5. HOST GENETICS

5.1 INTRODUCTION

The widespread resistance to anthelmintics seen in sheep nematodes in Australia and concerns about chemical residues in the environment and animal products has seen researchers begin developing new ways to combat parasitic infection. As has been discussed earlier (Chapter 1), one way has been the selection of animals showing resistance to their parasites.

Research has shown (Windon, 1990; Woolaston, 1992) that flocks selected for resistance to one genus of nematode parasite show side-resistance to other genera, however, studies of this nature have not investigated side-resistance within genera. In the genus *Haemonchus*, proportions of *Haemonchus placei* and *H. contortus* establishing in sheep can be influenced by host resistance (Le Jambre, 1983). Given equal proportions of *H. contortus* and *H. placei* larvae, *H. placei* is usually excluded from establishing in normal yearling sheep while equal proportions establish in immunosuppressed animals. If *H. contortus* and *H. placei* respond differently to the effects of host resistance, *T. colubriformis* and *T. vitrinus* may also show different responses. Adding to this, Eysker (1978) suggested that *T. colubriformis* and *T. vitrinus* were affected differently by host immune responses. He described *T. colubriformis* dominating in more immunocompetent hosts, older lambs and barren ewes, and *T. vitrinus* dominating in hosts that are less immunocompetent such as young lambs and lactating ewes.

It is well established that *Trichostrongylus* spp. prefer to live in the first three metres of the small intestine of their host but are commonly found in the first 10 metres. E. Roy and I. Beveridge (unpubl.) have evidence showing that *T. colubriformis* and *T. vitrinus* may compete for the "prime real estate" at the pylorus. If an infection of *T. colubriformis* or *T. vitrinus* is established before the other species enters the small intestine, the colonising species maintains the position nearest the pylorus, dominating in the first one metre. However, if both species arrive in the small intestine simultaneously *T. vitrinus* will dominate in the first one to three metres with *T. colubriformis* being pushed further down the gut to a less preferred position

An experiment was designed to examine the effect of host genetics on species proportion using sheep selected for resistance or susceptibility to nematode parasites and random-bred controls. It was also decided to look at establishment of *T. colubriformis* and *T. vitrinus* in sheep from the three selection lines, firstly to see if the simultaneous infection results of E. Roy & I. Beveridge could be repeated, and secondly, to see if host genetics had any effect on site preference in the small intestine.

5.2 MATERIALS AND METHODS

Twenty-eight yearling Merino rams from the CSIRO *Haemonchus* selection flock were used (Piper, 1987), seven from each of the three selection lines. The rams were made worm-free by dosing with the RDR of ivermectin (Ivomec, MSD AGVET), a combination treatment of benzimidazole and levamisole (Scanda, Coopers) and closantel (Razar, Coopers) before housing in pens. The rams were allowed to adapt to their

new surroundings for 6 weeks before being infected with 20 000 *T. colubriformis* L3 and 20 000 *T. vitrinus* L3.

Twenty-eight days post infection the rams were euthanased and the first ten metres of their small intestines collected in three sections, 1 (the first one metre), 2 (the second two metres) and 3 (the following seven metres). Each section of small intestine had its contents collected and was washed three times in water. The contents and washings were collected in jars and preserved in 70% ethanol. The volume of each jar was standardised to 500 ml and a total worm count conducted on 10% of that volume. Total worm number was estimated for each section before one hundred adult males from each section were identified to species using spicule morphology (Lapage, 1962).

Analyses were done to determine whether host resistance status had any effect on the number of adult *Trichostrongylus* spp. of each sex and the proportion of *T. colubriformis* and *T. vitrinus* adult males found in the rams. It was also determined whether position of either species in the small intestine was affected by host immunity.

5.3 RESULTS

The total number of worms found per metre for each of the three sections of the small intestine (Table 5.1) were log transformed and analysed using a repeated measures analysis of variance (Steel & Torrie, 1980). The total number of worms per metre for each section was found to differ significantly between sheep lines ($P<0.005$). Surprisingly, for the first two sections of the small intestine the sheep from the RB line had a higher

number of worms per metre than did those from the SUS line. Both these lines had fewer worms per metre than the sheep from the RES line.

The total number of worms per metre was also found to differ significantly along the length of the small intestine ($P<0.001$) for all three lines. Sheep from the SUS and RES lines showed a decrease in number of worms per metre moving away from the pylorus. However, sheep from the RB line had more worms per metre in section 2 than in section 1.

Table 5.1: Estimated total number (mean of seven sheep) of *Trichostrongylus* spp. per metre from three areas of the small intestine (1= first one metre; 2= second two metres; 3= next seven metres) and total number of *Trichostrongylus* spp. recovered from the three areas combined from Merino rams from CSIRO *Haemonchus* selection flocks (SUS= susceptible; RB= random bred; RES= resistant).

Sheep line	1	2	3	Total number
SUS	2404	2044	675	11316
RB	2797	3190	617	13353
RES	1196	859	229	4518

The percentages of *T. colubriformis* identified from each section of the small intestine (Table 5.2) were arcsine square root transformed and analysed using a repeated measures analysis of variance (Steel & Torrie, 1980). The percentage of *T. colubriformis* was found not to differ significantly between sheep lines ($P>0.5$). However, the percentage of *T. colubriformis* did differ significantly along the length of the small intestine for all three sheep lines ($P<0.001$). Sheep from all three lines showed an increase in the percentage of *T. colubriformis* moving away form the pylorus (Figure 5.1).

Table 5.2: Percentages (mean of seven sheep) of male *Trichostrongylus colubriformis* found in three sections of the small intestine (1= first one metre; 2= second two metres; 3= next seven metres) and percentage found in the three sections combined from Merino rams from CSIRO *Haemonchus* selection flocks (SUS= susceptible; RB= random bred; RES= resistant).

