SOME ASPECTS OF THE HOST-PARASITE RELATIONSHIP BETWEEN GOATS AND GASTROINTESTINAL NEMATODES

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ABSTRACT

Experiments were conducted investigating the acquisition, by goats, of resistance to *Haemonchus contortus* and *Trichostrongylus colubriformis*. Neither 5.5 or 14 months old Saanen goats given a trickle infection with *H. contortus*, which was terminated with anthelmintic after 10 and 14 weeks respectively, showed significant resistance to a challenge infection. Serum pepsinogen levels rose significantly as a result of infection. Serum gastrin levels also rose as a result of infection but, following challenge, were generally higher for naive goats than previously infected goats. There were no significant correlations between worm counts, gastrin and pepsinogen levels at the time of slaughter.

By contrast, a high level of resistance to *T. colubriformis* developed in 9 months old Saanen goats given trickle and two challenge infections. Goats exposed only to the two challenge infections developed worm burdens intermediate between, and significantly different from both trickle-infected and previously uninfected goats given just the second challenge. Both priming infections were removed with anthelmintic. Globule leukocyte (GL) counts in the proximal small intestine of trickle-infected goats were significantly higher than in goats given a single challenge infection, whilst counts for the group given two challenge infections were intermediate and not significantly different from other treatments. Nematode fecundity (eggs per female) and male:female ratios were also significantly decreased in the previously infected goats.

Ninety four percent of all *T. colubriformis* were found in the proximal 50% of the small intestine in both young goats and older goats. Male:female ratios increased and eggs/female nematode decreased with distance down the small intestine. Only about 50% of adult *T. colubriformis* were recovered from recently killed goats by opening and massaging the small intestine under running water.

Antiparasite activity of intestinal mucus from groups of Angora-cross goats killed 9, 18 or 27 days after infection with *T. colubriformis*, increased significantly in infected versus uninfected goats with no difference between infected groups. However, the establishment rate (57-67%) suggests little immunity was being expressed. Following infection, proximal small intestinal GL counts fell with time but there was still a clear negative relationship between GL counts and worm burdens. Mucosal mast cell and eosinophil counts showed no significant trends relative to duration of infection or worm

burden. Differential cell counts were made using a monoclonal antibody to sheep mast cells developed with diaminobenzidine, combined with haematoxylin and Biebrich's scarlet. This method was shown to be superior to others tried. In Angora-cross goats killed sequentially after removal of an infection with *T. colubriformis*, proximal small intestinal GL counts increased with time. However, antiparasite activity of intestinal mucus was consistently low. GL counts in all these Angora-cross goats were generally higher further down the intestine beyond the location of a large proportion of the *T. colubriformis* burden.

In Angora goats fitted with ileal cannulae and infected with *T. colubriformis*, antiparasite activity of ileal contents increased significantly with time after infection in all goats. However, the establishment rate (10.6-61.8%) indicated only a moderate degree of resistance was being expressed although a large proportion of established worms were inhibited L3s.

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CHAPTER ONE

LITERATURE REVIEW

1.1 GASTROINTESTINAL NEMATODE INFECTIONS IN GOATS

As providers of milk, meat, hair and skins, goats are immensely important in the world. In many developing countries they are traditionally herded and fed principally on browse. This presumably reduces the risk of them acquiring the large numbers of gastrointestinal nematodes usually associated with grazing pastures. Carrying fodder to goats which are housed or otherwise confined can have similar effects. When fed on natural or improved pastures, nematode parasitism is recognised as being of major importance in all age-groups and clinical observations suggest that goats fail to display the level of resistance to nematode infection that is seen in sheep grazing under similar conditions. The intriguing question which prompted this research is why this might be so. There has been little research on the subject. Indeed, much of the information on nematode infections in goats is fragmentary and difficult to integrate into a general interpretation of the immunological phenomena which modulate gastrointestinal parasitism in this species.

1.1.1. NEMATODE SPECIES INFECTING GOATS

The species of nematode occurring in the gastrointestinal tract of goats are essentially the same as those found in sheep (Brunsdon, 1960; Beveridge *et al.*, 1987; Chartier and Reche, 1992). Goats in New Zealand commonly suffer from clinical parasitism due to infections with *H. contortus*, *T. colubriformis* and *Ostertagia* spp. (Buddle *et al.*, 1988; Brunsdon, 1986). There is no evidence for strain adaptation specifically to goats or sheep, at least for *H. contortus* (Rahman and Collins, 1991b; Le Jambre and Royal, 1976; Preston and Allonby, 1978) although some minor differences in intensity of infection have been recorded between sheep and goats. For example, in comparison with sheep, *Nematodirus* eggs are rarely recorded in diagnostic faecal samples in New Zealand (McKenna, pers. com.) although Craig (1982) considers *Nematodirus* spp. to be important parasites of goats in Texas. *Trichostrongylus capricola* is more commonly found in goats than in sheep in Spain (Tarazona *et al.*, 1982) and was found to be common in a survey of feral goats in New Zealand (Brassell and Brunsdon, 1980). Hussein *et al.* (1985) demonstrated that

Haemonchus longistipes, although normally a parasite of camels, readily establishes in goats but not sheep.

1.1.2 DEVELOPMENT OF RESISTANCE TO GASTROINTESTINAL NEMATODES

There are a number of observational studies which generally suggest that goats must develop some immunity to gastrointestinal nematodes. Assoku (1981) monitored faecal egg counts of goats in Ghana and observed they declined over the first two years of life. Edwards and Wilson (1958) concluded that immunity to gastrointestinal nematodes gradually increased with age in West African Dwarf goats. Pomroy *et al.* (1986) made comparisons between nine months old New Zealand feral wethers, 12 months old New Zealand feral wethers and adult New Zealand feral does all being naturally exposed to infection on the same pasture. The does had significantly lower faecal egg counts on two occasions when they were examined. Although the design of this study is confounded by any effect of sex, the results suggest the acquisition of a measure of resistance to infection with age. A tendency for establishment rates of *H. contortus* to decrease with time during a trickle infection (Blackburn *et al.*, 1991) also suggests the development of some resistance.

Further evidence to support the contention that goats must be able to develop some resistance to gastrointestinal nematodes is the report that Angora goats could be readily selected for increased resistance to *H. contortus* (Warwick *et al.*, 1949) and that there were differences in resistance to this nematode between breeds. East African goats appeared more susceptible to this nematode than Galla and Saanen goats (Preston and Allonby, 1978) or Galla X Saanen goats (Griffin *et al.*, 1981). The finding that establishment of *H. contortus* was increased in these crossbred goats if they were concurrently infected with *Trypanosoma congolense*, also supports the view that they were normally able to develop some resistance (Griffin *et al.*, 1981).

Some studies, where goats of unspecified breeds were vaccinated with irradiated larvae of *H. contortus*, demonstrated substantial protection to challenge infections (Malviya and Tewari, 1983; Prasad and Singh, 1983a, 1983b) and further support the contention that at least some goats are able to develop some resistance. However, details of these 3 experiments are limited and do not indicate if goats were treated with an anthelmintic after vaccination which may have influenced their ability to reject a challenge infection (see Section 1.2.1).

Other studies have failed to demonstrate any development of resistance (Jansen, 1982; McCulloch and Kasimbala, 1968) or have shown an increased egg count with age (Richard *et al.*, 1990). Brunsdon (1986) generally found no difference in faecal egg counts between young goats in their first year of life and those one year older. Rahman and Collins (1990a) monitored does and kids for almost one year and found the mean faecal egg count of the does to be significantly higher than the kids. However, as these does were a milking breed, they were presumably lactating for at least part of this time and, in addition, goats were given anthelmintic treatment if their egg counts reached 2000 eggs per gram (epg) but no indication was given as to how many goats of each age were treated. Kettle *et al.* (1983) found no relationship between age and faecal egg count on 17 dairy goat farms. Here again no allowance appears to have been made for the lactational status of the goats.

Recent research on the use of novel antigens as parasite vaccines has shown that young goats can be substantially protected against *H. contortus* by a vaccine comprising antigens from the microvillous membrane of the gut of this parasite (Jasmer and McGuire, 1991) to which the host is not normally exposed or not by this route. This is, however, unusual and may bear little relationship to responsiveness to naturally acquired infections.

1.1.3. THE EFFECT OF NUTRITION

Goats kept on a low plane of nutrition were found to have higher faecal egg counts than those on a high plane of nutrition following infection with *H. contortus*, suggesting a difference in worm numbers and/or worm fecundity attributable to an effect on host immunity (Preston and Allonby, 1978). These egg counts were not, however, corrected for dry matter intake which may have affected them to some extent. In contrast, Blackburn *et al.* (1991) found no significant effect of the plane of nutrition on the establishment of *H. contortus* in goats.

1.1.4. COMPARISONS BETWEEN SHEEP AND GOATS

Comparisons between sheep and goats show sheep develop a more effective immune response to gastrointestinal nematodes. Pomroy *et al.* (1986) compared adult Romney sheep and New Zealand feral goats grazing a mixed grass and clover sward with no browse available and found significant differences in faecal egg counts between them. The faecal egg counts of goats were sufficiently high to require them to be given regular anthelmintic

treatment but this was not so with the sheep. Brunsdon (1986) observed faecal egg counts of lambs to fall spontaneously at about 10 months of age whereas those of goats continued at significantly higher levels. However, worm burdens in those sheep and goats which died of parasitism had a similar generic composition and were similar in magnitude when adjusted for liveweight of the host, suggesting goats are not more susceptible to gastrointestinal nematodes *per se*. Watson and Hosking (1989) compared young goats and lambs which were allowed to graze naturally infected pasture as well as being given pulsed infections of *T. colubriformis*. After 23 weeks, the young sheep had worm burdens which were significantly lower than those of the young goats.

Le Jambre (1984) compared yearling Merino and Angora wethers at different stocking rates on sown pasture. The sheep developed an effective immune response and had significantly fewer *H. contortus*, *T. colubriformis* and *O. circumcincta* but significantly more *Trichostrongylus axei*. The goats also proved to be more sensitive to the effect of stocking rate, suggesting that as they are forced to graze closer to the ground or to be less discriminating in their grazing, they ingest more infective larvae and therefore develop higher worm burdens.

Contrasting results indicating that sheep develop higher worm burdens than goats have also been reported. Al-Quaisy *et al.* (1987) infected Awassi sheep and an Iraqi Saanen-type goat with a sheep-derived strain of *H. contortus* and found the former had higher worm burdens. This may be a reflection of host-strain adaptation which has not been shown elsewhere, or a real reflection of a superior host resistance in the goats. Pathological changes were, however, more marked in the goats than the sheep (Al-Zubaidy *et al.*, 1987).

Stanton (1989) compared 6-7 months old goats and sheep given trickle infections of *H. contortus* and *T. colubriformis*. Goats had fewer worms than the sheep overall and this was particularly evident for *H. contortus*. A second experiment compared similar aged goats and sheep being naturally infected on pasture. Under these conditions, despite having larger nematode burdens than the sheep, goats still showed some indication of developing resistance to *H. contortus* after 10 weeks. This author suggested that part of the explanation for the difference in relative response between the pen and field experiments might be due to the rapid adaptation of the goats in the first experiment to pen conditions compared to the sheep and/or a difference in threshold worm burdens because the naturally infected animals received a much smaller challenge than those in the pen experiment. The nematode strains were the

same for both experiments. Because there has been no evidence to support the requirement for a threshold of worm exposure to induce resistance for *H. contortus* in sheep (see Section 1.2.5), other reasons are likely to apply for this species at least.

1.1.5. PERIPARTURIENT RELAXATION OF RESISTANCE (PPR)

Lloyd (1987) indicated that goats showed a PPR but did not support the claim with data. Rahman and Collins (1992) found a significant difference in faecal egg counts between lactating and non-lactating goats. However, the groups were grazed separately although pastures had a similar grazing history. Similarly, Vlassoff (1992) and Pomroy (unpublished) have noted that lactating does have higher faecal egg counts than dry does. Others have not observed this (Aken *et al.*, 1990). On balance, the weight of evidence suggests that there is a relaxation of immunity in lactating goats similar to that of sheep but it is likely to be less clearcut because of the relatively greater susceptibility of dry does to infection.

The following sections of the literature review are confined to dealing specifically with *H. contortus* and *Trichostrongylus colubriformis* as these are the two species used in experiments for this thesis. There is very little published information on host-nematode relationships of goats and of necessity this review will concentrate on information published for sheep. Similarly, in those parts of this review addressing immune mechanisms it will be necessary to discuss relevant information available from studies of host-parasite systems in rodents as well as in non-caprine ruminants.

1.2 THE DEVELOPMENT OF RESISTANCE TO *HAEMONCHUS CONTORTUS* IN SHEEP

The establishment and persistence of resistance to *H. contortus* is complex but for convenience an attempt will be made to examine each of the major components which have led to a degree of understanding of the overall picture in relation to this extensively studied host-parasite system.

1.2.1 THE EFFECT OF AGE

The methods and results of many experiments investigating the development of host resistance to *H. contortus* in sheep are summarised in Appendix 1a. It can be seen that although large numbers of permutations of

age, breed, immunization and challenge have been used, a general pattern has emerged. Where sheep are raised worm-free, they can develop an effective immune response from approximately 7-8 months of age. However, even at this age, not all sheep will do so reliably (Mulligan *et al.*, 1961; Benitez-Usher *et al.*, 1977; Altaif and Dargie, 1978b) and clinical haemonchosis can occur in adult sheep at pasture. The reason that younger sheep fail to develop an effective immune response is not clear.

Several experiments have shown that if the initial sensitising infection is not removed before challenge, the level of protection established is considerably greater and may even develop at a younger age than would otherwise be expected (Barger, 1988; Benitez-Usher *et al.*, 1977; Luffau *et al.*, 1985). Interestingly, in one study in which protection apparently followed initial infection at 2-3 months of age (Christie and Brambell, 1966), there is some evidence that not all worms were removed by the anthelmintic treatment given prior to challenge. In contrast, Dineen and Wagland (1966b) found that significant protection was evident only if the sensitising infection was removed. They considered this to be evidence of immunological exhaustion induced by the substantial primary worm burden which was established and that removal of the infection and associated delay in challenge allowed the animal to recover. Subsequent experiments provided further evidence for this (Wagland and Dineen, 1967). The difference between these and the other experiments mentioned above is the size of the sensitising infection, which was generally small where resistance was observed to be associated with an existing burden and thus unlikely to "exhaust" the immune response.

There is a suggestion, based on worm counts 26 days after infection, that older sheep (> 12 months of age) may be inherently less susceptible to *H. contortus* than lambs (≤ 9 months of age) (Knight and Rodgers, 1974) although an alternative explanation may be that the immune response is established more rapidly in these older sheep.

1.2.2 THE EFFECT OF GENETIC FACTORS

Male sheep have generally shown an increased susceptibility to infection with *H. contortus* as with other trichostrongylids (reviewed by Barger, 1993).

There is good evidence that some breeds of sheep are much better than others at resisting *H. contortus* after prior sensitisation (reviewed by Gray, 1991; see Appendix 1a). However, the rankings in various experiments are not

always consistent. Those breeds that are usually found in tropical areas, such as St Croix, Red Masai and Florida Native, generally tend to be more resistant than those from more temperate climates. However, most experiments do not take account of sire variation and single comparisons between breeds should be treated with caution (Gray, 1991). Indeed, within-breed variation is of approximately equal magnitude to variation among breeds (Gray *et al.*, 1987).

In recent years, selection within breeds has been used to improve the immune response to gastrointestinal nematodes, particularly *H. contortus*, with considerable success (Woolaston, 1990).

Several workers have shown decreased susceptibility to *H. contortus* of sheep of various breeds with HbA haemoglobin genotype compared to those with HbAB or HbB (Evans *et al.*, 1963; Jilek and Bradley, 1969; Allonby and Urquhart, 1976; Preston and Allonby, 1979) but others have failed to do so (Le Jambre, 1978; Albers and Gray, 1986; Kassai *et al.*, 1990; Luffau *et al.*, 1990; Bradley *et al.*, 1973). The reason for this dichotomy of results is not readily explained.

1.2.3 THE EFFECT OF NUTRITION

An inadequate diet may be detrimental to the development and/or the maintainance of resistance to *H. contortus* although there is some equivocal evidence. Abbott and Holmes (1990) successfully protected 7 month old Scottish Blackface sheep by vaccination with irradiated larvae, regardless of whether they were on a low or high protein diet. In contrast, Roberts and Adams (1990), using Australian Merinos, showed significantly increased egg counts in penned sheep fed a smaller ration although no allowance was made for faecal concentration in this comparison. Gordon (1948) also demonstrated that Australian Merinos fed on a lower plane of nutrition were slower to expel an existing *H. contortus* burden and then developed much larger burdens when reinfected. Similarly, Preston and Allonby (1978) found that sheep of several breeds fed a low protein diet had higher faecal egg counts and were slower to expel their nematode burdens than animals on a higher protein diet.

1.2.4 "SELF-CURE"

The term "self-cure" has been used to describe the abrupt rejection of established adult *H. contortus* following the intake of infective larvae. The term (as noted by Stewart, 1950) was first used in this context by Stoll (1929)

to describe the sudden drop in faecal egg counts in a pair of twin lambs grazing contaminated pasture. Gordon (1948) subsequently used the term in connection with dramatic falls in *H. contortus* faecal egg counts of sheep at pasture which coincided with periods when the pasture had "freshened" after rain. This would equate with ideal conditions for the development and/or migration of infective larvae onto herbage. These sheep were not resistant to reinfection as their faecal egg counts subsequently rose to very high levels and several died of acute haemonchosis.

Gordon (1967) found the majority of sheep expressing "self-cure" rejected the existing infection but allowed establishment of the new infection - so called "classical self-cure". A much smaller percentage also resisted establishment of a new infection. As few as 250 infective larvae have been found to induce "self-cure" but the success of such small challenges appears to be greater in sheep carrying a large worm burden (Gordon, 1967). Most reported experimental investigations into "self-cure" have used large challenges. Under grazing conditions in Australia naturally occurring "self-cure" is sporadic and unpredictable, and related to the weather conditions referred to earlier (Gordon, 1948; Dineen, 1978).

The phenomenon of "self-cure" has been observed in lambs as young as 4 months (Lopez and Urquhart, 1967). These particular animals subsequently developed very high worm burdens, supporting Gordon's (1948) conclusion that "self-cure" is not a "manifestation of resistance by the host".

Stewart (1953) showed that "self-cure" was closely associated with immediate hypersensitivity in as much as blood histamine levels were observed to rise and antihistamine drugs essentially prevented the sudden fall in egg counts. However, parenteral treatment of sheep with histamine had no effect on egg counts, although no measurements of histamine levels in the circulation or the abomasum were reported.

There appear to be no reports of "self-cure" in goats.

1.2.5 POPULATION DYNAMICS

Variable establishment rates of *H. contortus* in young lambs or naive older sheep have been reported. Mostly they are in the range of 40% - 60% (Barger and Le Jambre, 1988 ; Dineen and Wagland, 1966a; Barger *et al.*, 1985) but some are as high as 83% (Coadwell and Ward, 1981). In sheep which have

had sufficent exposure to induce an immune response, this establishment rate generally falls as host immunity develops. In Australian Merinos, it has been found to fall to levels of 3.5% or less (Barger *et al.*, 1985; Barger and Le Jambre, 1988). In both reports, analysis shows that establishment of infective larvae is not influenced by the rate of larval intake. The proportion of establishing larvae that are inhibited at the early fourth stage (EL4) increased as resistance developed although as larval establishment falls so eventually do numbers of EL4 (Barger *et al.*, 1985). Others, however, have not found EL4 numbers to increase with the development of resistance (Coyne and Smith, 1992).

Although Dineen *et al.* (1965) proposed that a threshold of antigenic information was required before the immune system started to mobilize an effective response against *H. contortus*, there has been no mention of this in subsequent investigations of population dynamics (Barger *et al.*, 1985; Barger and Le Jambre, 1988; Smith, 1988; Coyne *et al.*, 1991; Coyne and Smith, 1992).

Parasite fecundity does not appear to be affected by developing resistance (Coyne and Smith, 1992; Coyne *et al.*, 1991) although female worms recovered from resistant sheep have been noted to be slightly shorter (Coyne and Smith, 1992). Variable estimates of fecundity from 4700-12000 eggs/female/day have been made (Coyne and Smith, 1992; Dineen *et al.*, 1965). Another parameter that has been observed to vary with resistance for other trichostrongylids is male:female ratio (see Section 1.3.5) but this was observed to remain constant with time for *H. contortus,* suggesting that developing resistance did not kill male worms preferentially (Coyne *et al.*, 1991).

The instantaneous death rate of *H. contortus* has been estimated at between 0.01 and 0.07 worms per worm per day (Barger and Le Jambre, 1988; Coyne *et al.* 1991) and whilst the latter report found mortality increased with size of infection in the absence of further larval intake, the former found it had no effect. The death rate of older worms was also observed to increase as the rate of larval intake increased (Barger and Le Jambre, 1988). The lower figure of 0.01 gives a predicted half-life of 69 days with a mean life expectancy for individual worms of 100 days (Barger and Le Jambre, 1988) whilst for a death rate of 0.07 mean life expectancy is only calculated as 13.3 days (Coyne *et al.*, 1991).

In spite of the low establishment rate in adult sheep and the effect that high larval intakes have on the death rate of the nematodes, it is still

conceivable that significant burdens could establish in adult sheep if larval intakes are high and continue for long enough, particularly when combined with suboptimal nutrition. This could account for the occurrence of clinical haemonchosis in adult Australian Merinos as reported, for example, by Gordon (1948).

In the absence of reinfection, the duration of resistance following removal of the initial sensitising infection is relatively short. Wagland and Dineen (1967) showed a reduction in protection after 105-111 days; others have shown no protection after 9 weeks (Coyne and Smith, 1992) and 12 weeks (Jackson *et al.*, 1988).

Although it has been suggested that the challenge infection needs to be substantial to induce a protective immune response (Jackson *et al.*, 1988) others have found a challenge as low as 3000 larvae is sufficent to elicit protection (Dineen and Wagland, 1966b; Wagland and Dineen, 1967; Donald *et al.*, 1969).

1.3 THE DEVELOPMENT OF RESISTANCE TO *TRICHOSTRONGYLUS COLUBRIFORMIS* IN SHEEP

Many factors have been found to influence the establishment and persistence of resistance to *T. colubriformis*. The results of a large number of studies are summarised in Appendix 1b.

1.3.1 THE EFFECT OF AGE

Most of the reports noted in Appendix 1b indicate that an age of around 5-6 months is needed for the development of some measure of an effective immune response. This is consistent with the general observation that lambs younger than 6 months of age are usually less capable of developing a strong protective immunity to trichostrongylids than older lambs (Donald and Waller, 1982). However, evidence for an effective immune response has been observed as early as 12-17 weeks of age (Douch, 1988; Chiejina and Sewell, 1974a; Dineen *et al.*, 1978; Dineen and Windon, 1980). By 10 months of age, sheep can mount a very effective immune response rejecting >90% of a challenge infection (Gregg *et al.*, 1978; Gregg and Dineen, 1978). Nevertheless, examination of faecal egg counts from routine laboratory submissions in New Zealand suggests that it takes several months more for young sheep to express the solid immunity seen in mature adult sheep under field conditions (Stafford *et al.*, 1994). Such a gradual maturation of the immune response to *T.*

colubriformis is not generally seen under experimental conditions and may be a reflection of a variety of factors affecting resistance, including nutrition. Under New Zealand pastoral conditions it is not likely to be due to inadequate exposure to trichostrongylid larvae.

The presence or absence of worms does not appear to affect the resistance of young lambs to challenge infection as it does for *H. contortus* (Barger, 1988).

Dobson *et al.* (1990c) noted that age at the commencement of a continous primary infection affected the rate of development of this immune response. In sheep aged 36 weeks, establishment of a challenge infection decreased to negligible levels by 5 weeks whereas in 12 week-old sheep this took 9 weeks.

1.3.2 THE EFFECT OF GENETIC FACTORS

There appear to be no published reports of breed comparisons of resistance to *T. colubriformis* (Gray, 1991).

Genetic variability in the response of sheep to *T. colubriformis* within breeds allowed separation of lambs into "responders" and "non-responders" following vaccination with irradiated larvae and subsequent challenge (Dineen *et al.*, 1978; Windon and Dineen, 1980). Breeding lines have since been established to select for increased and decreased resistance to *T. colubriformis* and the heritability of responsiveness, based on faecal egg counts, has been shown to be 0.39±0.27 (Windon *et al.*, 1987). These two lines are continuing to diverge although the most dramatic response was seen in the first generation. Female lambs are more immunologically responsive being either more resistant than males in the "high responder" line or less resistant than males in the "low responder" line (Windon 1990). No mention has been made of the immune response of adult animals in these selection lines. In flocks selected for or against resistance to *H. contortus* in lambs this is also reflected in the same qualities of adult sheep (Gray, 1991).

There is evidence to suggest that selecting animals for resistance to one particular nematode species confers resistance to some other genera and species though not necessarily to the same extent. Young sheep selected for increased or decreased resistance to *H. contortus* also showed a difference in faecal egg counts following infection with *T. colubriformis* (Woolaston *et al.*,

1990). The results following *Haemonchus* infection of lines of sheep selected for resistance to *T. colubriformis* is less convincing but significant cross-protection was, however, achieved to infections with *Trichostrongylus rugatus, Trichostrongylus axei* and *Ostertagia circumcincta* (Windon *et al.*, 1987).

The lymphocyte antigens SY1a and SY1b have been found to be present with higher frequency in "high responder" sheep than in "low responder" sheep in *T. colubriformis* selection lines. These antigens are thought to be coded for by genes closely associated with the MHC (Outteridge *et al.*, 1985; Outteridge *et al.*, 1988).

Windon *et al.* (1984) demonstrated ewe lambs were more resistant than ram lambs following vaccination with high doses of irradiated larvae but no difference was observed following vaccination with low doses. Dobson *et al.* (1990d), however, could find no evidence that host sex plays a significant role in determining the development of the immune response against *T. colubriformis*. Nevertheless, experiments with laboratory animals indicate that male animals are generally more susceptible to nematode infection than females (Barger, 1993)

1.3.3 THE EFFECT OF NUTRITION

Wagland *et al.* (1984) showed that young lambs on a high plane diet had smaller *T. colubriformis* burdens than those on a low plane diet, suggesting that nutrition may be a significant factor in the development or maintenance of an immune response. This is consistent with numerous field reports of poorly fed adult sheep developing substantial worm burdens.

1.3.4. POPULATION DYNAMICS

DECLINE IN WORM ESTABLISHMENT

There is a threshold of worm exposure that has to be passed before substantial resistance to establishment occurs (Dineen, 1963; Donald *et al.*, 1964). In lambs sensitised with irradiated larvae, Windon *et al.* (1984) estimated the threshold vaccine dose to be about 4400 irradiated larvae given twice. As only female worms establish after irradiation, this was considered to equate to a worm burden of approximately 2-4000 worms (Dobson *et al.*, 1990a). Dobson *et al.* (1990a, 1990d) estimated the threshold to be approximately 2900-3500 established adult worms but the variability around this

threshold was large particularly at lower infection rates. Waller and Thomas (1981) observed a similar threshold phenomenon for *Trichostrongylus vitrinus* infections in lambs.

Emery *et al.* (1992a) extended this concept to one of "worm-days" of exposure and calculated, from published reports, a figure of around 1.8×10^5 "worm-days". In one infection allowed to continue for 8 weeks, sheep were exposed to 7.2×10^5 "worm-days" and displayed solid resistance. However, a series of larval infections truncated at 4 days exceeded this figure but did not protect sheep. The authors suggested this may indicate a need for exposure to a critical amount of antigen which is produced in larger quantities by adult worms. However, this concept is only useful if duration of infection is the principal factor in inducing resistance and ignores changes between larval and adult antigens (see below for discussion on stage-specific immunity).

Resistance to *T. colubriformis* is initially expressed as a suppression of establishment of larvae whilst adult worm numbers are unaffected (Gibson *et al.*, 1970; Gibson and Parfitt, 1972, 1973; Chiejina and Sewell, 1974a; Dobson *et al.*, 1990c). Decreased fecundity of female worms (Gibson and Parfitt 1972, 1973; Chiejina and Sewell 1974a; Dineen and Windon 1980b; Dobson *et al.*1990a) and a decrease in the ratio of male to female worms (Dineen and Windon, 1980b; Douch, 1989) also occur. Eventually adult worms are expelled.

An integrated series of models to describe the population dynamics of *T. colubriformis* in sheep has recently been published (Dobson *et al.*, 1990d) based on several related experiments (Dobson *et al.*, 1990a, 1990b, 1990c) summarised in Appendix 1b. These showed that once the threshold was achieved there was a similar rate of decline of establishment at the three dose rates investigated (1000, 3160 or 10,000 L3 per week). Once sheep are fully immune they reject most larvae within 3 days of infection (McClure *et al.*, 1992; Emery *et al.*, 1992a)

REJECTION OF ADULT WORMS

Dobson *et al.*(1990c) observed that rejection of adult worms occurs over a period of about 9 weeks commencing when establishment of incoming larvae declines to approximately 1%. Death rate of worms increases exponentially over this 9 week period and is largely controlled by host-derived factors and not worm age. A notable feature was the large variability in the onset of rejection, supporting the notion of genetic influences on the onset of the immune

response. In sheep exposed to a low rate of infection, the time to reach threshold levels is longer and therefore expulsion of adults is delayed relative to sheep given higher rates of infection. This pattern of population dynamics is consistent with the earlier work of Chiejina and Sewell (1974b) in which they showed that worm burdens were cumulative for 4 to 8 weeks after which establishment declined to negligible levels. This was followed by a fall in adult worm numbers between 8 to 15 weeks. Once the threshold is exceeded, adult worm expulsion would seem to be independent of infection rate and worm burden, which is contrary to earlier opinions (Dineen, 1963; Donald *et al.*, 1964) that suggested infections below the threshold would be continually tolerated.

STAGE-SPECIFIC IMMUNITY

Immunity induced by L3 and early L4 *T. colubriformis* appears to effectively protect sheep against larval challenge but is less effective against adult worms (Emery *et al.*, 1992a). Sheep immunised by transfer of adult *T. colubriformis* did not reject a challenge until 7-10 days post-infection which is much later than expected. This indicates the stage-specific nature of the rejection process (Emery 1992b).

NEMATODE FECUNDITY

Although Chiejina and Sewell (1974b) suggested that decreased fecundity preceded expulsion, Dobson (1990d) suggested they commenced essentially at the same time. Peak egg production has been estimated from models to be approximately 600-700 eggs per female per day (Dobson *et al.*, 1990a) but at higher infection rates the threshold exposure for the development of resistance may be reached before female worms have reached maturity thus limiting their egg production. No density-dependent effects on fecundity have been observed (Dobson, 1990d).

ARRESTED DEVELOPMENT

Arrested development of exsheathed L3 is generally accepted to be at least partly a consequence of the immune response (Eysker, 1978; Seaton *et al.*, 1989) and has been shown to increase as hosts become more resistant (Dobson *et al.*, 1990b).

The following sections deal with the immune mechanisms involved in the host's response to gastrointestinal nematodes by firstly considering the cellular response. Most of the work is based on parasitic infections of rodents although information from experiments with ruminants is included where possible.

1.4 T CELLS

Acquired immunity to gastrointestinal nematodes has been shown to be under lymphocyte control, particularly T cells. This has been demonstrated by adoptive transfer of lymphocytes from immune to naive animals in a variety of host-nematode systems (Wagland and Dineen, 1965; Nawa and Miller, 1978; Grencis and Wakelin, 1982; Smith *et al.*, 1984, 1986).

Of the various subsets of lymphocytes, T-helper (T_H) cells which express $CD4^+$ cell-surface markers, have been shown to play a pivotal role in mediating this immune response to gastrointestinal nematodes (Gill *et al.*, 1993). T_H cells have been divided into at least 4 subgroups in mice: T_Hp, T_H0, T_H1 and T_H2, each having distinct cytokine secretion patterns (reviewed by Mosmann and Moore, 1991). T_H1 and T_H2 subsets with similar cytokine secretion patterns to those in mice have also been identified in humans (reviewed by Romagnani, 1991) indicating this classification may be generally valid. It has been suggested that T_H1 cells are important in the control of intracellular parasites whereas T_H2 cells are more involved with extracellular organisms, particularly helminths (Janeway *et al.*, 1988; Finkelman and Urban, 1992). T_H1 cells are involved with delayed hypersensitivity responses whilst the characteristic host responses to helminth infections, including mastocytosis, eosinophilia and increased IgE and IgG_1 synthesis all appear to be induced by T_H2 cells and the cytokines they produce (see Table 1.1)(Finkelman *et al.*, 1991).

Cross-inhibitory behaviour is evident between T_H1 and T_H2 subsets and this is consistent with the inhibition of delayed hypersensitivity responses which occurs during a strong antibody response (Mosmann and Moore, 1991). Generation of the appropriate T_H cell response would appear to be important in the ability of different inbred strains of mice to control *Trichuris muris* (Else and Grencis, 1991) and *Leishmania major* (reviewed by Locksley and Scott, 1991). However, experiments in which various cytokines have been blocked with monoclonal antibodies have shown that this does not necessarily interfere with worm rejection (reviewed by Finkelman *et al.*, 1991). Nevertheless, Finkelman and Urban (1992) proposed that T_H2 responses are generally

protective and T_H1 are generally harmful to animals infected with intestinal helminths, and *vice versa* for intracellular parasites.

Cytokine[1]	Role/function
IL-4	induces IgE and IgG1 production (Coffman *et al.*, 1986; Snapper and Paul, 1987)
IL-5	induces eosinophilia (Finkelman *et al.*, 1991) and promotes IgA production (Haig and Miller, 1990)
IL-3 + IL-4	IL-4 augments IL-3 initiated mast cell proliferation (Smith and Rennick, 1986; Ihle *et al.*, 1983; Mosmann *et al.*, 1986; Rennick *et al.*, 1985; Madden *et al.*, 1991)

1. IL=Interleukin

Table 1.1 Recorded roles or functions of T_H2 cytokines

1.5 EOSINOPHILS

1.5.1 INTRODUCTION

Increased numbers of eosinophils in either tissues and/or blood are commonly associated with parasitic infections (Miller, 1984). Eosinophils differentiate in the bone marrow or within other haemopoietic foci and are then recruited to various tissues as differentiated cells (Haig and Miller, 1990). The main cytokine that specifically stimulates proliferation and differentiation of committed eosinophil progenitors is IL-5 (see Section 1.4). A large variety of eosinophil chemotactic factors which recruit eosinophils to particular tissue sites have been described including eosinophil chemotactic factors of anaphylaxis from mast cells, histamine, leukotriene B_4, IL-2, lymphocyte chemoattractant factor, platelet activating factor (PAF) and excretory-secretory molecules from nematodes (Kay, 1985; Wardlaw *et al.*, 1986; Weller, 1992; Klesius, 1993). Most eosinophils are found in tissues and not the circulation (McEwan, 1992). Research over the last 10-15 years in particular, has revealed the extraordinary functional complexity of these cells.

1.5.2 INFLAMMATORY MEDIATORS

Within their cytoplasm, eosinophils have numerous granules containing preformed inflammatory mediators which include eosinophil major basic protein, eosinophil cationic protein and eosinophil peroxidase. Those mentioned have been shown to be directly toxic to helminths (Butterworth *et al.*, 1979; McLaren *et al.*, 1981; Wassom and Gleich, 1979; Duffus *et al.*, 1980; McEwen, 1992). Eosinophils also have the capacity to exhibit a respiratory burst (Spry, 1985) which, it has been suggested, may play a role in damaging helminths (Kazura *et al.*, 1981).

As well as preformed mediators, eosinophils can also generate leukotrienes, particularly leukotriene C_4 (LTC_4)(Jörg *et al.*, 1982; Shaw *et al.*, 1985; Kajita *et al.* 1985; Weller *et al.* 1983) and PAF (Lee *et al.*, 1984) following appropriate stimulation of IgG and IgE surface receptors (Moqbel and MacDonald, 1990). These may have adverse effects on nematodes. Secretion of leukotrienes is an aspect of eosinophil activity that has received little experimental attention but may contribute to the immune response against helminths in the lumen of the gastrointestinal tract (see Section 1.8). This possibility has been disregarded by some authors, for example, Haig and Miller (1990), when summarising the role of eosinophils against helminths, referred only to their potential to damage or kill tissue-dwelling parasites.

1.5.3 EOSINOPHIL MODULATION OF INFLAMMATION

Eosinophils have the ability to modulate reactions due to IgE-mediated mast cell degranulation (Henderson *et al.*, 1982; Butterworth, 1984; Kay, 1985). However, to describe the role of eosinophils as "downregulators" of mast cells has been considered an oversimplification of their role in hypersensitivity responses (Rothwell, 1989) given their ability to also generate leukotrienes and PAF which can mediate inflammatory reactions (see Sections 1.5 and 1.7). In addition, eosinophil major basic protein can activate mast cells for histamine release (O'Donnell *et al.*, 1983). It is considered more likely that eosinophils will actually contribute to inflammatory injury in response to infectious agents rather than act primarily as "downregulators" (Spry, 1985). The situation is further complicated by the fact that eosinophils do not comprise a uniform cell population (see below).

1.5.4 PHENOTYPIC HETEROGENEITY

Eosinophils may be separated through density gradients into two populations referred to as normodense and hypodense. Hypodense eosinophils are more active metabolically (Winqvist *et al.*, 1982), express more IgG, IgE and complement receptors (Parillo and Fauci, 1978; Winqvist *et al*, 1982; Capron *et al.*, 1985), are more abundant in parasitised hosts (White *et al.*, 1986) and produce more LTC_4 (Kajita *et al.*, 1985). This heterogeneity may have some relevance, therefore, to their role in immune responses to helminths.

1.5.5 ANTI-HELMINTH ACTIVITY

A role in killing helminths has been demonstrated *in vitro* associated with degranulation of eosinophils and release of mediators onto the surfaces of *Schistosoma mansoni* (McLaren and Terry, 1982; Butterworth, 1984), *Trichinella spiralis* larvae (Grove *et al.*, 1977; Dessein *et al.*, 1981), *Strongylus vulgaris* larvae (Klei *et al.*, 1992), *Angiostrongylus cantonensis* (Yoshimura *et al.*, 1984) and *Dictyocaulus viviparus* larvae (Duffus, 1989). Hypodense eosinophils are more efficient in *in vitro* assays in killing *T. spiralis* larvae (Hamada *et al.*, 1992) and *S. mansoni* schistosomules (Capron *et al.*, 1984).

Eosinophils express low affinity FcE-RII receptors for IgE on their surfaces (Capron *et al.*, 1986) as well as receptors for IgG (Capron *et al.*, 1981), IgA (Spry *et al.*, 1992) and various complement components including C3a, C3b, C4 and C5a (Gerard *et al.* 1988; Muller-Eberhard, 1988; Goers *et al.*, 1984; Anwar and Kay, 1977). Attachment of eosinophils to helminths may be mediated by these various ligands either alone or in combination (Butterworth, 1984). Different inflammatory mediators have been shown to be selectively released depending on the receptors involved (reviewed by McEwan 1992).

Although eosinophils are often found in the parasitised gastrointestinal mucosa, nematodes in the lumen are unlikely to come into physical contact with sufficient numbers to suffer from significant damage through degranulation onto their cuticles (Moqbel and MacDonald, 1990; Dunn and Wright, 1985; Rothwell, 1989). In addition, depletion of eosinophils in infected mice had no influence on the expulsion of *T. spiralis* (Grove *et al.*, 1977) or *H. polygyrus* (Urban *et al.*, 1991) suggesting that neither physical contact nor production of mediators from eosinophils are essential for protection from at least these gastrointestinal nematode infections.

1.5.6 EOSINOPHIL RESPONSES IN RUMINANTS WITH GASTROINTESTINAL NEMATODES

In ruminants, the eosinophil responses to trichostrongylids have been investigated mainly in sheep but no consistent pattern has been found. This lends support to the view that these cells are not essential for the effectiveness of the immune response to gastrointestinal nematodes, but does not exclude their involvement.

An increase in eosinophils in gastrointestinal tissue has been described in adult sheep showing evidence of an effective immune response to *H. contortus* and *T. colubriformis* (O'Sullivan and Donald 1973). A similar marked increase in abomasal eosinophils occurred in adult sheep following infection with *H. contortus* but, in young lambs (< 14 weeks), there was only a slight increase in the 6 weeks following infection (Salman and Duncan, 1984). However, in a longitudinal study of *H. contortus* infections in 4 month-old lambs, eosinophil infiltration of the abomasal mucosa increased markedly 4 to 6 days post-infection whilst there was an almost complete absence of eosinophils from control worm-free sheep (Charleston, 1965). In another series of experiments in which lambs were given nine weekly doses of irradiated *H. contortus* larvae from two weeks of age and then challenged, there were more eosinophils in the abomasum in vaccinated animals but no apparent difference in *H. contortus* numbers (Salman and Duncan, 1985) indicating this cellular response was not necessarily associated with a protective immune response.

Significantly higher eosinophil counts in intestinal tissue developed in 5-10 month-old sheep vaccinated against *T. colubriformis* and given either an homologous or mixed field challenge compared with previously naive controls given a similar challenge (Douch *et al.* 1986; Douch, 1989). In contrast, Gregg *et al.* (1978) found no difference in intestinal eosinophil counts between 9-12 months-old sheep, which had been successfully vaccinated with irradiated *T. colubriformis*, and naive controls following challenge. However, a circulating eosinophilia was noted in the controls prior to challenge which suggests that some unrelated stimulus was responsible and possibly masked any effect of the parasites. Immune sheep given a trickle infection with *T. colubriformis* had an eosinophilia for the first six weeks which then resided to normal levels for the remaining four weeks of the experiment (Kimambo *et al.*, 1988b) which suggests eosinophils had no role in the continued resistance of these sheep. No tissue eosinophil counts were reported for this experiment to see if circulating levels were reflected in the mucosa. An attempt to vaccinate young lambs (up to 16

weeks-of-age) against *T. colubriformis* did not result in a difference in tissue eosinophil counts between vaccinated lambs which became resistant ("responders") and those that did not (Douch, 1988). Similarly, only a weak non-significant positive correlation was found between tissue eosinophil and worm counts in "responder" lambs which had been vaccinated and subsequently challenged with *T. colubriformis* (Dineen *et al.*, 1978).

Higher circulating eosinophil counts following infection with *T. colubriformis* have been observed in sheep genetically selected to be high "responders" (Dawkins *et al.*, 1989; Buddle *et al.*, 1992). For random-bred sheep, however, the correlation between circulating eosinophil counts and faecal egg counts is less clearcut than the difference between genetically selected lines (Dawkins *et al.*, 1989; Rothwell *et al.*, 1993).

It is interesting to note that higher tissue eosinophil counts have been reported from goats than from sheep given the same experimental trichostrongylid infections (Stanton, 1989; Al-Zubaidy *et al.*, 1987). As in sheep, there would seem to be a tendency for younger goats (less than about 8-9 months old) not to develop an eosinophil response to nematode infections (Fitzsimmons, 1966; Fitzsimmons *et al.*, 1968; Blackburn *et al.*, 1992). However, Rahman and Collins (1990b) found *T. colubriformis* infections resulted in an eosinophilia in 3-4 month-old goats but no difference was noted between two infection rates. In general little work has been done with goats.

1.6 MAST CELLS

1.6.1 INTRODUCTION

Mucosal mastocytosis is a common sequel to gastrointestinal nematodiasis. These cells are considered to be important as a source of immune mediators, both generated *de novo* and preformed, which are potentially capable of contributing to the expulsion of gastrointestinal nematodes. The major mediators which are preformed and stored within cytoplasmic granules include histamine, 5-hydroxytryptamine (5-HT), chondroitin sulphate and various proteases, whilst those generated *de novo* include Leukotriene B_4 (LTB_4), LTC_4, prostaglandins and PAF. Mediators with known or potential activity against helminths are considered in detail later in this review. Mast cells are also known to be able to secrete a variety of cytokines although their role is still uncertain (Gordon *et al.*, 1990).

Mast cells express high-affinity receptors for IgE and low-affinity receptors for IgG. Although different mediators have been reported to be released following binding and crosslinking of these receptors, the profile of mediators is considered more indicative of the mast cell phenotype (see below) than the receptor involved (Castells *et al.*, 1992). An increase in IgE receptors has been observed following infection of rats with *N. brasiliensis* (Chen and Enerback, 1992). In addition to these Ig receptors, a variety of other factors can induce mediator release including neuropeptides, C3a and C5a complement components, IL-1 and several cell-derived "histamine releasing factors" (see reviews by Huntley, 1992 and Siraganian, 1988). More recently stem cell factor, a ligand for the c-*kit* tyrosine kinase receptor on mast cells, has been shown to be able to induce mediator release *per se* as well as greatly enhancing IgE-dependent mediator release (Bischoff and Dahinden, 1992; de Paulis *et al.*, 1992).

Mast cells are derived from pluripotential haemopoietic stem cells that are located in bone marrow (Kitamura *et al.*, 1981) and it is thought that the cells which seed tissues from the bone marrow are probably progenitor cells (Jarrett and Haig, 1984). Mast cells are not, however, a homogeneous cell-type.

1.6.2 PHENOTYPIC HETEROGENEITY

Maximow, in 1905, (cited by Enerback, 1986) is credited with the first observation of mast cell heterogeneity as he noted that mast cells in rat intestinal mucosa differed morphologically from those occurring elsewhere. Mast cells have usually been classified, particularly in rodents, into two main categories based on their location: mucosal mast cells (MMCs) found in the mucosa of the gastrointestinal and respiratory tracts, and connective tissue mast cells (CTMCs) found elsewhere in the body such as the skin and serosa. Some of the principal features of these in rodents are summarised in Table 1.2. Within mucosae, MMCs are usually in the lamina propria (Galli, 1990).

However, classification based solely on location is not entirely satisfactory, even in rodents (Aldenburg and Enerbeck, 1988) and does not seem to be applicable to mast cells from humans (Schwartz, 1989).

	Rat		Mouse		
	Mucosal Mast Cell	**Connective Tissue Mast Cell**	**Mucosal Mast Cell**	**Connective Tissue Mast Cell**	**Bone marrow cultured mast cells**
Morphology	Small cells with few variable sized granules[1]	Larger cells of uniform shape and many uniform size granules[1]			
Migratory capacity	Yes[1]	No[1]			
Proteoglycan	Chondroitin sulphate[1]	Heparin[1]	Chondroitin sulphate[6]	Heparin[2,6]	Chondroitin sulphate[2,6]
Fixation	Anionic sites blocked by aldehyde fixation[1]	Anionic sites not blocked by aldehydes[1]			
Proteinase	Rat mast cell proteinase 2[1] Carboxypeptidase[2]	Rat mast cell proteinase 1[1]	Mouse mast cell protease 1 and 2[9]	Mouse mast cell protease 3, 4, 5 and 6. Carboxypeptidase A[9]	Mouse mast cell protease 5, 6 and 7. Carboxypeptidase A[9]
Control over proliferation	T cell dependent[1,3]	No proliferative immune response[1,3]	T cell dependent[2]	None[8]	T cell dependent[2]
Lifespan	Short; half-life <40days[1]	Long; half-life >60days[1], >6months[7]			
Alcian blue/safranin staining	Alcian blue +ve[1]	Safranin +ve[1]	Alcian blue +ve[6]	Safranin +ve[2]	Alcian blue +ve[6]
Berberine staining	Non-fluorescent[1]	Fluorescent[1]	No[6]	Yes[6]	No[6]

	Rat		Mouse		
	Mucosal Mast Cell	**Connective Tissue Mast Cell**	**Mucosal Mast Cell**	**Connective Tissue Mast Cell**	**Bone marrow cultured mast cells**
Histamine	+ve; 1/10th of CTMC[1] (1-2pg/cell)[3]	+ve[1] (15pg/cell from peritoneal cavity)[3]	Low[6]	High[6]	+ve; (0.4-0.6μg/10^6cells[3]; ie. low[6]
Serotonin		+ve[2]	Low[6]	Variable[6]	High[6]
Arachidonic metabolites	PGD2=LTC4 (20μg/10^6)[5]; $LTC_4 >> PGD_2$[7]	PGD2>>LTC4 (60μg:4-6μg/10^6)[5]			LTC4>>PGD2[5]

Key to References:

1. Enerback, 1986
2. Befus *et al.*, 1986
3. Stevens *et al.*, 1986
4. Miller *et al.*, 1989
5. Broide *et al.*, 1989
6. Galli, 1990
7. Siraganian, 1988
8. Huntley *et al.*, 1990
9. Castells *et al.*, 1992

Table 1.2. Morphological and functional characteristics of rodent mast cells

An alternative classification, based on their ability to bind cationic dyes such as toluidine blue following fixation in 4% formaldehyde, has been used in many species including humans (Enerback *et al.*, 1986), sheep (Chen *et al.*, 1990b), cattle (Chen *et al.*, 1990a) and pigs (Ashraf *et al.*, 1988) . Mast cells which are formalin-sensitive and lose their cationic dye-binding ability are generally considered to correspond to MMCs and those which do not to CTMCs.

1.6.3 FIXATION AND STAINING

One of the problems for those engaged in research on mast cells (and in turn for those reviewing the literature on them) is that they are difficult to fix and stain for identification and reliable counting. The most common approach to identifying mast cells is to make use of the metachromatic staining of their cytoplasmic granules. This metachromasia depends on the number of free acidic polyanionic groups of the proteoglycan and their availability after fixation. When a basic blue dye binds to these anionic groups in close proximity, aggregation of the dye occurs with a subsequent spectral shift from blue to purple (Askenase, 1980).

However, the level of staining of MMCs achieved, and the ease with which they can be identified, is very dependent on the fixative used. For rat tissues, Enerback (1966) chose isotonic formaldehyde acetic acid (IFAA) as the preferred mast cell fixative and proposed it as an alternative to Carnoy's fluid. It was shown to be superior to 4% formaldehyde, various concentrations of ethanol and Bouin's fluid. He found both Carnoy's fluid and basic lead acetate allowed some metachromatic staining although the staining time he used was only 4 minutes. Subsequently, Enerback (1986) showed that tissue preservation is adequate if fixation time in IFAA is restricted to 12 to 24 hours, followed by transfer to 70% ethanol for 12 hours before paraffin embedding and processing. The uptake of toluidine blue after aldehyde fixation can be improved by prolonged staining (e.g. 5 to 7 days) at a very low pH (Enerback and Wingren 1980). The ability of toluidine blue to stain CTMC was shown to be largely unaffected by the choice of fixative (Enerback, 1966).

Since 1966, the majority of authors have used Carnoy's fluid when counting intestinal mast cells in rodents (Miller *et al.*, 1981; Murray *et al.*, 1968). More recently, 4% paraformaldehyde has been used because it is as satisfactory as Carnoy's fluid and also allows staining for eosinophils and

immunocytochemistry for rat mast cell proteinase II (RMCP II)(Newlands *et al.* 1984).

In ruminants, corrosive formalin (=sublimate formal) has been used by several workers wishing to count MMCs (Miller *et al.*, 1967: Murray *et al.*, 1968; Dineen and Windon 1980a) and lead acetate by others (Gregg *et al.*, 1978; O'Sullivan and Donald, 1973). Corrosive formalin is considered a superior fixative for achieving brilliant staining with acid dyes and enhanced metachromasia (Drury and Wallington, 1967). IFAA has also been used for counting MMCs in sheep and cattle (Chen *et al.*, 1990a, 1990b) although it results in poor overall fixation (Miller *et al.*, 1967: Murray *et al.*, 1968). As in rodents, 4% paraformaldehyde has been used more recently to allow comcomitant staining of MMCs with toluidine blue and immunocytochemistry for sheep mast cell proteinase (SMCP) (Huntley *et al.*, 1992).

Immunocytochemical identification of mast cells using monoclonal antibodies to specific proteases has resulted in more MMCs being identified in human (Walls *et al.*, 1990) and mouse tissues (Miller unpublished, cited by Rothwell 1989) than by other procedures. However, in sheep slightly higher MMC counts were achieved with toluidine blue than with a monoclonal antibody to SMCP although the statistical significance of this difference was not indicated (Huntley *et al.*, 1992).

1.6.4 MAST CELL PROLIFERATION AND DIFFERENTIATION

It has been demonstrated that rodent-derived MMC populations are sensitive *in vitro* to growth factor/s from T cells which were subsequently identified as IL-3 potentially augmented with IL-4 (Schrader *et al.*, 1981; Razin *et al.*, 1981; Tertian *et al.*, 1981; Ihle *et al.*, 1983; Schrader, 1986; Haig *et al.*, 1982; Hamaguchi *et al.*, 1987). Experiments in rats infected with nematodes also suggest that the MMC response which usually occurs is T cell mediated (Mayrhofer and Fisher, 1979; Ruitenberg and Elgersma, 1976), at least partly, through the action of IL-3 (Abe and Nawa, 1988).

On the other hand, CTMCs exhibit no apparent dependence on T cells *in vivo* (Ruitenberg and Elgersma, 1976; Mayrhofer and Fisher, 1979). The presence of fibroblasts appears critical for the development of CTMCs *in vitro*, at least for mouse-derived cells (Levi-Schaffer *et al.*, 1986; Dayton *et al.*, 1988; Jarboe *et al.*, 1989). The fibroblast-derived factor responsible awaits identification (Galli, 1990) but is known not to be IL-3 or IL-4 (Jarboe *et al.*,

1989). It has not been possible to demonstrate this dependence in rat systems (Broide *et al.*, 1989).

A complication is that differentiation of mast cells is affected by the microenvironmental conditions in which they are found. Cells with MMC characteristics are capable of transforming to those with CTMC characteristics and *vice versa* (Kanakura *et al.*, 1988; Kobayashi *et al.*, 1986; Sonoda *et al.*, 1986).

More recently stem cell factor has been shown to be essential for mast cell development (Galli *et al.*, 1992).

There is some controversy as to whether or not mature mast cells are capable of division (Galli *et al.*, 1982; Hamaguchi *et al.*, 1987; Kanakura *et al.*, 1988). Miller and Jarrett (1971) reported both maturing and fully granulated MMCs in rats in mitosis 10 to 14 days after an infection with *N. brasiliensis* coinciding with rapid expansion of MMC numbers and expulsion of worms. However, it is possible that these "mitotic" cells were confused with reforming mast cells. Following degranulation, mast cells become considerably smaller and undergo nuclear and cytoplasmic blastogenesis where nuclear chromatin becomes completely condensed. After granules have reformed, the nuclear size increases and chromatin disperses (Dvorak, 1989).

1.6.5 TEMPORAL RELATIONSHIPS BETWEEN MASTOCYTOSIS AND NEMATODE INFECTIONS

Mastocytosis is claimed to be almost invariably provoked by nematode parasitism (Miller, 1984). Infection of rodents with *N. brasiliensis*, *T. spiralis* and *T. muris* generally results in early expulsion of a primary infection and rapid expulsion of a challenge infection (Lloyd and Soulsby, 1987). The temporal relationships between mastocytosis and expulsion of these nematodes from rodents vary according to different experimenters with peak numbers being present before, coinciding with, or after nematode expulsion (Keller, 1971; Kelly and Ogilvie, 1972; Nawa and Miller, 1979; Jarrett *et al.*, 1967; Alizadeh and Wakelin, 1982; Tronchin *et al.*, 1979; Brown *et al.*, 1981; Lee and Wakelin, 1982). For *N. brasiliensis* in rats, this temporal relationship has also been observed to vary with diet (Wells, 1962, 1368) and lactational status (Kelly and Ogilvie, 1972).

In studies with mast cell-deficient W/W^v mice, which lack functionally normal c-*kit* receptors on cells in the mast cell lineage (Galli *et al.*, 1992), it has been shown that expulsion of a primary *N. brasiliensis* infection is delayed (Crowle and Reed, 1981; Mitchell *et al.*, 1983) although these mice are as refractory as controls to second and third infections (Crowle and Reed (1981). Reconstitution of their mast cell precursors with bone marrow or spleen cells still resulted in a delayed expulsion of a primary infection (Crowle, 1983).

A delayed expulsion of adult worms was also demonstrated when W/W^v mice were infected with *T. spiralis* (Kamiya *et al.*, 1983; Ha *et al.*, 1983; Alizadeh and Murrell, 1984; Oku *et al.*, 1984) or *Strongyloides ratti* (Nawa *et al.*, 1985). However, unlike the situation observed with *N. brasiliensis* infections, the expulsion of a second infection of *T. spiralis* was also delayed (Alizadeh and Murrell, 1984) and expulsion of *T. spiralis* and *S. ratti* was as rapid as in normal mice following reconstitution with bone marrow from congenic mice (Ha *et al.*, 1983; Oku *et al.*, 1984; Nawa *et al.*, 1985). The reason for the difference in response between these two nematodes and *N. brasiliensis* is not clear but suggests that control mechanisms may vary between host-parasite systems.

In contrast to infection of mice with *N. brasiliensis*, *T. spiralis* and *T. muris*, primary infections of *Heligmosomoides polygyrus* in mice are chronically maintained for from 4 weeks to 8 months, with subsequent challenges being expelled at various times after reinfection depending on the host's genotype (reviewed by Monroy and Enriquez, 1992). As such, this host-parasite relationship more closely resembles trichostrongylid infections of ruminants than infection of rodents with nematodes such as *T. spiralis* and *N. brasiliensis*. During a primary infection of *H. polygyrus*, there is a non-specific suppression of mucosal mastocytosis which is attributed to lymphocyte suppression (Dehlawi *et al.*, 1987). Moreover, eventual expulsion of a primary infection is not associated with a mastocytosis (Dehlawi *et al.*, 1987). In secondary infections, a mastocytosis is seen but only around developing larvae in the mucosa (Adams and Monroy unpublished cited by Monroy and Enriquez, 1992; Dehlawi unpublished cited by Dehlawi *et al.*, 1987).

Considered together, all these studies suggest that MMCs appear to have some role in parasite expulsion but clearly other effector mechanisms, independent of mast cells, exist which are also capable of expelling intestinal nematodes.

1.6.6 MAST CELL PROTEASE RELEASE

Release of proteases from rodent MMCs has been observed to coincide with worm expulsion and may be a better indicator of MMC activity than changes in cell numbers (Miller *et al.*, 1986; Miller *et al.*, 1983b; Woodbury *et al.*, 1984; Newlands *et al.*, 1987; Huntley *et al.*, 1990).

Mast cell proteases tend to be specific for the species concerned, and the type of cell. For rodents these are shown in Table 1.2. These specific characteristics have been used to distinguish CTMCs from MMCs and to monitor MMC activity.

Sheep mast cell protease (SMCP) has been isolated from ovine MMCs and is equivalent to RMCP II of rats (Huntley *et al.*, 1986). This enzyme has been detected in the sera and gastric mucus of sheep immune to *H. contortus* and in gastric lymph of sheep immune to *O. circumcincta* following homologous challenges (Huntley *et al.*, 1987, 1992). This suggests that a release of SMCP occurs in response to the presence of the parasite analogous to that reported from rodents.

The function of these proteases is not clear. An increased permeability of intestinal mucosa has been shown to be highly correlated with circulating levels of RMCP II (King and Miller, 1984) and this protease has also been reported to be effective in releasing epithelial cells from intestinal slices *in vitro* (Woodbury, unpublished, cited by King and Miller, 1984). King and Miller suggested that the increased permeability may be due to the proteolytic activity of RMCP II on Type IV collagen of epithelial basement membrane. However, this hypothesis is based on the ability of mast cell proteinase recovered from rat skeletal muscle, which was presumably rat mast cell proteinase I (RMCP I) from CTMCs, to cleave Type IV collagen (Sage *et al.*, 1979). As there are substrate differences between RMCP I and II (Yoshida *et al.*, 1980) this hypothesis remains to be proved. RMCP II has also been observed to degrade collagenous components of nematode cuticle (McKean and Pritchard, 1989) suggesting it might have some role in damaging worms *in vivo*.

1.7 GLOBULE LEUKOCYTES

1.7.1 INTRODUCTION

The first full description of globule leukocytes (GLs) was probably that by Weill in 1919 in the tissues of dogs, cats, mice, rabbits and guinea pigs (cited by Gregory 1979). Taliaferro and Sarles (1939) were the first to report the association of GLs and parasitic infections (Gregory, 1979). Their presence has subsequently been associated with nematode, trematode, cestode and protozoal infections in a variety of animals (Pierce *et al.*, 1962; Whur, 1966; Miller *et al.*, 1967). Globule leukocytes were first described from the goat by Hill (1951).

Although Sommerville (1956) found no correlation between the location of GLs and nematodes in the gastrointestinal tract of sheep, others have done so in both rodents and sheep (Whur, 1966; MacDonald *et al.*, 1980; Dobson, 1966). Globule leukocytes are characteristically found within the epithelium of mucous surfaces (Miller *et al.*, 1967). In the intestine, GLs have been reported to be either concentrated in the mucosa over Peyer's patches (Gregory and Nolan, 1981) or conspicuously absent from this location (Brown *et al.*, 1981).

1.7.2 MORPHOLOGY

The principal histological characteristic of GLs is the large acidophilic granules found within their cytoplasm (Murray *et al.*, 1968). In cattle and sheep, these granules frequently indent the nucleus (Murray *et al.*, 1968) which is laterally placed within the cell (Huntley *et al.*, 1984). The size of the granules varies between species but they are generally larger than those in MMCs (Murray *et al.*, 1968).

1.7.3 DERIVATION

Ultrastructural examination and histochemistry have been used to demonstrate the derivation of GLs from MMCs in rats, cattle and sheep (Jarrett *et al.*, 1967; Miller *et al.*, 1967, Murray *et al.*, 1968). These authors described a spectrum of cells which ranged from typical MMCs through transitional cell types to mature GLs. Transitional cells contained a mixture of granule types which were ultrastructurally indistinguishable from those of

either MMCs or GLs. It was hypothesised that MMCs discharged amines during transition, with an accompanying alteration in the relationship between acid mucopolysaccharide and the basic protein of the granules, with the result that cytoplasmic granules of GLs show an affinity for acid dyes such as eosin. In addition granules fused during transition becoming larger. Thus GLs were considered to be, in effect, partially discharged MMCs (Miller and Jarrett, 1971). Huntley *et al.* (1984) confirmed these earlier findings by isolating MMCs, GLs and transitional cells from sheep infected with *O. circumcincta*, clearly demonstrating that they all shared similar granule and surface properties. The gradation of cell types between MMCs and GLs was very obvious in these isolated cells with the proportion of transitional cells remaining relatively constant at approximately 31 to 44% of the total population of all mast cell types. Interestingly, in a control group naturally infected on pasture, only MMCs and transitional cells but no GLs were seen.

The detection of RMCP II in both MMCs and GLs but not in CTMCs (Miller *et al.*, 1986) is further convincing evidence that GLs are derived from MMCs.

Nevertheless, as late as 1982 the relationship between MMCs and GLs was still considered speculative by at least some workers (Lee and Wakelin, 1982). The observation of mitoses in MMCs and GLs in parasite-induced mastocytosis in rodents (Miller and Jarrett, 1971; Ruitenberg and Elgersma 1979; Tronchin *et al.*, 1979) and two reports of GL neoplasms in a cat (Finn and Schwartz, 1972; Honor *et al.*, 1986) suggest that GLs are not end-stage derivatives of MMCs. However, there are no reports of GLs being cultured and most authors now accept that they are end-stage cells, the functions of which have yet to be discovered.

