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Analysis of the sheep/Haemonchus relationship

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Abstract

The kinetics of *Haemonchus contortus* (Barbers pole worm) infection in sheep is poorly understood. Elucidating the timing and stages of the sheep immune response to *Haemonchus* may lead to new strategies for controlling infection. To pursue this goal, four main sheep trials were conducted. These were designed to describe the course of infection (Experiment 1), to identify the timing of immunity on larval establishment and adult fecundity (Experiment 2A, 2B and 3), and to examine the effects of nutrition and immunosuppression on infection (Experiment 4).

These experiments successfully generated pathological symptoms of haemonchosis such as anaemia, and detected an immune response to the worms. Microarray results will identify genes involved in immunity to *Haemonchus* which will assist in breeding sheep which are resistant to infection. For the first time in *Haemonchus* infections, a correlation has been found between worm size and egg content. This effect which influences egg production appears to be linked to white blood cell numbers. This will help us to identify a mechanism for immune regulation of egg production which is an important factor in the spread of infection. Differential gene expression in *Haemonchus* has been demonstrated via differential display PCR using worms collected from immune and nonimmune sheep. Identification of such genes may provide new targets for parasite control.

Executive Summary

Gastrointestinal nematodes cost the Australian sheep industry $369 million annually or 8.7% of its total value (Sackett*, et al.* 2006). Barbers pole worm (*Haemonchus contortus*) is a blood feeding nematode that parasitises the abomasum. It is one of the three most important sheep roundworms in Australia (along with *Trichostrongylus* and *Ostertagia*) and represents a serious constraint to sheep production in regions with summer dominant rainfall such as northern NSW, south-eastern Queensland and the coastal regions of south-west WA. Its high fecundity leads to rapid contamination of pastures with larvae and high levels of ingestion cause acute death. Chronic blood loss caused by lighter *Haemonchus* burdens can result in anaemia, anorexia, reduction in body weight and wool growth, depression and death.

The costs of haemonchosis are related to reduced productivity, prophylaxis, treatment and sheep deaths. The continuing rise of anthelmintic resistance is a severe threat to the profitability of the Australian sheep industry. To develop improved and/or alternative methods for controlling *Haemonchus* such as genetic selection of resistant sheep, a better understanding of the host parasite relationship is essential. This is a dynamic relationship and altering it in favour of the host could help ameliorate parasitism.

The objectives of this project were;

- To describe parasite population dynamics and the host response to infection.
- To develop a method for serial sampling of worms and abomasal tissue from the same animal during an *Haemonchus* infection.
- To identify parasitological and molecular events associated with a switch to inhibition of larval establishment.
- To determine the morphological and molecular effects of host immunity on *Haemonchus.*
- To examine the influence of diet and immunosuppression on sheep immunity to *Haemonchus*.

The first step in this project was to understand the changes in parasite levels during an infection. For this reason, the first experiment examined the establishment of *Haemonchus* populations and the immune response by comparing sheep given a bolus dose of larvae with sheep given trickle doses of larvae prior to the bolus dose. Experiment 2A used staggered doses of ivermectin-resistant larvae as a marker to examine the timing of the immune response on the establishment of larvae. Permanent abomasal fistulas were implanted into 18 sheep and serial sampling of abomasal mucosa and *Haemonchus* was undertaken through these fistulas to enable cellular and molecular events to be observed in the same animal over time. The effect of sheep immunity on adult *Haemonchus* fecundity was examined in Experiment 2B. Experiment 3 repeated important time points identified in Experiment 2A to allow larger amounts of post-mortem material to be collected. In the final trial, sheep immunity to *Haemonchus* was observed under corticosteroid-induced immunosuppression and a reduced protein diet (Experiment 4).

These experiments successfully generated pathological symptoms of haemonchosis such as anaemia, and detected an immune response to the worms. Immunohistochemistry results showed general trends which are consistent with a Th2 (humoral) immune response. A Th2 response is associated with reduced nematode burdens in several species. In a parallel study in SheepGenomics, 41 microarrays have been performed using RNA from serial biopsies of abomasal mucosa in live sheep and abomasal mucosa collected at post-mortem. These will help to identify genes involved in immunity to *Haemonchus* which will assist in breeding sheep which are resistant to infection. The use of image analysis software to measure the area and perimeter of worms collected at post mortem has been highly effective. The most statistically significant reductions were measured from worms in trials where larval challenge continued closest to the slaughter date, suggesting that the presence of worms or recent worm exposure is required to maintain the immune response. A unique finding was a correlation between adult worm size and egg content. This leads to the conclusion that the immune regulation of control of egg production is manifest through the regulation of worm size and presumably growth which will help us to identify a mechanism. Differential gene expression in *Haemonchus* has been demonstrated via differential display PCR using worms collected from immune and nonimmune sheep. Twenty-five differentially expressed bands have been identified to date. Cloning and sequencing of such bands may lead to the identification of proteins which can be targeted for vaccination or chemical control.

Contents

1 Background

Sheep have several mechanisms to control parasite numbers. These include reducing establishment of larvae, inducing arrested development, stunting worms, expelling established worms and reducing the egg-laying capacity of worms. In terms of the development of host immunity, each developmental stage of *Haemonchus* may be considered a separate organism due to differences in behaviour, environmental niche and the stage specific antigens produced (Balic*, et al.* 2000; Balic*, et al.* 2000).

The effectors regulating the establishment, fecundity and rejection of worm populations vary according to the stage and persistence of worms present, ongoing exposure to larvae, and the physiological and immune status of the sheep.

Studies of helminthiasis from various species give us some understanding of the immune response. Th2 cytokines are associated with reduced nematode burdens in mice (Urban*, et al.* 1998; Schopf*, et al.* 2002), humans (Turner*, et al.* 2003) and sheep (Gill*, et al.* 2000). This response is characterised by $CD4^+$ helper T cells secreting IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Else and Finkelman 1998; Lacroux*, et al.* 2006).

When larvae penetrate the abomasal mucosa, there is an increase in the percentage of activated $CD4^+$ helper T cells, γδ-TCR^+ T cells and B cells in the abomasal mucosa. In a primary infection, this is observed at about five days post-infection (Balic*, et al.* 2000), whereas in a repeat infection significant recruitment can be observed by three days (Balic*, et al.* 2002). In both cases, there is also an increase in the number of activated $CD4^+$ helper T cells in the abomasal lymph node. However, significant increases in the percentage of γδ-TCR^+ T cells, $CD4^+$ $CD25^+$ T cells and B cells only occur in the abomasal lymph node during repeat infections (Balic*, et al.* 2002).

Seven days after *Haemonchus* infection, a six-times increase in the numbers of IgA-, IgG1- and IgM-containing cells was observed in the abomasal submucosa compared to uninfected control animals (Gill*, et al.* 1992). IL-5 from T-cells stimulates an eosinophilia in the blood and mucosa. Eosinophils possess receptors for components of the complement system, IgG, IgA and low-affinity receptors for IgE. *In vitro*, they accumulate around L3 larvae, and degranulate onto their surface, causing morphological damage to the larvae (Rainbird*, et al.* 1998). There are conflicting reports on the *in vivo* effects of eosinophils. One study found a weak statistical link between higher concentrations of IL-5, greater peripheral eosinophilia and lower faecal egg counts (Doligalska*, et al.* 1999). Another study found eosinophilia was associated with increased resistance to parasites in lines of Australian Merinos and New Zealand Romneys selected for resistance on the basis of low faecal egg counts (Hohenhaus and Outteridge 1995). In contrast, challenge experiments using *Haemonchus* (Fakae*, et al.* 2004) and *Teladorsagia* (Macaldowie*, et al.* 2003) in goats have not observed any significant differences in blood and tissue eosinophil counts between resistant and non-resistant animals.

The rate of establishment of adults in sheep with low *Haemonchus* immunity is estimated to be constant after trickle infection with L3 larvae regardless of the size of the infecting dose (Dineen and Wagland 1966; Barger and Le Jambre 1988). Subsequent to the

development of resistance 4-7 weeks later, most larvae penetrating the abomasal mucosa undergo developmental arrest or fail to establish. The mechanisms for these phenomena are unknown.

Loss of adult worms occurs at a very slow rate in the absence of further challenge with larvae (Barger and Le Jambre 1988). After the development of resistance, the loss of previously established adult worms increases with the rate of larval intake and the duration of the host's previous infections. By week 15 of an experiment dosing sheep weekly with 2400 or 4800 infective L3, less than 20 adult worms remained (Barger*, et al.* 1985).

The changes in the abomasum which occur after larval infection ceases do not seem to be maintained during infection with the adult parasite. A characteristic feature of adult *Haemonchus* burdens is an increase in the number of lamina propria and intraepithelial mast cells (Balic*, et al.* 2002). Expansion of mast cell populations in parasitised animals is dependent on cytokines produced by activated T cells, in particular IL-3 (Lantz*, et al.* 1998). In the context of a switch from anti-larval to anti-adult immunity, a negative correlation has been observed between tissue eosinophilia and mast cell-derived intraepithelial globule leukocyte numbers (Meeusen and Piedrafita 2003).

In sheep with significant numbers of mucosal mast cells and globule leukocytes, a single bolus of $1x10^6$ L3 can result in larval rejection within hours of infection, before they can enter the gastric mucosa (Miller 1996). This is known as rapid expulsion or immune exclusion and may remove any stage present in the abomasum. Rapid expulsion may account for 'self-cures' of adult *Haemonchus* infections, but self-cure has never been consistently reproducible under experimental conditions (Miller*, et al.* 1983; Jackson*, et al.* 1988; Rothwell 1989). Activation and degranulation of these cells by incoming larvae results in inflammation similar to type I hypersensitivity, increased water secretion and increased abomasal motility. This can be detected by release of sheep mast cell proteinase (SMCP or Chymase) into the serum (Huntley*, et al.* 1992; Douch*, et al.* 1996).

The mean size of worms and the number of worms are both important in the pathogenesis of *Teladorsagia* infection in lambs (Stear*, et al.* 1999). In this species worm length is positively associated with fecundity. There is a strong negative genetic correlation between faecal egg count and host bodyweight (-0.8) (Bishop*, et al.* 1996). Lambs control worm length but not worm number (Stear*, et al.* 1999). The major mechanism for controlling worm length appears to be parasite-specific local IgA (Smith*, et al.* 1985). The antigen specificity of the IgA response is important because a strong IgA response is not necessarily protective. There is extensive heterogeneity in antigens between third-stage, fourth-stage and adult *Teladorsagia*. Statistically significant relationships have been shown between IgA recognition of four epitopes on *Teladorsagia* antigens and variation in adult *Teladorsagia circumcinta* female worm length. Two of these epitopes are from adult parasites and two from fourth-stage larvae (McCririe*, et al.* 1997). Over 90% of the variation in adult *Teladorsagia circumcinta* female worm length can be accounted for by worm number, IgA response to fourth-stage larvae and specificity of the antibody response (Stear*, et al.* 1995). Similarly, there are distinct immune responses targeting *Trichostrongylus* larval stages and adults (Emery*, et al.* 1992). These can have a cumulative affect on adult morphology and fecundity. These

studies have not been conducted with *Haemonchus* and are a necessary prerequisite for rational control in sheep.

2 Project Objectives

This project aimed to improve understanding of the relationship between sheep and *Haemonchus*. It focused particularly on mechanisms associated with acquired immunity to larval establishment and a reduction in adult fecundity. The objectives were;

- To describe parasite population dynamics and the host response to infection.
- To develop a method for serial sampling of worms and abomasal tissue from the same animal during an *Haemonchus* infection.
- To identify parasitological and molecular events associated with a switch to inhibition of larval establishment.
- To determine the morphological and molecular effects of host immunity on *Haemonchus.*
- To examine the influence of diet and immunosuppression on sheep immunity to *Haemonchus*.

3 Methodology

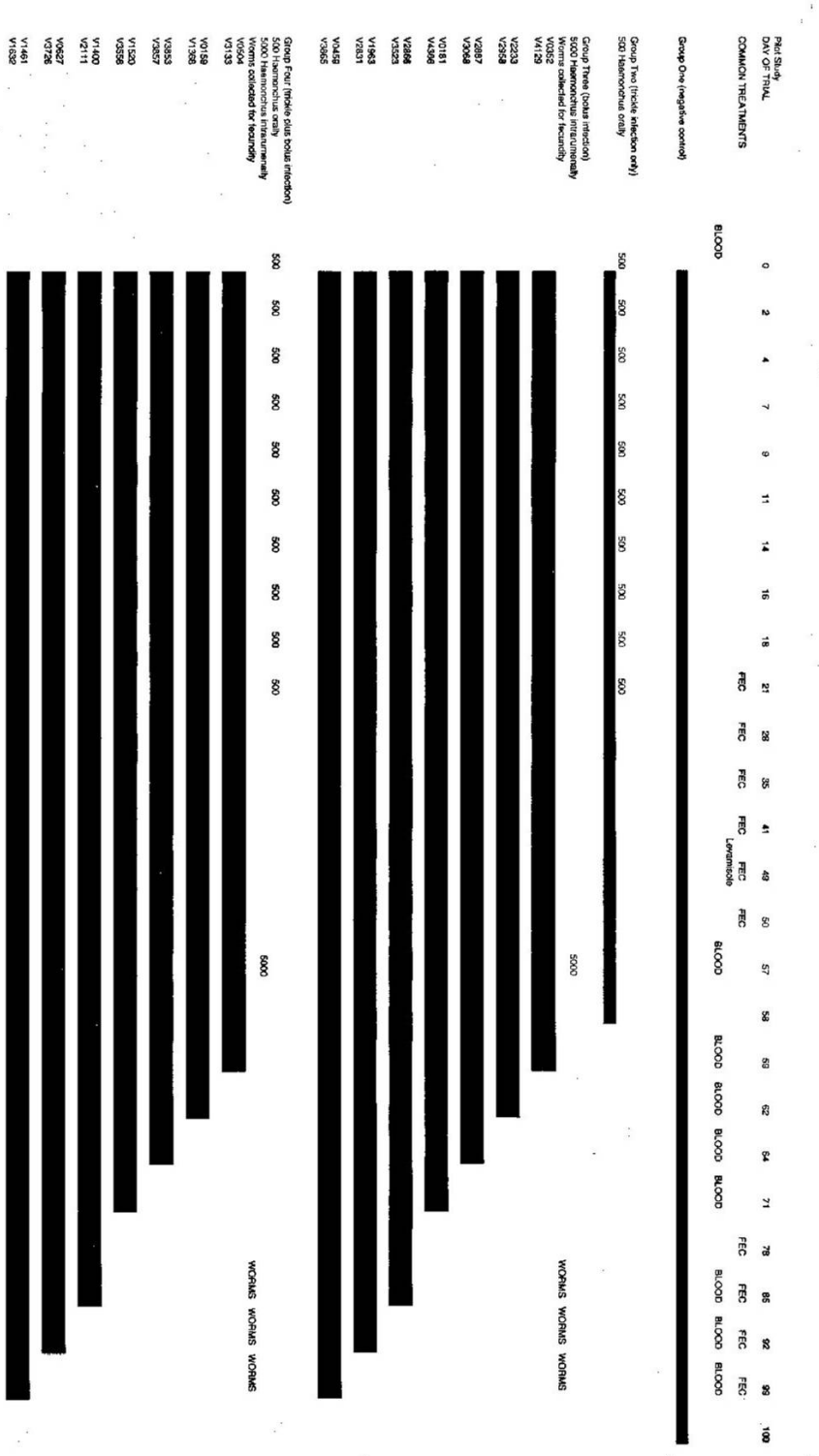
All sheep work was approved by The University of Sydney Animal Ethics Committee (Project Number N00/8-2003/3/3797).

3.1 Experimental Designs

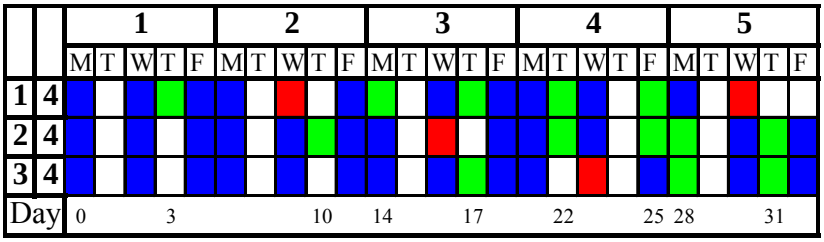
3.1.1 Design of Experiment 1

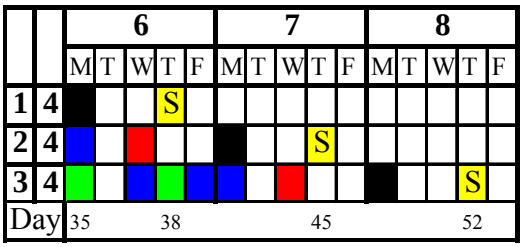
The duration of this trial was one hundred days. The four groups of sheep involved and their treatments are depicted in the Gantt chart on the next page. The non-linear scale of days along the top of the chart lists days where events in the trial occurred. Treatments common to all groups are listed beneath this. Group one was the negative control. These sheep received no worms and were slaughtered on day 100. Group two received ten doses of 500 L3 over 21 days and were slaughtered on day 58. Group three received a single bolus dose of 5000 L3 on day 57. Group four received the treatments of group two and three. The ear tag numbers of sheep in groups three and four are listed on the left of the chart and the horizontal bar for each pair ends on the day of slaughter. Abomasal contents were collected from every animal at slaughter. *Haemonchus* counts (total, adult and L3/L4) were performed in triplicate. These were averaged per animal and then per pair and graphed for each time point. Tissues collected at slaughter were lymph nodes (prescapular and abomasal), abomasal wall and abomasal folds. Samples were appropriately stored for DNA, RNA, ELISA, protein analysis, histology and immunohistochemistry. The days for blood collection, faecal egg counts (FEC) and collection of worms at slaughter for determination of egg loads in female worms (fecundity) are listed on the Gantt chart.

Bar chart showing the design of Experiment 1.



3.1.2 Design of Experiment 2A

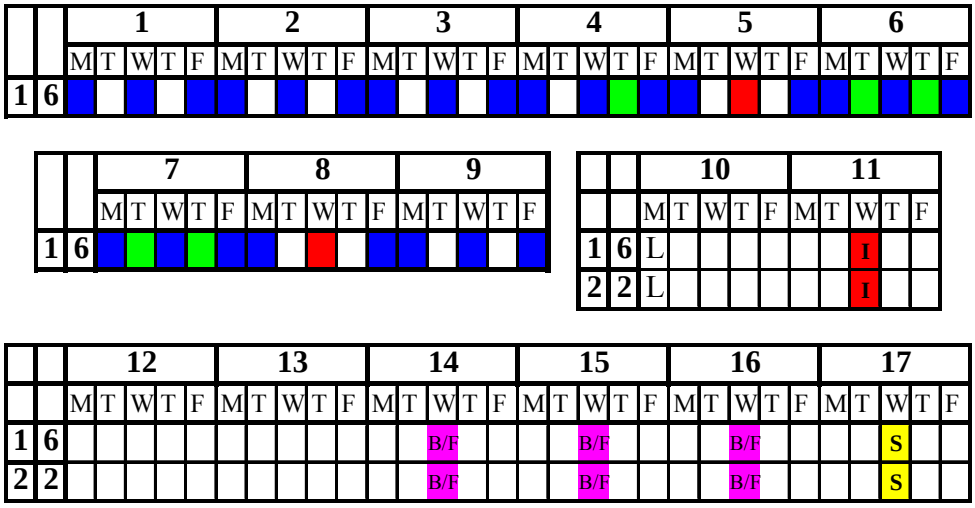




This trial ran for nine weeks and involved 12 sheep (n=4 per group). All sheep were surgically implanted with abomasal fistulas to enable mucosal biopsies to be taken (green cells). All groups were dosed with 500 ivermectin-sensitive L3 three times weekly (blue cells). On the days denoted by the red cells in the figure, the ivermectin-sensitive L3 were substituted with 500 ivermectin-resistant L3. Four days after the second resistant dose, each sheep was dosed with ivermectin (black cells). Eight days after the second resistant dose each group was slaughtered (yellow cells). At slaughter, any adults present should be from the first resistant dose, and any larvae present should be from the second resistant dose. This design enabled the effect of developing immunity to be observed on the establishment of the ivermectin-resistant larvae.

3.1.3 Design of Experiment 2B

Experiment 2B involved 8 sheep (six sheep in group one and two sheep in group 2). Group 2 was added as a control in week 10.

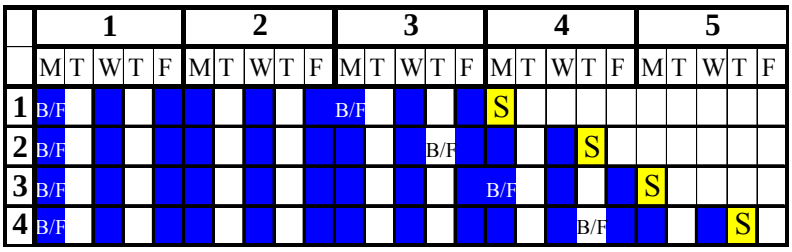


In contrast to Experiment 1, the sheep used for Experiment 2B were fistulated and immunity was induced by dosing with 500 *Haemonchus* L3 three times weekly for nine weeks in parallel with animals in Experiment 2A (McMaster strain - blue panels CAVR strain – red panels). Group one and two were drenched with levamisole in week 10 to remove all *Haemonchus* (L). 5000 L3 were administered intrarumenally in week 11 (I - red panel). Adult worms were then serially collected via the fistula in weeks 14, 15, 16 (pink panels) and 17 (post mortem – yellow panel).

3.1.4 Design of Experiment 3

Experiment 3 involved 30 sheep in six groups (n=5 per group) and consisted of two parts.

3.1.4.1 Experiment 3 Groups 1 to 4



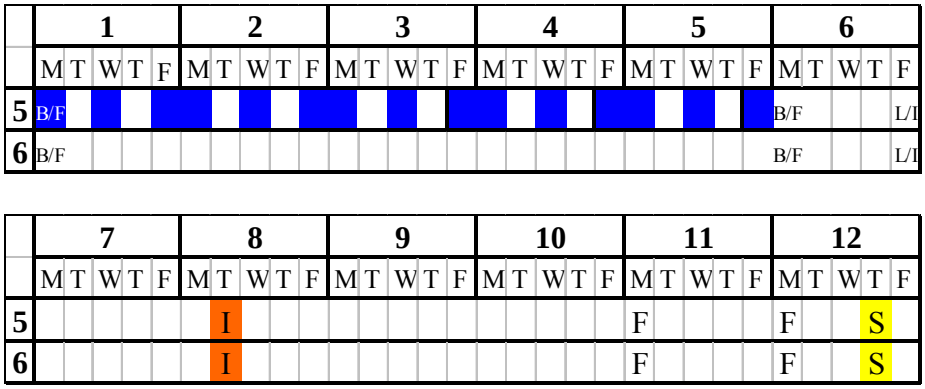
The first part of Experiment 3 consisted of groups 1 to 4. These were orally dosed with 500 L3 (McMaster strain) 3 times weekly for up to 5 weeks (blue panels). Groups were progressively slaughtered on Mondays and Thursdays in weeks four and five (S - yellow panels). These were the time points identified from Experiment 2A to be most significant in the switch to immunity against larval establishment with this infection regime.

Blood and faeces were collected from all animals at the beginning of the trial (B/F), one week pre-slaughter and at slaughter. Blood was fractionated into plasma, red cells and white cells. These samples were stored at -20^{O}C.

Samples collected at slaughter were abomasal mucosa and lymph nodes (abomasal, hepatic and pre-scapular). Samples of mucosa were frozen in liquid nitrogen and stored at -80^{O}C for protein and RNA, embedded in OCT (which was frozen and stored at -80^{O}C for immunohistochemistry), and fixed in 10% neutral-buffered formalin for histology with haematoxylin and eosin (general morphology and eosinophils) and toluidine blue (mast cells). Lymph nodes were frozen in liquid nitrogen and stored at -80^{O}C.

Samples of the adult worm population were collected from groups 3 and 4 for investigation by dd-PCR. Common samples collected from all sheep were tubes containing male worms only, female worms only and mixed worms. These were frozen in liquid nitrogen and stored at -80^{O}C. In addition, the sheep in each group with the highest faecal egg count had 40 male worms and 40 female worms collected and stored individually at -80^{O}C.

3.1.4.2 Experiment 3 Groups 5 and 6



The second part of Experiment 3 consisted of groups 5 and 6 and lasted for 12 weeks. Group 5 were orally dosed with 500 *Haemonchus* L3 (McMaster strain) 3 times weekly for 5 weeks (blue panels). Both groups were drenched with levamisole and ivermectin at the end of week 6, then infected with 5000 *Haemonchus* L3 (McMaster strain) by intrarumenal injection in week 8 (orange panels). Faecal egg counts (FEC) were done in week 11 and 12 (F) to confirm patency of infection. All sheep were slaughtered at the end of week 12 (S - yellow panels).

Blood and faeces were collected from all animals at the beginning of the trial (B/F), in week 6 and at slaughter. These samples and the material collected at post mortem, including worms, were processed and stored as for groups 3 and 4.

3.1.5 Design of Experiment 4

Experiment 4 ran for 12 weeks and involved 18 sheep (n=6 per group).

	1					2					3					4					5					6				
	M	T	W	T	F	M	T	W	T	F	M	T	W	T	F	M	T	W	T	F	M	T	W	T	F	M	T	W	T	F
1	B/F																									B/F				L/I
2	B/F																									B/F				L/I
3	B/F																									B/F				L/I

	7					8					9					10					11					12				
	M	T	W	T	F	M	T	W	T	F	M	T	W	T	F	M	T	W	T	F	M	T	W	T	F	M	T	W	T	F
1							I															F				F			S	
2							I															F				F			S	
3							I															F				F			S	

All groups were dosed with 500 ivermectin-sensitive H contortus L3 (McMaster strain) three times weekly for five weeks (blue panels). Group 1 was the control. From the beginning of week 6 (brown panels), group 2 received 0.5mg/kg dexamethasone (Trimedexil - Dexamethasone trimethylacetate) IM per week and group 3 received a diet calculated to contain 75% protein but similar energy content to the standard diet (these treatments are discussed further below). At the end of week 6, all sheep were drenched with levamisole and ivermectin. In week 8, all sheep were infected with 5000 H contortus L3 (McMaster strain) by intrarumenal injection (orange panels). Faecal egg counts (FEC) were done in week 11 and 12 (F) to confirm patency of infection. All sheep were slaughtered at the end of week 12 (S - yellow panels).

Blood and faeces were collected from all animals at the beginning of the trial (B/F), in week 6 and at slaughter. These samples and the material collected at post mortem were processed and stored as for Experiment 3. Abomasal contents were collected from every animal at slaughter. *Haemonchus* counts (total, adult and larval) will be performed to determine the effect of corticosteroid-induced immunosuppression and a reduced protein diet on sheep immunity to *Haemonchus.*

3.1.5.1 Group 2 (Corticosteroid Treatment)

The dose of corticosteroid chosen for group 2 was 0.5mg/kg dexamethasone IM per week. The dose used for this trial needed to be high enough to significantly improve the establishment of *Haemonchus* in immune sheep whilst avoiding the numerous adverse side effects of corticosteroids. We have experience with a dose of 0.1mg/kg dexamethasone to assist establishment of *Trichostrongylus* in sheep. This is an effective dose for this parasite and is well tolerated by the sheep. Various protocols using dexamethasone treatment to abrogate immune exclusion and expulsion of *Haemonchus* L3 in immune or genetically resistant sheep are reported in the literature. These vary from a low range of two doses of 0.5mg/kg dexamethasone within four days of each

other around infection (Adams 1986; Jackson*, et al.* 1988; Huntley*, et al.* 1992) to a mid range of 0.5mg/kg dexamethasone twice weekly for five weeks (Presson*, et al.* 1988) to a high dose of 1mg/kg dexamethasone three times per week for 10 to 15 weeks (Pena*, et al.* 2004). The protocol we chose (0.5mg/kg dexamethasone IM per week) is a mid-range dose and is the same as a protocol published for a study using *Trichostrongylus* in sheep (Douch*, et al.* 1996). We considered this to be the optimum balance between experimental effect and animal welfare.