Sheep Line	1	2	3	Total percentage
SUS	37	41	48	41
RB	36	43	52	44
RES	31	40	46	39

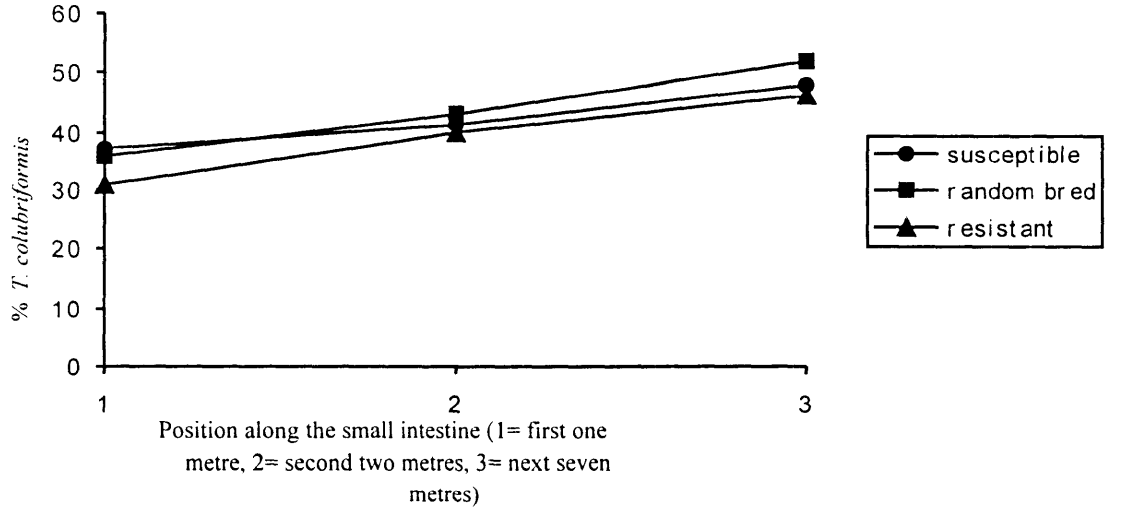

Figure 5.1: Percentage of *T. colubriformis* males found in each section of the small intestine of nematode susceptible, resistant and random bred sheep.

5.4 DISCUSSION

The results clearly show that there is no effect of host genetics on species proportion in a mixed infection with *T. colubriformis* and *T. vitrinus*. The percentage of *T. colubriformis* found in the small intestines of sheep

from each line did not differ significantly. This confirms results by Windon (1990) and Woolaston (1992) that a line of sheep selected for resistance, or susceptibility, to one nematode species also exhibits resistance, or susceptibility, to other nematode species. The sheep from the RES line used in this experiment were selected for resistance to *H. contortus* yet, as can be seen by their significantly lower worm burdens (Table 5.1), also exhibit resistance to *Trichostrongylus* spp.

Surprisingly, the sheep from the SUS line did not have significantly higher worms burdens than those from the RB line. Selection in this flock is based on FEC (Woolaston, Barger & Piper., 1990), so perhaps it is possible the sheep from the SUS line would have had higher FECs than those from the RB line, despite the actual worm burdens. Another possibility is that the rams from the SUS line were not as susceptible to nematode infection as was expected. The rams used for this experiment were not selected for breeding within the selection flock. While culling criteria for the CSIRO Haemonchus selection flock is not based solely on FEC, the rams selected for use in the line are usually the ones showing greater susceptibility, measured by means of higher FEC.

Results such as this, that host genetic resistance status does not affect species proportion, add to evidence that genetic resistance to nematode infection is more a generally boosted acquired immune response (Windon 1991), with resistant, or high responder animals, showing increased responses across a range of immunological functions (discussed in Chapter 1), and is not species specific.

However, LeJambre (1983) has shown that host resistance can affect species within a genus differently. *H. placei* cannot establish in a mixed

infection with *H. contortus* in immunologically normal sheep, it can do so only in immunocompromised sheep. The difference seen between his work and that of Windon (1991) and Woolaston (1992) could be explained the following way. *H. contortus* is a parasite of sheep while *H. placei* is a cattle parasite, whereas the species investigated by Windon (1991), Woolaston (1992) and in this work are all sheep specific parasites. *H. placei* will not normally establish in a mixed infection in sheep but the immunosuppression lowered the host immune response enough to allow the cattle parasites to survive with *H. contortus*. In other words, immunocompromisation is not the same as genetic susceptibility to parasite infection.

A definite trend can be observed looking at either the number of worms per metre or the percentage of *T. colubriformis* found in each section along the length of the small intestine. As would be expected, in most cases, the number of worms per metre decreased moving away from the pylorus. *Trichostrongylus* spp. prefer to live nearest the pylorus so it would be expected that more individuals would occur in the preferred habitat.

This was not the case in the sheep from the RB line, however. In most of these sheep the number of worms per metre was highest in section 2, the second two metres of the small intestine. One obvious explanation for this is that the contents of the small intestine were moved around after the death of the animal before the sections were separated from one another. While this explanation is possible I would consider it unlikely. Extreme care was taken with the handling of the sheep and intestines in an effort to make sure movement of the intestinal contents between sections did not occur. One operator processed the intestines of all sheep in the experiment and the sheep were processed in random order. If operator error were the explanation it

would be expected that a similar effect would also have been observed in the other sheep lines.

The percentage of *T. colubriformis* increased moving away from the pylorus. This is consistent with data from other workers (E. Roy and I. Beveridge, unpubl.). The explanation for this phenomenon is that *T. vitrinus* is a better competitor in the host and is able to prevent *T. colubriformis* from establishing close to the pylorus. The mechanism by which this occurs is unknown. Perhaps there is active exclusion, where *T. vitrinus* individuals physically prevent *T. colubriformis* from establishing. Perhaps *T. vitrinus* effect the habitat so that it is less suitable for *T. colubriformis*. It is known that *T. vitrinus* do more damage to the epithelial lining than do *T. colubriformis*, leaving more intraepithelial tunnels and totally eroding the intestinal villi (Beveridge *et al.*, 1989). Another alternative could simply be that *T. vitrinus* develop from L3 to adult faster than *T. colubriformis* and establish themselves more quickly. Other data from E. Roy and I. Beveridge (unpubl.) show that if one of these *Trichostrongylus* spp. reaches the small intestine first it establishes nearest the pylorus and the second species arriving must move further down the intestine.