1.7.4 ASSOCIATION OF GLOBULE LEUKOCYTES WITH MAST CELLS IN THE RESPONSE TO NEMATODES

In most reports involving rodents, GLs have been included with MMCs in mast cell counts and they have been found to comprise the majority of mast cells in mice infected with *T. muris* (Lee and Wakelin, 1982) and *T. spiralis* (Brown *et al.*, 1981). In rats infected with *N. brasiliensis*, GLs were observed to increase in parallel with MMCs but were fewer in number (Jarrett *et al.*, 1968; MacDonald *et al.*, 1980) whereas in similarly infected mice no increase in GLs occurred (Mitchell *et al.*, 1983).

Sommerville (1956) noted the association of GLs with chronic nematode parasitism in sheep and postulated a causal relationship. Subsequent studies have demonstrated a positive relationship between the presence of GLs and the immune response of sheep to *T. colubriformis* (Gregg *et al.*, 1978; Douch *et al.*, 1986; Douch, 1988; Douch, 1989; Dineen and Windon, 1980a) and *Oesophagostomum columbianum* (Dobson, 1966).

In contrast, MMC counts have generally failed to correlate with the *T. colubriformis* burdens or the immune status of sheep (Douch, 1988; Douch *et al.*, 1986; Gregg *et al.*, 1978).

Miller and Jarrett (1971) proposed that the proportion of GLs present is an indication of the extent of mast cell discharge that has occurred. O'Sullivan and Donald (1973) further suggested that large numbers of GLs in sheep are associated with an active host response and not merely the presence of nematodes. However, large numbers of MMCs and GLs are not required for an effective immune response against *H. contortus* (Huntley *et al.*, 1992).

Little has been published on GLs in goats. They were found to be more numerous in dairy goats than in Merino sheep exposed to similar infection regimes but there was no clearcut association with worm burden (Stanton, 1989).

A functional role for GLs has yet to be found even though they are in an ideal location to release mediators into the lumen of the gut.

Having now considered the various cells involved in the immune response, the following sections will consider immune mediators and their role or potential role in the response to gastrointestinal nematodes.

1.8 THE LEUKOTRIENES

1.8.1 INTRODUCTION

The sulphidopeptide leukotrienes C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4) are the components of what was originally described as slow reacting substance of anaphylaxis (SRS-A) (Moqbel and MacDonald, 1990). These sulphidopeptide leukotrienes (LTs) are derived from arachidonic acid by the

5-lipoxygenase pathway (see Fig 1.1). The relevant enzymes (5-lipoxygenase and LTC_4 synthase) for this pathway and hence production of LTC_4, are found in a variety of cell types and tissues including mast cells, eosinophils, basophils, monocytes, macrophages, brain, renal glomeruli, tracheal epithelium and myocardium (see Hansen 1989). In the helminth-infected gastrointestinal tract, the most likely sources are MMCs and eosinophils (Moqbel and MacDonald, 1990).

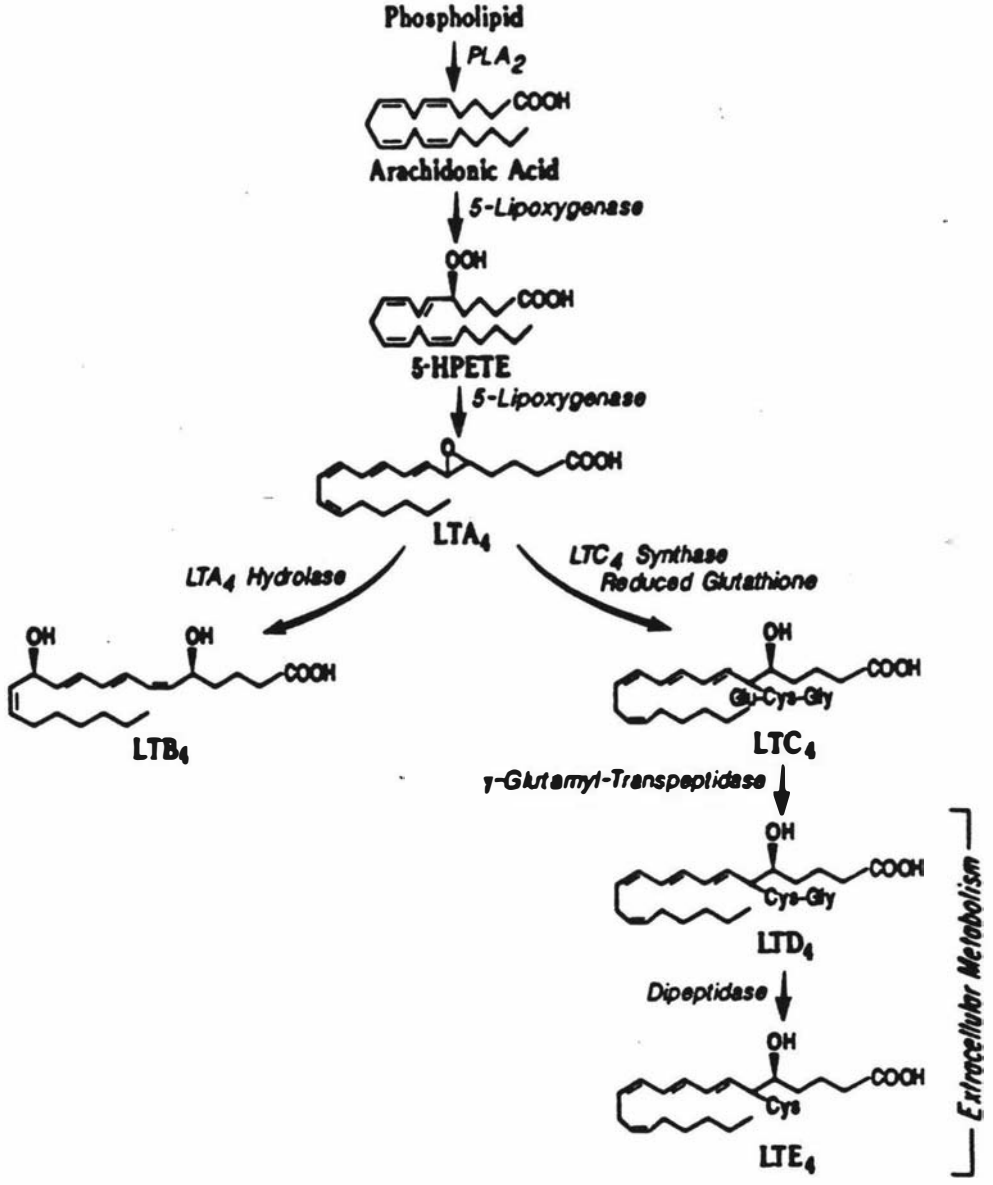


Figure 1.1. The 5-lipoxygenase pathway of leukotriene synthesis (from Lam and Austen, 1992).

1.8.2 BIOLOGICAL ACTIVITY

Leukotrienes are not stored preformed in granules but rather are produced *de novo* and released (Moqbel and MacDonald, 1990; Jones *et al.*, 1990). The stimulus for cells to produce and release LTs has only been investigated in a few instances and although not generally understood, it is accepted they are produced after transmembrane signalling or perturbation (Moqbel and MacDonald, 1990) and can involve antibody-cell interactions (Razin *et al.*, 1983; Heavey *et al.*, 1988; Rankin *et al.*, 1982; Rouzer *et al.*, 1982; Shaw *et al.*, 1985). Leukotriene C_4 appears to be the dominant leukotriene produced, at least initially, by various cells (Jakschik and Kuo, 1984; Krilis *et al.*, 1983; Moqbel *et al.*, 1986; Moqbel *et al.*, 1987).

The LTs are capable of inducing contraction of a variety of smooth muscle preparations such as guinea pig ileum and isolated airway smooth muscle (Piper, 1984) both of which are sensitive to picomolar concentrations. Leukotriene binding appears to be specific, involving saturable receptors which vary in density and distribution with the species and cell type involved (Hansen, 1989; Ford-Hutchinson, 1983; Bisgård *et al.*, 1982; Piper, 1984). Pharmacological activity varies between LTs. In terms of spasmogenic activity, LTD_4 is generally the most potent and LTE_4 the least potent but these differences vary depending on tissue and species (Krilis *et al.*, 1983; Burke *et al.*, 1982; Dahlén *et al.*, 1980; Hanna *et al.*, 1981; reviewed by Samhoun and Piper, 1986).

Within the host, LTs may also contribute to the inflammatory response by influencing vascular permeability as they have been shown to be able to augment vascular permeability in guinea pig skin (Drazen *et al*, 1980), human skin (Soter *et al*, 1983) and hamster buccal mucosa (Dahlén *et al*, 1981). They have also been noted, in conjunction with hydroxyeicosatetranoic acids, to enhance the production of mucus from goblet cells (Moqbel and MacDonald, 1990) which is a well-described host response to the presence of nematodes (see Section 1.12).

1.8.3 ANTI-NEMATODE ACTIVITY

Douch *et al.* (1983) described an assay of antiparasite activity which measured the ability of exsheathed trichostrongylid larvae to migrate out of an agar gel in which various test substances were included. In sheep considered

to be demonstrating an effective immune response (judged by low faecal egg counts in the face of a natural challenge), they showed that mucus from the abomasum and small intestine was capable of larval migration inhibition (LMI) whilst mucus from sheep considered to be showing a poor immune response (judged by high faecal egg counts in the face of a natural challenge) or sheep raised helminth-free demonstrated only slight LMI activity. Components of SRS-A were considered to be giving rise to this LMI activity. In a further study, Douch *et al.* (1986) found that high LMI levels were always associated with high GL and eosinophil counts but high GL counts were sometimes associated with low LMI and eosinophil counts. Interestingly, MMC numbers were not related to LMI activity.

Kimambo and MacRae (1988) confirmed the existence of LMI activity of small intestinal mucus from immune sheep challenged with *T. colubriformis* and also found that abomasal mucus from these same animals had significant LMI activity. In addition, they extended the use of this technique to demonstrate that ileal contents as well as mucus had LMI activity although Douch *et al.* (1983) had previously demonstrated some LMI activity in faeces.

Although Douch *et al.* (1983) originally suggested that SRS-A was responsible for LMI activity, Gray *et al.* (1992) reported no difference in LMI activity of intestinal mucus between genetically resistant and susceptible sheep although they did find the resistant lambs had higher levels of sulphidoleukotrienes in intestinal mucus. One possible explanation is that a substantial proportion of the LTs assayed were LTE_4 and thus had limited spasmogenic activity in the LMI assay.

An interesting question is whether or not LTs have an effect against nematodes *in vivo*. It is known that the effect of the immune response on nematode larvae is at least partly reversible. Rothwell (1989) reviewed the extensive literature on worm expulsion from the gastrointestinal tract and concluded that the expulsion process did not appear to involve irreversible damage to worms. Active larvae have been recovered from the faeces of immune sheep (Chiejina and Sewell, 1974b) and at least some of the larvae recovered from the caecum of a resistant sheep established when given to a susceptible sheep (Elliot, 1981). Similarly, adult *T. spiralis* and *S. ratti* recovered during the expulsion phase successfully re-established following transplantation into naive hosts (Kennedy and Bruce, 1981; Moqbel *et al.*, 1980). Binding of LTC_4 reaches an equilibrium, is saturable and reversible (see Lewis and Austen, 1988); nematodes could, therefore, conceivably recover from

their effect when removed from the source. Consistent with the argument that paralysis by LTs is an effector mechanism in expression of resistance to nematodes, is the observation that ingestion of dye by *N. brasiliensis*, which requires muscular activity, declined with parasite age *in vivo* but not *in vitro* (Bottjer and Bone, 1985a, 1985b) suggesting the developing immune response was affecting muscular activity. However, it was also suggested by this same group of researchers, that this reduced feeding could also be caused by antibody (Bottjer *et al.*, 1985).

1.9 BIOGENIC AMINES - HISTAMINE AND 5-HYDROXYTRYPTAMINE (5-HT)

As MMC numbers increase so do tissue histamine levels because, in most animals, it is essentially only found in MMC granules although in rodents a substantial proportion of stored histamine is also found in enterochromaffin-like cells in the mucosa (Chuang *et al.*, 1992). 5-HT is found primarily in enterochromaffin cells in the gastrointestinal tract and in rodents it is also found in mast cells (Siraganian, 1988). Reported increases in tissue amine levels are "not substantial" in a variety of laboratory animal-nematode models and tend to peak after expulsion has occurred (reviewed by Rothwell, 1989). The role of amines in the expulsion of nematodes is difficult to establish.

The pharmacological effects of histamine acting through H1 receptors are generally well established. These include smooth muscle contraction, increased permeability of blood vessels and increased exocrine secretions (Gorman and Halliwell, 1989). Histamine may also serve as a messenger in immune and non-immune inter-cellular signalling. Its effect is largely determined by the receptor type on the target cell, with signalling through H1 receptors usually having a stimulatory effect and through H2 receptors an inhibitory effect (reviewed by Falus and Merètey, 1992). The effects of histamine on H2 receptors include inhibition of lymphocyte proliferation, cell mediated toxicity, lymphokine production and induction of T suppressor cells (Befus and Bienenstock, 1982; Siraganian, 1988). However, McElroy and Befus (1987) treated rats with cimetidine, an H2 antagonist, and demonstrated they had larger *N. brasiliensis* burdens at 4 to 5 days after a primary infection which is contrary to expectations and suggests that some other undetermined histamine-dependent mechanism, acting through H2 receptors, is involved.

The biological actions of 5-HT include stimulation of secretion of water and sodium in rodent small intestine and contraction of rodent small intestinal

smooth muscle, as well as the ability to act as a neurotransmitter (Moqbel and MacDonald, 1990).

Increased levels of histamine, LTs but not 5-HT, have been found in intestinal mucus or contents associated with larval rejection of *T. colubriformis* in sheep (Jones *et al.*, 1990; Steel *et al.*, 1990) although LTC_4 is released in relatively greater quantities than histamine (Jones and Emery, 1991) and is relatively more spasmogenic (Hansen, 1989). However, Douch *et al.* (1984) found histamine levels in blood and intestinal contents did not correlate with LMI activity of intestinal and abomasal mucus in sheep and concluded it was not directly involved in worm expulsion. As noted earlier, in the self-cure response of sheep to *H. contortus* (see Section 1.2.4), histamine has been implicated as the effector chemical (Stewart, 1953).

Oral treatment of mice infected with *T. spiralis* with histamine resulted in decreased parasite fecundity (Stewart *et al.*, 1985) and infusion of guinea pig small intestine with histamine and 5-HT resulted in the expulsion of some of the *T. colubriformis* present but only from 5 to 9 days after infection (Rothwell *et al.*, 1974). These *in vivo* experiments are somewhat fragmentary and do not preclude histamine having some possible indirect effect on the nematode via other cells.

There have been only limited studies on the direct effect of biogenic amines on nematodes. Observations include: stimulation of ingestion by *T. colubriformis*, as demonstrated by uptake of the dye rhodamine B, following incubation with histamine, dopamine and 5-HT (Bone and Bottjer, 1985); contraction of the vulva and vagina or spicule extension following incubation with 5-HT (Croll, 1975); positive chemotaxis for male *N. brasiliensis* by histamine and 5-HT (Ward *et al.*, 1984); and no effect on mortality or egg laying following incubation of *H. contortus* with histamine (Stewart, 1953).

Rothwell (1989) reviewed the numerous publications utilising drugs to modify worm expulsion and concluded that, despite many reservations, drugs which antagonised the activity of histamine and 5-HT generally inhibit expulsion.

Some nematodes have been shown to contain 5-HT, including *T. colubriformis* (Fransden and Bone, 1987), ascarids (Mishra *et al.*, 1984) and *Caenorhabditis elegans* (Horvitz *et al.*, 1982). *Ascaris suum* had also been shown to be able to absorb 5-HT (Chauduri *et al.*, 1988). If this absorption is a

general phenomenon and 5-HT has a functional role, it is conceivable that an overload of 5-HT could have a detrimental effect on the parasite (Rothwell, 1989). Histamine has also been found in ascarids (Mishra *et al.*, 1984) although its function is not clear; as with 5-HT, any overload of histamine may affect its normal functioning in the nematode.

1.10 PROSTAGLANDINS

The prostaglandins are a family of mediators which have a variety of functions. Prostaglandins E_1, E_2 and prostacyclin have potent vasodilatory actions and are able to inhibit histamine release, whilst thromboxane is a potent vasoconstrictor (Moqbel and MacDonald, 1990).

Single intraduodenal injections of prostaglandin E_1 and prostaglandin E_2 were found to be highly effective in causing expulsion of *N. brasiliensis* from rats (Kelly *et al.*, 1974). Dineen and Kelly (1976) recorded peak levels of prostaglandin E on day 7 of *N. brasiliensis* infections in rats, which generally coincided with the onset of expulsion as worms were moving towards the posterior small intestine before their numbers declined rapidly from day 10. Kassai *et al.* (1980), however, failed to replicate these effects. Similarly, Rothwell *et al.* (1977) failed to demonstrate any involvement of prostaglandins in the expulsion of *T. colubriformis* from guinea pigs, as did Dutoit *et al.* (1979) with *T. spiralis* in rats or mice. These contradictory observations indicate that the role of prostaglandins in the immune response to nematodes needs further investigation.

Jones and Emery (1991) demonstrated there was an increase in the levels of prostacyclin and thromboxane B_2 in sheep infected with *T. colubriformis*. Both affect smooth muscle (Moncada and Vane, 1979) but their effects on nematodes are not known.

1.11 PLATELET-ACTIVATING FACTOR (PAF)

Platelet-activating factor (PAF) was originally described as a soluble degranulation product of rabbit basophils *in vitro* which aggregated rabbit platelets (Benveniste *et al*, 1972). Additional effects have since been described. It is known to induce bronchoconstriction but the mechanism is not entirely clear. Other effects of PAF include: causing microvascular leakage at substantially lower concentrations than histamine (Humphrey *et al.*, 1982); inducing mucus secretion in ferret trachea although this effect may be indirect

(Wirtz *et al.*, 1986); and inducing LTC_4 release from rat small intestine (Hseuh *et al.*, 1986). It is also a very potent eosinophil chemotactic agent (Wardlaw *et al.*, 1986).

A large number of different cell types from a variety of species are known to secrete PAF in response to a variety of stimuli. These include platelets, neutrophils, monocytes, macrophages, basophils, epidermal cells, mast cells, eosinophils and endothelial cells (reviewed by Barnes *et al*, 1988) of which the last three are most likely to be of relevance to gastrointestinal helminths (Moqbel and MacDonald, 1990).

Release from MMCs and eosinophils follows antibody-mediated activation (Mencia-Huerta *et al.*, 1983; Champion *et al.*, 1988). Endothelial cells will produce PAF following stimulation by histamine and LTs (Barnes *et al.*, 1988). Following intravenous challenge of *N. brasiliensis*-immune rats with worm antigen, PAF was observed in the intestinal contents, along with LTC_4 and RMCP II but its role in the immune response is uncertain (Moqbel *et al.*, 1989). The direct effect of PAF on nematodes has not been investigated.

1.12 MUCUS TRAPPING

Many nematode larvae, especially those of the trichostrongylids, enter the gland crypts of the abomasum or intestine to develop. They will, unavoidably, come into contact at an early stage with the mucus layer lining the epithelium. It has been suggested that mucus trapping of larvae with subsequent failure of these larvae to establish in their respective niches, in and on the epithelium, may be a significant component of the gastrointestinal immune response to nematodes (Dobson, 1967; Miller *et al.*, 1983a; Miller, 1984; Miller, 1987). However, mucus trapping has been shown to be not essential for expulsion of *T. spiralis* (Bell *et al.*, 1984; Carlisle *et al.*, 1991a, 1991b) or *N. brasiliensis* (Miller *et al.*, 1981).

Adult nematodes generally reside close to the epithelial surface and thus have continual contact with the mucus layer (Miller, 1984). As liquid feeders they will almost certainly ingest mucus and consequently any immune mediators or antibodies in mucus have access to both the cuticle and the intestinal tract of the nematode. Whether or not this is of any significance is not known.

Hyperplasia of goblet cells has been described from a large variety of different host-nematode infections (Wells, 1963; Uber *et al.*, 1980; Alizadeh and

Wakelin, 1982; Carroll *et al.*, 1984; Dobson, 1967; Jackson *et al.*, 1983; Angus and Coop, 1984) which implies an increase in mucus secretion. However, no correlation has been found between resistance to *T. colubriformis* and the number of goblet cells in sheep (Douch *et al.*, 1986). There are also some exceptions where goblet cell hyperplasia has not been observed, including *S. ratti* infections in rats (Mimori *et al* 1982) and *T. colubriformis* in guinea pigs (Rothwell unpublished, cited in Rothwell 1989). It has yet to be determined what factors mediate goblet cell hyperplasia and mucus secretion in nematode-infected animals (Tse and Chadee, 1991) or, if there is an increase in mucus production, whether it affects the parasites. Changes from acid to neutral mucin in goblet cells have also been observed (Koninkx *et al.*, 1988; Salmon and Duncan, 1985) which may have as yet undetermined effects, and could also influence the stability of some inflammatory mediators such as LTs.

1.13 SPECIFICITY OF NEMATODE EXPULSION

The role of antibodies as direct effectors in the expulsion of intestinal nematodes is unclear. Antibody reactions *per se* have not been reviewed principally because they are not considered to have a direct effector role in the expression of resistance, although they are involved with antibody-dependent cell-mediated cytoxicity mechanisms. Many potential effector cells have receptors for specific immunoglobulins such as those for IgE on mast cells. Although some studies of host/nematode systems in laboratory animals have demonstrated passive transfer of protection with serum from immune animals, others have not (reviewed by Miller, 1984). Passive transfer of either immune serum or cell-free intestinal lymph was shown not to be protective against *T. colubriformis* in sheep (Adams *et al.*, 1980). Of interest is the report by Jones and Ogilvie (1971) who noted that effective passive protection to *N. brasiliensis* was abolished in irradiated recipient rats, suggesting that it was not antibodies *per se* that were the effective component in the expulsion process. Nevertheless, the appearance of nematode specific antibodies in intestinal mucus is still proposed as being part of the mechanism for the rejection of *T. colubriformis* from sheep (McClure *et al.*, 1992)

There is clear evidence that rejection of one nematode species may affect another, indicating the non-specific nature of the actual expulsion process. In sheep immunized with irradiated *T. colubriformis* and subsequently challenged with this and related intestinal trichostrongylids, significant protection was afforded to an homologous challenge (81%). The levels of protection against *Trichostrongylus vitrinus* (34%) and *Nematodirus spathiger* (14%) were not

significant when these were given separately (although the former was approching significance (p=0.07)) but when all three species were given simultaneously the level of protection achieved was approximately 100% for all (Dineen *et al.* 1977). These findings also indicate that expulsion is triggered by specific antigens which are only shared between nematodes to a very limited extent, if at all, such as between closely related *Trichostrongylus* species.

Douch (1989) further investigated the level of cross-protection in sheep immunised only against *T. colubriformis*. Following natural challenge on pasture, substantial protection was evident to *T. colubriformis* (91%), *T. vitrinus* (56%), *N. spathiger* (91%), *Ostertagia* spp (42%) and *T. axei* (67%) but not to *Cooperia* spp.. Although not as convincing as the results shown by Dineen *et al.* (1977), they still support the hypothesis that the expulsion process is non-specific. The significant reduction in burdens of abomasal species "upstream" from the intestine suggests that a response was possibly generated by larvae migrating through the abomasum with or without cross-recognition of *T. axei* antigens. It was also considered possible that the cellular response from the intestine may extend into the abomasum.

Cross-inhibitory activity of intestinal mucus from sheep immune to *T. colubriformis* has been demonstrated *in vitro* to infective larvae of related trichostrongylid species (Douch *et al.*, 1983; Kimambo and MacRae, 1988) which again supports the non-specific nature of the expulsion process.

There are reports of antibodies apparently contributing directly to anti-parasite activity. For example, IgG1 would appear to suppress feeding by *T. colubriformis in vitro* possibly due to antibody binding to the stoma, cuticle and excretory pore of the worm (Bottjer *et al.*, 1985) and as McClure *et al.* (1992) demonstrated that IgG1 levels increased in intestinal mucus in immune sheep infected with *T. colubriformis*, it is possible they have a direct effect on the parasite. *In vitro* oviposition by *T. colubriformis* was also reduced by IgG from immune goats (90 days post-infection) (Bone and Klesius, 1986) and as reduced fecundity is a noted feature associated with a developing immune response, this may be of practical significance. Such examples, however, do not explain the non-specific nature of the expulsion process.

1.14 HYPOTHESIS FOR WORM EXPULSION

It is clear that infections with nematodes generate an immune response which will be affected by age, genetic factors and nutrition. Although a

considerable amount of research has been conducted, much of this has used nematode infections of rodents. Nematodes such as *T. spiralis*, *T. muris*, *S. ratti* and even *N. brasiliensis* and *H. polygyrus* have different life cycles and development patterns compared to trichostrongylid infections of ruminants and some models are of nematodes in abnormal hosts. The result is that some of these research findings should be extrapolated to ruminants with caution. Clearly, major humoral and cellular changes follow nematode infections resulting in the production of antibodies and various immunological mediators.

A hypothetical chain of events for the host reponse to lumen dwelling trichostrongylid nematodes could be as follows:

Excretory-secretory products from nematodes cross the mucosa and are presented to T cells and in some way trigger an appropriate T_H subset. The resulting production of cytokines subsequently drives the immune response with mastocytosis, increased tissue eosinophils and increased IgE, IgG_1 and IgA production. There is also an increase in goblet cells and presumably mucus secretion as well as pathological change in the mucosa itself. Release of inflammatory mediators will follow appropriate triggering by antigen cross-linking antibody on cell-membrane receptors or other stimulation of other cell-membrane receptors. Nematodes, especially incoming larvae, will be exposed to some of the inflammatory mediators released such as LTs. A further line of defence could be the mucus layer. Antibody may also contribute directly by binding to the stoma, excretory pore or cuticle and, in some as yet undefined manner, have a deleterious effect.

Studies on the host-parasite relationship between goats and gastrointestinal nematodes provides an ideal opportunity to contrast the host response with that of sheep, especially as sheep generally become resistant to these nematodes whereas goats appear to have a more variable host response.

Because of the dearth of information on host-parasite relationships between helminths and goats this study commenced by investigating the ability of goats to develop resistance to some gastrointestinal nematodes. This then provided some information to allow further study on the antiparasite activity of intestinal mucus and/or contents and the mast cell response to infections with *T. colubriformis*.

CHAPTER TWO

FAILURE OF YOUNG GOATS TO ACQUIRE RESISTANCE TO *HAEMONCHUS CONTORTUS*

2.1 INTRODUCTION

Haemonchus contortus is an important parasite of goats and sheep. It has been shown that sheep can develop appreciable host resistance to this nematode from about seven to eight months of age (see Section 1.2). Whether or not goats develop measurable host resistance to this nematode is uncertain. This chapter describes two experiments designed to investigate this.

2.2 MATERIALS AND METHODS

Experimental Schedule

- Experiment 2.1

Thirteen Saanen wethers were raised worm-free and fed a pelleted ration of lucerne, barley and bran (see Appendix 2a for formulation). Ten goats, varying in age by up to 42 days, were paired by age and randomly allocated to either group 1.1 or 1.2. At the beginning of the experiment, the goats' mean age was approximately 5½ months (157 days). The three remaining goats (A, B and C) were used to monitor the level of *H. contortus* infection during the main experiment.

The isolate of *H. contortus* used was obtained from a goat which had been grazed without contact with sheep. Adult nematodes were obtained post-mortem, ground to release their eggs and cultured in vermiculite to produce the initial infective larvae. These were cycled through a further worm-free goat to produce the infective larvae needed for the experiment. Larvae were stored at 10°C and were less than 2 months old when used.

Group 1.1 and goats A, B and C were dosed orally by syringe with 200 larvae, three times per week for 10 weeks. This was approximately 23 larvae/kg mean initial liveweight per week. Fourteen weeks after the first dose of larvae had been given (day 84), goats A and B were killed to assess parasite burdens. The remaining 11 goats were dosed twice, two days apart, with levamisole

hydrochloride (15mg/kg). Goat C was killed two days after the second dose of levamisole to check the efficacy of the anthelmintic.

At the beginning of week 15 (day 98) Groups 1.1 and 1.2 were challenged with 10,000 infective larvae, given by stomach tube and killed 30 days later.

- Experiment 2.2

Fifteen Saanen wethers were raised worm-free as above and then fed on meadow-hay during the experiment. They were 14 months old at the beginning of the experiment with only 6 days difference in age between them.

The *H. contortus* isolate used was a laboratory strain of ovine origin which was kindly provided by Dr P. Douch from the Wallaceville Animal Research Centre, Upper Hutt. This was also cycled through a further worm-free goat to provide the infective larvae needed for the experiment. Larvae were stored at 10°C and were less than 2 months old when used.

Goats were randomly allocated to either Group 2.1 (n=8) or Group 2.2 (n=7). Goats in Group 2.1 were dosed by stomach tube with 825 larvae once per week for 14 weeks with the exception of week 4, when only 300 larvae were given, and week 5 when no larvae were given. Overall this was also approximately 23 larvae/kg mean initial liveweight per week as in Experiment 2.1. One goat (goat 27) became severely anaemic. To ensure its survival, it was dosed orally with ivermectin (Ivomec Liquid^{R}, Merck Sharp & Dohme N.Z. Ltd; 300mcg/kg) in week 12 but still received larvae for the remaining two weeks.

All 15 goats were dosed with ivermectin at the beginning of week 15 (day 98) and challenged with 15,000 larvae, given by stomach tube, 6 weeks later (day 140). Twenty-eight days after challenge all goats were killed.

Because packed cell volumes (PCVs) of all animals were at the lower limit of normal value, sera from five goats were assayed for vitamin B12 levels in week 11. These showed a mean value of 115 pmol/litre which is considered marginal in sheep though no normal values for goats were available. Consequently, all goats were treated with 2mg hydroxocobalamin (Cobalex^{R}, Syntex Laboratories (NZ) Ltd) in weeks 12 and 21 of the experiment.

Parasitology

Faecal egg counts were estimated three times a week in Experiment 2.1 and once a week in Experiment 2.2 by a modified McMaster technique where each egg counted represented 50 eggs/g. At critical times, samples that yielded zero counts were also examined using a simple flotation method on 2g of faeces (See Appendix 2b for egg counting techniques).

Worm counts were carried out using standard procedures. In Experiment 2.1, counts were estimated from 10% aliquots of the total washings sieved through a 53 μm mesh to retain all larval and adult stages. In Experiment 2.2, a 1% aliquot of the washings was sieved as for Experiment 2.1 and a further 9% was sieved through a 250 μm mesh. In Experiment 2.2, therefore, counts of fourth stage larvae were based on 1% aliquots. All adult nematodes were counted by sex. Ten percent aliquots of both the small and large intestine were also examined after sieving through a 250 μm mesh.

After washing, the abomasum was digested in pepsin:HCl solution (see Appendix 2c). The same proportions as for the washings were examined for larvae i.e. 10% in Experiment 2.1 and 1% in Experiment 2.2.

Haematology

Approximately 10 ml of blood were collected from the jugular vein once a week in both experiments. Three ml was collected into EDTA and the remaining 7 ml allowed to clot for serum to be collected.

After red blood cells were lysed, total white cell numbers were estimated by an automatic counter (Cell-Dyn 900 Haematology Analyzer, Sequoia-Turner, California, U.S.A.). Blood smears were made at the time of blood collection and stained with Modified Wright's Stain (Sigma Chemical Company, Missouri, U.S.A.). Absolute differential white cell counts were estimated following identification of 100 cells in each smear.

Packed cell volumes (PCVs) were estimated by a standard microhaematocrit method. In Experiment 2.1, an additional 3 ml blood sample was collected 3 days later each week for an additional PCV estimation.

Serum Proteins

Albumin and total proteins were estimated weekly in both experiments with an automated biochemical analyser (Worthington Diagnostics Systems Inc., San Francisco, U.S.A.).

Pepsinogen

In both experiments, serum pepsinogen levels were estimated at intervals as possible indicators of mucosal damage. The method used was a modification of that of Uete *et al.* (1969; see Appendix 2d for method).

Gastrin

Gastrin was measured by radioimmunoassay as described by Simpson *et al.* (1993) using rabbit anti-human gastrin (G17) antibody. The minimal detectable level of the assay is reported as 1.6 fmol/ml, the coefficient of variation within assays as 10% and between assays as 21% (n=72). This anti-human gastrin antibody shows equal affinity for the sulphated and non-sulphated forms of human, ovine and porcine gastrin whether in the G34, G17 or G14 active molecular forms (Hansky and Cain, 1969). Although its use in goats has not been formally validated the amino acid sequence of gastrin (G17) is the same for sheep and goats (Anderson, 1985; Bonato *et al.*, 1986).

Assays were kindly performed by Mr J. Elgar, Dept of Physiology and Anatomy, Massey University.

Statistical Analysis

Experimental groups were compared separately during trickle and challenge infections by analysis of variance using the statistical software package SAS version 6.04 (SAS Institute Inc., U.S.A.). A major point of interest is the change in various parameters over the infection periods. In order to examine this, the independent variables modelled were group, time, goats (nested within group) and the interaction between group and time. The error term for overall comparison of the dependent variable between groups was goats nested within groups; the error term for the interaction of group with time was the residual error (see Cody and Smith, 1987). A significant interaction indicates that the change with time was different for the two treatments or, more figuratively, that when graphed the two lines were not

parallel. A significant difference between groups indicates the overall variation between groups was greater than the variation within groups.

Results following challenge were also analysed by multiple regression using Statistix version 4.0 (Analytical Software, U.S.A.) if analysis of variance had shown there was no interaction between group and time. Inclusion of interaction terms in the multiple regression was prevented in all cases due to problems with multicollinearity. Some comparisons were also made by two sample t-tests using Statistix version 4.0, as were correlations between some parameters. Worm counts were square root transformed (x+0.5) prior to analysis to stabilize the variances.

2.3 RESULTS

Parasitology

- Experiment 2.1

The pattern of faecal egg counts is shown in Figure 2.1 (see Appendix 2e for raw data). Strongylate eggs were first detected in one goat in Group 1.1 twenty-two days after the first dose of larvae and by 26 days infections were patent in all goats. At the time of anthelmintic treatment, the arithmetic mean egg count of goats in Group 1.1 was 800 epg (range 50 to 1650 epg). By 30 days after challenge, eggs were detected in four of five goats in Group 1.1 and in all goats in Group 1.2. Goat 9 had very low faecal egg counts throughout the experiment and these are shown separately in Figure 2.1. Small numbers of *Nematodirus* eggs were seen occasionally.

Total worm counts (including those for goats A, B & C) are given in Table 2.1. Although mean worm counts of Group 1.1 are lower than those of Group 1.2, when analysed by Student's t-test (see Table 2.2), the difference was not significant ($p>0.05$). No nematodes were found in goat C following levamisole treatment. There was also no difference in the male:female ratio between the two groups ($p>0.05$). No nematodes were seen in the small or large intestinal samples.

Goat Tag	Total L4	Total females	Total males	Total nematodes	male: female[1]
Group 1.1					
1	10	650	720	1380	1.11
4	-	1520	1310	2830	0.86
6	-	420	310	730	0.74
9	-	200	270	470	1.35
12	-	1730	1790	3520	1.03
Arithmetic mean				1786	
Group 1.2					
3	-	380	250	630	0.66
5	10	1490	1440	2940	0.97
7	-	2200	2200	4400	1.0
8	10	1920	1910	3840	0.99
10	-	1760	1950	3710	1.11
Arithmetic mean				3104	
Goat A	-	280	410	690	1.46
Goat B	30	140	160	330	1.14
Goat C	-	-	-	-	

1. based on adult nematodes only

Table 2.1. *H. contortus* burdens for Experiment 2.1

EXPERIMENT 1 - ANALYSIS OF SQUARE ROOT TRANSFORMED TOTAL WORM COUNTS BY GROUP USING TWO-SAMPLE T TESTS

GROUP	MEAN	SAMPLE SIZE	S.D.	S.E.
1.1	39.675	5	16.276	7.2787
1.2	53.706	5	16.569	7.4100

	T	DF	P
COMPARISON	-1.35	8	0.2137

	F	NUM DF	DEN DF	P
TESTS FOR EQUALITY OF VARIANCES	1.04	4	4	0.4866

CASES INCLUDED 10 MISSING CASES 0

Table 2.2. Statistical analysis of two sample T-test of the total worm counts of Experiment 2.1

-Experiment 2.2

The pattern of faecal egg counts is shown in Figure 2.2 (see Appendix 2f for raw data). Strongylate eggs were detected in the faeces of two goats in Group 2.1 at the beginning of the fifth week after the first dose of larvae but it was not until the 11th week that all goats in Group 2.1 had detectable eggs in their faeces. By week 14, the arithmetic mean faecal egg count of Group 2.1, excluding the goat dosed with ivermectin, was 1207 epg (range 450-3000 epg). After treatment with ivermectin, all egg counts fell to zero. Following challenge, no eggs were detected by day 28 when all goats were killed. Subsequently it was found that most of the worms recovered were immature adults and contained few eggs. As in Experiment 2.1, small numbers of *Nematodirus* eggs were seen occasionally.

Total worm counts are given in Table 2.3. No evidence of acquired host resistance in Group 2.1 was seen. Indeed the mean *Haemonchus* burden in Group 2.1 was higher than that in Group 2.2. The numbers of L4 *Haemonchus* were higher than in Experiment 2.1 but they were still a small proportion of the overall worm population. Fourth stage larvae contained the crystal formations described by Blitz and Gibbs (1971) suggesting they were inhibited. No worms were seen in the small or large intestinal samples.

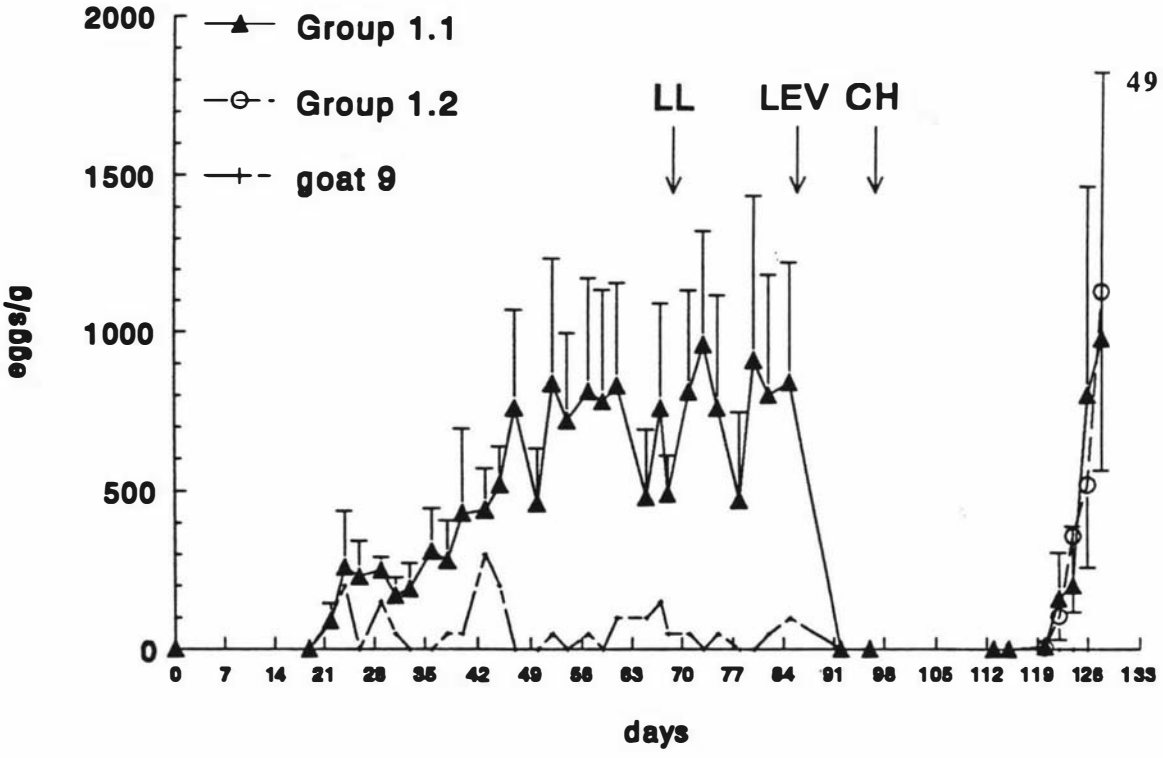


Figure 2.1. Faecal egg counts (mean ± s.e.) from Experiment 2.1. Group 1.1 = trickle-infected; Group 1.2 = control; LL = last larval dose of trickle-infection; LEV = levamisole treatment; CH = all goats challenged with 10,000 *H. contortus* L3s.

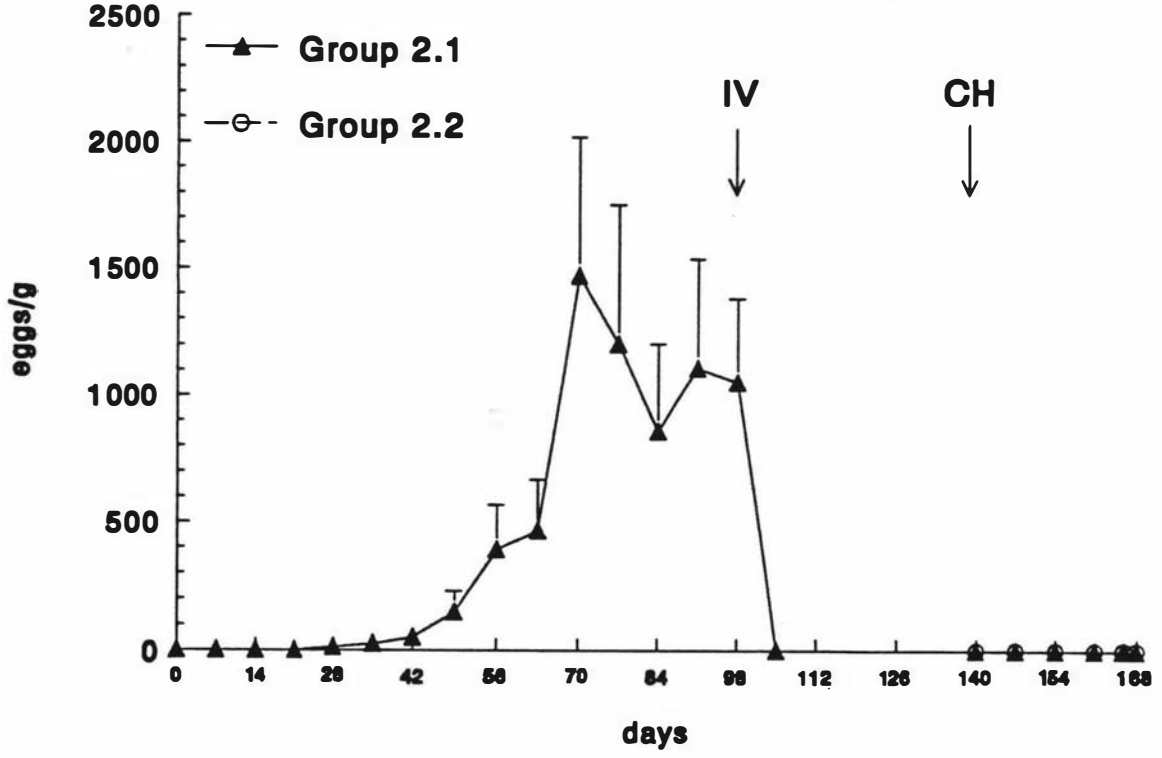


Figure 2.2. Faecal egg counts (mean ± s.e.) from Experiment 2.2. Group 2.1 = trickle-infected; Group 2.2 = control; IV = ivermectin treatment; CH = all goats challenged with 15,000 *H. contortus* L3s.

Goat Tag	Total L4	Total females	Total males	Total nematodes	male: female[1]
Group 2.1					
19	-	1040	740	1780	0.71
20	-	530	670	1200	1.26
22	-	1720	1780	3500	1.03
23	-	2310	2120	4430	.92
24	400	990	1030	2420	1.04
26	100	850	720	1670	.85
27	-	1790	1810	3600[2]	1.01
29	-	400	360	760	0.90
Arithmetic mean				2420	
Group 2.2					
15	200	1310	1380	2890	1.05
16	-	720	830	1550	1.15
21	800	910	990	2700	1.09
25	100	430	360	890	0.84
28	-	320	200	520	0.63
32	100	370	410	880	1.11
33	100	1950	1580	3630	0.81
Arithmetic mean				1823	

1. based on adult nematodes only
2. goat which required anthelmintic treatment in week 12 of trickle infection

Table 2.3. *H. contortus* burdens in Experiment 2.2

Haematology

- Packed cell volume

PCVs are shown for Experiments 2.1 and 2.2 in Figures 2.3 and 2.4 and statistical analyses in Appendices 2g and 2h respectively.

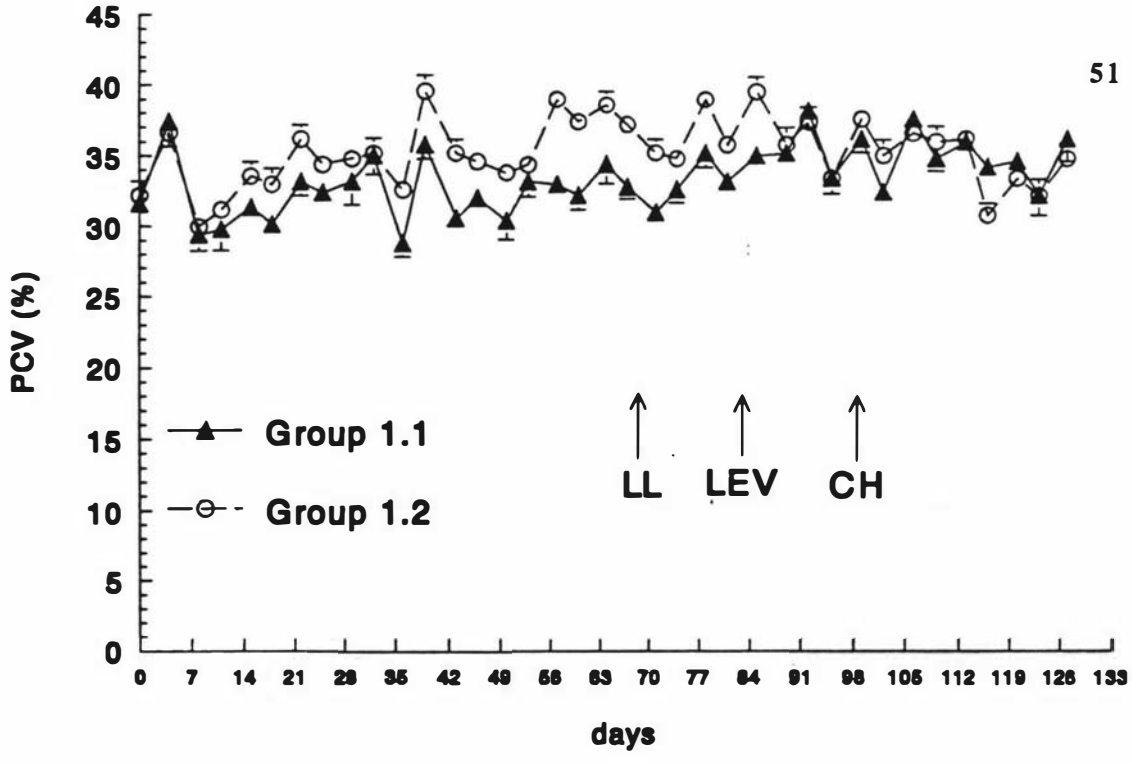


Figure 2.3. Packed cell volumes (mean ± s.e.) from Experiment 2.1. Group 1.1 = trickle-infected; Group 1.2 = control; LL = last larval dose of trickle-infection; LEV = levamisole treatment; CH = all goats challenged with 10,000 *H. contortus* L3s.

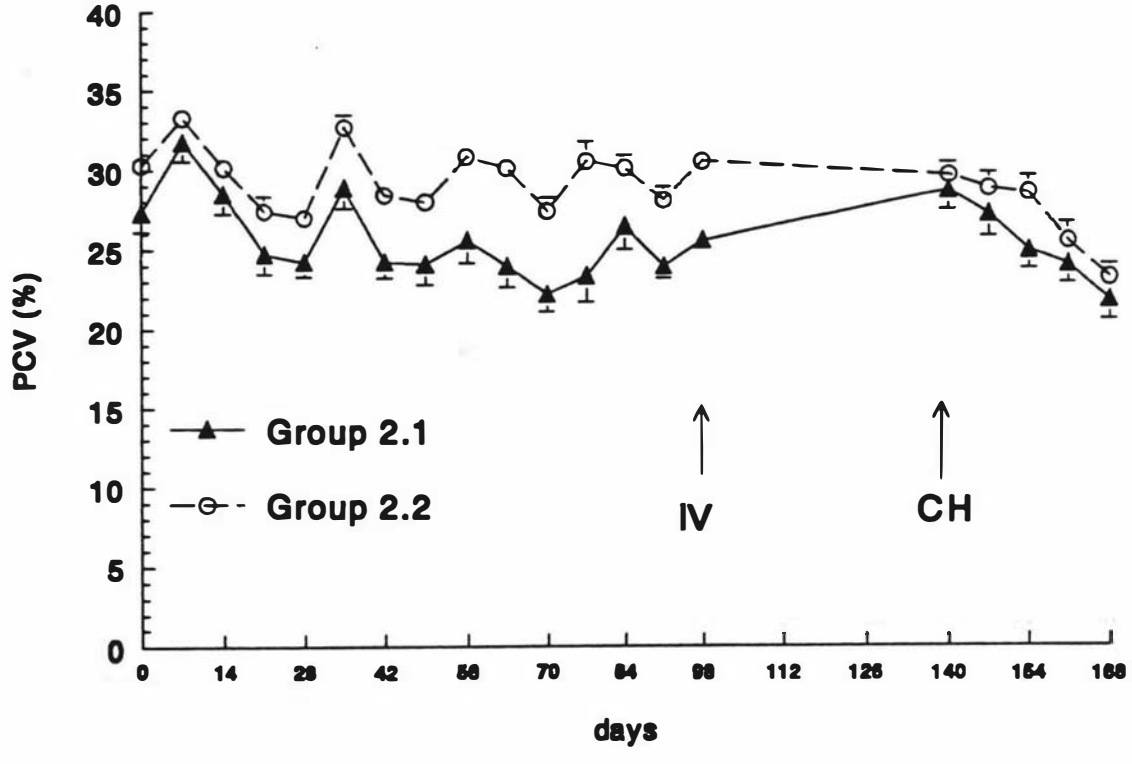


Figure 2.4. Packed cell volumes (mean ± s.e.) from Experiment 2.2. Group 2.1 = trickle-infected; Group 2.2 = control; IV = ivermectin treatment; CH = all goats challenged with 15,000 *H. contortus* L3s.

In Experiment 2.1, PCVs gradually diverged during the trickle infection which is reflected in the significant interaction ($p<0.01$) and the overall significantly lower values for the infected goats ($p<0.001$). At the end of the trickle infection (day 68) infected goats had a significantly lower PCV ($p<0.01$) but not at the time of anthelmintic treatment (day 85; $p>0.05$) or at the time of challenge ($p>0.05$). Following challenge there was some variation in PCVs between the two groups which is reflected in the significant interaction ($p<0.05$) between time and group. Multiple regression, to look for linear effect, was not attempted due to this interaction.

In Experiment 2.2, PCVs also declined in Group 2.1 during the trickle infection, especially in the goat which was dosed with ivermectin in week 12. Overall there was a significant interaction between group and time ($p<0.01$) and a significant difference between groups ($p<0.001$). At the end of the trickle infection there was a significant difference ($p<0.05$) between the two groups which disappeared, as in Experiment 2.1, by the time of the challenge infection. Following the challenge there was a significant decline in PCVs with time ($p<0.001$). There was also a significant effect of group ($p<0.05$) following challenge although at slaughter there was no difference between the two groups ($p>0.05$). The significant effect of group, lack of interaction, but absence of differences at the beginning and end of the challenge infection reflects the almost parallel decline of PCVs in both groups as seen in Figure 2.4.

- Differential white blood cell counts

Changes in counts for the various types of cells and their statistical analyses are shown in Appendices 2g and 2h for Experiments 2.1 and 2.2 respectively.

In Experiment 2.1 during the trickle infection, no significant effects of infection were seen on total white cell, neutrophil, monocyte or basophil counts. There was, however, sufficient variation of eosinophil counts with time to generate a significant interaction ($p<0.05$) between group and time which probably reflects the higher eosinophil counts for Group 1.1 on day 56 and perhaps 78 and/or the higher counts for group 1.2 seen on days 1 and 7, but two sample t-tests on these days showed no significant differences between groups ($p>0.05$).

Following challenge in Experiment 2.1, a group's previous infection history had no significant effect on any cell count except for basophil counts

for which there was a significant effect of group ($p<0.05$) which reflects the higher counts seen for Group 1.2. Eosinophil counts were increasing and the effect of time was approaching significance ($p=0.06$) but there was considerable variation between counts.

In Experiment 2.2 during the trickle infection, no significant effects of treatment were seen for total white cell, neutrophil, monocyte and basophil counts. For lymphocyte counts, the difference between the two groups changed with time which is reflected in a significant interaction ($p<0.01$) between group and time. For eosinophil counts, the relationship between the two groups also changed with time as counts in Group 2.1 initially rose, fell back to low levels around day 63 and then rose and fell again. This is reflected in both a significant interaction ($p<0.01$) between group and time, and an overall higher eosinophil count ($p<0.05$) for Group 2.1.

Following challenge in Experiment 2.2, eosinophil counts rose significantly ($p<0.001$) in both groups and there was no significant difference between them. Lymphocyte counts showed a downward trend after challenge tending to converge towards the end of the experiment. Although lymphocyte counts for Group 2.1 were slightly, but not significantly, higher than Group 2.2 on the day of challenge, over the post-challenge period both the downward trend with time ($p<0.05$) and the effect of groups were significant ($p<0.01$). No significant between-group effects were seen for counts of other cell types. Total white cell counts in Group 2.2 tended to decrease whilst those in Group 2.1 fluctuated even though they remained higher. These trends are reflected in a significant interaction ($p<0.05$) between time and group.

Serum Proteins

The changes in, and statistics for, total protein and albumin levels seen during the two experiments are shown in the figures and tables in Appendices 2g and h for Experiments 2.1 and 2.2 respectively.

In Experiment 2.1 during the trickle infection, there were no significant effects of group for either total protein or albumin. Following challenge, total proteins, when controlled for time, were significantly higher ($p<0.01$) for Group 1.1 but no differences were seen between groups for albumin although they did decrease slightly with time ($p<0.01$).

In Experiment 2.2 during the trickle infection, total proteins and albumin levels generally diverged slightly, with Group 2.1 being lower in both cases. This is reflected in a significant interaction between time and group ($p<0.01$) for both these parameters. Following challenge, both albumin and total protein showed a slight but significant decrease with time ($p<0.001$). For albumin the difference between the two groups fluctuated enough to result in a significant interaction ($p<0.05$). Although albumin of Group 2.2 was lower than Group 2.1 at slaughter, this difference was not significant ($p<0.05$).

Pepsinogen

Serum pepsinogen levels for Experiment 2.1 are shown in Figure 2.5. and their statistical analyses in Appendix 2g. During the trickle infection, the mean values for Group 1.1 increased to a maximum at the time of anthelmintic treatment with a subsidiary peak at around 5 weeks. Overall there was a significant interaction between time and group ($p<0.001$) and a significant difference ($p<0.001$) between the 2 groups. The pepsinogen levels during the primary infection period were also analysed by paired t-tests to determine when the difference became significant. A conservative approach was used to calculate the t-statistic because of the problems in using multiple sequential paired t-tests where successive data sets are not independent. This involved using the largest observed standard deviation for each group during the 10 week period as the standard deviation for all paired observations during this period. Even with this more conservative assessment, the differences in pepsinogen values between Group 1.1 and Group 1.2 were significant ($p<0.05$) from the fourth week until the goats were dosed with levamisole.

Pepsinogen levels for Experiment 2.2 are shown in Figure 2.6. and their statistical analyses in Appendix 2h. Levels plateaued in Group 2.1 from about week 6 at a mean level of approximately 0.7 IU. When analysed as for Experiment 2.1, there was a significant interaction between time and group ($p<0.001$) and an overall significant difference ($p<0.001$) between the 2 groups. When sequentially compared as in Experiment 2.1, pepsinogen values for the two groups were significantly different ($p<0.05$) from week 4 of the infection period until all goats were dosed with ivermectin.

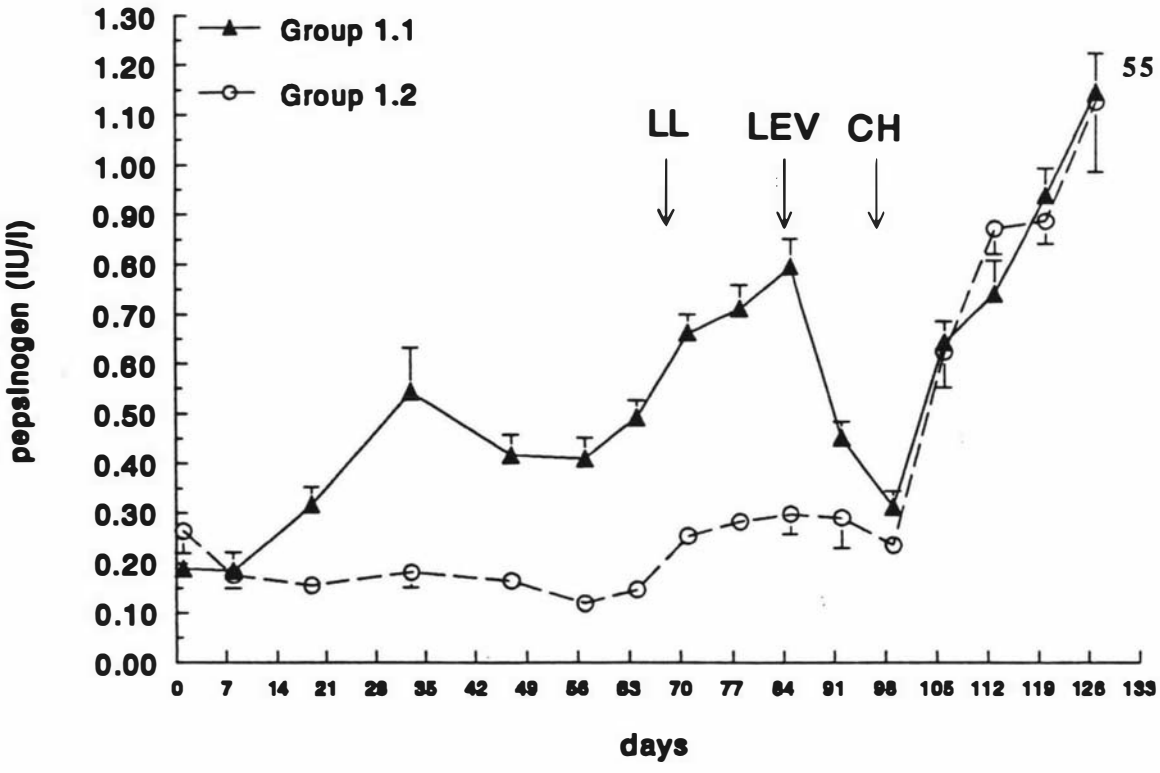


Figure 2.5. Serum pepsinogen values (mean ± s.e.) from Experiment 2.1. Group 1.1 = trickle-infected; Group 1.2 = control; LL = last larval dose of trickle-infection; LEV = levamisole treatment; CH = all goats challenged with 10,000 *H. contortus* L3s.

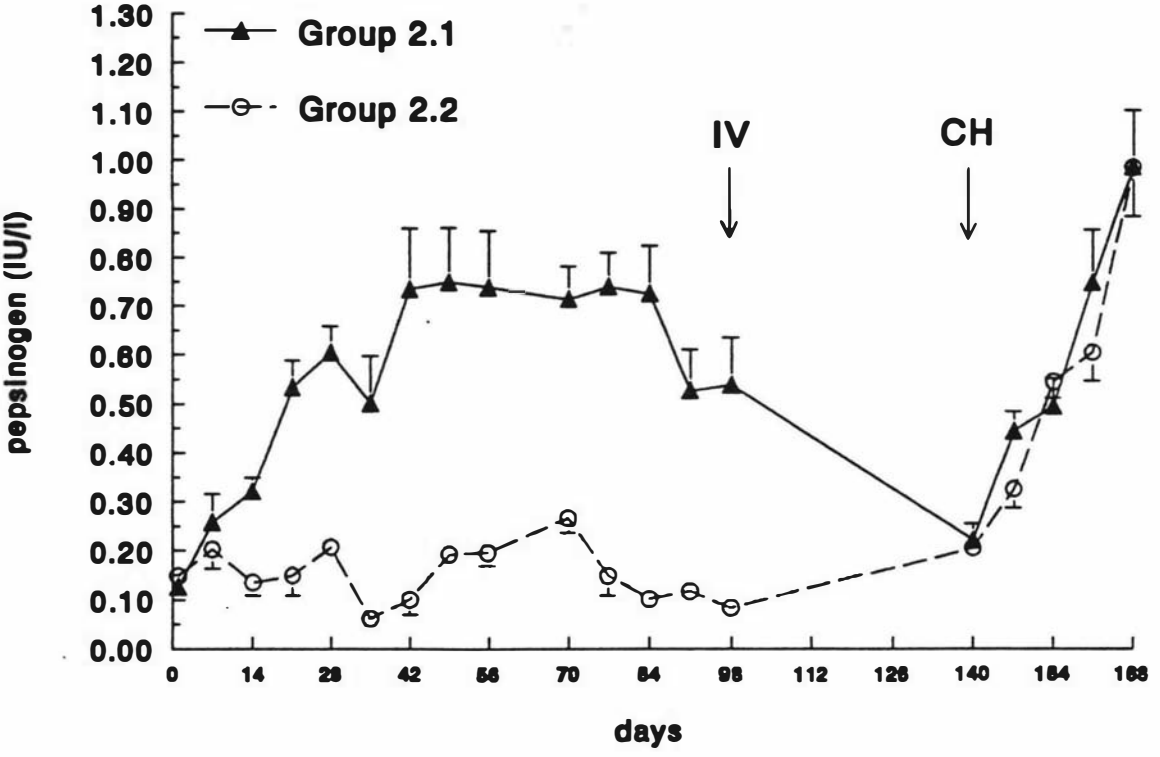


Figure 2.6. Serum pepsinogen values (mean ± s.e.) from Experiment 2.2. Group 2.1 = trickle-infected; Group 2.2 = control; IV = ivermectin treatment; CH = all goats challenged with 15,000 *H. contortus* L3s.