3.1.5.2 Group 3 (Reduced Protein Diet)

Rather than preparing two special diets for Experiment 4 (i.e. high and low protein), the diet of groups 1 and 2 needed to be the same as that used in Experiments 1 to 3 to enable comparison of results between trials. Thus a reduced protein diet was formulated for Group 3 of Experiment 4.

The standard diet throughout these trials was 800g per day of 'Progress 10' and was the diet used for groups 1 and 2. 'Progress 10' is a mixed feed produced by Wagga Seed and Produce (116 Gurwood Street Wagga NSW 2650 tel 6 931 7037 fax 6 931 7039). The formula is listed below;
300kg white chaff
300kg lucerne chaff
200kg Progress 100 (extruded pellets manufactured from soybeans, peas, faba beans, black sunflower, corn, clover, wheat, vitamin and minerals. Min crude protein 20%)
20kg molasses

Our intention was to provide Group 3 with a Progress 10 based diet containing a similar energy content but with 70-75% of the protein content (Roberts and Adams 1990; Datta*, et al.* 1998). Nutritional advice was obtained from Dr Lewis Kahn (Senior Research Scientist Animal Science University of New England Armidale) and Todd Middlebrook (Technical Services Manager Weston Animal Nutrition). Dried molasses was the lowest protein feed that could be sourced so this was used as a high energy, low protein additive to dilute the normal diet. The preparation purchased was Molatein (34% molasses sugars, 8% protein. Manufacturer - International Animal Health Products. Distributor – Kensington Produce Pty Ltd 2/19a Baker Street Banksmeadow NSW 2019 tel 2 9666 7755)

By feeding 630g Progress 10 and Molatein mixed in a 2:1 ratio, each sheep would consume 70-75% of the crude protein and around 90% of the metabolisable energy in the normal diet (800g Progress 10). The exact calculations were determined by analysing the feed contents.

FEEDTEST is a commercial feed analysis service operated by the Victorian Department of Primary Industries. To assess the variability of the feed, samples of Progress 10 and a 2:1 ratio of Progress 10 to Molatein were submitted for feed analysis on 3.7.06 and 6.11.06. The results for dry matter (DM), crude protein (CP) and metabolisable energy (ME) are shown below;

Progress 10

	3.7.06	6.11.06	Mean
dry matter (%)	86.1	86.5	86.3
crude protein (% of DM)	14.1	14.6	14.4
metabolisable energy (MJ/kg DM)	11.2	11.6	11.4

800g Progress 10 contains 690.4g DM, 99.1g crude protein and 7.9 MJ of ME.

Progress 10 : Molatein (2:1 ratio)

	3.7.06	6.11.06	Mean
dry matter (%)	89.3	89.5	89.4
crude protein (% of DM)	12.9	12.7	12.8
metabolisable energy (MJ/kg DM)	12.2	12.2	12.2

630g of Progress 10 : Molatein (2:1 ratio) contains 563.2g DM, 72.1g crude protein and 6.9 MJ of ME. This ration provided 72.8% of the crude protein and 87.3% of the ME in the normal ration (800g progress 10).

Group 3 sheep were fed 630g of Progress 10 : Molatein (2:1 ratio) in individual pens on weekdays. To help maintain bodyweight and normal digestive function, these sheep were group fed the normal ration (800g Progress 10) on weekends.

3.2 Sheep Anaesthesia and Surgery for Abomasal Fistulation

Premedication.
a) Buprenorphine (Temgesic = 300µg/ml) 1ml intramuscular

Ten minutes after dosing with Buprenorphine, the sheep was placed in left lateral recumbency and the right jugular vein was clipped and disinfected using 0.5% chlorhexidine in 70% ethanol. An intravenous catheter was inserted, then plugged and flushed with sterile saline. The catheter was sutured securely in place.

b) Acepromazine (2mg/ml preparation) 0.05-0.1mg/kg intravenously (IV). Injection volume for a 30kg sheep = 0.75 to 1.5ml

Induction.
5% Thiopentone IV slowly to effect (approximately 3.5mL). Once anaesthesia was induced, the sheep was placed in dorsal recumbency with its head extended. By holding the larynx with one hand, a cuffed endotracheal tube could be passed into the trachea then tied in place. The cuff was inflated and the rebreathing circuit attached.

Maintenance.
3 to 5% Isofluorane on 1.5 to 2 litres per minute O_2.

Procedure.
The right side of the sheep was clipped from the middle of the thorax to the wing of the pelvis. The skin was alternately disinfected using povidine iodine then 0.5% chlorhexidine in 70% ethanol three times each. A 7cm paracostal incision was made approximately 7cm caudal to last rib and the abomasum was exteriorised. Two 30cm nylon stay sutures were temporarily placed through the fundus and held up by an assistant using clamped haemostats to prevent spillage of abomasal contents. A stab incision was made through the wall of the abomasum between the stay sutures whilst avoiding obvious blood vessels. The fistula was inserted through this incision (this was the open end of a 20ml syringe barrel through a curved oval plastic collar - see picture Appendix 8.1). The fistula (pointed end of the syringe barrel) was exited through a stab incision in the abdominal wall between the laparotomy wound and the last rib. The serosal surface of the abomasum was wiped with a sterile swab moistened with saline and the abomasum replaced. A standard three layer laparotomy wound closure was used;
a) a continuous layer of 2-0 Biosyn placed through the peritoneum and transversus abdominus muscle
b) a continuous layer of 2-0 Biosyn placed through the internal and external abdominal oblique muscles.
c) medium nylon (Vetafil) was used to close the skin wound with a cruciate suture pattern then covered in wound gel and sterile swabs. The swabs were held in place with a bandage wrapped around the abdomen.

A large rubber plumbing washer was placed around the exterior portion of the fistula and against the skin to provide protection. A purpose cut perspex collar was placed around

the fistula next to the rubber and held in place with a rubber castration ring. The end of the syringe was cut with a hacksaw and the edges filed smooth. The plunger of the syringe was then inserted into the barrel and cut to leave one centimetre protruding. The barrel and plunger were then drilled eccentrically. A screw was placed through the hole and a washer was used to hold the screw in place.

Post-operative medication.
0.4mL ketoprofen (40mg) intramuscular
2mL oxytetracycline (200mg) intramuscular

3.3 Laboratory Protocols

3.3.1 Immunohistochemistry (IHC)

Preparation of gelatine-coated slides

Prepare 500mls of coating solution containing 2.5g gelatine and 0.25g of chromium potassium sulphate in distilled water. Heat gently to dissolve. Avoid bubbles on surface. Dip slides in coating solution then allow to dry.

Frozen sections.

Tissues collected at post-mortem were stored long term at -80^{O}C and placed at -20^{O}C overnight before cutting. Sections were cut onto gelatine-coated slides. After air drying for 30 minutes they were fixed in cold 100% ethanol for 10 minutes then air dried again. Slides could be stored in slide boxes at -20^{O}C for up to two weeks. If stored, the box must be warmed to room temperature before opening to avoid condensation on the slides which can result in detachment of sections.

Immunohistochemistry protocol.

This protocol was developed in conjunction with protocols from Sue McClure (CSIRO Armidale) and David Piedrafita/Garry Barcham (Centre for Animal Biotechnology (CAB) Monash University).

Mouse anti-ovine primary antibodies were purchased from Serotec;

- CD1 (clone number 20.27) (Cortical Thymocytes, Langerhan's Cells, Dendritic Cells)
- CD4 (clone number 44.38) (T Cell Subset (helper), Thymocyte subset, Monocytes, Macrophages)
- CD8 (clone number 38.65) (T Cell Subset (Cytotoxic), Thymocyte Subset, NK Cells)
- CD45RA (clone number 20.96) (B Cells, T Cell Subset (naive), Monocytes).

The CD4 supernatant obtained from David Piedrafita is a cocktail of two CD4 antibodies, obtained from clone number 44.38 and clone number 44.97.

These were detected with a polyclonal rabbit anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (DakoCytomation)

Immunohistochemistry commenced by rehydrating sections in Tris-buffered saline (TBS). From this point it is essential that sections do not dry out so all staining should proceed within a humidified chamber.

Block Endogenous Peroxidase

*1% hydrogen peroxide in TBS for 10 minutes.

Block Fc receptors and non-specific background

*4% skim milk powder (SMP) + 1% bovine serum antigen (BSA) + 5% normal rabbit serum (NRS) in TBS (blocking solution) for 30 minutes with at room temperature.
*Tip off blocking solution.

Primary antibody

*Primary antibodies were diluted 1:100 in blocking solution. 100μl was applied for 2 hours at room temperature.
*Rinse in TBS.

Secondary antibody

*The secondary antibody was diluted 1:500 in blocking solution. 100μl was applied for 30 minutes at room temperature.
*Rinse in TBS.

Detection with DAB (3,3´-diaminobenzidine tetrahydrochloride)

*DAB stock was prepared as 25mg/ml and stored in 200µl aliquots at -20^{O}C.
*Thaw aliquot and mix with 5ml phosphate-buffered saline (PBS).
*Add 5µl 30% hydrogen peroxide (final concentration of 0.03%).
*Add 100µl of DAB to all slides and incubate at room temperature until optimal staining achieved.
*Rinse in water.

Counterstaining

*30 seconds in Haematoxylin
*Rinse in water
*2 minutes in Scott's blueing solution (0.2% sodium bicarbonate + 2% magnesium sulphate in distilled water)
*Dip in water

Dehydrating slides

*Change to dry slide holder
*Dips in 70% ethanol, 95% ethanol, 100% ethanol, 50% ethanol/Xylene, 100% Xylene

Place 2-3 drops of xylene-based mounting medium on slide and add coverslip.
Squeeze out air bubbles and air dry.

3.3.2 Histology

Image Pro Plus 5.1 software was tested for image analysis.

Prescapular lymph node, abomasal lymph node, abomasal folds and abomasal wall were collected from all animals in Experiment 1 and stored in formalin for H&E staining, and Bouin's fixative for Giemsa Chromotrope staining. The H&E staining was used for general histological comparison (e.g. total cell counts and nuclear proportions) and the Giemsa Chromotrope was used to count eosinophils and mast cells. In all subsequent trials, Toluidine Blue stained sections cut from formalin fixed tissue was found to be superior for counting mast cells and eosinophils could be counted in H&E stained sections.

3.3.3 Dissolution of Individual Female *Haemonchus* and Egg Counting.

1. Worms collected at post mortem and preserved in iodine and photographed for area and perimeter measurements using image analysis software (Image-Pro Plus version 5.1).
2. After image analysis individual worms were placed in clean eppendorf tubes with 100μl of 4% bleach (stock solution is 12.5% sodium hypochlorite) and vortexed every 2 minutes until the worm dissolved.
4. 1400μl of water was added to stop degradation of the eggs (tubes could be stored at $4^{O}C$ for several days at this point if required).
5. Each sample was mixed thoroughly and a 500μl aliquot was counted in a Whitlock universal counting chamber.
6. The result obtained was multiplied by 3 to give the individual worm fecundity.

3.3.4 Exsheathment of L3

1. Flasks of L3 were stood at room temperature for 30 minutes.
2. The supernatant was aspirated and the sedimented larvae were transferred into a 50ml tube.
3. Larvae were counted and diluted to 1000 per ml.
4. 1ml of bleach (12.5% sodium hypochlorite) was added per 30ml of larval solution (0.4% final concentration).
5. Larval solution was incubated for 5 minutes whilst aerating slowly to prevent settling.
6. A drop of larvae was examined microscopically for evidence of exsheathment. This was detectable as larvae wriggling out of their sheaths, discarded sheath casings and curling of the tail in free larvae compared to the sharp, pointed tails present in ensheathed larvae.
7. Once 90% of larvae had exsheathed, the mixture was poured through a mini Baermann filter (70μM) and the filter transferred over a tube containing 50ml of saline so as to be in contact with the saline in the absence of air bubbles.
8. The larvae were left for 30 minutes at $37^{O}C$ to migrate through the filter then the supernatant was aspirated down to the sedimented larvae.

3.3.5 Axenization of L3 and Culture to L4

Note – Axenization means purification of larvae by removal of micro-organisms

Solutions

GKN salt solution

	Weight (grams)		
NaCl	8.18		
KCl	0.37		
Na_2HPO_4	2.84	(or $Na_2HPO_4.2H_2O$	3.56g)
$NaH_2PO_4.2H_2O$	0.78		
Glucose	1.8		

Add distilled water to 800mls. Adjust pH to 7.2. Top up to one litre.

Penicillin/Streptomycin (Penstrep)

	Weight (grams)
Streptomycin sulphate	1.0
Benzylpenicillin	0.6

Add distilled water to 50mls. Filter sterilise (0.2μM filter) and store in 5ml volume at -20^{O}C.

Complete GKN solution

GKN salt solution	95ml
Amphotericin (250μg/ml)	40μl
Penstrep	5ml

50x supplement for RPMI tissue culture medium

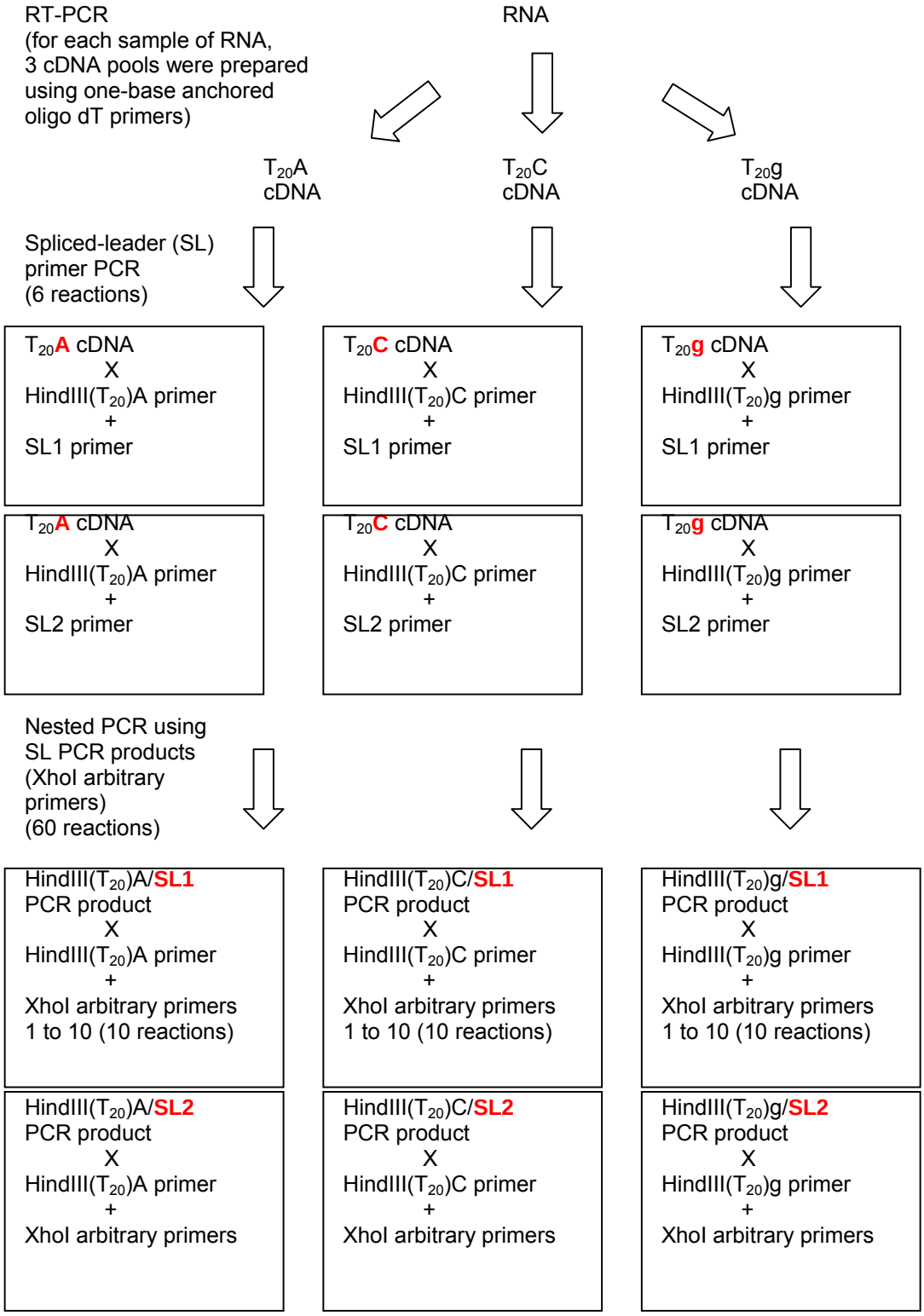
	Weight (grams)
Streptomycin sulphate	0.1
Benzylpenicillin	0.06
Glutamine	0.29
$NaHCO_3$	0.84

Make up to 40ml with distilled water. Add 10ml of concentrated HCl slowly whilst stirring. Filter sterilise (0.2μM filter) and store in 5ml volumes at -20^{O}C.

1. The solution of exsheathed L3 was diluted with 5 parts fresh made complete GKN solution.
2. This was incubated at 37^{O}C for 30 minutes. This process was repeated twice by aspirating and replacing the supernatant over the sedimented larvae with fresh solution.
3. After the third wash in complete GKN solution, the supernatant was removed and the larvae were washed in unsupplemented RPMI medium.
4. Once the larvae had sedimented, the RPMI wash was removed, then the larvae were counted and diluted to 1000 per ml in complete RPMI containing 20% sheep serum.

5. Each well of a 12-well plate was seeded with 2000 larvae. Each well was topped up to 3mls with complete RPMI containing 20% sheep serum and cultured within a vacuum chamber at $37^{O}C$ in an atmosphere of 85% N_2, 10% CO_2 and 5% O_2.
6. Progression to L4 was determined microscopically by the presence of mouth parts and pharyngeal motility.

3.3.6 Differential Display PCR (ddPCR) Flowchart



The cDNA generated by RT is amplified by PCR. The 3′ (reverse) primer used for PCR has the same one base-anchored oligo-dT sequence at its 3′ end as the corresponding anchored oligo-dT used in RT. Additionally, a HindIII restriction sequence was added to the 5′ end of each oligo-dT primer used for PCR to provide an option for cloning PCR products. The additional sequence also makes the primers longer and more efficient in PCR (by raising the primer melting temperature).

Two different sets of 5′ (forward) primers were used in a two stage dd-PCR strategy. The first stage amplifies the entire transcriptome with spliced leader (SL) sequence primers. Stage one produces a mixture of products that is too complex to resolve on a gel and produces a smear (Brehm*, et al.* 2003). The second stage is a nested PCR using arbitrary primers to separate the transcriptome into sub-pools from which bands can be identified via electrophoresis.

Two trans-spliced leader sequences have been identified in *Haemonchus* mRNA transcripts, SL1 and SL2. These are identical to the sequences in *C.elegans*, where more than 80% of mRNAs are trans-spliced with SL1 (Redmond and Knox 2001). Using primers targeted to the SL sequences in the first stage of PCR provides a large volume of PCR product to serve as template for a nested PCR (stage 2). This approach allows cDNA to be conserved, which needs to be resynthesised by RT once depleted. This is particularly important in cases where limited starting material is available (e.g. a single worm). The sequences of all primers used are included in Appendix 4.

The number of arbitrary 5′ primers used to analyse transcription profiles in published studies has ranged from 5 (Boschi and Vergara 1998) to 26 (Bauer*, et al.* 1993). Ten arbitrary 5′ (forward) primers were designed for this study. These use the terminal 3′ variable region of the ten arbitrary primers listed in the Delta Differential Display PCR Kit (Clontech) with a XhoI restriction sequence added to the 5′ end. Clontech selected the terminal variable regions to avoid secondary structures, complementarity and slippage during PCR whilst favouring common sequence motifs in the coding regions of genes. The XhoI restriction sequence was added because XhoI is the most economical restriction enzyme which has the same digestion buffer as HindIII and a recognition sequence on the opposite side of the cloning site to Hind III in the pCR 2.1-TOPO cloning vector (Invitrogen). pCR 2.1-TOPO will be used to clone bands of interest.

Adding a XhoI recognition sequence to the final nine bases from the Delta kit arbitrary primers created 15-16 mer primers with melting temperatures around 50^{O}C. This is similar to the melting temperature of the HindIIIT20(A/C/g) reverse primers. PCR works optimally when the primers used have similar melting temperatures. Each of the six PCR reactions from stage 1 (2 SL 5′ primers x 3 HindIII one base anchored 3′ primers = 6 reactions), is reamplified in stage 2 with the same 3′ primers and each of the ten 5′ arbitrary primers (6 stage 1 reactions x 10 arbitrary primers = 60 reactions).

3.3.7 RNA Purification

This protocol uses using Tri-Reagent, Heavy phase-lock gel tubes, 1-bromo-3-chloropropane (BCP) and Qiagen RNeasy columns.

Partially thaw eppendorf tube containing worms on ice.
Add 1ml of Tri Reagent and mix/disperse using an autoclaved eppendorf pestle.
Homogenise with 20G needle and syringe.
Leave 5 minutes room temperature to dissociate nucleoprotein complexes.

Add 100µl BCP per 1ml of Tri-reagent.
Shake vigorously for 15 seconds.
Leave at room temperature for 10 minutes.

Pre-spin required number of heavy PLG tubes – 3000rpm for 2 minutes (to spin gel to bottom of tube).

Cool on ice.
Add sample to PLG tubes and centrifuge at 12000xg for 15 minutes at 4 ^{O}C.

Transfer 80% of aqueous phase into new tubes to avoid residual interphase.
Add 500µl isopropanol per 1ml of Tri-reagent.
Leave tube at room temperature for 5 minutes.
Centrifuge tubes at 12000xg for 8 minutes at 4^{O}C.

Discard supernatant.
Wash in 1ml of 70% ethanol (EtOH).
Air dry pellet for 3-5 minutes.

Resuspend in 50µl dH_2O.

RNeasy Column (Qiagen)

NOTE – binding capacity of column = 100µg = approx. 30mg / 27mm^3 tissue.
DO NOT EXCEED.

To 50µl RNA solution, add;
600µl RLT buffer with 6µl 2-ME
600µl 70% ethanol

Spin successively 15s x 8000g (Place 625µl on spin column per spin)

ON COLUMN DNase DIGESTION

Add 350µl RW1 buffer
Centrifuge for 15s x 8000g

Add 10µl DNase I stock solution to 70µl RDD buffer (from RNase-Free DNase set). Mix by gentle inversion.
Pipette 80µl solution directly onto membrane and leave at room ^{O}C for 15 minutes.

Add 350µl RW1 buffer
Centrifuge for 15s x 8000g

NEW COLLECTION TUBE

Add 500µl RPE buffer
Centrifuge for 15s x 8000g
Add 500µl RPE buffer
Centrifuge for 15s x 8000g

NEW COLLECTION TUBE

Centrifuge for 60s at full speed

NEW COLLECTION TUBE

Spin successively 15s x 8000g into same tube (Add 30µl dH_2O per spin)

60µl RNA

Add 700µl RW1 buffer
Centrifuge for 15s x 8000g

NEW COLLECTION TUBE

Add 500µl RPE buffer
Centrifuge for 15s x 8000g
Add 500µl RPE buffer
Centrifuge for 15s x 8000g

NEW COLLECTION TUBE

Centrifuge for 60s at full speed

NEW COLLECTION TUBE

Successive spins 15s x 8000g into same tube (Add 30µl dH_2O per spin)

Final solution is 60µl RNA

3.3.8 RNA Electrophoresis in 1% Agarose.

Wash gel tanks, combs, measuring cylinders and volumetric flask in 0.3% pyroneg, spray with RNaseZAP, and rinse with Baxter water for irrigation.
Wipe inside top of gel tank with RNaseZAP.
Add 500mg of agarose to 49ml dH_2O + 1ml 50x TAE for mini-gel (8 lanes).
Heat in microwave until dissolved.
Cool while swirling flask under tap.
Prepare 250mls of TAE running buffer for mini-gel tank.
Run 2-5µl per sample mixed with 3µl 10x loading buffer containing ethidium bromide.
Load 4µl RNA marker (1µg per µl)
Run 60V for one hour.

3.3.9 Reverse Transcription (RT)

3.3.9.1 First-Strand cDNA Synthesis Using SuperScript III for RT-PCR.

SuperScript III is used in a 20µl reaction volume with 10pg-5µg of total RNA or 10pg-0.5µg of mRNA. Three separate reactions each using a different one-base anchored oligo-dT primer are used to produce three different cDNA pools. Invitrogen recommend using 1µl of 50µM oligo $dT_{20.}$

1. Add the following components to a nuclease-free microcentrifuge tube:

5µl of 10µM T20G (5'-TTTTTTTTTTTTTTTTTTTG- 3') (Tube 1)
5µl of 10µM T20A (5'-TTTTTTTTTTTTTTTTTTTA- 3') (Tube 2)
5µl of 10µM T20C (5'-TTTTTTTTTTTTTTTTTTTC- 3') (Tube 3)

To each tube, add the following;
5µl 2mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH)
2µl RNA solution
(12µl total)

2. Heat mixture to 65°C for 5 minutes (heating block) and incubate on ice for at least 1 minute.

3. Collect the contents of the tube by brief centrifugation.

4. Prepare master mix (x tubes + 0.5x) containing the following volumes for each tube:

4µl 5X First-Strand Buffer
1µl 0.1 M DTT
1µl RNaseOUT. Recombinant RNase Inhibitor
1µl of SuperScript. III RT (200 units/µl)
1µl dH_2O
8µl total

4. Mix by pipetting gently up and down.

5. Incubate at 50°C for 60 min. (thermocycler)

6. Inactivate the reaction by heating at 70°C for 15 min.

Use 1μl as a template for amplification in each 25 μl of PCR reaction.

3.3.9.2 First-Strand cDNA Synthesis Using Fermentas for RT-PCR.

RevertAid is used in 30μl reaction volume with 0.1μg-5μg of total RNA or 10ng-0.5μg of mRNA. Three separate reactions each using a different one-base anchored oligo-dT primer are used to produce three different cDNA pools. Fermentas recommend using 500ng oligo $dT_{18.}$

1. Add the following components to a nuclease-free microcentrifuge tube:

8μl of 10μM T20G (5'-TTTTTTTTTTTTTTTTTTTG- 3') (Tube 1) (approx. 480ng)
8μl of 10μM T20A (5'-TTTTTTTTTTTTTTTTTTTA- 3') (Tube 2)
8μl of 10μM T20C (5'-TTTTTTTTTTTTTTTTTTTC- 3') (Tube 3)

+

2μl RNA solution
10μl total

Prepare master mix (x tubes + 0.5x) containing the following volumes for each tube:

6μl 5x reaction buffer
1.5μl 10mM dNTPs
9μl dH2O
16.5μl total

2. Heat mixture to 72°C for 3 minutes. Centrifuge and incubate on ice for 5 min.

3. Collect the contents of the tube by brief centrifugation.

4. Prepare master mix (x tubes + 0.5x) containing the following volumes for each tube:

0.6μl RNaseOUT. Recombinant RNase Inhibitor
2μl of RevertAid M-MuLV RT (200 units/μl)
2.6μl total

4. Mix by pipetting gently up and down.

5. Incubate at 37°C for 2 hours, then 50°C for 10 minutes then 95°C for 5 minutes. (thermocycler)

6. Centrifuge briefly, divide into 2 x 15µl aliquots and store at -80°C.

Use 1µl as a template for amplification in each 25 µl of PCR reaction.