Overall, this experiment shows that while host genetics can play an important role in total number of *Trichostrongylus* spp. present, it has no effect on species proportion in a mixed *T. colubriformis*/*T. vitrinus* infection. Both the number of *Trichostrongylus* spp. and the species proportion are affected by distance from the pylorus irrespective of host genetics.

6. COMPETITION AND POPULATION DENSITIES IN FREE-LIVING STAGES

6.1 INTRODUCTION

The common species of gastrointestinal nematodes infecting sheep occur together as free-living larvae in faeces on pasture. The larvae of these species feed on bacteria and, depending on larval density, could reasonably be expected to compete for resources (food and space). *Haemonchus contortus* is a highly fecund species with each female laying many more eggs than each *Trichostrongylus* sp. female. Perhaps *T. colubriformis* and *T. vitrinus* cope with this potential competition differently with one species surviving better in the presence of *H. contortus* than the other. Since *T. colubriformis* and *T. vitrinus* overlap in their requirements for larval development it would be expected that they are in competition with each other.

If these species do compete with each other as free-living larvae, it would be expected that competition would be heightened as population density increased. Experiments were carried out to determine whether competition between the free-living stages of these two *Trichostrongylus* species and *Haemonchus contortus* occurs by looking for changes in species proportion in faecal culture over a range of population densities. A similar experiment was designed to show the effect of competition between the free-living stages of *T. colubriformis* and *T. vitrinus*.

6.2 MATERIALS AND METHODS

Seven Merino wether weaners were made worm-free by dosing with RDR of ivermectin (Ivomec, MSD AGVET), a combination of benzimidazole and levamisole (Scanda, Coopers) and closantel (Razar, Coopers). Six sheep were dosed with 30 000 infective larvae, two with *Trichostrongylus colubriformis* (McMaster BZ-susceptible), two with *T. vitrinus* (laboratory strain supplied by E. Roy, University of Melbourne) and two with *Haemonchus contortus* (Kirby strain). The remaining animal was used as a source of worm-free faeces for the duration of the experiment. The infected sheep were given 1.5 ml Dexapent (5 mg/ml dexamethasone sodium phosphate) at the time of infection and three days post infection to aid larval establishment.

Twenty-eight days post infection faeces were collected from the six infected sheep over a twenty-four hour period. The faeces were soaked in water, mixed to a thin paste then strained through cheesecloth. This mixture, 200 to 300 ml, was made up to one litre with a saturated sodium chloride solution and poured into a shallow tray. A plastic sheet was carefully laid over the top of the faeces/salt solution and left to stand for no more than 15 minutes. The eggs floated to the top of the solution and adhered to the plastic sheet. They were then washed off in water.

6.2.1 Experiment One:

The four treatment groups in Experiment One were 1000, 10 000, 50 000 and 100 000 eggs per gram (90% *H. contortus* 10% *T. colubriformis*) each of which had three replicates. The appropriate numbers of eggs of each species were placed into McCartney bottles (30 mL volume), each containing

two grams of worm-free faeces, and cultured at 26°C for 7 days. Larvae were recovered from the cultures, using an inversion technique. Each jar was filled to the top with water covered with a Petri dish and inverted. The Petri dish was then filled with water and the jar and dish were left to stand at room temperature for approximately 18 hours. After this time the water from the dish and the jar were collected and stored in tubes at 10°C. Each tube was standardised to 10 ml and the total number of L3 was counted in a 100 µl aliquots (1%), this number was multiplied by 100 to give an estimate of total number of L3 recovered from the culture. One hundred individuals were placed on a microscope slide, stained with iodine and the proportion of each species present was determined by morphological examination (Georgi, 1980).

6.2.2 Experiment Two

Experiment Two was set up as for Experiment One, however, *T. vitrinus* eggs were used instead of *T. colubriformis* eggs. The results of Experiments One and Two were compared to determine whether competition with *H. contortus* affects the two *Trichostrongylus* spp. differently. Experiments One and Two were conducted simultaneously, in the same incubator.

6.2.3 Experiment Three:

Experiment Three was similar in design to both Experiments One and Two. However, the four treatment groups were 1 000, 10 000, 15 000 and 25 000 epg and the eggs used in each group were 50% *T. colubriformis* and 50% *T. vitrinus*. One hundred individuals were identified to species using a PCR based species identification technique (Gasser *et al.*, 1993; Hoste *et al.*, 1993; Gasser *et al.*, 1994; Stevenson *et al.*, 1995; Hoste *et al.*, 1995). The results of this experiment were analysed to determine whether one species would

dominate in the cultures and whether this would change or not with increased population density.

6.3 RESULTS

All three experiments showed that the percentage of eggs developing to L3 within the seven day incubation period was reduced as the population density increased (Table 6.1). However, in Experiment One the percentage of eggs developing to L3 was higher at 100 000 epg than at 50 000 epg. Percentage development to L3 was higher at 1 000 epg in Experiments One and Two (73% and 83%, respectively) than in Experiment Three (58%), the experiment looking at competition between the two *Trichostrongylus* species.

Due to the small sample size counted from each replicate, the replicates for each treatment group having the highest and lowest estimated number of L3 recovered were tested to determine whether the counts fitted the Poisson distribution. The counts were plotted against each other on a graph with square root scales on each axis (Georgi, 1980), the theory being that if the sampling technique is satisfactory, 95% of the duplicate counts will fall within two lines drawn on the graph. This was the case for the L3 count data, indicating that the technique used for the estimation of L3 numbers in each replicate was an accurate representation of the actual numbers.

Table 6.1: Percentage of eggs developing to L3 in 7 days and proportion of L3 identified as *T. colubriformis*, *T. vitrinus* or *H. contortus* from each experiment at each of four population densities (mean of three replicates).