On the day of challenge in both experiments, the pepsinogen values in the trickle-infected animals had dropped to levels not significantly different ($p>0.05$) from those of controls. Following challenge, pepsinogen values rose steeply and significantly ($p<0.001$) in a linear fashion in both experiments. There were no significant differences between groups in their respective experiments.

Data from both experiments considered separately or combined showed no significant correlation between pepsinogen and faecal egg counts at the end of the trickle infection (Table 2.4) or between terminal pepsinogen and total worm counts (Table 2.5).

Gastrin

Serum gastrin levels for Experiments 2.1 and 2.2 are shown in Figures 2.7 and 2.8 and their statistical analyses in Appendices 2g and 2h respectively. Overall there were significantly higher circulating gastrin levels in infected animals during the trickle infections in Experiment 2.1 ($p<0.01$) and Experiment 2.2 ($p<0.05$). In Experiment 2.1, but not 2.2, there was also a significant interaction between group and time ($p<0.001$).

Following challenge, gastrin levels were generally higher for the naive goats than the previously infected animals. Analysis showed a significant effect of time and group for Experiment 2.1 ($p<0.05$). For Experiment 2.2 there was a significant interaction between time and group ($p<0.01$) in Experiment 2.2 which reflects the greater rise of values for Group 2.2 than Group 2.1. At the time of slaughter, the difference between the two groups in Experiment 2.2 was approaching significance ($p=0.1$) when analysed by a two sample t-test and overall the difference between the two groups following challenge was significant ($p<0.05$).

Correlations between gastrin, pepsinogen and faecal egg counts at the end of the trickle infections are shown in Table 2.4 and for gastrin, pepsinogen and worm counts at the times of slaughter in Table 2.5. None of these correlations were significant.

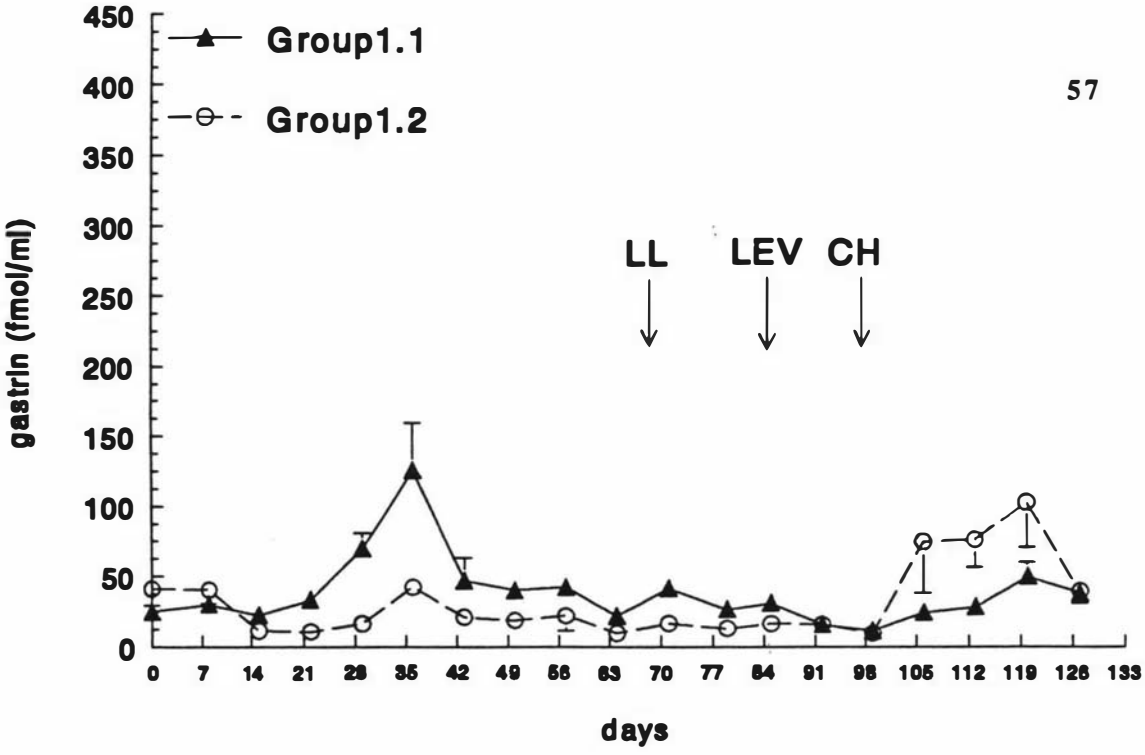


Figure 2.7. Serum gastrin values (mean ± s.e.) from Experiment 2.1. Group 1.1 = trickle-infected; Group 1.2 = control; LL = last larval dose of trickle-infection; LEV = levamisole treatment; CH = all goats challenged with 10,000 *H. contortus* L3s.

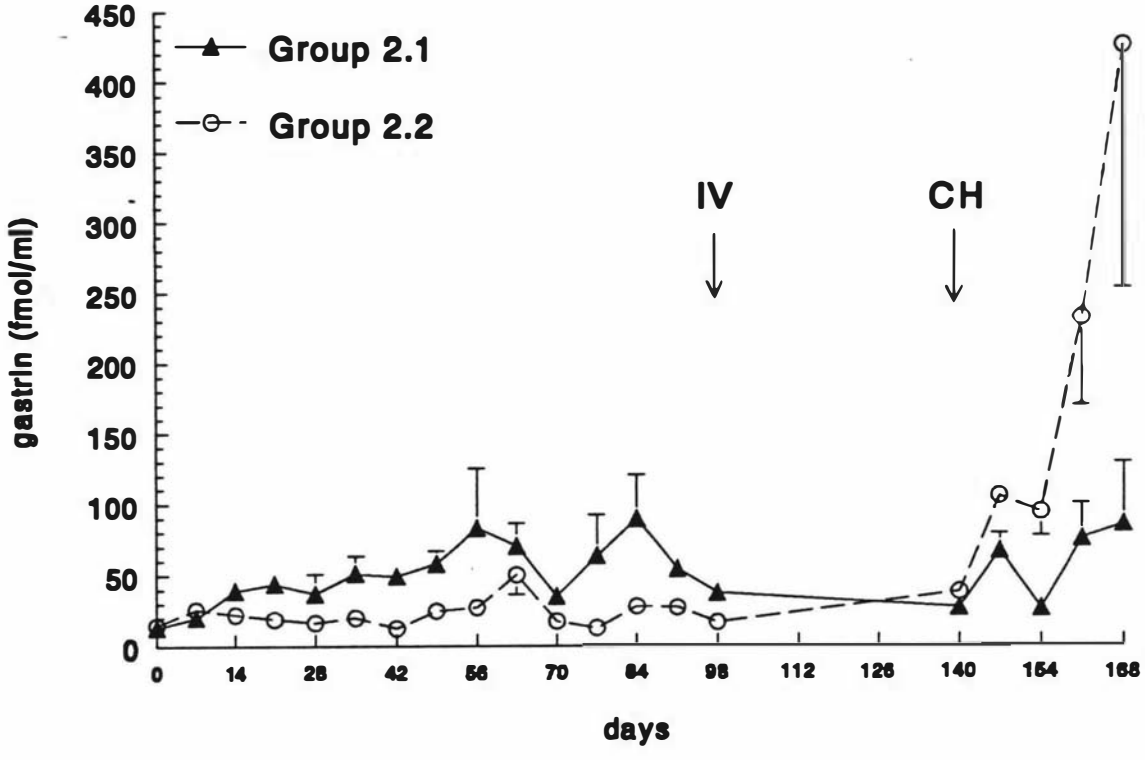


Figure 2.8. Serum gastrin values (mean ± s.e.) from Experiment 2.1. Group 2.1 = trickle-infected; Group 2.2 = control; IV = ivermectin treatment; CH = all goats challenged with 15,000 *H. contortus* L3s.

Correlations at the end of the trickle infection in Experiment 2.1 (n=5)		
	gastrin	faecal egg count
faecal egg count	-0.21	
pepsinogen	0.75	-0.24
Correlations at the end of the trickle infection in Experiment 2.2 (n=8)		
	gastrin	faecal egg count
faecal egg count	0.19	
pepsinogen	0.04	0.57
Correlations at the end of the trickle infection for the combined data of both Experiments 2.1 and 2.2		
	gastrin	faecal egg count
faecal egg count	0.14	
pepsinogen	-0.07	0.28

Table 2.4 Correlations between faecal egg counts, pepsinogen values and gastrin values at the end of the trickle infections.

Correlations at the time of slaughter in Experiment 2.1 (n=10)		
	gastrin	worm count
worm count	-0.29	
pepsinogen	0.54	-0.24
Correlations at the time of slaughter in Experiment 2.2 (n=15)		
	gastrin	worm count
worm count	-0.39	
pepsinogen	0.24	-0.10
Correlations at the time of slaughter for the combined data of both Experiments 2.1 and 2.2 (n=25)		
	gastrin	worm count
worm count	-0.31	
pepsinogen	0.10	-0.11

Table 2.5 Correlations between total worm counts, pepsinogen values and gastrin values at the time of slaughter

2.4 DISCUSSION

Parasitology

The poor and variable establishment of *H. contortus* in the control groups in both experiments is difficult to explain, especially as in Experiment 2.1 a caprine isolate was used, and in both experiments the challenge dose was given by stomach tube to preclude any effect of the closure of the oesophageal groove on establishment. Others have reported low or variable establishment rates of *H. contortus* in goats (Colglazier *et al.*, 1967; Watson, 1986; Al-Quaisy *et al.*, 1987; McKenna and Watson, 1987; Rahman and Collins. 1990d; Rahman

and Collins, 1991a; Hosking and Watson, 1993)). However, Le Jambre and Royal (1976) compared goats and young sheep grazing the same infested pasture and found no difference in *Haemonchus* burdens between the two species although only 3 animals of each species were compared. Similarly, no difference in establishment was seen when goats were infected with sheep-derived or goat-derived *H. contortus* (Rahman and Collins, 1991b). The very long prepatent period seen in Experiment 2.2 might be accounted for by the fact that the isolate originated from sheep. There has been one report that suggest a goat-derived strain developed faster than a sheep-derived strain (Rahman and Collins, 1991b) but whether this reflects a difference in host-derivation or just strains is not clear.

In Experiment 2.1, there was a tendency for the previously infected goats to have lower worm burdens than the controls following challenge but, with the small group sizes and within-group variation, the difference did not reach statistical significance. At the time of challenge, these animals were approximately nine months old. By analogy with sheep, one might have expected to see some measure of acquired resistance under the regime of infection used. However, even in Experiment 2.2, where the animals were 18 months old when challenged, there was still no evidence of resistance having been induced. The results cannot be explained in terms of the general inability of goats of this age to effectively resist gastrointestinal nematode infections. In a further, similar experiment involving Saanen wethers but infected with *Trichostrongylus colubriformis* (see Chapter 3), a very high level of acquired resistance to homologous challenge at 12 months of age was obtained.

A possible explanation is the size of the challenge dose. Immune exclusion of larvae appears to be threshold dependent as immune sheep challenged with $1x10^5$ and $1x10^6$ larvae excluded most whilst those challenged with $1x10^4$ did not (Jackson *et al.*, 1988). However, a closer examination of this report shows that the control animals challenged with $1x10^4$ had about a 20% establishment rate which is lower than the 40-60% usually seen in naive sheep (see Section 1.2.5) and lower than the 50-60% establishment in naive challenge-control sheep given $1x10^5$ or $1x10^6$ larvae. The establishment for vaccinated sheep given $1x10^4$ larvae was 21% which is higher than the 15% and 5% for the groups given $1x10^5$ and $1x10^6$ larvae respectively but these two higher dose rates are artificially high and lower establishment is not surprising. No statistical comparison between these establishment rates was given. Examination of Appendix 1a shows that most experimental challenges given as a single dose have been around 10,000 to 50,000 larvae as in the experiments with goats

reported here. In Appendix 1a there are several reports of experiments where the sensitising infection is removed before challenge and where challenges of this same order have resulted in significantly lower *H. contortus* burdens in immune sheep (Christie *et al.*, 1964b; Dineen and Wagland, 1966b; Donald *et al.*, 1969; Zajac *et al.*, 1990).

The response of goat 9 in Group 1.1 is interesting. This goat exhibited an apparent inherent resistance to *H. contortus* as its faecal egg count (shown in Figure 2.1) was consistently low and after challenge this goat had the lowest worm burden in this experiment. Genetic variation has been observed in the response to gastrointestinal nematodes in other ruminants especially sheep, and specific breeding programs established to select for resistant animals (Woolaston, 1990). Other individual goats expressing a high level of host resistance under field conditions, have been observed (Soe and Pomroy, unpublished). These animals may represent genetically resistant individuals and offer hope for future selection programs.

The small number of *Nematodirus* eggs observed would probably have resulted from infection acquired from low level contamination of the straw bedding or, in Experiment 2.2, the hay the goats were fed. The numbers were evidently too small to be detected by the examination of 10% aliquots.

Whether the present trials indicate an inability of goats generally to develop resistance to *Haemonchus*, or a requirement for longer primary exposure to the parasite, or a larger challenge to express resistance, is impossible to say. Certainly, under field conditions in New Zealand, clinical haemonchosis is commonly seen in adult goats suggesting that effective resistance to *Haemonchus* is either not easily established or is difficult to maintain. It would be interesting to know if these results apply to other breeds of goat.

In these experiments, the *H. contortus* burden was removed before challenge. It has been suggested that goats, as with young sheep, may demonstrate a level of protection if the sensitising infection is not removed (Hosking and Watson, 1993). The observation that the faecal egg counts in the present experiments tended to reach a peak and then plateau until anthelmintic treatment was given, is consistent with such an hypothesis. In addition, there is some evidence that goats may be successfully protected following vaccination with irradiated *H. contortus* (see Section 1.1.2). However, it was not stated if these sensitising infections were removed before challenge.

Pepsinogen

The mechanism inducing pepsinogen release into circulation is not just a simple leakage through loose cell junctions but is considered to be multifactorial and may include endocrine or paracrine regulation (McKellar, 1993). In Experiment 2.1, values rose to a peak at about week 5 then fell slightly and rose again (Figure 2.5). The plateau seen in pepsinogen values of Group 2.1 during primary infection period suggests that a steady state had developed despite continuing intake of larvae. No reason can be given for the difference between experiments.

Pepsinogen responses after challenge were similar in both experiments. They increased rapidly and by 7 days levels were significantly higher than preinfection. This is consistent with increases in circulating pepsinogen levels seen in sheep occurring soon after *H. contortus* L4 larvae emerge from the gland crypts on or about day 5 (Coop 1971). A similar rapid increase has also been seen in young field-reared goats given a single moderate challenge with *H. contortus* (Fox *et al.*, 1991).

Nevertheless, the increases in pepsinogen values seen in both experiments were relatively small. Pepsinogen values >3.0 IU/litre (Armour, 1970) or >2.6 IU/litre (Brunsdon 1971) have been considered diagnostic of ostertagiosis in cattle, and similar increases have been recorded from sheep with large *O. circumcincta* burdens (Anderson *et al.*, 1985). However, much lower levels of the order of 0.4-1.0 IU/litre have been recorded from sheep given massive single infections with *H. contortus* (Coop, 1971) and goats with relatively small *H. contortus* burdens (Fox *et al.*, 1991), although calves infected with *H. contortus* did show a rise to >3 IU/litre (Shoo and Wiseman, 1986).

The lack of correlation between pepsinogen levels and faecal egg counts or postmortem worm numbers suggests pepsinogens may not be helpful in diagnosing abomasal parasitism, or at least haemonchosis, in goats.

Gastrin

The resting values of <50 fmol/ml in control goats during both the trickle infections are similar to those recorded by others in goats (Fox *et al.*, 1991), sheep (Anderson *et al.*, 1981) and cattle (Berghen *et al.*, 1993). The control of gastrin production by G cells in the abomasum is complex but production is known to increase with gastric nematodosis and often to very high

levels (Titchen, 1982). Increases in serum gastrin have been observed in goats infected with *O. ostertagi* (Markovits, 1987) and *H. contortus* (Fox *et al.*, 1991) but not *T. colubriformis* (Reinemeyer *et al.*, 1991). In both the present experiments, serum gastrin levels slowly increased in the trickle-infected goats relative to the control animals but were never more than 3X control levels. This is similar to the peak rise of 3.9X recorded for lambs exposed to a trickle infection of 2000 *O. circumcincta* daily (Fox *et al.*, 1988) but less than the high values seen when large infections of *Ostertagia* species have been given to sheep (Anderson *et al.*, 1981; Reynolds *et al.*, 1979) and calves (Pitt *et al.*, 1988; Hilderson *et al.*, 1991). The peak observed at day 35 in Experiment 2.1 may have been simply a result of parasitism as circulating gastrin levels have been observed to peak and then gradually fall away in sheep given a single infection (Nicholls *et al.* 1988) or a trickle-infection (Fox *et al.*, 1988) of *H. contortus*. However, a possible contributing factor is the time of feeding in relation to blood sampling which may have varied, as the decision to assay serum gastrin was made at the completion of the experiment. Feeding can increase circulating gastrin levels by a factor of 2X for more than an hour in sheep (Reynolds *et al.*, 1991). In Experiment 2.2, goats were always bled before feeding and no similar peak was seen.

The pattern of gastrin release following challenge is difficult to interpret as it was slightly different in the two experiments although levels for naive goats were always higher than those of previously infected animals; this was particularly obvious in Experiment 2.2. This suggests some conditioning of the gastrin response by previous infection although previously infected goats did not have significantly fewer worms. Lower peak gastrin levels have also been reported in sheep reinfected with *O. circumcincta* although the two sheep concerned had substantially lower worm counts than sheep infected once (Anderson *et al.*,1981).

The overall sizes of the gastrin responses following challenge, particularly for groups 1.2 and 2.2, were similar to those recorded for much larger *Haemonchus* infections in sheep (Nicholls *et al.*, 1988; Fox *et al.*, 1988) which suggests that gastrin production may not be related to worm burden. This is consistent with the poor correlations between faecal egg counts and gastrin at the end of the trickle infections and between total worm counts and gastrin levels at slaughter. It has been suggested that estimating gastrin may be of value in the diagnosis of caprine haemonchosis because of the correlation of pepsinogen and gastrin observed in goats ($r=0.77$; $p<0.001$; Fox *et al.* 1991) and sheep ($r=0.70$; $p<0.001$; Fox *et al.*, 1988). However, these authors did not

present correlations with faecal egg counts or any other parasitological parameters to support the use of either for this purpose. In the present experiments, no significant correlations between gastrin and pepsinogen were observed nor were there any correlations between either of these two parameters and faecal egg counts or worm counts. This suggests neither may be useful diagnostically. Similar poor correlations between pepsinogen and gastrin responses have also been observed in parasitised cattle (Pitt *et al.*, 1988; Berghen *et al.*, 1993).

Haematology

The declines in PCV in infected goats noted during the trickle infections in both experiments and after challenge in Experiment 2.2 were small but are consistent with the blood-sucking nature of *H. contortus*. The failure to observe differences between the two groups following challenge reflects the similarity in worm burdens as blood loss would be expected to be a direct function of worm numbers. Others have observed the fall in PCV in goats infected with *Haemonchus* to be related to faecal egg counts (Arzoun *et al.*, 1983). The greater fall following challenge in Experiment 2.2 than in Experiment 2.1 may have been influenced by the difference in plane of nutrition in the two experiments as worm burdens were similar in both. It also suggests that the feeding activity of this strain was not affected by its ovine origin even though egg production was restricted. Rahman and Collins (1991b) observed that a goat-derived strain caused a greater fall in PCV than a sheep strain ("McMaster susceptible"), but the latter had been a laboratory strain for many years which may have reduced its pathogenicity (Anderson, *pers. comm.*).

The most notable change recorded in white cell counts was the rise in eosinophil numbers seen in Experiment 2.2. Interestingly, during the trickle and challenge infections in Experiment 2.1, eosinophil counts did not increase significantly overall; this is similar to the lack of an eosinophil response recorded by Rahman and Collins (1991b) in young goats. The rise seen during the trickle infection in Experiment 2.2 indicates that the immune system was responding to the infection in these older goats even though this was not reflected in control of worm numbers after challenge. The absence of a difference between the two groups after challenge in both experiments is consistent with the lack of an effective immune response, although the rise observed in previously naive goats in Group 2.2 suggests an eosinophil response is not necessarily dependent on previous exposure to infection.

Rahman and Collins (1991b) reported that total white cell counts fell significantly in goats infected with *H. contortus* and this was presumably due to the fall in lymphocyte counts recorded. A similar significant fall in lymphocyte count was seen in older goats in Experiment 2.2 after challenge but not in Experiment 2.1 although the trend was in the same direction. The biological significance of this is not clear. Rahman and Collins (1991b) also reported a significant rise in neutrophil counts but this did not occur in the present experiments.

In both experiments, the observed falls in serum albumin levels are mainly attributable to blood loss. However, further loss of albumin may result from mucosal leakage due to damage to the mucosa or separation of the *zona occludens* between the epithelial cells. The pattern of total protein levels following challenge was dissimilar between the two experiments and is not readily explained. Interestingly, a feature of total serum proteins and albumins in these experiments is the small variation between individual goats which makes group differences more readily apparent.

CHAPTER THREE

DEVELOPMENT OF RESISTANCE TO *TRICHOSTRONGYLUS COLUBRIFORMIS* IN GOATS (EXPERIMENT 3.1)

3.1 INTRODUCTION

It has been shown that sheep can develop resistance to *Trichostrongylus colubriformis* from 5-6 months of age (see Section 1.3). This resistance is initially expressed over a period of several weeks when larval establishment falls while adult worm numbers are unaffected. Following this is a period during which adult worms are also expelled (Dobson *et al.*, 1990a). A similar pattern has been observed for other *Trichostrongylus* spp. in sheep (Waller and Thomas, 1981). Other observed effects of developing resistance against *T. colubriformis* are decreased fecundity of female nematodes (Gibson and Parfitt, 1972, 1973; Chiejina and Sewell 1974b; Dineen and Windon, 1980b; Wagland *et al.*, 1984), a decrease in the ratio of male to female worms (Dineen and Windon, 1980b), and an increase in numbers of inhibited L3s (Dobson *et al.*, 1990b).

This chapter reports an experimental investigation into the establishment of resistance to *T. colubriformis* in young goats.

3.2 MATERIALS AND METHODS

Experimental schedule

Saanen wethers were raised nematode-free in pens. At the beginning of the experiment they were approximately 9 months old with a range of 30 days between the youngest and oldest. These animals were raised as a group with those used in Experiment 2.2.

The *T. colubriformis* used were an isolate of ovine origin passaged through a goat to provide infective larvae. The isolate was kindly provided by Dr P. Douch, Wallaceville Animal Research Centre. Larvae were stored at room temperature and were less than two months old when used. Viability was assessed by observing motility. At all times it was considered to be very high (>90%).

Initially 10 goats, including three sets of twins (from a pool of 32 goats), were selected for the trial. One sibling from each twin was randomly allocated to either Group 1 or Group 2. The remaining 4 goats were then randomly allocated to make two equal groups.

Two further goats (A and B) were included in Group 1 to assess the accumulated burden acquired following the initial infection period.

Group 1 goats were dosed by stomach-tube once a week for 10 weeks with 10,000 *T. colubriformis* infective larvae. Group 2 were not infected. In week 11, goats A and B were killed to determine worm counts and the remaining 10 goats were dosed with 300mcg/kg ivermectin. Faecal egg counts of the infected goats fell to zero.

Two weeks later (week 13), each goat in Groups 1 and 2 was challenged with 50,000 infective larvae given by stomach tube. Twenty eight days later (week 17), unlike the animals of Group 2, all animals in Group 1 had zero faecal egg counts. To confirm this unexpectedly unequivocal result, the 10 goats in Groups 1 and 2 were again dosed with 300 mcg/kg ivermectin together with a further 5 goats (Group 3) randomly selected from the remaining nematode-free goats. In week 19, all 15 goats were challenged with 50,000 infective larvae and killed 28 days later.

Parasitology

Faecal egg counts were estimated weekly throughout the experiment using a modified McMaster technique (see Appendix 2b). In week 17, the faeces of goats in Group 1 were also examined by simple flotation.

At necropsy, 6 x 1-2 cm blocks of small intestine at one metre intervals distal to the pylorus and 2 x 1-2 cm blocks at 1 metre intervals proximal to the ileocaecal junction were removed for histological studies. Results of these will be reported in Chapter 7. The remaining small intestine was opened and washed thoroughly under running water. A 1% aliquot of the washings was sieved through a 53 µm mesh to retain all adult and larval stages. A further 9 percent aliquot was sieved through a 250 µm mesh. Counts of larval stages were thus based on 1 percent aliquots and adults on 10 percent aliquots. The abomasum was also opened and washed thoroughly. 10 percent aliquots of these abomasal washings were counted after sieving through a 250um mesh. Male and female adult worms were counted separately. The numbers of eggs

in utero per female worm were counted in 50 worms from each goat. All washings were preserved in 10% formalin.

Haematology and serum proteins

Ten ml of blood were collected each week from Groups 1 and 2 and from week 18 onward, from Group 3. Albumin and total protein were estimated as described in Chapter 2. In addition 5 ml of blood were collected into EDTA from each goat in Groups 1 and 2 in Week 10 for differential white cell counts.

Statistical analysis

Statistical comparisons of worm counts and eggs per female were made by analysis of variance using Statistix version 4.0: worm count data were transformed square root (x + 0.5) beforehand. Male to female ratio data were analysed by the Mann-Whitney U-test by comparing Group 3 vs (Groups 1+2). Serum protein data were analysed as in Section 2.2. Haematological comparisons were made with two-sample t-tests using Statistix version 4.0.

3.3 RESULTS

Parasitology

Arithmetic mean faecal egg counts (± range) are shown in Fig 3.1 with raw data in Appendix 3a and terminal egg counts after the second challenge in Table 3.1. Total *T. colubriformis* burdens, eggs/female and male to female ratios, where it was possible to calculate them, are also shown in Table 3.1. Faecal egg counts in Group 1 rose steeply, peaked in week 7 and then tended to gradually fall until the goats were treated with ivermectin although there was only a small difference in mean egg counts between weeks 5 and 11. No eggs were detected in the faeces of any goat in Group One 28 days after the first challenge infection (week 17) by either the modified McMaster technique or simple flotation. The worm counts of goats A and B (Table 3.1) indicate that moderate burdens established in Group 1 during the initial infection period. Worm burdens of all groups were significantly different from each other ($p<0.05$).

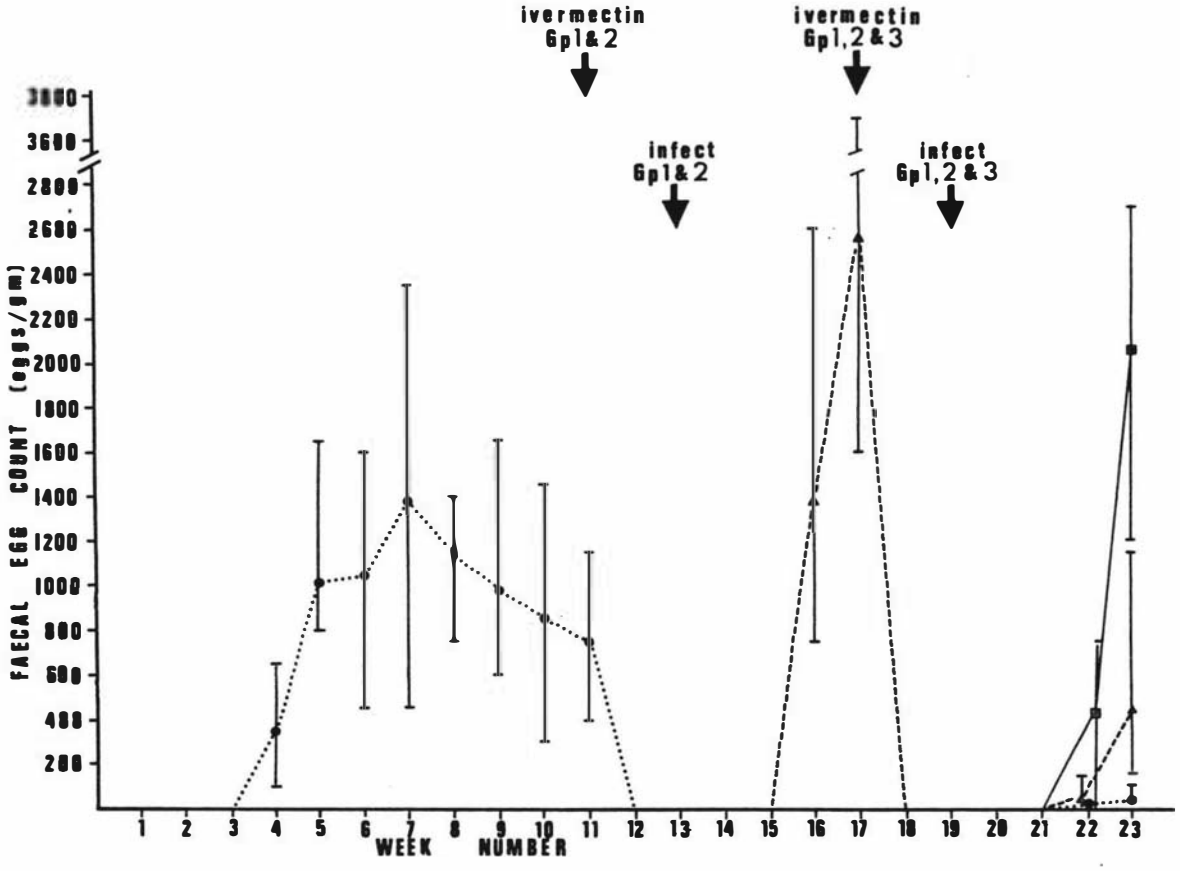


Figure 3.1. Faecal egg counts (± range) of Experiment 3.1. Group 1 (n = 5; ● .... ●) trickle infected with 10,000 *T. colubriformis* per week x 10 and challenged twice; Group 2 (n = 5; ▲ ---- ▲) challenged twice; Group 3 (n = 5; ■ ———— ■) challenged once. Each challenge comprised 50,000 *T. colubriformis*.

Group	Goat	Worm counts				Eggs per female	Male: female ratio	Final egg count (eggs/g)
		Small Intestine		Abomasum	Total			
		L4	Adult	Adult				
1	3	0	0	0	0	-	-	0
	5	0	40	0	40	-	-	0
	6	0	0	0	0	-	-	0
	9	0	720	10	730	5.4	0.76	50
	10	0	990	0	990	8.7	0.77	100
					352#			
2	1	0	2470	0	2470	4.2	0.47	150
	2	700	2400	0	3100	6.7	0.59	200
	4	0	8860	0	8860	8.1	1.15	350
	8	0	5700	0	5700	6.6	0.75	350
	11	0	10460	10	10470	13.3	0.70	1150
					6120#			
3	7	0	10430	0	10430	16.5	0.94	2100
	17	0	10210	0	10210	16.8	1.11	2450
	18	0	15200	50	15250	12.5	1.10	1200
	30	0	12060	60	12120	13.3	1.11	1850
	31	0	13850	10	13860	18.1	1.09	2700
					12374#			
	A	0	6260	0	6260	5.6	1.05	950
	B	0	9490	0	9490	6.9	1.15	1200

Table 3.1. *T. colubriformis* worm counts, eggs per female, male:female ratios and final egg counts of Groups 1, 2 and 3 (# = arithmetic mean)

The numbers of eggs per female nematode were significantly lower in Group 2 than in Group 3 ($p<0.01$). A significantly lower male to female ratio ($p<0.05$) is also apparent in Groups 1 and 2 combined compared with Group 3. Correlation coefficients between various terminal parasitological parameters are shown in Table 3.2.

	worm count	eggs/g	eggs/female
eggs/g	0.79		
eggs/female	0.81	0.96	
male:female	0.76	0.65	0.67

Table 3.2 Correlation coefficients at the time of slaughter for goats with an egg count >0 epg. All correlations are highly significant ($p<0.01$).

Haematology

In week 10 there were significantly ($p<0.001$) more circulating eosinophils in Group 1 (mean $0.59x10^9$/l ± s.e. $0.07x10^9$) than 2 (mean $0.05x10^9$/l ± s.e. $0.02x10^9$) but no differences between them for total neutrophil, lymphocyte, monocyte or basophil counts.

Serum Proteins

Means and standard errors for total protein and albumin levels are shown in figures in Appendix 3b, as are their respective statistical analyses.

During the trickle infection no difference in total protein levels between Groups 1 and 2 was seen. Following the first challenge, a significant interaction between group and time ($p<0.01$) was generated by the more rapid initial fall in levels in Group 1. Following the second challenge the fluctuations were sufficiently different between groups to generate a significant interaction between time and group ($p<0.01$) but no obvious pattern was apparent except for a decline with time which would be reflected in the significant effect of time ($p<0.001$).

During the trickle infection, albumin levels declined in the infected goats; this is reflected in a significant interaction ($p<0.001$) between time and group and significantly lower levels ($p<0.05$) overall in Group 1. At Week 11, a two-sample t-test showed the albumin levels of Group 1 were significantly lower ($p<0.01$) than those of Group 2.

The difference in albumin levels between the 2 groups had diminished by the time of the first challenge (Week 13) although still significant ($p<0.05$). Following the first challenge, albumin levels in Group 1 were significantly lower

($p<0.05$) overall although levels in Group 2 declined whilst those of Group 1 stabilized so that by the end of this first challenge (Week 17) they were not different, indeed values for Group 1 were higher than Group 2 but not significantly so. This convergence was also reflected in a significant interaction ($p<0.001$) between group and time.

At the time of the second challenge, albumin levels of Group 2 were significantly lower than those in Groups 1 and 3 but by the end of the experiment this difference was no longer apparent ($p>0.05$). There was a general decline overall in albumin levels with time following the second challenge which is reflected in the significant effect of time ($p<0.001$) and a significant interaction between time and group ($p<0.001$) which presumably reflects the greater fall in Group 3 goats than in Group 2 animals which was in turn greater than in those in Group 1. A correlation between the fall in albumin levels during the second challenge and various terminal parasitological parameters showed a significant correlation with male to female ratio ($r=0.7315$; $n=12$; $p<0.01$) but not with worm count, egg count or eggs per female worm.

3.4 DISCUSSION

The development of substantial resistance to *T. colubriformis* is clearly evident. Group 1 goats apparently strongly resisted the first challenge (following the 10-week infection period) as seen by their failure to develop patent infections, and also the second challenge as indicated by post mortem worm counts. Group 2, which had previously only experienced a single infection with 50,000 larvae, had worm burdens intermediate between Groups 1 and 3 following the second challenge. This suggests that Group 2 animals had developed partial resistance at a rate consistent with the time frame for the development of host resistance to *T.colubriformis* in sheep (Chiejina and Sewell, 1974b; Dobson *et al.*, 1990a). The lower fecundity and male to female ratio are also consistent with trends noted with developing resistance to similar infections in sheep (see Dineen and Windon, 1980b; Dobson *et al.*, 1990a).

The tendency of Group 1 faecal egg counts to peak and decline slowly during the trickle infection suggests Group 1 goats were already expressing a measure of resistance during this period. This pattern is, however, not dissimilar from that noted for both experiments in Chapter 2 where no difference in worm count was noted after challenge. If the threshold for development of resistance in goats is similar to that for sheep, it would be

expected to be easily surpassed in the second week of the trickle infection with 10,000 larvae/week. Therefore, larval establishment would be expected to fall from Week 2 with adult worm burdens eventually peaking and then falling. Faecal egg counts would follow adult worm burdens with the additional factor of declining fecundity contributing to their more rapid fall. The gradual decline in faecal egg counts in the present experiment is similar to that described for a trickle infection of 2000 L3/day in sheep (Dobson *et al.*, 1990a).

The overall mean establishment rate in naive goats of approximately 25% is lower than the 65% (range 30-80%) reported in sheep (Dobson *et al.*, 1990a). However, the intestine was processed immediately after death and, although washed vigorously, it was not digested. Some worms would not have been dislodged (see Chapter 4) and this would have reduced the overall recovery. Nevertheless, the establishment, as recorded, was similar to that reported by others in goats (Rahman and Collins, 1990c). It might also be expected that a percentage of the worm population in the resistant goats would be inhibited as third stage larvae (Dobson *et al.*, 1990b; Eysker 1978) but none were seen. Presumably these larvae would be not easily released without digesting the mucosa. It has also been noted that in the presence of formalin, which was used to preserve washings in this experiment, third stage larvae coil tightly and are difficult to see (Dobson *et al.*, 1990a).

The results of this experiment contrast with those described in Chapter 2 in which a similar experimental design was used to investigate the development of resistance to *H. contortus*. The difference in the response to these two nematodes is difficult to explain and clearly further work is needed. It is interesting to note that on a property where field observations of faecal egg counts suggested the acquisition of a measure of host resistance with age (Pomroy *et al.*, 1986), *H. contortus* is rarely seen (Pomroy, unpublished). These results also contrast with those of Watson and Hosking (1989), who failed to demonstrate any evidence of host resistance in slightly younger, 6-8 month-old, Saanen goats which were grazed on pasture and also received pulsed infections of *T. colubriformis*. Indeed, the goat egg counts were continuing to climb at a steady rate throughout their experiment.

In young sheep, the correlation between faecal egg counts and worm burdens has been variably reported to be not significant (Sangster *et al.*, 1979) or highly significant (Douch *et al.*, 1984). In the present experiment the strong correlation is similar to the latter report in sheep but may be due, in part, to

a quirk of the data in that the animals fall into three clusters which would have a large influence on the overall correlation.

The increase in eosinophil counts in infected goats seen at Week 10 is consistent with the eosinophilia generally associated with trichostrongylid infections in other ruminants and similar to the findings in Experiment 2.2 and is indicative of an active immune response (see Section 1.5.6).

The decrease in levels of albumin in this experiment is similar to that recorded in sheep infected with *T. colubriformis* (Coop *et al.*, 1976). This may be attributable to leakage of plasma proteins through damaged epithelium and loose tight-cell junctions (Barker, 1975b; Barker, 1973) and an increased catabolic rate of albumin which is presumably required to compensate for loss of endogenous nitrogen (Steel *et al.*, 1980; Parkins *et al.*, 1989; Bown *et al.*, 1991). Falls in albumin levels were generally larger in goats with higher worm burdens although this trend was not significant, at least not as measured at the time of slaughter. Interpretation of the decline in albumin levels is complicated by the tendency for all levels to fall over the course of the experiment. The reasons for this are unknown. However, a commercial standard and a pooled serum sample which were analysed on each sampling day did not vary appreciably indicating it was not a fault with the assay. The significant correlation between albumin levels and male to female ratio is interesting. It suggests that males may be more affected by the immune response which is also associated with decreased albumin levels.

The reasons for the interactions between time and group for total protein levels particularly after the second challenge is not clear. In part, it will be because levels of other serum proteins (e.g. globulins) which were not measured, may have risen during infection, as has been reported to occur in sheep (Steel *et al.*, 1980) and this may mask changes in the albumin component of total protein.

CHAPTER FOUR

THE DISTRIBUTION OF *TRICHOSTRONGYLUS COLUBRIFORMIS* IN THE SMALL INTESTINE (EXPERIMENT 4.1)

4.1 INTRODUCTION

In order to study the mast cell response in the intestinal mucosa and larval migration inhibitory activity of small intestinal mucus, some appreciation of the distribution of *T. colubriformis* in the small intestine was required. No information on this in goats had been published when the investigations described in this chapter were carried out. The paper by Rahman and Collins (1990c) appeared subsequently.

The distribution of *T. colubriformis* in sheep has been well described. Although early reports considered *T. colubriformis* to be normally distributed in the proximal small intestine (Tetley, 1937; Sommerville, 1963), a later more definitive set of experiments (Barker, 1974) found maximum numbers occurred in the first, second and third metres of the small intestine of eight, three and three lambs respectively with over 90% of the worm burden in the first six metres. This is similar to the later report of Rahman and Collins (1990c) who examined the distribution of *T. colubriformis* in young goats and found the majority were located in the proximal 3 metres of the small intestine. Time after infection appeared to have no influence on this distribution in either goats (Rahman and Collins, 1990c) or sheep (Barker, 1975c).

4.2 MATERIALS AND METHODS

Experimental schedule

Young goats (kids)

Ten 5 month-old 7/8 Angora X 1/8 New Zealand feral kids were purchased commercially. They had been raised on pasture. All were treated with ivermectin (>0.2mg/kg) and shown to be worm-free by a faecal flotation of 2 gms of faeces seven days later. They were housed in concrete pens with straw bedding and fed meadow hay *ad lib*.

On Day 1 all 10 goats were infected with 20,000 viable *T. colubriformis* larvae given by stomach tube. This amounted to approximately 1250 larvae/kg

liveweight. The larvae used had been cultured four months previously and stored at 10°C. Viability was assessed by larval motility and estimated at 96%.

All goats were killed on Day 28 with an overdose of pentobarbitone.

Older goats

These were the goats in Groups A.1 and B.4 in Experiment 7.1 (n = 8). They were infected and killed as described in Section 7.2.

Parasitology

Young goats

The intestine was ligated at the abomasal pylorus and removed. The small intestine was separated from the mesentery with scissors and then gently laid out with sufficent tension to reduce slack but avoid stretching. The intestine was then divided into 1 metre lengths and stored frozen at -20°C. Total intestinal lengths were recorded. After thawing, these metre lengths were opened longitudinally and immersed in 150mls of pepsin/HCl (see Appendix 2c) for 2 hours at 37°C. The digested material was then sieved through a 53µm sieve to retain all larval and adult stages. *T. colubriformis* were then counted in a 10% subsample. Males and females were counted separately with five males per metre retained to confirm the species was *T. colubriformis*, and ten females being retained per metre to count eggs *in utero*. Metre segments were counted in sequence until the sum of the last two counts was less than 1% of the total counted so far.

Older goats

See Section 7.2

Statistical analysis

To make valid comparisons between goats and estimate the mean distribution and because the total small intestinal lengths varied, it was necessary to standardise worm counts relative to the total intestinal length by converting the counts per metre to numbers of nematodes in each successive one-tenth of the total length. How this was achieved is best explained with an example. Suppose the total intestinal length was 17 m; counts for the proximal one-tenth (1.7 m) were derived from all of those from the first 1 m counted and

70% of those from the second metre. Counts for the second tenth comprised the remaining 30% from the second metre, all of those from the third and 40% of those from the fourth; and so on. This had the effect of slightly smoothing out the trends evident along the intestine. In the older goats this was even more so, because it was necessary to pool the digests of the second and third metres as they had both been scraped to collect mucus, and these scrapings had been combined. These worm counts were then converted to a proportion of the total worm count in each goat.

It was not possible to allocate eggs per female to one-tenth proportions of length as above and these were considered per metre.

Linear regression was used to analyse the eggs per female worm data and the separate regression lines for both groups of goats were compared as described by Zar (1984). Male to female ratio was analysed by analysis of variance. Data were analysed using Statistix version 4.0.

4.3 RESULTS

Length of small intestine

The mean lengths (range) for the young and older goats were 16.75 metres (14.5-19.5) and 17.75 metres (15.0-23.0) respectively. A two sample t-test showed their was no significant difference between the two age groups ($p=0.43$).

Parasitology

The arithmetic mean worm count in young goats (± s.e.) was 14,270 (± 386.2) and in old goats 23,126 (± 1387.8). Although the variation between worm counts was quite small within age groups, it did vary between the two age groups, with only one count overlapping. All males identified were *T. colubriformis*.

The distributions of the proportion of worm counts by proportion of length are shown in Fig. 4.1 for young goats and Fig. 4.2 for older goats with the means for the two shown together in Fig. 4.3. Most worms are concentrated in the proximal few metres of the small intestine with numbers decreasing with distance from the pylorus.

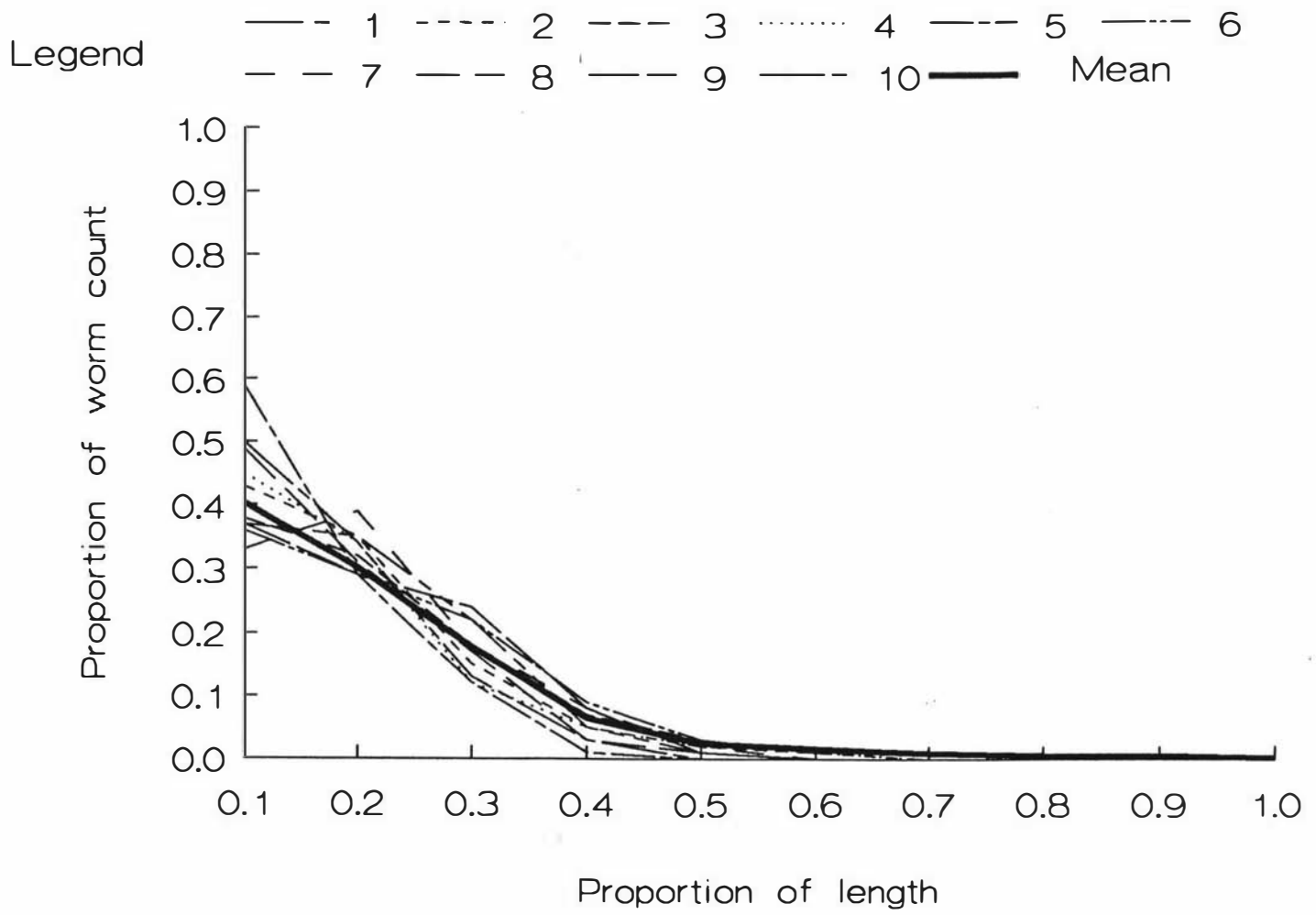


Figure 4.1. The distribution of the proportion of worm counts by the proportion of small intestinal length for young goats (n = 10) in Experiment 4.1.

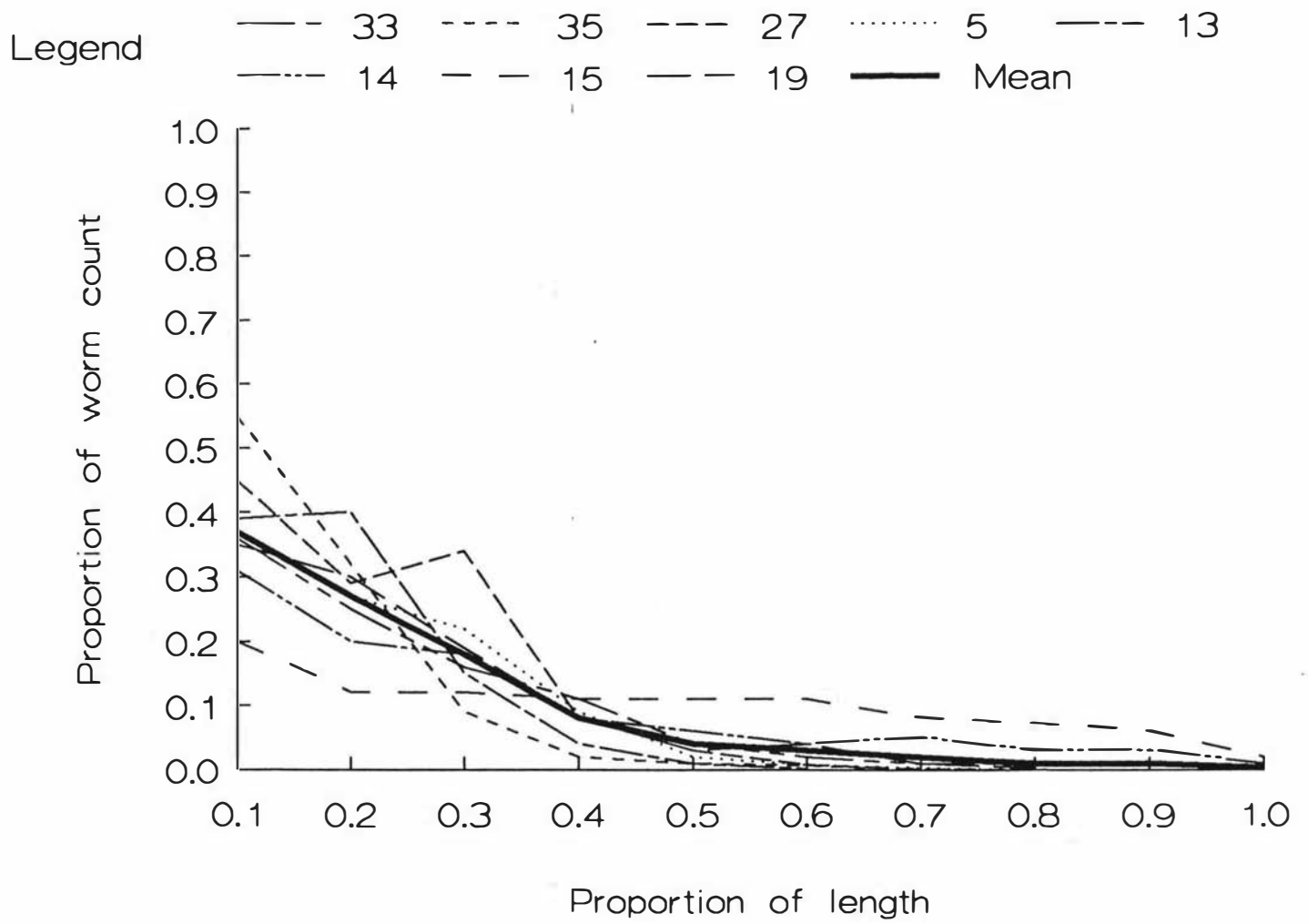


Figure 4.2. The distribution of the proportion of worm counts by the proportion of small intestinal length for older goats (n = 8) in Experiment 4.1

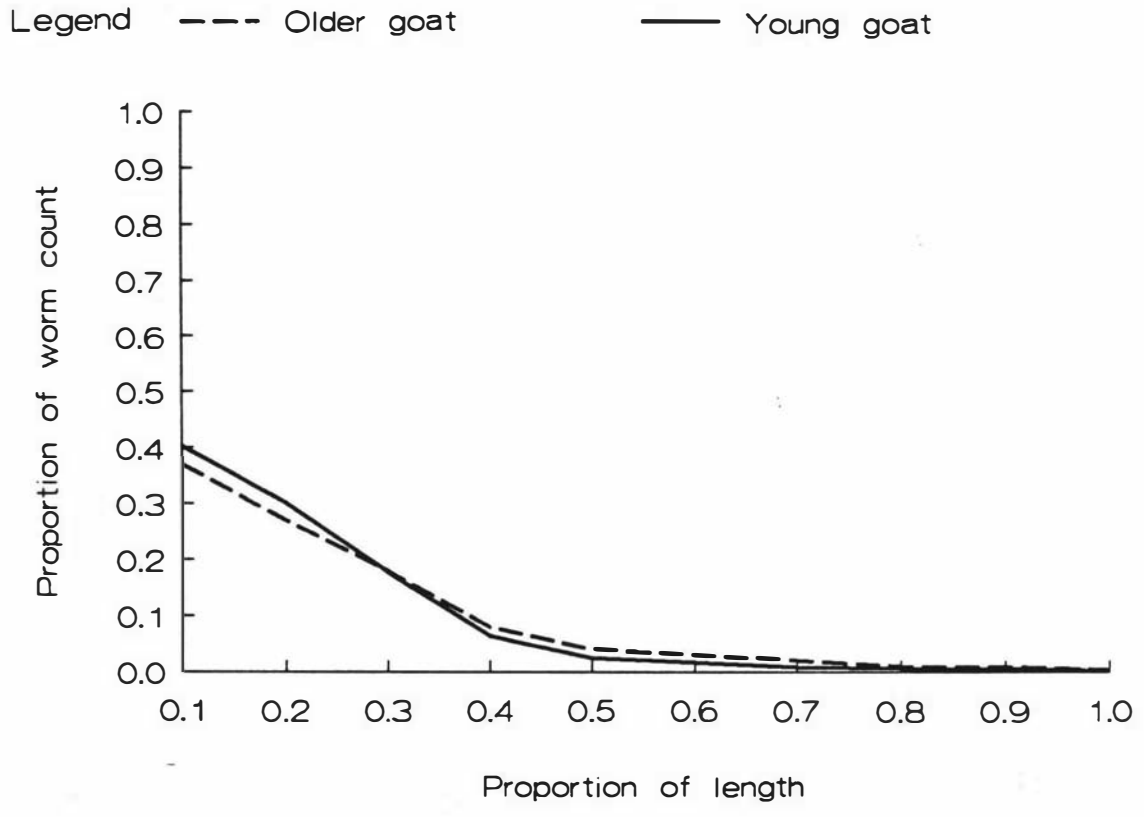


Figure 4.3. The mean distribution of the proportion of worm counts by the proportion of small intestinal length for young goats (n = 10) and older goats (n = 8) in Experiment 4.1.

The distribution of nematodes in both groups of goats is obviously very similar. As some older goats e.g. Goats 14 and 15, had a slight tendency to have more worms further down the small intestine, the lines fitted through the means are slightly different.

The mean proportions of nematodes found per one-tenth proportion of the small intestine for all goats are shown in Appendix 4a. From this it can be seen that at least 94% of all *T. colubriformis* were found in the proximal 50% of the small intestine.

Male to female ratio

Data were only analysed for the first six tenths of the small intestine as there were limited data beyond this, especially in young goats. Parametric analysis was not possible because of the highly significant difference in the variances ($p<0.0001$) as determined by Bartlett's test. These ratios were analysed by a Kruskal-Wallis non-parametric analysis of variance and found to be significant ($p<0.001$). Data for the two age groups were considered together as they appeared to be similar and the increased group size allowed greater statistical power.

Non-parametric multiple comparisons were conducted in a fashion paralleling the Tukey test by using rank sums instead of means (Zar, 1984). These are shown in Table 4.1. There were significant differences between one tenth proportions indicating there was a higher ratio of males:females further down the small intestine.

Length (in tenths)	Mean male:female ratio	Mean rank	Rank order	Sample size
1	.875	38.7^{a}	3	18
2	.852	35.1^{a}	2	18
3	.837	30.4^{a}	1	18
4	1.13	57.9^{ab}	4	18
5	1.49	76.8^{b}	6	16
6	1.55	75.5^{b}	5	12
Total		50.5		100

Table 4.1. Multiple comparison of male:female ratio for all goats (n=18). Mean ranks with different superscripts are significantly different ($p<0.05$)

Eggs per female nematode

Scatter plots for eggs per female nematode and distance are shown in Figures 4.4 and 4.5 for young and older goats respectively. Data were only included for those metre segments where at least 10 female nematodes were available. Comparison of regression lines fitted through this data show they are different ($p<0.001$). In both age groups eggs per female nematode declined with distance from the pylorus ($p<0.001$).

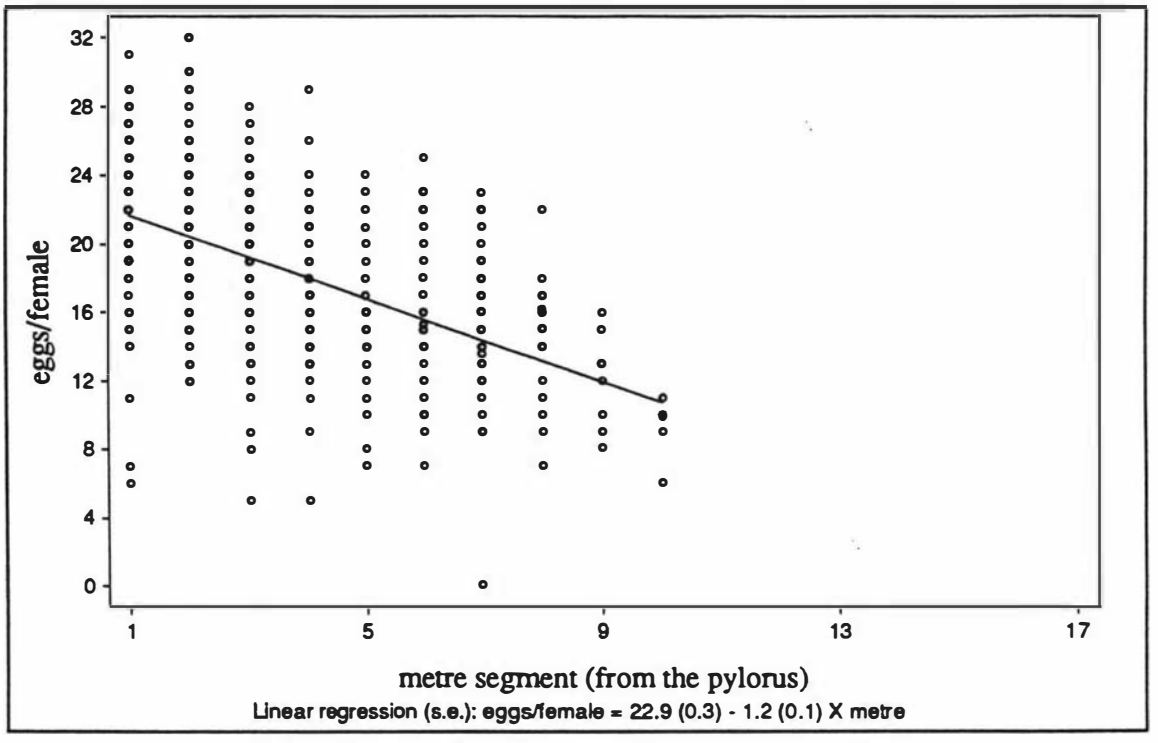


Figure 4.4. Scatter plot of eggs/female by distance down the small intestine for young goats in Experiment 4.1. Each symbol may represent >1 data point.

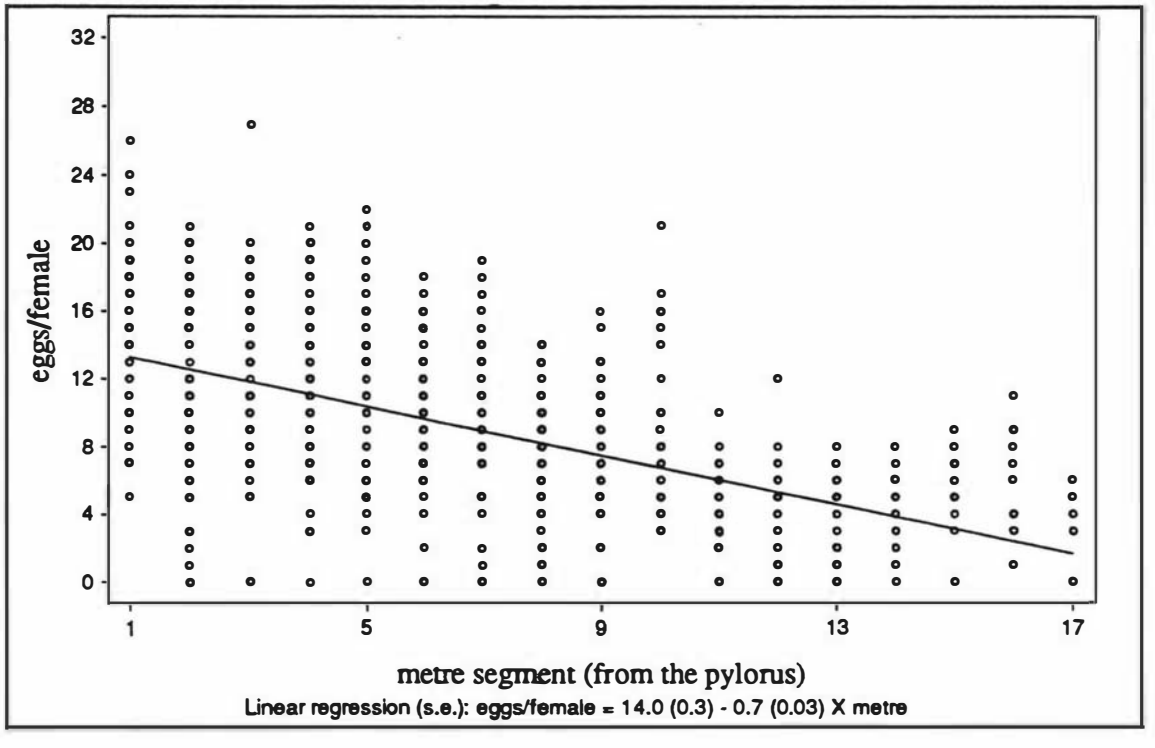


Figure 4.5. Scatter plot of eggs/female by distance down the small intestine for older goats in Experiment 4.1. Each data point may represent >1 data point.

4.4 DISCUSSION

The distribution of *T. colubriformis* reported here is consistent with the findings of Rahman and Collins (1990c) although they expressed their distances from the pylorus in metres and gave no indication of the total lengths of the small intestines. In sheep, both Tetley (1937) and Sommerville (1963) described the distribution of *T. colubriformis* as "normal" implying that few worms were found in that region of the small intestine immediately posterior to the pylorus although examination of data in the former report shows this is a misuse of the term in the strict statistical sense as most nematodes were found immediately posterior to the pylorus. Sommerville (1963) did not present data to support his description of the distribution as "normal". The distribution reported by Barker (1974) in sheep is consistent with the findings reported here and by Rahman and Collins (1990c) which is that the maximum number of *T. colubriformis* was usually found in the anterior metre or one-tenth portion of the small intestine.