3.3.10 Primer Sequences used for RT, dd-PCR and Sequencing.

Reverse transcription

T_{20}A	(5'-TTTTTTTTTTTTTTTTTTTA- 3')
T_{20}C	(5'-TTTTTTTTTTTTTTTTTTTC- 3')
T_{20}g	(5'-TTTTTTTTTTTTTTTTTTTG- 3')

dd-PCR 3´(Reverse) primers

HindIII(T_{20})A	5'-AAGCTTTTTTTTTTTTTTTTTTTA- 3'
HindIII(T_{20})C	5'-AAGCTTTTTTTTTTTTTTTTTTTC- 3'
HindIII(T_{20})g	5'-AAGCTTTTTTTTTTTTTTTTTTTG- 3'

Spliced leader 5´(Forward) primers

H.contortus SL1 (22mer)	5'-GGTTTAATTACCCAAGTTTGAG- 3'
H.contortus SL2 (22mer)	5'-GGTTTTAACCCAGTATCTCAAG-3'

XhoI Arbitrary 5´(Forward) primers

XhoArb 1 (15mer)	5'-CTCGAGTGCTGGGGA-3'
XhoArb 2 (16mer)	5'-CTCGAGTCGGTCATAG-3'
XhoArb 3 (15mer)	5'-CTCGAGTGCTGGTGG-3'
XhoArb 4 (15mer)	5'-CTCGAGTGCTGGTAG-3'
XhoArb 5 (16mer)	5'-CTCGAGGATCTGACTG-3'
XhoArb 6 (15mer)	5'-CTCGAGTGCTGGGTG-3'
XhoArb 7 (15mer)	5'-CTCGAGTGCTGTATG-3'
XhoArb 8 (15mer)	5'-CTCGAGTGGAGCTGG-3'
XhoArb 9 (15mer)	5'-CTCGAGTGTGGCAGG-3'
XhoArb 10 (15mer)	5'-CTCGAGGCACCGTCC-3'

pCR 2.1-TOPO Sequencing Primers

M13 Forward (-20)	5´-GTAAAACGACGGCCAG-3´
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´

3.3.11 dd-PCR Protocol

Low annealing temperatures are used due to the non-specificity of the primers. Typical annealing temperatures are 36-38^{O}C (Graf*, et al.* 1997) and 40-42^{O}C (Malhotra*, et al.* 1998). A hot start and an increase in annealing temperature from 40^{O}C to 42^{O}C after the initial five cycles were included to improve specificity.

PCR Reaction

Note: For multiple reactions with common components, prepare a master mix of the components common to all reactions to reduce pipetting errors.

For each 25µl reaction, add;
*1µl cDNA OR 0.5µl PCR product
*200nM Amplification primer 1 (1µl x 5µM)
*200nM Amplification primer 2 (1µl x 5µM)

22.5µl Platinum® Blue PCR SuperMix (contains 1 U Platinum® *Taq* DNA Polymerase)

2. Mix gently and layer 1.2 drops (~50µl) of silicone oil over the reaction.

3. Incubate tubes in a thermal cycler at 94°C for 3 min to completely denature the template and activate the enzyme.

4. Program thermal cycler as below; (Lid =0^{O}C)

1. T=94 ^{0}C	0:03:00
2. T=94 ^{0}C	0:00:30
3. T=40 ^{0}C	0:02:00
4. T=72 ^{0}C	0:00:30
5. Go to 2	Rep 5
6. T=94 ^{0}C	0:00:30
7. T=42 ^{0}C	0:02:00
8. T=72 ^{0}C	0:00:30
9. Go to 6	Rep 35
10. T=72 ^{0}C	0:10:00

Final extension 72°C for 10 minutes. 10 minutes at 4°C. End program.

5. Analyse PCR products on agarose. If DNA present, go to PAGE.

3.3.12 Polyacrylamide Gel Electrophoresis (PAGE)

Prepare 5% polyacrylamide mixture containing 1.6M Urea

Urea	1.92g
30% acrylamide	3.333mls
5x TBE	4mls
10% ammonium persulfate	160µl
TEMED	20µl
dH_2O	to 20mls

5X TBE

	1 L	2 L
Tris	54 g	108
Boric Acid	27.5g	55.0g
0.5M EDTA	20mL	40mL

Pour gel and allow it to set for one hour.

Load 11µl of PCR product with 3µl of urea loading buffer per lane of gel and electrophorese for 20 minutes in TBE buffer at 70V followed by 2.5 hours at 110V. Silver stain gel following electrophoresis.

Urea loading buffer
http://genetics.mgh.harvard.edu/szostakweb/resources/Public%20Protocols/denaturepage/index.html

Urea (8 M)	4.8g
EDTA (20 mM)	400µL 0.5M
50mM Tris pH 7.5 (5 mM)	1mL
Bromophenol blue	0.5 g
dH_2O	to 10 mls

3.3.13 Silver Staining Methods

Silver staining (Bassam, *et al*. 1991)

Fixation	10% acetic acid (20 minutes – can leave overnight)
Rinse	dH_2O (3 x 5 minutes)
Impregnate	0.1% $AgNO_3$, 150µl 37% HCOH/100mls (60 minutes)
Rinse	dH_2O (3 x 5 minutes)
Develop	3% Na_2CO_3, 150µl 37% HCOH/100mls, $Na_2S_2O_3.H_2O$ 0.6mg/100mls (5 minutes – should begin developing very quickly)
Stop	10% acetic acid (5 minutes)

This protocol was modified slightly in the development step. A three times increase in $Na_2S_2O_3.H_2O$ to 1.8mg/100mls resulted in less background and clearer staining of bands.

Silver staining (Qu*, et al.* 2005)

Impregnate	25% ethanol, 1% HNO3, 0.2% $AgNO_3$ (5-10 minutes)
Rinse	dH_2O (3 minutes)
Develop	3% Na_2CO_3, 0.2% HCOH (2-5 minutes)
Stop	10% acetic acid (2-5 minutes)

3.3.14 Crush and Soak DNA Fragment Purification from Acrylamide

(http://genetics.med.harvard.edu/~cepko/protocol/mike/D5.html)

Solutions

Crush and Soak Solution

	50mL	100mL
500 mM NH4OAc	1.65g NH4OAc	3.3g NH4OAc
0.1% SDS	0.05g SDS	0.1g SDS
0.1 mM EDTA	10µl 500 mM EDTA	20µl 500 mM EDTA

bring up to 50/100ml with dH_2O
store at room temperature

3 M NaOAc pH 5.2

	50mL	100mL
anhydrous sodium acetate	12.3g	24.6g

pH to 5.2 with acetic acid and bring up to 50/100 ml with dH_2O
store at room temperature

Procedure
Cut desired band out of gel.
Crush the gel with an eppendorf pestle
Add 300µl crush and soak solution and incubate overnight at 37°C.
Spin in the centrifuge for 10 minutes at 14,000 rpm.
Transfer supernatant to new tube.
Add 30µl of 3M NaOAc,
Add 6µl Fermentas glycogen as a carrier (20µg/µl).
Add 750µl of ethanol. Mix gently but thoroughly.
Incubate the mixture at -20°C overnight.
Centrifuge the mixture for 10-15min at 10,000rpm. Discard the supernatant.
Rinse the pellet with cold 70% ethanol. Air-dry the pellet.
Dissolve DNA in 20µl nuclease-free water or TE buffer.

NOTE

*5µg of Glycogen (0.25µl) forms a visible pellet in a microcentrifuge tube.
*Recommended final Glycogen concentration in a precipitation mixture is 0.05-1µg/µl (< 1µl of Glycogen per 20µl of the precipitation solution).
*Up to a final concentration of 8µg/µl, Glycogen does not interfere with most downstream applications e.g. PCR, DNA sequencing, bacterial transformation.
*6µl Fermentas glycogen (20 µg/µl = 120 µg) in 1080µl precipitation solution = 0.11 µg/µl. In final 20µl suspension of DNA, it will equal 6 µg/µl glycogen.
*Longer incubation at lower temperature provides better recovery of nucleic acids.
*Avoid over-drying the pellet, as it then takes more time to dissolve.

3.3.15 Protocols for Cloning and Sequencing

TOPO cloning reaction (Invitrogen)

The table below describes the reagents for the TOPO cloning reaction (6µl) for eventual transformation into chemically competent *E.coli* (e.g. TOP10F′ one shot *E.coli*).

Reagent	
Fresh PCR product	0.5 to 4µl
200mM NaCl 10mM $MgCl_2$ salt solution	1µl
Sterile water	Add to a total volume of 5µl
TOPO vector	1µl (keep it in ice)
Final volume	6µl

Mix gently and incubate for 15 minutes at room temperature.

One shot chemical transformation protocol

Each transformation requires one vial of competent cells and three selective plates.
Warm one vial of S.O.C bacterial growth medium.
Warm selective plates (50µg ampicillin per ml of luria broth agar) at 37^{O}C for 30 minutes.
Spread 40µl of 40mg/ml X-gal and 40µl of 100mM IPTG (required for blue/white screening) on each LB plate and incubate at 37^{O}C until ready for use.
Thaw on ice 1 vial of one shot cells for each transformation.

Add 2µl of the TOPO cloning reaction into a vial of one shot chemically competent *E.coli* and mix gently.
Incubate on ice for 12 minutes.
Heat shock the cells for 30 seconds at 42^{O}C without shaking.
Immediately transfer the tubes to ice.
Add 250µl of room temperature S.O.C medium.
Cap the tube tightly and shake the tube horizontally (200rpm) at 37^{O}C for 1 hour.
Spread 40µl, 80µl and the remaining volume onto three prewarmed selective plates and incubate at 37^{O}C.
Pick approximately 10 white or light blue colonies for analysis.
Save stocks of positive bacterial broth at -80^{O}C DNA.

Sequencing.
DNA will be sent to SUPAMAC for sequencing (Sydney University Prince Alfred Macromolecular Analysis Centre http://www.supamac.com/). SUPAMAC recommends the Marligen (old Concert) or Viogene kits from Diagnostic Technology for plasmid purification. Sequencing primers are listed in Appendix 4. The specifications for sequencing reactions are listed below;

Dye-Terminator Reactions

Reagent	DS templates
Primer	5-10 pmol
DNA	1-3µg depending on the size of the plasmid
dH2O	As needed
Final volume	16.0µl

Samples are submitted in liquid form in labelled PCR tubes in strips of eight.

4 Results and Discussion

4.1 Experiment 1

Aim - To describe the kinetics of *Haemonchus* infection in sheep. A bar chart showing the design of Experiment 1 and a description of materials and methods are included in Section 3.1.1.

4.1.1 Haematology

Appendix 8.3 shows the group average of RBC count in all animals in each group at the time of each blood collection. There was an affect of housing on the red cell count, as it declined in all groups between day 0 and the second blood sample at day 57. This included groups one and three, which received no worms during this period. From this point, the average count in group one fluctuated around a similar level to the end of the trial and it was the same on days 57 and 99 (9.4 $x10^{12}$/L). At day 57, the average red cell count in groups three and four were 10.0 and 7.5 respectively. At day 99, these values were 6.2 (group three) and 6.7 (group four), demonstrating a significant anaemia.

Since individual sheep were serially sampled, statistical validity was best achieved by comparing values from the first time point after acclimatisation to the animal house (day 57) with a later time point where a sufficient interval had elapsed to enable it to be independent, whilst having sufficient animals remaining to provide statistical power. Day 85 was selected as the second time point to provide a time interval of 28 days and six sheep in groups 3 and 4. The change in group means between days 57 and 85 for these groups was compared by Mann-Whitney U test (Appendix 8.4). The mean group values on day 85 in groups 1 (negative control), 3 and 4 were compared by REML (Restricted Maximum Likelihood). REML has advantages over ANOVA and similar models by being able to cope well with unbalanced designs and with correlated data arising from sampling the same unit repeatedly i.e. repeated measures data. A table showing comparisons with a significant difference is included (Appendix 8.5). The values in this table are the LSD for a significant difference at the $p<0.05$ level. Eosinophil and basophil counts were excluded from this analysis because the raw data for these parameters did not discriminate between individuals with sufficient sensitivity.

Summary of haematology

- This experiment successfully generated pathological symptoms of haemonchosis such as anaemia and established a suitable pen model of the disease using primary and secondary infections.
- The bolus dose of 5000 L3 caused more severe haematological changes in group 3 than group 4.
- Between days 57 and 85, the mean group 3 RBC, haemoglobin (Hb) and haematocrit (Ht) decreased significantly more thaThe n those of group 4. This is clear in the graph of RBC (Appendix 8.3). These results were reflected in Hb and Ht.

- For mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), the result was reversed. The group 3 mean value increased significantly more than group 4.
- There was no significant difference between group 3 and group 4 at day 85 for any haematological parameter.
- RBC, Ht and MCH in groups 3 and 4 were significantly different to group 1 at day 85.

4.1.2 Parasitology

Worm counts were performed on abomasal contents obtained from sheep at slaughter. The average count for each pair in groups three and four per slaughter date was calculated for total larval counts, adult worms and total worm counts (Appendix 8.6). Statistical analysis using REML showed no significant difference when comparing groups on the same day. The worm burdens did change significantly over time.

The graphs of total worm count and adult worm burden show similar patterns of increase. The numbers of larvae recovered rose sharply in both groups after the 5000 L3 bolus. At the second slaughter date (19.7.04), the average burden in group 3 (2967) was almost twice that of group 4 (1550). This showed establishment of larvae was inhibited by a response in group 4 which was primed by the trickle dose of L3 administered prior to the post-Levamisole bolus dose (5000 L3). The purpose of Experiment 2A was to determine the timing and mechanism of this response. Similar numbers of worms were subsequently counted in each group as the larval population declined through the remaining kill days.

Worms were also collected to measure their responses to host immunity. Worms were photographed and the areas and perimeters of their silhouettes were calculated using Image-Pro Plus version 5.1 and taken as a measure of size. After photographing, they were dissolved in hypochlorite to release their eggs which were then counted. The protocol for dissolving worms and counting eggs in individual females is described in Section 3.3.3.

There was a statistically significant correlation between mean worm area, perimeter and fecundity (Appendix 8.7). This was calculated for groups 3 and 4 using 60 worms from each group (6 sheep per group x 10 worms). Such correlation leads to the conclusion that the immune regulation of control of egg production is manifest through the regulation of size and presumable growth. For the first time with *Haemonchus* infections this will help us identify a mechanism.

There was no significant difference in worm values (area, perimeter and fecundity) between groups 3 and 4 (Appendix 8.8). These were analysed using a Nested ANOVA. This approach allows variance to be partitioned between treatment groups (among groups), among subgroups within groups (sheep) and within subgroups (worms). There were no statistically significant treatment effects. Most of the variance was among and within subgroups.

Spearman rank correlation showed a significant inverse correlation between haematological parameters and worm fecundity (see Appendix 8.9);
absolute lymphocyte count (n=12) and worm fecundity (p= 0.005, r= -0.75)
differential lymphocyte count (n=12) and worm fecundity (p=0.009, r= -0.72)

The logical next step is to collect and immunophenotype lymphocytes. This can be done with lymphocytes purified from blood and/or abomasal lymph. This is the first time a link has been demonstrated between numbers of immune cells and worm fecundity in haemonchosis. It may be a direct or indirect effect but is an important lead in piecing together aspects of the host parasite relationship.

There were no significant correlations found between haematology and FEC using Spearman rank correlation;
absolute lymphocyte count (n=24) and FEC (p= 0.97, r= -0.008)
differential lymphocyte count (n=24) and FEC (p=0.29, r= -0.23)

4.1.3 Immunohistochemistry (IHC)

The protocol used for cutting and staining frozen sections for immunohistochemistry is described in Section 3.3.1. The primary antibodies used for this work were mouse anti-ovine CD1 (dendritic cells, B cells and monocytes), CD4 (helper T lymphocytes), CD8 (cytotoxic T lymphocytes) and CD45RA (B lymphocytes, natural killer cells and naïve T lymphocytes). These were purchased from Serotec. The Serotec CD4 antibody did not stain as described and a CD4 antibody cocktail was obtained from the laboratory of David Piedrafita at the Centre for Animal Biotechnology (CAB) at the University of Melbourne (now at Monash University). Primary antibodies were detected with a polyclonal rabbit anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (DakoCytomation). Examples of staining with these antibodies are shown in Appendix 8.10. Negative control sections stained only with this secondary antibody were included in every staining.

After considerable development and a visit to the Melbourne laboratories to seek advice, the following protocols were established. ICC was performed on frozen sections cut from abomasal folds. The sheep used in these studies were;

- Three sheep from group one (negative control - sheep # V1469, V1796, V4062).
- Three sheep from group two (trickle infection only – sheep # V0830, V2419, V4380).
- Both sheep from each of the first pair (V4129, V0352), fourth pair (V4366, V0181), sixth pair (V1963, V2831) and seventh (last) pair (V3865, V0459) in group three.
- Both sheep from each of the first pair (V0504, V3133), fourth pair (V1520, V3558), sixth pair (V0627, V3726) and seventh (last) pair (V1632, V1461) in group four (trickle plus bolus infection).

ICC sections were initially being analysed with image analysis software (Image-Pro Plus version 5.1). This software uses models of colour such as RGB (red, green, blue) or HSI (hue, saturation, intensity). Required colours (e.g. brown produced by HRP) can be

defined and gated on, then automatically counted and results exported to an Excel spreadsheet. With this approach we had the potential to analyse a large number of slides quickly. However, we encountered the difficulty of non-uniform staining. Gating that worked well for one area of one section was not accurate for the next area or section. To define and change gating regularly removes the advantage of this approach and most slides were counted manually.

Ten representative fields were photographed and counted (at 40x magnification each field measures 440µm x 330µm) from four separate sections of abomasal mucosa per animal per antibody. These data are graphed in Appendix 8.11. Due to the high variability of these data, no statistically significant differences were detected, however, general trends are apparent.

Group 2 was a control for group 4. For each antibody, the value for the group 4 pair at day 59 is similar to the pooled value for group 2 at day 58.

CD1 positive cells were very low in groups 3 and 4 at day 59. They are very high in group 3 at day 72, and rise only slightly in group 4 at the same time point. They taper off to the level of group 1 at day 99.

CD1 is a marker on the professional antigen presenting cells (APCs) - dendritic cells and B cells. It is also on monocytes, which differentiate into macrophages. These cells are efficient phagocytes which migrate to an infection site in response to pathogen-associated molecular patterns (PAMPs) (Meeusen and Piedrafita 2003). An increase in these cells would be expected following the bolus dose of 5000 L3. This may be more pronounced in group 3 since this was their first exposure. In contrast, group 4 was immunised with a trickle infection which may have predisposed to a more mature immune response involving cells downstream to APCs. By day 99 the larvae had left the mucosa as adults and the population of CD1 positive cells declines in both groups.

The most interesting results involved CD4, CD8 and CD45RA cells. CD4 and CD45RA cells are higher in group 4 than group 3 at all time points and at day 99, groups 3 and 4 are higher than the negative control group (group 1 - day 100). CD45R shows a consistent upward trend across time in both groups. CD8 cells in groups 3 and 4 trend downwards from day 59 to day 99 to match the range of the negative control group. These results are indicative of a switch away from a CD8-cytotoxic response towards a humoral (Th2) response mediated by CD4 cells and involving B-lymphocytes. A Th2 response is associated with reduced nematode burdens in mice (Urban*, et al.* 1998; Schopf*, et al.* 2002), humans (Turner*, et al.* 2003) and sheep (Gill*, et al.* 2000).

Summary of Immunohistochemistry

- Immunohistochemistry has required extensive development and optimisation.
- General trends are present which are consistent with a Th2 (humoral) response.
- It is difficult to objectively quantitate a heterogenous process such as the immune response to parasitic infection. Throughout the abomasum, different areas are in varying stages of infection and immune response. Within the same section, some

fields contain aggregates of positive cells and other fields contain no positive cells. This lead to the high variability of counts present in these data.
- Careful selection of abomasal tissue at post mortem according to gross pathology and obvious parasite burden may help reduce variability between animals. Use of positive (e.g. clearly parasitised folds from the fundic region) and negative (e.g. grossly normal folds from the pyloric region) tissue from each animal would help to define background.
- Similar trends and problems have been encountered by David Piedrafita and Joanne Kemp (Monash University) in SheepGenomics.

4.1.4 Tissue Weights

If cellular recruitment to tissues could be demonstrated, it may contribute to an understanding of the mechanism of resistance. Cell recruitment could be quantitated as either a difference in the weight of tissues from different groups, or a difference in the density of cells in tissues from different groups.

There were no statistically significant differences in the weights of the left prescapular lymph node (LPSLN), right prescapular lymph node (RPSLN) and abomasal lymph nodes (ALN) between groups three and four (data not shown). The weight of abomasal mucosa from different animals can not be meaningfully compared due to natural variation in the size of the abomasum

4.1.5 Histology

4.1.5.1 Total Cell Counts

The cell density in H&E stained paraffin embedded sections from abomasal mucosa and lymph nodes from different treatment groups was compared to determine whether there was a difference in cellular recruitment. These data were collected using image analysis software (Image-Pro Plus version 5.1).

For each tissue, five representative fields were photographed and counted (at 40x magnification each field measures 440µm x 330µm). In each image, the darkly stained nuclei of cells were gated on. The total number of nuclei and the proportion of the total area that was occupied by nuclei were determined for each field and averaged per tissue. For lymph nodes, this was calculated separately in both follicular and interfollicular regions. These data were collected for all animals in group 1 (V1469, V1796, V3402, V4062), all animals in group 2 (V0830, V2419, V4144, V4380), group 3 pair 4 (V0181, V4366) group 3 pair 7 (V0459, V3865), group 4 pair 4 (V1520, V3558) and group 4 pair 7 (V1461, V1632). These data are included in Appendix 8.12.

Image-Pro Plus was used to measure the proportion of tissue made up of nuclei. Whilst the problems encountered using Image-Pro Plus for immunohistochemistry were reduced in H&E sections by the greater uniformity of staining, even within dark objects such as nuclei the software detects regions of heterogeneity. If the sensitivity of detection is increased, the overall slide background signal also increases. Due to this

heterogeneity, the darker parts of a single nucleus can be recognised as separate nuclei. The settings used determined how adjacent spots were discriminated and how many times each nucleus was counted. Small adjustments in the settings gave widely variable results for the total number of cells present in an image so total cell number was considered too subjective to be reliable.

Since there are two animals at each time point in group 3 and 4, formal statistical analysis can not been undertaken at this time. The nuclear proportions range from 0.45 to 0.5 (45-50%) in lymph nodes but is 0.35 to 0.4 (35-40%) in abomasal mucosa, which probably reflects the relative water content of these tissues. In all tissues the nuclear proportion in group 4 declines from day 71 to day 99. It is level or increased in group 3 in the lymph nodes but not in the abomasal mucosa.

In all samples, the range of values for group 3 and 4 are close to those in the control groups (1 and 2), implying there was little change with treatment. This suggests that recruitment of cells into tissues is more likely to result in an increase in the volume of tissue rather than an increase in the density of the cell population. We conclude that cell density remains within a limited range and natural biological variation makes changes in the weight of tissues between different treatment groups difficult to quantitate with small group sizes.

4.1.5.2 Mast Cells and Eosinophils

Eosinophils and mast cells were manually counted in paraffin-embedded histological sections of abomasal folds stained with giemsa chromotrope. These were from the same animals used for ICC (see above) Ten representative fields were photographed and counted (at 40x magnification each field measures 440µm x 330µm) from four separate sections of abomasal mucosa. These data are shown in Appendix 15.

Clear trends were not evident with eosinophils and mast cells and the standard deviations are more pronounced than for cells identified by ICC. The value obtained for the animal in group 4 at day 59 is similar to the pooled value for group 2 (group 4 control) at day 58. At day 99, only the eosinophils in group 3 appear markedly different to the negative control group at day 100. Group 4 eosinophils and mast cells for both groups at day 99 were similar to the negative control group at day 100. Eosinophils increased in group 4 to day 72 then plateaued to day 99. Mast cells increased to day 72 then declined to day 99 in group 4, whilst gradually increasing across the three time points in group 3.

Tissue eosinophil counts were difficult to quantitate. The cells accumulate around larvae at early time points and in the submucosal space at day 99. Numbers in the submucosal space were minimal at day 59 and increased markedly with time of infection as the tissue became oedematous. These observations made it difficult to select representative fields in a non-biased fashion. For this reason, blood eosinophil counts may be more reliable than tissue counts.

4.1.6 ELISA

In collaboration with Ross Windon at CSIRO Armidale, serum ELISAs have been completed on all animals in groups 1, 3 and 4 that were alive on day 0, 57, 62 and 85 for IgG1, IgG2, IgA and IgE. ELISA was also performed on homogenates of abomasal mucosa, abomasal lymph node and prescapular lymph node collected from each animal at post-mortem. Tissue (lymph node and abomasal homogenates) ELISA included IgM in addition to IgG1, IgG2, IgA and IgE.

The ELISAs were performed as titrations of each sample. The titres calculated for each sample are the mid point of the straight line section of the curve. Each plate had a common standard that was used to calculate a corrected value to take into account the between plate variation.

These ELISA data were analysed by Associate Professor Peter Thompson and biometry honours student Kristy Mann using a REML (Restricted Maximum Likelihood) approach. REML has advantages over ANOVA and similar models by being able to cope well with unbalanced designs and with correlated data arising from sampling the same unit repeatedly i.e. repeated measures data.

No statistically significant difference ($p<0.05$) was detected between groups at any time point for serum or tissue ELISA results. Statistical differences were only detected in immunoglobulin levels between some of the tissues. IgA was higher in abomasal mucosa than prescapular lymph node. This is because IgA is part of mucosal immunity. IgG1 was significantly higher in lymph nodes than abomasal mucosa. This is because IgG1 is the major serum immunoglobulin. IgE was higher in abomasal lymph node than the other tissues. IgE is scarce in serum and is found predominantly on the surface membrane of basophils and mast cells. This implies a higher population of these cells in abomasal lymph node than other tissues.

In some cases, the lack of detectable difference between groups may be due to the frequency and timing of sampling. For example, IgA and IgE are local antibodies that may appear in plasma as spikes at specific times after infection. If blood is not sampled at these time points, IgA and IgE responses may not be seen in the plasma.

Antibody responses to *Haemonchus* infection are usually much lower than the response to *Trichostrongylus*, especially to adult antigens (Ross Windon - personal communication). Combined with the large variations observed between individuals from the same group at the same time point (commonly ranging from two to ten times difference), significant differences are unlikely to be revealed with small group sizes.