Species 1 /Species 2	Population Density (epg)	% L3 in 7 days	% Species 1	% Species 2
Hc/Tc	1 000	**73**	**88**	**12**
Hc/Tc	10 000	**59**	**84**	**16**
Hc/Tc	50 000	**12**	**75**	**25**
Hc/Tc	100 000	**22**	**15**	**85**
Hc/Tv	1 000	**83**	**88**	**12**
Hc/Tv	10 000	**72**	**89**	**11**
Hc/Tv	50 000	**16**	**93**	**7**
Hc/Tv	100 000	**12**	**92**	**8**
Tc/Tv	1 000	**58**	**61**	**39**
Tc/Tv	10 000	**47**	**42**	**58**
Tc/Tv	15 000	**39**	**28**	**72**
Tc/Tv	25 000	**27**	**12**	**88**

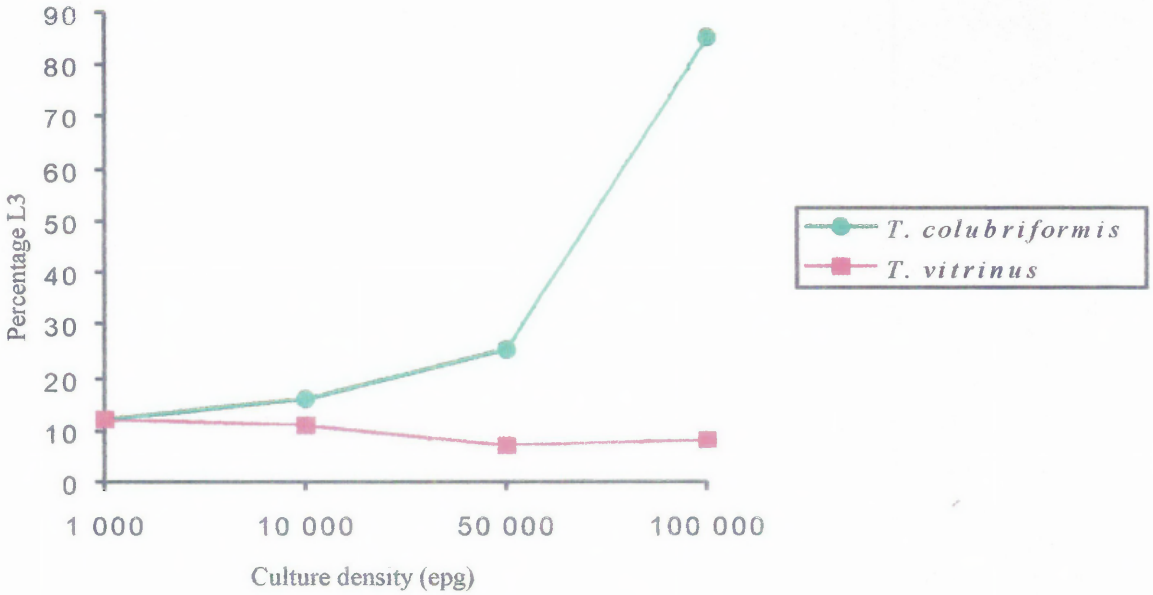

Figure 6.1: Comparison of the variation in percentage of *T. colubriformis* or *T. vitrinus* L3 recovered from faecal cultures, containing 90% *H. contortus* eggs, over four population densities.

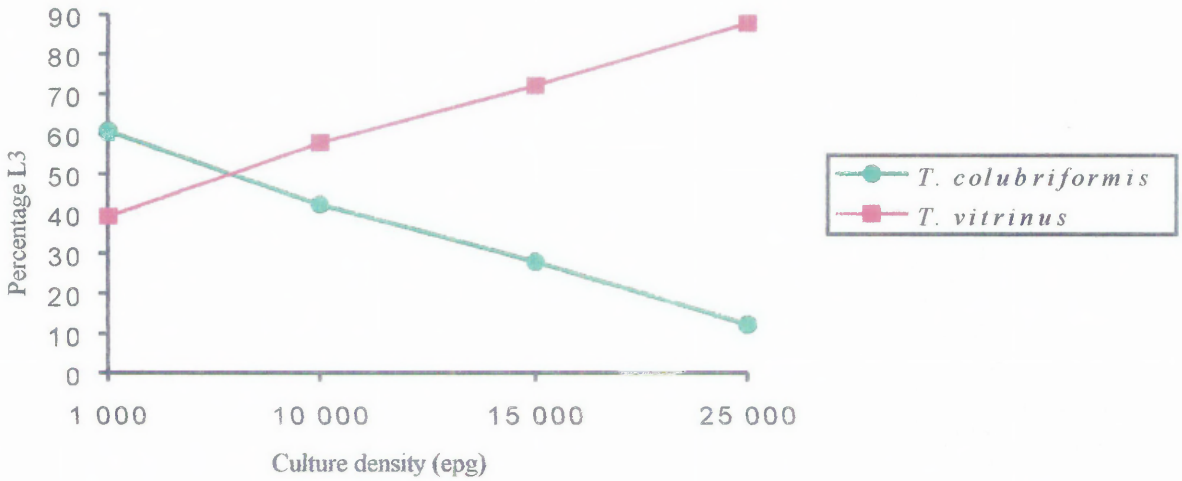

Figure 6.2: Variation in percentage of *T. colubriformis* and *T. vitrinus* L3 recovered from faecal cultures, containing 50% *T. colubriformis* and 50% *T. vitrinus* eggs, over four population densities.

The proportion of L3 identified as *T. colubriformis* or *T. vitrinus* from Experiments One and Two, the experiments including *H. contortus*, were compared as a way of determining whether one of the *Trichostrongylus* species was better able to survive in the presence of a large number of *H. contortus* competing for resources, as could be seen in faecal culture or in the field. The linear regression analysis (Steel & Torrie, 1980) of the proportion of *H. contortus* in the L3 against log proportion of *T. colubriformis* and *T. vitrinus* eggs, shows that the proportion of *T. colubriformis* developing to L3 in the culture with 90% *H. contortus* eggs was significantly different from the proportion of *T. vitrinus* developing under the same conditions.