The reason for *T. colubriformis* choosing this anterior site is uncertain. Davey (1938) hypothesised that they may lodge in the first position they find suitable. However, Barker (1974) argued that the proportion of larvae which ultimately establish in the first two metres is lower than in succeeding metres where a plateau of establishment of available larvae occurs, and that this lower establishment may be because some larvae had not completely exsheathed before leaving the abomasum. Both these hypotheses assume little movement of adults post-infection even though they may be long-lived. The extent to which adult *T. colubriformis* move in the intestine is not known.

Barker (1974) found there was no influence of crowding on establishment. This is consistent with the lack of variation in distribution reported here given the variation in total worm counts from 12,360 to 25,880.

Counting eggs *in utero* is an established measurement of the fecundity of nematodes. The reduction in eggs per female with increasing distance from the pylorus suggests that location in the anterior small intestine is more suitable. A decrease in fecundity with distance from the region of the small intestine with most worms, was also noted by Sukhdeo (1991) for *T. spiralis* in rats. Being located in their preferred site implies optimal physiological conditions which would allow maximal metabolic activity including egg production. Davey (1938) observed *T. colubriformis* was more tolerant of low pH and bile salts than *Nematodirus spathiger*, *Nematodirus filicollis* and *Cooperia*

curticei which are found further down the small intestine where the pH is neutral and more stable and the concentration of bile salts is lower. Davey (1938) did not, however, establish optimum concentrations for either the intestinal pH or bile salt concentration to indicate why this anterior position was the preferred site for *T. colubriformis*. Sukhdeo and Croll (1981) showed that bile was important for the establishment of *H. polygyrus* in mice and that larvae would preferentially establish in regions to which the bile had been had been redirected surgically.

It has been observed that decreased fecundity of *T. colubriformis* is one effect of an immune response by the host (see Section 1.3). The significant difference in eggs per female nematode between the two different ages of goats suggests some evidence of an immune response in the older animals which could be expected, although the establishment rate was similar in both groups. However, incorporated with the difference in age of goats is the difference in challenge given to the two groups of goats with the resulting variations in total worm burdens. These two groups were also infected at different times with different batches of larvae although they were the same strain. It was not possible to separate these two confounding variables from the available data. However, no density dependent effects on fecundity have been noted for *T. colubriformis* (Dobson *et al.*, 1990a) and it is reasonable to assume the effect seen was due to an immune response in the older goats.

Tetley (1937) and Sommerville (1963) noted one sheep of ten and five of 26 respectively in which the distribution was not "normal" in that worms were unevenly distributed along the small intestine. In the present experiment there is a very consistent distribution for the young goats but two older goats (Goats 14 and 15) were particularly notable in having *T. colubriformis* present along the whole length of their small intestine with the terminal one-tenth having more than one percent of the total worm count. However, even in these two older goats the general pattern of most worms being concentrated in the anterior small intestine was still apparent and no obvious secondary peaks further down occurred. The reason for the erratic distributions noted by Tetley (1937) and Sommerville (1963) was not determined. It has been observed that *T. colubriformis* redistributed into the caudal small intestine of guinea pigs, probably associated with a developing immune response (Connan, 1966). The slight change in distribution noted in these goats may be a manifestation of this same phenomenon as it was only found in the older, presumably more immune goats.

The occurrence of a higher male:female ratio further down the intestine probably reflects a greater adaptibility of male nematodes to the less than optimum conditions encountered further down the intestine.

CHAPTER FIVE

A STUDY ON THE HOST RESPONSE OF FIELD-REARED GOATS TO INFECTION WITH *TRICHOSTRONGYLUS COLUBRIFORMIS* AND REPEATABILITY OF FAECAL EGG COUNTS OVER SUCCESSIVE INFECTIONS (EXPERIMENT 5.1)

5.1 INTRODUCTION

This experiment provides information on the response of adult field-reared Angora goats to *T. colubriformis*. It was also used to assess the repeatability of faecal egg counts over successive infections and hence determine how useful the egg count of one infection would be for estimating the worm count, or at least the ranking of worm counts in a group, in a subsequent infection. A problem with assessing LMI of intestinal mucus and relating that to worm counts is the difficulty in estimating the numbers of worms removed with these mucus samples. This is particularly so when goats are killed within the prepatent period as in Experiment 7.1.

5.2 MATERIALS AND METHODS

Experimental schedule

Twenty-nine 18 month to 2 year-old 7/8th Angora X 1/8th New Zealand feral wether goats were purchased from a commercial farm. On arrival they were treated with >300 µg/kg ivermectin to remove existing worm burdens. They were housed in concrete pens with straw bedding and fed hay *ad lib.*.

All goats were infected with 35,000 infective larvae on three occasions. The first two infections were terminated after 21 and 28 days respectively with a treatment of 300 µg/kg ivermectin. All goats were killed 28 days after the third infection with an overdose of pentobarbitone. A three week period was allowed between successive infections. Goats were housed for almost two months before the first infection.

The same isolate of *T. colubriformis* was used as in Experiment 3.1. Larvae were cultured separately for each infection, stored at 10°C and were less than one month-old at the time of use. As before, motility was used as a measure of viability and assessed as ≥ 95% in all infections.

Parasitology

Faecal egg counts were estimated weekly using the technique described in Appendix 2b.

At slaughter, the small intestine was removed and separated from the mesentery with scissors. 1-2 cm segments of small intestine were removed for histological examination at the same locations as in Experiment 3.1. The histological results will be reported in Chapter 7.

The remainder of the small intestine was opened and washed thoroughly. The tissue was then frozen until it was later digested in pepsin:HCl for two hours as described in Appendix 2c. One-tenth of the washings and digest was removed for counting and sieved separately using the procedure described for Experiment 2.2. Larvae were, therefore, estimated from 1% aliquots and adults from 10% aliquots. Counting washings and digests separately allowed an assessment of the proportion of the nematode burden recovered by each procedure. Adults were counted separately by sex.

The number of eggs per female nematode was estimated in 50 female nematodes randomly selected from each goat.

The abomasums were also removed and washed thoroughly with a 10% aliquot being sieved through a 250 μm mesh and all nematodes recovered being counted.

Statistical analysis

The rankings of the three sets of egg counts were compared by Spearman rank correlations. Faecal egg counts were compared by analysis of variance of square root transformed data ($x + 0.5$). This transformation was necessary to normalise the distribution of counts. These analyses were carried out using Statistix version 4.0. The Kendall coefficient of concordance, which allows comparison of more than two ranks was calculated as described by Zar (1984).

5.3 RESULTS

Raw data on worm counts and faecal egg counts are shown in Appendix 5a. Spearman rank correlations for the egg counts of the three infections were determined and are shown in Table 5.1. As egg counts for the first infection

are only available up to Day 21, these final egg counts are correlated with both Day 21 and Day 28 counts for the second and third infections.

	Infection 1 - Day 21	Infection 2 - Day 21	Infection 2 - Day 28	Infection 3 - Day 21
Infection 2 - Day 21	0.45*			
Infection 2 - Day 28	0.71***	0.61***		
Infection 3 - Day 21	0.49**	0.66***	0.75***	
Infection 3 - Day 28	0.59**	0.68***	0.74***	0.78***

Table 5.1. Spearman rank correlations between egg counts for 3 successive infections. (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

The Kendall coefficient of concordance (W) for final egg counts ($W=0.79$; $df=28$; $p<0.001$) for each infection showed there was a very strong association between them but the same analysis of 21 day egg counts showed they were not significantly associated ($W=0.13$; $df=28$; $p>0.05$).

Arithmetic mean egg counts (± standard errors) and statistical significance of comparisons of square root transformed counts are shown in Table 5.2.

Worm counts, eggs per female, egg counts, male:female ratios and the ratio of worm recovery between digests and washings are shown in Appendix 5a. The arithmetic mean worm count (± standard error) was 13,650 (±1037) with a range from 1620 to 24,710. This represents a mean establishment of 39% (range 0.05% to 71.5%). In all animals, substantial numbers of nematodes were recovered in the digests, in many cases more than found in the washings.

	N	Mean (eggs/g)	standard error
1st infection			
- Day 21	29	3357^{ag}	330
2nd infection			
- Day 21	29	1485^{b}	261
- Day 28	29	3826^{a}	479
3rd infection			
- Day 21	29	1272^{b}	237
- Day 28	29	2560^{a}	344

Table 5.2. Arithmetic mean egg counts for the three infections. Means which share the same superscript are not significantly different ($p>0.05$) when square root transformed data were compared. Data were not compared within an infection.

Correlations between terminal parasitological parameters are shown in Table 5.3.

	Worm count	Male:female ratio	Eggs/female
Male:female ratio	0.46^{*}		
Eggs/female	0.46^{*}	0.46^{*}	
1st infection - FEC Day 21	0.37^{*}	0.41^{*}	0.44^{*}
2nd infection - FEC Day 21	0.30	0.35	0.66^{**}
2nd infection - FEC Day 28	0.41^{*}	0.49^{**}	0.72^{***}
3rd infection - FEC Day 21	0.49^{**}	0.58^{**}	0.78^{***}
3rd infection - FEC Day 28	0.56^{**}	0.53^{**}	0.84^{***}

Table 5.3 Correlations between parasitological parameters following the third infection (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; FEC = faecal egg count).

5.4 DISCUSSION

The absence of Day 28 egg counts for the first infection is unfortunate but, interestingly, the mean egg count at Day 21 is similar in magnitude to the Day 28 counts for the next two infections and significantly higher than their 21 day counts. This suggests that there was greater susceptibility to the first infection and establishment at that time may have been greater, fecundity higher, patency achieved sooner, or some combination of these. Indeed, the 21 day counts in the first infection were remarkably high given that the goats were field-reared and at least 18 months old, and so might have been expected to have acquired a substantial level of resistance. Their apparently high levels of susceptibility suggests they had not done so or that the two month period between housing and first infection was sufficient for some relaxation of resistance to occur as seen with resistance to *H. contortus* in sheep after about nine weeks (see Section 1.2.5). However, immune sheep have been shown to maintain their solid resistance to *T. colubriformis* over a worm-free period of 24 weeks before being rechallenged (Kimambo *et al.*, 1988b) so, by analogy, it could be expected that goats would be able to maintain their resistance for at least two months. Whatever the explanation, as shown in Experiment 3.1 (Group 2), a reasonable degree of resistance would be expected to develop following a single infection of *T. colubriformis* which could explain the lower faecal egg counts in the second and third infections.

In young sheep, significant rank correlations of egg counts for successive infections with *T. colubriformis* or *N. spathiger* have been reported (Dineen *et al.*, 1965; Windon *et al.*, 1980; Douch *et al.*, 1984) but the information from goats is more limited. In young goats up to two years of age the correlations of egg counts one week apart was very high, whereas a poor correlation was observed between egg counts six months apart (Vlassoff, 1992). No explanation was offered but it is possible that extraneous factors such as nutrition etc., may have affected their degree of host resistance or that different species of nematodes were dominant at different times.

Although there was strong concordance between final egg counts of the three infections and a reasonably high correlation of final egg counts with worm counts, these relationships do not seem precise enough to allow egg counts from a previous infection to be used as a predictor of worm burden in a subsequent infection where associations between worm burden (which may be difficult to assess accurately) and other parameters such as LMI are of interest.

It is clear that washing an opened small intestine under running water whilst massaging manually with fingers is not very effective in removing *T. colubriformis* from a very recently killed goat as, on average, only about 50% of the nematodes were removed - the remainder were presumably sufficiently anchored between epithelial cells to resist dislodgement. Others have reported a poor recovery of *T. colubriformis* from sheep by only washing (Coop *et al.*, 1976) although Barker (1974) found no advantage in digesting over just flushing and kneading the intestine. Routine diagnostic worm counts are usually performed on intestines from animals which have died or, if killed for the purpose, are not processed for some time after death, thus allowing some autolysis of the epithelium to occur and a much higher recovery to be achieved.

The mean establishment in this experiment of 39% is higher than that reported for the control goats in Experiment 3.1. However, as the intestine was not digested in the latter, it may be assumed that the recovery from the control goats in that experiment represents only about half of those actually present. The real establishment in those control goats was probably about 50%. Although the mean establishment of 39% in the present experiment is, therefore, probably slightly lower, it still suggests that few goats had developed the degree of resistance seen in Experiment 3.1 or which would be expected in sheep of a similar age and exposure to *T. colubriformis* (Dobson *et al.*, 1990a, 1990d). The range in establishment of the third infection from 0.05% to 71.5% does indicate that some goats had developed solid host resistance whilst others showed no evidence of any, despite their similar histories and exposure to experimental infection. There may have been some effect of nutrition as the goats in the present experiment were only fed hay whilst those in Experiment 3.1 were on a concentrate diet, but the most likely explanation is the difference in breed of goat.

The correlations between eggs/female, egg counts, male:female ratio and worm counts are all consistent with the more resistant animals having smaller worm burdens, relatively fewer males and decreased fecundity per female worm. This follows the pattern for the development of resistance previously outlined for *T. colubriformis* in sheep (see Section 1.3) and shown in Experiment 3.1. The correlation between terminal egg count and worm count ($r=0.56$) is lower than that in Experiment 3.1. This may be due to the difference in experimental design or may be analogous to the poorer correlations seen in older than in younger sheep (McKenna 1981). Third stage larvae were detected only in three goats and in two of these L4s were also found. This indicates that very few larvae were inhibited and remained so until slaughter after 28 days. As in

sheep, the proportion of inhibiting larvae would be expected to increase as overall establishment decreased (Dobson *et al.*, 1990b), but there is no indication that this occurred in these goats. Immunity of the host has been considered to be the main cause of *Trichostrongylus* sp. inhibiting in sheep (Eysker, 1978; Seaton *et al.*, 1989; Dobson *et al.*, 1990b).

The *T. colubriformis* burden in the abomasum was approximately 0.6% of the total which is consistent with that reported for sheep (Barker, 1974) but less than the mean figure of 2.2% (range 0 - 7.8%) previously reported for goats (Rahman and Collins, 1990c). However, for practical purposes, the abomasal burden of *T. colubriformis* was small and was ignored in subsequent experiments.

CHAPTER SIX

THE USE OF A MONOCLONAL ANTIBODY AND TRADITIONAL STAINING METHODS IN COUNTING MAST CELLS AND EOSINOPHILS IN INTESTINAL MUCOSA (EXPERIMENT 6.1)

6.1 INTRODUCTION

The purpose of this experiment was to establish the usefulness of a monoclonal antibody for detecting mast cells, including GLs, and eosinophils in goat intestinal tissue. This monoclonal antibody has been shown to detect a component of the granule wall of some cells of the myeloid line of sheep including mast cells and eosinophils (Shaw *et al.*, 1994). The problems of staining mast cells have been discussed in Section 1.6.3. In recent times, immunocytochemistry has been used to identify MMCs, often with better success than with traditional fixative/stain combinations (Walls *et al.*, 1990)(Miller unpublished, cited by Rothwell 1989).

In this experiment two fixation procedures and three staining methods were compared.

6.2 MATERIALS AND METHODS

Goats

Four goats were used. Goats 1 to 3 were adult Angora females which had been reared under conventional pasture conditions. Goat 4 was an Angora X New Zealand feral wether which had been used to cycle *T. colubriformis* continuously for two years. It had been kept in a pen with *ad lib.* hay during this time.

Fixation

All goats were killed with an overdose of pentobarbitone. Their small intestines were removed immediately after death. One set of tissues was taken approximately 1 to 2 metres from the pylorus and a second approximately 1 to 2 metres distal to this. At each site, two approximately 1.5 cm blocks of intestine were removed. Each was opened along its mesenteric border and stapled to a piece of card which was then floated tissue side down in fixative. One block was fixed in 10% neutral buffered formalin and the other in an iso-

osmotic solution of 0.6% formalin + 0.5% acetic acid (IFAA)(Enerback, 1966). Four to 12 hours later the tissues fixed in IFAA were transferred to 70% alcohol as described by Enerback *et al.* (1986).

After approximately 24 hours, tissues were removed from the centre of both blocks and routinely embedded in paraffin.

Staining

Sections were cut at 3 μm from blocks orientated to give a transverse section of the intestine.

Formalin-fixed tissue

One section from each formalin-fixed tissue block was stained with Gill's haematoxylin and eosin (formalin/HE) - see Appendix 6a for methodology.

A second section (formalin/MAB) was probed with a murine monoclonal antibody (MABC8) raised against sheep mast cells which was kindly provided by Dr P. Douch. Staining was achieved by probing the tissue with a 1:10 dilution of the supernatant from the mouse hybridoma cell culture, detecting the attached antibody with horse-radish peroxidase-labelled rabbit antimouse polyclonal antibody and then developing the subsequent reaction with peroxide using diaminobenzidine (DAB) which gives a brown colouration. This was followed by counterstaining with Weigert's haematoxylin combined with Biebrich's scarlet as described in Luna's method for erythrocytes and eosinophil granules (Luna, 1968). Precise methodology is given in Appendix 6c.

A third section was stained with toluidine blue (formalin/TB) - see Appendix 6b for methodology.

IFAA-fixed tissue

One section was stained with toluidine blue (IFAA/TB).

A second section was probed with the monoclonal antibody (IFAA/MAB), except that the MABC8 was used at a dilution of 1:160 and 1:200 to avoid overstaining. In addition, after developing with DAB the sections were immersed in 3% $K_2Cr_2O_7$ at 60°C for 15 minutes before counterstaining

with Luna's method. The $K_2Cr_2O_7$ acts as a mordant (Drury and Wallington, 1967) with eosinophil granules to assist uptake of Biebrich's scarlet.

Cell definitions

The cells of interest were MMCs, GLs and eosinophils. Each technique allowed identification of different features of some of these cells. They were identified using the following criteria.

Toluidine blue (see Figures 6.1 and 6.2)

Cells were considered to be MMCs and counted if they comprised a substantial collection of dense blue-violet metachromatically stained granules. These were very obvious against the pale blue, lightly stained background. Not all MMCs counted had a nucleus visible. GLs were identified in IFAA/TB sections by their intraepithelial location and by the presence of a small number of intensely stained blue-violet granules within an otherwise unstained cytoplasm. Eosinophils were not identifiable with this stain. In formalin/TB sections GLs did not stain and were not counted.

Mast cell monoclonal antibody counterstained with Luna's method

A. Formalin fixation (see Figure 6.3)

The MMCs appeared brown and granular whilst GL granules which were large and unevenly sized, had a dull reddish-brown colouration due to the combination of DAB and Biebrich's scarlet. Eosinophils were identified by the presence of small even-sized granules with a similar dull reddish-brown colouration. The presence of a light blue polymorphic nucleus was an additional feature that usually assisted differentiation of eosinophils from small MMCs.

B. IFAA fixation (see Figure 6.4)

The MMCs had a less granular appearance than in formalin-fixed tissue. GL granules did not stain with Biebrich's scarlet and the cells were identified by the presence of large DAB-stained granules and their intraepithelial location.

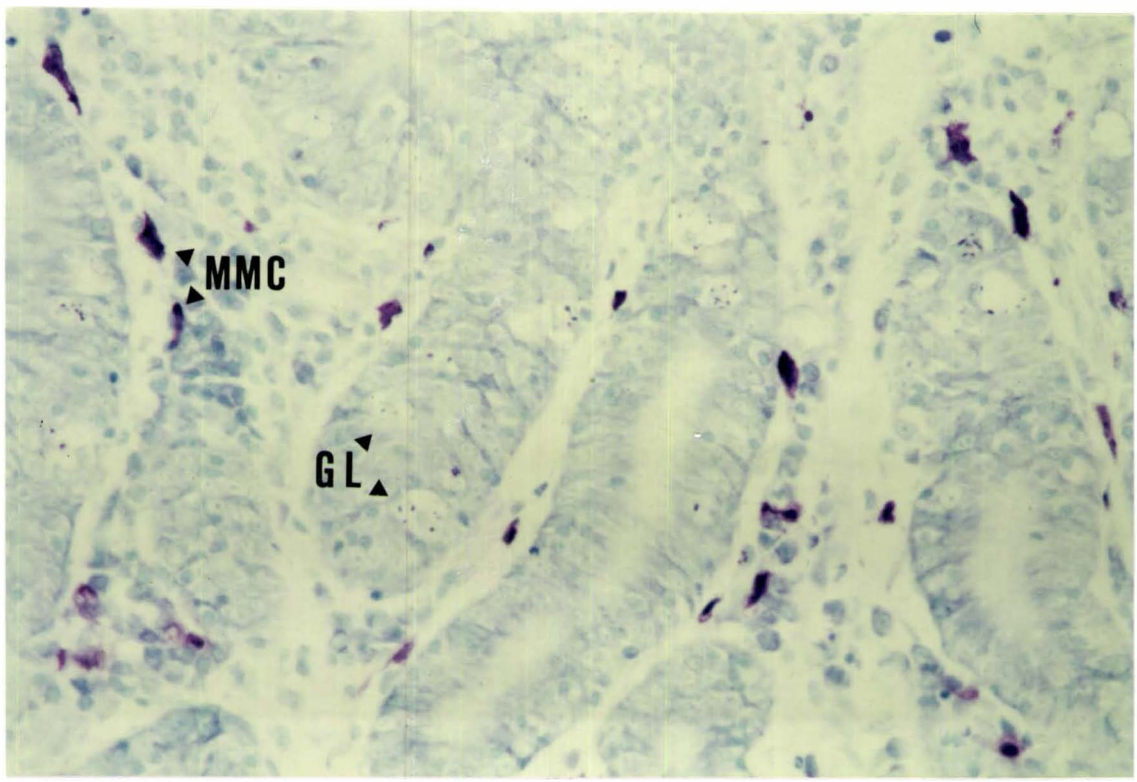


Figure 6.1. Tissue fixed in IFAA and stained with toluidine blue (430 x magnification). MMC = mucosal mast cell; GL = globule leukocyte.

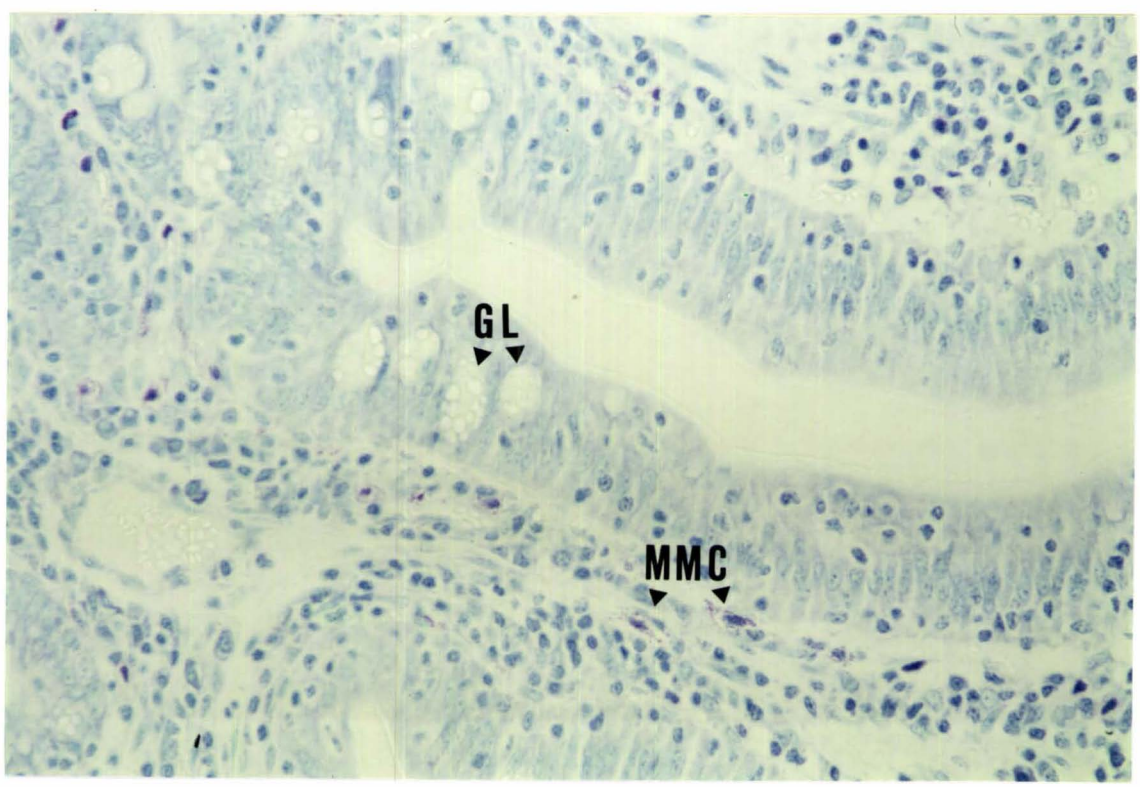


Figure 6.2. Tissue fixed in formalin and stained with toluidine blue (430 x magnification). MMC = mucosal mast cell; GL = globule leukocyte.

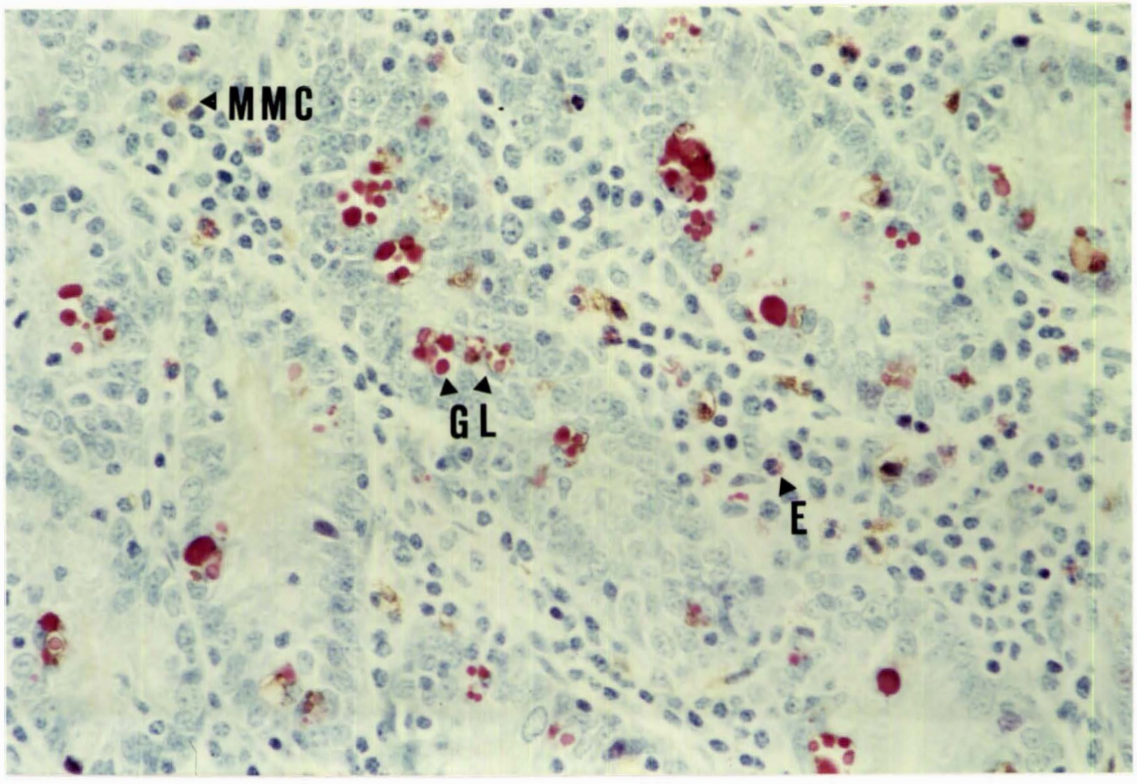


Figure 6.3. Tissue fixed in formalin, probed with the monoclonal MABC8, stained with diaminobenzidine and counterstained with Weigert's haematoxylin and Biebrich's scarlet (430 x magnification). MMC = mucosal mast cell; GL = globule leukocyte; E = eosinophil.

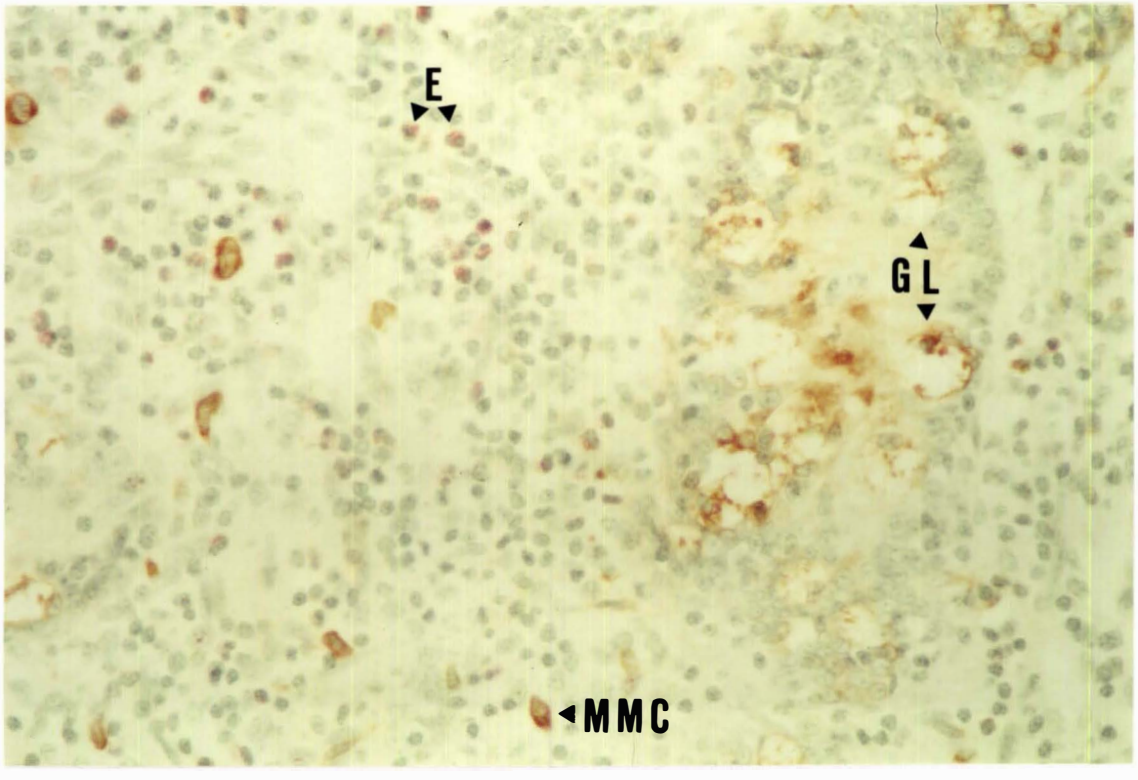


Figure 6.4. Tissue fixed in IFAA, probed with the monoclonal MABC8, stained with diaminobenzidine and counterstained with Weigert's haematoxylin and Biebrich's scarlet (430 x magnification). MMC = mucosal mast cell; GL = globule leukocyte; E = eosinophil.

Fluorescence microscopy with haematoxylin and eosin (see Figures 6.5 and 6.6)

Cells were considered to be GLs and were counted if they had a least three large fluorescent granules of uneven size. Eosinophils and MMCs could not be identified with this technique. Although eosinophil granules did fluoresce, they did so poorly and were often difficult to see and were not counted.

Counting

All cells were counted at a magnification of 500 X using a graticule eyepiece which covered an area of 0.408 mm^2.

Cells in 20 fields were included in each count and five counts were made per tissue section. The search pattern adopted was a battlement type and moved progressively and systematically across the section. Only cells in the mucosa were counted. The exception to this counting routine was the formalin/TB section where only one count of 20 fields per section was made and only MMCs were counted.

Haematoxylin and eosin (H&E) stained sections were examined under incident fluorescence (Olympus BH2-RFL) using a wide band pass 490 nm wavelength exciter filter which combined with the use of 515 nm and 530 nm barrier filters, caused the eosin-stained granules in globule leukocytes to fluoresce bright yellow. The same magnification and graticule eyepiece were used as before.

Statistics

Cell counts were compared by analysis of variance (SAS version 6.04) with the respective cell counts as the independent variable analysed against the two class variables, tissue block and fixative/stain combination. Correlations were also analysed (Statistix version 4.0).

6.3 RESULTS

The cell counts, correlation coefficients and statistical analyses for the various fixatives and staining techniques, except formalin/TB, are summarised in Tables 6.1 and 6.2 respectively.

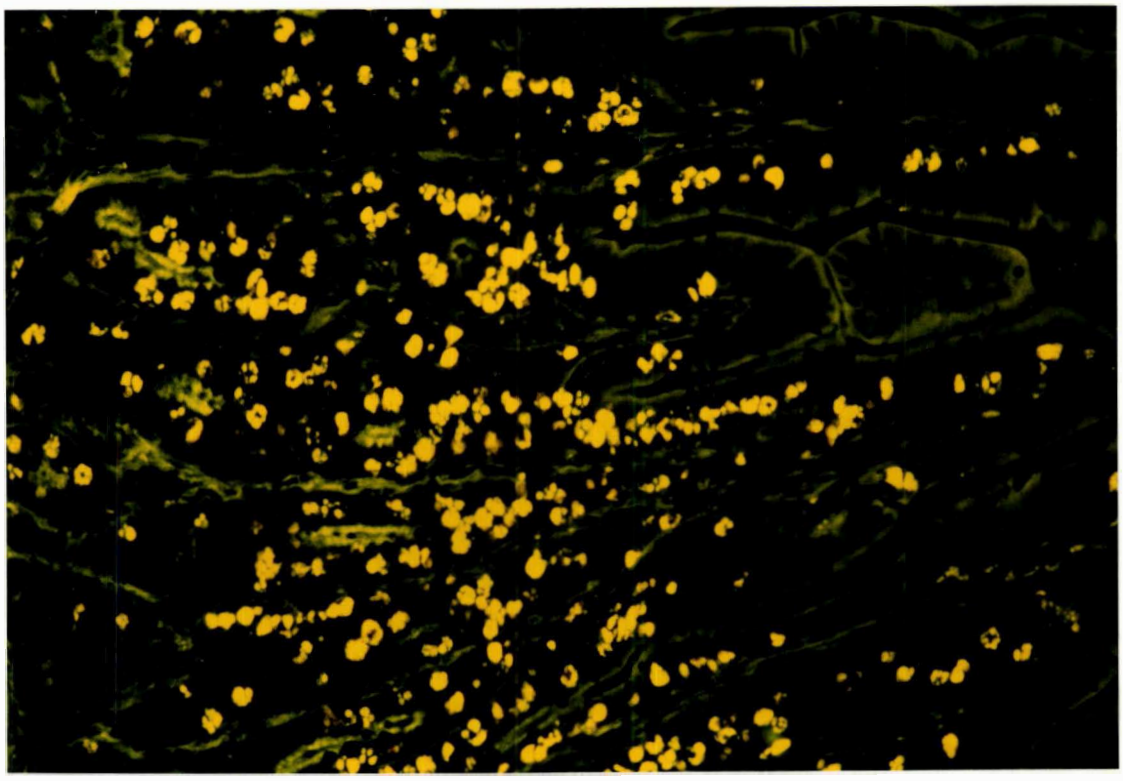


Figure 6.5. Tissue fixed in formalin, stained with haematoxylin and eosin and observed with fluorescence microscopy (215 x magnification).

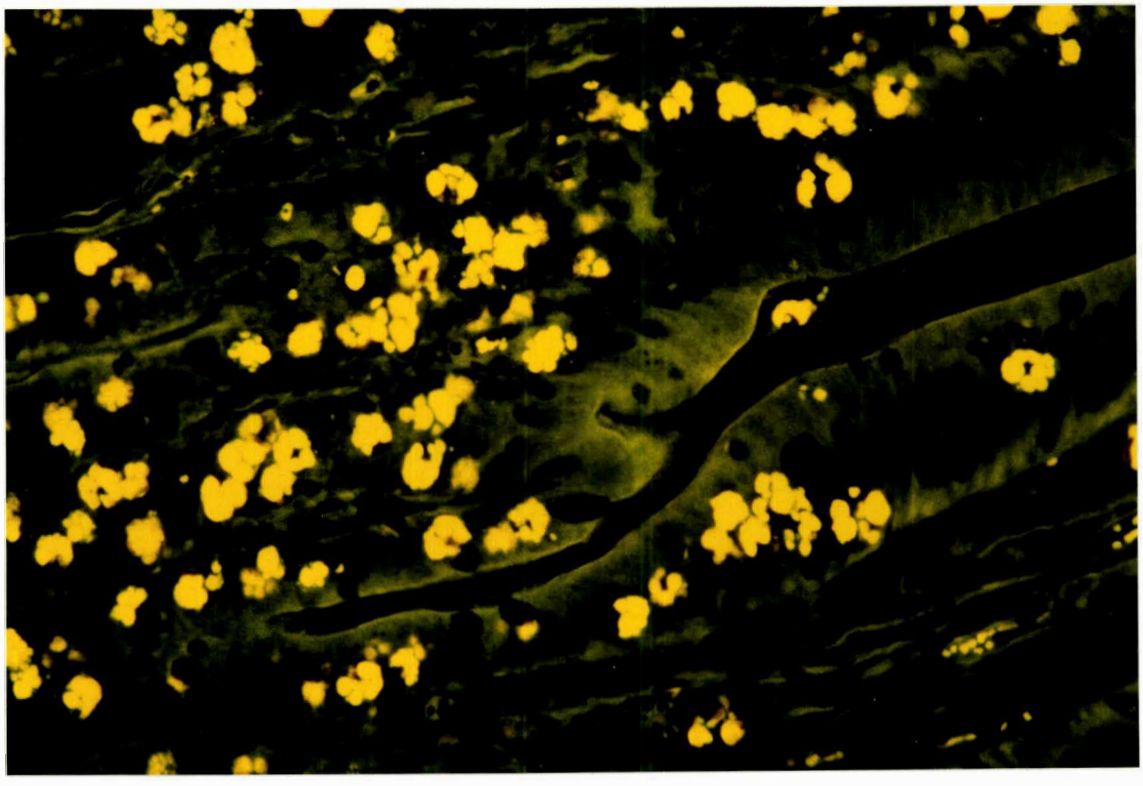


Figure 6.6. Tissue fixed in formalin, stained with haematoxylin and eosin and observed with fluorescence microscopy (430 x magnification).

Goat No.	Tissue block	Mucosal mast cells			Globule leukocytes				Eosinophils	
		formalin	IFAA[1]		formalin		IFAA		formalin	IFAA
		mab + Luna's[2]	mab + Luna's	tol. blue[3]	mab + Luna's	haem. & eosin[4]	mab + Luna's	tol. blue	mab + Luna's	mab + Luna's
1	1	108.6 (12.7%)[5]	164.2 (8.4%)	133.6 (12.5%)	45.8 (58.0%)	44.8 (37.2%)	36.8 (68.6%)	25.5 (50.4%)	425.2 (31.5%)	356.1 (21.2%)
	2	164.4 (13.9%)	209.5 (13.4%)	194.8 (13.8%)	100.5 (39.5%)	85.0 (21.7%)	107.1 (18.9%)	95.6 (25.0%)	729.6 (19.4%)	472.0 (19.4%)
2	1	228.9 (10.4%)	270.6 (11.7%)	251.9 (15.9%)	55.4 (42.3%)	72.5 (28.7%)	67.6 (24.0%)	34.8 (58.0%)	159.3 (26.8%)	107.1 (18.2%)
	2	203.7 (15.1%)	266.9 (11.5%)	281.8 (12.5%)	108.1 (24.5%)	113.0 (7.9%)	100.0 (33.3%)	54.7 (33.0%)	175.5 (23.8%)	131.6 (20.9%)
3	1	152.7 (12.0%)	207.6 (13.8%)	200.0 (16.1%)	233.8 (34.7%)	208.3 (24.8%)	75.2 (34.7%)	75.3 (25.8%)	121.6 (43.2%)	80.7 (54.9%)
	2	152.7 (18.8%)	232.1 (13.5%)	172.5 (15.4%)	146.1 (7.0%)	137.5 (15.6%)	129.2 (13.4%)	101.7 (41.4%)	58.8 (26.5%)	101.2 (38.9%)
4	1	215.7 (14.5%)	226.0 (10.7%)	319.8 (10.2%)	233.3 (15.8%)	199.0 (14.3%)	219.6 (11.4%)	132.1 (8.3%)	180.6 (27.3%)	148.5 (26.3%)
	2	237.5 (6.3%)	233.3 (11.0%)	275.7 (10.0%)	235.3 (9.8%)	228.7 (24.7%)	188.2 (18.6%)	152.7 (12.9%)	465.9 (19.8%)	338.9 (37.3%)
Ranking in decreasing order within cell types[6]		3[b]	2[b]	1[a]	1[b]	2[b]	3[1]	4[1]	1[a]	2[1]
mean CV[7]		13.0	11.8	13.3	29.0	21.9	27.9	31.9	27.3	29.6

Key: 1 Iso-osmotic formaldehyde (0.6%) and acetic acid
2 Tissue initially probed with sheep mast cell monoclonal antibody, detected with diaminobenzidene and counterstained with Luna's method
3 Toluidine blue
4 Haematoxylin and eosin
5 Mean cell count per mm^2 (coefficent of variation)
6 Those columns within cell types sharing the same letter are not significantly different ($p<0.05$)
7 Mean coefficent of variation %

Table 6.1. Comparison of mean cell counts (n=5) per mm^2 for different fixative/stain combinations

	Mucosal mast cells		Globule leukocytes			Eosinophils
	formalin/ MAB	IFAA/ MAB	formalin/ MAB	IFAA/ MAB	IFAA/ TB	formalin/MAB
MMCs						
IFAA/MAB	0.79**					
IFAA/TB	0.90**	0.67*				
GLs						
IFAA/MAB			0.73*			
IFAA/TB			0.81**	0.92**		
formalin/ HE			0.99**	0.72*	0.80**	
Eosinophils						
IFAA/MAB						0.98**

Table 6.2. Correlation coefficients between mean cell counts using different fixative/stain combinations (n = 8; * = $p<0.05$; ** = $p<0.01$)

Mucosal mast cells

In the mucosa, MMCs were found only within the lamina propria. They appeared to vary in size depending on their location with those near the base of the mucosa being larger and generally staining more intensely than MMCs in the villi. This varying intensity of staining was similar for formalin/MAB, IFAA/MAB and IFAA/TB. After formalin fixation, both granules and cell wall were distinctly stained. With IFAA fixation, the staining of individual cells was more diffuse and there was often some faint background staining. Nevertheless significantly ($p<0.05$) more MMCs were counted in IFAA-fixed sections. However, the CVs were similar for all procedures (see Table 6.1) indicating that the techniques were of similar repeatability.

MMC counts in formalin/TB sections averaged only 23% (range 9-44%) of those in IFAA/TB sections and the cells seen were often unevenly and palely stained.

A small number of small cells with 3 to 5 metachromatically stained granules in a pale blue cytoplasm, were also observed in the lamina propria of

some IFAA/TB sections. Their identity is unknown but they were distinctly different from MMCs and GLs, and were not counted.

Globule leukocytes

In all tissue sections examined, GLs were nearly always located in the epithelium which assisted with identifying them, particularly with both staining combinations of IFAA-fixed tissues. GLs in formalin/TB sections did not stain and could not be recognised with certainty and so were not counted.

GL counts in formalin/MAB sections were significantly higher than with both staining techniques on IFAA-fixed tissue. For all fixative/stain combinations, the CVs were similar but generally larger than for MMCs.

As they were unstained apart from a few small granules, GLs in IFAA/TB sections were very difficult to identify if obscured by overlying cells.

In IFAA/MAB sections, because of the failure of GL granules to take up Biebrich's scarlet there were difficulties in separating MMCs from GLs, particularly for cells located at or about the basement membrane of the epithelium and where GLs were not located in the epithelium.

Eosinophils

Eosinophils were unevenly distributed throughout the mucosa and were often present in clusters within the lamina propria. They were never seen in the epithelium.

Formalin/MAB resulted in significantly ($p<0.05$) higher counts than IFAA/MAB.

If sections were stained as for GLs in formalin/MAB, eosinophils were well-stained. If overstained with DAB they could be confused with MMCs. With IFAA-fixed tissues they stained less satisfactorly with Biebrich's scarlet even with the use of a mordant.

Basophils

Occasionally, cells with polymorphic nuclei and granules that stained with DAB but not Biebrich's scarlet were observed in the lamina propria. Their

morphology was consistent with that of basophils. MABC8 is known to be also able to stain neutrophils and basophils but this staining appears to be fixation dependent, at least for the former (Shaw *et al.*, 1994). Lung tissues from a goat with a massive infiltration of neutrophils were fixed and stained as for the formalin/MAB procedure. There was no uptake of MAB by the neutrophils indicating that the polymorphonuclear cells observed in the intestine were probably basophils. There were too few of them to make counts and meaningful comparisons.

6.4 DISCUSSION

The morphology of mast cells staining with MABC8 was very similar to that of cells staining with toluidine blue in IFAA-fixed tissue. The location, variation in size, and intensity of staining were also very similar. This, combined with the significant correlations between cell counts using the various techniques (see Table 6.2), indicates that MABC8 was detecting a similar component of MMCs, GLs and eosinophils in goats as in sheep. These findings would suggest a substantial, but perhaps not absolute, genetic conservation of this component between these two species. Thus, MABC8 can be usefully used to identify these cell types in goats. However, it only appears to be able to detect MMCs with cytoplasmic granules. Recently derived MMCs or cells which have degranulated and are in the early stages of reforming granules would be difficult to detect.

In formalin/MAB stained sections, the distinction between MMCs and GLs was clear-cut as the GL granules stained a dull reddish-brown. However, overstaining with DAB inhibited the uptake of Biebrich's scarlet. This emphasises the necessity to ensure sufficient DAB uptake to allow MMCs to be identified, particularly those paler-staining smaller cells in the villi, whilst avoiding overstaining. The combination of MABC8 dilution and DAB staining duration as detailed in Appendix 6c, produced reliable, repeatable results.

As might be predicted from other reports (see Section 1.6.3), the majority of mast cells in the mucosa did not stain with toluidine blue after formalin fixation. However, the distinction between MMCs that are sensitive to formalin fixation and those that are not is not clearcut. Many mast cells that were counted with formalin/TB were only faintly and irregularly stained compared to MMCs in IFAA/TB tissues, suggesting that toluidine blue uptake in the formalin fixed-tissue, even in cells that did stain, was partly inhibited.

As can be seen from the numbers of MMCs and GLs counted, there is a reverse ranking of superiority of fixative/stain combinations (see Table 6.1). This is not likely to be due to misclassification as the two cell types are essentially found in different locations, even though a few cells, particularly around the basement membrane in gland crypts cut tangentially, could be confused, especially with the IFAA/MAB combination where GL granules do not stain with Biebrich's scarlet. Because of the clarity of staining and ease of counting, formalin/MAB was the superior combination of those tested for counting all three cell types simultaneously.

It was apparent that IFAA resulted in better preservation of epitopes for the monoclonal antibody as it was necessary to use a much lower dilution (1:160 to 1:200).

Transitional cells between MMCs and GLs were rarely observed in any combination. Some of the transitional cells noted by Huntley *et al.* (1984) had only one or two granules with a different staining pattern, with the remainder being either typical MMC or GL granules. Such cells would be difficult to detect in tissue sections. In formalin/MAB sections, the uptake of Biebrich's scarlet by GL granules was too variable to be sure if some granules were not staining. Douch *et al.* (1986) promoted the use of fluoresence of granules to count GLs in formalin/HE tissues. These authors considered that cells containing even one fluorescent granule were "immature" GLs which would presumably equate to transitional cells. However, the use of this criterion in goats would result in a large number of other cell types, such as some lymphocytes or eosinophils with eosinophilic cytoplasmic granules which also fluoresce, being included in the GL count.

CHAPTER SEVEN

THE DYNAMICS OF LARVAL MIGRATION INHIBITORY ACTIVITY, MAST CELLS AND EOSINOPHIL COUNTS IN GOATS INFECTED WITH *TRICHOSTRONGYLUS COLUBRIFORMIS*

7.1 INTRODUCTION

The objectives of the experiments in this chapter were to investigate the dynamics of larval migration inhibitory activity of abomasal and intestinal mucus, as well as mast cell and eosinophil counts in goats infected with, or recovering from infections with *T. colubriformis*. This information was then integrated with that obtained from histological studies of goats used in experiments previously described in Chapters 3 and 5. In addition, information on the development of *T. colubriformis* in goats was obtained.

7.2 MATERIALS AND METHODS

7.2.1 Experiment 7.1: Comparison of LMI, mast cell and eosinophil counts at intervals during and after infection with *T. colubriformis*

Experimental Schedule

The experiment was divided into two parts. Part A was designed to examine the dynamics of eosinophil and mast cell numbers in the mucosa, and antiparasite activity of intestinal mucus in animals following termination of a *T. colubriformis* infection. Part B was designed to investigate changes in these same parameters in goats which were subsequently reinfected with *T. colubriformis*. The schedules for infection and slaughter are shown in Table 7.1.

Two groups of 1 year-old goats were purchased from separate commercial sources. One group of twelve 1/2 Angora X 1/2 New Zealand feral wethers was used for Part A and a second group of twenty four 3/4 Angora X 1/4 New Zealand feral wethers was used for Part B. All goats had been grazed continously throughout life. They were treated with ivermectin (>0.2 mg/Kg) on arrival and then housed in pens and fed a diet of meadow hay *ad lib*.

Day	Part A	Part B
0	all goats infected with 35,000 L3	all goats infected with 35,000 L3
28	3 goats killed (Group A1); remainder treated with ivermectin (0.2mg/kg)	all goats treated with ivermectin (0.2mg/kg)
35	3 goats killed (Group A2)	
42	3 goats killed (Group A3)	
49	3 goats killed (Group A4)	
58 (day 0, 2nd infection)		3 goats killed (Group B1.1); 15 infected with 35,000 L3 and 6 left uninfected
67 (day 9, 2nd infection)		5 infected (Group B2) and 2 uninfected goats (Group B1.2) killed
76 (day 18, 2nd infection)		5 infected (Group B3) and 2 uninfected goats (Group B1.3) killed
85 (day 27, 2nd infection)		5 infected (Group B4) and 2 uninfected goats (Group B1.4) killed

Table 7.1 Experimental schedule for Experiment 7.1

For Part A, the 12 goats were randomly allocated to four groups of three (Groups A1, A2, A3, A4). For Part B, goats were restrictively randomised into experimental groups on the basis of faecal egg counts 28 days after the first infection (see Table 7.1).

Post-mortem procedure and selection of blocks for histology

Goats were killed with an overdose of pentobarbitone and were immediately necropsied. Six X 1-2cm blocks of small intestine were taken at one metre intervals distal to the pylorus as in Experiment 3.1 and labelled M1 to M6 respectively. Two X 1-2cm blocks were taken at one metre intervals proximal to the ileocaecal valve and labelled M-1 and M-2 respectively. Blocks were fixed and processed as for formalin-fixed tissue in Section 6.2.

In order to collect mucus for LMI assay, the second and third metre segments were immediately stored on ice. They were flushed with 25mls of cold PBS and then opened longitudinally. The mucosa was gently scraped with a rounded piece of perspex to collect intestinal mucus. This was diluted 1:1 by volume with PBS, mixed by stirring and then sieved through a 250μm aperture sieve. The mucus+PBS filtrate was centrifuged at 200 g for 5 minutes. The supernatant was recovered and stored frozen (for LMI assay) at -20°C as were the sediment, residue from the sieve and the flushings (for parasite counting). The remainder of the small intestine, now ligated into one metre lengths, as well as the scraped second and third metre segments, was also stored at -20°C for worm counting.

Tissue blocks were removed from the pylorus (Abo-P) and fundus (Abo-F) of the abomasum, stapled to card and fixed as for the small intestine. The remainder of the abomasum was quickly rinsed in PBS and then the whole surface was scraped as described above to collect mucus which was diluted 1:1 by volume with PBS and stored at -20°C.

Parasitology

Faecal egg counts were estimated weekly (see Appendix 2b for method).

For all goats, worm counts and eggs per female worm were estimated as in Section 4.2. The flushings, sieve residue and sediment after mucus collection from metres 2 and 3 were pooled, sieved and subsequently processed as for the remainder of the small intestine. For uninfected goats, the first 6 metres including the flushings, sieve residue and sediment after mucus collection were examined.

Larval migration inhibition (LMI)

For simplicity, mucus+PBS will be referred to as mucus. Samples were stored at -20°C for about 8 months before assay. LMI activity of mucus was assayed as described in Appendix 7b. Ten control gels with PBS as the test substance were also run on each occasion for comparison of larval recovery. LMI was expressed as an LMI-index which was the ratio of the mean number of larvae migrating from the test gels to the mean number of larvae migrating from PBS control gels i.e. the lower the LMI-index the greater the LMI activity. This is different from the way in which LMI activity was originally

described but was necessary as more larvae migrated from the test gels than controls on several occasions.

Histology

H&E stained sections of each tissue block were prepared. GLs were counted at 500X in 20 fields of 0.031 mm^2 using fluorescence microscopy as in Section 6.2. In addition, MMCs, GLs and eosinophils were counted in M1, M2, M3 and M-1 using the sheep mast cell monoclonal antibody (MABC8) as described in Section 6.2.

7.2.2 Experiment 7.2: GL numbers in tissues from Experiment 3.1

This experiment concerns the GL numbers in intestinal tissues from animals used in the experiment described in Chapter 3. Group 1 was trickle-infected and subsequently given two challenges with *T. colubriformis*. Group 2 received only the two challenges whilst Group 3 received only the second challenge. Blocks of small intestine were removed at necropsy as previously described (Section 3.2) and fixed in 10% neutral buffered formalin. Sections were cut and stained with H&E as described in Section 6.2. GLs were counted as in Experiment 7.1 using fluorescence microscopy. Counts were made in 20 fields examined in a series of passes across the sections.

7.2.3 Experiment 7.3: GL numbers in tissues from animals used in Experiment 5.1

This experiment concerns the GL numbers in intestinal tissues from animals used in the experiment described in Chapter 5. 1-2 cm blocks of small intestine were taken as previously described (Section 5.2) and fixed in 10% neutral buffered formalin as described in Section 6.2. GL counts were made on H&E stained sections as described for Experiment 7.1.

7.2.4 Experiment 7.4: Stability of LMI activity with storage

Two adult goats which had been grazing pasture and were naturally infected with nematodes, were slaughtered. The small intestines were immediately removed and stored on ice and their contents recovered and pooled. These were mixed by a 5 minute agitation with a stomacher (Colwarth Stomacher 400, Seward, United Kingdom) and then frozen in aliquots at -20°C.

At varying intervals (see Table 7.5) samples were thawed and assayed for LMI activity as described in Appendix 7b. Ten control and test gels were run on each occasion.

7.2.5 Statistical analysis

As appropriate, data were analysed by analysis of variance, Tukey test for paired comparisons, paired t-tests, linear regression and correlation using Statistix, version 4.0. Power analysis was undertaken using Power Analysis and Sample Size, version 1.0 (Klintze, Utah, 1991).

7.3 RESULTS

7.3.1 Experiment 7.1

One control goat in Group B1.3 died during the experiment. All data for this animal were excluded from analysis.

Parasitology

Worm counts are shown in Table 7.2. Counts were analysed untransformed as transformation did not improve their normality. Total worm counts of the 4 infected groups did not differ significantly ($p>0.05$).

Comparison of male:female ratios shows that the ratio for Group B2 was significantly lower than Groups B3 and B4 ($p<0.05$) but there was no significant difference between these latter two groups ($p>0.05$).

Faecal egg counts are shown in Appendix 7a. For statistical analysis they were transformed $\log_{10}(x + 1)$ to normalise their distributions. Counts for Days 21 and 28 of the second infection were not significantly different ($p>0.05$) although only 4 pairs of data were available. Mean number of eggs per female in the first metre of the intestine from Groups A1, B3 and B4 were also not significantly different ($p>0.05$) when compared by analysis of variance.

Additional parasitological results for Groups A1 and B4 from this experiment are given in Experiment 4.1 (older goats).

Group	L3 (s.e.)	L4 (s.e.)	Adults (s.e.)	Male:female (s.e.)	Total nematodes (s.e.)	Establishment % (s.e.)	Eggs/female in metre 1 (s.e.)
A1 (n=3)	0	0	23530 (1600.7)	0.85^{ac} (0.061)	23530^{a} (1600.7)	67^{a} (4.5)	13.5^{a} (2.34)
A2 (n=3)	0	0	3 (3.3)	-	3 (3.3)	NA	NA
A3 (n=3)	0	0	3 (3.3)	-	3 (3.3)	NA	NA
A4 (n=3)	0	0	0	-	0	NA	NA
B1 (n=8)	0	0	4 (2.6)	-	4 (2.6)	NA	NA
B2 (n=5)	206 (201.4)	10,090 (2564.4)	9658 (2064.4)	0.49^{a} (0.113)	19954^{a} (1076.5)	57^{a} (3.1)	0
B3 (n=5)	0	2 (2)	22190 (2948.4)	0.84^{bc} (0.052)	22192^{a} (2949.1)	63^{a} (8.4)	12.2^{a} (2.72)
B4 (n=5)	0	0	22890 (2160.0)	0.90^{bc} (0.031)	22890^{a} (2160.0)	65^{a} (6.2)	14.3^{a} (1.75)

Table 7.2. Worm counts, establishment and eggs per female for Experiment 7.1. Within columns, groups sharing the same superscript are not significantly different when compared by analysis of variance and Tukey test (α=0.05). NA = not applicable.

Larval migration inhibition

The mean recovery of larvae of those present in PBS control gels (± s.e.) was 57% (± 0.2%; n=60). When gels were left for a further 30 hours only another 8% (± 0.2%; n=10) were recovered.

The number of larvae recovered using abomasal and intestinal mucus as test substances, expressed as a proportion of larvae recovered from PBS control gels, is the LMI-index. These are shown for Part A in Figure 7.1 and Part B in Figure 7.2. It can be seen that, in general, when no worms were present, larval recoveries were higher from gels with mucus than from control gels. This was particularly evident with assays of abomasal LMI in both Parts A and B.

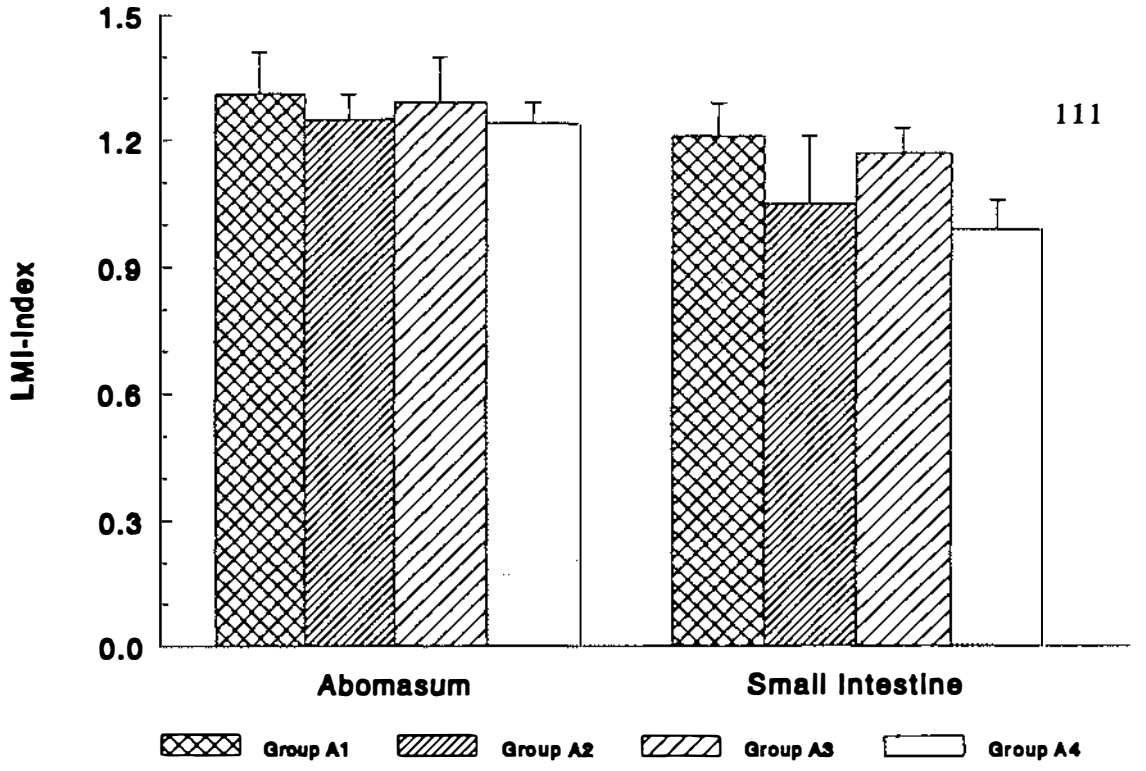


Figure 7.1. Experiment 7.1: LMI-index (mean ± s.e.) of goats in Part A. All goats infected with 35,000 *T. colubriformis* and treated with ivermectin 28 days later. Groups A1, A2, A4 and A4 killed 0, 7, 14 and 21 days after ivermectin treatment (n = 3 for each group).

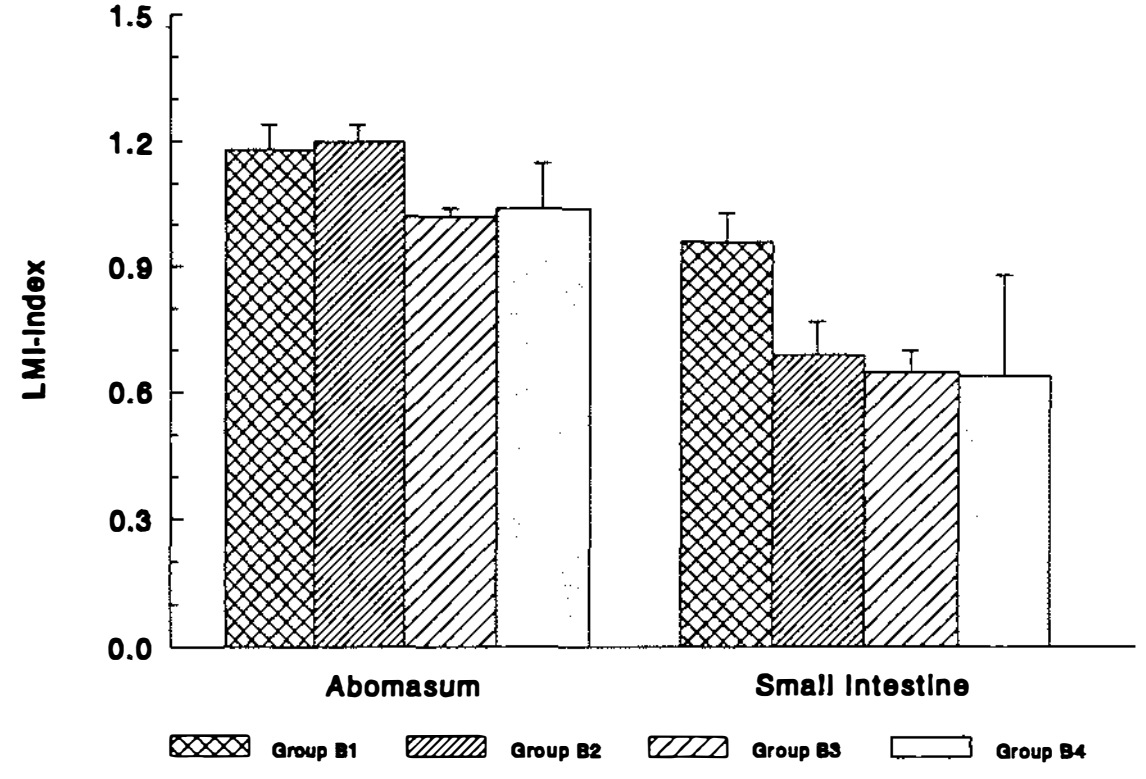


Figure 7.2. Experiment 7.1: LMI-index (mean ± s.e.) of goats in Part B. Group B1 (n = 8) killed on day 0. All other goats infected with 35,000 *T. colubriformis* and killed 9, 18 or 27 days later from Groups B2 (n = 5), B3 (n = 5) and B4 (n = 5).