4.2 Development of the Abomasal Fistula Technique

Development of the fistulation and endoscopy approach evolved with the project. Our initial approach was to collect biopsy samples for histology and a single fistula approach was developed. A 10mL syringe barrel was used and this was externalised through the diaphragm via the ventral extremity of intercostal space IX. Both Terumo and Livingstone brand syringes were tested and Terumo was found to have superior strength and flexibility for use as a fistula. Biopsy samples of abomasal mucosa were obtained via the 3mm wide port of the endoscope. There was no leakage of abomasal contents observed but healing was delayed in places where protruding tissue was entrapped between ribs and the syringe barrel. A biopsy report is included in the appendices (Appendix 8.2).

With the change of emphasis to proteomics and RNA work we had to reassess our needs. Each milligram of abomasal mucosa would be expected to provide 2-4µg of total RNA (http://www.ambion.com/techlib/tips/rna-conversions.html) and 100-250µg of total protein (Litvak*, et al.* 1998). We required 10-20µg of RNA for each microarray experiment and 0.5-1mg of protein per 2D gel. To provide ample material for multiple experiments, we aimed to obtain two separate biopsy samples of 50-100mg each, saving one for RNA extraction and one for protein extraction. This would require five to ten bites using the endoscopic biopsy tool and is impractical.

To increase the amount of biopsy material recoverable, we tried inserting two abomasal fistulas into each of four sheep (Experiment 2A control experiment 1 Group 2). One fistula was placed in the fundus and the second was placed in the pylorus. This enabled the simultaneous passage of instruments through one fistula and the endoscope through the other. While the fistulae were sound it was difficult to locate the instruments within such an active organ.

We finally settled on a single fistula using a 20mL syringe barrel (Section 3.2). This provided a 6mm gap beside the endoscope which allowed alligator forceps to be used to grab folds within the field of vision of the endoscope. Folds were lifted into the base of the fistula and portions removed. The endoscope could then be used to locate fresh areas for subsequent biopsy.

4.2.1 Administration of Larvae via the Abomasal Fistula

Protocols were established in our laboratory to exsheath L3 (Section 3.3.4) and to culture L3 to L4 (Section 3.3.5). Exsheathed L3 and cultured L4 were used to infect fistulated sheep.

One sheep was infected with 5000 L3 by intrarumenal injection eighteen days prior to surgery. Strongyle eggs were present in the faeces fifteen days post-surgery. This demonstrated that an existing infection can survive the anaesthesia and surgery for implanting an abomasal fistula.

A second sheep was infused with 5000 exsheathed L3 via the abomasal fistula, and strongyle eggs were detected in the faeces after 17 days. This showed that larvae

obtained and exsheathed in the lab can be used to establish an infection in fistulated sheep.

In two other sheep, larvae were cultured for either four or eight days *in vitro* then infused. Strongyle eggs were observed in the faeces twenty days after infection with larvae cultured *in vitro* for four days. This shows that larvae can retain the ability to establish an infection after four days in culture. This period provides an opportunity to examine treatments in culture that may diminish this ability (such as RNAi) and lead to new strategies for controlling haemonchosis. Since the time to patency was similar to that for the exsheathed L3, it implies that little of the development required for reproductive maturity occurs *in vitro*.

Five weeks post-infection, no strongyle eggs were found in the faeces of the sheep infused with larvae cultured *in vitro* for eight days. This sheep was subsequently infected with exsheathed L3 via the fistula as a positive infection control and eggs were detected in the faeces twenty-three days post-infection. This result demonstrated that this sheep was susceptible to infection and that after eight days in culture the first batch of larvae infused were unable to establish an infection.

4.3 Experiment 2A.

Aim - To sample tissues from sheep during the window when worm establishment is inhibited by host responses. Worms were also collected to measure their responses to host immunity. A chart showing the design of Experiment 2a and a description of materials and methods are included in Section 3.1.2.

4.3.1 Haematology

A haematology profile was obtained from each blood sample and these results have been tabulated from all samples taken during this trial. Group averages for red blood cells at each time point have been graphed (Appendix 8.14). In line with the developing anaemia these values decline in all groups throughout the trial then show a recovery after the drench with Ivermectin. The three groups in this trial were slaughtered on day 38, 45 and 52 (weekly). Until day 32, the groups were replicates except for the timing of the two doses of Ivermectin-resistant larvae (CAVR), so there was no treatment effect to be measured with additional haematology.

4.3.2 Parasitology

4.3.2.1 Worm Establishment

Two control experiments were performed for this experiment. Control Experiment 1 was designed to determine whether the sheep immune response to ivermectin-sensitive (McMaster strain) and ivermectin-resistant (CAVR strain) *Haemonchus* is equivalent and also whether fistulation affected the model. This experiment used three groups of four sheep. Group 2 sheep were surgically implanted with two abomasal fistulas each. The development of the fistulation procedure is discussed in Methodology Section 3.2. Groups 1 and 3 did not have fistulas. All sheep were given two intrarumenal doses of 5000 L3 three weeks apart, and then slaughtered eight days after the second dose. Group 1 was infected with ivermectin-sensitive L3 (McMaster strain). Groups 2 and 3 were infected with ivermectin-resistant L3 (CAVR strain). One sheep in group 1 was removed from the experiment due to non-parasitic disease.

Control Experiment 2 was designed to more accurately define the ratio of adults to larvae to help select appropriate tissues for future microarrays. It replicated the first control except that it used two doses of 500 L3 given orally. This allowed us to confirm the control ratios of adults to larvae in non-immune sheep and help set the protocol for Experiment 3.

The control experiments confirmed that the protocol of infecting 3 weeks apart allowed the recovery of adults and larvae at post mortem as a measure of relative establishment. Included in the appendices are worm counts and ratios of larvae to adults from Control Experiment 1 (Appendix 8.15), Control Experiment 2 and Experiment 2A (Appendix 8.16).

In non-immune sheep where both infections establish at the same rate the expected ratio is 1:1, but because larvae are more difficult to recover and count, ratios would be expected to be lower. The ratio of larvae to adults from Group 3 (CAVR strain with no fistulas) was 0.87:1. The ratio for group 1 was 0.42:1 and for group 2, 1.32:1. There was a significant difference between group 1 and group 3 (p= 0.05) but not between group 2 and group 3 (p= 0.11) (These are two-tailed p values obtained from t-tests assuming equal variance and using log10 transformed ratios from all individuals in each group). This indicates that fistulation did not alter the development of immunity for the CAVR isolate.

Using these control ratios, sheep from Experiment 2A with low ratios of larvae to adults were identified (Appendix 8.16). Sheep numbers 2212, 2225 (group one), 2211, 2224 (group two) and 2229 (group three) are examples of sheep sampled within the window when host responses appear to be inhibiting larval establishment.

4.3.2.2 Response to Host Immunity

The FECs from each group after drenching with Ivermectin are shown in Appendix 8.17. The mean FEC in Group 3 was significantly reduced compared to Group 1 (p=0.03) and approaching significance compared to Group 2 (p=0.06).

The adults remaining in Groups 1 and 2 after Ivermectin were administered as L3 in weeks 2 and 3 of the trial respectively. The adults remaining in Group 3 were given as L3 in week 4 of the trial. Based on this result, the protocol of dosing sheep with 500 L3 three times per week was producing a switch from immune naivety to inhibition of establishment of larvae around 3.5 to 4.5 weeks. This is the reason the subsequent trial (Experiment 3) was designed with slaughters half weekly starting at the beginning of week 4, enabling large amounts of material to be collected at post-mortem during this critical period in the immune response.

Nested ANOVA showed no statistically significant treatment effect (among groups) on worm area, perimeter or fecundity for this experiment, although the p values among groups for area (0.1) and perimeter (0.08) were approaching significance. For each worm parameter, Group 3 had the lowest overall mean values, followed by group 2 then group 1 (Appendix 8.18). This implies that these values decline as sheep receive more trickle infections and agrees with published findings that immune responses to adult worms are related to ongoing larval challenge (Barger*, et al.* 1985; Barger and Le Jambre 1988). Graphs of the mean worm data per sheep are shown in Appendix 8.19.

There was a statistically significant correlation between mean worm area, perimeter and fecundity (Appendix 8.20). This was calculated using 40 female worms for Groups 1 and 2 and 28 female worms for Group 3.

The greatest difference between mean worm values in each group occurs in the sheep with the lowest FEC. The difference between animals in each group decreases as sheep FEC increases. These observations are most likely linked to the level of immune control over the parasite by the sheep. As this control is relaxed, the results from each group become more similar.

4.3.3 ELISA

Using the same techniques as Experiment 1, the same antibody ELISAs were completed with Experiment 2A samples in collaboration with Ross Windon at CSIRO Armidale. Serum ELISAs were completed on all animals in groups 1, 2 and 3 that were alive on day 0, 22, 38, 45 and 52. ELISAs were was also performed on homogenates of abomasal mucosa, abomasal lymph node and prescapular lymph node collected from each animal at post-mortem. These data were analysed using the REML (Restricted Maximum Likelihood) approach used for Experiment 1 ELISA.

This yielded similar results to Experiment 1. No statistically significant difference ($p<0.05$) was detected between groups at any time point for serum or tissue ELISA results. Statistical differences were only detected in immunoglobulin levels between some of the tissues. IgG1 was significantly higher in lymph nodes than abomasal mucosa.

4.3.4 Microarray

We currently have 30 sets of microarray data (6 biopsies x 5 sheep) from Experiment 2A. The sheep selected had the lowest larval establishment rates from the second dose of CAVR L3, indicative of the most effective immune exclusion (Group 1 – sheep 2212 and 2225, Group 2 – sheep 2211 and 2224, Group 3 2229). In addition, we have 11 sets of control data comparing the affects of fistulation vs. non-fistulation, infection with ivermectin sensitive (McMaster) vs. ivermectin resistant (CAVR) strains and pre-infection vs. post-infection.

Biostatistical analysis of the microarray results is in progress in SheepGenomics. This will show which genes are differentially expressed in immune and non-immune animals and provide targets for further validation by real time PCR. Our initial microarray analyses were discussed in detail in AWI HRIP Milestone Report project SG215 – 1/7/06 (TMS-2).

4.4 Experiment 2B.

Aim - To examine the effect of host immunity on adult worm fecundity. A chart showing the design of Experiment 2b and a description of materials and methods are included in Section 3.1.3.

4.4.1 Parasitology

The overall group mean values for worm area, perimeter and fecundity in group 1 and the two control animals (group 2) at post mortem (27.7.05) are shown in Appendix 8.21. The mean worm values per sheep are shown in Appendix 8.22. At the earlier time points (13.7.05 and 20.7.05) only one to two worms could be recovered via the abomasal fistula from each sheep and could not be interpreted meaningfully so data are based on the later timepoints.

The graphs show the mean worm area and fecundity from the two control sheep (sheep #2231 FEC 13640 and sheep #2232 FEC 15440) are very similar to the two immune sheep with comparable FEC (sheep #2228 FEC 10280 and sheep # 2217 FEC 16760) and have a similar range for individual worm fecundity. With unequal numbers in control and treatment groups and only two animals in the control group formal statistical analysis is not appropriate. In both control and immune groups, the mean values for worm area, perimeter and individual fecundity increase with increasing FEC.

There was a statistically significant correlation between mean worm area, perimeter and fecundity (Appendix 8.23). This was calculated using 38 female worms selected from four animals in group 1.

4.5 Experiment 3

Aim - To repeat important time points identified in Experiment 2A to allow larger amounts of post-mortem material to be collected. A chart showing the design of Experiment 3 and a description of materials and methods are included in Section 3.1.4.

4.5.1 Parasitology

Worm areas, perimeters and fecundity were measured from fifteen individual female adult worms per sheep collected from group 5 and group 6 at post mortem (13.7.06). The mean values per group are tabulated in Appendix 8.24. The mean values per sheep are graphed in Appendix 8.25. Whilst the overall mean values for each parameter in the group 5 (immune) sheep were lower than group 6 (control), there were no statistically significant differences between the groups ($p>0.05$ among groups Nested ANOVA).

There was a statistically significant correlation between mean worm area, perimeter and fecundity in worms from groups 5 and 6 (Appendix 8.26). This was calculated using 73 worms per group.

4.5.2 Microarray

The slaughter of these sheep at 3-4 weeks was timed to coincide with the switch to immunity against larval establishment. Use of samples from these animals for microarray will be based on results from Experiment 2A microarray data.

4.6 Experiment 4

Aim - To test the hypothesis that corticosteroid-induced immunosuppression and a reduced protein diet abolish normal immune affects on *Haemonchus* burdens, size and fecundity. The experimental design is explained in Section 3.1.5.

4.6.1 Parasitology

FECs were calculated for all sheep in the trial on 6.11.06 (Slaughter date). These are included in Appendix 8.27. The group 1 (control) FEC was significantly higher than groups 2 and 3. There was no significant difference between groups 2 and 3.

The finding that the final FECs were lower in the two groups treated in a way designed to lower immunity was surprising. There may be several explanations for this. A temporary reduction in gut integrity may accompany the inflammation of a more competent immune response (Group 1) and initially accelerate worm development. Alternatively, the stress on worms of a more competent immune response may encourage egg production prior to death. A longer duration of trial would test these outcomes.

There may also be a nutritional explanation for this result in group 3. Low carbohydrate diets result in a greater loss of body weight, reduction in immunity and increase in worm burdens than low protein diets (Sue McClure - personal communication). The molasses used to dilute protein would have increased soluble carbohydrate. This may have offset any immune detriment caused by the reduced protein. Crude protein may also need to be severely deficient to measurably suppress immunity (e.g. 7% crude protein). This raises the question of whether nutrition is more important during the primary trickle or the secondary bolus infection. However, nutritional affects would not account for the difference between groups 1 and 2.

Worm counts were performed on abomasal contents obtained from sheep at slaughter. The average count for each group was calculated for total larval counts (Appendix 8.28) and adult worms (Appendix 8.29). Group 2 had a significantly higher number of larvae. There was no statistically significant difference between the groups in adult worm numbers. This implies the corticosteroid treatment in group 2 lead to some delay in larval development, as observed elsewhere (Adams 1986).

Worm areas, perimeters and fecundity were measured from thirty individual female adult worms per sheep. The mean values per group are tabulated in Appendix 8.30. The mean values per sheep are graphed in Appendix 8.31. There were no statistically significant differences between the groups ($p>0.05$ among groups Nested ANOVA - Appendix 8.30). The p-value among groups for worm perimeter (0.07) was approaching significance and mean worm perimeter and area was highest in group 2. This implies a relaxation in immunity to adult growth in group 2, which is counterintuitive to the observation of delayed larval development. Adams (1986) concluded the paradoxical suppression of larval development observed during corticosteroid treatment is due to preferential inhibition of suppressor lymphocytes compared to helper lymphocytes, as reported in other immunological systems (Bradley and Mishell 1981). This results in a

net increase in immune activity against the larvae. However, whilst there are multiple immune pathways to target larval stages, adult *Haemonchus* graze on the surface of the mucosa and the major mechanism for controlling worm length appears to be parasite-specific local IgA (Smith*, et al.* 1985; Stear*, et al.* 1999). The finding that adult worms were largest in group 2 implies this immune pathway was adversely affected by corticosteroids in this trial.

There was a statistically significant correlation between mean worm area, perimeter and fecundity in worms from groups 5 and 6 (Appendix 8.32). This was calculated using 180 worms per group. The correlations for all groups from all experiments are tabulated in Appendix 8.33.

4.6.2 Immunohistochemistry (IHC)

The protocol used for cutting and staining frozen sections for immunohistochemistry is described in Section 3.3.1. The antibodies used were as described for Experiment 1 (Section 4.1.3).

Ten representative fields were photographed and counted (at 40x magnification each field measures 440µm x 330µm) from four separate sections of abomasal mucosa per animal (n=4) per antibody. These data are shown in Appendix 8.34. Due to the high variability of these data, no statistically significant differences were present.

Counts for CD1, 4 and 8 were highest in Group 1, and declined through Groups 2 and 3. CD45R was also highest in Group 1, but increased slightly from Group 2 to Group 3. These results are not indicative of any trends.

4.7 Analysis of *Haemonchus* Transcription Profile

The purpose of this work is to attempt to identify ways in which worms respond to immunity imposed on them, by their host. We are interested in genes unregulated during infection and genes specifically differentially regulated in worms in immune sheep.

Worms were collected from sheep in groups 5 and 6 of Experiment 3 for analysis of gene expression. A chart showing the design of Experiment 3 groups 5 and 6 and a description of materials and methods are included in Section 3.1.4.2. Common samples collected from all sheep were tubes containing male worms only, female worms only and mixed worms. These were snap frozen in liquid nitrogen and stored at -80^{O}C. In addition, we chose the sheep in each group with the highest faecal egg count and collected and stored 40 male worms and 40 female worms individually. This approach will provide the maximum opportunity to look at gene expression profiles within different subpopulations of the parasite.

In the absence of comprehensive genome sequence differential display polymerase chain reaction (dd-PCR) was chosen as the most appropriate technology and is under development to analyse transcription profiles. A flow chart showing the dd-PCR strategy is included in Section 3.3.6.

4.7.1 RNA Extraction

Mixed populations of worms were selected from sheep #1516 (group 5 – immune group – FEC 7640) and sheep #1099 (group 6 – control group – FEC 13320) for RNA extraction. These sheep were chosen because their FECs were closest to the group means (group 5 mean FEC 7656, group 6 mean FEC 12696). These worm populations are likely to show transcription profiles most representative of the treatment effects rather than confounding factors such as initial larval establishment.

RNA was extracted from worms as described in Section 3.3.7. It was necessary to add a DNase step to this protocol because any residual genomic DNA can serve as a PCR template and provide false positives. DNase-treated RNA samples were stored prior to reverse transcription (RT). When this RNA was used in PCR as a negative control for genomic DNA contamination, no bands were detected on gels confirming that this RNA extraction method effectively removes genomic DNA.

4.7.2 Reverse Transcription (RT)

ddPCR is a method used to identify and characterise gene expression in two comparative groups. It is based on reverse transcription (RT) with anchored oligo-dT primers (Liang and Pardee 1993). Oligo-dT primers can be anchored by one or two bases. One base-anchored oligo-dT primers were used for this study. The use of one base-anchored oligo-dT primers (20 T nucleotides followed by either an A, C or G nucleotide 5′→3′) reduces the number of RT reactions required for each RNA sample from twelve (oligodT + A/C/g + A/C/g/T) to three (oligodT + A/C/g) and minimises

redundancy and under-representation caused by degeneracy of the primers (Liang*, et al.* 1994).

RT was optimised using two different protocols and sources of reverse transcriptase, SuperScript III (Invitrogen) and RevertAid (Fermentas). Both are derived from Moloney Murine Leukaemia Virus (M-MuLV) and are genetically modified to reduce RNase H activity (which degrades RNA in RNA:DNA hybrids). These protocols are included in Section 3.3.9.

4.7.3 Differential Display Polymerase Chain Reaction (dd-PCR)

To identify differentially expressed genes, comparisons are made between PCR products amplified under the same conditions using templates (DNA derived from RNA) from different populations of worms (i.e. corresponding boxes on the flowchart in Section 3.3.6 obtained from different RNA samples). When dd-PCR products are separated by electrophoresis, differentially transcribed genes appear as different intensities of the equivalent band in different samples. These bands can be excised from the gel, purified, reamplified by PCR then cloned and sequenced for identification.The PCR protocol used is shown in Section 3.3.11. A one year Bachelor of Science (Vet) student (Kwan Yun) at the University of Sydney is undertaking part of this work.

4.7.4 Detection of PCR Products

Silver staining of polyacrylamide gels is a relatively simple procedure reported to achieve sensitivity of 0.44ng DNA in denaturing (1.6M or 7M urea) polyacrylamide gel electrophoresis (PAGE) and 3.5ng DNA in nondenaturing PAGE (Qu*, et al.* 2005). Two silver staining methods were tested (Section 3.3.13). The method of Bassam was found to be preferable (Bassam*, et al.* 1991) but with a slight modification in the development step. A three times increase in $Na_2S_2O_3.H_2O$ from 0.6 to 1.8mg/100mls resulted in less background and clearer staining of bands. The sensitivity of this method is reported to be 1pg/mm^2 band cross-section.

4.7.5 Recovery and Reamplification of Differentially Expressed Bands

Differential display has been achieved with worms from sheep #1516 (group 5 – immune) compared with sheep #1099 (group 6 – control). The comparison has been made for all SL1 products from the three cDNA pools (i.e. 30 reactions from 1516 vs 30 reactions from 1099) (stage 1 PCR - HindIII(T_{20})A/C/g primer x SL1 primer) (stage 2 PCR - HindIII(T_{20})A/C/g primer x XhoI arbitrary primers 1 to 10). Twenty-five differentially expressed bands are shown in Section 8.35. The seventeen most prominent bands without differential intensity are shown in Section 8.36. Genes that are highly expressed in both populations are also of interest because they reveal general responses to parasitism and can be compared to expressed sequence tag (EST) databases.

Silver stained gels were air dried between stretched cellophane overnight and each band of interest was excised with a new scalpel blade and placed in a separate

eppendorf tube. We failed to recover DNA from these bands for reamplification after, gel slices were boiled in water for 15 minutes (Cui*, et al.* 2001; Brehm*, et al.* 2003; Heider*, et al.* 2006) then soaked in water at room temperature for 24 hours (Boschi and Vergara 1998) and samples of supernatant taken at each time point.

A more complicated recovery method was then used named the crush and soak technique (http://genetics.med.harvard.edu/~cepko/protocol/mike/D5.html). This is detailed in Section 3.3.14. Two bands have been successfully reamplified using this technique – a differentially expressed 100 bp band upregulated in worms from sheep 1516 (Stage 2 primers = HindIII(T_{20})g + XhoI arbitrary 9) and the equally prominent 450 bp band (Stage 2 primers = HindIII(T_{20})g + XhoI arbitrary 7). Highly prominent bands such as the 450 bp band can be excised and extracted from 1% agarose gels if a large volume of sample is loaded (e.g. 25μl). This approach has also been successful using the crush and soak technique. Differentially expressed bands are not sufficiently abundant to resolve on agarose.

4.7.6 Cloning and Sequencing

We have provided ourselves with two options for pursuing cloning of bands which can be cleanly reamplified. pCR 2.1-TOPO (Invitrogen) is the first approach and employs the cloning vector covalently bound to the topoisomerase I enzyme. This ligates the terminal A added to PCR products by *Taq* DNA polymerase to the T overhang on the cloning vector. As a second option, the HindIII and XhoI restriction sites added to the primers used for PCR can be digested and the DNA ligated into the corresponding sites on the vector with T4 DNA ligase.

TOP10F′ (or DH5α) *E.coli* will be transformed and positive clones identified with the X-gal and IPTG system. Broths grown from positive colonies will be screened via PCR using SL1/2 primer (5′) and M13 forward primer (3′) and cross referenced with the expected size of product. Plasmid DNA purified from positive broths will be sequenced to identify the selected band. This protocol is shown in Section 4.7.6.

Summary of progress on dd-PCR for analysing transcription profiles

- These methods have required extensive development and optimisation.
- Worms have been collected from sheep in groups 5 and 6 of Experiment 3 for analysis of gene expression. Other worms are stored for future analysis.
- RNA has been successfully extracted.
- Each RNA sample has been reverse transcribed into three separate cDNA pools.
- A two stage PCR protocol has been developed to amplify and preserve cDNA pools.
- Differential gene expression has been demonstrated (25 genes).
- A successful protocol for silver staining acrylamide gels has been developed.
- Methods for recovering and reamplifying bands of interest have been developed (PAGE / agarose with crush and soak technique).
- Two strategies have been implemented to clone bands of interest (Topoisomerase I on the pCR 2.1-TOPO construct and HindIII/XhoI double

digestion of PCR products and pCR 2.1-TOPO construct then ligation with DNA ligase).

- A method for transforming/screening bacteria and sequencing constructs has been compiled and is in process.
- This work is being continued as part of a one year Bachelor of Science (Vet) project at the University of Sydney.

5 Success in Achieving Objectives

1. To describe parasite population dynamics and the host response to infection.

Experiment 1 examined the establishment of *Haemonchus* populations and the immune response by comparing sheep given a bolus dose of larvae only (group 3) with sheep given trickle doses of larvae prior to the bolus dose (group 4). This experiment successfully generated pathological symptoms of haemonchosis such as anaemia, and detected an immune response to the worms. Immunohistochemistry results showed general trends which are consistent with a Th2 (humoral) immune response which is associated with reduced nematode burdens in several species. A significant inverse correlation was found between blood lymphocyte counts and worm fecundity. This is the first time a link has been demonstrated between numbers of immune cells and worm fecundity in haemonchosis.

2. To develop a method for serial sampling of worms and abomasal tissue from the same animal during an *Haemonchus* infection.

The abomasal fistulation technique was initially developed using two different brands of 10ml syringe and tested by infusing *in vitro* cultured larvae into the abomasum. It was optimised during the first control experiment for Experiment 2A using double fistulas. In Experiment 2A fistulas constructed from a 20ml syringe were implanted into 18 sheep and serial sampling of abomasal mucosa and *Haemonchus* was undertaken through these fistulas. Biopsy of abomasal mucosa via the fistula was more successful than recovery of worms.

3. To identify parasitological and molecular events associated with a switch to inhibition of larval establishment.

Experiment 2A used staggered doses of ivermectin-resistant larvae to examine the timing of the immune response on the establishment of larvae. According to FEC results, a switch from immune naivety to inhibition of establishment of larvae was occurring in this experiment around 3.5 to 4.5 weeks. In a parallel study in SheepGenomics, 41 microarrays were performed using RNA from serial biopsies of abomasal mucosa taken before, during and after these time points. These will help to identify genes involved in inhibition of larval establishment. Experiment 3 replicated the treatment protocol of Experiment 2A with slaughters in weeks 4 and 5 to allow larger amounts of material to be collected at post-mortem.

4. To determine the morphological and molecular effects of host immunity on *Haemonchus.*

Image analysis software was used to measure the area and perimeter of worms collected at post mortem. The most statistically significant reductions were measured from worms in trials where larval challenge continued closest to the slaughter date, suggesting that the presence of worms or recent worm exposure is required to maintain the immune response. A unique finding was a correlation between adult worm size and

egg content. This leads to the conclusion that the immune regulation of control of egg production is manifest through the regulation of worm size and presumably growth which will help us to identify a mechanism.

Differential gene expression in *Haemonchus* has been demonstrated via differential display PCR using worms collected from immune and nonimmune sheep. Twenty-five differentially expressed bands have been identified to date. Cloning and sequencing of such bands may lead to the identification of proteins which can be targeted for vaccination or chemical control.

5. To examine the influence of diet and immunosuppression on sheep immunity to *Haemonchus*.

In Experiment 4, sheep immunity to *Haemonchus* was observed under corticosteroid-induced immunosuppression and a reduced protein diet. Corticosteroid treatment appeared to slow larval development yet enable adults to grow larger. A longer duration of trial and either a reduction in carbohydrate content of the diet or a more severe protein deficiency may have been necessary to obtain clearer nutritional results.

6 Impact on Meat and Livestock Industry – now & in five years time

The outcomes from this project will make their major contribution to Industry in the 3-5 year time frame as the endpoints are not yet complete. The investigation was an essential prerequisite for developing chemical and biological control of *Haemonchus* through analysis of the host-parasite relationship and had 3 major outcomes impinging on further research within GINTIP and SheepGenomics.

Firstly, identification of differentially-expressed parasite proteins activated or suppressed during immune attack will lead to:

- new chemical targets (ongoing for GINTIP)
- development of assays which monitor development of immunity and potentially discriminate between resistant and susceptible sheep (relevant to SheepGenomics-SG)

Secondly, the large volume of correlated and complementary host and parasite phenotypic data has raised the intensity of *Haemonchus* measures to that for other gastrointestinal parasites such as *Trichostrongylus* and *Ostertagia.* This enables greater precision and flexibility for measures of parasite resistance, especially with commonalities of host resistance on worm establishment, growth and fecundity. Direct application into SG will assist fine-mapping QTL studies with a broader range of relevant resistance measures than simply FEC and PCV alone.