The percentage of both *T. colubriformis* and *T. vitrinus* L3 recovered from mixed cultures with *H. contortus* remained close to the 10% found in the eggs at the lower population densities of 1 000 and 10 000 epg. However, as population density increased to 50 000 and 100 000 epg the proportion of recovered L3 identified as *T. colubriformis* increased to 85% while that of recovered L3 identified as *T. vitrinus* remained around 10% (Table 6.1, Figure 6.1).

Complications were encountered during the amplification of the ITS-2 rDNA region used for species identification. Many of the larvae would not amplify at all and some that did amplify did not cut with either DraI or Hin*f*I, the enzymes used for species identification. Fresh L3 were picked for amplification with extreme care taken at each step to ensure that the larvae were still in their microfuge tubes and that they were completely lysed before being used as template DNA. More individuals were amplified at this stage, but there were still many individuals not giving a PCR product. This process

was continued until a minimum of 50 individuals from each replicate had been amplified successfully.

Individuals that had been successfully amplified but that would not cut with either restriction enzyme were incubated with alternative restriction enzymes, Rsa1 and Vsp1, known to cut both ITS-2 from both *T. colubriformis* and *T. vitrinus* (Gasser *et al.*, 1994). None of these products were cut by these alternative enzymes. These PCR products were then cleaned by ethanol precipitation and a further attempt to cut them with Dra1 and Hin*f*1 was made. After cleaning all products could be identified as either *T. colubriformis* or *T. vitrinus*.

Chi-square analyses were conducted on the results of Experiment Three, comparing the proportion of recovered L3 identified as *T. colubriformis* or *T. vitrinus* (mean of three replicates) to the proportion of 50% in the eggs. The proportion of *T. vitrinus* was significantly greater ($P<0.005$) at the higher population densities of 15 000 and 25 000 epg (Table 6.1, Figure 6.2), while there was no significant difference ($P>0.05$) in species proportion of the L3 isolated from cultures at the lower population densities of 1 000 and 10 000 epg.

6.4 DISCUSSION

At lower population densities (1 000 and 10 000 epg) *T. colubriformis* and *T. vitrinus* appeared to compete equally with *H. contortus*. However, as the population density increased the percentage of surviving L3 found to be *T. vitrinus* did not change whereas that of *T. colubriformis* increased

significantly. This result suggests that *T. colubriformis* can compete well with the highly fecund *H. contortus*, increasing its proportion of the population by eight times. In contrast, *T. vitrinus* did not increase its proportion of the population at these high population densities; it maintained the proportion seen in the eggs. As the population density increased the proportion of eggs of all species developing to L3 in seven days decreased, so although *T. colubriformis* increased its proportion relative to that of *H. contortus* it did not necessarily increase the percentage of its total eggs developing to L3.

From the results of Experiments One and Two it could be suggested that perhaps competition with *H. contortus* is not the factor leading to the increase in *T. colubriformis* proportion at all, rather population density has an effect. However, the results of Experiment Three dispel this theory. At the higher population densities (15 000 and 25 000 epg) in Experiment Three, the species increasing in proportion in the population was *T. vitrinus* rather than *T. colubriformis*. Although these population densities are not as high as those seen in the experiments involving *H. contortus* they are high for a *Trichostrongylus* infection. This suggests that while *T. colubriformis* performs better than *T. vitrinus* in the presence of *H. contortus* at high population densities, it is at a competitive disadvantage when competing with its related species at high population densities. Further experiments would be necessary to determine which factor, or combination of factors, (eg. food, space, oxygen, toxic excretory products, physical interference) were leading to these results.

The apparent difference in the ability of *T. colubriformis* and *T. vitrinus* to "cope" with *H. contortus* at high population densities could be a factor

contributing to findings (Chapter 3) indicating that the prevalence of *T. vitrinus* has increased in the New England region of NSW in recent years. Previously *T. colubriformis* has been considered the dominant *Trichostrongylus* species in this region (Southcott, Major & Barger, 1976) but more recently up to 60% of the *Trichostrongylus* spp. identified from some paddocks have been *T. vitrinus*. Until the release of the narrow spectrum anthelmintic, closantel, *H. contortus* was a dominant species on pasture in this region. Now it is rare on properties where closantel resistance has not developed. Population peaks are usually seen in *T. vitrinus* during the winter months in winter rainfall zones of Australia. However, the reduction of *H. contortus* populations in non-seasonal high rainfall ones may allow a longer period for *T. vitrinus* larval development and thus contribute to its increase in these environments.

7. CULTURE TEMPERATURE

7.1 INTRODUCTION

The free-living larval stages of the trichostrongylid nematodes infecting livestock occur together in dung and on pasture. However, climatic distribution suggests that each species has a different temperature preference. *T. colubriformis* and *H. contortus* are generally found to dominate when the weather is warmer, or in warmer, summer rainfall regions (Southcott *et al.*, 1976; Beveridge & Ford, 1982) while *T. vitrinus* and *Tel. circumcincta* dominate in cooler weather and in cooler, winter rainfall regions (Anderson, 1972, 1973; Callinan, 1979; Beveridge & Ford, 1982).

Knowledge of this difference in temperature preference for egg hatching and larval development may be important when culturing larvae from faeces in the laboratory. Most faecal cultures are incubated at 25°C to 27°C, a temperature range which may inadvertently select for *T. colubriformis* and *H. contortus* and against *T. vitrinus* and *Tel. circumcincta*. Any type of selection pressure exerted during the culturing process could distort species proportion data collected from the cultures. Dobson, Barnes, Birclijin & Gill (1992) showed that mixed *T. colubriformis*/*Tel. circumcincta* cultures incubated at 22°C or 25°C, for six to ten days, tend to overestimate the number *T. colubriformis* present and underestimate the number of *Tel. circumcincta*.

Apart from protecting against inadvertent selection of a particular species in a mixed culture, a more detailed knowledge of culture temperature

preferences could also lead to methods of obtaining cultures dominated by the species of interest.