There were no significant group differences in abomasal LMI in either part.

In Part A there was no difference in intestinal LMI between goats killed at the time of anthelmintic treatment and those killed at any of the subsequent time intervals. If anything, the proportionate larval recoveries from mucus gels were actually higher for Group A1 than for Groups A2, A3 and A4.

In Part B, analysis of variance of intestinal LMI showed that the difference in LMI-index between infected and uninfected goats was approaching significance ($p=0.09$). As there was no significant difference in intestinal LMI-index between the uninfected goats in Parts A and B ($n=17$) these were pooled. Subsequent comparison showed a significant difference ($p<0.05$) between infected and uninfected goats but not between the 3 infected groups of Part B.

Correlation coefficients of LMI-index with parasitological parameters are given in Appendix 7c. Of note are:

(i) the general lack of significant correlations between intestinal LMI-index and total worm count on an individual group basis although all are positive;

(ii) the significant positive correlation between intestinal LMI-index and total worm count for the combined data of Groups B2+B3+B4;

(iii) there were no significant correlations between abomasal LMI-index and any parasitological parameter;

(iv) the significant negative correlation between male:female ratio and intestinal LMI-index in Group B2.

Mucosal mast cells

MMC counts are shown in Figures 7.3 and 7.4 for Parts A and B respectively.

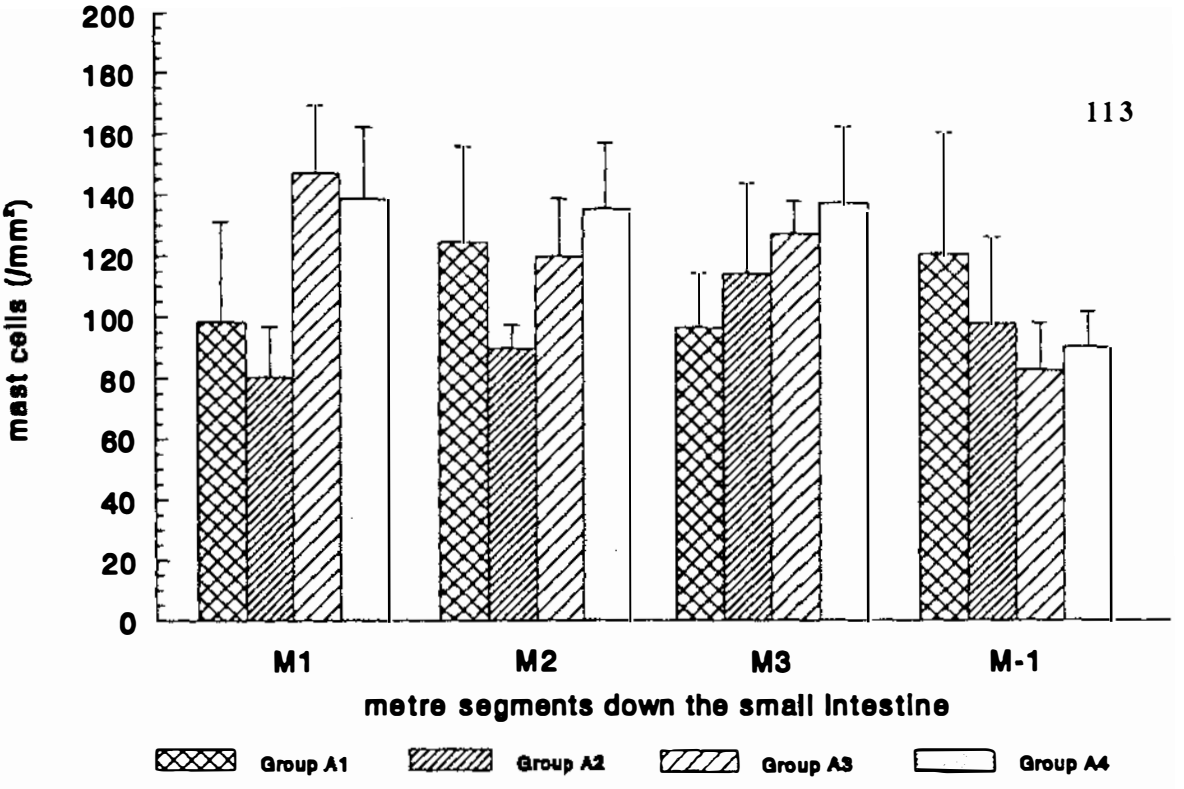


Figure 7.3. Experiment 7.1: Mucosal mast cell counts (mean ± s.e.) of goats in Part A. All goats infected with 35,000 *T. colubriformis* and treated with ivermectin 28 days later. Groups A1, A2, A4 and A4 killed 0, 7, 14 and 21 days after ivermectin treatment (n = 3 for each group).

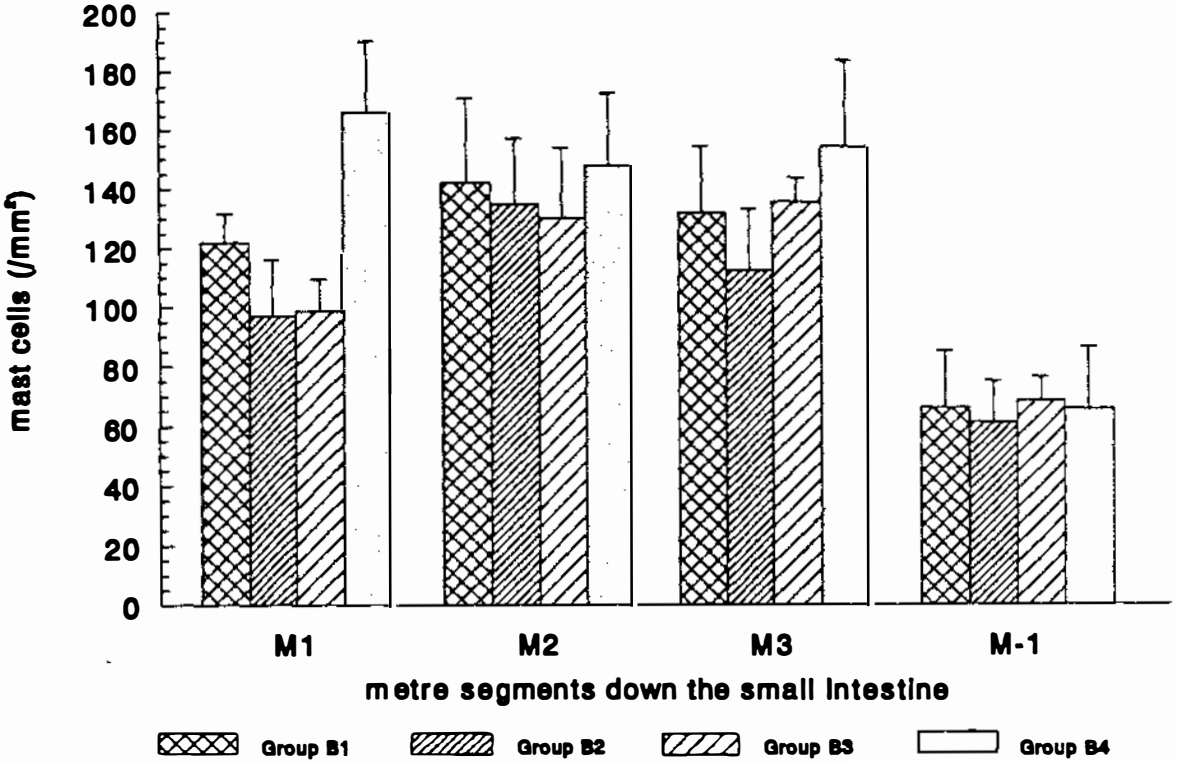


Figure 7.4. Experiment 7.1: Mucosal mast cell counts (mean ± s.e.) of goats in Part B. Group B1 (n = 8) killed on day 0. All other goats infected with 35,000 *T. colubriformis* and killed 9, 18 or 27 days later from Groups B2 (n = 5), B3 (n = 5) and B4 (n = 5).

As no significant trend with time was apparent following linear regression analysis, data from Groups B1.1 - B1.4 were combined to form Group B1.

Linear regression showed no significant relationship between counts for each metre or the sum of the first three metres (MMC123), and days after the end of the first infection for Part A, or days after reinfection for Part B.

Correlation coefficients for MMC counts with worm counts and LMI-index for Part B are given in Appendices 7c and 7d. Of note are:

(i) most correlations are not significant;

(ii) the significant negative correlations between M1 MMC and abomasal LMI-index for Groups B2+B3+B4 (Appendix 7c);

(iii) the significant negative correlations between M1 MMC and Group B4 worm count (Appendix 7d).

Globule leukocytes

As shown previously (Section 6.3), when raw GL counts were corrected to counts/mm^2 and compared by paired t-tests there was no significant difference between counts using H&E staining or monoclonal antibody ($p>0.05$) whether compared in all goats ($n=35$) or in Part A ($n=12$) or Part B ($n=23$) analysed separately. The correlation coefficients between both counting methods for each metre segment in this experiment are also all highly significant (see Appendix 7e).

Part A

Globule leukocyte counts for each metre were generally lowest in Group A1 (see Figures 7.5a and 7.5b). Following anthelmintic treatment GL counts tended to increase. This is particularly apparent for the first three metres. Analysing counts obtained using the monoclonal antibody, there was a significant linear regression ($p<0.05$) with "day after anthelmintic treatment" for M1, M3 and the sum of metres 1 to 3 (GL123). Using counts obtained with H&E stained tissues there was only a significant linear regression ($p<0.05$) for M3 with GL123 approaching significance ($p=0.07$). The difference between

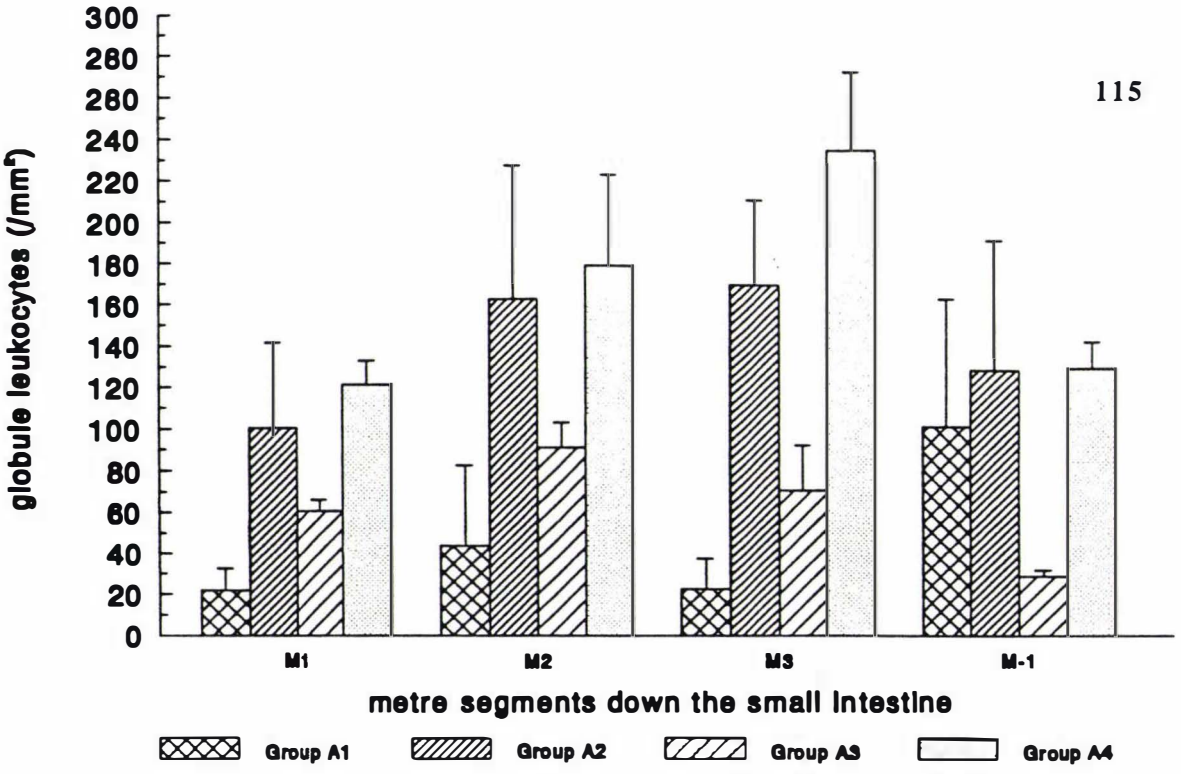


Figure 7.5a. Experiment 7.1: Globule leukocyte counts (mean ± s.e.) of goats in Part A; stained using a monoclonal antibody. All goats infected with 35,000 *T. colubriformis* and treated with ivermectin 28 days later. Groups A1, A2, A4 and A4 killed 0, 7, 14 and 21 days after ivermectin treatment (n = 3 for each group).

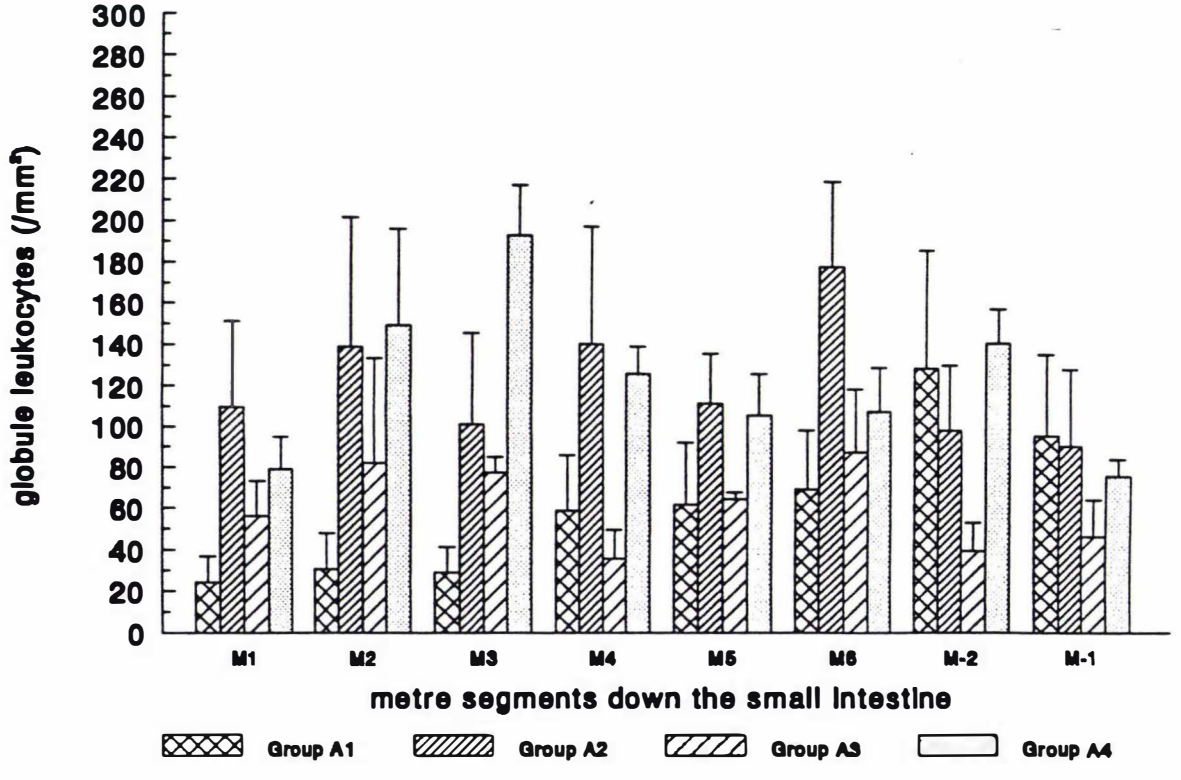


Figure 7.5b. Experiment 7.2: Globule leukocyte counts (mean ± s.e.) of goats in Part A; stained with H & E. All goats infected with 35,000 *T. colubriformis* and treated with ivermectin 28 days later. Groups A1, A2, A4 and A4 killed 0, 7, 14 and 21 days after ivermectin treatment (n = 3 for each group).

staining methods probably results from variations in cell distribution within and between sections.

Part B

No trend was evident in counts in uninfected goats over time and so data from Groups B1.1 - B1.4 were combined as Group B1 for comparison.

In general, in infected goats, GL counts in the first six metres decreased with time after infection (Figures 7.6a and 7.6b) but group variances were large so that differences between groups were non-significant although approaching significance with counts combined over the first 3 metres (monoclonal antibody sections $p=0.1$; H&E sections $p=0.06$). The only individual group difference that was significant was between Groups B1 and B4 for M1.

Linear regression of GL counts by "day after infection" revealed a significant regression ($p<0.05$) for M1 and M2 as well as GL123 for counts made with both the monoclonal antibody and H&E staining. In addition, with H&E staining, there was also a significant regression for counts in M3 and one approaching significance ($p=0.07$) in M4.

Correlations of GL counts with worm counts, LMI-index and MMC counts for Part B are listed in Appendices 7c, 7d and 7f. Of note are:

(i) the significant positive correlation for proximal small intestinal sections in Group B2 with abomasal LMI-index and the significant negative correlations for proximal small intestinal sections with intestinal LMI-index for Group B3 and the combined counts for Groups B3+B4 (Appendix 7c);

(ii) the consistently negative correlations with worm counts for proximal small intestinal sections with many being significant (Appendix 7d);

(iii) the general lack of correlation with MMC counts except for significant positive correlations with MMC counts of Group B4 and M-1 of the combined counts of Groups B2+B3+B4 (Appendix 7f).

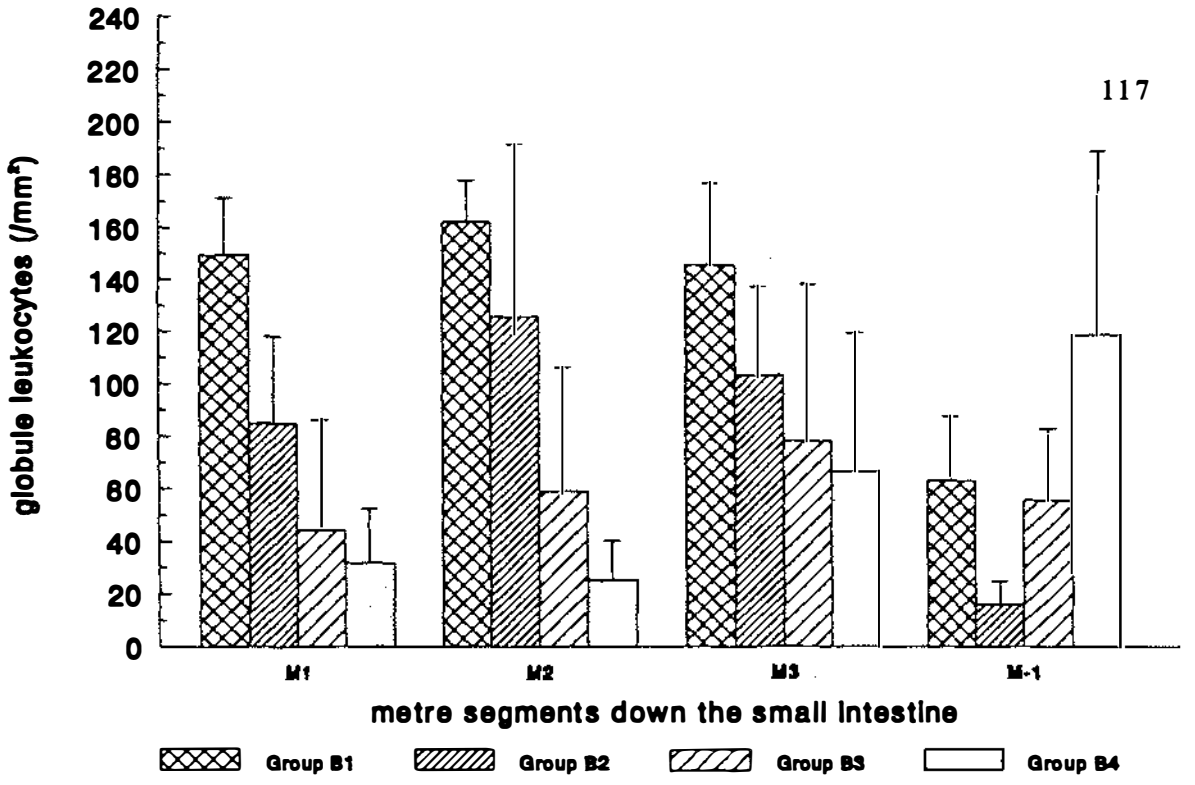


Figure 7.6a. Experiment 7.1: Globule leukocyte counts (mean ± s.e.) of goats in Part B; stained using a monoclonal antibody. Group B1 (n = 8) killed on day 0. All other goats infected with 35,000 *T. colubriformis* and killed 9, 18 or 27 days later from Groups B2 (n = 5), B3 (n = 5) and B4 (n = 5).

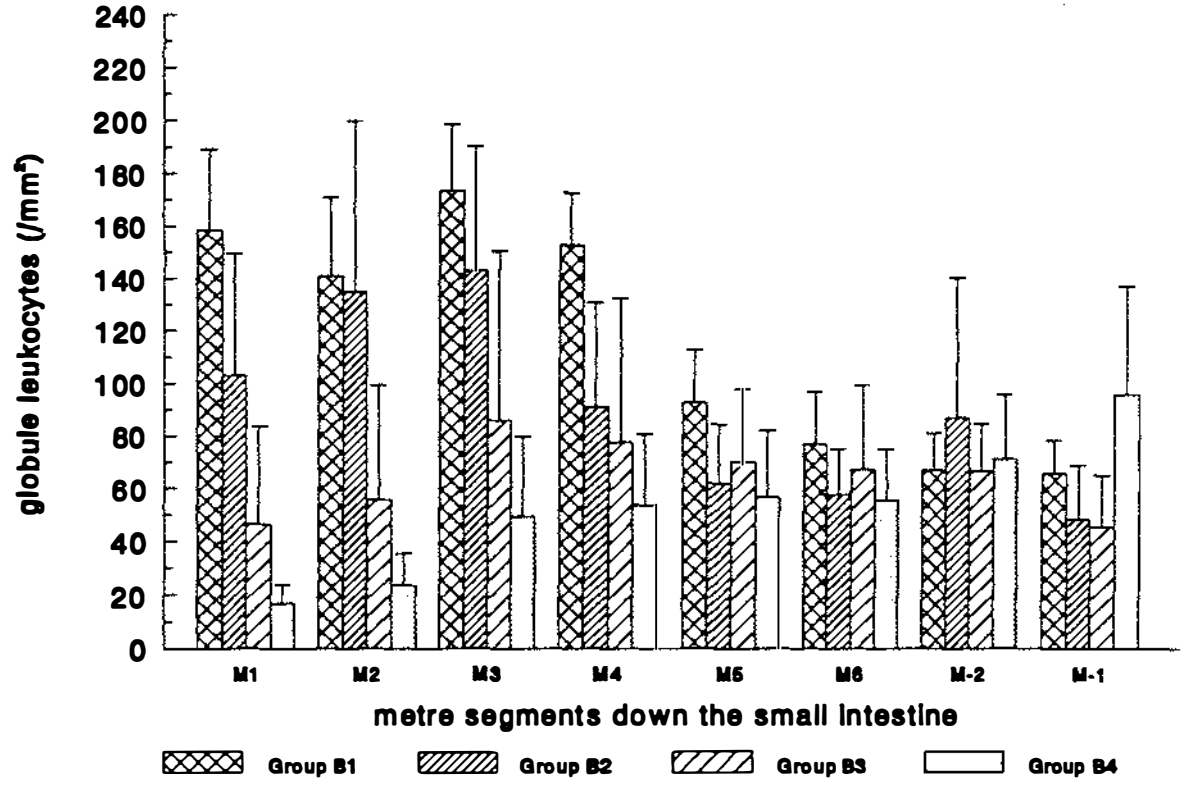


Figure 7.6b. Experiment 7.1: Globule leukocyte counts (mean ± s.e.) of goats in Part B; stained with H & E. Group B1 (n = 8) killed on day 0. All other goats infected with 35,000 *T. colubriformis* and killed 9, 18 or 27 days later from Groups B2 (n = 5), B3 (n = 5) and B4 (n = 5).

Eosinophils

Counts are shown in Figures 7.7 and 7.8. It can be seen that following removal of the nematode burden, counts increased slightly before dropping to lower levels by days 14 and 21. This contrasts with the consistently low counts seen in Part B. Counts for each of the first 3 metre-sections in Part B did not differ significantly.

Correlations between eosinophil counts and LMI-index, worm counts, MMC counts and GL counts are listed in Appendices 7c, 7d and 7f. Of note are:

(i) the general lack of correlations with LMI-index except for the significant positive correlations for counts in proximal small intestinal sections with abomasal LMI-index for Group B2 (Appendix 7c);

(ii) all correlations between M1, M2, M3 and worm count were negative but only M1 Group B3 and the combined counts of Group B2+B3+B4 for M1 were significant (Appendix 7d);

(iii) the significant positive correlation with GL counts for M1 and M3 Group B2, M1 Group B3 and M1 and M2 for Groups B2+B3+B4 (Appendix 7f).

Contribution of various parameters to the variability of worm counts

The contribution of various parameters to the variation in total worm counts in Groups B2, B3 and B4 was assessed by linear regression. The stepwise regression analysis of total worm count with day post-infection (DAY), the sum of eosinophils in the first 3 metres (EOS123), MMC123 and GL123 is shown in Table 7.3a. Multicollinearity prevented inclusion of any interaction terms. The dominant and only significant independent variable is GL123. Although the partial correlation coefficient of intestinal LMI-index (LMI-SI) with total worm count is positive as expected, the partial F test for this variable is not significant ($p>0.05$). However, power analysis for a multiple regression with a sample size of 15, an initial R^2 of 0.7 and the desire to test if an additional variable will add 0.05 to this R^2 with $\alpha=0.05$, shows the chance of including an appropriate variable is only 34%.

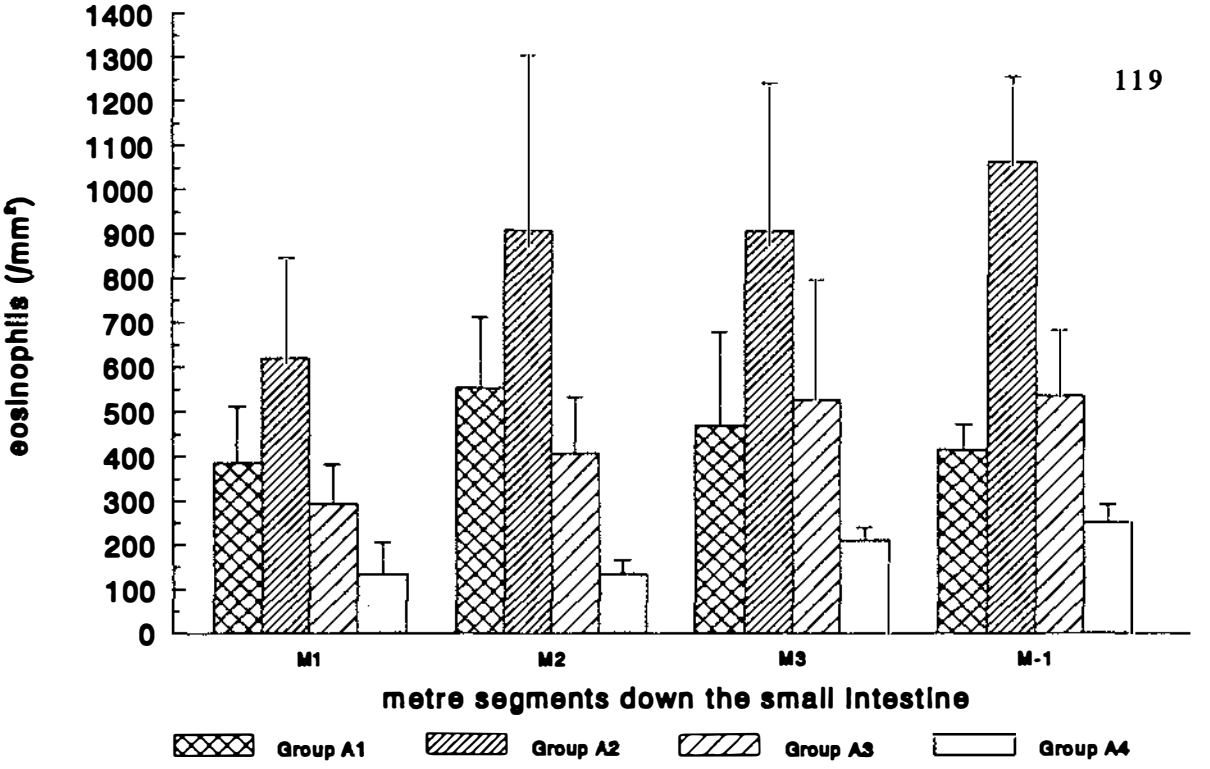


Figure 7.7. Experiment 7.1: Eosinophil counts (mean ± s.e.) of goats in Part A. All goats infected with 35,000 *T. colubriformis* and treated with ivermectin 28 days later. Groups A1, A2, A4 and A4 killed 0, 7, 14 and 21 days after ivermectin treatment (n = 3 for each group).

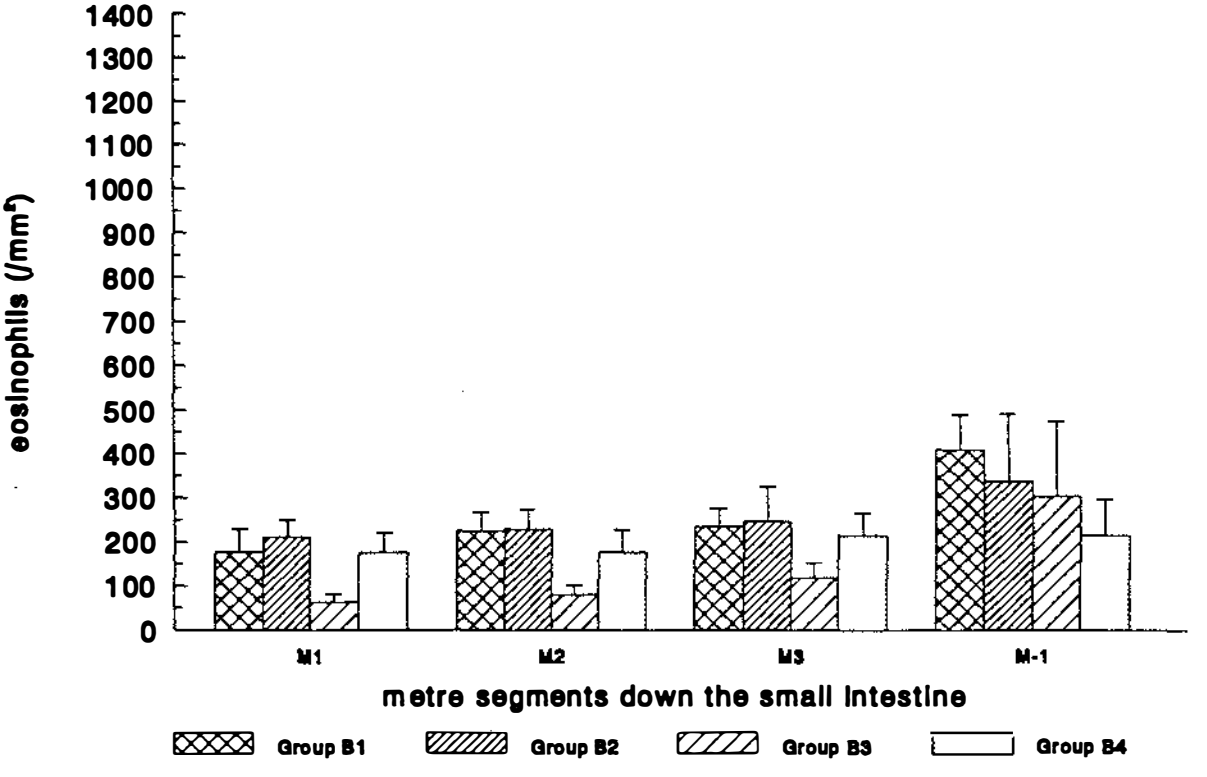


Figure 7.8. Experiment 7.1: Eosinophil counts (mean ± s.e.) of goats in Part B. Group B1 (n = 8) killed on day 0. All other goats infected with 35,000 *T. colubriformis* and killed 9, 18 or 27 days later from Groups B2 (n = 5), B3 (n = 5) and B4 (n = 5).

This suggests the likelihood of falsely excluding an extra variable in this regression is quite high given the sample size.

STEPWISE REGRESSION OF Total Worm Count
UNFORCED VARIABLES: DAY EOS123 MMC123 GL123 LMI-SI
F TO ENTER 4.00
F TO EXIT 4.00

STEP	VARIABLE	COEFFICIENT	T	R SQ	MSE
1	CONSTANT	21677.3	17.73	0.0000	2.243E+07
2	CONSTANT	24863.6	33.20	0.7847	5.200E+06
	GL123	-18.9210	-6.88		

RESULTING STEPWISE MODEL

VARIABLE	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	24863.6	748.911	33.20	0.0000	
GL123	-18.9210	2.74853	-6.88	0.0000	1.0

CASES INCLUDED 15 R SQUARED 0.7847 MSE 5.200E+06
MISSING CASES 0 ADJ R SQ 0.7682 SD 2280.25

VARIABLES NOT IN THE MODEL

VARIABLE	PARTIAL CORRELATIONS	T
DAY	-0.0001	-0.00
EOS123	0.3012	1.09
MMC123	-0.1203	-0.42
LMI-SI	0.4048	1.53

KEY:
DAY = day after infection; EOS123 = sum of eosinophil counts in the first 3 metres; MMC123 = sum of MMC counts in the first 3 metres; GL123 = sum of GL counts (using monoclonal antibody) in the first 3 metres

Table 7.3a. Stepwise regression analysis of total worm counts of goats in Groups B2, B3 and B4, Experiment 7.1.

Contribution of various parameters to the variability of intestinal LMI-index

The contributions of various parameters in Groups B2, B3 and B4 towards intestinal LMI were assessed by linear regression. The best subsets regression analysis of LMI-SI with DAY, EOS123, MMC123 and GL123 is shown in Table 7.3b. As before, multicollinearity prevented inclusion of any interaction terms. The two significant predictors of LMI-SI are GL123, with a small negative regression coefficient and EOS123, which is only just significant for α=0.05, with a small but positive regression coefficient. However, power analysis suggests that the likelihood of inappropriately excluding another variable is high with this small sample size.

STEPWISE REGRESSION OF LMI-index of small intestinal mucus
UNFORCED VARIABLES: DAY EOS123 MMC123 GL123
F TO ENTER 4.00
F TO EXIT 4.00

STEP	VARIABLE	COEFFICIENT	T	R SQ	MSE
1	CONSTANT	0.66047	10.53	0.0000	0.05898
2	CONSTANT	0.76778	11.40	0.3385	0.04202
	GL123	-6.373E-04	-2.58		
3	CONSTANT	0.63050	7.36	0.5299	0.03235
	EOS123	4.550E-04	2.21		
	GL123	-9.298E-04	-3.66		

RESULTING STEPWISE MODEL

VARIABLE	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.63050	0.08571	7.36	0.0000	
EOS123	4.550E-04	2.058E-04	2.21	0.0472	1.4
GL123	-9.298E-04	2.540E-04	-3.66	0.0033	1.4

CASES INCLUDED 15 R SQUARED 0.5299 MSE 0.03235
MISSING CASES 0 ADJ R SQ 0.4516 SD 0.17986

VARIABLES NOT IN THE MODEL

VARIABLE	PARTIAL CORRELATIONS	T
DAY	-0.3807	-1.37
MMC123	-0.2635	-0.91

KEY:
DAY = day after infection; EOS123 = sum of eosinophil counts in the first 3 metres; MMC123 = sum of MMC counts in the first 3 metres; GL123 = sum of GL counts (using monoclonal antibody) in the first 3 metres

Table 7.3b. Stepwise regression analysis of LMI-index of small intestinal mucus of goats in Groups B2, B3 and B4, Experiment 7.1.

7.3.2 Experiment 7.2

Parasitological results are given in Chapter 3.

GL counts are shown in Figure 7.9. For M1, M2 and M3 counts are generally highest for Group 1, then Group 2 with Group 3 being lowest. Group 1 counts are significantly higher ($p<0.05$) than those of Group 3 whilst counts of Group 2 are intermediate and not significantly different ($p>0.05$) from those of Groups 1 or 3.

GL counts along the intestine increase to a maximum at metres 3 or 4 and then decline. However, the differences between counts in the first five metres were not significant in any group.

Linear regression of GL123 by group shows the latter is a significant predictor ($p<0.05$) of GL count (adjusted $R^2 = 0.37$). Linear regression of square root transformed worm counts by group also shows the latter is a significant predictor ($p<0.01$) of worm count (adjusted $R^2 = 0.85$). Inclusion of GL123 as an additional independent variable only increased this adjusted R^2 to 0.86 and its partial regression coefficient was not significant ($p>0.05$).

However, as before, power analysis for n=15, indicates that the likelihood of incorrectly excluding an appropriate extra variable is high. Nevertheless, these findings indicate that history of prior exposure to *T. colubriformis* was most important with little additional variation accounted for by variation in GL counts within groups.

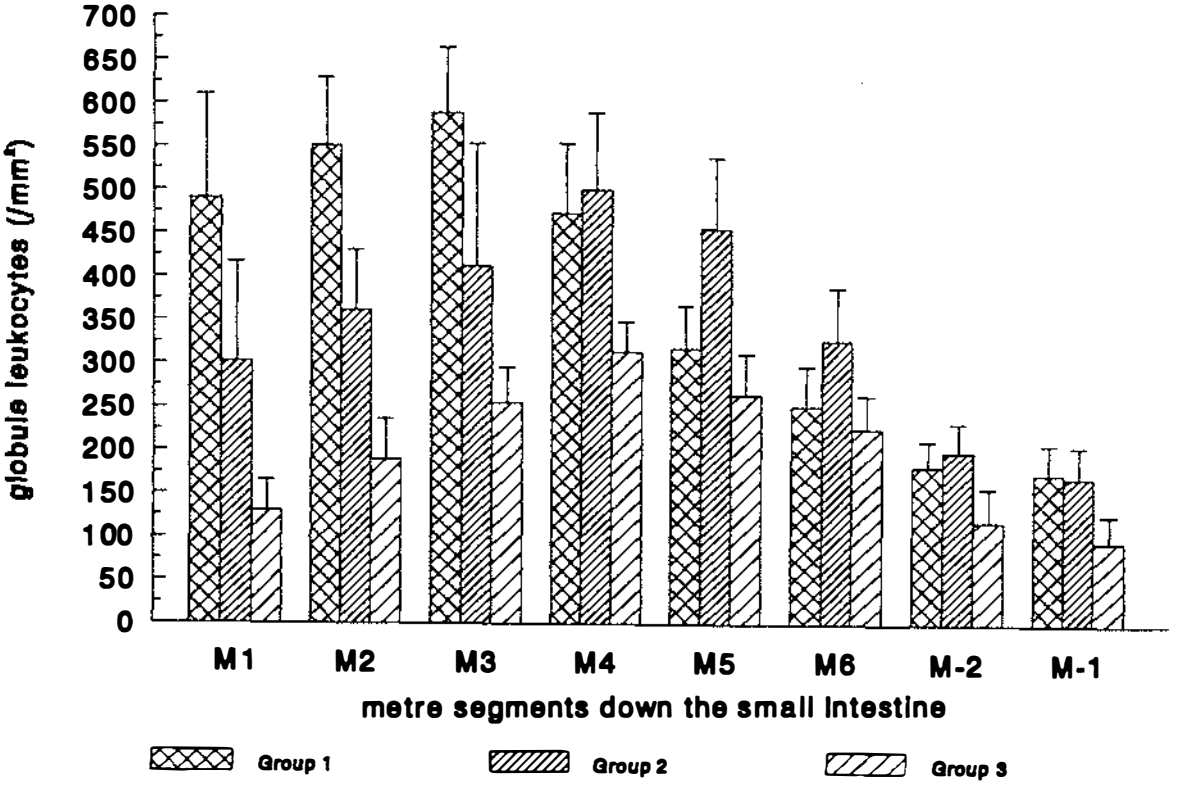


Figure 7.9. Experiment 7.2: Globule leukocyte counts (mean ± s.e.), stained using H & E. Group 1 (n = 5) trickle-infected with 10,000 *T. colubriformis* per week x 10 and challenged twice; Group 2 (n = 5) challenged twice; Group 3 (n = 5) challenged once. Each challenge comprised 50,000 *T. colubriformis* and all goats killed 28 days after the final challenge.

7.3.3 Experiment 7.3

Parasitological results are given in Chapter 5. GL counts are shown in Figure 7.10. Again counts tended to peak in metres 3 and 4.

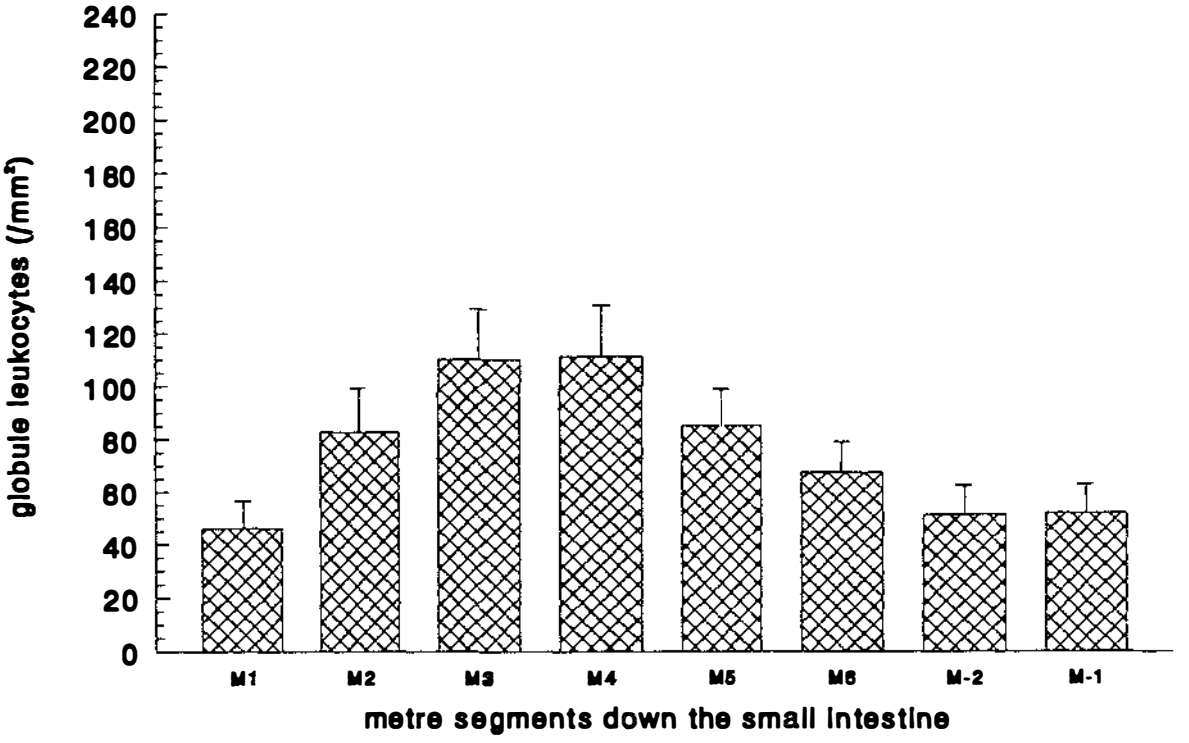


Figure 7.10. Experiment 7.3: Globule leukocyte counts (mean ± s.e.), stained using H & E. All goats (n = 29) challenged with 35,000 *T. colubriformis* and killed 28 days later.

Correlations of GL counts with other variables are given in Table 7.4. GL counts, especially GL123, are generally significantly negatively correlated with eggs per female and to a lesser extent faecal egg counts but not with total worm counts. For multiple regression analysis, worm counts and egg counts were examined for normality and and the former found not to require transformation. Egg counts were transformed to their square roots as this was superior to $\log_{10}$ transformation for normalising their distribution. Analysis showed that GL123 was not a significant predictor ($p>0.05$) of worm counts or the square roots of terminal egg counts.

Distance down the small intestine	Parasitological parameters			
	terminal egg counts	total worm counts	eggs per female	male:female
1 metre	-0.49**	-0.28	-0.59**	-0.37*
2 metres	-0.34*	-0.18	-0.45**	-0.15
3 metres	-0.18	-0.15	-0.36*	-0.11
4 metres	-0.22	-0.04	-0.38*	-0.07
5 metres	-0.34*	-0.24	-0.51**	-0.21
6 metres	-0.28	-0.42*	-0.35*	-0.31
minus 2 metres	-0.36*	-0.08	-0.43**	-0.19
minus 1 metre	-0.42*	-0.11	-0.65**	-0.24
sum of counts for the first 3 metres (GL123)	-0.34*	-0.21	-0.49**	-0.21

Table 7.4. Correlations of various parasitological parameters with GL counts for Experiment 7.3 (n=29; *=p<0.05; **=p<0.01; all considered as one-tailed tests).

7.3.4 Experiment 7.4

The LMI activity at varying intervals is shown in Table 7.5

Days in storage at -20°C	Mean LMI-index (standard error)	Difference between means[1]	Difference between control and test gels[2]
0	0.62 (0.043)	a	p<0.01
14	0.85 (0.047)	b c	p=0.06
57	0.54 (0.031)	a	p<0.01
118	0.67 (0.043)	a b	p<0.01
211	0.92 (0.031)	c	p>0.1
432	1.29 (0.071)	d	p<0.01[3]

Table 7.5. Experiment 7.4 - LMI-index of small intestinal contents at varying intervals of storage at -20°C. (1 = rows sharing the same letter are not significantly different (p<0.05); 2 = probability of larval recovery from PBS and control gels being the same on that day; 3 = larval recovery from test gels > PBS control gels on this occasion).

7.4 DISCUSSION

Larval Migration Inhibition

In Experiment 7.1, LMI activity was detected in the small intestinal mucus from the infected goats in Part B but not in that from goats in Group A1 which had received a single experimental infection 28 days previously, or others in Part A following removal of the infection.

As the assays for LMI activity were carried out on Part A and Part B samples after approximately 10 and 8 months respectively, the possible effect of storage on LMI activity needs to be considered at this point. Although the intervals between assays of the pooled material in Experiment 7.4 were longer than ideal, and the result at day 14 appears out of line with the overall trend, the results suggest that some loss of activity in the samples in Experiment 7.1 would have occurred in 8-10 months. If that is so, there may have been some activity in samples from Part A goats at the time of collection and greater activity in those from Part B. Other workers using this assay have also stored samples at -20°C (Douch *et al.*, 1983, 1984, 1986) or -12°C (Kimambo and MacRae, 1988) prior to assay but have not indicated the period of storage. It would appear that the agent(s) responsible for the inhibitory activity are not stable under these conditions and that assays should be carried out as soon as possible after collection.

In spite of the fact that there may have been loss of activity of the samples, the level of inhibition recorded in intestinal mucus from the infected goats in Groups B2-B4 (mean 34%) is similar to that reported for mucus from relatively resistant sheep with low faecal egg counts (Douch *et al.*, 1983, 1984). However, the level of establishment of infection in the goats (mean 63%; range 31-77%) was similar to that expected in naive sheep (Dobson *et al.*, 1990a) suggesting little or no resistance was being expressed. This level of activity was present only 9 days after infection and raises the question as to the role in parasite rejection of the inhibitory substances being assayed. In this connection, it is interesting to note that no difference in LMI activity was found in naturally infected sheep of genetically resistant and susceptible lines but the resistant animals had higher levels of $LTC_4/D_4/E_4$ (Gray *et al.*, 1992). Clearly further work is needed to determine the biological significance of LMI as assessed *in vitro*.

The absence of inhibitory activity from abomasal mucus is not surprising as very few adult *T. colubriformis* would be expected to have been in that organ to stimulate release of immune mediators. The passage of larvae through the abomasum may have provided sufficient antigenic stimulation to initiate mediator release in that organ. However, if this occurred it would be within the first 48 hours following challenge and could be expected to have subsided by Day 9.

The greater recovery of larvae from the test gels from uninfected goats in Experiment 7.1 compared with control gels was unexpected. At the time it was thought that it might have been caused by evaporation during the assay increasing the concentration of PBS which could have adversely affected the activity of the larvae. This would be more marked in control gels in which mucus was replaced by PBS. However, evidence that mucus from at least some uninfected goats may be stimulatory is presented and discussed further in Chapter 8 and this may be a more likely explanation. It is notable that the pooled sample which was assayed after being stored for approximately 14 months (Experiment 7.4) also showed evidence of stimulatory activity (mean LMI-index 1.29), perhaps after all inhibitory substances had become inactive. Stimulatory activity has not been noted in previous reports using this assay. In the original report (Douch *et al.*, 1983) 89.8-98.6% of larvae had migrated from the agar by 18 hours which would, therefore, not allow stimulatory activity to be detected. The reason for fewer larvae migrating from agar in Experiment 7.1 is unclear but has allowed the possible expression of stimulatory activity.

Mucosal Mast Cells (MMC)

MMC counts (excluding GLs) showed no trends relative to duration of infection or significant correlations with worm count or LMI-index except for Group B4 M1 and worm count ($r=0.92$; $p<0.05$). This one exception is likely to be a chance event as correlations of MMC counts in M2 and M3 with worm count in that group are low. This general lack of correlation between MMC counts and LMI-index is consistent with previous observations in sheep (Douch *et al.*, 1986). However, as the monoclonal antibody mainly recognises components of the granule wall, if granules are absent, such as in immature or fully discharged mast cells, these cells would not be recognised but may still be able to secrete some mediators such as leukotrienes. The negative correlation between abomasal LMI-index and M1 MMC counts for the combined data of Groups B2+B3+B4 is in the expected direction assuming that mast cells are the source of inhibitory substances but, as correlations with counts in M2 and

lower sections are not significant, and as there are no significant correlations between GL counts in the abomasum and abomasal LMI-index, it is difficult to explain and may simply be due to chance.

Globule Leukocytes (GLs)

GL counts were similar using both counting techniques. In Experiment 7.1, results from Part B showed mean GL counts for the first 3 metres decreased with increasing duration of infection and results from Part A show they then increased following the removal of an infection. This decline in GL numbers following infection is similar to that recorded in partly immune young lambs given a challenge infection of *T. colubriformis* (Gregg *et al.*, 1978). In contrast, in immune sheep challenged with *T. colubriformis*, GL counts were observed to increase by day 1, then decline to day 5 and subsequently recover to be very high at days 8 and 14, whilst immune sheep that were not challenged had small numbers present (McClure *et al.*, 1992). If a similar initial peak in GL numbers occurred early in Experiment 7.1 it would not have been observed. However, it is clear that these goats did not mount an effective immune response to the challenge infections although the apparent reduction in eggs per female worm suggests that at least some control of fecundity was being exerted. They would be more analogous to the partly immune lambs involved in the report by Gregg *et al.* (1978) than to immune sheep. An actual fall in GL numbers over the 28 day period was, nevertheless, surprising given that a significant degree of LMI activity was detected.

GLs have been described as partially degranulated MMCs and an indirect measure of MMC activity (see Section 1.7). Thus, if MMCs are a principal source of antiparasitic chemicals such as leukotrienes, a decline in GL numbers suggests that little MMC degranulation is occurring. Another factor which may influence GL numbers is epithelial cell turnover and their potential rate of loss. GLs are characteristically located in the epithelium at about the level of the top of the gland crypts. This suggests they must be mobile cells or they would be more evenly distributed along the villi. However, if increased cell turnover occurs following increased sloughing of epithelial cells due to parasitism, there may be an increased loss of GLs as a result. Although techniques for quantifying the losses of epithelial cells are not available (Parkins and Holmes, 1989), in trichostrongylid infections in sheep, an increased loss of mucus and epithelial cells is suspected to occur (Bown *et al.*, 1991) and an increased number of epithelial cells in mitosis have been observed in intestinal crypts (Barker, 1975a). The decline by Day 5 of GL numbers in the small intestine

of immune sheep challenged with *T. colubriformis* (McClure *et al.*, 1992) suggests their half-life is short under these conditions. As no increase in MMC numbers occurred in the challenged goats in Experiment 7.1, then a drop in GL numbers might be expected.

Even though GL numbers declined with time in infected goats in Experiment 7.1 while worm burdens did not differ significantly, there was still a clear negative relationship between GL count and worm burden as has been reported to occur in sheep (Gregg *et al.*, 1978; Douch *et al.*, 1986; Douch, 1988). This was particularly apparent for GL counts for the first 6 metres of intestine of goats killed at Day 18 (Group B3) and, to a lesser extent, at Day 27 (Group B4). The correlations for Group B3 are particularly high and consistent (see Appendix 7d), much more so than for Groups B2 and B4. It is interesting to note that the correlation between GL counts in the proximal intestine and LMI-index is also stronger for Group B3. The explanation for this may be that Day 18 happened to coincide with a time when these parameters were optimally related, though the biological implications of this, if any, are far from clear.

The significant positive correlations between MMC and GL numbers for Group B4 for M1, M2 and M-1 (Appendix 7f) are contrary to findings in sheep (Douch 1988). If there is a short half-life for GLs, this strong correlation may indeed reflect mast cell activity at that time, as residual GLs present earlier, could have been lost. In addition, as there was only a limited immune response, as indicated by the high nematode establishment, low MMC and GL counts are to be expected. The positive correlation for the combined data of Groups B2-B4 between M-1 GL and MMC counts may reflect a slower turnover of cells at this distal location, since no nematodes are located at this site and there should be no mucosal damage. However, these individual cases of significant correlations may also be due to chance.

One interesting relationship that is difficult to explain is the strong positive correlation between abomasal LMI-index and GL counts in the abomasal fundus (Abo-F) and most sections down the intestine of Group B2 goats (Appendix 7c). This indicates that where more larvae were recovered from the gels containing abomasal mucus i.e. less inhibition, more GLs were observed. This is inconsistent with the usually described relationship between GL numbers and LMI (see Section 1.8), particularly as the mean LMI-index was >1 for this group.

Given the significant correlation between worm count and intestinal LMI-index for the combined data of Groups B2-B4 ($r=0.67$; $p<0.01$) i.e. higher worm count associated with less inhibitory activity, it is perhaps surprising to find that best-subsets linear regression failed to show such a significant relationship. It could be argued that a simple correlation oversimplifies the situation by omitting other factors contributing to worm counts but the apparent inconsistency may be more a reflection of the effects of the small data set on the power of the regression.

The group mean GL numbers in Experiment 7.2 (in tissues from goats in Experiment 3.1), particularly for M1, M2 and M3, was in the same relative ranking, as the resistance of the goats as determined by worm counts. Clearly, an increase in GL numbers, especially in the region where most nematodes are located, occurs in goats that develop resistance to *T. colubriformis*. The increment in GL numbers for these proximal 3 metres was similar between Groups 1 and 2, and between Groups 2 and 3 which is consistent with Group 2 being only partly immune. The high numbers observed in Group 1 after 28 days were seen despite very low worm numbers being present. Assuming most of the infection was expelled very early, this suggests that the antigenic challenge from the remaining nematodes was still enough to stimulate MMC production and degranulation, or that the half-life of existing GLs was longer when few worms were present as cell turnover in the infected epithelium would be slower.

The GL counts for Group 3, Experiment 7.2 (challenge control) were similar to those of uninfected goats in Experiment 7.1 (Groups A2, A3, A4 and B1) whilst the GL counts for Group 1, Experiment 7.2 (trickle-infected and challenged twice) are approximately 3X higher. The slight difference in counting technique between the 2 experiments should not affect this comparison. Assuming there is no effect of breed, this suggests that Group 3 goats did not show a decrease in GL counts following infection as occurred in Experiment 7.1. Perhaps over this period of 28 days the Group 3 goats were already beginning to mount an immune response which countered any fall in GL numbers that might have otherwise occurred.

Unfortunately, most other studies in ruminants where GLs have been counted, have not reported GL numbers in terms of tissue area so as to be comparable with GL counts in these experiments. Stanton (1989) recorded larger numbers of GLs in goats than sheep of similar ages either exposed to trickle infections with *H. contortus* and *T. colubriformis* or exposed to infections

at pasture. In the former, the goats harboured fewer nematodes than sheep and in the latter the reverse was the case. The counting procedure used by Stanton (1989), which was to select fields only at the luminal surface, would have tended to inflate counts relative to a randomised system but the numbers were still considerably lower than those seen in the Saanen goats exhibiting substantial resistance to *T. colubriformis* in Experiment 7.2. It is also interesting to note that the counts seen in these resistant goats are similar to those recorded from the abomasums of immune sheep given an homologous challenge with *H. contortus* (Huntley *et al.*, 1992).

The mean establishment of the challenge infection in goats in Groups A1 and B4 in Experiment 7.1 and in Experiment 7.3 were approximately 67%, 57% and 39% respectively 27-28 days post-infection. Interestingly, GL counts reflect this difference as those of Experiment 7.3 are larger than those of Groups A1 or B4 suggesting that the difference may well have been due to differences in levels of resistance.

The relative pattern of counts in the proximal intestine is similar for most infected groups in all 3 experiments. Counts tended to increase down the intestine to a peak and then decrease. In Group 1, Experiment 7.2 they peaked at M3, in Experiment 7.3 at M4, for Groups 2 and 3 in Experiment 2 at M5, whilst for Group A1 they continued to increase down to M6. The pattern of counts in Part B, Experiment 7.1 is interesting. In addition to having overall lower GL counts with increasing age of infection, the peak count also tended to move distally with increasing age of infection. Groups B1 (uninfected) and B2 (9 days) peaked at M2, B3 (18 days) at M3, and B4 (27 days) at M5. The pattern in the uninfected goats in Part A, Experiment 7.1 is less clearcut and may, in part, be due to smaller group sizes and/or result from the random allocation of goats to groups which may not have resulted in a uniform distribution of goats with similar immune responsiveness.

If GL counts reflect mast cell secretory activity in these locations, they suggest that such activity is not maximal where most *T. colubriformis* are located. If mediators such as leukotrienes are secreted into the lumen and are active against nematodes, then it might be expected they would be secreted proximally to allow maximum contact with the nematodes as they move distally with the contents. Mediators secreted further down the intestine would not come in contact with the majority of *T. colubriformis*. Stanton (1989) also reported higher GL counts in sheep and goats distal to the location of nematode burdens that apparently stimulated them though their resistance

status was not clearly established. In contrast, Douch *et al.* (1986) considered that, in immunised sheep, the GL distribution reflected the presumed *T. colubriformis* distribution, but this would not appear to be the case in goats showing little resistance to infection. There were, however, similarities between the distribution of GLs in resistant sheep and goats in Group 1, Experiment 7.2 which suggests that in resistant animals GL distribution may parallel nematode distribution. Interestingly, unimmunised, non-resistant sheep had peak GL numbers at a slightly more distal location than in immunised resistant sheep (Douch *et al.*, 1986).

If the presence of nematodes stimulates increased cell turnover in the mucosa which results in a shorter half-life for GLs, the numbers of GLs in regions where the nematodes are most numerous may not reflect mast cell activity. Indeed, if the half-life of GLs is in some way related to the number of nematodes in that section of small intestine, then lower numbers of GLs in the first few metres would be expected. This highlights the problems of using intermittent histological examinations to monitor dynamic states and of assuming mast cell secretory activity is proportional to the number of mast cells observed, which may not be so (Woodbury *et al.*, 1984).

Eosinophils

In sheep a significant positive correlation has been reported between LMI activity of mucus and eosinophil cell counts (Douch *et al.*, 1986). In Experiment 7.1, eosinophil numbers did not appear to be significantly correlated with either worm counts or intestinal LMI-index but did appear to have some positive relationship with GL counts in the proximal small intestine of infected goats (see Appendix 7f). However, this was only apparent on Days 9 and 18 after challenge, and even though eosinophil counts remained at preinfection levels over the course of the infection. Linear regression significantly connected eosinophil counts to LMI-SI ($p=0.047$) but the direction of the relationship is positive i.e. more eosinophils coinciding with less inhibitory activity. The partial regression coefficient of 4.66×10^{-4} is very small but nevertheless suggests that eosinophils were not contributing to LMI. If it is assumed that the number of cells present is an indication of their activity, which is not necessarily true, and if intestinal LMI activity is principally due to the action of leukotrienes, then this would suggest that eosinophils, although known to be able to secrete leukotrienes, were not doing so. Analysis of interactions was not possible but might explain this unexpected result.

The tendency for more eosinophils to be present 7 days after anthelmintic treatment (Group A2) in Part A of Experiment 7.1 and for eosinophil counts to then fall away is based on very small numbers of animals and there are large variations within groups. Thus it needs to be re-examined before any conclusions are drawn.

Parasitology

Examination of worm counts of goats in Part B, Experiment 7.1 shows the development of this isolate was very similar to that described by others in goats (Fitzsimmons, 1966), sheep (Monnig, 1927; Barker, 1975c) and calves (Douvres, 1957). The significantly lower male:female ratio of Group B2 compared to other infected groups in Part B, indicates that females completed their final moult before males. This would not appear to have been noted by others studying the parasitic development of *T. colubriformis*. The significant negative correlation between male:female ratio and intestinal LMI-index ($r=-0.95$; $p<0.01$) for Group B2 further suggests the immune response may have influenced the relative development rate of male and female nematodes.

There was also no significant difference in eggs per female in the first metre of the small intestine, between female nematodes recovered at Day 18 (Group B3) or Day 27 (Group B4) indicating that fecundity was similar at both times. This is supported by the fact that faecal egg counts at these times were not significantly different ($p>0.05$) although only 4 pairs of counts were available. In comparison with the challenge control goats (Group 3) of Experiment 7.2 (see Chapter 3), however, these mean eggs per female counts were slightly lower (15.4 and 13.2 eggs/female respectively) and were substantially lower than the mean counts in the first metre of the small intestine for the young goats in Experiment 4.1 (21.5 eggs/female). This would suggest that fecundity had peaked by Day 18 and was precluded from reaching its potential of over 20 eggs per female by an immune response. That eggs were present in 98% of female worms in the first metre of goats in Group B3 killed 18 days after infection also indicates the prepatent period reported here is somewhat shorter than that suggested by Monnig (1927) in sheep, or by Rahman and Collins (1990c) in goats. This may just reflect a difference between the strains used.

