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ANTHELMINTIC EFFECTS OF TROPICAL SHRUB LEGUMES IN RUMINANT ANIMALS

Thesis submitted by

Keryn Joy Cresswell BVSc, MTVSc

in October 2007

for the degree of Doctor of Philosophy

in the Australian Institute of Tropical Veterinary and Animal Science

School of Veterinary and Biomedical Sciences

James Cook University

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics*, *Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval numbers A687_01, A751_02, A826_03).

Keryn Cresswell October 2007

CONTRIBUTIONS OF OTHERS

Associate Professor Esala Teleni assisted with the design of the research presented in this thesis, provided editorial advice and assistance on the preparation of the thesis and is a co-author on papers arising form this thesis.

Dr Pat Pepper performed the statistical analysis of the data presented in Chapters 5 and 6 of this thesis.

Funds to support this project came from JCU IRA accounts maintained by Associate Professor Esala Teleni.

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Dr Yvette Cunningham provided me with statistical advice and Dr Pat Pepper performed the statistical analysis for Chapters 3 and 4. Ms Jessica Viduka assisted with proof-reading.Their assistance is gratefully acknowledged.

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ABSTRACT

Gastrointestinal (GI) nematode parasitism causes major economic losses and animal health problems in farmed livestock and thus poses a serious challenge to livestock production worldwide. Parasite control strategies historically have relied heavily on anthelmintic drenches, but with the emergence of anthelmintic resistance, new strategies are required. A number of forage species containing high concentrations of condensed tannins have been shown to have anthelmintic properties. The aim of the current study was to determine whether selected tropical legumes used as protein supplements for livestock, and in particular *Calliandra calothyrsus*, might also be useful for the control of GI nematode parasites.

A pilot study was undertaken initially to determine the effects of *Calliandra*, when fed to lambs as a sole diet, on burdens of *Haemonchus contortus* or *Trichostrongylus colubriformis* and on egg production by adult worms. Lucerne (*Medicago sativa*) pellets were used as a high protein control diet and Mitchell grass (*Astrebla* species) hay was used as a low protein control diet. The effects of diet on the worms were assessed by faecal egg counts and post-mortem worm counts. Worm burdens of each species and egg production by *H. contortus* were similar in lambs on all three diets. However, egg production of *T. colubriformis* was reduced by 85-90 % by feeding *Calliandra* to the host lambs and the number of eggs *in utero* of female *T. colubriformis* was significantly increased.

These results were confirmed and extended in a larger-scale feeding experiment, in which the Mitchell grass diet was omitted. A number of haematological parameters were measured to examine the nutritional status, resistance and resilience of the host lambs in response to the worm burdens. Because pH was expected to affect the ability of condensed tannins to bind to worms, the pH was recorded immediately after slaughter in each segment of the GI tract to examine the relationship between pH and faecal egg counts. Worm burdens of *H. contortus* were similar in lambs fed *Calliandra* (2237 ± 395 worms) and lucerne (1861 ± 230 worms). Worm burdens of *T. colubriformis* were also similar in lambs fed *Calliandra* (5718 ± 339 worms) and

lucerne (4861 ± 452 worms). However, egg production by both *H. contortus* and *T. colubriformis* were reduced by 64-84 % and 24-68 % respectively. There were no improvements in any of the haematological parameters measured attributable to the different diets, indicating that reduced egg counts were due to a direct toxic effect of the *Calliandra* diet and not due to improved resistance or resilience to the worms. The variation in pH in each of the GI segments was too small to identify any relationship between pH and reduced egg output by female worms.

A study was then conducted to confirm that the anthelmintic effects of *Calliandra* were due to the condensed tannins in the plant. Condensed tannins were extracted and purified from *Calliandra* leaves and incubated with *H. contortus* and *T. colubriformis* eggs and larvae in *in vitro* assays. Egg hatch and larval development rates were determined. *Calliandra* tannins delayed hatching and reduced egg hatch and larval development in both species. Development of infective larvae was almost completely inhibited at tannin concentrations above 300 µg/mL.

In vitro assays were also used to screen a number of other tropical legumes for anthelmintic activity. Crude extracts of nine legume species, including *Calliandra* and lucerne pellets, and two different fractions of purified *Calliandra* were incubated with *H. contortus* and *T. colubriformis* eggs and larvae. In addition to *Calliandra*, *Leucaena leucocephala* and *Desmanthus virgatus* were identified as having possible anthelmintic activity against nematode eggs and larvae. The two *Calliandra* fractions both had similar anthelmintic activity to the *Calliandra* crude extract.