Thirdly, measures of gene activation through microarray analysis of host tissue at critical times during the host-parasite relationship (eg 4-5 weeks after infection when L3 establishment is suppressed by developing host resistance) is directed at discovery of resistance gene markers and product identification (in SG).

Each of the project outcomes has important potential applications and effort has been made to integrate the current findings into ongoing research projects in industry parasite programs to capitalize on the project outcomes.

7 Conclusions and Recommendations

7.1 Conclusions

1. Trends present in immunohistochemistry from Experiment 1 are consistent with a Th2 (humoral) response. Variability within and between animals may be reduced leading to more stringent statistical results by counting sections from both grossly parasitised and non-parasitised abomasal mucosa from each animal.
2. A significant inverse correlation was found between blood lymphocyte counts and worm fecundity. This is the first time a link has been demonstrated between numbers of immune cells and worm fecundity in haemonchosis. This link could be further investigated by immunophenotyping lymphocytes purified from blood and/or abomasal lymph.
3. Histological analyses of tissues for mast cells, eosinophils and total cell numbers have not shown clear trends between groups in Experiment 1. Histology has not been as informative as immunohistochemistry.
4. Blood samples for ELISA should be taken closer to the times of infection. In experiment 1, blood was collected on day 0, 57, 62 and 85. The trickle infections occurred during day 0 to 21 and the bolus dose was given on day 57. These blood collections time points may not have been close enough to the infection times although antibody responses may not be good indicators of responses to *Haemonchus* infection.
5. The use of image analysis software to measure worm areas and perimeters has been highly effective. The use of this software for immunohistochemistry and histology is limited by variability of staining within and between slides.
6. Each of the trials conducted detected a statistically significant correlation between mean worm area, perimeter and fecundity. Such correlation leads to the conclusion that immune regulation of control of egg production is manifest through the regulation of size and presumably growth. For the first time with *Haemonchus* infections this will help us to identify a mechanism.
7. The most statistically significant effects on adult worms were measured from experiments where larval challenge of sheep continued up to the slaughter date suggesting that this is required to maintain the immune response.
8. The results of Experiment 2A showed our protocol of dosing sheep with 500 L3 three times per week was producing a switch from immune naivety to inhibition of establishment of larvae around 3.5 to 4.5 weeks. RNA from samples of abomasal mucosa biopsied before, during and after this window was used for microarray which may identify the molecular mechanisms. Large amounts of material were collected around these time points in the subsequent slaughter experiment (Experiment 3).

9. Abomasal fistulation is an effective method for obtaining mucosal biopsies for genomic and proteomic studies but was not effective in our hands for recovering *Haemonchus*.
10. Abomasal fistulation could be used as a route for delivering cultured *Haemonchus* which have received treatments such as RNAi. This approach could lead to new strategies for controlling haemonchosis.
11. In Experiment 4, extending the period between bolus (secondary) infection and slaughter from 4 weeks to 6 or 7 weeks with weekly monitoring of FECs may have produced more insightful results. Corticosteroid treatment appeared to be retarding larval development but encouraging adult growth.
12. Differential gene expression between Haemonchus from immune and non-immune sheep has been demonstrated using dd-PCR. The materials and methods are in place to clone and sequence these genes.

7.2 Recommendations

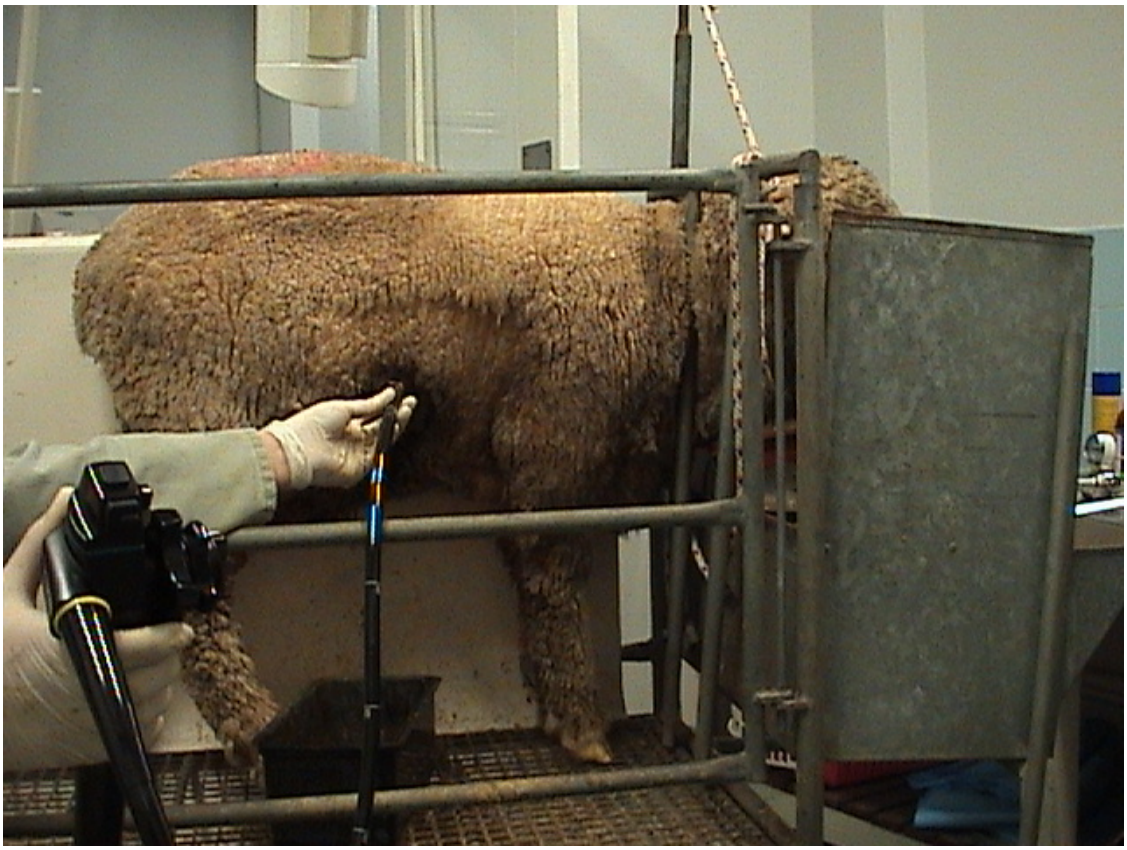
1. Completion of the worm gene expression studies will complement the physical and fecundity data in revealing the effects of the host on worms. This information is vital if we are to manipulate the host-parasite relationship to improve parasite control in the future. Differentially expressed genes should be communicated and evaluated as chemotherapeutic or diagnostic targets (ongoing) for Industry application.
2. Phenotypic correlates should be directly communicated to SG as alternative resistance measures for QTL and fine-mapping studies relevant to the search for resistance/susceptibility markers.
3. Microarray data from sheep samples in this project which are currently being analysed in the SG program should be communicated to the parasitology program(s) within MLA/AWI to maximize integration and utilization of the combined datasets. Blood and faeces collected at the beginning of Experiment 3 and 4, during these trials and at slaughter will become valuable resources to identify proteins/biomarkers associated with resistance to infection once genomic studies have identified target genes.
4. The results should be published.

8 Appendices

8.1 Materials used for abomasal fistula

This photograph shows the body of an abomasal fistula constructed from a Terumo syringe. The open end of the syringe with a curved oval plastic collar was placed inside the abomasum. A new transparent collar can be seen at the top of the picture. The brown discolouration present in the collars of the assembled fistulas developed whilst *in situ*. The pointed end of the syringe barrel was exited through a stab incision in the abdominal wall between the laparotomy wound and the last rib. A large rubber plumbing washer was placed around the exterior portion of the fistula and against the skin to provide protection. A purpose cut perspex collar was placed around the fistula next to the rubber and held in place with a green rubber castration ring. These held the fistula in place during healing enabling adhesions to form between the abomasum and the peritoneum. The washer and perspex collar were removed after one week to facilitate cleaning of the fistula sight. The pointed end of the syringe was cut with a hacksaw and the edges filed smooth. The plunger of the syringe was then inserted into the barrel and cut to leave one centimetre protruding. The barrel and plunger were then drilled eccentrically. A screw was placed through the hole and a washer was used to hold the

screw in place. The plunger prevented spillage of abomasal contents and could be easily removed to access the interior of the abomasum, as shown below.



This picture shows a sheep with an abomasal fistula temporarily constrained inside a metabolism crate during endoscopy.

8.2 Biopsy report from first fistulation approach

VETERINARY PATHOLOGY DIAGNOSTIC SERVICES

Faculty of Veterinary Science, Building B14.
University of Sydney, NSW. 2006.
Telephone 9351 7170

BIOPSY REPORT

Date: 21st January 2005

Clinic: Research

Clinician: Kate McMaster

Our Ref: SN 1096/04 and 1097/04

Animal: Sheep

History: Surgically placed fistula in abomasal wall.

Specimen: Skin and abomasal wall associated with the fistula.

MACROSCOPIC FINDINGS:

Selected by researcher.

MICROSCOPIC FINDINGS:

1096.04 – The sections are composed of tissue covered by abomasal wall (including mucosa, submucosa and muscularis to which is adherent a large area of mature, vascular, fibrous connective tissue. In one section the abomasal mucosa is mildly dysplastic. The fibrous connective tissue is continuous with the dermis of normal skin on the other side of the section. Within this connective tissue there is a moderate multifocal lymphoid inflammatory response and the remains of suture material surrounding which is a granulomatous inflammatory response.

1097.04 – This section is of similar structure to 1096.04 described above. In this section there is ulceration of the abomasal mucosa with a moderate suppurative inflammatory response with occasional eosinophils, lymphocytes and plasma cells present. There is also a mild inflammatory response consisting of lymphocytes, plasma cells and occasional eosinophils surrounding vessels and follicles within the skin.

DIAGNOSIS:

1096.04 Wound repair of the fistula site associated with mild chronic inflammation

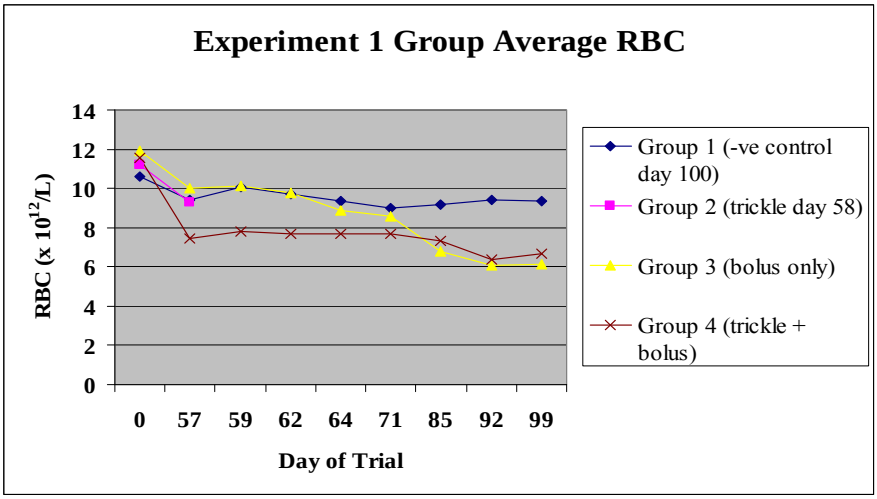
1097.04 Wound repair of the fistula site with associated moderate chonic-active inflammation and abomasal mucosal ulceration at the fistula site.

COMMENT:

There has been good wound repair in 1096.04 with a low grade chronic inflammation associated with the wound. In 1097.04, while there has still been good wound repair however this has been associated with a much more substantial active inflammatory process occurring and ulceration of the abomasal mucosa.

Katrina Bosward BVSc, BSc (Vet), PhD, Dip VCS.

8.3 Group average of RBC count in all animals in each group at time of blood collection



8.4 Statistical analysis of changes in absolute haematological values from day 57 to day 85 in Experiment 1

Change in absolute values from day 57 to day 85

	Group 3 Mean±SE (n=6)	Group 4 Mean±SE (n=6)	Mann Whitney U-test p value
RBC	↓3.22±0.72	↓0.52±0.27	***0.01***
Hb	↓24.83±6.22	↓4.83±2.56	***0.02***
Ht	↓0.08±0.02	↓0.02±0.01	***0.03***
MCV	↑3.50±0.76	↑0.33±0.84	***0.04***
MCH	↑1.33±0.21	↑0.33±0.21	***0.03***
MCHC	↑1.17±2.65	↑1.5±3.35	0.86
Platelets	↓43±81.8	↓168.33±118.97	0.70
WCC	↓0.67±0.55	↓0.98±0.48	0.73
Neut ab	↓0.72±0.34	↓0.57±0.08	0.98
Neut %	↓8.3±1.4%	↓6.2±2.5%	0.68
Lym ab	↓0.02±0.15	↓0.26±0.41	0.56
Lym %	↑5.2±2.4%	↑7.0±3.1%	0.72
Mon ab	0±0.08	↓0.15±0.08	0.26
Mon %	↑1.7±1.2%	↓1.3±1.4%	0.25

8.5 Experiment 1 Mean group haematology values on day 85

Mean group values on d85	Group 1 Mean±SD (n=4)	Group 3 Mean±SD (n=6)	Group 4 Mean±SD (n=6)
RBC	9.15±0.77	6.82±1.21	7.33±0.61
Hb	100.5±6.95	77.83±14.15	85.0±7.38
Ht	0.31±0.03	0.24±0.04	0.27±0.03
MCV	34±1.83	35.83±2.48	36.16±3.19
MCH	11±0	11.5±0.55	11.5±0.84
MCHC	324.75±10.34	319.17±9.58	321.16±9.52
Platelets	556.25±133.41	578±47.73	363.33±256.77
WCC	4.68±1.03	4.68±0.90	4.18±0.86
Neut ab	1.75±0.53	1.53±0.47	1.1±0.19
Neut %	37.5±6.9%	33.5±10.4%	26.3±2.7%
Lym ab	2.48±0.90	2.65±0.93	2.7±0.76
Lym %	52.0±8.8%	55.5±13.2%	63.8±6.2%
Mon ab	0.38±0.24	0.42±0.15	0.3±0.14
Mon %	9.0±7.7%	8.8±3.1%	7.7±3.9%

Significant differences between groups at day 85.

Data	Group comparison	Difference in Means at Day 85	LSD ($p<0.05$)
RBC	1 vs 3	2.33	1.46
RBC	1 vs 4	1.82	1.34
Ht	1 vs 3	0.07	0.05
Ht	1 vs 4	0.04	0.04
MCH	1 vs 3	0.5	0.05
MCH	1 vs 4	0.5	0.04

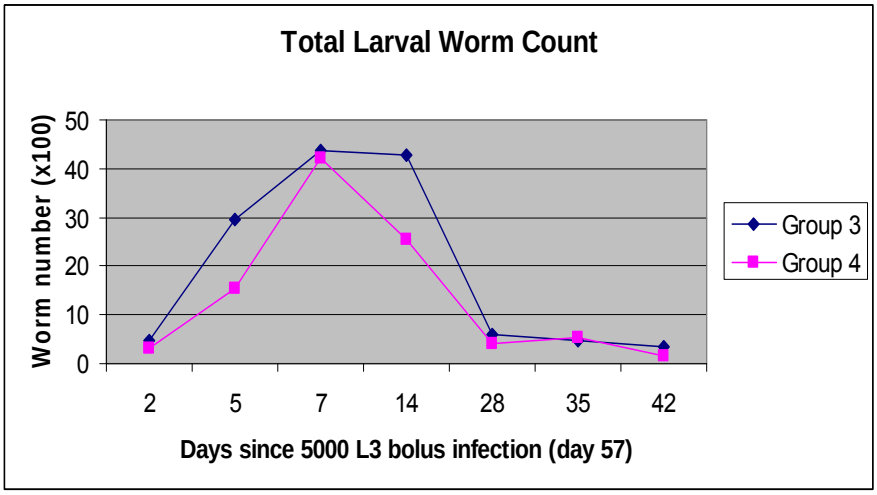
8.6 Experiment 1 Worm counts for groups 3 and 4

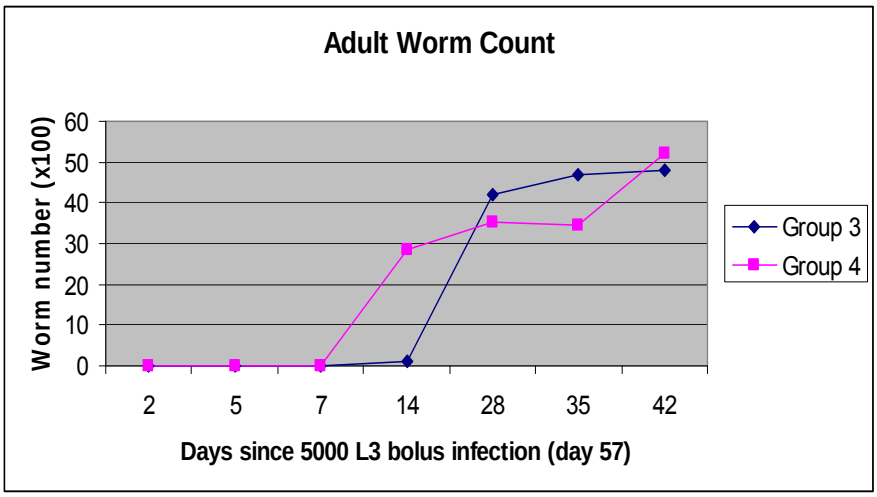
Experiment 1 Group 3

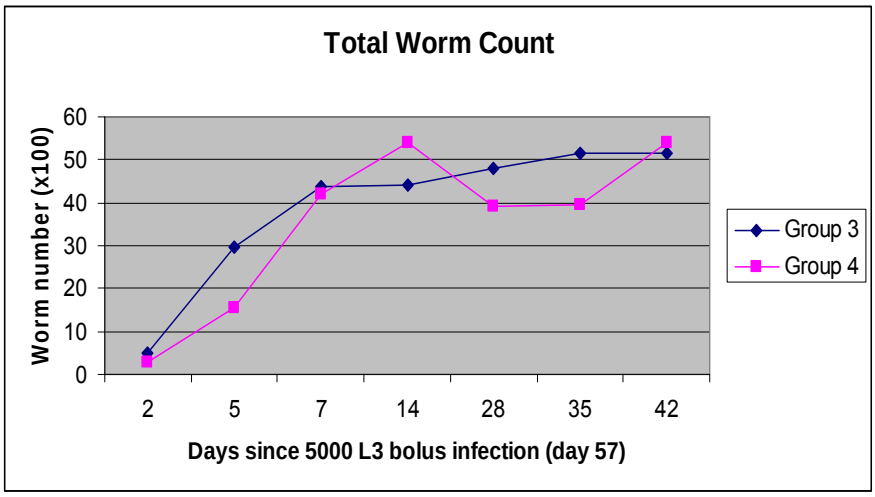
Date	Day of Trial	Days since 5000 L3 bolus infection (day 57)	Total Larval Count (x100)	Adult Count (x100)	Total Worm Count (x100)
16.07.04	59	2	4.8	0.0	4.8
19.07.04	62	5	29.7	0.0	29.7
21.07.04	64	7	43.8	0.0	43.8
28.07.04	71	14	42.8	1.2	44.0
11.08.04	85	28	6.0	41.8	47.8
18.08.04	92	35	4.8	46.8	51.7
25.08.04	99	42	3.5	48.2	51.7

Experiment 1 Group 4

Date	Day of Trial	Days since 5000 L3 bolus infection (day 57)	Total Larval Count (x100)	Adult Count (x100)	Total Worm Count (x100)
16.07.04	59	2	3.0	0.0	3.0
19.07.04	62	5	15.5	0.0	15.5
21.07.04	64	7	42.2	0.0	42.2
28.07.04	71	14	25.5	28.3	53.8
11.08.04	85	28	4.0	35.2	39.2
18.08.04	92	35	5.3	34.3	39.7
25.08.04	99	42	1.7	52.2	53.8


Total Larval Worm Count
Worm number (x100)
0
10
20
30
40
50
2
5
7
14
28
35
42
Days since 5000 L3 bolus infection (day 57)
Group 3
Group 4


Adult Worm Count
Worm number (x100)
0
10
20
30
40
50
60
2
5
7
14
28
35
42
Days since 5000 L3 bolus infection (day 57)
Group 3
Group 4


Total Worm Count
Worm number (x100)
0
10
20
30
40
50
60
2
5
7
14
28
35
42
Days since 5000 L3 bolus infection (day 57)
Group 3
Group 4

8.7 Experiment 1 Spearman correlations between worm area, perimeter and fecundity

Group 3 (bolus only) 60 worms (6 sheep x 10 worms)

	Mean area	Mean perimeter
Mean perimeter	0.82	
Mean fecundity	0.66	0.49

Group 3 95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.71 to 0.89	
Mean fecundity	0.48 to 0.79	0.26 to 0.66

Group 3 p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	P<0.0001	P<0.0001

Group 4 (trickle + bolus) 60 worms (6 sheep x 10 worms)

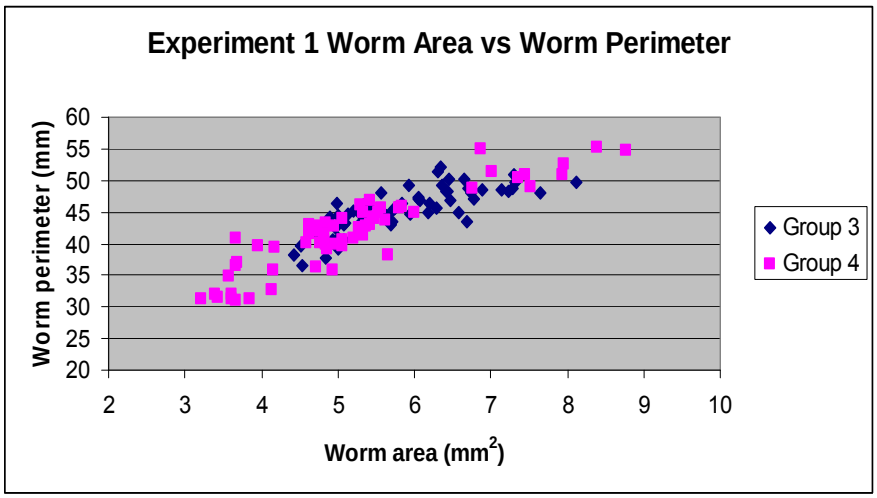
	Mean area	Mean perimeter
Mean perimeter	0.88	
Mean fecundity	0.80	0.78

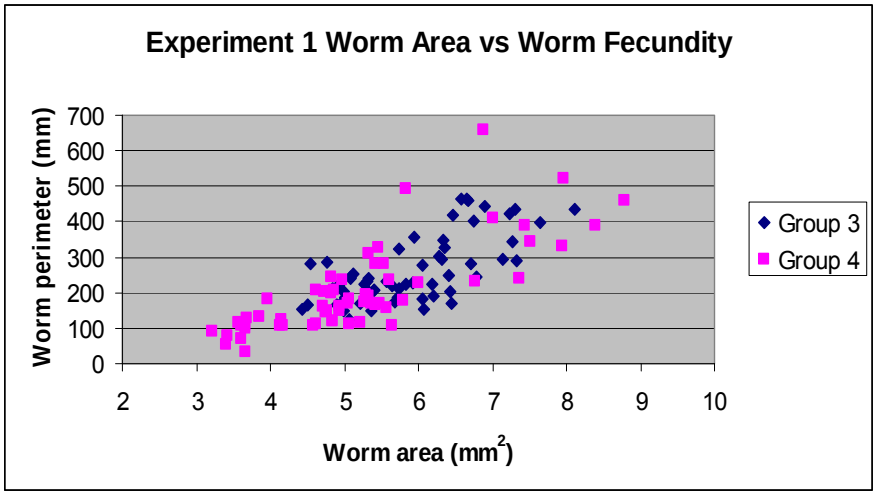
Group 4 95% confidence interval

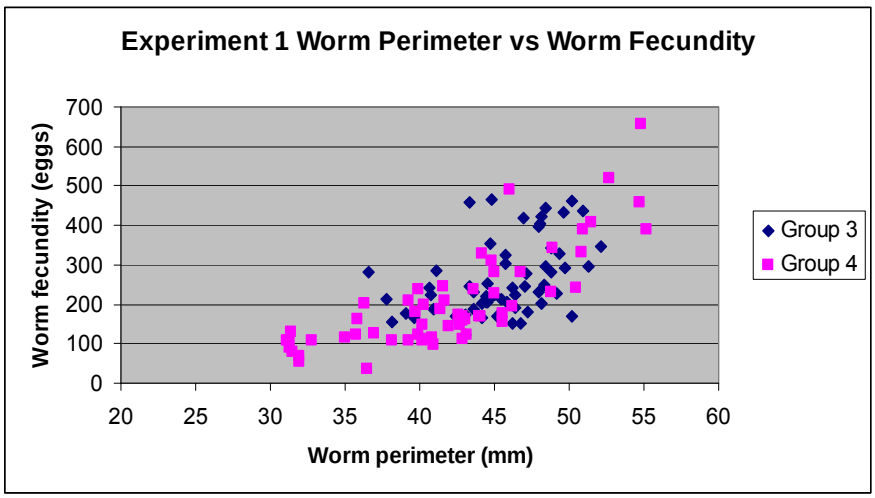
	Mean area	Mean perimeter
Mean perimeter	0.80 to 0.93	
Mean fecundity	0.69 to 0.88	0.66 to 0.87

Group 4 p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	P<0.0001	P<0.0001


Experiment 1 Worm Area vs Worm Perimeter
Worm perimeter (mm)
60
55
50
45
40
35
30
25
20
2
3
4
5
6
7
8
9
10
Worm area (mm2)
Group 3
Group 4


Experiment 1 Worm Area vs Worm Fecundity
Worm perimeter (mm)
700
600
500
400
300
200
100
0
2
3
4
5
6
7
8
9
10
Worm area (mm2)
Group 3
Group 4


Experiment 1 Worm Perimeter vs Worm Fecundity
Worm fecundity (eggs)
700
600
500
400
300
200
100
0
20
25
30
35
40
45
50
55
60
Worm perimeter (mm)
Group 3
Group 4

8.8 Experiment 1 Mean worm values from the final six sheep of groups 3 and 4 with 95% CI

	Overall group mean	95% confidence interval
Group 3 mean worm area	262.42	166.09-358.75
Group 4 mean worm area	204.6	92.64-316.56
Group 3 mean worm perimeter	5.89	5.1-6.68
Group 4 mean worm perimeter	5.21	3.88-6.53
Group 3 mean worm fecundity	45.48	42.84-48.13
Group 4 mean worm fecundity	41.9	35.36-48.44