Two experiments were designed to investigate culture temperature preferences of four species of gastrointestinal nematodes affecting ruminants, *T. colubriformis*, *T. vitrinus* , *H. contortus* and *Tel. circumcincta*. In Experiment One eggs were cultured from Merino sheep with natural pasture infections consisting of *T. colubriformis*, *T. vitrinus* and *Tel. circumcincta*. In Experiment Two eggs were cultured from Merino sheep artificially infected with single species infections of *T. colubriformis*, *T. vitrinus*, *H. contortus* or *Tel. circumcincta*. A second objective of Experiment One was to determine whether *T. colubriformis*, *T. vitrinus* and *Tel. circumcincta* have similar sex ratios and rates of egg production.

7.2 MATERIALS AND METHODS

7.2.1 Experiment One

Two Merino wether weaners were made worm-free by treatment with the RDR of ivermectin (0.2 mg/kg, Ivomec®). The weaners were allowed to become infected by grazing a paddock previously grazed by tracer sheep for four weeks. The tracer sheep worm counts suggested, the paddock was contaminated with *T. colubriformis*, *T. vitrinus* and *Tel. circumcincta*. The infected animals were housed in pens for two weeks before being euthanased. Faeces were collected from both animals for the 24 hours immediately prior to euthanasing and the animals were not fed during this time.

The contents of the abomasum and small intestine were collected for examination of the worm species present. The contents were standardised to a volume of 500 ml, a total worm count was conducted on a 50 ml aliquot of the contents and the total worm burden was estimated. Adult male *Teladorsagia* spp. and *Trichostrongylus* spp. were collected and identified to species by spicule morphology (Lapage, 1958). Adult females were collected and identified to species using a DNA-based species identification technique (Gasser *et al.*, 1993; Hoste *et al.*, 1993; Gasser *et al.*, 1994; Stevenson *et al.*, 1995; Hoste *et al.*, 1995). For identification of both males and females, 100 individuals from each genus were examined.

Faecal egg counts (FEC) were conducted on the faeces from each sheep using a modified McMaster technique. Eggs were then separated from 28 grams of faeces from each animal, using a saturated sodium chloride solution flotation technique (described in Chapter 6). One hundred eggs from each animal were identified to species using the DNA-based identification method.

The remaining faeces from each animal were divided between four jars. One jar from each animal was incubated for 21 days at 5°C followed by seven days at 27°C, 14 days at 20°C, or seven days at either 27°C or 35°C. Follwing 21 days of incubation at 5°C, approximately half of the faeces from the first jar were placed in a Baerman apparatus, no larvae were isolated, so the incubation temperature was raised to 27°C for seven days. At the end of the incubation period L3 were recovered from the cultures by an inversion method (described in Chapter 6). A 10% aliquot of the recovered L3 from each jar was counted to

estimate the number of L3 present before 100 individuals were identified to species using the PCR-based species identification technique.

The number and proportion of males of each species were calculated and compared to the proportions of females, larvae and eggs. The results were analysed to determine whether culture temperature affects the proportion of each species developing to L3. Analyses were also carried out to determine differences in the proportion of males and females of each species and the relative number of eggs released by each species over a 24 hour period.

7.2.2 Experiment Two

Eight Merino wether weaners were made worm free by treatment with two times the RDR of Ivomec™ (0.4 mg/kg ivermectin) and Scanda™ (4.53 mg/kg oxfendazole; 9.6 mg/kg levamisole) and housed indoors. Two weeks after dosing two sheep were infected, via intraruminal injection, with 20 000 *T. colubriformis* L3 (McMaster BZ-susceptible), two with 20 000 *T. vitrinus* L3 (laboratory strain supplied by E. Roy, University of Melbourne), two with 20 000 *Tel. circumcincta* L3 ("Chiswick" field strain) and two with 5 000 *H. contortus* L3 (Kirby strain). After 28 days, when the infections were patent, faeces from each animal were collected over a 24 hour period. The faeces from each pair infected with the same species were combined and mixed thoroughly. FEC were conducted on the mixed faeces to estimate the number of eggs going into each culture.

Fifty grams of faeces were cultured using the following conditions; 5°C for 14, 21 and 28 days, 10°C for 7, 14 and 21 days, 20°C for 7 and 14 days,

27°C for 7 days and 35°C for 7 days. All four nematode species were cultured under all conditions. Standard sized culture jars (500 mL volume) were used and 15 ml of distilled water was added to each culture. There were three replicates for each set of culture conditions

After the appropriate incubation duration, L3 were collected from the cultures using an inversion technique (described in Chapter 6). Larvae from aliquots equaling 10% of the volume were stained with iodine and examined, under a coverslip, on a slide under a compound microscope. All L3 in the aliquots were counted and the number recorded for each replicate under each set of culture conditions.

The results were analyzed to determine the effect of the different culture conditions on the percentage of eggs developing to L3 within the culture duration. Results were compared with the standard culture conditions of 27°C for seven days.

The standard culture conditions of 27°C for seven days were used as the basis for comparison for Experiment Two because the results from Experiment One showed the greatest percentage development to L3 under these conditions for the three species investigated and that species proportion was not significantly altered. The culture temperatures and durations chosen for Experiment Two were chosen on the basis of the results from Experiment One.

7.3 RESULTS

7.3.1 Experiment One

The ratio of females to males was calculated (from Table 7.1) as 2.3:1 for *T. colubriformis*, 0.7:1 for *T. vitrinus* and 1.4:1 for *Tel. circumcincta*. Rates of egg laying were only calculated for *T. colubriformis* and *Tel. circumcincta* because no eggs were identified as being *T. vitrinus*. Egg laying rates were calculated using the total mass of faeces collected over 24 hours, the number of females estimated for each species and the proportion of eggs identified as *T. colubriformis* or *T. vitrinus*. For *T. colubriformis* the egg laying rate was determined to be 342.9 eggs per female per 24 hours and for *Tel. circumcincta* to be 321.6 eggs per female per 24 hours.