In an attempt to determine the mode of action of *Calliandra* condensed tannins against GI nematodes, a staining technique was developed to identify tannins histologically in mammalian and nematode tissues. Haematoxylin & Eosin (H&E), ferric chloride, butanol-H_2SO_4 and vanillin-HCl techniques were examined, initially using plant tissues, and then optimised for use in mammalian tissues. The H &E and vanillin-HCl techniques were then used to stain GI tissues and nematodes obtained from *Calliandra*-fed lambs. In the ovine GI tissue, condensed tannins were present in the lumen and in macrophages and giant cells in the lamina propria, particularly in the abomasum. Condensed tannins were observed on the outside of the cuticle of intact adult *H. contortus* and *T. colubriformis*, mainly in adherent digesta. Condensed

tannins were also observed in the pharynx and intestine of sectioned *H. contortus*, but no condensed tannins were observed in the reproductive tract.
A final experiment was conducted to determine the effects of *Calliandra* on the development, establishment and reproductive capacity of nematodes derived from eggs exposed to the legume in the host diet but with no subsequent exposure as adults. Eggs of *H. contortus* and *T. colubriformis* obtained from *Calliandra*-fed or lucerne-fed donor lambs were cultured *in vitro* to obtain infective larvae, which were used to infect groups of recipient lambs. Worm egg and larval production from the donor lambs, and adult worm egg output and worm burdens of the recipient lambs, were monitored. Although worm egg production and larval production from the donor lambs was reduced by the *Calliandra* diet, the ratio of eggs to larvae was also reduced. It was not clear whether this was due to problems with the experimental technique or to selection pressure exerted on the worms by the *Calliandra* diet. Worm burdens were higher in the recipient lambs receiving larvae from the *Calliandra*-fed donors. Egg production by the female worms derived from *Calliandra*-fed donors may have been reduced, but the results were not clear.

Calliandra, in addition to its value as a source of protein for tropical livestock, may be useful in reducing egg production by GI parasitic nematodes, thus reducing larval contamination of tropical pastures and infection rates of livestock. *Calliandra* therefore has potential as an anthelmintic alternative in tropical regions.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
A	Abomasum
ACh	Acetyl choline
BNF	Buffered neutral formalin
Cae	Caecum
CCK	Cholecystokinin
Co	Colon
CP	Crude protein
D	Duodenum
DM	Dry matter
DOM	Digestible organic matter
DOMI	Digestible organic matter intake
DPX	Distrene, Plasticiser, Xylene (tissue mounting medium)
DW	Dry weight
EAA	Essential amino acids
ECL	Enterochromaffin-like cell
ECT	Extractable condensed tannins
ED_{50}	Concentration required to prevent 50 % of eggs from hatching
EHA	Egg hatch assay
EHI	Egg hatch inhibition
epg	Eggs per gram
ES	Excretory/secretory
FAA	Formalin-Alcohol-Acetic acid (plant fixative)
FCT	Fibre-bound condensed tannins
FW	Fresh weight
GI	Gastrointestinal
H & E	Haematoxylin and Eosin
I	Ileum
IgG	Immunoglobulin G
IVOMD	*In vitro* organic matter digestibility
J	Jejunum
JCU	James Cook University
LAP	Leucine aminopeptidase
LD_{50}	Concentration required to prevent 50 % eggs developing to the L_3
LDA	Larval development assay
LDI	Larval development inhibition
ME	Metabolisable energy
MGH	Mitchell grass hay
N	Nitrogen
NAN	Non-ammonia nitrogen
NEAA	Non-essential amino acids
NPM	Nutritional Physiology and Metabolism
NPY	Neuropeptide Y
OCT	Optimum cutting tempetature medium
OM	Organic matter

LIST OF ABBREVIATIONS continued

Abbreviation	Definition
PCT	Protein-bound condensed tannins
PCV	Packed cell volume
PED	Protein entering the duodenum
PEG	Polyethylene glycol
pgp	P-glycoprotein
PLE	Protein-losing enteropathy
PPR	Periparturient rise in faecal egg counts
R	Rumen
s	Seconds
SVBS	School of Veterinary and Biomedical Sciences
TCT	Total condensed tannins
VFA	Volatile fatty acids

CHAPTER 1

GENERAL INTRODUCTION

One of the biggest constraints on livestock production in both temperate and tropical areas today is gastrointestinal (GI) nematode parasitism. Clinical nematode infections result in failure to thrive, illness and death of livestock, causing significant losses to farmers. Subclinical infections cause more insidious losses of production. This may include reduced growth rates (Enterocasso *et al*., 1986; Kimambo *et al*., 1988b), decreased wool production (McDonald, 1979) and reduced milk yields (Ploeger *et al.*, 1989; Hoste & Chartier, 1993; Gross *et al.*, 1999). Effective parasite control programs are therefore vital to maintain the economic viability of farming systems and to promote optimal animal welfare.