The correlations of total worm counts with faecal egg counts at Days 21 and 28 of the first infection in Experiment 7.1 were poor being -0.41 and 0.51 respectively. The correlation of other parameters with these egg counts was

also poorer than with final worm counts which supports the conclusion reached in Chapter 5 that faecal egg counts from a previous infection are of less use than worm counts where the immune response to an infection is being investigated. (see p 90)

CHAPTER EIGHT

THE DYNAMIC PATTERN OF LARVAL MIGRATION INHIBITION ACTIVITY IN ILEAL CONTENTS OF GOATS WITH ILEAL CANNULAE THAT WERE INFECTED WITH *TRICHOSTRONGYLUS COLUBRIFORMIS* (EXPERIMENT 8.1)

8.1 INTRODUCTION

The objective of this experiment was to investigate the dynamic pattern of LMI activity by monitoring this in the ileal contents of cannulated goats infected with *T. colubriformis*. A problem with Experiment 7.1 was that different groups of goats were observed at different time intervals and individual variability within groups precluded some possible changes being seen.

8.2 MATERIALS AND METHODS

Experimental schedule

Six adult mixed-age Angora does which had been in the same flock for at least a year were used in this experiment. They were held in metabolism crates and restrained with leather halters to prevent them interfering with their cannulae. They were fed mainly lucerne chaff with some meadow hay during the first infection, and chaffed meadow hay during the second.

The goats were surgically fistulated and the cannulae inserted 6 weeks before the start of the experiment.

All goats were treated with a combination of anthelmintics prior to surgery but a small number of eggs were detected in the faeces of Goats 3 and 4 on Day -13. All goats were treated again which removed this persistent infection as judged by the absence of eggs in faecal egg counts and faecal floats 10 and 13 days later and through until the first infection was patent.

On Day 0, all goats were infected with 35,000 viable infective larvae of *T. colubriformis*. These larvae had been cultured approximately one month before and stored at 10°C. Viability was assessed by observing larval motility.

This first infection was terminated with oral ivermectin on Day 28 with a supplementary dose of oxfendazole on Days 38 and 39 as Goats 4 and 5 still had very small numbers of eggs in their faeces.

On Day 48 (day 0 for the second infection), the goats were again infected with 35,000 viable infective larvae of *T. colubriformis* using the same batch of larvae as for the first infection.

On day 78 (30 days after the 2nd infection) all goats were killed.

Larval migration inhibition activity of ileal contents was determined as described in Appendix 7b. These assays commenced 5 days before each infection and were then run at 2 or 3 day intervals until the infection was terminated or the goats were killed. All assays were done on the day of collection.

In addition to the 6 Angora goats, a six-month old Saanen wether, raised under worm-free conditions, became available prior to the second infection as a result of another unrelated experiment finishing. Its faeces had been regularly examined by faecal floatation and no nematode eggs seen. Small intestinal contents from this worm-free Saanen goat were also assayed on each occasion during the second infection with the intention of using it as an uninfected control.

Cannulae

T-shaped cannulae were constructed from medical grade silastic tubing with an outside diameter of 12 mm and an inside diameter of 6 mm. The top of the T was made with a 1/2 section approximately 6-7cm long, suitably smooth and rounded. The body of the T which protruded through the abdominal wall was 5cm long and stopped with a rubber bung.

The cannulae were inserted into the terminal ileum approximately one metre proximal to the ileocaecal valve. The bodies of the cannulae protruded through the right mid-lumbar flank. These were originally held in place with a disc of soft plastic against the skin and a rubber elastrator ring above that to hold it in place. After the fistulae had fibrosed, only the elastrator ring was required.

The cannula of Goat 6 was lost internally on Day 6 of the first infection. It was subsequently found at necropsy to be lodged about 0.5 metres distal to the fistula. The intestine at this point was thickened and firmly adherent to the abdominal wall. There was also an ulcer apparently created by one of the wings of the "T". This goat appeared to clinically unaffected until Day 24 of the first infection when she became anorexic and febrile. She was treated with antibiotics for 4 days by which time both her temperature and appetite were within normal limits and remained so until the end of the experiment. A replacement cannula made of rubber with an expanded perforated end which could be constricted to allow it to be inserted into the fistula without requiring surgery was subsequently used in this goat.

Collection of ileal contents

Dialysis tubing with a flat width of 23mm (Union Carbide 6-8000 molecular weight), proved suitable for collecting ileal contents. Lengths of about 25cm were knotted at one end and held in place over the cannulae with rubber elastrator rings. Prior to placement, the cannulae were manually cleared of digesta and flushed with 10ml of sterile PBS. The tubing was then left in place for 1 to 1.5 hours.

The worm-free Saanen wether was killed on Day 48. Its small intestinal contents were collected and processed as described for intestinal contents in Experiment 7.4.

Necropsy procedure

Goats were killed with an overdose of pentobarbitone. They were immediately necropsied. The small intestine from each animal was removed and separated into 1 metre lengths. Six tissue blocks from the proximal small intestine and 2 from the distal small intestine were removed, fixed and labelled as described in Experiment 7.1.

Mucus was collected from the second and third metre segments as in Experiment 7.1. The mucus+PBS filtrate was stored frozen at -20°C and is referred to as mucus in the following text. The period of storage before LMI-assay was 41 days. The only variation from the procedure used in Experiment 7.1 was that the whole filtrate, not just the supernatant following centrifugation, was used as the test substance.

The remainder of the small intestine, now ligated into 1 metre lengths, as well as the scraped 2nd and 3rd metre segments, flushings from these segments, and material retained on the sieve after mucus processing, were stored at -20°C for worm counting.

Cell counting

GLs were counted by fluorescence microscopy as described in Section 6.2

Parasitology

Faecal egg counts during each infection were estimated weekly until patency was expected and then every 2 to 3 days, including the final day of each infection. If counts revealed no eggs a faecal float was also performed (see Appendix 2b for these methods).

Nematodes present were counted in a 10% aliquot following digestion of thawed intestinal segments in pepsin+HCl for 2 hours as described in Section 4.2. The worms present in a 10% aliquot of the flushings and sieve residue from the mucus collection were also counted. The first 3 metres were processed together and then groups of 2 metres were processed until the last 2 metres contained less than 1% of the cumulative total worm count already counted.

Ten inhibited L3 larvae per goat were measured with a micrometer eyepiece.

The number of eggs *in utero* was counted in 10 female worms taken from the first 3 metres in each goat if that many were present, otherwise in all females recovered.

Statistical analysis

Correlations, analysis of variance, Tukey tests for multiple comparison and linear regressions were performed using Statistix version 4.0.

8.3 RESULTS

Parasitology

Terminal faecal egg counts for each infection, eggs per female worm and worm counts are shown in Table 8.1. A notable feature is the higher egg counts achieved in the first infection compared with the second. The latter coincided with a high percentage of inhibited L3 recovered at necropsy. This count of L3s would be an underestimate as the flushings and scrapings from metres 2 and 3 were sieved through a 250μm sieve which may not have retained all L3 stages. A finer sieve was not used as the occurrence of L3s was not anticipated.

The inhibited L3s had a mean length of 709μm (n=60; range 579-863μm; s.d. 50.36μm). They were predominantly found in the first 3 metres of the small intestine and very few beyond 6 metres. No parasitic stages were observed within gland crypts in any of the sections examined histologically.

Faecal egg counts were highly correlated with worm counts (see Appendix 8b) but this is exaggerated to some extent by the ranges of both that were seen with these six goats. Male:female ratios and eggs/female worm were also significantly correlated ($p>0.05$) suggesting that an increase in the proportion of males in the population was associated with an increased egg production by female worms.

Goat number	1	2	3	4	5	6
Faecal egg counts						
Faecal egg count 28 days after the 1st infection	5050	900	200	2050	100	1650
Faecal egg count 28 days after the 2nd infection	2900	0	0	50	0	250
Worm counts						
-adults (% establishment as adults)	20660 (59.0)	760 (2.2)	620 (1.8)	470 (1.3)	50 (0.1)	3210 (9.2)
-male:female ratio of adults	0.88	0.41	0.51	0.88	0.66	0.76
-L4s	0	0	0	110	0	0
-inhibited L3s (% of total count)	990 (4.6)	2960 (79.6)	4680 (88.3)	4680 (88.9)	9510 (99.4)	6640 (67.4)
-total (% establishment)	21650 (61.8)	3720 (10.6)	5300 (15.1)	5260 (15.0)	9560 (27.3)	9850 (28.1)
-eggs/female (s.e.)	6.1 (0.50)	4.3 (1.18)	3.8 (0.77)	7.8 (0.59)	5.0 (2.08)	6.9 (0.45)
Larval Migration Inhibition-Index						
-mucus	0.14	0.35	0.21	0.96	0.44	0.21
-contents preslaughter (Day 76)	0.20	0.46	0.61	0.61	0.27	0.11
-contents 7 days after the 2nd challenge (Day 55)	0.49	0.00	0.50	0.19	0.49	0.39
-minimum for contents in the 2nd infection	0.20	0.00	0.23	0.15	0.10	0.11

Table 8.1 Faecal egg counts, worm counts, eggs per female worm and larval migration inhibitory activity of intestinal mucus and ileal contents of goats challenged with 35,000 *T. colubriformis*, given anthelmintic treatment after 28 days, rechallenged as before after 20 days and killed after a further 28 days

Larval migration inhibition

The LMI-indices were calculated as in Experiment 7.1 and are shown in Figure 8.1. The terminal and maximum LMI-indices of intestinal contents in the second infection, as well as the LMI-indices of mucus are listed in Table 8.1. During the first infection, the mean LMI-index of contents decreased steadily over the 28 day period. During the second infection, the LMI-index was unchanged after 2 days but then decreased more quickly than in the first infection. Thereafter, the mean tended to plateau at about the lower limit seen in the first infection but with pronounced individual variation.

The LMI-index of contents for the three assays preceding each infection (5, 3 and 0 days before infection) were analysed separately and then compared between the two infections by analysis of variance. No significant differences ($p>0.05$) were found and in subsequent analyses Day 0 figures for each infection were taken as the respective mean of their three preinfection assays.

Analysis of variance followed by Tukey's test for multiple comparisons showed that LMI-index was significantly different ($p<0.05$) from respective preinfection levels on Days 8, 17, 20, 23 and 28 for the first infection and 7, 9, 21 and 28 days after the second challenge (see Appendix 8a for detailed comparisons).

The consistency of the ileal contents varied sporadically during the two infection periods. On occasions, the contents were almost clear (see Figure 8.2) and apparently mucoid with very little digesta present (see Figure 8.1). This generally coincided with a low LMI-index i.e. high inhibitory activity. On two occasions during the second infection, Goat 4 had clear mucoid intestinal contents which also contained flocculent material identified histologically as fibrin. Necrotic epithelial cells, red blood cells and nematodes were found in these fibrin clots.

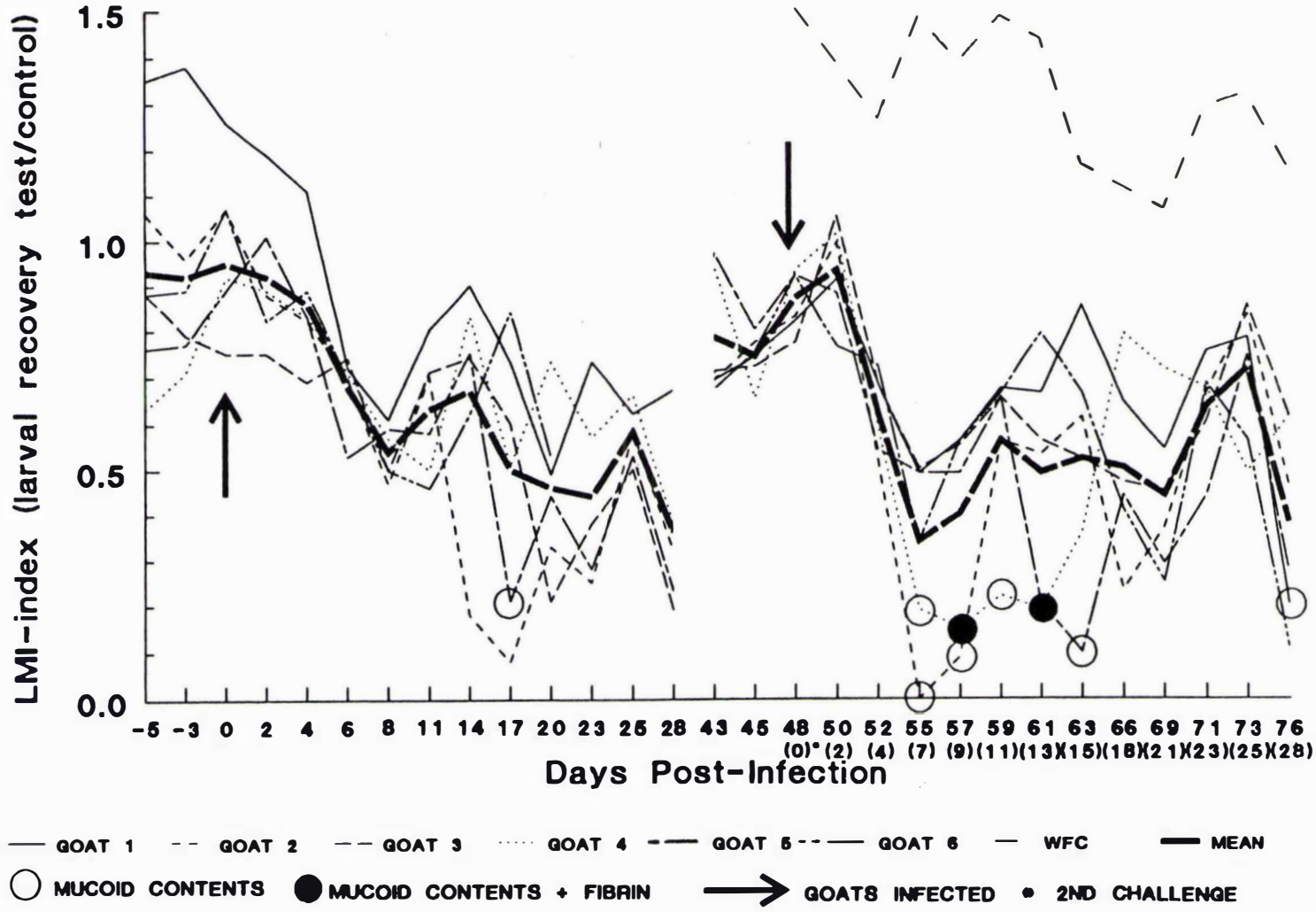


Figure 8.1. LMI-index of intestinal contents of goats (n = 6) infected with 35,000 *T. colubriformis* on Day 0 and again on Day 48 after being treated with ivermectin on Day 28. WFC = worm-free control goat killed on Day 48. Mean = mean LMI-index of 6 infected goats.

Figure 8.2. Comparison of samples of intestinal contents on day 59. Sample from goat 4 (4th from left) shows lack of digesta and clear mucoid nature of contents.

Correlations between some LMI values and other parasitological parameters are shown in Appendix 8b. Of note are:

(i) an almost significant negative correlation ($0.05<p<0.1$) between terminal LMI-index of contents and total worm count i.e. more inhibitory activity associated with more nematodes recovered at necropsy;

(ii) a negative, but non-significant correlation between mucus LMI-index and total worm count;

(iii) no significant correlations of total worm count with contents LMI-index at Day 55 (7 days after the second challenge when the mean index was lowest) or with minimum contents LMI-index in individual goats but

both these correlations were positive i.e. less inhibitory activity associated with more nematodes recovered at necropsy;

(iv) a poor correlation of terminal contents LMI-index with mucus LMI-index (see Figure 8.3).

However, any correlations with worm count will be influenced by the high count for Goat 1 and relatively low counts in other goats.

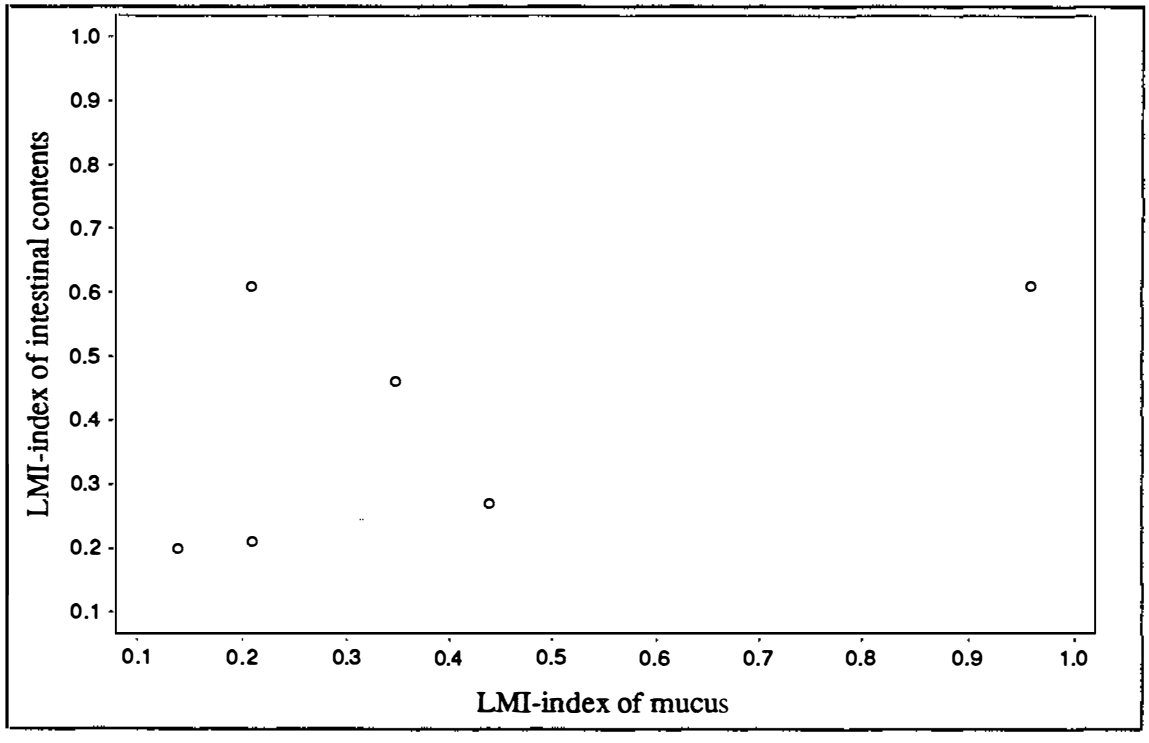


Figure 8.3. Comparison of the LMI-index of intestinal mucus collected at necropsy with LMI-index of intestinal contents collected 2 days pre-slaughter.

The LMI activities of the small intestinal contents of the worm-free Saanen are shown in Figure 8.1. These are all greater than 1.0. Although there is pronounced variability between assays, the values follow a downward trend with time in storage which is significant by linear regression of LMI with time ($p<0.05$; $R^2=0.37$).

Histology

GL counts are shown in Figure 8.4. and correlations with other parameters in Appendix 8b. Correlations were tested as one-tailed tests for

correlations that were in the expected direction and as two-tailed tests otherwise. Of note from Appendix 8b are:

(i) no significant correlations between GL counts in the proximal 6 metres of the small intestine and any parasitological parameters except for significant negative correlations ($p<0.05$) of GL counts in M4 and M6 with eggs per female;

(ii) a general tendency for all correlations between GL counts and LMI-index values to be positive even though none reached significance i.e. more GLs associated with less inhibitory activity;

(iii) a significant negative correlation between GL counts in M-1 and terminal faecal egg counts and adult worm counts;

(iv) globule leukocyte counts at different levels in the intestine were generally significantly correlated within goats.

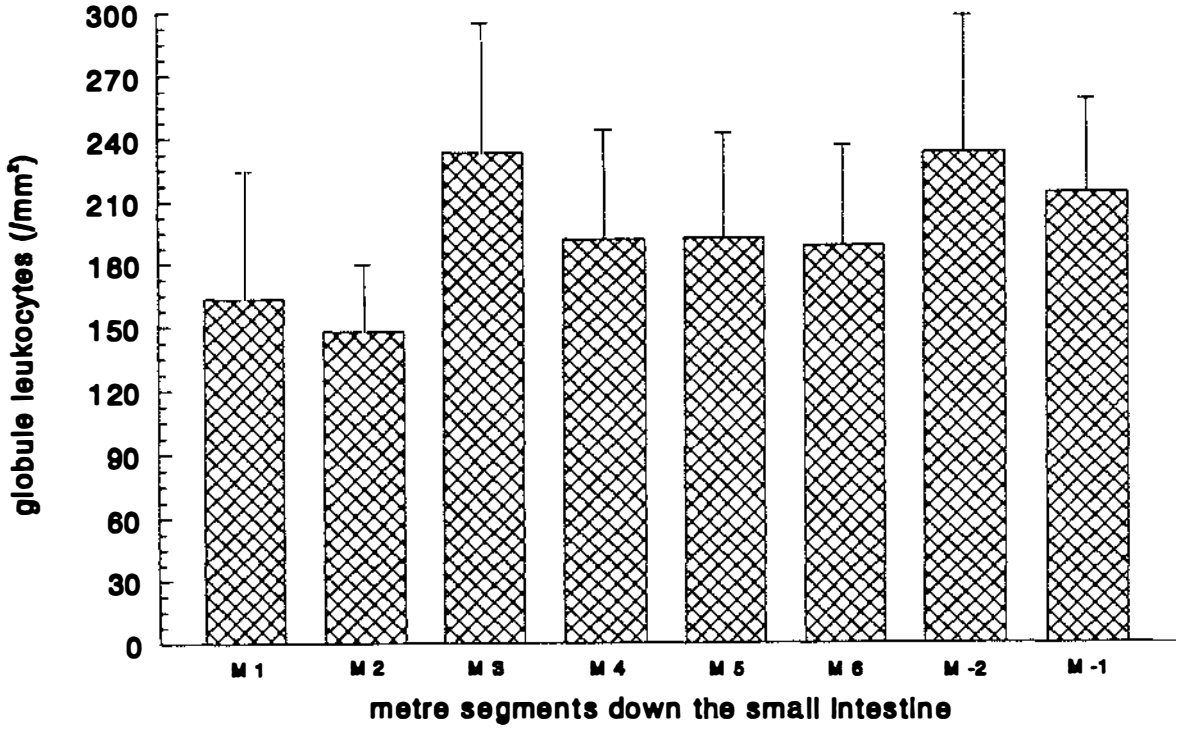


Figure 8.4. Globule leukocyte counts (mean ± s.e.) of sections taken at metre intervals down the small intestine.

8.4 DISCUSSION

Ileal contents from infected goats clearly inhibited 3rd stage larval motility *in vitro*. The level of inhibitory activity in intestinal contents only increased slowly during the 28 day period of the first infection. In immune sheep most *T. colubriformis* larvae are expelled within 1 day of infection (McClure *et al.*, 1992). Whether any larvae were expelled from these goats this rapidly is unknown, but 3 of the 6 had low egg counts of <1000 epg in the first infection suggesting relatively few nematodes survived to patency. However, if the components responsible for *in vitro* inhibitory activity are involved in rapid expulsion, they would have made little contribution to any such rapid expulsion that occurred in these goats.

The low faecal egg counts and worm counts 30 days after the second infection in five of the six goats suggests a level of host resistance in these animals which is generally more substantial than that seen in crossbred Angora goats in Experiments 7.1 and 7.3. Another parameter which suggests the immune response in these goats is having an effect is the relatively low number of eggs per female worm. The significant positive correlation of eggs per female with male:female ratio (see Appendix 8b) is also consistent with both being depressed in immune sheep (see Section 1.3.4) and goats (see Experiment 3.1). If larvae were expelled early in the second infection and it is assumed that this is linked to an increase in inhibitory activity, it is possible that expulsion occurred later than has been seen in sheep (McClure *et al.*, 1992) since the sudden increase in inhibitory activity (decrease in LMI-index) occurred between four and seven days after infection. By this time the larvae would have entered the mucosa and be moulting to L4s (Monnig, 1927; Douvres, 1957). However, the LMI-index pattern over this period in Goat 1 which showed little evidence of acquired resistance was similar to Goats 3 and 5 which did show resistance.

The situation is complicated by the occurrence of a high proportion of inhibited L3 stages in the goats which were more resistant. The proportion of inhibited L3 stages to adults that established is consistent with that described in immune sheep (Dobson *et al.*, 1990a). It is conceivable that the percentage inhibition may also have been influenced by the fact that the same batch of larvae was used for both challenges so that the time in storage was several weeks longer for the second. However, there appears to be no published work linking environmental factors with inhibition of this parasite.

The correlation of the LMI-index of ileal contents at Day 55 and the minimum LMI-index of contents for each goat in the second infection with worm count at slaughter was in the expected direction i.e. more inhibition (lower LMI-index) associated with fewer worms. However, by the end of the experiment, this relationship seemed to have reversed in that some of the goats which had a low worm burden and previously had more marked contents LMI activity (lower LMI-index) then the others, showed relatively less at the last sampling. Although the relationship is not absolute, this could be taken to indicate that continued release of mediators requires more adult parasites than present in some of these animals. However, the small numbers of goats studied and the pronounced individual variability which is apparent (Figure 8.1) precludes any firm conclusions about this. Most previous studies in sheep have been with animals receiving continuous infections which would continue to stimulate production of components of inhibitory activity (Douch *et al.*, 1983; Douch *et al.*, 1984; Kimambo and MacRae, 1988; Gray *et al.*, 1992; Gamble and Zajac, 1992) or their adult worm burdens were larger than those reported here in the more resistant goats (Douch, 1989).

There is a suggestion from faecal egg counts that goats were more resistant to the second infection than the first i.e. that this first exposure may have primed their ability to resist the second challenge as in Group 2 goats in Experiment 3.1. The results suggest that the goats were not expressing their full potential resistance which may reflect either inadequate field exposure or some decline in resistance in the period following removal from pasture or both, although by analogy with sheep resistance to *T. colubriformis* would not decline over the relatively short period of time between housing and the first challenge (Kimambo *et al.*, 1988b). Again, by analogy with sheep, mast cell numbers may have declined over the period between housing and first challenge (McClure *et al.*, 1992; Huntley *et al.*, 1992). As they are considered to be the source of components of LMI, this may be the reason for the relatively gradual development of LMI activity after the first challenge. In contrast, the time interval between termination of the first infection and the second challenge was short so that mast cell numbers would not be expected to have declined substantially which may account for the more rapid fall in LMI-index at this time.

The correlation between terminal LMI-index of contents and mucus (see Appendix 8b and Figure 8.3) is positive but not as strong as might have been expected. The assay of LMI in mucus after 41 days in storage at -20°C would not be likely to have had a major effect on activity (see Experiment 7.4). The

fact that LMI activity of the ileal contents would potentially reflect mediators produced throughout the length of the small intestine whereas mucus was only collected from the second and third metre segments may well contribute to the weakness of the correlation. Variability in rate of passage of intestinal contents and the variability in the assay itself are factors which may have had an influence. These may also account for the differences in LMI-index of contents between consecutive days in individual goats.

The proportion of mucus in the ileal fluid would appear to have a profound influence on the LMI activity of that sample and suggests the active components are closely associated with the mucus and/or secreted at the same time. An increase in goblet cell numbers and mucus production has been reported from parasitised sheep (see Section 1.12). However, the tendency for recovered ileal contents to be almost clear of digesta on occasions was unexpected. These mucoid samples did not just reflect a slow collection of contents through the fistula as the collection tubes often filled very rapidly on these occasions. All goats generally ate well during the second infection so flow of ruminal contents into the abomasum and eventually the ileum would be expected to be relatively constant day-to-day. Therefore, these sporadic clear mucoid collections imply sporadic increases in mucus production which were largely restricted to the time after larvae had re-entered the intestinal lumen and were maturing as adults.

The appearance of fibrin clots which had bound epithelial cells, red blood cells and nematodes in Goat 4 suggests considerable damage and protein leakage in the proximal intestine. No evidence of fibrinous inflammation was seen histologically in Goat 4 or any other goat in these series of experiments that would explain such an observation. Loss of epithelium was observed in some sections but was considered to be an artefact produced during sectioning. No previous mention of fibrin clots in the intestinal contents would appear to have been made in studies on the pathology or nutritional physiology of gastrointestinal nematode infections in ruminants. However, epithelial cell erosion has been observed in sheep infected with *T. colubriformis* (Barker, 1975a; Gregg *et al.*, 1978; McClure *et al.*, 1992) as well as enteric plasma loss (Steel *et al.*, 1990; Barker 1973; Poppi *et al.*, 1986; Bown *et al.*, 1991) so it is possible that sufficient fibrinogen could be released to produce visible clots. The inflammation was not considered likely to have come from the fistula wound as this was not obviously inflamed at the time and appeared to have healed and fibrosed well when examined at necropsy.

There was no significant difference between GL counts at different metre intervals and no obvious pattern for GL counts down the small intestine, contrary to what was seen in Experiment 7.2 and 7.3. This may result from the differing levels of resistance to *T. colubriformis* seen in this group of 6 goats which could obscure any pattern. The GL counts are of similar magnitude to those in uninfected goats in Experiment 7.1 and in control goats (Group 3) in Experiment 7.2 but much lower than in resistant goats (Group 1) in Experiment 7.2. This leads to the conclusion that no obvious mastocytosis occurred or at least that there was little conversion of MMCs to GLs. Despite this there was obvious LMI activity present in both mucus and ileal contents. Moreover, there was generally a positive, although not significant, correlation between LMI-index and GL counts which is the reverse of the expected relationship i.e. less migration inhibition *in vitro* associated with higher GL counts. Such a trend is contrary to findings in sheep and in goats in Experiment 7.1 and is difficult to explain given the hypothesis that mast cells are likely sources of the components of LMI activity.

The correlations of GL counts with eggs per female and with male:female ratio are negative as expected. Interestingly, these correlations are stronger and only become significant for eggs per female in the terminal metre segments of the small intestine, not the proximal segments where the nematodes are located. This is also difficult to explain and may be simply a chance occurrence.

The LMI activity of contents is of a similar magnitude to LMI levels reported in immune sheep which successfully rejected a daily challenge of *T. colubriformis* (Kimambo and MacRae, 1988). It is also of a similar magnitude to the LMI recorded in Experiment 7.1. As already discussed in Chapter 7, this degree of LMI activity would appear to have little effect on adult worms in goats except perhaps to restrict egg laying. It is difficult to associate LMI activity with the tendency for larvae to be inhibited in their development in immune goats (or sheep). Any such influence would need to occur in the first three days which is before LMI-index had changed substantially from preinfection levels. Moreover, Goat 1 which had the lowest percentage of inhibited L3s, had LMI values similar to those of other goats in this period following challenge, yet these latter two goats had very high degrees of inhibition.

The LMI activity of the ileal contents of the worm-free Saanen goat is interesting. Considerably larger numbers of larvae were recovered than from

PBS control gels indicating stimulatory activity of the contents, apparently caused by some agent that was labile as activity declined with storage at -20°C. Since the animals were all on the same diet, a dietary source of this stimulatory activity is not likely. Unfortunately, only one animal was available and thus it was not possible to repeat the investigation. Nevertheless, it is interesting to note that contents from Goat 1 also showed stimulatory activity at the beginning of the experiment. The fact that stimulatory activity decreased with storage indicates that the recovery of more larvae from test than from PBS control gels is not due to any effects of changing PBS concentration as suggested in Chapter 7. However, the raised LMI-index in contents stored for 432 days (Experiment 7.4) is inconsistent with this trend. Clearly, further investigation of this stimulatory activity is needed.

Inhibition of *Trichostrongylus* spp. is known to be at the L3 stage (Eysker, 1978). The measurements of the inhibited larvae in this experiment are consistent with those given for the parasitic third stage of *T. colubriformis* by Douvres (1957) and Monnig (1927).

CHAPTER NINE

GENERAL DISCUSSION

At the commencement of this study little was known about the response of goats to gastrointestinal nematodes. The initial aim was to quantify the ability of goats to develop resistance to *H. contortus* and *T. colubriformis* following a trickle-infection exposure. Further experiments were then designed to investigate the dynamic pattern of some elements of the host-parasite relationship with *T. colubriformis*.

The results of this work clearly demonstrate that some goats can develop substantial resistance to *T. colubriformis*. Unfortunately, worm-free Saanen goats were only available for the early experiments and of necessity, later experiments were conducted with Angora-type goats raised under field conditions. Interestingly, the differing results obtained with Saanen wethers in Experiment 3.1 versus later experiments with Angora and Angora-cross goats suggests there may be breed differences in the ability of goats to develop resistance to this parasite. It was only with the older does in Experiment 8.1 that some evidence of reasonably effective host resistance was shown by Angoras. These experiments are, of course, not strictly comparable given that the Saanens were raised worm-free and experimentally exposed and challenged, whereas the Angoras were field-reared and evidence of their resistance status was based on estimates of larval establishment. That Saanens should be more capable of developing resistance is consistent with their origin in the temperate zones of Europe where they are likely to be exposed to high levels of parasitism. In contrast, Angoras evolved in the dry plateau of Central Anatolia (Mason, 1981) which would be an area of low nematode challenge where resistance to nematode parasitism would not be as important for survival as in Europe. However, even though this quantitative difference exists between breeds it is reasonable to assume that the fundamental immune processes that are operating should be similar within this host species.

Another factor which appeared to show some consistency between experiments was the relatively poor resistance to infection shown by Angoras when first challenged after removal from the field, compared to later challenges. This was apparent to a varying extent in Experiments 5.1, 7.1 and 8.1. Although their earlier exposure to *T. colubriformis* infective larvae was unknown, it is not unreasonable to assume it would have repeatedly exceeded the estimated threshold for sheep (of about 4000 infective larvae) given the

conditions under which they were previously grazed. A similar comparison can be made between the Saanens in Experiment 3.1, which were housed and experimentally infected in a very controlled manner, with those in the report of Watson and Hosking (1989) which were both raised on pasture and kept on pasture during the experiment and did not express obvious resistance to *T. colubriformis*. Although this comparison is confounded by the slightly younger age of the grazed goats, it does question the effect on the subsequent immune response, of artificially raising goats worm-free before experimental infection, versus natural infection on pasture from birth. However, there does not appear to be any suggestion from reports of experimental work with sheep that there is a difference in responsiveness between those with early natural exposure to infection and those raised worm-free.

Stress of being housed is one possible reason for a decline in the immune response of the Angora and Angora-cross goats. However, on each occasion there was no discernible difference in demeanour at the commencement of an experiment or later on. Penned goats demonstrate a dominance hierarchy that is more obvious than with sheep but this does not seem to change with time after an initial, reasonably brief, settling-in period.

The response of goats to *H. contortus* is in distinct contrast to that to *T. colubriformis*. The failure to develop resistance to this abomasal nematode was surprising, given the response to *T. colubriformis*, and clearly warrants further study. This would seem to be more likely an effect of nematode species rather than the organ where the parasite resides, as the response of various antigen presenting cells, T cells and other components of the immune response should not vary appreciably between the abomasum and small intestine. That solid resistance should be demonstrated to *T. colubriformis* but none to *H. contortus* questions the "commonality" of the immune response to these two related trichostrongylid nematodes. However, the poor establishment of both strains of *H. contortus* in controls compared to the levels usually reported in sheep (see Section 1.2.5) does question the applicability of these results to goats under field conditions. Nevertheless, haemonchosis is a common problem for goats of all ages in the field. The isolate of *H. contortus* used in Experiment 2.2 does not appear to be have been well-adapted to goats. However, no specific data are available on this strain's development in sheep. Little experimental work has been done with *O. circumcincta* in goats. Appropriate studies with this species would address the issue as to whether there was a difference in immune response between the small intestine and abomasum. It would also provide a further contrast with development of resistance to *T.*

colubriformis as well as *H. contortus*. As a non blood-sucking nematode it more closely parallels *T. colubriformis* than *H. contortus* and would not be susceptible to any immune mediators found only in blood which may possibly affect *H. contortus*.

In the studies designed to monitor antiparasite activity of mucus or contents and cellular changes in the mucosa, the cellular changes, particularly those of mast cells were of special interest. Goats can clearly mount a mast cell response to the presence of nematodes which is indicated by the number of GLs present in Experiment 3.1. However, the relationship between GL numbers and antiparasite activity of intestinal mucus was confounded by a tendency for GL numbers to decline with duration of infection when Angora-type goats, with an apparently less successful immune response, were infected with *T. colubriformis*. The actual function, if any, of GLs is still undetermined but it seems unlikely that they are simply effete, partly degranulated MMCs. As in sheep, MMC numbers *per se* do not appear to vary with the level of parasitism which, if GLs do only come from MMCs, illustrates the deficiencies of point estimates of a dynamic state. There is no evidence from Experiment 7.1 that eosinophils make a substantial contribution to an immune response in goats. However, circulating eosinophil levels were elevated in both Experiments 2.1 and 3.1 suggesting that in these particular Saanen goats the numbers of eosinophils in the mucosa may have differed between trickle-infected and control groups.

The LMI assay as used here to study the host's response to *T. colubriformis* has the appeal that it measures a direct effect of part of the host's response on the nematode itself. In doing so, the assumption is that components of the host's response are sufficiently stable to survive a three hour incubation at 37°C during which they permanently affect the larvae, or that they also persist for many more hours in agar during the overnight incubation at room temperature. As both leukotrienes and components of LMI activity are considered to produce a reversible paralysis of larvae (Douch *et al.*, 1983) then, if leukotrienes are involved, they must presumably remain stable in the agar. The assay conditions are obviously artificial and it may well be that components of the host's immune response do not survive unchanged during this procedure. There are no reports of anyone measuring levels of any of the likely individual components of this inhibitory activity (e.g. leukotrienes) during the assay or in similar conditions.

It is considered that the effector response to the presence of *T. colubriformis* is multifactorial (McClure *et al.*, 1992). The results of Experiments 7.1 and 8.1 both suggest that LMI activity does not necessarily reflect the ability of the host to reject *T. colubriformis* and, in this respect, are similar to the results of Gray *et al.* (1992). The rapid rejection observed by McClure *et al.* (1992) in immune sheep may not have occurred in these goat experiments thus allowing nematodes to establish. These same authors proposed that rejection of adults may involve other mechanisms and these may not be measured by the LMI assay. Taken together, there is considerable scope for further dissection of the components responsible for LMI activity (and parasite rejection) in ruminants. The putative factor(s) which actually stimulate larval migration from gels during the assay is an example of just such a component.

The parasitological findings in this series of experiments generally suggest that *T. colubriformis* develops similarly in sheep, cattle and goats although the strain used here in goats may possibly have a slightly shorter minimum prepatent period. The establishment of only 25% of the infective dose noted in Experiment 3.1 after the strain had been passaged once through goats, compared with 71%, 39% and 63% in the young goats of 4.1 and in older goats in 5.1 and 7.1 respectively, further suggests this strain became better adapted to goats after being passaged through them several times. However, the worm counting procedure used in Experiment 3.1 did not include pepsin digestion and may have underestimated the infection by as much as a half.

The absence of inhibited larvae in Experiment 5.1 compared to the large percentage found in Experiment 8.1 is interesting as some goats in 5.1 had very low worm counts. If host regulation is important for inducing inhibition as suggested by Dobson *et al.* (1990b), then some inhibited L3s would have been expected in these animals. The difference between these two experiments suggests host immunity is not necessarily involved, in agreement with the earlier report by Waller *et al.* (1981), and that environmental conditions for free-living larval stages (such as time in storage) may be important as they can be for other nematode species (Gibbs, 1986). The appearance of inhibited L4 *H. contortus* in Experiment 2.2 in the absence of detectable host resistance also suggests that environmental conditions had influenced the tendency of these larvae to inhibit.

These studies have illustrated that some goats can develop solid resistance to *T. colubriformis* but the situation with *H. contortus* appears to be

different suggesting some variation in the immune response. The work has also provided some insight into the mucosal mast cell response of goats and the production of antiparasite substances from the mucosa. It provides baseline data for future direct comparisons between goats and sheep which could be particularly rewarding in determining patterns of mast cell recruitment, mediator release and the role of GLs.

APPENDIX 1a

A table of experimental results detailing the development of resistance to *H. contortus*

Age when sensitised[1]	Method of sensitising[2]	Anthelmintic given	Time to challenge after last sensitising dose[4]	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
7 mths	10,000 L3 - irradiated with 10KR (x-ray)	no	117 days	8000	>99%	not stated	Jarrett *et al.*, 1959
7 mths	10,000 L3 - irradiated with 20KR (x-ray)	no	117 days	8000	>99%	not stated	"
7 mths	10,000 L3 - irradiated with 40KR (x-ray)	no	117 days	8000	90% (pns)	not stated	"
7 mths	10,000 L3 - irradiated with 60KR (x-ray)	no	117days	8000	>99%	not stated	"
7 mths	10,000 L3	no	117 days	8000	70% (pns)	not stated	"
8-9 mths	2x10,000 L3 -35 days apart - irradiated with 40KR (x-ray)	no	29 days	10,000	99%	not stated	Jarrett *et al.*, 1961
8-9 mths	2x10,000 L3 -35 days apart irradiated with 40KR (x-ray)	no	29 days	50,000	>99%	not stated	"
7-8 mths	2000 L3 day 0 + 5000 L3 day 35 (all irradiated with 60KR - x-ray)	no	35 days	10,000	70% (pns) 6 very resistant 4 developed moderate burdens	MxB L	Mulligan *et al.*, 1961
2-5 mths	2x1500 L3 - 30 days apart	no	30 days	5x1000 - daily	0%	DH	Manton *et al.*, 1962
2-5 mths	30x100 L3 - over 60 days	no	30 days	5x1000 - daily	0%	DH	"
10-13 mths	2x4500 L3 - 30 days apart	no	30 days	5x3000 - daily	>99%	D	"
10-13 mths	2x4500 L3 - 30 days apart	no	30 days	5x3000 - daily	>99%	D	"
5.5-6 mths	14x10,000 L3 - daily	PTZ day 19	18-71 days	46 daily doses of 10,000	FEC 96% lower than control 4 wks after the start of challenge	3/4SB	Christie *et al.*, 1964a

Age when sensitised[1]	Method of sensitising[2]	Anthelmintic given	Time to challenge after last sensitising dose[4]	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
6-6.4 mths(26-27.5wks)	10x10,000 L3-daily	TBZ on day 11 & 15	12 days	51,000	58% (p<.001)	3/4SB	Christie *et al.*, 1964b
1.5 - 3mths at start	3000 L3 as single dose or as 100 L3/d for 30 days	not relevant	not challenged	not challenged	incr. EL4 in trickle infected vs single dose group (pns)	3/4Mx 1/4BL	Dineen *et al.*, 1965
10-14 mths	2x10,000 L3 30 days apart - irradiated with 40KR (x-ray)	no	30 days	50,000	84-100% (pns)	SB	Bitakaramire, 1966
2.2-3.0 mths (66-92 days)	25,000 L3 on day 1, 2, 3, 4, 5, 6, 18, 19, 21, 22, 23, 24, 25, 26	TBZ on day 7, 11, 27, 31	15 days	50,000	71% (p<.001)	3/4SH -SB	Christie & Brambell, 1966
3.6-4.5 mths (111-138 days) at start	500, 1000, 1500, 2000, 2500 or 3000 L3	TBZ day 56 and 63 and again after 1st challenge on day 126 and 133	1st challenge on day 70, 2nd challenge of on day 140	3000 day 70 and 3000 day 140	no effect on worm count from either challenge in any sensitisation group. Slight incr. EL4	MxB L	Dineen and Wagland, 1966a
6.8-10.5 mths (206-321 days)	(3000 L3/2weeks)x6	TBZ 90 days after first sensitizing dose	14 days	3000	80% (p<.001)	MxB L	Dineen and Wagland, 1966b
"	"	no	"	"	not significant (challenge was additive)	"	"
3-4 mths	1x10,000 or 2x10,000 L3 30 days apart - all irradiated with 40KR (x-ray)	no	35 or 60 days	20,000	no protection in either group (pns)	SB	Urquhart *et al.*, 1966
1.2-2.1 mths (5-9 wks)	2x10,000 L3 4 wks apart - irradiated with 40KR (x-ray)	no	30 days	10,000	not significant	SB	Urquhart *et al.*, 1966
3-3.9 mths (13-17 wks)	2x10,000 L3 4 wks apart - irradiated with 40KR (x-ray)	no	30 days	10,000	not significant	"	"

Age when sensitised[1]	Method of sensitising[2]	Anthelmintic given	Time to challenge after last sensitising dose[4]	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
4.8-5.8 mths (21-25 wks)	2x10,000 L3 4 wks apart - irradiated with 40KR (x-ray)	no	30 days	10,000	64% ($p<.05$)	"	"
birth to adult	natural infection on pasture	TBZ when penned. Pasture control group not treated	0 to 30 days	10,000	establishment rate: 16-20% in anthelmintic treated sheep but 10% in pasture control group when adjusted for existing burden. (pns)	EAM	Lopez & Urquhart, 1967
6.5-7 mths	2X10,000 L3 - X-irradiated	no	30 days	10,000	32% (pns)	"	"
6.5-7 mths (reared on pasture)	"	TBZ before vaccination	30 days	10,000	0%	"	"
24 mths	"	no	30 days	10,000	86% (pns)	"	"
3.1-5.2 mths (95-158 days)	(3000 L3/week)x6	TBZ 7 and 14 days after the last sensitising dose	21, 35, 63 or 119 days	3000	21 days, 14%; 35 days, 50%; 63 days, 46%; 119 days,10%. Overall sensitised < controls ($p<.001$). Max. effect seen 35-63 days ($p<.05$)	MxB L	Wagland and Dineen, 1967
3.3-6.0 mths (100-182 days) at the start	(3000 L3/2 weeks)x4	TBZ 14 days after last sensitising dose	98 days	3000	21% < challenge control (ns)	M	Donald *et al.*, 1969
"	(3000 L3/2 weeks)x4 and 3000 L3 22 days later	TBZ 8 days before and 14 days after the last sensitising dose	76 days	"	31% < challenge control (ns)	"	"
"	"	TBZ 8 days before and 68 days after the last sensitising dose	"	"	9% < challenge control (ns)	"	"

Age when sensitised[1]	Method of sensitising[2]	Anthelmintic given	Time to challenge after last sensitising dose[4]	Challenge (L3)	Protection from challenge[4]	Breed[3]	Reference
"	(3000 L3/2 weeks)x4 and 3000 L3 76 days later	TBZ 8 days before and 14 days after the last sensitising dose	22 days	"	31% < challenge control (ns)	"	"
"	(3000 L3/2 weeks)x4	TBZ 90 days after the last sensitising dose	98 days	"	82% < challenge control ($p<.01$)	"	"
"	(3000 L3/2 weeks)x4 and 3000 L3 22 days later	TBZ 8 days before the last sensitising dose	76 days	"	43% < challenge control (ns) & 11% < sensitised unchallenged control (ns)	"	"
"	(3000 L3/2 weeks)x4 and 3000 L3 76 days later	TBZ 62 days before the last sensitising dose	22 days	"	15% > challenge control (ns) & 58% > sensitised unchallenged control ($p<.05$)	"	"
"	(3000 L3/2 weeks)x4	no	98 days	"	95% < challenge control ($p<.01$) & 93% < sensitised unchallenged control ($p<.05$) [self-cure]	"	"
5 mths	standardised dose based on liveweight	no	not challenged	not challenged	worm establishment: FN 15.5%, R 33.3% ($p<.05$)	R & FN	Radhakrishnan *et al.*, 1972
3,6,9,21 mths	12,375 L3	no		not challenged	worm count: 21mths < 3,6,9 mths ($p<.05$)	PD	Knight & Rodgers, 1974
3,12,23,32-35 mths	12,375 L3	no		not challenged	worm count: 12, 32-35 mths < 23 mths < 3 mths ($p<.05$)	"	"
2.3-3.2 mths (10 - 14 weeks)	2x10,000 L3 4 wks apart - irradiated with 60KR (^{60}Co)	TBZ week 3 and 7	28 days	10,000	not significant	SB	Benitez-Usher *et al.*, 1976

Age when sensitised[1]	Method of sensitising[2]	Anthelmintic given	Time to challenge after last sensitising dose[4]	Challenge (L3)	Protection from challenge[4]	Breed[3]	Reference
"	2x100,000 L3 4 wks apart - irradiated with 60KR (^{60}Co)	"	"	"	"	"	"
"	2x1,000,000 L3 4 wks apart - irradiated with 60KR (^{60}Co)	"	"	"	"	"	"
2.3 mths (10 wks) at start	10,000 L3 at wk 28 & 32 (age 38 & 42 wks) - irradiated with 60KR (^{60}Co)	TBZ on week 27,31 and 35	28 days	10,000	not significant	SB	"
"	1-2000 L3 given at weeks 0,4,8,12,16,20 & 24 and then 10,000 irradiated (60KR ^{60}Co) L3 at wk 28 & 32 (age 38 & 42 wks)	"	"	"	"	"	"
7.8-8.8 mths (34-38 weeks)	2x10,000 L3 4 wks apart - irradiated with 60KR (^{60}Co)	no	28 days	10,000	97% ($p<.001$)	SB	"
"	"	TBZ three wks after each sensitising infection	"	"	not significant	"	"
2.3 mths (10 wks) at start	1-200 L3 given on weeks 0,4,8,12,16 and 20 and then 10,000 irradiated (60KR ^{60}Co) L3 at wk 24 & 28 (aged 34 & 38 wks)	TBZ on weeks 3,7,11,15,19 and 23	28 days	10,000	46% not significant	SB	"
"	"	TBZ on week 23 only	"	"	88% ($p<.001$)	"	"
"	10,000 L3 irradiated with 60KR (^{60}Co) - at wk 24 & 28 (aged 34 & 38 wks)	"	"	"	96% ($p<.001$)	"	"

Age when sensitised[1]	Method of sensitising[2]	Anthelmintic given	Time to challenge after last sensitising dose[4]	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
7 months	350 L3/Kg	TBZ day 35	53 days	10,000	no sig. protection but sensitised were 20% smaller ($p<.001$) & removed less blood ($p<.001$). No sig. diff. between HbAA & HbAB	SB	Altaif and Dargie, 1978
8 mths	500 L3/Kg	no	28 days	20,000	SB had 75% less worms than FD ($p<.001$) SB had 50% less worms than SB challenge controls (only 2 control sheep)	SB, FD	"
14-15 mths	10,000x2 L3 28 days apart - all irradiated with 60KR (^{60}Co)	no	28 days	10,000	95% ($p<.01$)	SxGF	Smith & Angus 1980
1.4-3 mths (6-13 wks)	10,000x2 L3 28 days apart - all irradiated with 60KR (^{60}Co)	no	28 days	10,000	not significant (1 lamb < 10% control burden)	"	"
1.4-3.2 mths (6-14 wks)	10,000x4 L3 14 days apart - all irradiated with 60KR (^{60}Co)	no	14 days	10,000	not significant (3 lambs < 10% control burden)	"	"
6-7 mths	5000 L3	no	1st chall - 385 days 2nd chall - 700 days	1st & 2nd chall - 10,000	1st chall - 66% decrease in FEC ($p<.01$) 2nd chall - 65% decrease in FEC ($p,<.01$)	M	Adams & Beh, 1981

Age when sensitised[1]	Method of sensitising[2]	Anthelmintic given	Time to challenge after last sensitising dose[4]	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
from 6 mths (pasture reared)	600, 1200, 2400 or 4800 L3/wk for 15 wks	no	not challenged	not challenged	establishment & %EL4 not affected by larval intake. Establishment decreased from 45% in wks 1 & 4 to very low levels in wks 10 & 13 ($p<.001$). EL4 increased from 0% to approx 70% in wk 12 ($p<.001$). Worm burdens increased in proportion to infection level until wk 9 when 600 & 1200 plateaued whereas 2400 & 4800 fell to low levels	M	Barger *et al.*, 1985
1.4-3.7 mths (6-16 weeks)	6000 L3	LEV day 42	56 days	12,000	SC < FN & B < DO ($p<.05$)	B, DO, FN, SC	Courtney *et al.*, 1985
3.2-4.2 mths (14-18 weeks)	6000 L3	LEV day 35	42 days	12,000	SC < 3/4SC < FN, B, DO & DO challenge controls ($p<.05$)	B, DO, FN, SC, 3/4SC	"
7-9 mths	DP pasture reared, ZI worm-free	IV at start	not stated	1600X3	ZI < DP ($p<0.01$)	DP, ZI	McKenzie, 1987
up to 4 mths (pasture reared)	natural infection on pasture	each month 4 lambs given OXF+LEV 2 wks before slaughter when 4 worm-free tracers put on pasture. These were compared to continuously grazed lambs	lambs continuously being infected on pasture except for tracers which were put on pasture for 2 weeks before slaughter	natural infection on pasture	worm burdens in drenched lambs = tracer lambs until 8 mths. Continuously grazed < both tracer and drenched lambs until 8 mths of age ($p<.05$)	M	Barger, 1988

Age when sensitised[1]	Method of sensitising[2]	Anthelmintic given	Time to challenge after last sensitising dose[4]	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
9-11 mths	10,000 L3 then after 6 weeks given 5x10,000/wk for 6 weeks	FBZ 1 day after last sensitising infection	7, 42 and 84 days	$1X10^6$ L3	83% ($p<0.001$), 94% ($p<0.001$) and 0% respectively	GFX S	Jackson *et al.*, 1988
"	"	"	7 days	$1X10^4$, $1X10^5$ or $1X10^6$ L3	0%, 77% ($p<0.01$) and 91% ($p<0.001$) respectively	"	"
up to 6-7 mths	natural infection on pasture	only at Day 0	6 days	25 or 100L3/wk	establishment rate did not decline. Wk 2 was 59% and wk 11 was 43%	M	Stanton, 1989
7 mths	10,000x2 L3 28 days apart (all irradiated with 40 KR)	no	28 days	10,000	97% protection on high protein diet ($p<.05$) & 99% protection on low protein ($p<.05$)	SB	Abbott & Holmes, 1990
from 5 mths	500x3 L3/wk for 17 wks. Fed either 400gms or 600gms of pellets/day	no	not challenged	not challenged	from wk 8 FECs of 600gms/day were < 400gms/day ($p<.05$). From wk 13 FECs of 600gms/day had dropped to low levels whereas 400gms/day remained high	M	Roberts & Adams, 1990
9-10 mths	20,000 L3	LEV 63 days after sensitising infection	77 days	16,000	76% protection; no difference between breeds	DHX R, SC, FN	Zajac *et al.*, 1990
2 mths	2500 L3	FBZ 42 days after sensitising infection	45 days	2500	FEC of SC(10 epg) < DH(241 epg)	DH, SC	Gamble and Zajac, 1992

KEY

1. Includes the period during which sensitisation occurred plus the age variation of experimental animals. All animals were raised under worm-free conditions unless stated otherwise. If ages were not stated in months they were converted by dividing days by (365/12 = 30.42) and weeks by (52/12 = 4.33). In these cases the original description is given in brackets.
2. This describes the method used to attempt to stimulate an immune response
3. TBZ = thiabendazole; PTZ = phenothiazine; FBZ = fenbendazole; OXF = oxfendazole; LEV = levamisole

4. This is expressed as a percentage reduction of a control group's worm count: ns = not significant; pns = probability not stated in the reference; FEC = faecal egg count; epg = eggs/g
5. B = Barbados; BL = Border Leicester; D = Downs; DO = Domestic (1/2S or DH, 1/4FD, 1/4R); DH = Dorset Horn; DP = Dorper; EAM = East African Merino; FD = Finn Dorset; FN = Florida Native; GF = Greyface; M = Merino; PD = Polled Dorset; R = Rambouillet; S = Suffolk; SB = Scottish Blackface; SC = St Croix; SH = Scottish Hill; ZI = Zimbabwean indigenous

APPENDIX 1b

A table of experimental results detailing the development of resistance to *T. colubriformis*

Age when sensitised[1]	Primary/sensitising infection schedule[2]	Anthelmintic given following primary infection[3]	Time to challenge after primary infection	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
8-10 weeks at start	24X10,000 L3/week	TBZ or PTZ weekly, every 4 weeks, every 12 weeks, once at 24 weeks or none	start week 24 (32-34 weeks of age)	10X20,000/week	all groups except weekly TBZ resistant to challenge: "4 weekly" & "12 weekly" group resistant to reinfection after 12 weeks: untreated group showed gradually falling FEC but still had substantial WC at week 36	not stated	Gibson *et al.*, (1970)
8, 12, 16, 20, 24, 28, 32, 36 weeks at start	10,000 L3/weekX36 - 8 groups starting at different ages indicated	no	not challenged	not challenged	sheep over 24 weeks better able to develop resistance to infection	not stated	Gibson and Parfitt, (1972)
11-16 weeks at start	10,000 L3/week - sheep killed at 5 weekly intervals X 9	no	not challenged	not challenged	WC increased up to 20 weeks after first infection (between 33 and 38 weeks of age) then decreased	DH	Gibson and Parfitt, (1973)
3 weeks at start	L3/day doubled every 7 days starting at 10 L3/day up to 5000 L3/day which was then maintained up to 20 weeks	no	not challenged	not challenged	WC cumulative during 1st 12 weeks then refractory to new infection ie. at 15 weeks of age. Adult burdens persisted	DHXF	Chiejina and Sewell, (1974a)
3, 5 or 6 mths at start	30,000 L3/week for up to 20 weeks - sheep killed at intervals	no	not challenged	not challenged	FEC peaked 28-40 days and declined exponentially. Mean peak FEC and subsequent rate of fall varied inversely with age at start	DHXF	Chiejina and Sewell, (1974b)

Age when sensitised[1]	Primary/sensitising infection schedule[2]	Anthelmintic given following primary infection[3]	Time to challenge after primary infection	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
6-9 mths	2X20,000 L3 - 21 days apart - γ-irradiated with 50KR (Co^{60})	TBZ (time after sensitisation not stated)	39 days	20,000	81% ($p<0.01$)	M	Dineen *et al.* (1977)
3 mths or 10 mths	2X20,000 L3 - 14 days apart - γ-irradiated with 50KR (Co^{60})	no	21 days	4X10,000/week	38% and 92% protection (WC) (53% and 91% reduction in total egg output) from challenge for "3 mth" and "10 mth" groups	M	Gregg *et al.* (1978)
9-10 mths	3X20,000 L3 - 14 days apart - γ-irradiated with 50KR (Co^{60})	half treated with TBZ - Day 49	28 days - Day 56	one-half 40,000; second-half 4X10,000/week	protection (WC) > 98% all vaccinated sheep	M	Gregg and Dineen (1978)
12-14 weeks	2X30,000 - 14 days apart - γ-irradiated with 50KR (Co^{60})	no	3 weeks (17 weeks old) or 23 weeks (37 weeks old)	4X10,000/week	10/16 "17 week-olds" were responders; 12/14 "37 week-olds" were responders (responder = lower than 99% confidence interval of control WC): these ratios were not significantly different	M	Dineen *et al.*, (1978)
1-4.5 mths	30,000 L3 total - γ-irradiated with 50KR (Co^{60}); divided at 1, 2 &/or 3 months: all combined with a sequential infection as for Chiejina and Sewell 1974a till 4.5 mths including control	LEV at 4.5 mths - Day 98	52 days (25.5 weeks old) - Day 150	10,000	no difference between vaccination regimes or sequential infection alone: ewe FEC < ram FEC ($p<0.05$): % responders greater for those vaccinated earlier (responder = FEC < lower 95% confidence interval of control)	M	Windon *et al.*, (1980)

Age when sensitised[1]	Primary/sensitising infection schedule[2]	Anthelmintic given following primary infection[3]	Time to challenge after primary infection	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
8-12 weeks	2X20,000 L3 - 14days apart - γ-irradiated with 50KR (Co^{60})	LEV - 4 weeks after 2nd sensitising dose	5 weeks (17 weeks old)	20,000	56% decrease in wether WC ($p<0.001$): wether responder WC < non-responder WC ($p=0.063$): ewe responder FEC < non-responder FEC ($p<0.05$)	M	Dineen and Windon, (1980)
"	"	"	"	"	eggs in utero, male/female ratio and worm length were less in responders ($p<0.05$)	"	Dineen and Windon, (1980)
18-27 weeks at start	300, 950, 3000, 9500 or 30,000 L3/week X24	no	no	no	FEC proportional to infection for 8-10 weeks then declined abruptly between 16-20 weeks with 300 L3 group being slower to fall; worm burdens decreased after egg counts	M, MXBL, MXS,	Steel *et al.* (1980)
17-21 wks	2X80,000 L3 - 4 weeks apart - γ-irradiated with 50KR (Co^{60}); sheep fed either a high or low plane diet	LEV (25 weeks of age)	35 days	30,000	78% (WC) high plane (pns); 53% (WC) low plane (pns); difference between the two groups ($p<0.03$)	M	Wagland *et al.*, (1984)
12-16 weeks	2X(5000 or 20,000 or 80,000)L3 - 28 days apart - γ-irradiated with 50KR (Co^{60})	LEV (20 weeks of age)	35 days	5000 or 80,000	ram lambs less responsive to vaccination than ewe lambs; threshold vaccine dose is about 2X4400; protection generally improved with increasing size of vaccine dose	M	Windon *et al.* (1984)
8-12 weeks	2X(10,000 or 40,000 or 80,000 or 160,000) - 28 days apart - γ-irradiated with 50KR (Co^{60})	LEV (16 weeks of age)	35 days	20,000		M	"

Age when sensitised[1]	Primary/sensitising infection schedule[2]	Anthelmintic given following primary infection[3]	Time to challenge after primary infection	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
15 mths	2X20,000 - 14 days apart - γ-irradiated with 50KR (Co^{60})	no	0 days	natural infection on pasture for 9-10 weeks	vaccinated < unvaccinated ($p<0.05$ at 9 weeks; $p=0.1$ at 10 weeks)	M	"
from 4 months (pasture reared)	natural infection on pasture	each month 4 lambs were given OXF+LEV 2 weeks before slaughter when 4 worm-free tracers were put on pasture for 2 weeks	lambs continously infected on pasture.	natural infection on pasture	OXF+LEV group WC < tracer WC ($p<0.05$) at 8 months but no difference at 7 months	M	Barger (1988)
8-10, 12-14 and 16-18 weeks	2X2000 L3/Kg for the 3 age groups - 16 days apart	TBZ 12 days after each sensitising infection	14 days	same as sensitising	sensitised FEC < control FEC ($p<0.01$) all three groups; sensitised WC < control WC ($p<0.01$)for 12-14 and 16-18 week groups only; proportion of responders was similar in each group (about 0.5)	R	Douch (1988)
5 mths at start	34X2500 L3 - 1 week apart	no	24 weeks	10X2500 L3 - 1 week apart	FEC peaked at week of sensitising infection and reduced to 0 at week 14; no eggs detected after challenge	SXF	Kimambo *et al.* (1988a, 1988b)
5-6 mths	2X200,000 L3 - 15 days apart + a further 20,000	TBZ 12 days after each sensitising infection	0 days	natural infection on pasture for 4 weeks	87% ($p<0.001$)	R	Douch (1989)
21 weeks at start	1000, 3160 or 10,000 L3/week	no	no	no	establishment fell to <5% in 7, 10 and 14 weeks respectively	M	Dobson *et al.* (1990a)
birth to 19 weeks on pasture	natural infection on pasture	LEV+TBZ when housed at 19 weeks of age	7 days	10,000/week for up to 5 weeks	nil	M	Dobson *et al.* (1990b)

Age when sensitised[1]	Primary/sensitising infection schedule[2]	Anthelmintic given following primary infection[3]	Time to challenge after primary infection	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
birth to 35 weeks on pasture	"	LEV+TBZ when housed at 35 weeks of age	"	"	100%	M	Dobson *et al.* (1990b)
12, 20 or 36 weeks at start	10,000 L3/week	not relevant	not relevant	not relevant	decline in establishment rate to negligible levels was in proportion to age at start. In "12 week-old" took 9 weeks: in "36 week-old" took 5 weeks	M	Dobson *et al.* (1990b)
25 weeks at start	1000, 3160, 5620 or 10,000 L3/week	"	"	"	loss of adults occurred at approx. the same time (7-9 weeks) for the 3 highest dose rates whereas 1000 L3/week was delayed 5 weeks. Rejection period was the same for all (approx. 9 weeks)	M	Dobson *et al.* (1990c)
6 mths at start	30,000 L3 in Weeks 1, 14, 19, 26 and 39	LEV at Weeks 13 and 42	28 days (Week 43)	5000 or 8500 adults	89% or 72% protection from adult challenge (pns)	M	Emery *et al.*, (1992a)
8 mths at start	4X100,000 L3 @ 10 or 14 day intervals	LEV given either 4, 7 or 10 days after each primary infection	3-7 days after final LEV	30,000 L3	0% (4X4 days); 70% (4X7 days; $P<0.01$); 91% (4X10 days; $p<0.001$)	M	"
8 mths at start	5X100,000 L3 @ 10 day intervals	LEV 7 days after each primary infection	8-9 days after final LEV	30,000 L3 or 8000 adults into the duodenum	>90% to L3 challenge; 55% to adult challenge (pns)	M	"
8 mths	20,000 L3 or 2X20,000 γ-irradiated with 50KR (Co^{60}) 28 days apart or 9000 adult surgically transferred	LEV - Week 8	7 days after LEV	20,000 L3	84%, 85% or 64% respectively (pns); all but one sheep in adult transfer group considered immune (< 95% confidence interval)	M	Emery *et al.*, (1992b)

Age when sensitised[1]	Primary/sensitising infection schedule[2]	Anthelmintic given following primary infection[3]	Time to challenge after primary infection	Challenge (L3)	Protection from challenge[4]	Breed[5]	Reference
12 mths	9000 adults surgically transferred	no	14 weeks	30,000 L3 into duodenum	sensitised sheep had significantly fewer worms after 10 days but not significantly different at 2,3 or 6 days	M	"
12 mths	9000 adults surgically transferred	LEV at 14 weeks	10 days after LEV	30,000 L3 into duodenum	sensitised sheep had significantly fewer worms after 7 and 10 days but not after 3 or 5 days	M	"

KEY

1. Includes the period during which sensitisation occurred plus the age variation of experimental animals. All animals were raised under worm-free conditions unless stated otherwise
2. This describes the method used to attempt to stimulate an immune response
3. TBZ = thiabendazole; PTZ = phenothiazine; LEV = levamisole
4. This is generally expressed as a percentage reduction of a control group's worm count (WC) or faecal egg count (FEC) unless stated otherwise: ns = not significant; pns = probability not stated in the reference
5. BL = Border Leicester; DH = Dorset Horn; F = Finn; M = Merino; R = Romney; S = Suffolk

APPENDIX 2a

Formulation of pelleted ration

This ration comprises:

60% lucerne meal
20% barley meal
20% bran

As lucerne meal has 8.9 ppm Cu and barley meal has 6.2 ppm Cu it is desirable to have 2 ppm Mo and about 0.35% SO_4 to avoid copper toxicity. This can be achieved by adding sodium molybdate and sodium sulphate to the diet.