Nested ANOVA Group 3 vs 4 log 10 transformed worm areas

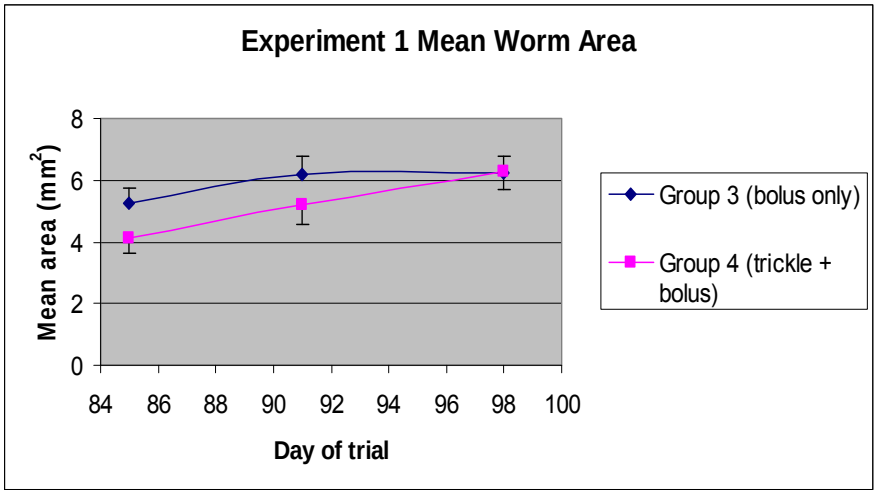
	p-value	% of variance
among groups (3 vs 4)	0.217	8.7
subgroups within groups (sheep)	0.000	69.0
within subgroups (worms)		22.2

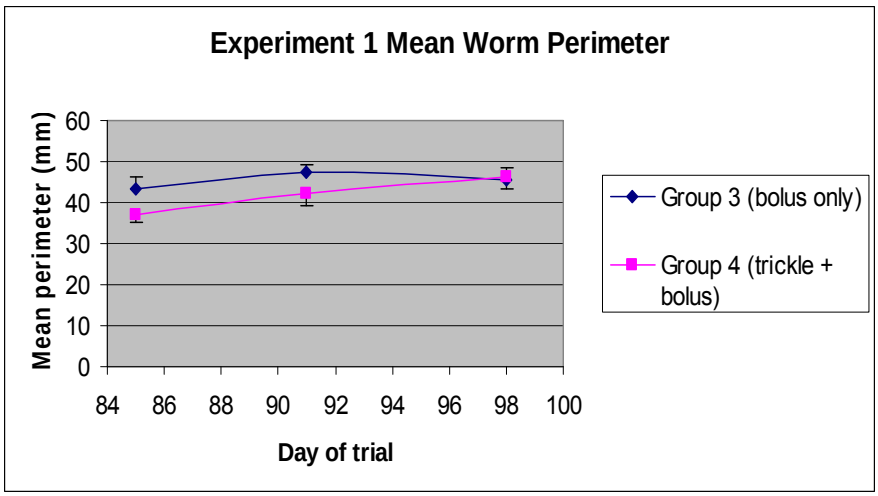
Nested ANOVA Group 3 vs 4 log 10 transformed worm perimeters

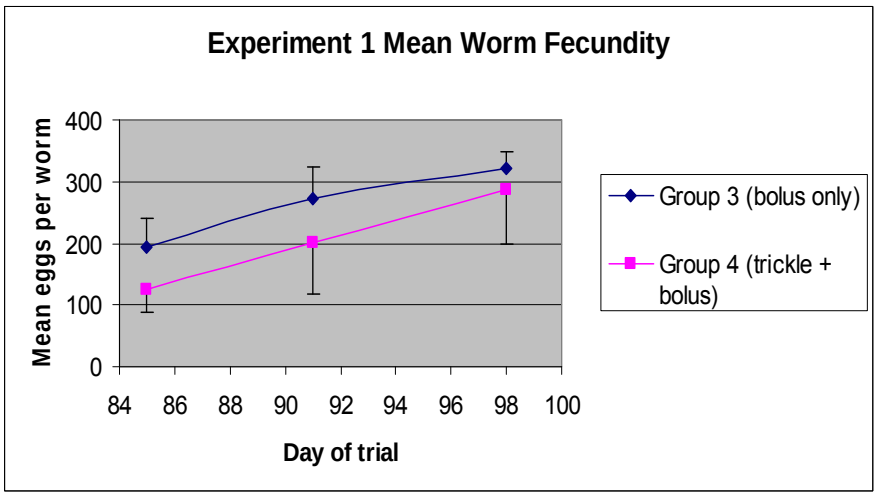
	p-value	% of variance
among groups (3 vs 4)	0.214	9.1
subgroups within groups (sheep)	0.000	69.2
within subgroups (worms)		21.8

Nested ANOVA Group 3 vs 4 log 10 transformed worm fecundity

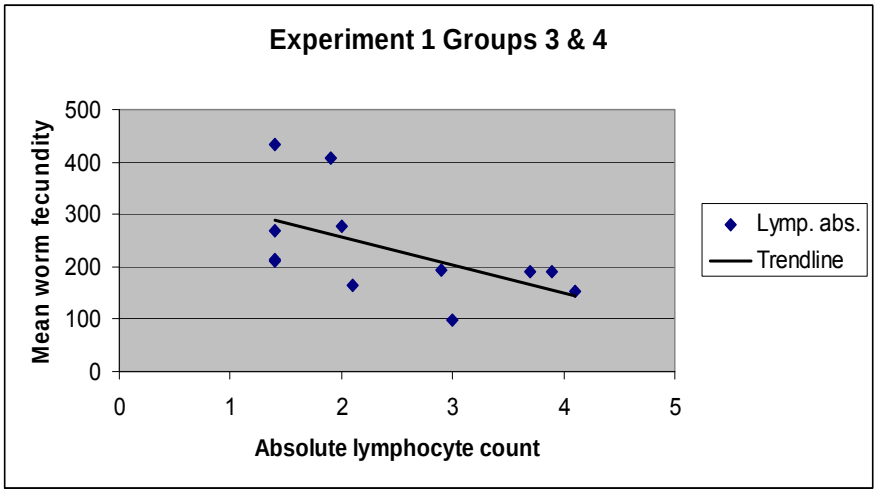
	p-value	% of variance
among groups (3 vs 4)	0.162	12.3
subgroups within groups (sheep)	0.000	54.1
within subgroups (worms)		33.6

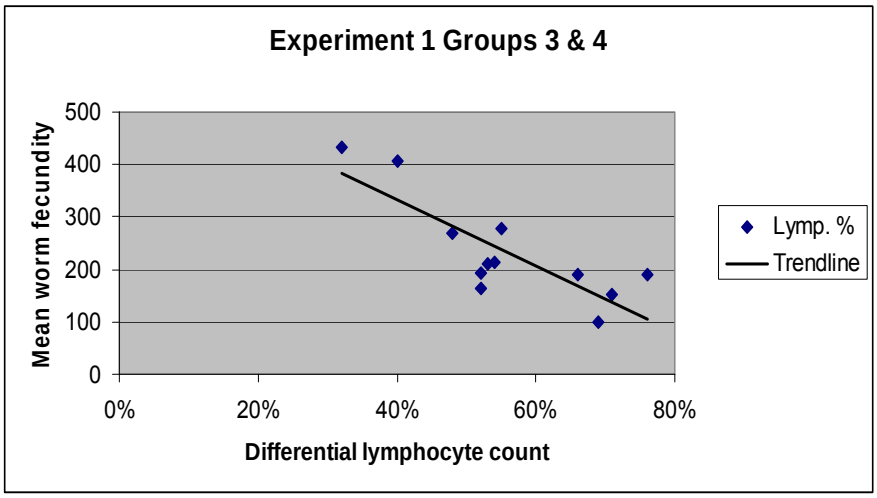

Experiment 1 Mean Worm Area
Mean area (mm2)
0
2
4
6
8
84
86
88
90
92
94
96
98
100
Day of trial
Group 3 (bolus only)
Group 4 (trickle + bolus)


Experiment 1 Mean Worm Perimeter
Mean perimeter (mm)
0
10
20
30
40
50
60
84
86
88
90
92
94
96
98
100
Day of trial
Group 3 (bolus only)
Group 4 (trickle + bolus)


Experiment 1 Mean Worm Fecundity
Mean eggs per worm
0
100
200
300
400
84
86
88
90
92
94
96
98
100
Day of trial
Group 3 (bolus only)
Group 4 (trickle + bolus)

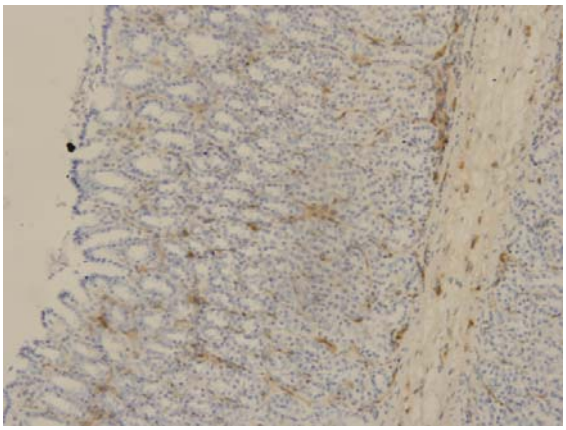
8.9 Experiment 1 Correlations between mean worm fecundity and lymphocyte counts



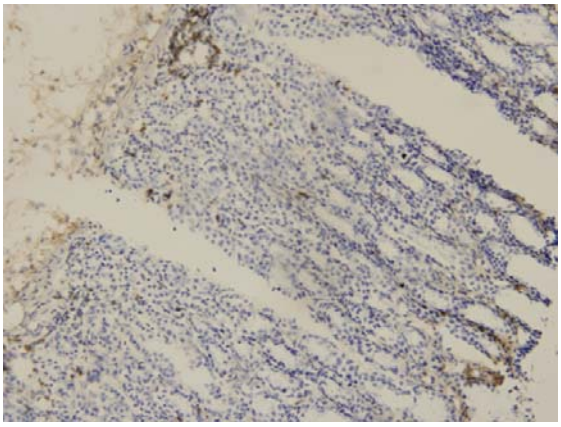


8.10 Experiment 1 IHC staining of abomasal mucosa

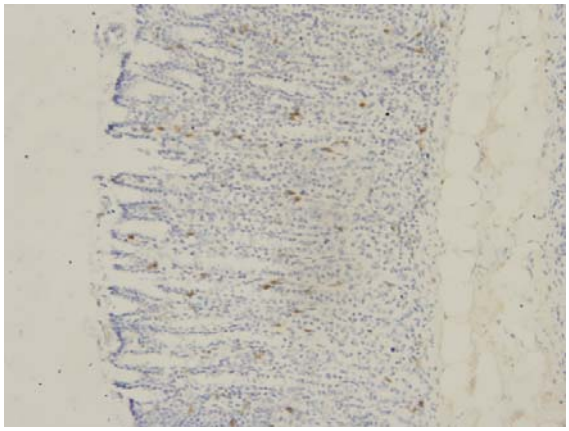
CD1 (Sheep #1520 group 4 pair 4). Cells (stained brown) are scattered throughout the mucosa and muscularis mucosae.



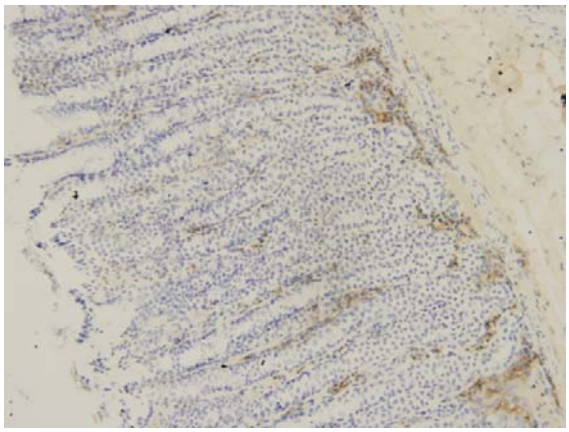
CD4 (Sheep #1632 group 4 pair 7). There are aggregations of cells at the base and tips of the villi with scattered cells throughout the mucosa.



CD8 (Sheep #4366 group 3 pair 3). Cells are distributed evenly in the mucosa.

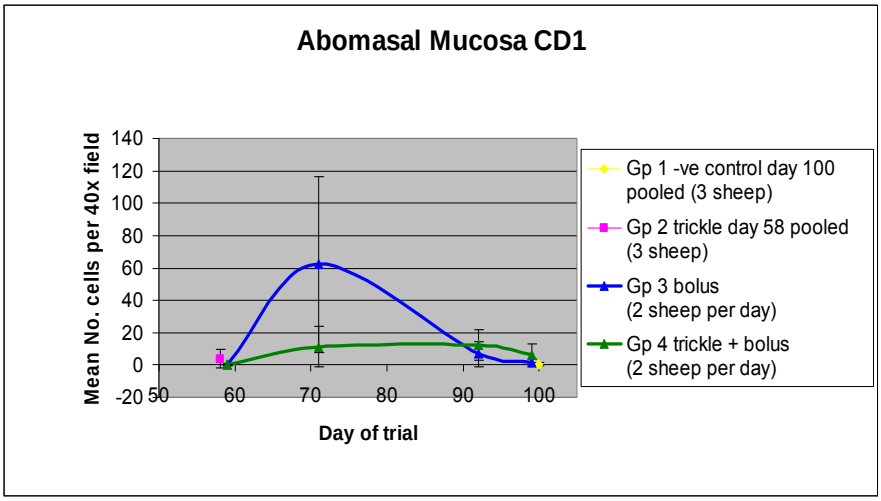


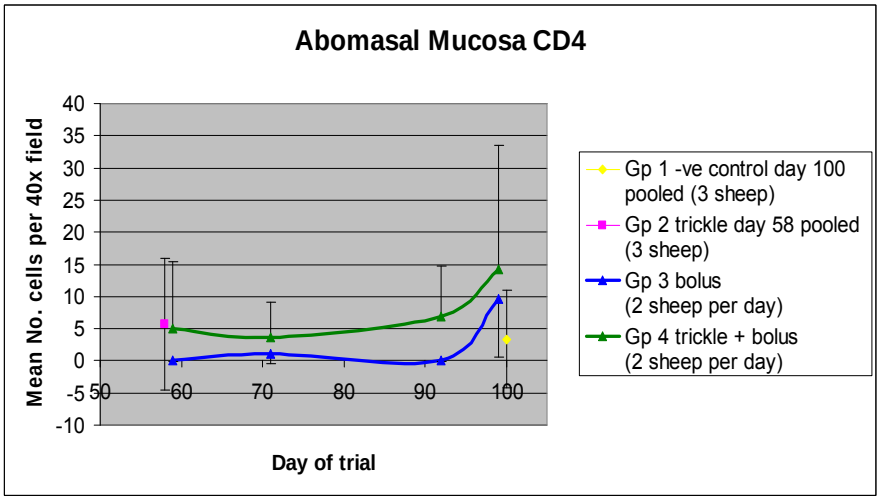
CD45RA (Sheep #1632 group 4 pair 7). Aggregates of cells are present at the base of the villi and lines of cells are present within the mucosa.

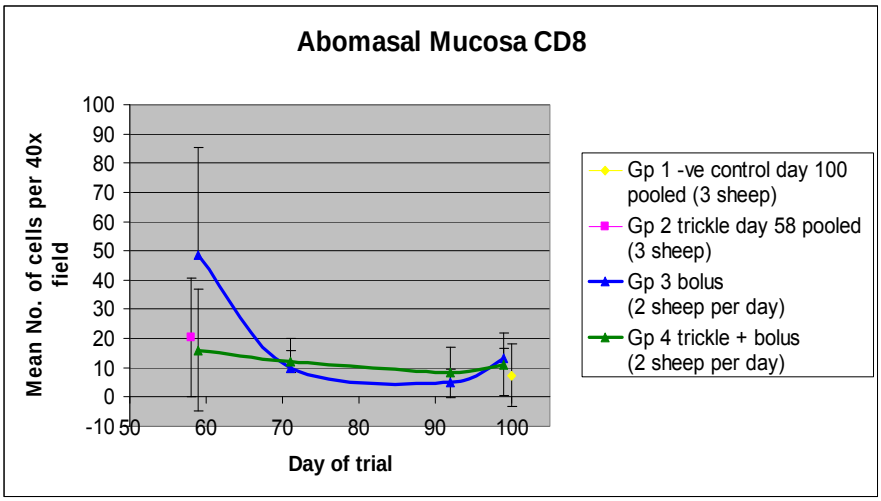


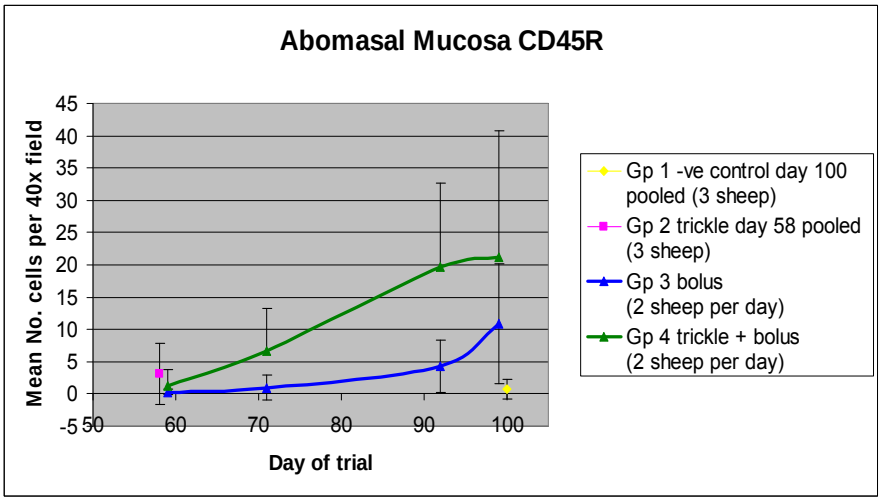
8.11 Experiment 1 IHC results

Bars on graph are standard deviations





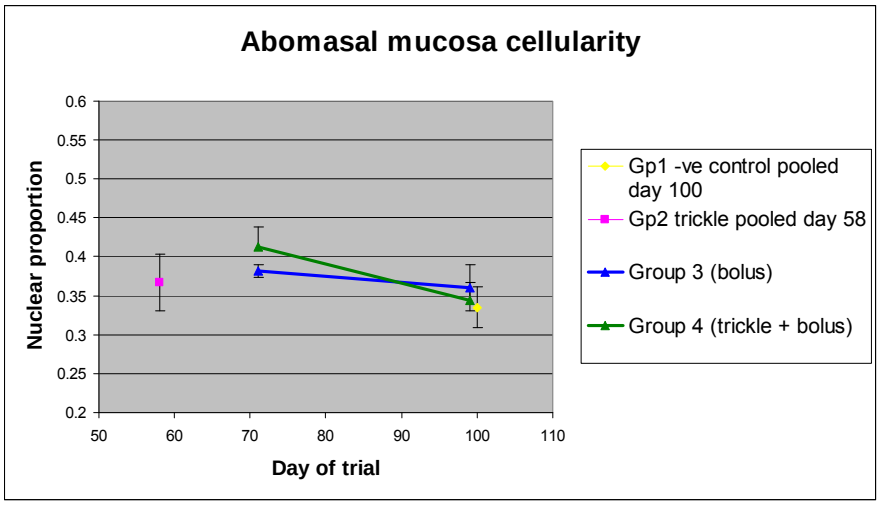

Abomasal Mucosa CD8
Mean No. of cells per 40x field
100
90
80
70
60
50
40
30
20
10
0
-10
50
60
70
80
90
100
Day of trial
Gp 1 -ve control day 100 pooled (3 sheep)
Gp 2 trickle day 58 pooled (3 sheep)
Gp 3 bolus (2 sheep per day)
Gp 4 trickle + bolus (2 sheep per day)

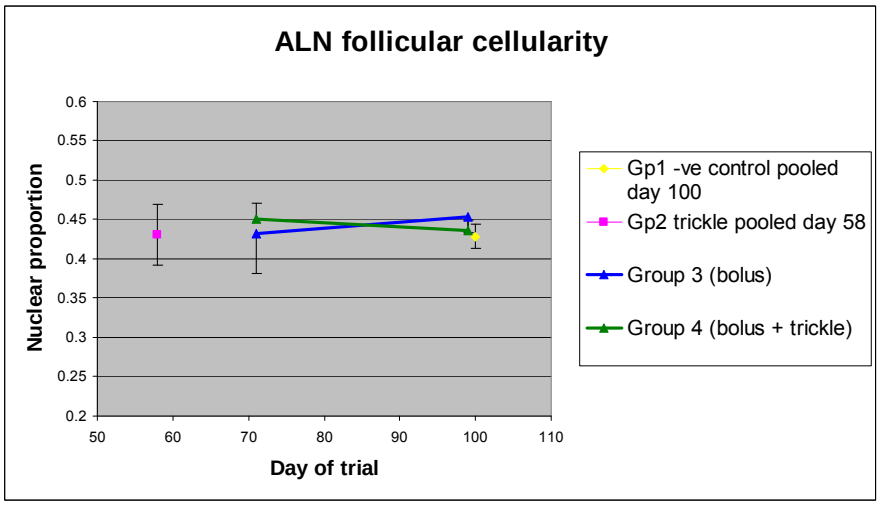

Abomasal Mucosa CD45R
Mean No. cells per 40x field
45
40
35
30
25
20
15
10
5
0
-5
50
60
70
80
90
100
Day of trial
Gp 1 -ve control day 100 pooled (3 sheep)
Gp 2 trickle day 58 pooled (3 sheep)
Gp 3 bolus (2 sheep per day)
Gp 4 trickle + bolus (2 sheep per day)

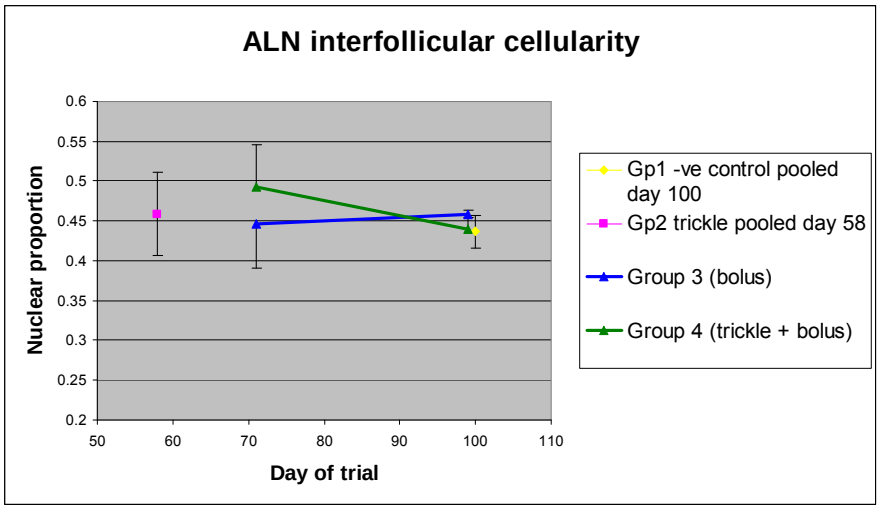
8.12 Experiment 1 Histology total cell counts

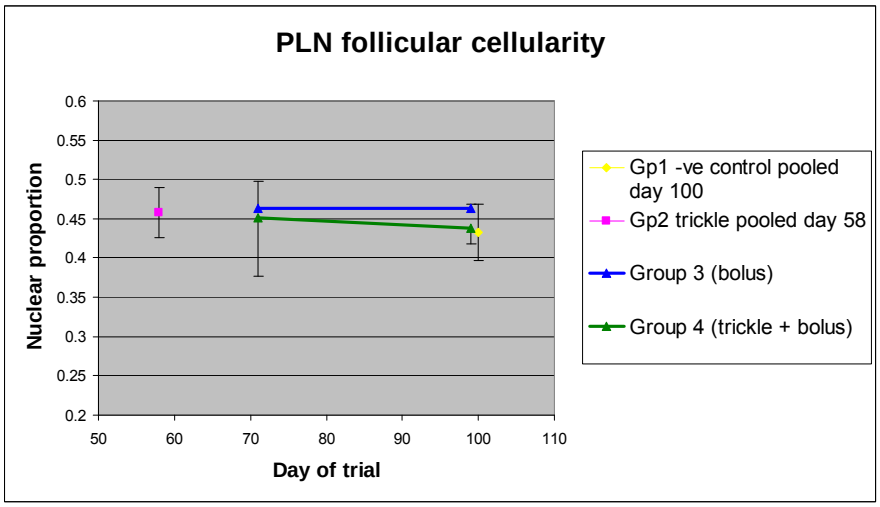
Bars on graph are standard deviations

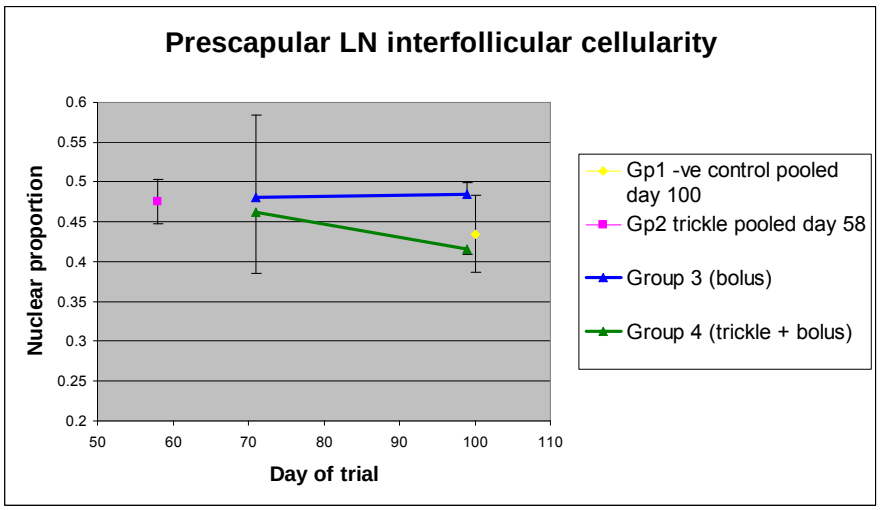
PLN = prescapular lymph node ALN = abomasal lymph node





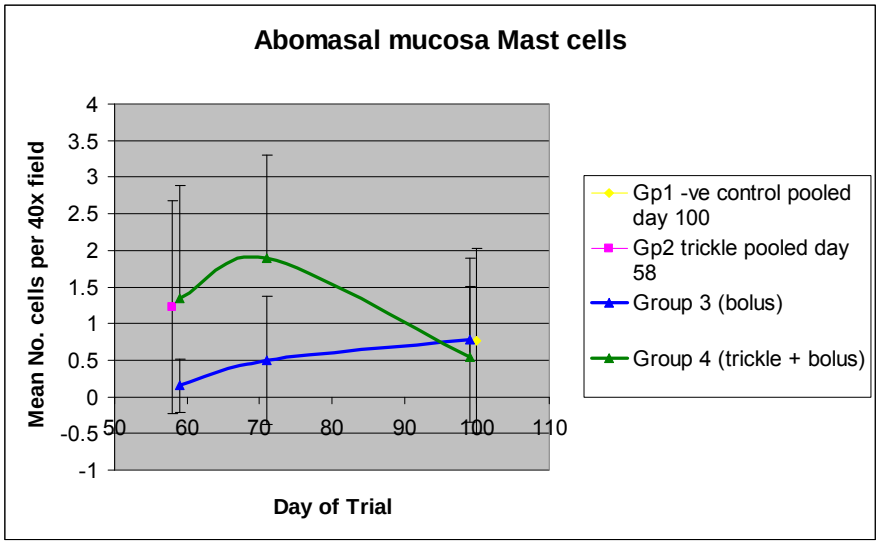

ALN interfollicular cellularity
Nuclear proportion
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
50
60
70
80
90
100
110
Day of trial
Gp1 -ve control pooled day 100
Gp2 trickle pooled day 58
Group 3 (bolus)
Group 4 (trickle + bolus)