A Chi-square analysis (Elliot, 1979) was performed on the numbers of indivduals identified as each species (Table 7.2), comparing numbers of L3 of each species identified from each set of culture conditions and the numbers of each species identified from the eggs. Because of the very small number of *T. vitrinus* identified in the infection, *T. vitrinus* counts were combined with those of *Tel. circumcincta* for the analysis. It was considered feasible to combine the results for *T. vitrinus* and *Tel circumcincta* due to their similar climatic preferences. The results of the analysis showed that species composition of the eggs and after culturing for 14 days at 20°C or seven days at 27°C were not significantly different ($P>0.05$). Species compositions after culturing for 21 days at 5°C followed by seven days at 27°C or after culturing for seven days at 35°C were significantly different ($P<0.05$), indicating that these culture conditions had a significant effect on species proportion. After incubating at

5°C *Tel. circumcincta* was the dominant species in the infection, whereas after incubating at 35°C *T. colubriformis* dominated the infection.

The standard culturing conditions of seven days at 27°C gave the highest percentage of eggs developing to L3 (Table 7.3). When compared to this level of development using a Chi-square analysis, development at 5°C, 20°C and 35°C was significantly lower ($P<0.005$).

Table 7.1: Numbers and proportions of adult male and female nematodes identified from the abomasum and small intestine of two Merino wether weaners.

	T. colubriformis	*T. vitrinus*	*Tel. circumcincta*	Other
No. males	123	44	670	3
% males	22.9	4.8	72.0	0.3
No. females	283	32	960	3
% females	22.2	2.5	75.1	0.2

Table 7.2: Numbers of individuals identified as *T. colubriformis*, *T. vitrinus* or *Tel. circumcincta* from each treatment group (total number counted in parentheses).

	T. colubriformis	*T. vitrinus*	*Tel. circumcincta*
Eggs	31 (160)	0 (160)	129 (160)
Larvae @ 5°C	0 (100)	2 (100)	98 (100)
Larvae @ 20°C	17 (100)	1 (100)	82 (100)
Larvae @ 27°C	28 (100)	3 (100)	69 (100)
Larvae @ 35°C	89 (92)	0 (92)	3 (92)

Table 7.3: Percentage of individuals identified as *T. colubriformis*, *T. vitrinus* or *Tel. circumcincta* from each treatment group.

	T. colubriformis	***T. vitrinus***	***Tel. circumcincta***	**% eggs developed to L3**
Eggs	19.4	0	80.6	-
L3 @ 5°C	0	2.0	98.0	1.1
L3 @ 20°C	17.0	1.0	82.0	31.2
L3 @ 27°C	28.0	3.0	69.0	56.0
L3 @ 35°C	96.7	0	3.3	1.3

7.3.2 Experiment Two

From the FEC on the combined faeces of the two sheep infected with each species and the total mass of faeces added to each culture, the total number of eggs in each culture was estimated at 149 972 for *T. colubriformis*, 135 063 for *T. vitrinus*, 74 953 for *Tel. circumcincta* and 154 930 for *H. contortus*. The numbers of L3 emerging from each culture and the percentage of eggs that developed to L3 within the stipulated culture duration are listed in Tables 7.4 and 7.5.

The number of L3 emerging from each set of culture conditions was compared to the standard culture conditions of seven days incubation at 27°C using a Chi-square analysis (Elliot, 1979). The results of the analyses showed that incubating *T. colubriformis* at 5°C or 10°C for any culture duration, 20°C for 14 days or 35°C for seven days significantly altered the number of L3 emerging from the cultures ($P<0.005$). The cooler incubation temperatures decreased the number of L3 developing while the warmer temperatures,

including 20°C for 14 days, increased the number of L3. Incubating at 20°C for seven days did not produce a significant effect ($P>0.05$).

The development to L3 of *T. vitrinus* was significantly affected by culturing at 5°C or 10°C for any culture duration, 20°C for 14 days and 35°C for seven days ($P<0.005$). Incubating cultures at 20°C for seven days had no significant effect ($P>0.05$). Of the culture conditions having a significant effect, only 20°C for 14 days increased the number of L3 developing.

The development of *Tel. circumcincta* eggs to L3 was significantly affected by all variations on culture conditions ($P<0.005$). However, while statistically significantly different, there is less variation in the number of L3 developing when incubated at 5°C for 21 or 28 days, 10°C for 21 days or 20°C for seven or 14 days than was seen in the other species examined. Incubation at 5°C for any duration, at 10°C for seven or 14 days or 20°C or 35°C for seven days decreased the number of L3 developing, while incubating at 10°C for 21 days or 20°C for 14 days increased the number.

All culture condition variations examined significantly affected development of *H. contortus* to L3 compared to the standard culture conditions of 27°C for 7 days ($P<0.005$). There was no development to L3 during incubation at 5°C or 10°C for any culture duration and development was decreased when cultures were incubated at either 20°C or 35°C.

Table 7.4: Numbers of L3 counted for each of 10 culture conditions for *Trichostrongylus colubriformis*, *T. vitrinus*, *Teladorsagia circumcincta* and *Haemonchus contortus* (mean of 3 replicates-estimated from 10% counted).

Culture conditions	No. *T. colubriformis*	No. *T.vitrinus*	No. *Tel. circumcincta*	No. *H. contortus*
14 days @ 5°C	167	3033	2300	0
21 days @ 5°C	233	19200	11333	0
28 days @ 5°C	233	19433	11233	0
7 days @ 10°C	18500	21367	3433	0
14 days @ 10°C	21567	31800	8800	0
21 days @ 10°C	22533	58700	14667	0
7 days @ 20°C	83167	83033	11967	55367
14 days @ 20°C	139400	96133	13667	55600
7 days @ 27°C	90133	80633	12967	77000
7 days @ 35°C	99367	4567	100	48133

Table 7.5: Percentage of eggs of each species that developed to L3 in the specified culture duration, calculated from FEC and L3 count data (mean of 3 replicates estimated from 10% counted).