To achieve this the following can be added:

	sodium molybdate anhydrous	3.98 g/tonne
or	sodium molybdate ($2H_2O$)	4.80 g/tonne
	sodium sulphate anhydrous	4.4 kg/tonne
or	sodium sulphate ($7H_2O$)	8.5 kg/tonne
or	glaubers salt ($NaSO_4.10H_2O$)	10.1 kg/tonne

APPENDIX 2b

Modified McMaster technique for counting strongylate eggs

2 g of faeces are mixed manually with 28 ml of saturated NaCl (specific gravity 1.2) in a bowl and then sieved through a coarse mesh (aperture about 0.85 mm). Eggs are counted in two 0.15 ml aliquots using a McMaster counting slide (Paracount-EPG, Olympic Equine Products, Issaquah, U.S.A.) with the result that each egg counted represents 50 eggs/g. For a more sensitive test where the count was 0 a simple faecal floatation was used. To achieve this the remainder of the filtrate is placed in a universal bottle, topped up to form a meniscus, a coverslip placed on top and left for 15 minutes. This is then removed in a positive upward motion, placed on a microscope slide and examined at 100X for the presence of eggs

APPENDIX 2c

Pepsin digest technique (pepsin/HCl)

Sections of small intestine or the abomasum were added to the following:

pepsin (1200 units/g, Riedel-de Haën, Germany)	20 g
water	600 ml
concentrated HCl	10 ml

Tissue to be digested was incubated with the pepsin/HCl for 2 hours at 37°C. 150 ml was allowed per 1 metre length of small intestine.

APPENDIX 2d

Pepsinogen assay

This is a modification of the method described by Uete *et al.* (1969).

Principle

Serum pepsinogen is converted by dilute HCl to pepsin, which then splits the serum proteins to give peptides possessing tyrosine end groups which are soluble in trichloracetic acid (TCA). Folin-ciocalteau reagent gives a colour reaction with the tyrosine end groups, which, under the given conditions, is proportional to the concentration of pepsinogen in serum.

Method

1. Into each of two centrifuge tubes labelled "test" and "control" pipette:
   0.5 ml serum
   1.5 ml 0.1 N HCl
   1.0 ml water
2. To the control tube immediately add 2.0 ml 10% TCA to deproteinise.
3. Incubate test tube for 3 hours in a 37°C waterbath, then add 2.0 ml 10% TCA.
4. Let tubes stand 10 minutes then centrifuge.
5. Pipette 2 ml supernatant into clean tubes for Test samples,
   pipette 2 ml water into a tube for the Blank,
   pipette 1.0 ml working standard, 0.2 ml distilled water and 0.8 ml 10% TCA into tube for Standard.
6. Add 4 ml 0.5 N NaOH to all tubes, then 1.0 ml dilute Folin-Ciocalteau reagent and mix.
7. Read absorbance within 3-20 minutes at 700 nm.

Calculation

$$\frac{\text{Test - Control}}{\text{Standard - Blank}} \times \frac{0.2^{a}}{} \times \frac{1000}{0.2^{b}} \times \frac{1}{180^{c}}$$

$$= \frac{T - C}{S - B} \times 5.5 \quad \text{IU/litre at 37°C}$$

a = Moles tyrosine in standard
b = volume of serum coloured up
c = incubation time in minutes

Reagents

Stock Tyrosine Standard Stable for at least 4 weeks	- dissolve 181 mg tyrosine in 0.1 N HCl and make to 500 ml.
Working Standard	- dilute stock solution 1:10 before use
Folin-Ciocalteu Reagent	- dilute commercial stock reagent (Folin-Ciocalteu phenol reagent, BDH, U.K.) with 2 volumes of distilled water before use.

Appendix 2e

Faecal egg counts of Experiment 2.1

	Group 1.1					Group 1.2					Monitor Goats		
Goat No.	**1**	**4**	**6**	**9**	**12**	**3**	**5**	**7**	**8**	**10**	**A**	**B**	**C**
Day													
0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0	0
22	50	0	0	100	300	0	0	0	0	0	450	100	50
24	150	0	0	200	950	0	0	0	0	0	600	200	0
26	100	300	100	0	650	0	0	0	0	0	750	550	100
29	150	300	300	150	350	0	0	0	0	0	950	0	250
31	100	250	350	50	100	0	0	0	0	0	350	50	100
33	150	150	500	0	150	0	0	0	0	0	400	350	100
36	250	50	600	0	650	0	0	0	0	0	1400	400	150
38	150	50	700	50	450	0	0	0	0	0	900	600	50
40	150	50	1450	50	450	0	0	0	0	0	950	400	150
43	250	150	650	300	850	0	0	0	0	0	1400	450	100
45	450	400	650	200	900 (200)[2]	0	0	0	0	0	1450 (150)	400 (50)	350
47	800	350	1850	0	800 (50)	0	0	0	0	0	2500 (100)	600	300
50	500 (50)	200	1000	0	600	0	0	0	0	0	1100	650	400
52	nf[3]	300	1750	50	1250	0	0	0	0	0	1000	600	300
54	450	400	1350	0	1400	0	0	0	0	0	700	400	150
57	nf	350	1400	50	1450	0	0	0	0	0	300	150 (50)	400
59	300	400	1300	0	1900 (200)	0	0	0	0	0	750	50	300
61	500	700 (50)	2050 (150)	100	800	0	0	0	0	0	300	150	300

	Group 1.1					Group 1.2					Monitor Goats		
Goat No.	**1**	**4**	**6**	**9**	**12**	**3**	**5**	**7**	**8**	**10**	**A**	**B**	**C**
Day													
65	300	450 (50)	1300 (150)	100	250	0	0	0	0	0	700	600	250 (50)
67	1000	400 (50)	1950 (50)	150	300 (50)	0	0	0	0	0	1950 (200)	1100 (50)	1200 (100)
68	450 (50)	550	750	50	650	0	0	0	0	0	1700	550	450
71	200	1200	1800	50	800	0	0	0	0	0	1100	400	1000
73	450 (50)	900	2100	0	1350 (100)	0	0	0	0	0	1000	850 (150)	850 (50)
75	250	400	2000 (50)	50	1100	0	0	0	0	0	1750	300	550
78	150	100	1500	0	600	0	0	0	0	0	900	50	400
80	250	50	2650 (50)	0	1600	0	0	0	0	0	1100	150	150
82	400	100	1800	50	1650	0	0	0	0	0	350	100	300
85	350 (50)	300 (50)	2100	100	1350	0	0	0	0	0	-	-	550
92[1]	0	0	0	0	0	0	0	0	0	0	-	-	-
96[1]	0	0	0	0	0	0	0	0	0	0	-	-	-
113[1]	0	0	0	0	0	0	0	0	0	0	-	-	-
115[1]	0	0	0	0	0	0	0	0	0	0	-	-	-
120[1]	0	0	0	0	39	0	0	20	6	0	-	-	-
122	50	0[1]	0[1]	0[1]	750	1[1]	1[1]	400	50	7[1]	-	-	-
124	50	11[1]	2[1]	0[1]	950	0[1]	0[1]	1300	350	150	-	-	-
126	250	250	50	0[1]	3450	0[1]	50	1400	350	800	-	-	-
128	350	200	0[1]	0[1]	4350	0[1]	50	3100	1100	1400	-	-	-

1. faecal float at this time
2. *Nematodirus* spp. eggs shown in brackets
3. nf = no faeces

Appendix 2f

Faecal egg counts of Experiment 2.2, Chapter 2

	Group 2.1								Group 2.2						
Goat No.	**19**	**20**	**22**	**23**	**24**	**26**	**27**	**29**	**15**	**16**	**21**	**25**	**28**	**32**	**33**
Day															
0[1]	0	0	0	0	0	0	0	0	0 (1)[2]	0	0	0	0	0 (2)	0 (1)
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	1 (1)[1]	0 (1)[1]	0[1]	100	0 (1)[1]	0 (1)[1]	0 (1)[1]	0[1]	0	0	0	0	0	0	0
35	0	0	0	200	0	0	0	0	0	0	0	0	0	0	0
42	0	0	50	300	0	0	50	0	0	0	0	0	0	0	0
49	0	0	0	650	200	0	250	100	0	0	0	0	0	0	0
56	250	0	400	1450	0	0	750	300	0	0	0	0	0	0	0
63	250 (50)	0	350	1650	0	200	1000	300	0	0	0	0	0	0	0
70	700 (50)	50	1500	4500	0	850	3000	1200	0	0	0	0	0	0	0
77	50	250	1250	2150	350	700	4650[3]	250	0	0	0	0 (50)	0	0 (50)	0
84	700	200	650	2950	100	750	0	1550	0	0	0 (100)	0	0	0	0
91	1250	400	1450	3900	600	600	0	700	0	0	0 (50)	0	0	0	0
98	1150	450	1750	3000	600	800	0	700	0	0	0	0	0	0	0
137[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
140[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
147[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
154[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
154[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Group 2.1								Group 2.2						
Goat No.	**19**	**20**	**22**	**23**	**24**	**26**	**27**	**29**	**15**	**16**	**21**	**25**	**28**	**32**	**33**
Day															
161[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
168[1]	0	0	0	2	0	0	0	0	1	0	0	1	0	1	0

1. faecal float at this time
2. *Nematodirus* spp. egg count shown in brackets
3. this goat was treated at this time

Appendix 2g

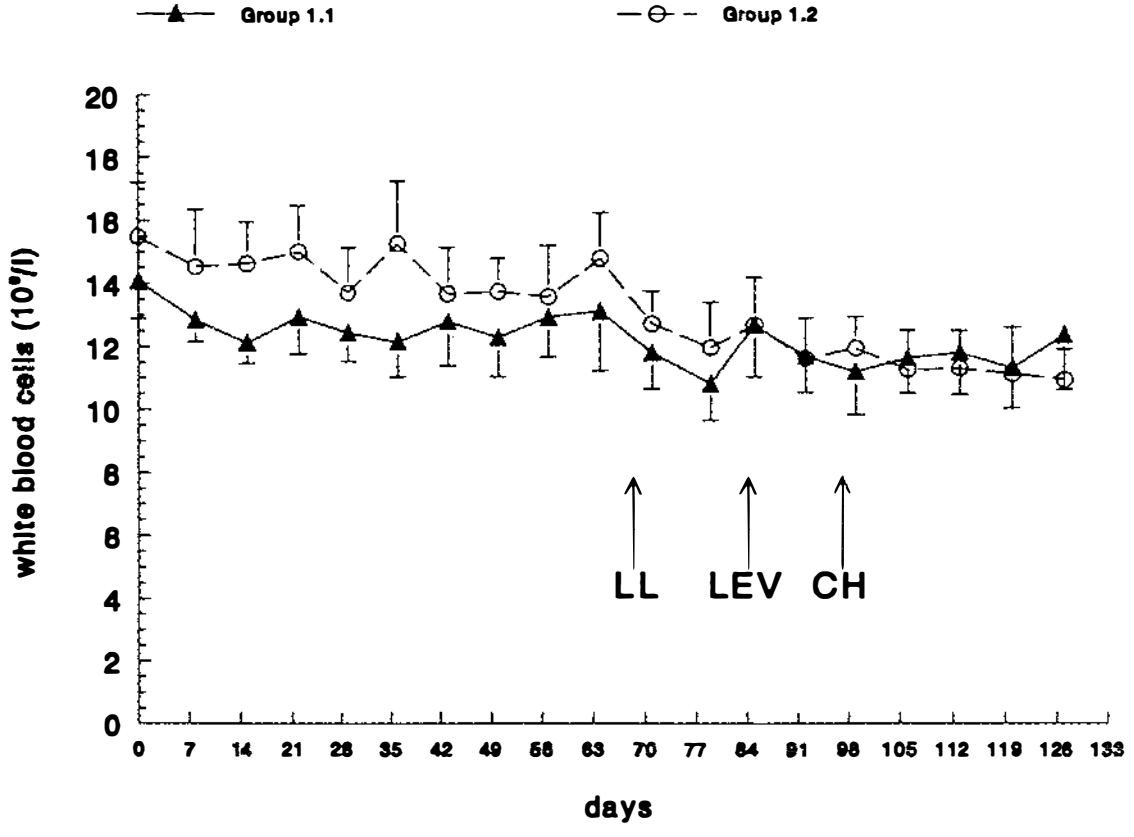
Graphs and Statistical Analyses for Blood Parameters in Experiment 2.1

Keys for the following graphs:

LL = last larvae of the trickle infection given here
LEV = levamisole given here
CH = challenge given here
Group 1.1 = trickle infected
Group 1.2 = control

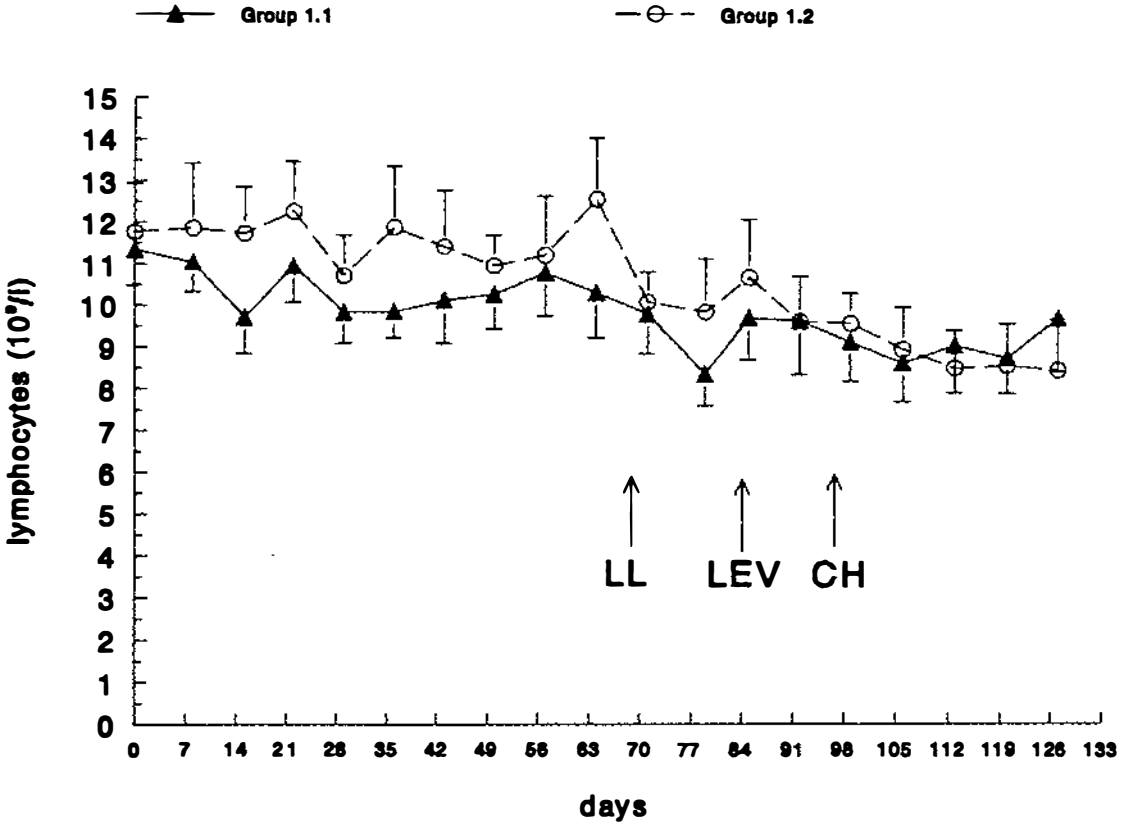
Total White Blood Cells - Experiment 2.1

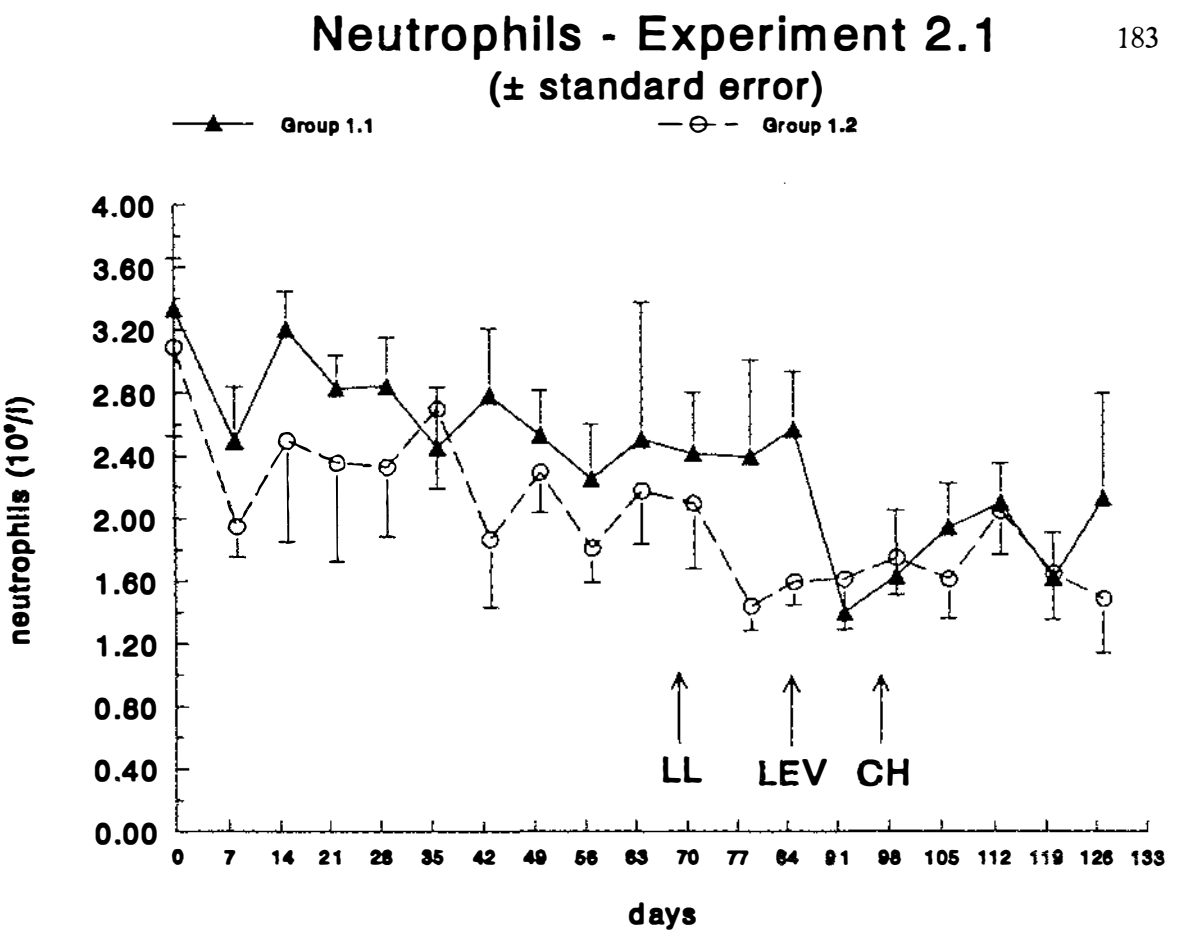
(± standard error)

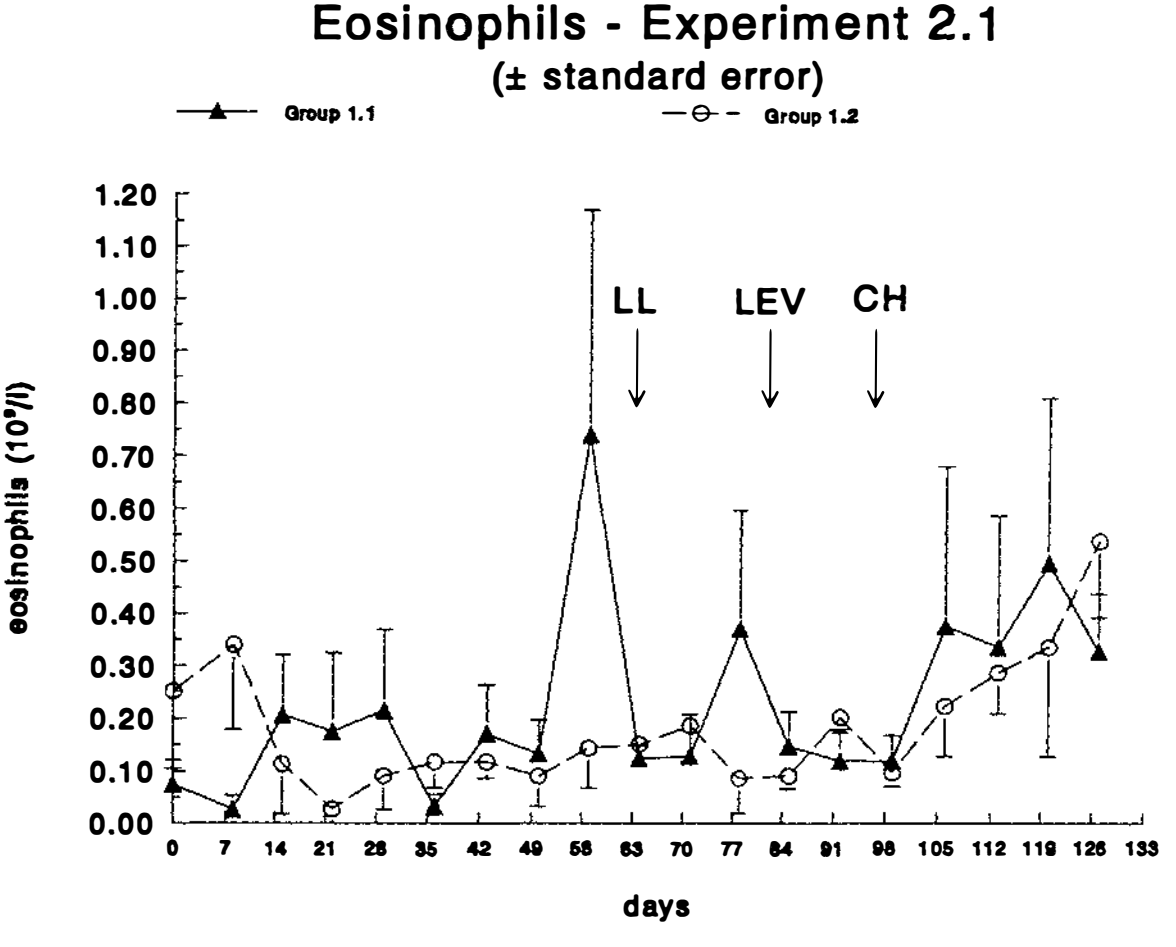


Lymphocytes - Experiment 2.1

(± standard error)

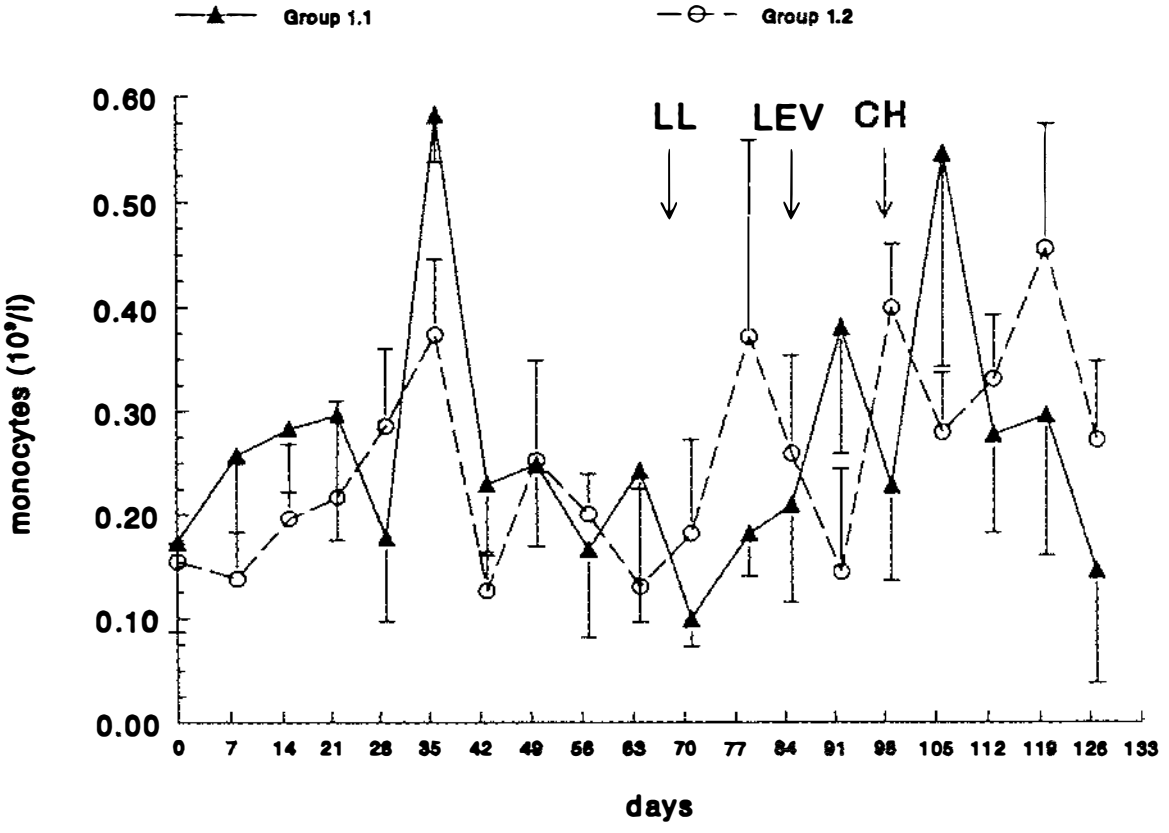



Neutrophils - Experiment 2.1
(± standard error)
Group 1.1
Group 1.2
neutrophils (10⁹/l)
4.00
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
LL
LEV
CH
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112 119 126 133
days


Eosinophils - Experiment 2.1
(± standard error)
Group 1.1
Group 1.2
eosinophils (10⁹/l)
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
LL
LEV
CH
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112 119 126 133
days

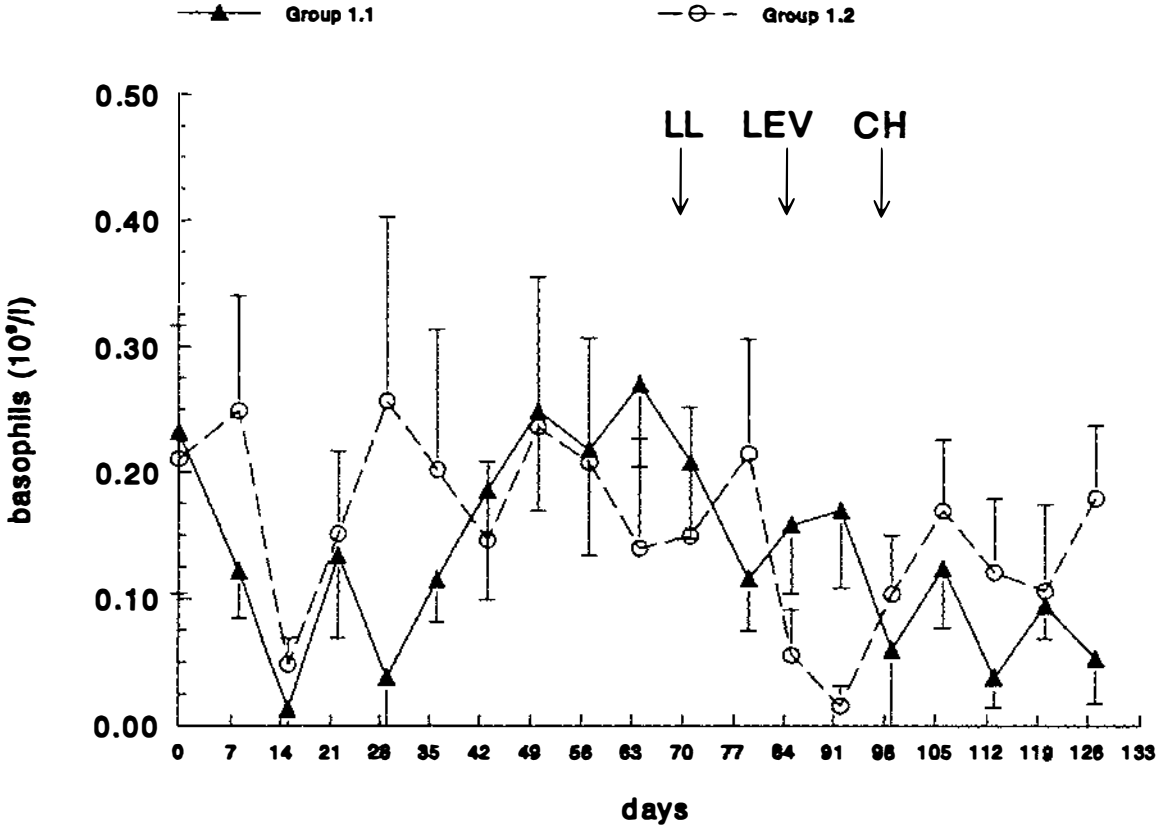
Monocytes - Experiment 2.1

(± standard error)



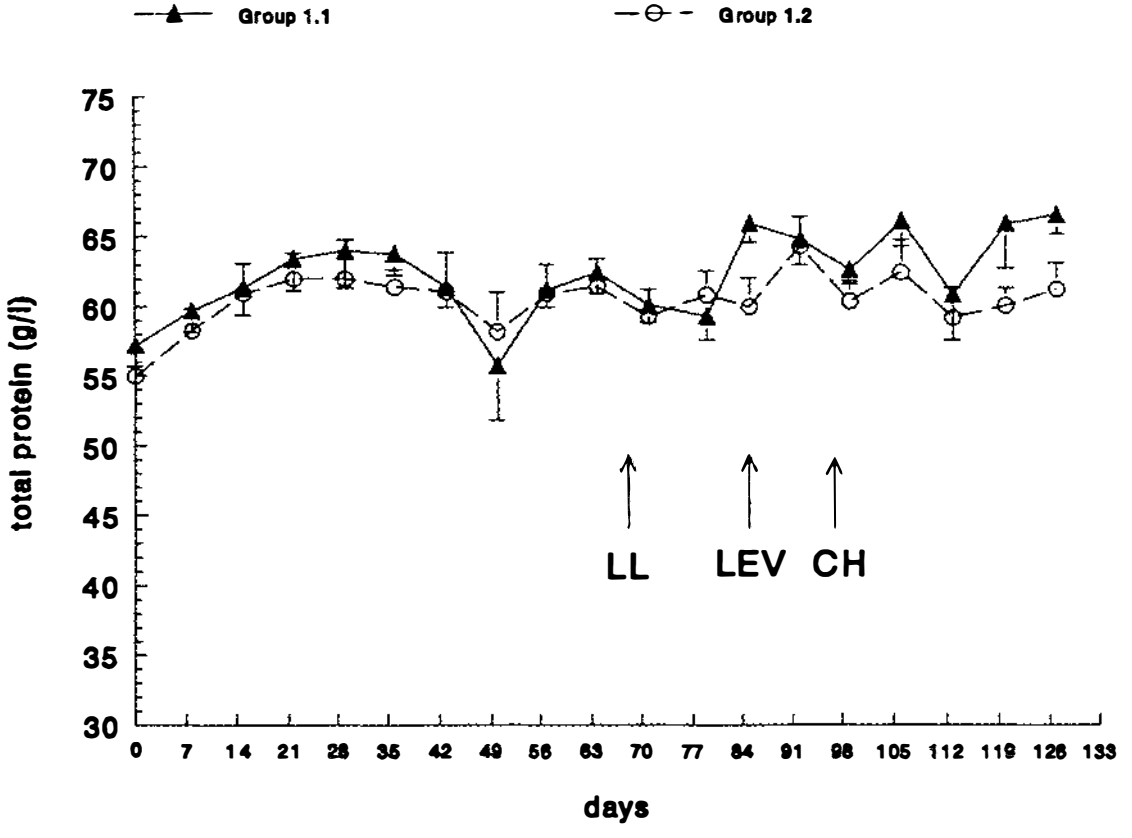
Basophils - Experiment 2.1

(± standard error)



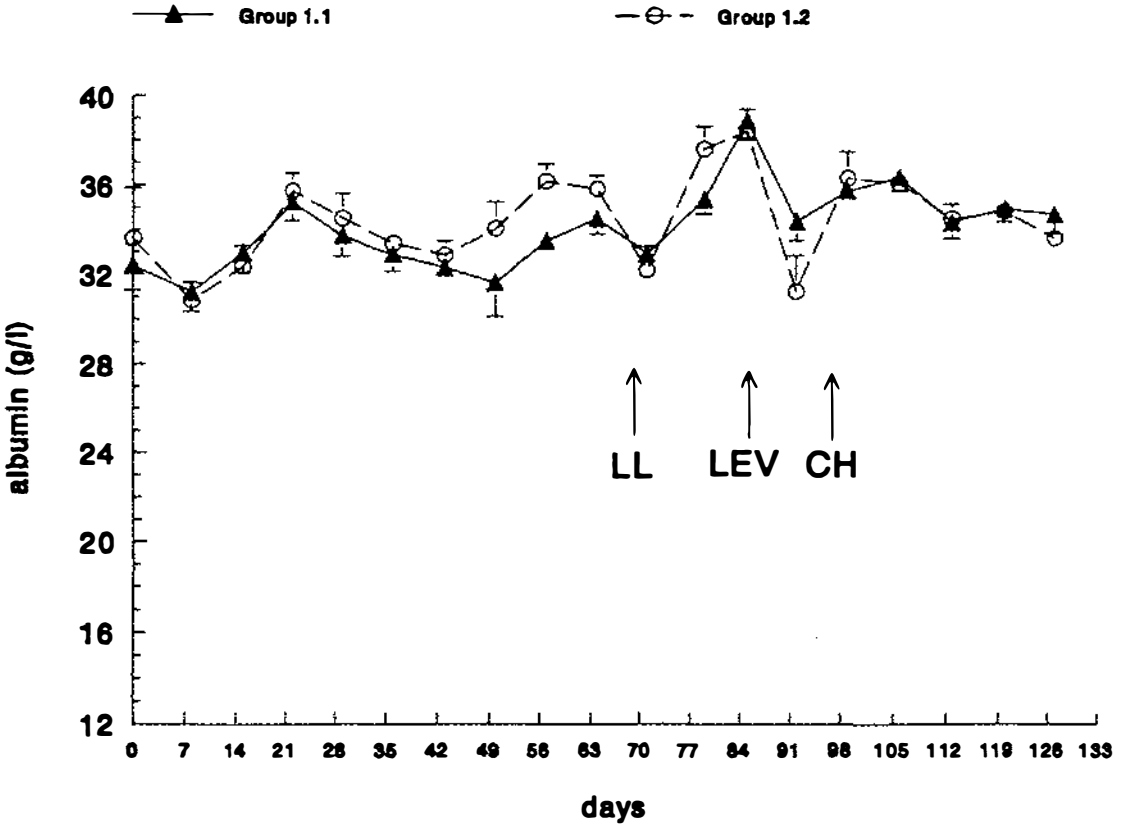
Total Protein - Experiment 2.1

(± standard error)



Albumin - Experiment 2.1

(± standard error)



Experiment 2.1 - Statistical analyses

White blood cells

Analysis of variance of white blood cells during the trickle infection using SAS version 6.04.

(week = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF TOTAL WHITE BLOOD CELLS DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: WBC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	1045.694154	31.687702	25.07	0.0001
Error	96	121.341538	1.263974		
Corrected Total	129	1167.035692			

R-Square	C.V.	Root MSE	WBC Mean
0.896026	8.479612	1.124266	13.2584615

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	68.2587692	68.2587692	54.00	0.0001
GOAT(TRT)	8	870.6984615	108.8373077	86.11	0.0001
WEEK	12	87.0896923	7.2574744	5.74	0.0001
TRT*WEEK	12	19.6472308	1.6372692	1.30	0.2339

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	68.25876923	68.25876923	0.63	0.4512

ANALYSIS OF TOTAL NEUTROPHILS DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: NEUTROPHILS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	55.88604876	1.69351663	3.74	0.0001
Error	93	42.11241207	0.45282164		
Corrected Total	126	97.99846083			

R-Square	C.V.	Root MSE	NEUT Mean
0.570275	29.67257	0.672920	2.26781890

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1.32272716	1.32272716	2.92	0.0908
GOAT(TRT)	8	30.03891048	3.75486381	8.29	0.0001
WEEK	12	18.62770500	1.55230875	3.43	0.0003
TRT*WEEK	12	5.79501673	0.48291806	1.07	0.3972

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1.32272716	1.32272716	0.35	0.5692

ANALYSIS OF TOTAL EOSINOPHILS DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: EOSINOPHILS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	4.76026465	0.14425044	2.32	0.0008
Error	93	5.77459706	0.06209244		
Corrected Total	126	10.53486172			

R-Square	C.V.	Root MSE	EOS Mean
0.451858	147.4871	0.249184	0.16895276

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.08970216	0.08970216	1.44	0.2324
GOAT(TRT)	8	2.19443449	0.27430431	4.42	0.0001
WEEK	12	0.99706016	0.08308835	1.34	0.2108
TRT*WEEK	12	1.46983494	0.12248625	1.97	0.0355

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.08970216	0.08970216	0.33	0.5831

ANALYSIS OF TOTAL BASOPHILS DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: BASOPHILS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	33	2.12008343	0.06424495	2.11	0.0028
Error	93	2.83659992	0.03050107		
Corrected Total	126	4.95668335			

R-Square	C.V.	Root MSE	BAS Mean
0.427722	74.86663	0.174646	0.23327559

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.01071293	0.01071293	0.35	0.5549
GOAT(TRT)	8	0.90063058	0.11257882	3.69	0.0009
WEEK	12	0.83730364	0.06977530	2.29	0.0134
TRT*WEEK	12	0.36023629	0.03001969	0.98	0.4698

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.01071293	0.01071293	0.10	0.7656

ANALYSIS OF TOTAL NEUTROPHILS DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: LYMPHOCYTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	693.1288213	21.0039037	15.97	0.0001
Error	93	122.3298657	1.3153749		
Corrected Total	126	815.4586869			

R-Square	C.V.	Root MSE	LYMPH Mean
0.849986	11.03527	1.146898	10.3930236

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	92.81884176	92.81884176	1.40	0.2699

ANALYSIS OF TOTAL MONOCYTES DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: MONOCYTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	33	1.66384527	0.05041955	2.04	0.0041
Error	94	2.32601829	0.02474488		
Corrected Total	127	3.98986355			

R-Square	C.V.	Root MSE	MONO Mean
0.417018	94.66851	0.157305	0.16616406

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00819827	0.00819827	0.33	0.5663
GOAT(TRT)	8	1.02457286	0.12807161	5.18	0.0001
WEEK	12	0.34957881	0.02913157	1.18	0.3107
TRT*WEEK	12	0.27147311	0.02262276	0.91	0.5361

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00819827	0.00819827	0.06	0.8066

Analysis of variance of white blood cells following the challenge infection using SAS version 6.04

(week = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF TOTAL WHITE BLOOD CELLS FOLLOWING THE CHALLENGE

General Linear Models Procedure

Dependent Variable: WBC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	312.0752000	18.3573647	18.63	0.0001
Error	32	31.5248000	0.9851500		
Corrected Total	49	343.6000000			

R-Square	C.V.	Root MSE	WBC Mean
0.908251	8.645882	0.992547	11.4800000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1.5488000	1.5488000	1.57	0.2190
GOAT(TRT)	8	303.4912000	37.9364000	38.51	0.0001
WEEK	4	1.0880000	0.2720000	0.28	0.8912
TRT*WEEK	4	5.9472000	1.4868000	1.51	0.2229

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1.54880000	1.54880000	0.04	0.8449

ANALYSIS OF TOTAL NEUTROPHILS FOLLOWING THE CHALLENGE

General Linear Models Procedure

Dependent Variable: NEUTROPHIL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	14.88329112	0.87548771	2.22	0.0255
Error	32	12.64057720	0.39501804		
Corrected Total	49	27.52386832			

R-Square	C.V.	Root MSE	NEUT Mean
0.540741	35.00560	0.628505	1.79544000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.38473992	0.38473992	0.97	0.3311
GOAT(TRT)	8	12.38935720	1.54866965	3.92	0.0025
WEEK	4	1.14991372	0.28747843	0.73	0.5796
TRT*WEEK	4	0.95928028	0.23982007	0.61	0.6604

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.38473992	0.38473992	0.25	0.6316

ANALYSIS OF TOTAL EOSINOPHILS FOLLOWING THE CHALLENGE

General Linear Models Procedure

Dependent Variable: EOSINOPHIL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	4.21975602	0.24822094	2.15	0.0304
Error	32	3.69950848	0.11560964		
Corrected Total	49	7.91926450			

R-Square	C.V.	Root MSE	EOS Mean
0.532847	107.6335	0.340014	0.31590000

Source	DF	Type III SS	Mean Square	F Value	Pr > F

TRT	1	0.01503378	0.01503378	0.13	0.7208
GOAT(TRT)	8	3.29826752	0.41228344	3.57	0.0046
WEEK	4	0.67986460	0.16996615	1.47	0.2343
TRT*WEEK	4	0.22659012	0.05664753	0.49	0.7430

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.01503378	0.01503378	0.04	0.8533

ANALYSIS OF TOTAL BASOPHILS FOLLOWING THE CHALLENGE

General Linear Models Procedure

Dependent Variable: BASOPHILS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	0.24406482	0.01435675	1.28	0.2669
Error	32	0.35943840	0.01123245		
Corrected Total	49	0.60350322			

R-Square	C.V.	Root MSE	BAS Mean
0.404413	100.6106	0.105983	0.10534000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.04860962	0.04860962	4.33	0.0456
GOAT(TRT)	8	0.14504160	0.01813020	1.61	0.1598
WEEK	4	0.03100812	0.00775203	0.69	0.6042
TRT*WEEK	4	0.01940548	0.00485137	0.43	0.7845

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.04860962	0.04860962	2.68	0.1402

ANALYSIS OF TOTAL LYMPHOCYTES FOLLOWING THE CHALLENGE

General Linear Models Procedure

Dependent Variable: LYMPHOCYTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	183.1897385	10.7758670	11.57	0.0001
Error	32	29.7960095	0.9311253		
Corrected Total	49	212.9857480			

R-Square	C.V.	Root MSE	LYMPH Mean
0.860103	10.80206	0.964948	8.93300000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.7118631	0.7118631	0.76	0.3884
GOAT(TRT)	8	174.6290929	21.8286366	23.44	0.0001
WEEK	4	3.1410938	0.7852735	0.84	0.5081
TRT*WEEK	4	4.7076887	1.1769222	1.26	0.3045

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.71186312	0.71186312	0.03	0.8612

ANALYSIS OF TOTAL MONOCYTES FOLLOWING THE CHALLENGE

General Linear Models Procedure

Dependent Variable: MONOCYTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	1.44647482	0.08508675	1.77	0.0802
Error	32	1.53956520	0.04811141		
Corrected Total	49	2.98604002			

R-Square	C.V.	Root MSE	MONO Mean
0.484412	67.87867	0.219343	0.32314000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.02971922	0.02971922	0.62	0.4377
GOAT(TRT)	8	0.84400000	0.10550000	2.19	0.0550
WEEK	4	0.24220932	0.06055233	1.26	0.3065
TRT*WEEK	4	0.33054628	0.08263657	1.72	0.1704

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.02971922	0.02971922	0.28	0.6100

Multiple regression of white blood cell counts following challenge in Experiment 2.1 using Statistix version 4.0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF TOTAL WHITE BLOOD CELL COUNTS

PREDICTOR

VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	12.0250	4.74216	2.54	0.0146	
GROUP	-0.35200	0.76303	-0.46	0.6467	1.0
TIME	-0.00100	0.26977	-0.00	0.9971	1.0

R-SQUARED	0.0045	RESID. MEAN SQUARE (MSE)	7.27768
ADJUSTED R-SQUARED	-0.0379	STANDARD DEVIATION	2.69772

SOURCE	DF	SS	MS	F	P
REGRESSION	2	1.54890	0.77445	0.11	0.8993
RESIDUAL	47	342.051	7.27768		
TOTAL	49	343.600			

CASES INCLUDED 50 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF NEUTROPHIL COUNTS

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	1.90152	1.33555	1.42	0.1611	
GROUP	-0.17544	0.21489	-0.82	0.4184	1.0
TIME	0.00924	0.07598	0.12	0.9037	1.0

R-SQUARED	0.0143	RESID. MEAN SQUARE (MSE)	0.57725
ADJUSTED R-SQUARED	-0.0277	STANDARD DEVIATION	0.75977

SOURCE	DF	SS	MS	F	P
REGRESSION	2	0.39328	0.19664	0.34	0.7130
RESIDUAL	47	27.1306	0.57725		
TOTAL	49	27.5239			

CASES INCLUDED 50 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF EOSINOPHIL COUNTS

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	-0.94261	0.69325	-1.36	0.1804	
GROUP	-0.03468	0.11155	-0.31	0.7572	1.0
TIME	0.07709	0.03944	1.95	0.0566	1.0

R-SQUARED	0.0769	RESID. MEAN SQUARE (MSE)	0.15553
ADJUSTED R-SQUARED	0.0377	STANDARD DEVIATION	0.39437

SOURCE	DF	SS	MS	F	P
REGRESSION	2	0.60932	0.30466	1.96	0.1524
RESIDUAL	47	7.30994	0.15553		

TOTAL 49 7.91926

CASES INCLUDED 50 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF BASOPHIL COUNTS

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	-0.02747	0.19091	-0.14	0.8862	
GROUP	0.06236	0.03072	2.03	0.0480	1.0
TIME	0.00231	0.01086	0.21	0.8325	1.0

R-SQUARED	0.0814	RESID. MEAN SQUARE (MSE)	0.01179
ADJUSTED R-SQUARED	0.0423	STANDARD DEVIATION	0.10860

SOURCE	DF	SS	MS	F	P
REGRESSION	2	0.04914	0.02457	2.08	0.1359
RESIDUAL	47	0.55436	0.01179		
TOTAL	49	0.60350			

CASES INCLUDED 50 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LYMPHOCYTE COUNTS

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	10.4230	3.73185	2.79	0.0075	
GROUP	-0.23864	0.60047	-0.40	0.6929	1.0
TIME	-0.06659	0.21230	-0.31	0.7552	1.0

R-SQUARED	0.0054	RESID. MEAN SQUARE (MSE)	4.50703
ADJUSTED R-SQUARED	-0.0369	STANDARD DEVIATION	2.12298

SOURCE	DF	SS	MS	F	P
REGRESSION	2	1.15529	0.57764	0.13	0.8800
RESIDUAL	47	211.830	4.50703		
TOTAL	49	212.986			

CASES INCLUDED 50 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF MONOCYTE COUNTS

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.66667	0.43636	1.53	0.1333	
GROUP	0.04876	0.07021	0.69	0.4908	1.0

TIME	-0.02451	0.02482	-0.99	0.3285	1.0

R-SQUARED	0.0301	RESID. MEAN SQUARE (MSE)	0.06162
ADJUSTED R-SQUARED	-0.0112	STANDARD DEVIATION	0.24824

SOURCE	DF	SS	MS	F	P
REGRESSION	2	0.08979	0.04490	0.73	0.4880
RESIDUAL	47	2.89625	0.06162		
TOTAL	49	2.98604			

CASES INCLUDED 50 MISSING CASES 0

Total Protein and Albumin

Analysis of variance of total protein and albumin during the trickle infection with SAS version 6.04

(week = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF TOTAL PROTEIN DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: TOTAL PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	2106.027385	63.819012	7.35	0.0001
Error	96	833.271385	8.679910		
Corrected Total	129	2939.298769			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.716507	4.858211	2.946169	60.6430769

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	38.995692	38.995692	4.49	0.0366
GOAT(TRT)	8	1345.384615	168.173077	19.37	0.0001
WEEK	12	598.778769	49.898231	5.75	0.0001
TRT*WEEK	12	122.868308	10.239026	1.18	0.3087

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	38.99569231	38.99569231	0.23	0.6430

ANALYSIS OF ALBUMIN DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: ALBUMIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	717.6123077	21.7458275	6.40	0.0001
Error	96	326.3384615	3.3993590		
Corrected Total	129	1043.9507692			

R-Square	C.V.	Root MSE	ALBUMIN Mean
0.687401	5.410509	1.843735	34.0769231

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	19.8510769	19.8510769	5.84	0.0176
GOAT(TRT)	8	149.3135385	18.6641923	5.49	0.0001
WEEK	12	507.3587692	42.2798974	12.44	0.0001
TRT*WEEK	12	41.0889231	3.4240769	1.01	0.4485

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	19.85107692	19.85107692	1.06	0.3326

Analysis of variance of total protein and albumin following challenge in Experiment 2.1 using SAS version 6.04

(week = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF TOTAL PROTEIN FOLLOWING THE CHALLENGE

General Linear Models Procedure

Dependent Variable: TOTAL PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	1019.687200	59.981600	8.83	0.0001
Error	32	217.412000	6.794125		
Corrected Total	49	1237.099200			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.824257	4.170754	2.606554	62.4960000

Source	DF	Type III SS	Mean Square	F Value	Pr > F

TRT	1	178.2272000	178.2272000	26.23	0.0001
GOAT(TRT)	8	680.2800000	85.0350000	12.52	0.0001
WEEK	4	127.2592000	31.8148000	4.68	0.0043
TRT*WEEK	4	33.9208000	8.4802000	1.25	0.3106

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	178.2272000	178.2272000	2.10	0.1857

ANALYSIS OF ALBUMIN FOLLOWING THE CHALLENGE

General Linear Models Procedure

Dependent Variable: ALBUMIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	80.96320000	4.76254118	2.46	0.0137
Error	32	61.94400000	1.93575000		
Corrected Total	49	142.90720000			

R-Square	C.V.	Root MSE	ALBUMIN Mean
0.566544	3.950796	1.391312	35.2160000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.25920000	0.25920000	0.13	0.7168
GOAT(TRT)	8	42.55200000	5.31900000	2.75	0.0198
WEEK	4	34.38520000	8.59630000	4.44	0.0057
TRT*WEEK	4	3.76680000	0.94170000	0.49	0.7455

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.25920000	0.25920000	0.05	0.8308

Multiple regression of total protein and albumin following challenge in Experiment 2.1 using Statistix 4.0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF TOTAL PROTEIN

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	62.1760	8.29461	7.50	0.0000	
GROUP	-3.77600	1.33463	-2.83	0.0068	1.0

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TIME            0.35200       0.47186         0.75       0.4594      1.0

R-SQUARED           0.1541      RESID. MEAN SQUARE (MSE)    22.2656
ADJUSTED R-SQUARED  0.1181      STANDARD DEVIATION          4.71864

SOURCE        DF       SS           MS          F        P
----------    ---   ----------   ----------   -----   ------
REGRESSION     2      190.618      95.3088     4.28    0.0196
RESIDUAL      47      1046.48      22.2656
TOTAL         49      1237.10

CASES INCLUDED 50   MISSING CASES 0


UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF ALBUMIN

PREDICTOR
VARIABLES    COEFFICIENT    STD ERROR     STUDENT'S T       P       VIF
---------    -----------    ---------     -----------    ------    -----
CONSTANT        43.9150       2.78232         15.78       0.0000
GROUP          -0.14400       0.44769         -0.32       0.7491      1.0
TIME           -0.49900       0.15828         -3.15       0.0028      1.0

R-SQUARED           0.1761      RESID. MEAN SQUARE (MSE)    2.50527
ADJUSTED R-SQUARED  0.1410      STANDARD DEVIATION          1.58281

SOURCE        DF       SS           MS          F        P
----------    ---   ----------   ----------   -----   ------
REGRESSION     2      25.1593      12.5797     5.02    0.0106
RESIDUAL      47      117.748      2.50527
TOTAL         49      142.907

CASES INCLUDED 50   MISSING CASES 0
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Packed cell volume

Analysis of variance of PCV during the trickle infection using SAS version 6.04

(rep = time; trt = group; goat(trt) = goat nested within group)

```
      ANALYSIS OF PCV DURING THE TRICKLE INFECTION

                    General Linear Models Procedure

Dependent Variable: PCV
                                    Sum of          Mean
Source                    DF        Squares        Square    F Value     Pr > F

Model                     57     1988.228000     34.881193       9.56     0.0001

Error                    192      700.288000      3.647333

Corrected Total          249     2688.516000

                  R-Square           C.V.       Root MSE            PCV Mean
```

0.739526	5.624335	1.909799	33.9560000	

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	492.804000	492.804000	135.11	0.0001
GOAT(TRT)	8	146.112000	18.264000	5.01	0.0001
REP	24	1176.416000	49.017333	13.44	0.0001
TRT*REP	24	172.896000	7.204000	1.98	0.0063

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	492.8040000	492.8040000	26.98	0.0008

Analysis of variance of PCV following the challenge infection using SAS version 6.04

(rep = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF PCV FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: PCV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	442.7555556	17.7102222	5.55	0.0001
Error	64	204.4000000	3.1937500		
Corrected Total	89	647.1555556			

R-Square	C.V.	Root MSE	PCV Mean
0.684156	5.132087	1.787107	34.8222222

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.7111111	0.7111111	0.22	0.6386
GOAT(TRT)	8	116.0000000	14.5000000	4.54	0.0002
REP	8	261.3555556	32.6694444	10.23	0.0001
TRT*REP	8	64.6888889	8.0861111	2.53	0.0185

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.71111111	0.71111111	0.05	0.8303

Pepsinogen

Analysis of variance of pepsinogen during the trickle infection using SAS version 6.04

(week = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF PEPSINOGEN DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: PEPSINOGEN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	3.04386441	0.12175458	25.24	0.0001
Error	64	0.30872498	0.00482383		
Corrected Total	89	3.35258939			

R-Square	C.V.	Root MSE	PEPSIN Mean
0.907914	22.07607	0.069454	0.31461111

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1.31890028	1.31890028	273.41	0.0001
GOAT(TRT)	8	0.18343822	0.02292978	4.75	0.0001
WEEK	8	0.91230029	0.11403754	23.64	0.0001
TRT*WEEK	8	0.62922562	0.07865320	16.31	0.0001

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1.31890028	1.31890028	57.52	0.0001

Analysis of variance of pepsinogen following the challenge infection with SAS version 6.04

(week = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF PEPSINOGEN DURING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: PEPSINOGEN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	4.44840554	0.26167091	11.15	0.0001
Error	32	0.75130304	0.02347822		
Corrected Total	49	5.19970858			

R-Square	C.V.	Root MSE	PEPSIN Mean
0.855511	20.36984	0.153226	0.75222000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00071442	0.00071442	0.03	0.8626
GOAT(TRT)	8	0.18544616	0.02318077	0.99	0.4640
WEEK	4	4.19756828	1.04939207	44.70	0.0001
TRT*WEEK	4	0.06467668	0.01616917	0.69	0.6051

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00071442	0.00071442	0.03	0.8650

Multiple regression of pepsinogen following challenge using Statistix version 4.0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF PEPSINOGEN

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	-2.47357	0.26517	-9.33	0.0000	
TIME	0.02865	0.00226	12.67	0.0000	1.0
GROUP	-0.00756	0.04477	-0.17	0.8666	1.0

R-SQUARED	0.7735	RESID. MEAN SQUARE (MSE)	0.02506
ADJUSTED R-SQUARED	0.7639	STANDARD DEVIATION	0.15830

SOURCE	DF	SS	MS	F	P
REGRESSION	2	4.02194	2.01097	80.25	0.0000
RESIDUAL	47	1.17777	0.02506		
TOTAL	49	5.19971			

CASES INCLUDED 50 MISSING CASES 0

Gastrin

Analysis of variance of gastrin during the trickle infection with SAS version 6.04

(week = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF GASTRIN DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: GASTRIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	33	78524.19419	2379.52104	6.03	0.0001
Error	94	37117.77456	394.86994		
Corrected Total	127	115641.96875			

R-Square	C.V.	Root MSE	GASTRIN Mean
0.679029	61.49141	19.87133	32.3156250

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	14221.56897	14221.56897	36.02	0.0001
GOAT(TRT)	8	8671.75094	1083.96887	2.75	0.0091
WEEK	12	36297.79692	3024.81641	7.66	0.0001
TRT*WEEK	12	18984.06749	1582.00562	4.01	0.0001

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	14221.56897	14221.56897	13.12	0.0068

Analysis of variance of gastrin following challenge infection with SAS version 6.04

(week = time; trt = group; goat(trt) = goat nested within group)

ANALYSIS OF GASTRIN FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: GASTRIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	72193.96160	4246.70362	4.50	0.0001
Error	32	30208.46720	944.01460		
Corrected Total	49	102402.42880			

R-Square	C.V.	Root MSE	GASTRIN Mean
0.705002	65.41650	30.72482	46.9680000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	8924.48000	8924.48000	9.45	0.0043
GOAT(TRT)	8	30356.05280	3794.50660	4.02	0.0021
WEEK	4	27730.46480	6932.61620	7.34	0.0003
TRT*WEEK	4	5182.96400	1295.74100	1.37	0.2654

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	8924.480000	8924.480000	2.35	0.1637

Multiple regression of gastrin following challenge using Statistix version 4.0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF GASTRIN

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	-152.592	79.1659	-1.93	0.0600	
GROUP	26.7200	12.0727	2.21	0.0318	1.0
TIME	8.86000	4.26834	2.08	0.0434	1.0

R-SQUARED	0.1638	RESID. MEAN SQUARE (MSE)	1821.87
ADJUSTED R-SQUARED	0.1282	STANDARD DEVIATION	42.6834

SOURCE	DF	SS	MS	F	P
REGRESSION	2	16774.4	8387.22	4.60	0.0149
RESIDUAL	47	85628.0	1821.87		
TOTAL	49	1.024E+05			

APPENDIX 2h

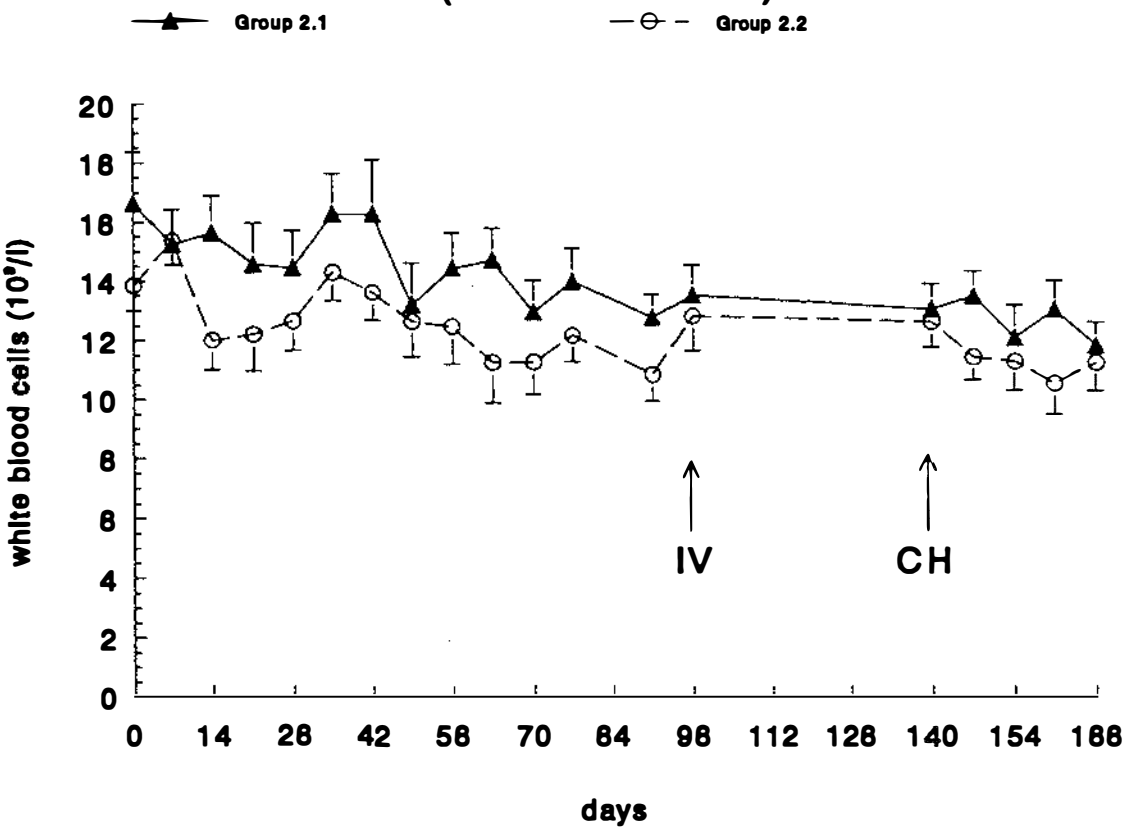
Graphs and Statistical Analyses for Blood Parameters in Experiment 2.2