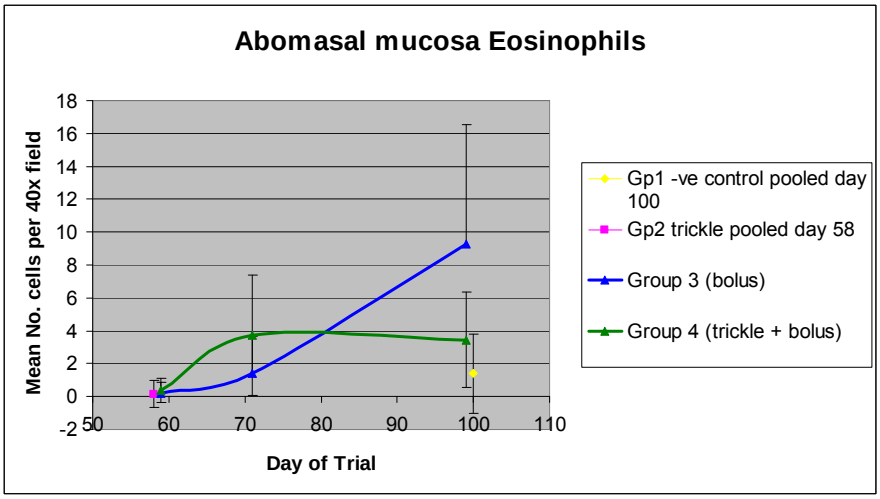

PLN follicular cellularity
Nuclear proportion
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
50
60
70
80
90
100
110
Day of trial
Gp1 -ve control pooled day 100
Gp2 trickle pooled day 58
Group 3 (bolus)
Group 4 (trickle + bolus)


Prescapular LN interfollicular cellularity
Nuclear proportion
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
50
60
70
80
90
100
110
Day of trial
Gp1 -ve control pooled day 100
Gp2 trickle pooled day 58
Group 3 (bolus)
Group 4 (trickle + bolus)

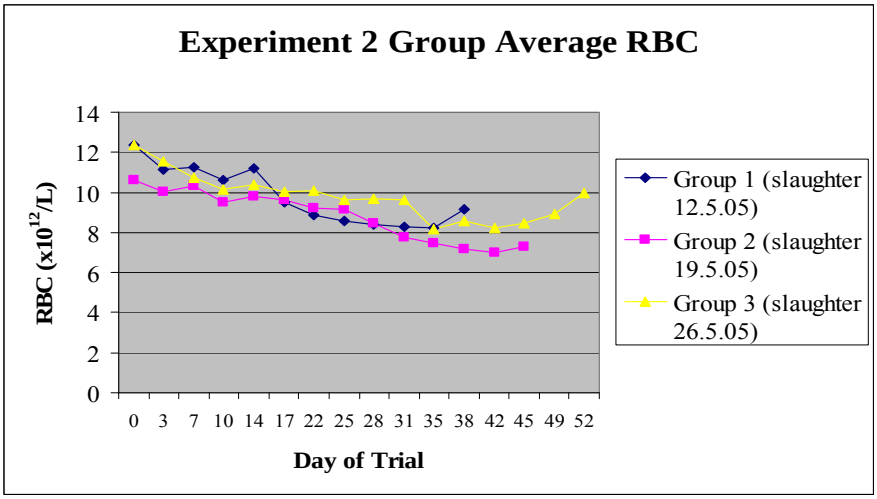
8.13 Experiment 1 Histology counts - mast cells and eosinophils

Bars on graph are standard deviations





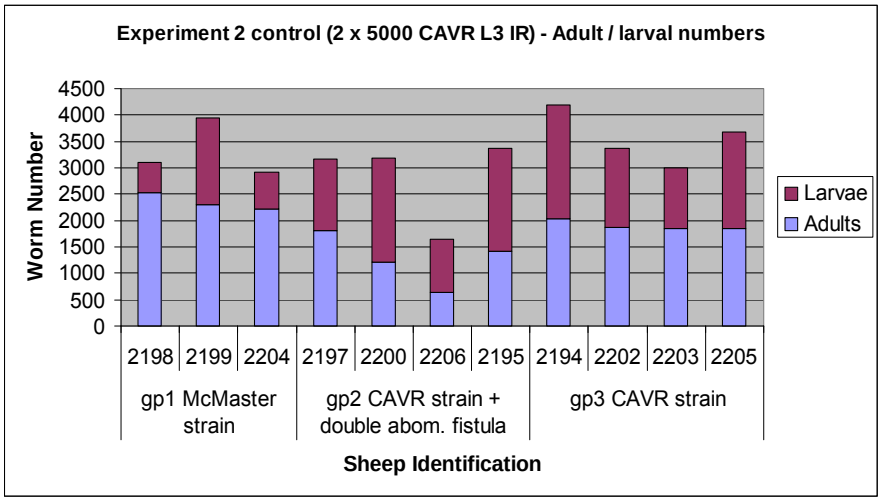
8.14 Experiment 2A Group mean RBC count in all animals in each group at time of blood collection



8.15 Experiment 2A (Control experiment number 1) Ratios of larval to adult worms

Group	1	1	1	2	2	2	2
Number	2198	2199	2204	2197	2200	2206	2195
Adults	2520	2300	2220	1800	1220	640	1420
Larvae	580	1640	700	1360	1960	1000	1940
Ratio	0.23	0.71	0.32	0.76	1.61	1.56	1.37

Group	3	3	3	3
Number	2194	2202	2203	2205
Adults	2040	1880	1840	1840
Larvae	2160	1480	1160	1840
Ratio	1.06	0.79	0.63	1.00



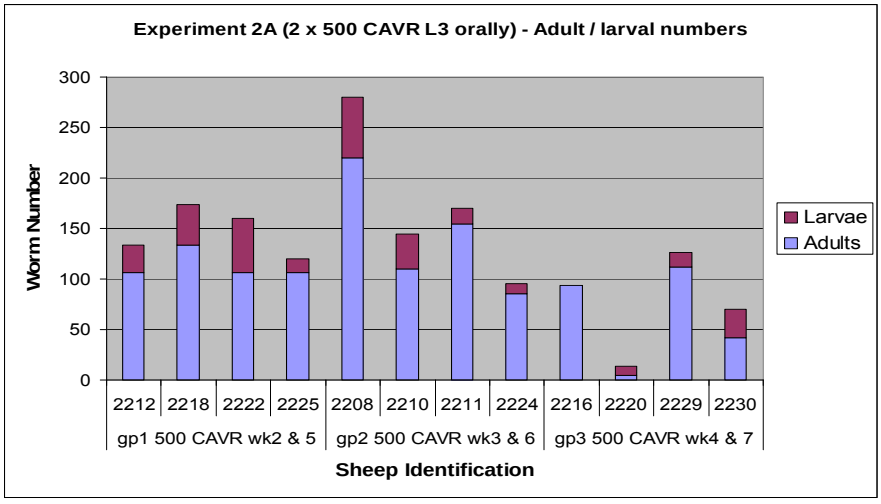
8.16 Experiment 2A (Control experiment number 2) and Experiment 2A Ratios of larval to adult worms

Sheep with ratios in bold were selected for microarray analysis due to inhibition of larval establishment.

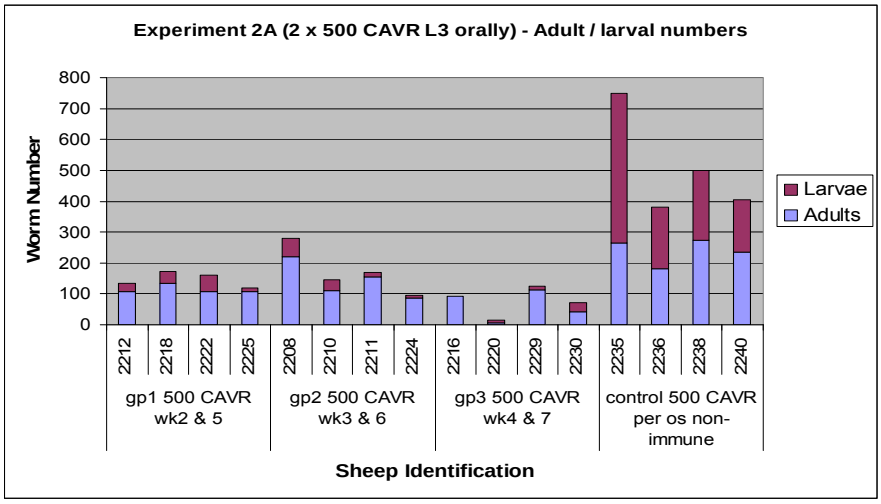
Group	1	1	1	1	2	2	2	2
Number	2212	2218	2222	2225	2208	2210	2211	2224
Adults	107	133	107	107	220	110	155	85
Larvae	27	40	53	13	60	35	15	10
Ratio	**0.25**	0.30	0.50	**0.12**	0.27	0.32	**0.10**	**0.12**

Group	3	3	3	3	Control expt #2	Control expt #2	Control expt #2	Control expt #2
Number	2216	2220	2229	2230	2235	2236	2238	2240
Adults	93	5	112	42	265	180	275	235
Larvae	0	9	14	28	485	200	225	170
Ratio	0.00	1.8	**0.13**	0.67	1.83	1.11	0.82	0.72

Graph showing Experiment 2A.



Graph showing Experiment 2A and Control Experiment 2 together.

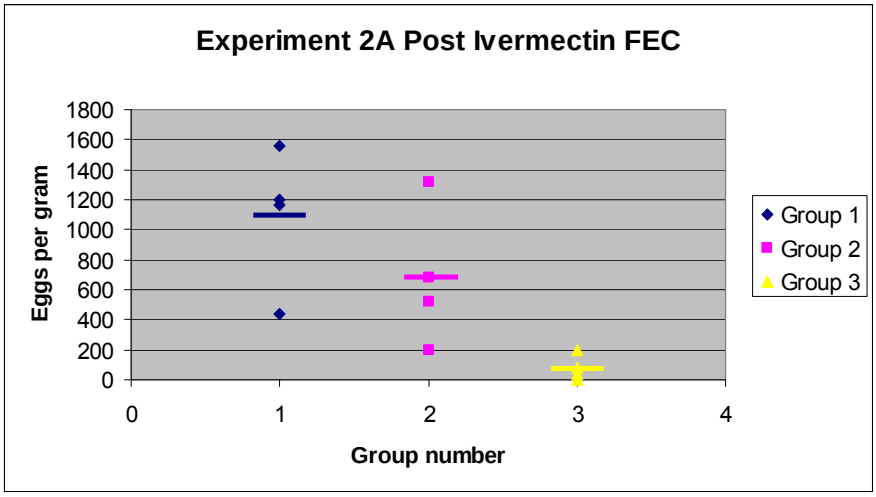


8.17 Experiment 2A Post Ivermectin FEC

	Group 1 (11.5.05)	Group 2 (18.5.05)	Group 3 (25.5.05)
	1560	1320	200
	1160	520	40
	440	680	80
	1200	200	0
Mean	**1090**	**680**	**80**

p-values from group comparisons (Mann-Whitney U tests)

	Group 2	Group 3
Group 1 mean FEC	0.49	***0.03***
Group 2 mean FEC		0.06



8.18 Experiment 2A Overall group means with 95% CI

	Overall group mean	95% confidence interval
Group 1 mean worm area	5.61	6.36-4.87
Group 2 mean worm area	4.99	6.46-3.52
Group 3 mean worm area	3.73	4.60-2.86
Group 1 mean worm perimeter	45.02	49.34-40.69
Group 2 mean worm perimeter	41.59	48.20-34.98
Group 3 mean worm perimeter	35.04	39.96-30.12
Group 1 mean worm fecundity	176.4	241.59-111.11
Group 2 mean worm fecundity	124.3	184.52-64.08
Group 3 mean worm fecundity	76.8	147.75-5.85

Nested ANOVA Groups 1, 2 and 3 log 10 transformed worm areas

	p-value	% of variance
among groups (1 vs 2 vs 3)	0.099	29.5
subgroups within groups (sheep)	0.000	38.2
within subgroups (worms)		32.4

Nested ANOVA Group 1, 2 and 3 log 10 transformed worm perimeters

	p-value	% of variance
among groups (1 vs 2 vs 3)	0.077	30.8
subgroups within groups (sheep)	0.000	31.6
within subgroups (worms)		37.6

Nested ANOVA Group 1, 2 and 3 log 10 transformed worm fecundity

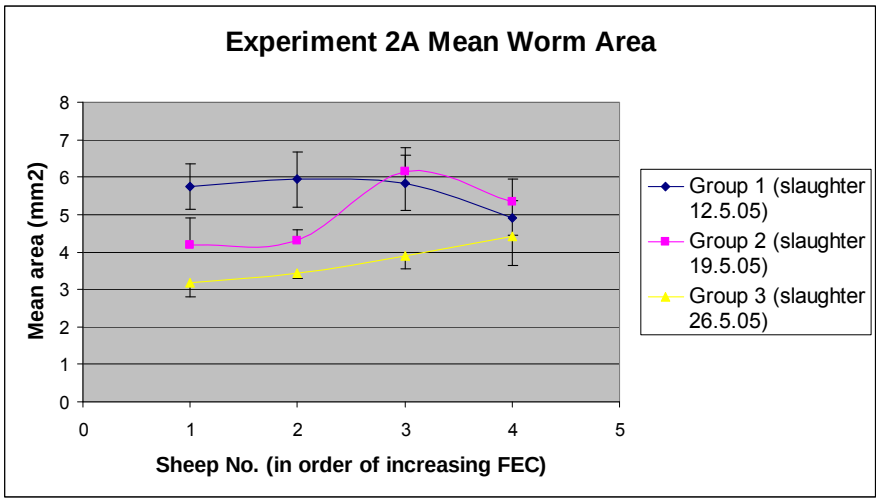
	p-value	% of variance
among groups (1 vs 2 vs 3)	0.140	16.7
subgroups within groups (sheep)	0.000	27.2
within subgroups (worms)		56.1

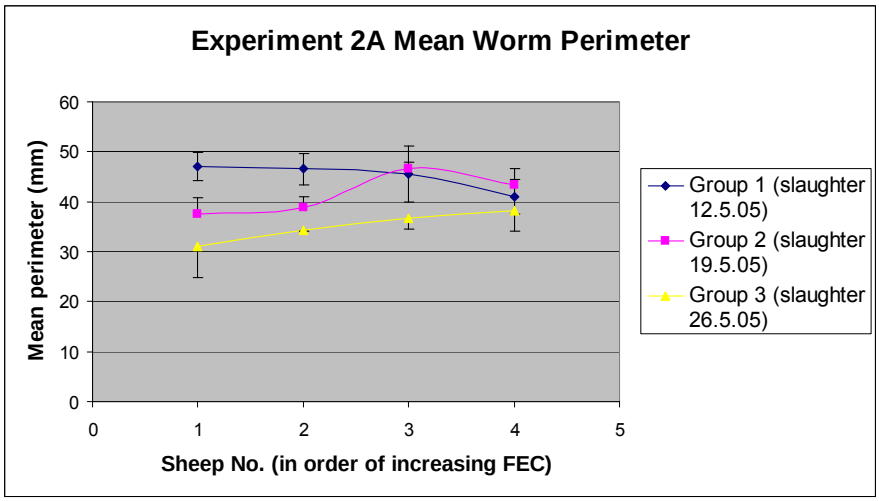
8.19 Experiment 2A Mean worm areas, perimeters and individual fecundity per sheep

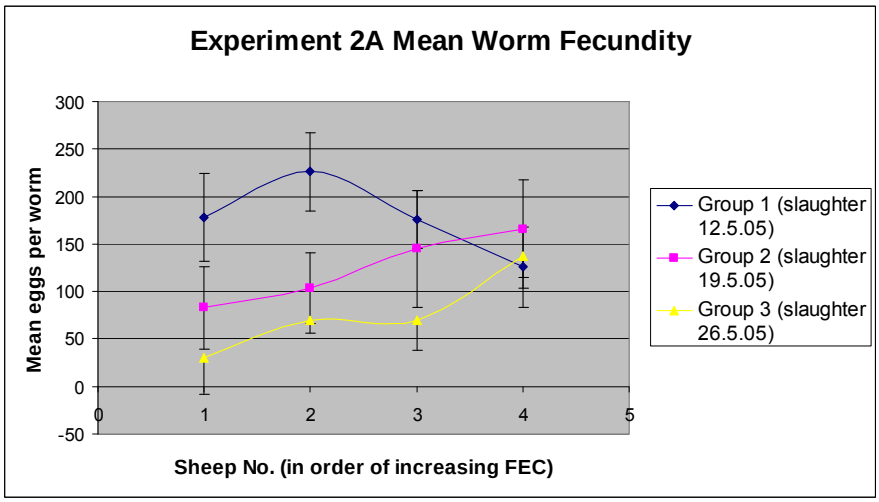
Bars on graph are standard deviations

Slaughter dates;
Group 1 12.5.05
Group 2 19.5.05
Group 3 26.5.05

Group 1	FEC 11.5.05	Group 2	FEC 18.5.05	Group 3	FEC 25.5.05	# on graph
2222	440	2224	200	2230	0	1
2218	1160	2210	520	2220	40	2
2225	1200	2211	680	2229	80	3
2212	1560	2208	1320	2216	200	4




Experiment 2A Mean Worm Perimeter
Mean perimeter (mm)
60
50
40
30
20
10
0
0
1
2
3
4
5
Sheep No. (in order of increasing FEC)
Group 1 (slaughter 12.5.05)
Group 2 (slaughter 19.5.05)
Group 3 (slaughter 26.5.05)


Experiment 2A Mean Worm Fecundity
Mean eggs per worm
300
250
200
150
100
50
0
-50
0
1
2
3
4
5
Sheep No. (in order of increasing FEC)
Group 1 (slaughter 12.5.05)
Group 2 (slaughter 19.5.05)
Group 3 (slaughter 26.5.05)

8.20 Experiment 2A Spearman correlations between worm area, perimeter and fecundity

Group 1 (slaughter date 12.5.05) 40 worms (4 sheep x 10 worms)

	Mean area	Mean perimeter
Mean perimeter	0.92	
Mean fecundity	0.52	0.42

95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.85 to 0.96	
Mean fecundity	0.24 to 0.72	0.12 to 0.65

p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	0.0005	0.0070

Group 2 (slaughter date 19.5.05) 40 worms (4 sheep x 10 worms)

	Mean area	Mean perimeter
Mean perimeter	0.94	
Mean fecundity	0.53	0.55

95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.89 to 0.97	
Mean fecundity	0.25 to 0.73	0.28 to 0.74

p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	0.0004	0.0002

Group 3 (slaughter date 26.5.05) 28 worms (4 sheep x 10 worms – 12 worms)

	Mean area	Mean perimeter
Mean perimeter	0.85	
Mean fecundity	0.65	0.49

95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.69 to 0.93	
Mean fecundity	0.36 to 0.83	0.14 to 0.74

p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	0.0002	0.0074

8.21 Experiment 2B Overall group means with 95% CI

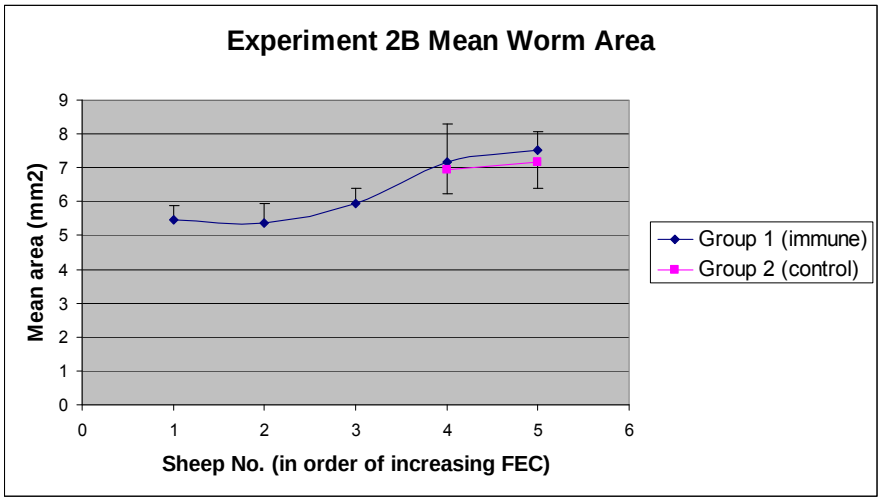
	Overall group mean	95% confidence interval
Group 1 mean worm area	6.30	7.53-5.07
Group 2 mean worm area	7.07	8.50-5.64
Group 1 mean worm perimeter	48.39	52.97-43.80
Group 2 mean worm perimeter	52.48	52.83-52.12
Group 1 mean worm fecundity	296.14	432.27-160.01
Group 2 mean worm fecundity	318.40	578.88-57.92

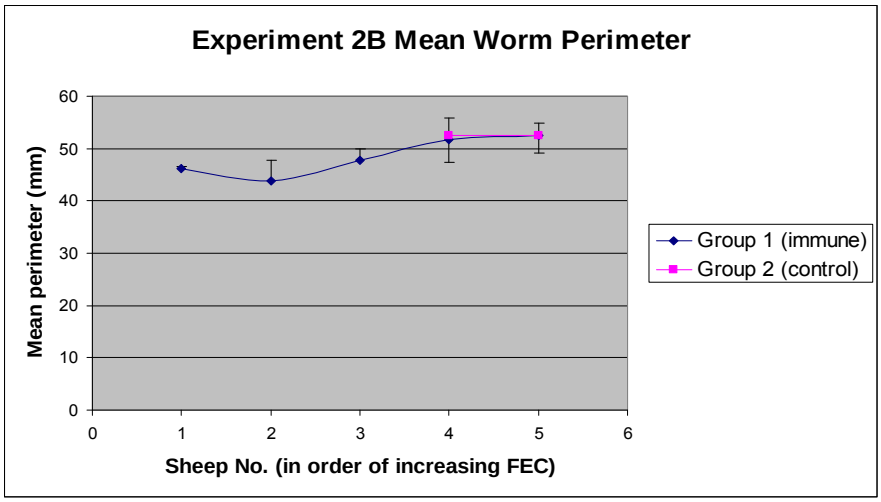
8.22 Experiment 2B Mean worm areas, perimeters and individual fecundity per sheep

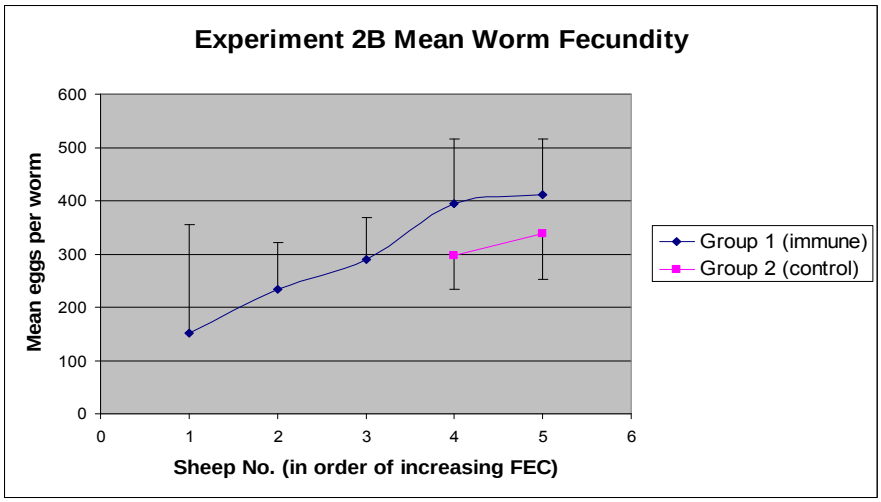
Bars on graph are standard deviations

Slaughter date 27.7.05

Group 1	FEC 27.7.05	Group 2	FEC 27.7.05	# on graph
2213	640			1
2221	4520			2
2209	6800			3
2228	10280	2231	13640	4
2217	16760	2232	15440	5




Experiment 2B Mean Worm Perimeter
Mean perimeter (mm)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Sheep No. (in order of increasing FEC)
Group 1 (immune)
Group 2 (control)


Experiment 2B Mean Worm Fecundity
Mean eggs per worm
600
500
400
300
200
100
0
0
1
2
3
4
5
6
Sheep No. (in order of increasing FEC)
Group 1 (immune)
Group 2 (control)

8.23 Experiment 2B Spearman correlations between worm area, perimeter and fecundity

Group 2B (sheep #2209, 2217, 2221, 2228) 38 worms

	Mean area	Mean perimeter
Mean perimeter	0.84	
Mean fecundity	0.62	0.62

Group 2B 95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.71 to 0.92	
Mean fecundity	0.37 to 0.79	0.37 to 0.79

Group 2B p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	P<0.0001	P<0.0001

8.24 Experiment 3 Mean worm values with 95% CI

	Overall group mean	95% confidence interval
Group 5 mean worm area	5.45	4.91-6.00
Group 6 mean worm area	6.34	5.06-7.62
Group 5 mean worm perimeter	41.56	39.73-43.39
Group 6 mean worm perimeter	44.37	40.09-48.64
Group 5 mean worm fecundity	594.14	497.23-691.04
Group 6 mean worm fecundity	719.33	331.24-1107.43

Nested ANOVA Group 5 vs 6 log 10 transformed worm areas

	p-value	% of variance
among groups (5 vs 6)	0.143	19.1
subgroups within groups (sheep)	0.000	56.6
within subgroups (worms)		24.2

Nested ANOVA Group 5 vs 6 log 10 transformed worm perimeters

	p-value	% of variance
among groups (5 vs 6)	0.144	16.3
subgroups within groups (sheep)	0.000	47.7
within subgroups (worms)		36.1

Nested ANOVA Group 5 vs 6 log 10 transformed worm fecundity

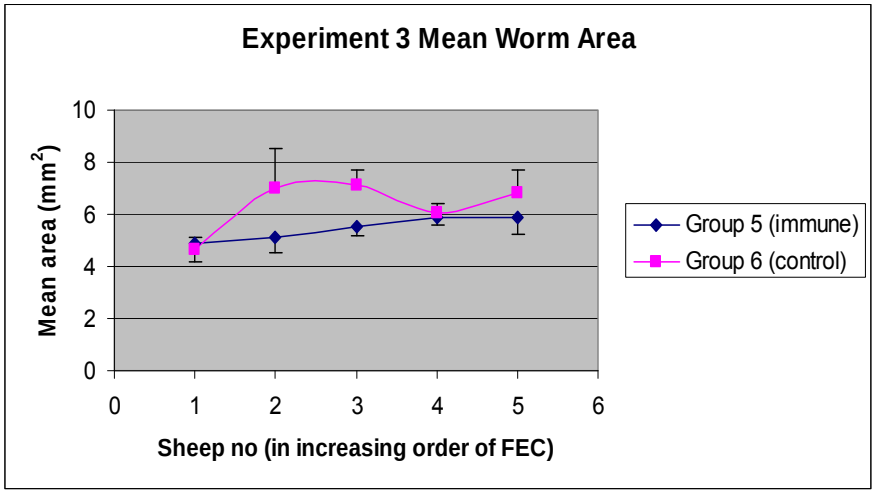
	p-value	% of variance (approx)
among groups (5 vs 6)	0.764	0.2
subgroups within groups (sheep)	0.000	70.0
within subgroups (worms)		29.8