Culture conditions	% *T. colubriformis*	% *T.vitrinus*	% *Tel. circumcincta*	% *H. contortus*
14 days @ 5°C	0.1	2.5	3.3	0
21 days @ 5°C	0.2	16.0	16.2	0
28 days @ 5°C	0.2	16.2	16.0	0
7 days @ 10°C	12.3	17.8	4.9	0
14 days @ 10°C	14.4	26.5	12.6	0
21 days @ 10°C	15.0	48.9	21.0	0
7 days @ 20°C	55.4	61.5	16.0	35.7
14 days @ 20°C	92.9	71.2	18.2	35.9
7 days @ 27°C	60.1	59.7	17.3	49.7
7 days @ 35°C	66.2	3.4	0.1	31.1

7.4 DISCUSSION

Experiment One has shown that incubation temperature significantly affected species proportion in faecal culture. The commonly used culture conditions of 27°C for seven days did not significantly alter species proportions and gave the best egg hatch results. A lower culture temperature of 20°C for 14 days most closely represented the species proportions seen in the adult population although the percentage which hatched was reduced by nearly 25%. When culture temperature was raised to 35°C for seven days species proportion was significantly changed, selecting for *T. colubriformis*. A significant change in species proportion was also observed when cultures were incubated for 21 days at 5°C before incubation at 27°C for seven days, however, in this instance selection was for *T. vitrinus/Tel. circumcincta*. For both increased and decreased culture temperatures percentage egg hatch was reduced to around 1% development to L3 in the stipulated culture duration.

Experiment Two has shown that culture conditions can have a significant effect on development from egg to L3 in the four trichostrongylid species examined. If this result were transferred to a mixed culture instead of the single species cultures used here, it would translate to a significant effect on species proportion. Reinforcing the results of Experiment One, increased culture temperature led to an increase in *T. colubriformis*, while a decrease in culture temperature led to an increase in *T. vitrinus* and *Tel. circumcincta*.

H. contortus was observed to be a much more sensitive species. There was no development to L3 at all in the *H. contortus* cultures incubated at 5°C or

10°C and a significant reduction in development to L3 when incubated at 20°C or 35°C.

From the results of Experiment One it can be concluded that, in a laboratory situation, incubating at 27°C for seven days should not significantly alter the species proportion present in the eggs, and the greatest percentage development to L3 should be achieved. This result is different from that seen by Dobson *et al.* (1992) where incubation at 25°C for seven days caused the number of *Tel. circumcincta* present to be underestimated while that of *T. colubriformis* was overestimated.

The strains used by Dobson *et al.* (1992) included a laboratory strain (*T. colubriformis,* McMaster susceptible) and a strain from Victoria (*Tel. circumcincta,* JRO) while the strains used in Experiment One were field strains obtained when the tracer sheep were grazing on the CSIRO Pastoral Research laboratory paddocks near Armidale, NSW. It has previously been shown that different strains of the same species can exhibit markedly different temperature preferences for hatching (Crofton & Whitlock, 1965b; Crofton, Whitlock & Glazer, 1965; LeJambre & Whitlock, 1976), which could well translate into differences in preferences for development to L3. Strain difference could be the reason for the different findings, as the strain used for Experiment Two did not yield as many L3 after incubation at 27°C for seven days as did the field strain used in Experiment One.

An egg hatch experiment conducted by Crofton (1965) measured the temperature range over which eggs from a number of nematode species would hatch and the time it took them to do so. The species examined included *H.*

contortus, *Tel. circumcincta* and *T. vitrinus* and their temperature ranges for hatching were 9°C to 36°C, 4°C to 34°C and 8°C to 36°C, respectively. As would be expected, Crofton's results showed that the time taken to hatch increased as temperature decreased.

In the current experiments, no *H. contortus* L3 were found when the cultures were incubated at 10°C, even for 21 days, yet Crofton (1965) found them to hatch at 9°C. An explanation for this difference, besides strain variability is that every egg that hatches does not necessarily develop to L3. Dobson *et al.* (1992) have shown that low recoveries of L3 from faecal cultures is due to death at the L1 and L2 stages. Both Experiment One and Two, however, did show *Tel. circumcincta* individuals developing to L3 at 35°C, above the temperature Crofton (1965) found them to be able to hatch and *T. vitrinus* developing to L3 at 5°C, below Crofton's hatch limiting temperature.

Strain difference seems to be an obvious choice of explanation in these cases. Crofton & Whitlock (1965a) have described strains of *Tel. circumcincta* from the United States and the United Kingdom with egg hatch ranges of 10°C to 38°C and 4°C to 34°C, respectively. The strains used in the current experiments would appear to extend over most of the range of both strains investigated by Crofton & Whitlock (1965a). They were able to develop to L3 over the range of 5°C to 35°C which suggests they could hatch beyond this range.

From Experiment Two, a lower culture temperature of 20°C and an extended culture duration of 14 days, significantly increased the number of both *Trichostrongylus* species and *Tel. circumcincta*. Species proportions of L3 can

be manipulated to achieve a culture predominantly of the species of choice. A culture dominated by *T. colubriformis* can be achieved by incubating at 35°C for seven days. The percentage development to L3 of *T. colubriformis* under these conditions was higher at 66.2% than that at the standard culture conditions while percentage development of the other three species was significantly reduced.

H. contortus are likely to be totally removed from a mixed culture by incubating at a temperature of 10°C or lower. These temperatures will still allow development of the other three species, although that of *T. colubriformis* is very significantly reduced, even with an extended culture duration. Incubating at 10°C for 21 days will allow development to L3 for both *T. vitrinus* and *Tel. circumcincta* at reasonable levels (an increased rate of development for *Tel. circumcincta*). None of the culture conditions examined could differentiate between the two species preferring a colder development temperature, *T. vitrinus* and *Tel. circumcincta*, or between the two species preferring a warmer development temperature, *T. colubriformis* and *H. contortus*.

Extrapolating these data to a natural paddock environment gives results that would be expected knowing the epidemiology of the four species examined. Both *T. colubriformis* and *H. contortus* are active in the field during the warmer months of the year and were shown to have a greater percentage development from egg to L3 at warmer culture temperatures, with the reverse being true for both *T. vitrinus* and *Tel. circumcincta*. Similarly, it is known that development is slowed at cooler temperatures, a fact that was echoed in the results with percentage development at cooler culture temperatures reaching

levels closer to those at standard conditions when the culture duration was extended.