Keys for the following graphs:

IV = ivermectin given here
CH = challenge given here
Group 2.1 = trickle infected
Group 2.2 = control

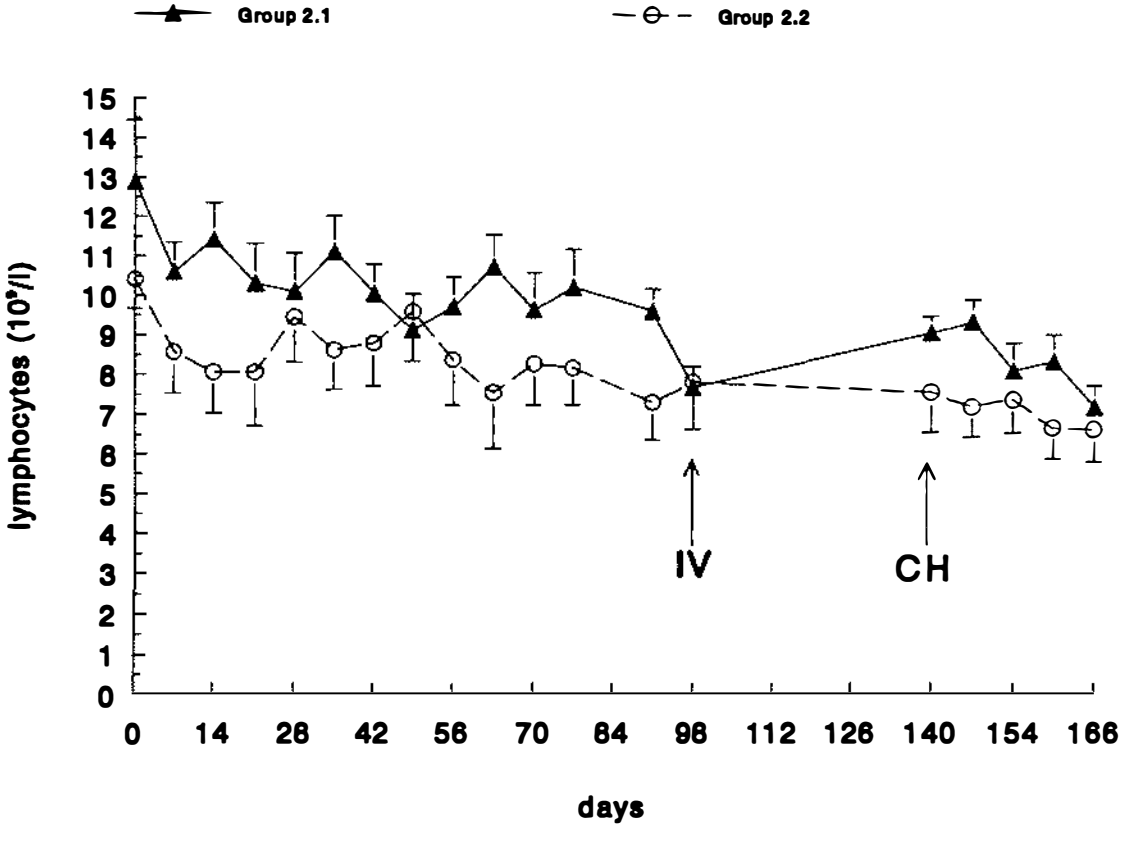
Total White Blood Cells - Experiment 2.2

(± standard error)

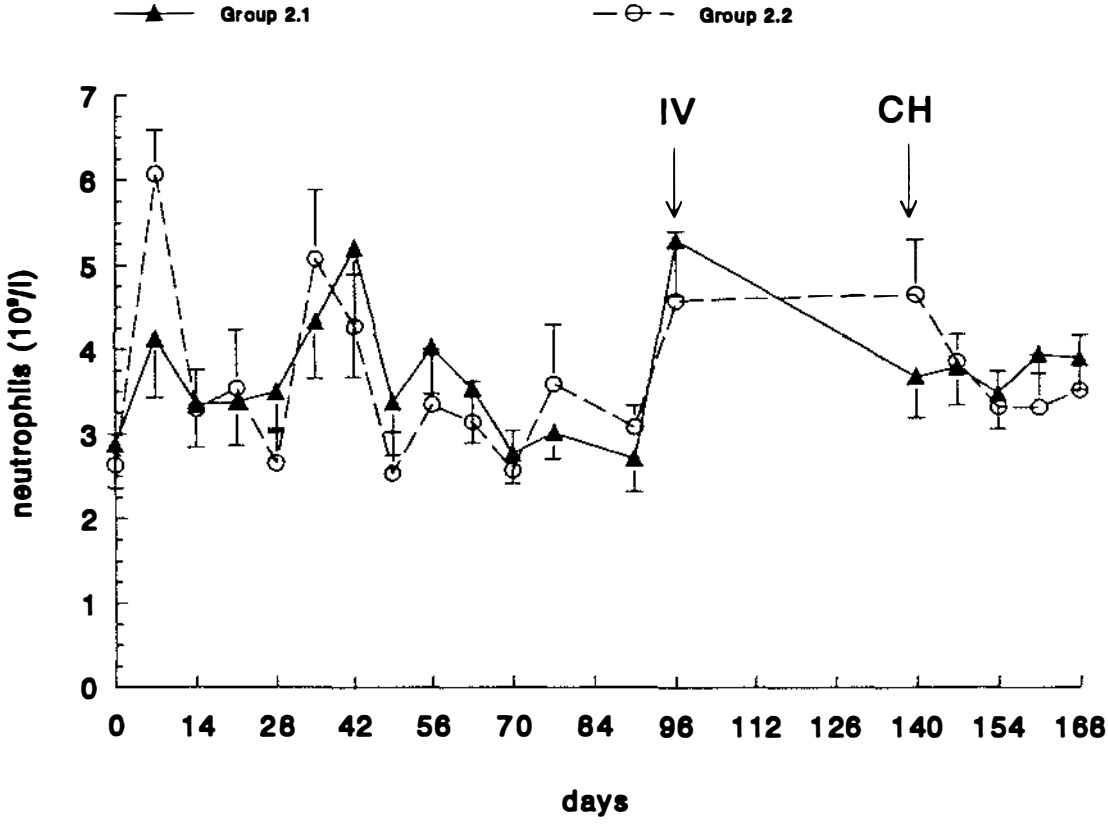


Lymphocytes - Experiment 2.2

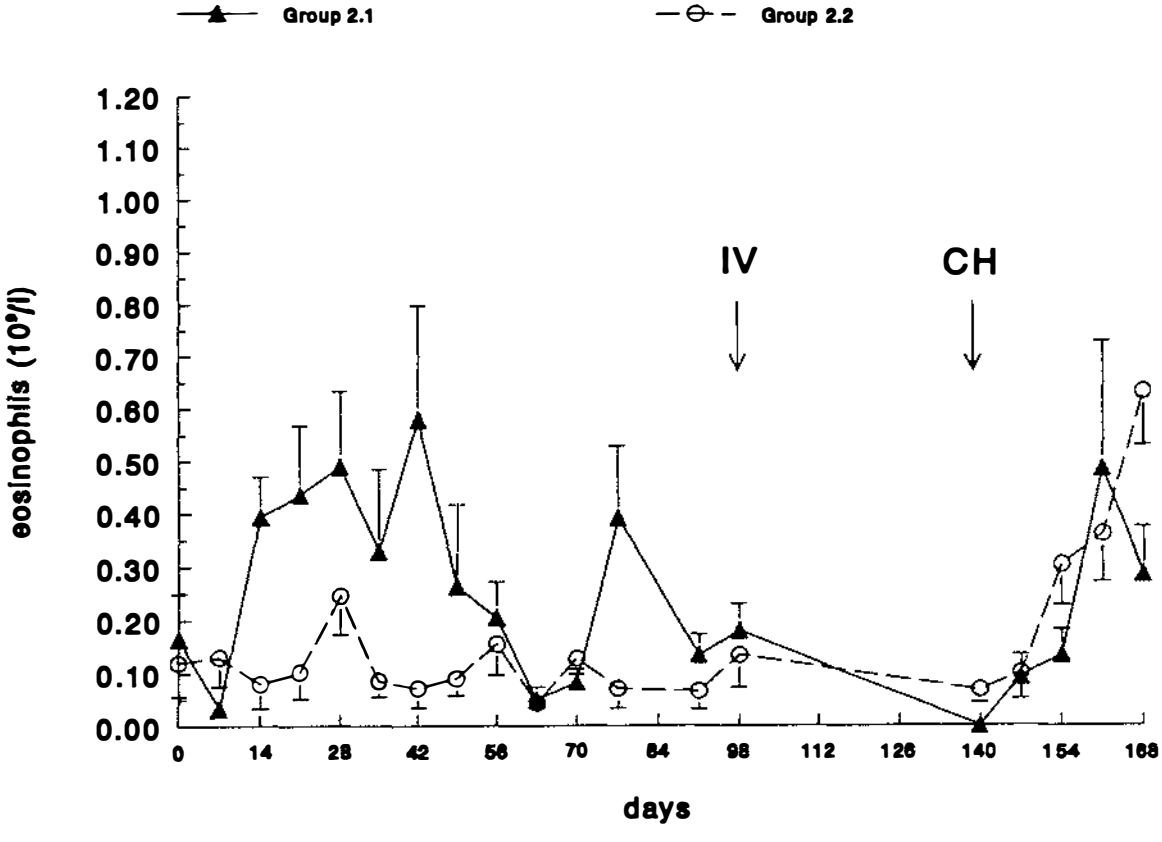
(± standard error)



Neutrophils - Experiment 2.2
(± standard error)

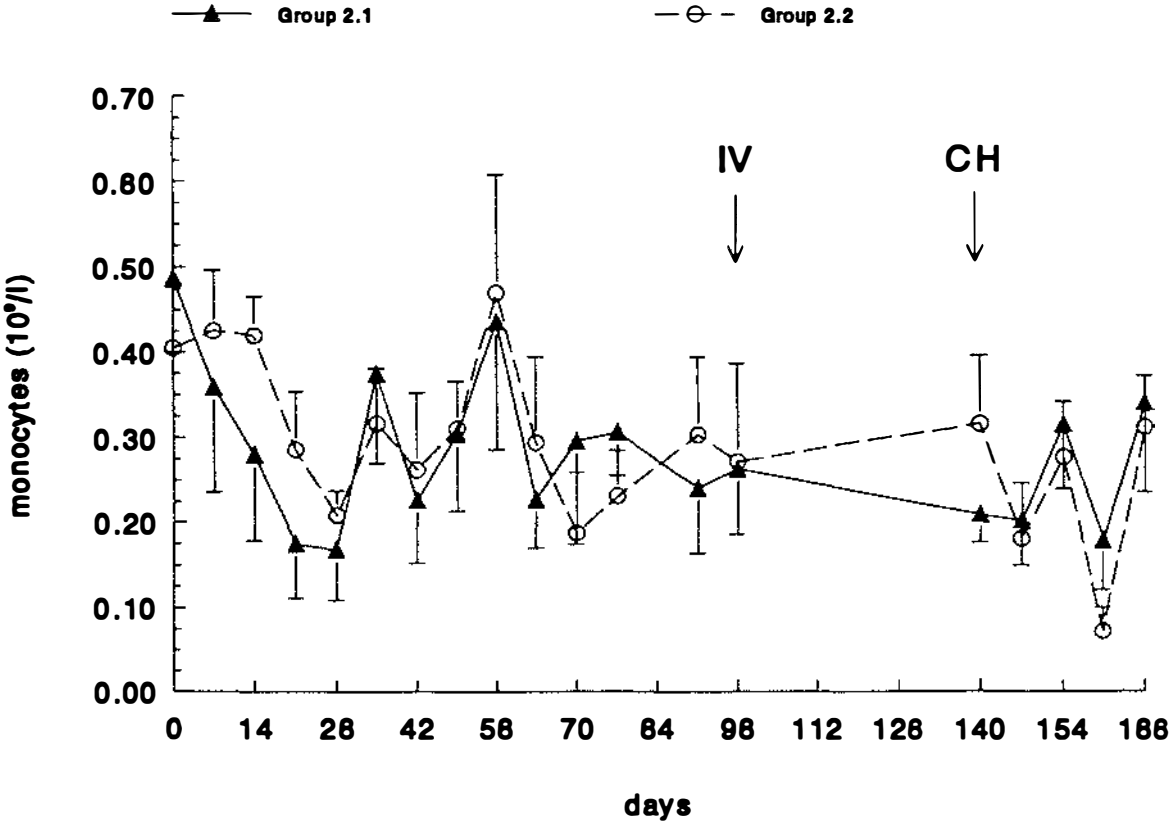


Eosinophils - Experiment 2.2
(± standard error)



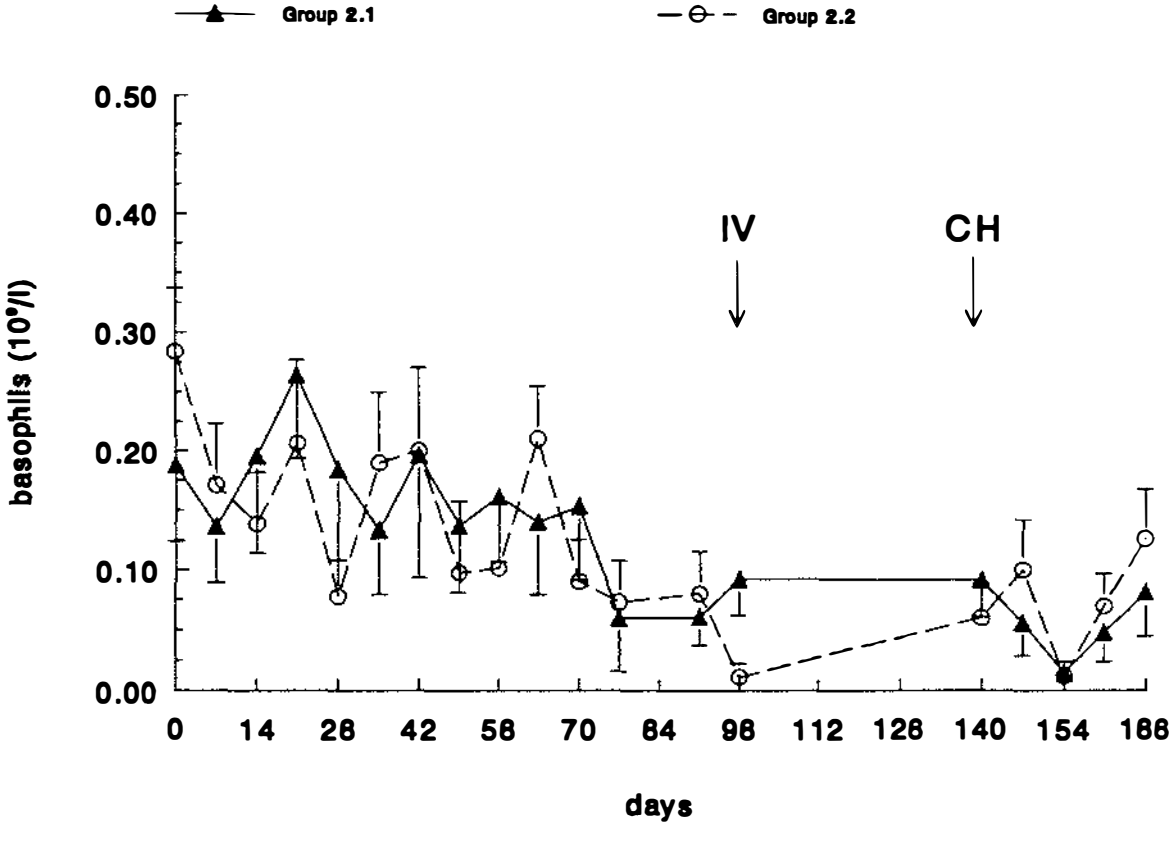
Monocytes - Experiment 2.2

(± standard error)



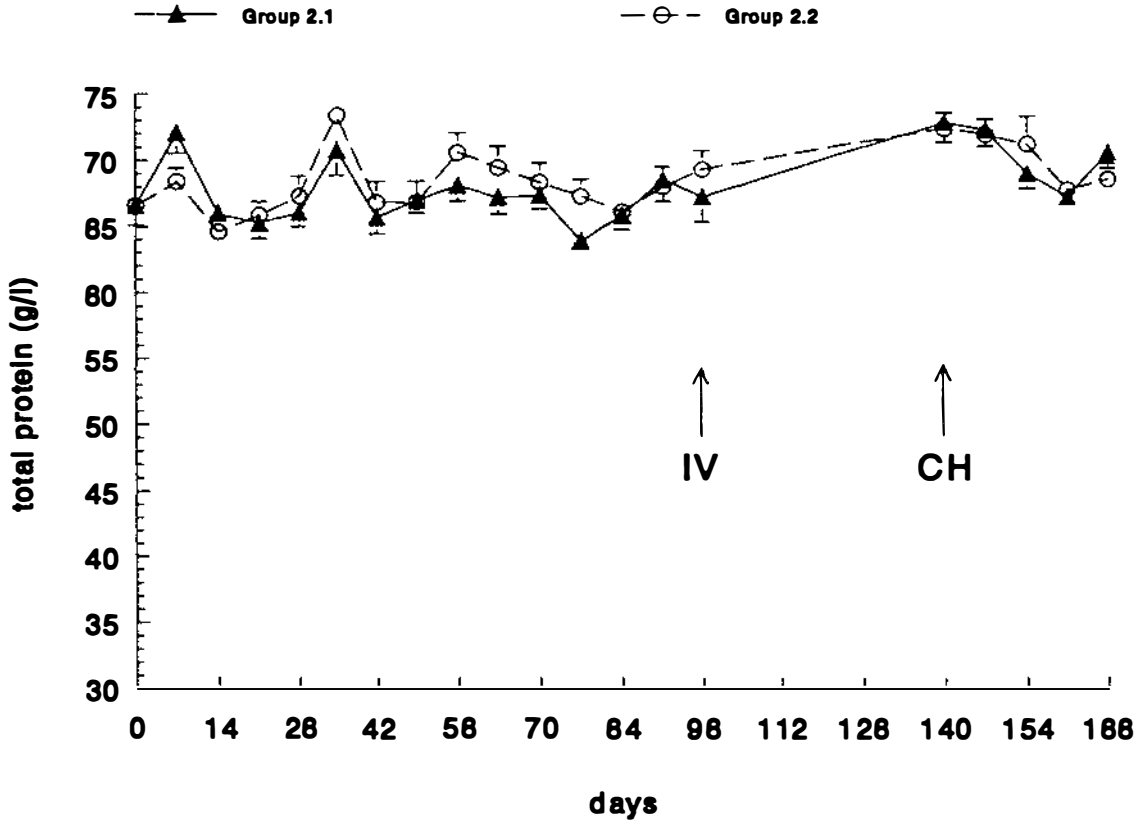
Basophils - Experiment 2.2

(± standard error)



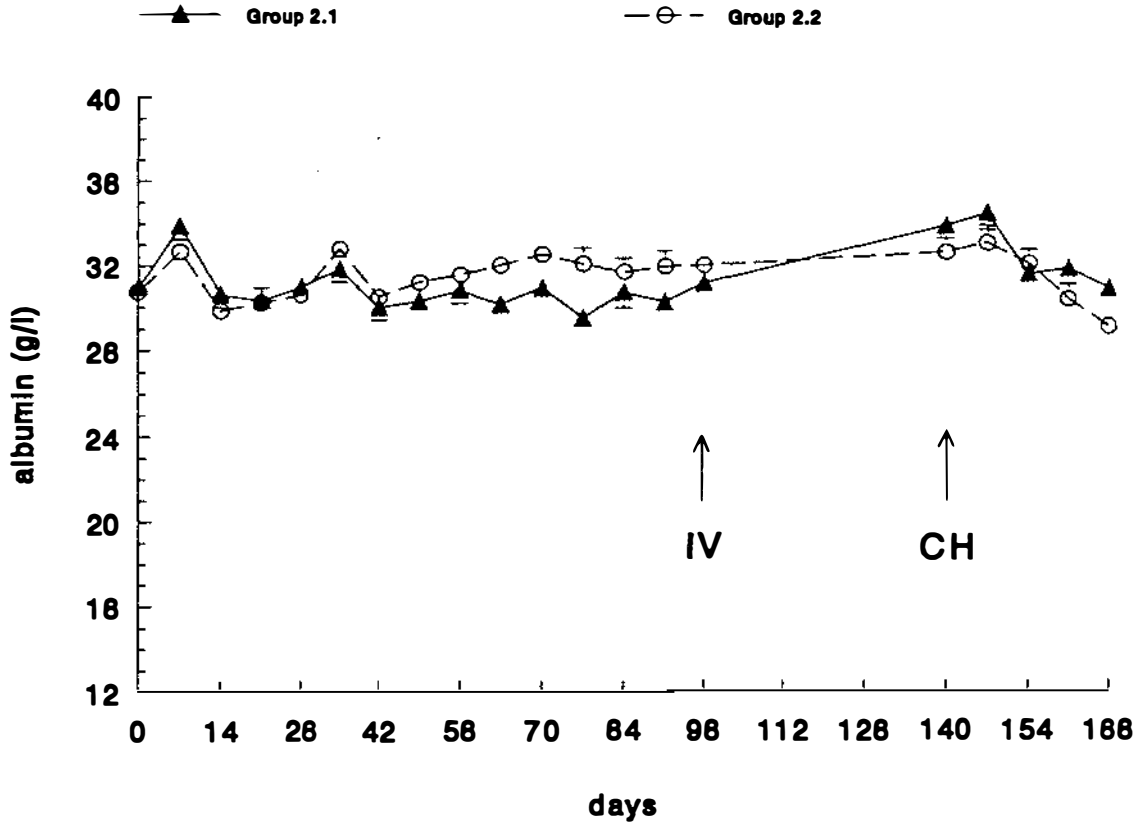
Total Protein - Experiment 2.2

(± standard error)



Albumin - Experiment 2.2

(± standard error)



Experiment 2 - Statistical analyses

White blood cells

Analysis of variance of white blood cells during the trickle infection using Sas version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

ANALYSIS OF TOTAL WHITE BLOOD CELLS DURING THE TRICKLE

General Linear Models Procedure

Dependent Variable: WBC

Source	DF	Sum of Squares	F Value	Pr > F
Model	40	2035.82190136	18.98	0.0001
Error	169	453.07505102		
Corrected Total	209	2488.89695238		

R-Square	C.V.	WBC Mean
0.817962	11.95479	13.69619048

Source	DF	Type III SS	F Value	Pr > F
TRT	1	197.60164626	73.71	0.0001
GOAT(TRT)	13	1526.20530612	43.79	0.0001
WEEK	13	257.11513946	7.38	0.0001
TRT*WEEK	13	53.33799660	1.53	0.1109

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	F Value	Pr > F
TRT	1	197.60164626	1.68	0.2171

ANALYSIS OF NEUTROPHILS DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: NEUTROPHIL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	40	375.542279	9.388557	4.64	0.0001
Error	169	341.939961	2.023313		

R-Square	C.V.	Root MSE	NEUT Mean
0.523417	39.05323	1.42243	3.6422905

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.321379	0.321379	0.16	0.6907
GOAT(TRT)	13	204.035041	15.695003	7.76	0.0001
WEEK	13	142.429838	10.956141	5.41	0.0001
TRT*WEEK	13	30.716727	2.362825	1.17	0.3071

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.32137938	0.32137938	0.02	0.8884

ANALYSIS OF EOSINOPHILS DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: EOSINOPHILS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	40	7.19709308	0.17992733	3.53	0.0001
Error	169	8.62590823	0.05104088		
Corrected Total	209	15.82300131			

R-Square	C.V.	Root MSE	EOS Mean
0.454850	117.0235	0.22592	.19305714

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1.31728633	1.31728633	25.81	0.0001
GOAT(TRT)	13	2.54305284	0.19561945	3.83	0.0001
WEEK	13	1.65921501	0.12763192	2.50	0.0038
TRT*WEEK	13	1.48610686	0.11431591	2.24	0.0099

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1.31728633	1.31728633	6.73	0.0222

ANALYSIS OF BASOPHILS DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: BASOPHILS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	40	1.49583806	0.03739595	1.65	0.0150
Error	169	3.82048923	0.02260645		
Corrected Total	209	5.31632728			

R-Square	C.V.	Root MSE	BAS Mean
0.281367	103.9967	0.150354	0.14457619

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00770181	0.00770181	0.34	0.5602
GOAT(TRT)	13	0.66606026	0.05123540	2.27	0.0090
WEEK	13	0.64426503	0.04955885	2.19	0.0118
TRT*WEEK	13	0.18963231	0.01458710	0.65	0.8131

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00770181	0.00770181	0.15	0.7045

ANALYSIS OF LYMPHOCYTES DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: LYMPHOCYTES

Source	DF	Sum of Squares	F Value	Pr > F
Model	40	1391.90620685	16.61	0.0001
Error	169	354.02281097		
Corrected Total	209	1745.92901781		

R-Square	C.V.	LYMPH Mean
0.797230	15.37204	9.41544286

Source	DF	Type III SS	F Value	Pr > F
TRT	1	154.77962762	73.89	0.0001
GOAT(TRT)	13	1024.76734912	37.63	0.0001
WEEK	13	144.59969094	5.31	0.0001
TRT*WEEK	13	62.27239322	2.29	0.0083

Tests of Hypotheses using the Type III MS for
GOAT(TRT) as an error term

Source	DF	Type III SS	F Value	Pr > F
TRT	1	154.77962762	1.96	0.1846

ANALYSIS OF MONOCYTES DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: MONOCYTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	40	2.64649270	0.06616232	1.18	0.2369
Error	169	9.49804523	0.05620145		
Corrected Total	209	12.14453792			

R-Square	C.V.	Root MSE	MONO Mean
0.217916	77.98793	0.237068	0.30398095

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.01679310	0.01679310	0.30	0.5854
GOAT(TRT)	13	1.06711797	0.08208600	1.46	0.1370
WEEK	13	1.28004058	0.09846466	1.75	0.0546
TRT*WEEK	13	0.26789237	0.02060711	0.37	0.9783

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.01679310	0.01679310	0.20	0.6585

Analysis of variance of white blood cells following the challenge infection using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

TOTAL WHITE BLOOD CELL COUNT RESPONSE FOLLOWING THE CHALLENGE
General Linear Models Procedure

Dependent Variable: WBC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	431.9608667	19.6345848	18.65	0.0001
Error	52	54.7530000	1.0529423		
Corrected Total	74	486.7138667			

R-Square	C.V.	Root MSE	WBC Mean
0.887505	8.493514	1.026130	12.0813333

Source	DF	Type III SS	Mean Square	F Value	Pr > F

TRT	1	29.9552595	29.9552595	28.45	0.0001
GOAT(TRT)	13	369.8586071	28.4506621	27.02	0.0001
WEEK	4	18.9048667	4.7262167	4.49	0.0034
TRT*WEEK	4	13.2424667	3.3106167	3.14	0.0217

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	29.95525952	29.95525952	1.05	0.3236

NEUTROPHIL RESPONSE FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: NEUTROPHILS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	75.07724095	3.41260186	4.02	0.0001
Error	52	44.08917857	0.84786882		
Corrected Total	74	119.16641952			

R-Square	C.V.	Root MSE	NEUT Mean
0.630020	24.56719	0.920798	3.74808000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.01212780	0.01212780	0.01	0.9053
GOAT(TRT)	13	65.30690052	5.02360773	5.92	0.0001
WEEK	4	4.66861895	1.16715474	1.38	0.2548
TRT*WEEK	4	5.58950258	1.39737564	1.65	0.1762

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.01212780	0.01212780	0.00	0.9616

EOSINOPHIL RESPONSE FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: EOSINOPHILS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	4.52758312	0.20579923	3.15	0.0003
Error	52	3.39282843	0.06524670		
Corrected Total	74	7.92041155			

R-Square	C.V.	Root MSE	EOS Mean
0.571635	104.4180	0.255434	0.24462667

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.16426256	0.16426256	2.52	0.1186
GOAT(TRT)	13	1.72983979	0.13306460	2.04	0.0354
WEEK	4	2.18394925	0.54598731	8.37	0.0001
TRT*WEEK	4	0.46909389	0.11727347	1.80	0.1434

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.16426256	0.16426256	1.23	0.2867

BASOPHIL RESPONSE FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: BASOPHIL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	0.20876122	0.00948915	1.61	0.0808
Error	52	0.30664177	0.00589696		
Corrected Total	74	0.51540299			

R-Square	C.V.	Root MSE	BAS Mean
0.405045	116.9653	0.076792	0.06565333

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00444754	0.00444754	0.75	0.3891
GOAT(TRT)	13	0.12300544	0.00946196	1.60	0.1141
WEEK	4	0.06724162	0.01681040	2.85	0.0328
TRT*WEEK	4	0.01558471	0.00389618	0.66	0.6221

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00444754	0.00444754	0.47	0.5050

LYMPHOCYTE RESPONSE FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: LYMPHOCYTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	22	268.8993225	12.2226965	15.49	0.0001
Error	52	41.0249417	0.7889412		
Corrected Total	74	309.9242642			

R-Square	C.V.	Root MSE	LYMPH Mean
0.867629	11.41785	0.888224	7.77925333

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	33.0492654	33.0492654	41.89	0.0001
GOAT(TRT)	13	208.2731216	16.0210094	20.31	0.0001
WEEK	4	19.9442225	4.9860556	6.32	0.0003
TRT*WEEK	4	6.4252067	1.6063017	2.04	0.1029

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	33.04926539	33.04926539	2.06	0.1746

MONOCYTE RESPONSE FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: MONOCYTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	1.06731112	0.04851414	1.62	0.0778
Error	52	1.55659986	0.02993461		
Corrected Total	74	2.62391099			

R-Square	C.V.	Root MSE	MONO Mean
0.406763	71.78699	0.173016	0.24101333

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00595714	0.00595714	0.20	0.6574
GOAT(TRT)	13	0.58534824	0.04502679	1.50	0.1475
WEEK	4	0.39591192	0.09897798	3.31	0.0173
TRT*WEEK	4	0.08913848	0.02228462	0.74	0.5661

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00595714	0.00595714	0.13	0.7219

Multiple regression of white blood cells following challenge using Statistix version 4.0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF NEUTROPHILS

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	5.44262	1.83319	2.97	0.0041	
GROUP	-0.02549	0.29597	-0.09	0.9316	1.0
TIME	-0.09748	0.10441	-0.93	0.3536	1.0

R-SQUARED	0.0121	RESID. MEAN SQUARE (MSE)	1.63512
ADJUSTED R-SQUARED	-0.0154	STANDARD DEVIATION	1.27872

SOURCE	DF	SS	MS	F	P
REGRESSION	2	1.43748	0.71874	0.44	0.6516
RESIDUAL	72	117.729	1.63512		
TOTAL	74	119.166			

CASES INCLUDED 75 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF EOSINOPHILS

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	-1.87856	0.40372	-4.65	0.0000	
GROUP	0.09381	0.06518	1.44	0.1544	1.0
TIME	0.11680	0.02299	5.08	0.0000	1.0

R-SQUARED	0.2791	RESID. MEAN SQUARE (MSE)	0.07930
ADJUSTED R-SQUARED	0.2591	STANDARD DEVIATION	0.28161

SOURCE	DF	SS	MS	F	P
REGRESSION	2	2.21060	1.10530	13.94	0.0000
RESIDUAL	72	5.70981	0.07930		
TOTAL	74	7.92041			

CASES INCLUDED 75 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF BASOPHILS

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	-0.01207	0.12058	-0.10	0.9206	
GROUP	0.01544	0.01947	0.79	0.4305	1.0
TIME	0.00324	0.00687	0.47	0.6385	1.0

R-SQUARED	0.0117	RESID. MEAN SQUARE (MSE)	0.00707
ADJUSTED R-SQUARED	-0.0158	STANDARD DEVIATION	0.08411

SOURCE	DF	SS	MS	F	P
REGRESSION	2	0.00602	0.00301	0.43	0.6605

RESIDUAL	72	0.50938	0.00707
TOTAL	74	0.51540	

CASES INCLUDED 75 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LYMPHOCYTES

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	15.8720	2.71010	5.86	0.0000	
GROUP	-1.33060	0.43754	-3.04	0.0033	1.0
TIME	-0.36125	0.15435	-2.34	0.0220	1.0

R-SQUARED	0.1698	RESID. MEAN SQUARE (MSE)	3.57361
ADJUSTED R-SQUARED	0.1467	STANDARD DEVIATION	1.89040

SOURCE	DF	SS	MS	F	P
REGRESSION	2	52.6241	26.3121	7.36	0.0014
RESIDUAL	72	257.300	3.57361		
TOTAL	74	309.924			

CASES INCLUDED 75 MISSING CASES 0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF MONOCYTES

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.14221	0.27294	0.52	0.6040	
GROUP	-0.01786	0.04407	-0.41	0.6864	1.0
TIME	0.00735	0.01555	0.47	0.6376	1.0

R-SQUARED	0.0054	RESID. MEAN SQUARE (MSE)	0.03625
ADJUSTED R-SQUARED	-0.0223	STANDARD DEVIATION	0.19039

SOURCE	DF	SS	MS	F	P
REGRESSION	2	0.01407	0.00703	0.19	0.8240
RESIDUAL	72	2.60984	0.03625		
TOTAL	74	2.62391			

CASES INCLUDED 75 MISSING CASES 0

Total protein and albumin

Analysis of variance of total protein and albumin during the trickle infection using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

ANALYSIS OF TOTAL PROTEIN DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: TOTAL PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	42	2534.906837	60.354925	11.56	0.0001
Error	182	950.095119	5.220303		
Corrected Total	224	3485.001956			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.727376	3.382303	2.284798	67.5515556

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	32.234229	32.234229	6.17	0.0139
GOAT(TRT)	13	1557.845060	119.834235	22.96	0.0001
WEEK	14	773.015725	55.215409	10.58	0.0001
TRT*WEEK	14	169.797592	12.128399	2.32	0.0057

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	32.23422937	32.23422937	0.27	0.6127

ANALYSIS OF ALBUMIN DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: ALBUMIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	42	489.1700984	11.6469071	12.17	0.0001
Error	182	174.1678571	0.9569662		
Corrected Total	224	663.3379556			

R-Square	C.V.	Root MSE	ALBUMIN Mean
0.737437	3.139386	0.978247	31.1604444

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	25.1830865	25.1830865	26.32	0.0001
GOAT(TRT)	13	266.6055357	20.5081181	21.43	0.0001
WEEK	14	137.6989873	9.8356420	10.28	0.0001
TRT*WEEK	14	57.8035206	4.1288229	4.31	0.0001

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F

TRT	1	25.18308651	25.18308651	1.23	0.2879

Analysis of variance of total protein and albumin following the challenge infection using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

ANALYSIS OF TOTAL PROTEIN FOLLOWING CHALLENGE

General Linear Models Procedure

Dependent Variable: TOTAL PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	660.9010143	30.0409552	4.17	0.0001
Error	52	374.9997857	7.2115343		
Corrected Total	74	1035.9008000			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.637996	3.809985	2.685430	70.4840000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.0030857	0.0030857	0.00	0.9836
GOAT(TRT)	13	364.9977143	28.0767473	3.89	0.0002
WEEK	4	256.4103476	64.1025869	8.89	0.0001
TRT*WEEK	4	36.0060810	9.0015202	1.25	0.3022

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.00308571	0.00308571	0.00	0.9918

ANALYSIS OF ALBUMIN FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: ALBUMIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	318.3408714	14.4700396	14.84	0.0001
Error	52	50.7199286	0.9753832		
Corrected Total	74	369.0608000			

R-Square	C.V.	Root MSE	ALBUMIN Mean
0.862570	3.078216	0.987615	32.0840000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	21.9459429	21.9459429	22.50	0.0001
GOAT(TRT)	13	142.0868571	10.9297582	11.21	0.0001
WEEK	4	142.1355381	35.5338845	36.43	0.0001
TRT*WEEK	4	12.1499381	3.0374845	3.11	0.0227

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	21.94594286	21.94594286	2.01	0.1800

Multiple regression of total protein and albumin following challenge

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF TOTAL PROTEIN

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	93.9255	6.35217	14.79	0.0000	
GROUP	-0.01286	0.80265	-0.02	0.9873	1.0
TIME	-0.15210	0.04045	-3.76	0.0003	1.0

R-SQUARED	0.1641	RESID. MEAN SQUARE (MSE)	12.0260
ADJUSTED R-SQUARED	0.1409	STANDARD DEVIATION	3.46785

SOURCE	DF	SS	MS	F	P
REGRESSION	2	170.030	85.0152	7.07	0.0017
RESIDUAL	72	865.870	12.0260		
TOTAL	74	1035.90			

CASES INCLUDED 75 MISSING CASES 0

Packed cell volume

Analysis of variance of PCV during the trickle infection using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

ANALYSIS OF PCV DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: PCV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	42	3014.857341	71.782318	26.84	0.0001
Error	182	486.698214	2.674166		
Corrected Total	224	3501.555556			

R-Square	C.V.	Root MSE	PCV Mean
0.861005	5.936906	1.635288	27.5444444

TRT	1	894.667163	894.667163	334.56	0.0001
GOAT(TRT)	13	1060.788393	81.599107	30.51	0.0001
WEEK	14	905.675119	64.691080	24.19	0.0001
TRT*WEEK	14	130.012897	9.286635	3.47	0.0001

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	894.6671627	894.6671627	10.96	0.0056

Analysis of variance of PCV following challenge using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

ANALYSIS OF PCV FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: PCV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	916.9871429	41.6812338	11.13	0.0001
Error	52	194.6928571	3.7440934		
Corrected Total	74	1111.6800000			

R-Square	C.V.	Root MSE	PCV Mean
0.824866	7.374108	1.934966	26.2400000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	60.4800000	60.4800000	16.15	0.0002
GOAT(TRT)	13	423.2000000	32.5538462	8.69	0.0001
WEEK	4	415.9204762	103.9801190	27.77	0.0001
TRT*WEEK	4	15.4938095	3.8734524	1.03	0.3983

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	60.48000000	60.48000000	1.86	0.1960

Multiple regression of PCV following challenge using Statistix version 4.0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF PCV

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF

CONSTANT	59.9733	5.46617	10.97	0.0000	
GROUP	1.80000	0.69070	2.61	0.0111	1.0
TIME	-0.23619	0.03481	-6.79	0.0000	1.0

R-SQUARED	0.4232	RESID. MEAN SQUARE (MSE)	8.90519
ADJUSTED R-SQUARED	0.4072	STANDARD DEVIATION	2.98416

SOURCE	DF	SS	MS	F	P
REGRESSION	2	470.507	235.253	26.42	0.0000
RESIDUAL	72	641.173	8.90519		
TOTAL	74	1111.68			

CASES INCLUDED 75 MISSING CASES 0

Pepsinogen

Analysis of variance of pepsinogen during the trickle infection using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

ANALYSIS OF PEPSINOGEN DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: PEPSINOGEN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	40	16.29661744	0.40741544	23.69	0.0001
Error	169	2.90586715	0.01719448		
Corrected Total	209	19.20248460			

R-Square	C.V.	Root MSE	PEPSIN Mean
0.848672	35.67177	0.131128	0.36759524

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	8.73362503	8.73362503	507.93	0.0001
GOAT(TRT)	13	3.04806392	0.23446646	13.64	0.0001
WEEK	13	2.23514327	0.17193410	10.00	0.0001
TRT*WEEK	13	2.01861096	0.15527777	9.03	0.0001

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	8.73362503	8.73362503	37.25	0.0001

Analysis of variance of pepsinogen following the challenge infection using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)
ANALYSIS OF PEPSINOGEN FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: PEPSINOGEN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	6.73411549	0.30609616	14.63	0.0001
Error	52	1.08817566	0.02092645		
Corrected Total	74	7.82229115			

R-Square	C.V.	Root MSE	PEPSIN Mean
0.860888	25.86783	0.144660	0.55922667

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.03879040	0.03879040	1.85	0.1792
GOAT(TRT)	13	1.37958234	0.10612172	5.07	0.0001
WEEK	4	5.19825146	1.29956287	62.10	0.0001
TRT*WEEK	4	0.10059333	0.02514833	1.20	0.3212

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	0.03879040	0.03879040	0.37	0.5559

Multiple regression of pepsinogen following the challenge infection using Statistix version 4.0

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF PEPSIN

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	-3.41942	0.35546	-9.62	0.0000	
GROUP	-0.04559	0.04492	-1.01	0.3135	1.0
TIME	0.02627	0.00226	11.61	0.0000	1.0

R-SQUARED	0.6534	RESID. MEAN SQUARE (MSE)	0.03766
ADJUSTED R-SQUARED	0.6438	STANDARD DEVIATION	0.19406

SOURCE	DF	SS	MS	F	P
REGRESSION	2	5.11094	2.55547	67.86	0.0000
RESIDUAL	72	2.71135	0.03766		
TOTAL	74	7.82229			

CASES INCLUDED 75 MISSING CASES 0

Gastrin

Analysis of variance of gastrin during the trickle infection using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

ANALYSIS OF GASTRIN DURING THE TRICKLE INFECTION

General Linear Models Procedure

Dependent Variable: GASTRIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	42	205791.8054	4899.8049	5.36	0.0001
Error	182	166430.6962	914.4544		
Corrected Total	224	372222.5016			

R-Square	C.V.	Root MSE	GASTRIN Mean
0.552873	81.84461	30.23995	36.9480000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	42088.6046	42088.6046	46.03	0.0001
GOAT(TRT)	13	104267.7717	8020.5978	8.77	0.0001
WEEK	14	38040.3493	2717.1678	2.97	0.0004
TRT*WEEK	14	18600.3182	1328.5942	1.45	0.1331

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	42088.60460	42088.60460	5.25	0.0393

Analysis of variance of gastrin following the challenge infection using SAS version 6.04

(TRT = group; WEEK = time; GOAT(TRT) = goat nested within treatment)

ANAYSIS OF GASTRIN FOLLOWING THE CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: GASTRIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	1376717.628	62578.074	2.76	0.0014
Error	52	1177607.054	22646.290		
Corrected Total	74	2554324.682			

R-Square	C.V.	Root MSE	GASTRIN Mean

0.538975	133.2229	150.4868		112.958667

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	275646.2464	275646.2464	12.17	0.0010
GOAT(TRT)	13	406650.0154	31280.7704	1.38	0.2002
WEEK	4	473825.9079	118456.4770	5.23	0.0013
TRT*WEEK	4	263543.0146	65885.7536	2.91	0.0302

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	275646.2464	275646.2464	8.81	0.0109

APPENDIX 3a

Faecal egg counts for Experiment 3.1

	Group 1					Group 2					Monitor Goats	
	3[1]	5	6	9	10	1	2	4	8	11	A	B
Week												
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	350	650	250	400	100	0	0	0	0	0	550	350
5	900	950	800	1650	750	0	0	0	0	0	1250	1050
6	1600	950	1100	450	1100	0	0	0	0	0	700	1550
7	2250	750	450	2350	1100	0	0	0	0	0	1350	2500
8	1400	1100	750	1200	1250	0	0	0	0	0	850	2000
9	900	600	600	1150	1650	0	0	0	0	0	850	1000
10	1450	500	300	800	1200	0	0	0	0	0	1600	800
11	1150	400	500	750	950	0	0	0	0	0	950	1200
12	0	0	0	0	0	0	0	0	0	0		
13	0	0	0	0	0	0	0	0	0	0		
14	0	0	0	0	0	0	0	0	0	0		
15	0	0	0	0	0	0	0	0	0	0		
16	0	0	0	0	0	750	1150	2600	900	1500		
17	0[2]	0[2]	0[2]	0[2]	0[2]	1600	2950	3700	2250	2300		
18	0	0	0	0	0	0	0	0	0	0		
19	0	0	0	0	0	0	0	0	0	0		
20	0	0	0	0	0	0	0	0	0	0		
21	0	0	0	0	0	0	0	0	0	0		
22	0	50	0	0	0	0	0	0	0	150		
23	0	0	0	50	100	150	200	350	350	1150		

	* Group 3				
	7[1]	17	18	30	31
Week					
17	0	0	0	0	0
18	0	0	0	0	0
19	0	0	0	0	0
20	0	0	0	0	0
21	0	0	0	0	0
22	750	400	750	250	0
23	2100	2450	1200	1850	2700

Key: 1 = goat number
2 = faecal float

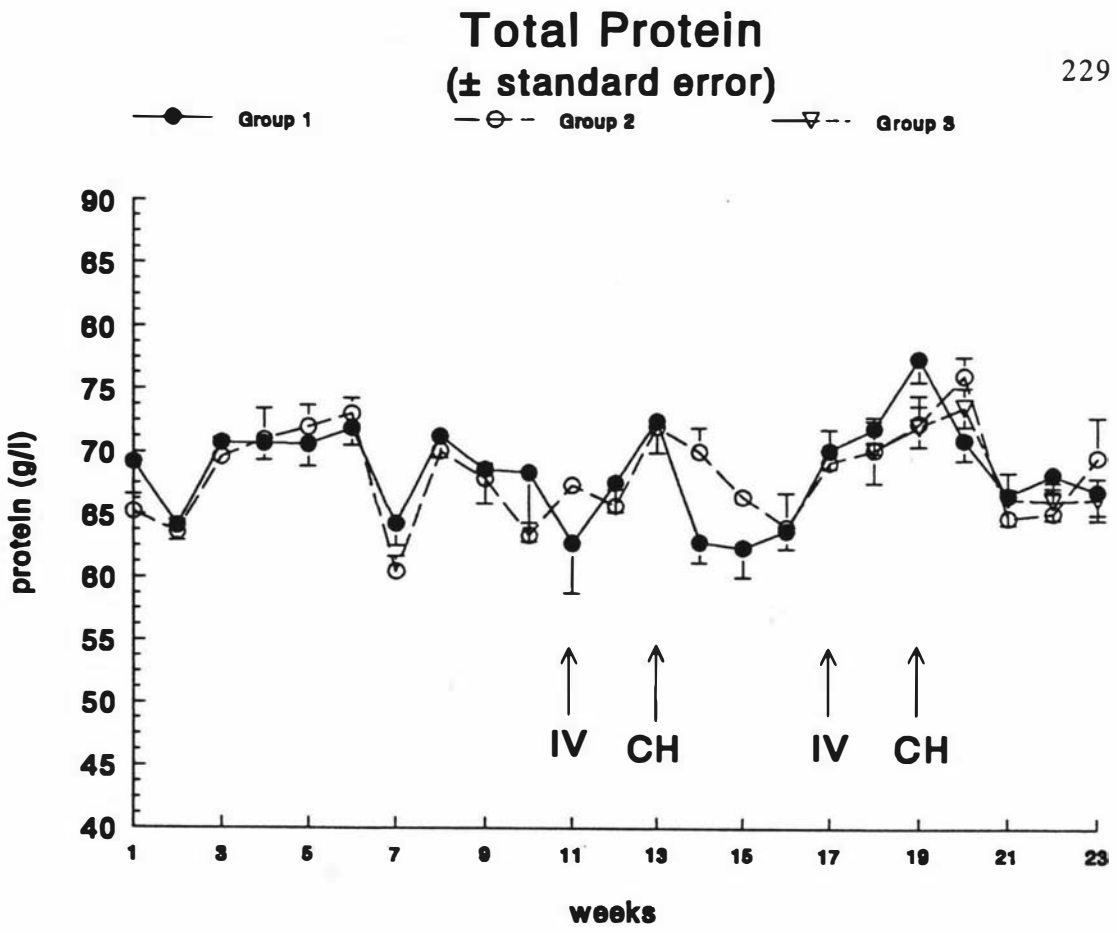
* Group 1 (n=5) trickle infected with 10,000 *T. colubriformis* per week x 10 and challenged twice; Group 2 (n=5) challenged twice; Group 3 (n=5) challenged once. Each challenge comprised 50,000 *T. colubriformis*.

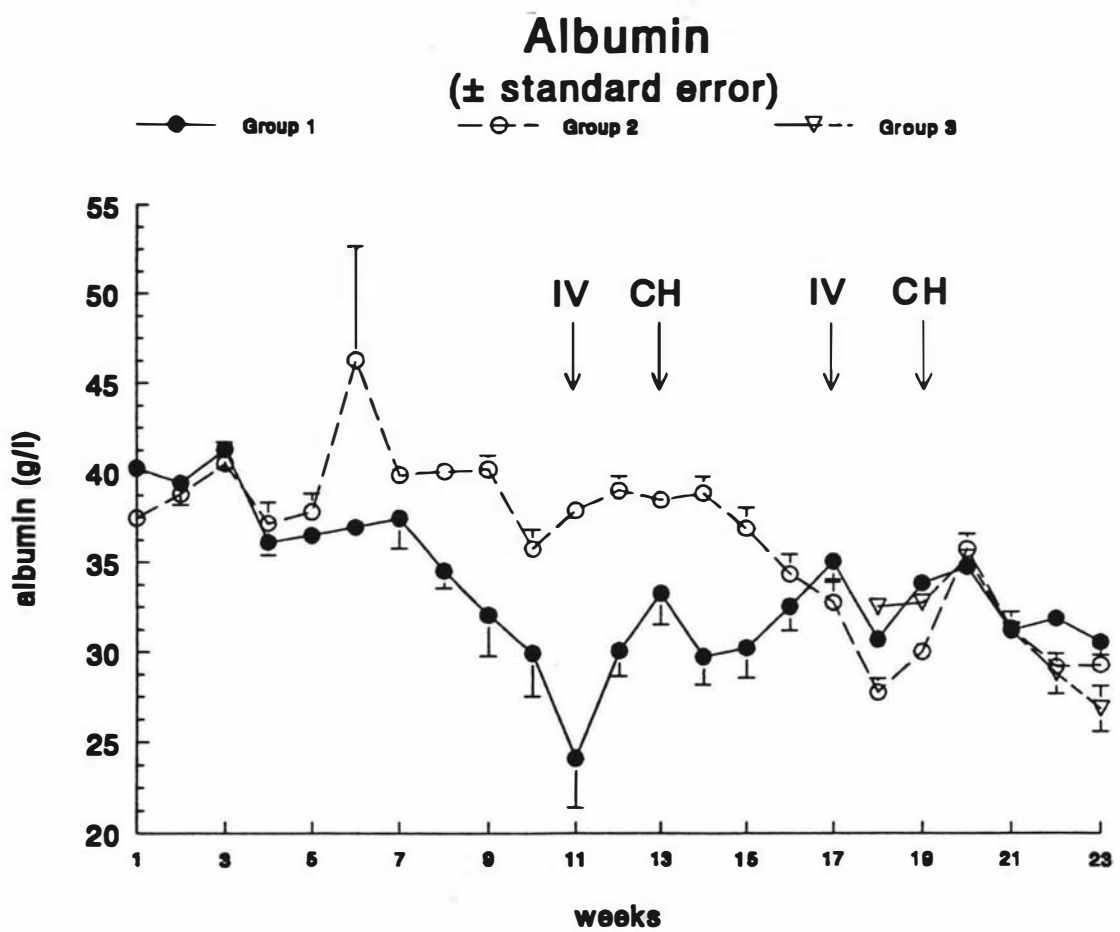
APPENDIX 3b

Graphs and Statistical Analyses for Serum Proteins in Experiment 3.1

Keys for the following graphs:

IV = ivermectin given here
CH = challenge given here
Group 1 = trickle infected plus two single challenges
Group 2 = two single challenges
Group 3 = single challenge (challenge control)


Total Protein
(± standard error)
Group 1
Group 2
Group 3
protein (g/l)
90
85
80
75
70
65
60
55
50
45
40
IV
CH
IV
CH
1
3
5
7
9
11
13
15
17
19
21
23
weeks


Albumin
(± standard error)
Group 1
Group 2
Group 3
albumin (g/l)
55
50
45
40
35
30
25
20
IV
CH
IV
CH
1
3
5
7
9
11
13
15
17
19
21
23
weeks

Analysis of total protein and albumin during the trickle infection using SAS version 6.04.

(Key for variable names: TRT = group; WEEK = time; GOAT(TRT) = goat nested within group)

ANALYSIS OF TOTAL PROTEIN DURING THE TRICKLE INFECTION BETWEEN GROUP 1 AND 2

General Linear Models Procedure

Dependent Variable: PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	1905.068455	65.692016	4.20	0.0001
Error	80	1250.846182	15.635577		
Corrected Total	109	3155.914636			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.603650	5.815988	3.954185	67.9881818

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	17.840818	17.840818	1.14	0.2886
GOAT(TRT)	8	561.413818	70.176727	4.49	0.0002
WEEK	10	1132.505636	113.250564	7.24	0.0001
TRT*WEEK	10	193.308182	19.330818	1.24	0.2812

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	17.84081818	17.84081818	0.25	0.6277

ANALYSIS OF ALBUMIN DURING THE TRICKLE INFECTION BETWEEN GROUPS 1 AND 2

General Linear Models Procedure

Dependent Variable: ALBUMIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	2373.684818	81.851201	5.57	0.0001
Error	80	1176.544727	14.706809		
Corrected Total	109	3550.229545			

R-Square	C.V.	Root MSE	ALBUMIN Mean
0.668600	10.27760	3.834946	37.3136364

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	425.324455	425.324455	28.92	0.0001
GOAT(TRT)	8	307.523273	38.440409	2.61	0.0136
WEEK	10	1003.380545	100.338055	6.82	0.0001
TRT*WEEK	10	637.456545	63.745655	4.33	0.0001

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	425.3244545	425.3244545	11.06	0.0104

Analysis of total protein and albumin during the first challenge infection using SAS version 6.04.

(Key for variable names: TRT = group; WEEK = time; GOAT(TRT) = goat nested within group)

ANALYSIS OF TOTAL PROTEIN DURING THE 1ST CHALLENGE INFECTION BETWEEN GROUPS 1 AND 2

General Linear Models Procedure

Dependent Variable: PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	1247.843400	73.402553	11.71	0.0001
Error	32	200.602400	6.268825		
Corrected Total	49	1448.445800			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.861505	3.721517	2.503762	67.2780000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	52.2242000	52.2242000	8.33	0.0069
GOAT(TRT)	8	557.2736000	69.6592000	11.11	0.0001
WEEK	4	514.8968000	128.7242000	20.53	0.0001
TRT*WEEK	4	123.4488000	30.8622000	4.92	0.0033

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	52.22420000	52.22420000	0.75	0.4118

ANALYSIS OF ALBUMIN DURING THE FIRST CHALLENGE INFECTION BETWEEN GROUPS 1 AND 2

General Linear Models Procedure

Dependent Variable: ALBUMIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	674.7018000	39.6883412	11.27	0.0001
Error	32	112.7232000	3.5226000		
Corrected Total	49	787.4250000			

R-Square	C.V.	Root MSE	ALBUMIN Mean
0.856846	5.476682	1.876859	34.2700000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	211.7682000	211.7682000	60.12	0.0001
GOAT(TRT)	8	225.5688000	28.1961000	8.00	0.0001
WEEK	4	38.1860000	9.5465000	2.71	0.0474
TRT*WEEK	4	199.1788000	49.7947000	14.14	0.0001

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	211.7682000	211.7682000	7.51	0.0254

Analysis of total protein and albumin during the second challenge infection using SAS version 6.04.

(Key for variable names: TRT = group; WEEK = time; GOAT(TRT) = goat nested within group)

ANALYSIS OF TOTAL PROTEIN DURING THE 2ND CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	1867.898667	71.842256	11.17	0.0001
Error	48	308.855200	6.434483		
Corrected Total	74	2176.753867			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.858112	3.650595	2.536628	69.4853333

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	2	16.8450667	8.4225333	1.31	0.2796
GOAT(TRT)	12	710.0288000	59.1690667	9.20	0.0001
WEEK	4	933.5512000	233.3878000	36.27	0.0001
TRT*WEEK	8	207.4736000	25.9342000	4.03	0.0010

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	2	16.84506667	8.42253333	0.14	0.8688

ANALYSIS OF ALBUMIN DURING THE SECOND CHALLENGE INFECTION

General Linear Models Procedure

Dependent Variable: ALBUMIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	540.8874667	20.8033641	11.47	0.0001
Error	48	87.0344000	1.8132167		
Corrected Total	74	627.9218667			

R-Square	C.V.	Root MSE	ALBUMIN Mean
0.861393	4.264676	1.346557	31.5746667

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	2	33.1922667	16.5961333	9.15	0.0004
GOAT(TRT)	12	79.1536000	6.5961333	3.64	0.0007
WEEK	4	356.6058667	89.1514667	49.17	0.0001
TRT*WEEK	8	71.9357333	8.9919667	4.96	0.0002

Tests of Hypotheses using the Type III MS for GOAT(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	2	33.19226667	16.59613333	2.52	0.1223

APPENDIX 4a

Mean Proportion of Nematodes in the Small Intestine of Goats in Experiment 4.1

Proportion of the small intestine	Young Goats (s.e.)	Older Goats (s.e.)
0 → .1	0.427 (0.0256)	0.372 (0.0359)
.1 → .2	0.327 (0.0104)	0.269 (0.0188)
.2 → .3	0.173 (0.0136)	0.181 (0.0268)
.3 → .4	0.054 (0.0082)	0.076 (0.0111)
.4 → .5	0.016 (0.0034)	0.039 (0.0117)
.5 → .6	0.005 (0.0022)	0.028 (0.0129)
.6 → .7	0.001 (0.0013)	0.019 (0.0104)
.7 → .8		0.014 (0.0087)
.8 → .9		0.013 (0.0126)
.9 → 1		0.004 (0.0025)

APPENDIX 5a

Parasitological Results for Experiment 5.1

Goat	Abomasal worm count	Small intestinal worm count				Total worm count	male: female	Faecal egg count - 1st infection	Faecal egg count - 2nd infection		Faecal egg count - 3rd infection	
	adult	L3	L4	adult	digest: wash			Day 21 (epg)	Day 21 (epg)	Day 28 (epg)	Day 21 (epg)	Day 28 (epg)
2	30	0	0	12480	.72	12510	1.10	7500	50	10250	1400	2250
3	10	0	0	15400	.82	15410	.96	4200	750	2350	1400	1500
12	20	0	0	24710	.32	24730	.86	4800	1700	4400	2000	4350
15	10	0	0	18230	.58	18240	.89	4700	650	4500	800	1500
16	0	0	0	21750	.19	21750	.98	3550	4750	7000	4350	5200
18	20	0	0	16320	.31	16340	.72	3300	3600	4200	900	4350
19	0	600	200	12190	2.05	12990	.79	2000	900	1650	300	1650
21	0	600	1100	12920	.49	5490	1.06	6750	5150	9050	2150	6600
23	20	0	0	14240	.37	14260	.99	7300	2050	5850	1250	3450
24	0	0	0	3790	.41	3790	.73	1250	0	50	150	400
28	0	0	0	15950	1.41	15950	1.00	3900	1800	3650	650	3150
30	10	0	0	13280	.55	13290	1.11	5000	2750	4050	1450	4750
34	10	0	0	18760	.42	18770	.92	3650	650	3800	100	2300
39	40	0	0	21270	.32	21310	1.06	4400	1500	6800	3250	3350
48	0	0	0	13890	.52	13890	.99	1700	350	850	100	600
49	10	0	0	11770	.56	11780	.94	2500	100	700	900	950
52	0	0	560	2500	1.0	3060	.82	50	0	50	0	350

Goat	Abomasal worm count	Small intestinal worm count				Total worm count	male: female	Faecal egg count - 1st infection	Faecal egg count - 2nd infection		Faecal egg count - 3rd infection	
	adult	L3	L4	adult	digest: wash			Day 21 (epg)	Day 21 (epg)	Day 28 (epg)	Day 21 (epg)	Day 28 (epg)
54	0	100	0	7540	1.05	7640	.93	2400	850	1850	250	850
56	0	0	0	12980	.75	12980	.91	3100	550	2500	150	2250
59	0	0	0	12960	1.79	12960	1.00	1850	950	1350	350	900
62	20	0	0	18880	.39	18900	.89	1600	50	1450	700	1500
63	0	0	1010	5560	1.31	6570	.66	2300	1050	2550	600	550
65	20	0	100	16310	.53	16430	.9	2650	450	4800	1700	5300
75	0	0	0	15390	2.63	15390	1.09	2950	3050	3000	2050	3300
77	0	0	0	18230	.80	18230	.92	4150	1700	5500	2000	3100
82	20	0	0	15020	.67	15040	1.07	1750	2150	6900	2400	2850
85	0	0	100	1520	.52	1620	.62	3550	1350	3700	200	150
86	30	0	0	8780	1.99	8810	.68	1450	600	2250	250	750
89	0	0	0	13130	1.95	13130	1.17	3050	3550	5900	5100	6050

APPENDIX 6a

Gill's haematoxylin and eosin (Gill *et al.*, 1974).

Stains

1. Gill's haematoxylin:

haematoxylin	4gm
distilled water	700ml
ethylene glycol	250ml
sodium iodate	0.6gm
aluminium sulphate	36gm
acetic acid	50ml

Mix in order given. Add acid after all solids have dissolved. Maintain acid content by adding 1 drop of acetic acid per 100mls of stain weekly (change monthly). Requires no differentiation. Can be used at once.

2. Alcoholic eosin:

1% aqueous eosin (GI 45380)	100mls
1% aqueous phloxine	10mls
95% ethanol	880mls
acetic acid	5mls

Change this solution weekly.

Method

1. take to water
2. Gill's haematoxylin for 3 minutes
3. running water for 30 seconds
4. Scott's tapwater for 30 seconds
5. running water for 1 minute
6. alcoholic eosin for 1 to 2 minutes
7. running water for 30 seconds
8. dehydrate briskly through alcohols without pause
9. clear and mount