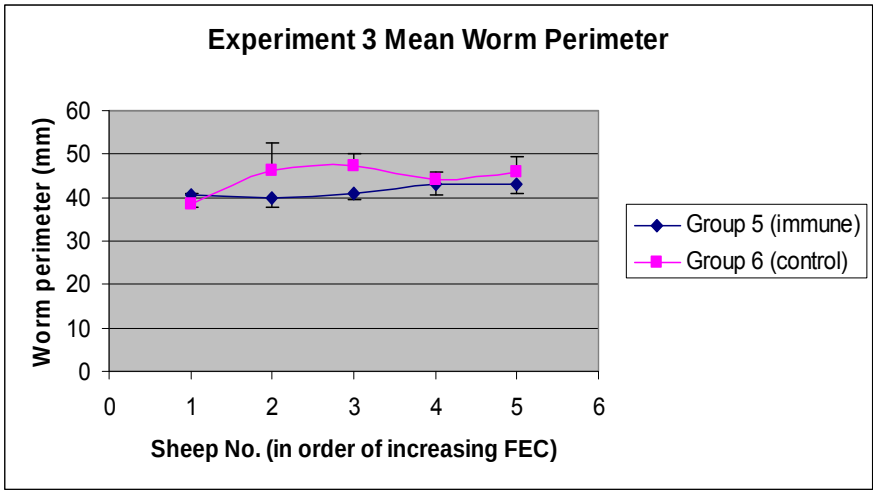
8.25 Experiment 3 Mean worm areas, perimeters and individual fecundity per sheep

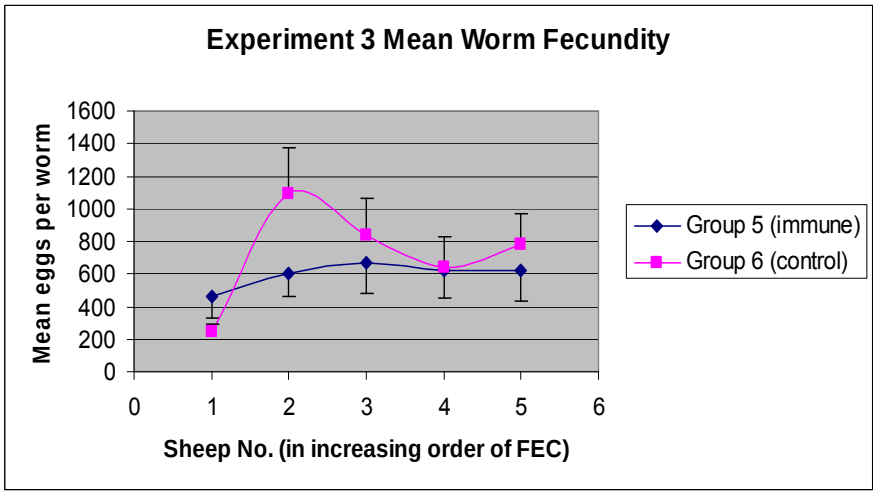
Bars on graphs are standard deviations

Slaughter date 13.7.06

Group 5	FEC 10.7.06	Group 6	FEC 10.7.06	# on graph
V 3434	2760	V 0910	3880	1
V 1275	6000	V 2932	7520	2
V 1516	7640	V 1099	13320	3
V 1341	10000	V 0333	16000	4
V 3225	11880	V 0669	22760	5




Experiment 3 Mean Worm Perimeter
Worm perimeter (mm)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Sheep No. (in order of increasing FEC)
Group 5 (immune)
Group 6 (control)


Experiment 3 Mean Worm Fecundity
Mean eggs per worm
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
Sheep No. (in increasing order of FEC)
Group 5 (immune)
Group 6 (control)

8.26 Experiment 3 Spearman correlations between worm area, perimeter and fecundity

Group 5 (immune) 73 worms (5 sheep x 15 worms – 2 worms)

	Mean area	Mean perimeter
Mean perimeter	0.85	
Mean fecundity	0.54	0.40

95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.77 to 0.91	
Mean fecundity	0.34 to 0.69	0.18 to 0.58

p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	P<0.0001	0.0005

Group 6 (non-immune) 73 worms (5 sheep x 15 worms – 2 worms)

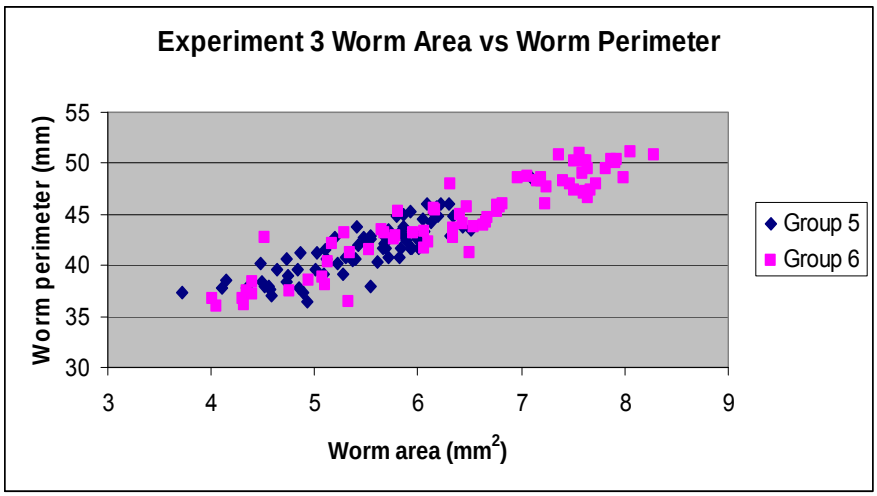
	Mean area	Mean perimeter
Mean perimeter	0.92	
Mean fecundity	0.85	0.78

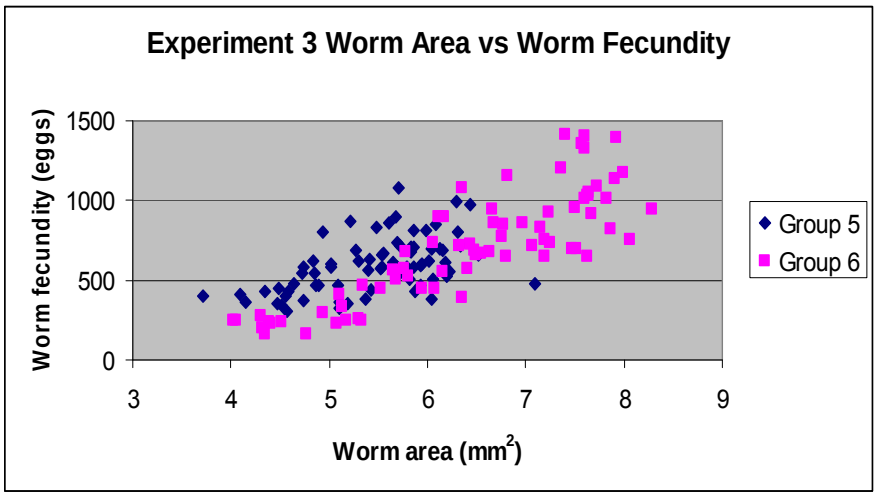
95% confidence interval

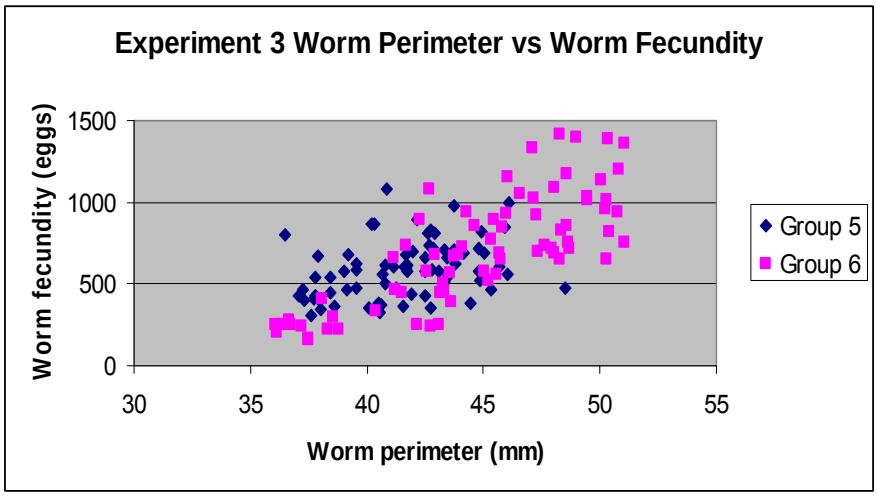
	Mean area	Mean perimeter
Mean perimeter	0.87 to 0.95	
Mean fecundity	0.76 to 0.90	0.66 to 0.86

p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	P<0.0001	P<0.0001


Experiment 3 Worm Area vs Worm Perimeter
Worm perimeter (mm)
55
50
45
40
35
30
3
4
5
6
7
8
9
Worm area (mm2)
Group 5
Group 6


Experiment 3 Worm Area vs Worm Fecundity
Worm fecundity (eggs)
1500
1000
500
0
3
4
5
6
7
8
9
Worm area (mm2)
Group 5
Group 6


Experiment 3 Worm Perimeter vs Worm Fecundity
Worm fecundity (eggs)
1500
1000
500
0
30
35
40
45
50
55
Worm perimeter (mm)
Group 5
Group 6

8.27 Experiment 4 Mean FEC with 95% CI

	Overall group mean	95% confidence interval
Group 1 mean FEC	21680	16980-26380
Group 2 mean FEC	14947	10779-19115
Group 3 mean FEC	10627	6278-14975

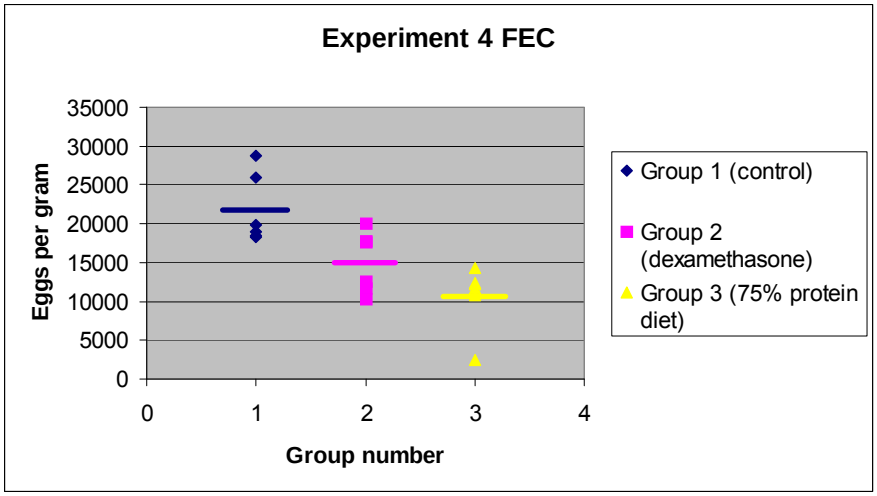
p-values from group comparisons (Mann-Whitney U tests)

	Group 2	Group 3
Group 1 mean FEC	***0.03***	***0.002***
Group 2 mean FEC		0.18

p-values from group comparisons (REML)

	Difference in Means	LSD (p<0.05)	Sig Diff
Group 1 vs 2	6733	5289	Yes
Group 2 vs 3	4320	5289	No
Group 1 vs 3	12720	5289	Yes

FEC 6.11.06 (Slaughter date)

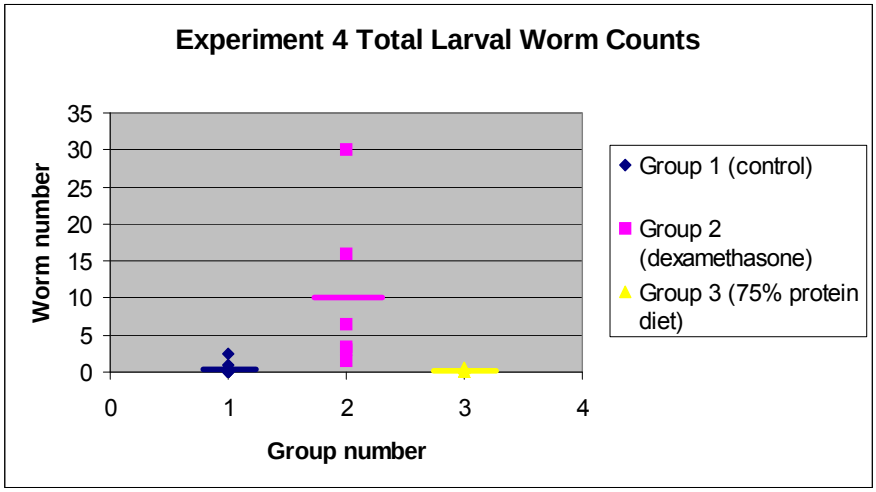


8.28 Experiment 4 Mean larval worm counts with 95% CI

	Overall group mean	95% confidence interval
Group 1 mean larval worms	0.7	0-2.4
Group 2 mean larval worms	10.1	0-31.8
Group 3 mean larval worms	0.2	0-0.6

p-values from group comparisons (Mann-Whitney U tests)

	Group 2	Group 3
Group 1 mean larval worms	***0.004***	0.47
Group 2 mean larval worms		***0.002***

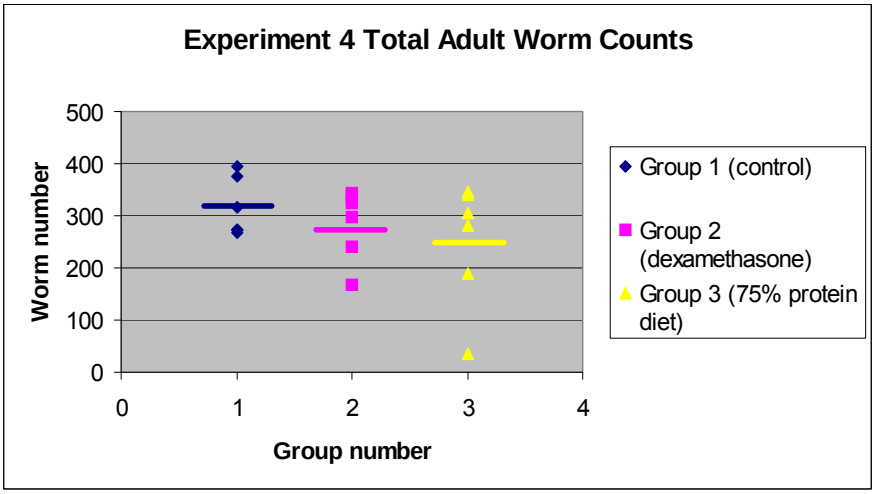


8.29 Experiment 4 Mean total adult worm counts with 95% CI

	Overall group mean	95% confidence interval
Group 1 mean adult worms	317	258-375
Group 2 mean adult worms	285	213-357
Group 3 mean adult worms	250	125-375

p-values from group comparisons (Mann-Whitney U tests)

	Group 2	Group 3
Group 1 mean adult worms	0.70	0.59
Group 2 mean adult worms		0.94



8.30 Experiment 4 Mean worm values with 95% CI

	Overall group mean	95% confidence interval
Group 1 mean worm area	7.72	7.18-8.27
Group 2 mean worm area	7.86	7.58-8.13
Group 3 mean worm area	7.24	6.31-8.17
Group 1 mean worm perimeter	54.34	52.56-56.12
Group 2 mean worm perimeter	56.25	55.50-57.00
Group 3 mean worm perimeter	52.97	49.06-56.82
Group 1 mean worm fecundity	1252.05	1018.05-1486.05
Group 2 mean worm fecundity	1211.38	1076.97-1345.80
Group 3 mean worm fecundity	1084.60	850.77-1318.43

Nested ANOVA Groups 1, 2 and 3 log 10 transformed worm areas

	p-value	% of variance
among groups (1 vs 2 vs 3)	0.206	6.5
subgroups within groups (sheep)	0.000	49.9
within subgroups (worms)		43.6

Nested ANOVA Group 1, 2 and 3 log 10 transformed worm perimeters

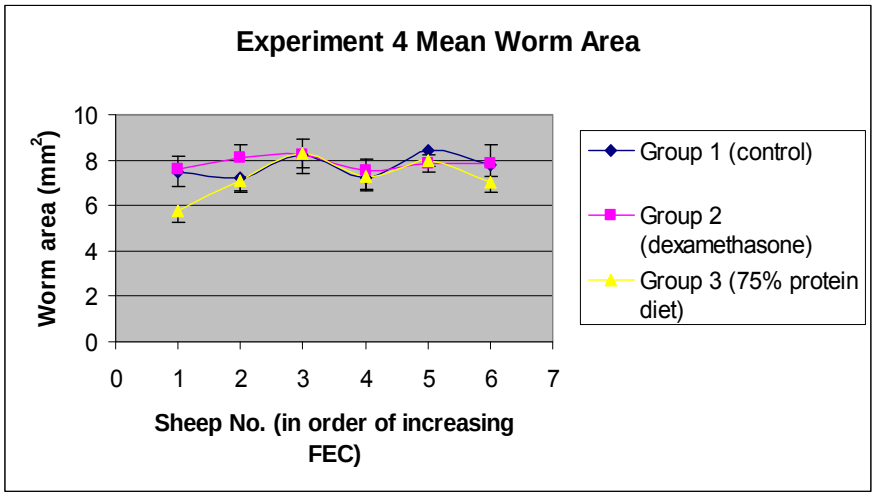
	p-value	% of variance
among groups (1 vs 2 vs 3)	0.069	14.0
subgroups within groups (sheep)	0.000	36.3
within subgroups (worms)		49.7

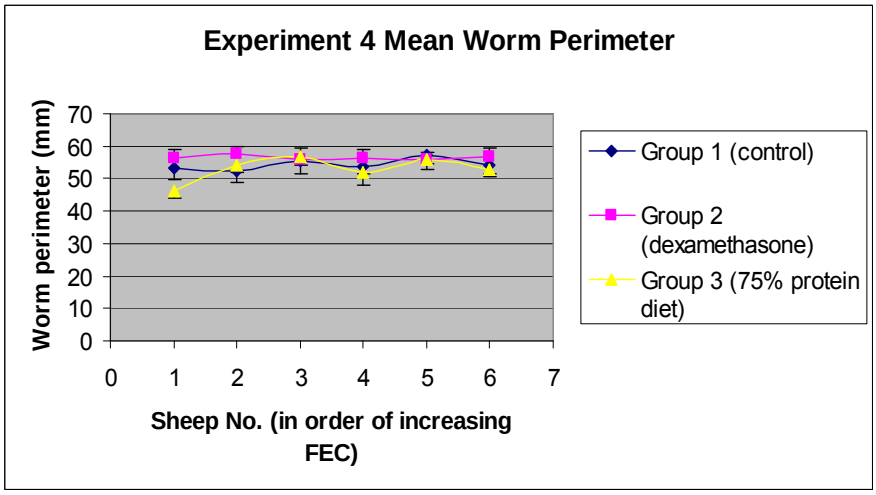
Nested ANOVA Group 1, 2 and 3 log 10 transformed worm fecundity

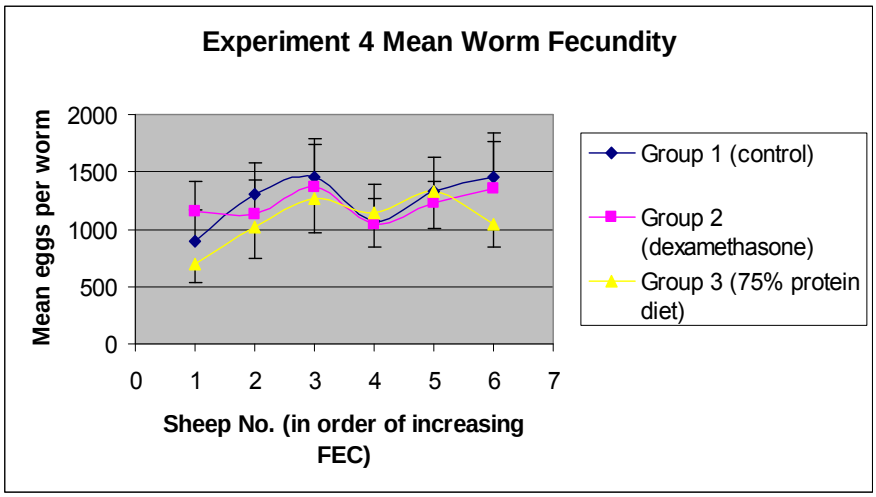
	p-value	% of variance
among groups (1 vs 2 vs 3)	0.371	0.4
subgroups within groups (sheep)	0.000	33.9
within subgroups (worms)		65.7

8.31 Experiment 4 Mean worm areas, perimeters and individual fecundity per sheep

Bars on graphs are standard deviations






Experiment 4 Mean Worm Fecundity
Mean eggs per worm
2000
1500
1000
500
0
0
1
2
3
4
5
6
7
Sheep No. (in order of increasing FEC)
Group 1 (control)
Group 2 (dexamethasone)
Group 3 (75% protein diet)

8.32 Experiment 4 Spearman correlations between worm area, perimeter and fecundity

Group 1 (control) 180 worms (6 sheep x 2 replicates x 15 worms per replicate)

	Mean area	Mean perimeter
Mean perimeter	0.85	
Mean fecundity	0.50	0.34

95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.80 to 0.89	
Mean fecundity	0.37 to 0.60	0.20 to 0.47

p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	P<0.0001	P<0.0001

Group 2 (corticosteroids) 180 worms (6 sheep x 2 replicates x 15 worms per replicate)

	Mean area	Mean perimeter
Mean perimeter	0.68	
Mean fecundity	0.51	0.25

95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.59 to 0.75	
Mean fecundity	0.39 to 0.61	0.11 to 0.39

p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	P<0.0001	0.0006

Group 3 (75% protein) 180 worms (6 sheep x 2 replicates x 15 worms per replicate)

	Mean area	Mean perimeter
Mean perimeter	0.88	
Mean fecundity	0.67	0.52

95% confidence interval

	Mean area	Mean perimeter
Mean perimeter	0.84 to 0.91	
Mean fecundity	0.58 to 0.74	0.40 to 0.62

p-values

	Mean area	Mean perimeter
Mean perimeter	P<0.0001	
Mean fecundity	P<0.0001	P<0.0001

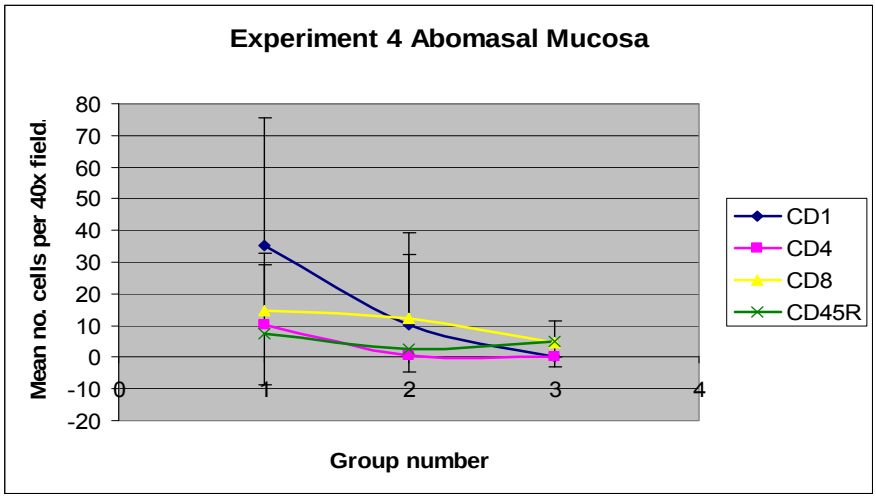
8.33 Table showing all correlations between worm area, perimeter and fecundity

Group	Area/perimeter	Area/fecundity	Perimeter/fecundity
Ex 1 Gp 3 (bolus)	0.82	0.66	0.49
Ex 1 Gp 4 (trickle + bolus)	0.88	0.80	0.78
Ex 2A Gp 1	0.92	0.52	0.42
Ex 2A Gp 2	0.94	0.53	0.55
Ex 2A Gp 3	0.85	0.65	0.49
Ex 2B	0.84	0.62	0.62
Ex 3 Gp 5 (immune)	0.85	0.54	0.40
Ex 3 Gp 6 (non-immune)	0.92	0.85	0.78
Ex 4 Gp 1	0.85	0.50	0.34
Ex 4 Gp 2	0.68	0.51	0.25
Ex 4 Gp 3	0.88	0.67	0.52
Mean	**0.86**	**0.62**	**0.51**

8.34 Experiment 4 IHC results

Bars on graph are standard deviations

Group	Value	CD1	CD4	CD8	CD45R
Grp 1 control	mean	35.4	10.3	14.6	7.4
	std dev	40.5	19.2	18.4	16.0
Grp 2 Dexameth.	mean	10.2	0.6	12.4	2.4
	std dev	28.6	2.4	19.7	6.8
Grp 3 75% protein	mean	0	0.1	4.6	4.9
	std dev	0	0.2	6.9	8.4



8.35 Differentially expressed bands

HindIII(T_{20})A + XhoI arbitrary 1-10

Band size	Upregulated in	Stage 1 primers	Stage 2 primers
550 bp	1099 (control)	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 1
650 bp	1516 (immune)	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 1
220 bp	1516 (immune)	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 2
280 bp	1516 (immune)	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 2
450 bp	1099 (control)	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 7
200 bp	1516 (immune)	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 9
200 bp	1099 (control)	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 10

HindIII(T_{20})C + XhoI arbitrary 1-10

Band size	Upregulated in	Stage 1 primers	Stage 2 primers
>2000 bp	1099 (control)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 2
>2000 bp	1099 (control)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 2
400 bp	1516 (immune)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 4
1000 bp	1516 (immune)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 4
500 bp	1516 (immune)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 5
400 bp	1099 (control)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 8
500 bp	1099 (control)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 8
>2000 bp	1516 (immune)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 8
1100 bp	1516 (immune)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 8
1150 bp	1516 (immune)	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 8

HindIII(T_{20})g + XhoI arbitrary 1-10

Band size	Upregulated in	Stage 1 primers	Stage 2 primers
620 bp	1099 (control)	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 1
250 bp	1516 (immune)	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 5
150 bp	1516 (immune)	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 6
400 bp	1516 (immune)	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 6
100 bp	1516 (immune)	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 9
400 bp	1099 (control)	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 9
400 bp	1099 (control)	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 10
650 bp	1099 (control)	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 10

8.36 Prominent bands of equal intensity in samples from 1099 (control) & 1516 (immune)

HindIII(T_{20})A + XhoI arbitrary 1-10

Band size	Stage 1 primers	Stage 2 primers
300 bp	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 1
350 bp	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 3
400 bp	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 4
200 bp	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 5
350 bp	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 6
400 bp	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 8
500 bp	HindIII(T_{20})A + SL1	HindIII(T_{20})A + XhoI arbitrary 8

HindIII(T_{20})C + XhoI arbitrary 1-10

Band size	Stage 1 primers	Stage 2 primers
1000 bp	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 3
1050 bp	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 3
300 bp	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 6
150 bp	HindIII(T_{20})C + SL1	HindIII(T_{20})C + XhoI arbitrary 9

HindIII(T_{20})g + XhoI arbitrary 1-10

Band size	Stage 1 primers	Stage 2 primers
200 bp	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 1
800 bp	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 5
2000 bp	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 5
450 bp	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 7
300 bp	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 8
420 bp	HindIII(T_{20})g + SL1	HindIII(T_{20})g + XhoI arbitrary 8

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