 1981; Burden *et al.*, 1983). Failure to detect a drop in eosinophil levels may, again, have been attributable to the altered timing of the infection protocols.

An *H. diminuta*-associated depression of the characteristic eosinophilia of rats infected with *F. hepatica*, may operate under certain experimental conditions. In general, there was more evidence to suggest that prior infection with *H. diminuta* could prevent an increase in the number of circulating eosinophils following *F. hepatica* challenge (experiments 2, 3 and 6). The ability of a challenge *H. diminuta* infection to deplete an existing *F. hepatica*-induced eosinophilia was less obvious (Experiments 1, 6 and 7). How, and when, this response occurs, and whether it could impair the effectiveness of the host at killing secondary fluke infections, remains to be answered. Far more detailed investigations of the *F. hepatica/H. diminuta/* rat system are necessary before categorical conclusions can be drawn.

## 7.7 SUMMARY

(1) Under certain experimental conditions, the circulating eosinophilia observed in *Fasciola hepatica*-infected rats is either partially or wholly reduced by combined infection with

*Hymenolepis diminuta*, administered either before, concurrently or after *F. hepatica*. The effect was less noticeable, however, when *H. diminuta* was administered to rats with immature fluke infections.

(2) In experiments 1 and 2, rats with concurrent infections exhibited eosinophil levels comparable to those of uninfected control rats, both in the intestines, and in the blood throughout the duration of the experiments. This suggested that the reduction was not due to increased sequestration of eosinophils to the intestine.

(3) Examination of rat bone marrow provided some evidence of the suppression of eosinophil production in the bone marrow of rats following mixed infection with *F. hepatica* and *H. diminuta*.

(4) Despite the apparent involvement of eosinophils in host resistance to secondary *F. hepatica* infections, rats with mixed *F. hepatica/H. diminuta* infections did offer resistance to challenge with *F. hepatica* comparable to that of controls. The discovery that flukes were slightly larger in the rats with the mixed species infections suggested possible synergistic effects. The significance of these results were, however, not clear as a marked impairment of the eosinophilic response was not detected in this experiment.

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