Since the advent of safe and effective anthelmintic drugs such as phenothiazine in 1939 and the benzimidazoles a few years later, anthelmintic drenching programmes have played the predominant role in parasite control. The initial success of these drugs in controlling parasites led to their wide acceptance and extensive use by farmers. Unfortunately, the adverse consequences of such widespread use were not long in following, and strains of nematodes resistant to commonly used anthelmintics soon emerged. In Australia alone, by the early 1990s, 80 per cent of farms in the major sheep raising areas had resistance problems to both the benzimidazole and levamisole/morantel groups of drenches (Waller *et al*., 1995) and anthelmintic resistance was identified as the most serious obstacle to livestock production in this country (Australian Agricultural Council, 2003). The introduction of regional worm control programmes may have slowed the development of resistance but has been unable to halt it. A similar situation existed in most other countries and at present the incidence of resistant strains to all drench families is increasing worldwide (Waller, 1997a). Anthelmintic resistance in cattle nematodes, although currently much less of a problem than in small ruminants, is increasingly reported and must not be ignored (Williams, 1997).

The growing problem of anthelmintic resistance and the dearth of novel anthelmintic prospects have made it imperative that alternative systems of parasite control be

developed. There is also growing consumer awareness of the issue of drug residues in meat, and some concern that anthelmintics, the avermectins in particular, persist in the faecal environment for some time and may have detrimental effects on non-target species (McKeller, 1997). Furthermore, in developing countries, the cost of anthelmintics puts them beyond the reach of most smallholder farmers, leading to unavoidable production losses due to uncontrolled parasitism. There is a great need, therefore, to identify inexpensive, practical and sustainable methods of parasite control that avoid or minimise the use of chemical drenches.

A possible alternative to the use of commercially available anthelmintics is to utilise compounds with anthelmintic properties that may occur naturally in certain forage species commonly fed to livestock. There is evidence that temperate forage species containing condensed tannins may be useful in this regard, and it therefore seems likely that tropical forage species containing condensed tannins may also have anthelmintic properties. There are a number of shrub legume species that are already widely incorporated into livestock feeding regimes in tropical regions, but little research has been carried out to determine the effects of these feeds on GI nematodes. Shrub legumes grow readily in tropical agricultural regions and have the potential to be an affordable and sustainable component of parasite control programmes if their anthelmintic properties can be demonstrated.

The aims of the current study were to investigate the effects of condensed tannins in *Calliandra calothyrsus* on different life-cycle stages of two parasites important in tropical areas, *T. colubriformis* and *H. contortus*, and to identify other tropical legumes with the potential to reduce production losses in ruminants due to GI nematode parasitism.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Gastrointestinal nematodes of importance in the tropics

The most important GI nematodes of sheep and goats in tropical areas are *Haemonchus contortus*, *Trichostrongylus* species, *Oesophagostomum columbianum, Bunostomum trigonocephalum* and *Gaigeria pachyscelis* (Urquhart *et al*., 1996). Those of importance in cattle include *H. placei*, *Cooperia punctata*, *C. pectinata*, *B. phlebotomum* and *Oe. radiatum*. Occasionally, clinical infections of *O. ostertagi*, *T. axei* or *C. oncophera* occur in cooler tropical regions, but these are generally of more importance in temperate areas (Winks *et al.*, 1983; Bowman, 1995).

2.1.1 Haemonchus species

In sheep and goats, adult *H. contortus* are 2 – 3 cm long and live in the abomasum. The females may lay in excess of 10,000 eggs per day (Bowman, 1995). Eggs pass out in the faeces from two to three weeks after infection, containing embryos that develop into L_1 larvae. After hatching, the free-living L_1 feed on bacteria and eventually moult to the L_2 stage. The second moult is incomplete, so that the cuticle of the L_2 larva forms a sheath around the L_3. The sheath prevents the L_3 from feeding, so that it must rely on body stores of nutrients, but also ensures it can survive desiccation and changes in environmental temperatures. The development from eggs to the infective L_3 may occur in five days under favourable conditions, or several months in cooler climates. The infective larvae exsheath in the rumen and complete development to the adult stage by moulting twice in the abomasal mucosa close to the gastric glands. Adults attach to the surface of the mucosa, where they feed on blood from mucosal vessels. Daily blood loss due to worms feeding and due to leakage from the damaged mucosa amounts to 0.05 mL per worm (Clark *et al.*, 1962), and in sheep, worm burdens in clinical cases range from 2000 - 20,000 worms. The predominant clinical sign of haemonchosis is thus a progressive

anaemia, which if left untreated, can result in the death of the host animal. Compensatory erythropoiesis is often observed in infected animals, but protein and iron losses into the GI tract, combined with low feed intakes, eventually result in exhaustion of the bone marrow. Extremely heavy worm burdens result in sudden death from acute haemorrhagic gastritis, whereas small burdens cause chronic low-level blood loss accompanied by weight loss and weakness. Diarrhoea is not usually a feature of haemonchosis, but faeces are dark in colour due to the presence of blood. Ventral oedema may also occur (Urquhart *et al*., 1996).

Haemonchosis in cattle is very similar to the disease in sheep, with the exception that cattle greater than two years of age are generally immune to re-infection (Urquhart *et al*., 1996).

2.1.2 Trichostrongylus species

A number of species of *Trichostrongylus* can occur in sheep, the most important in the tropics being *T. axei*, an abomasal parasite, and *T. colubriformis* and *T. vitrinus,* which inhabit the proximal one fifth of the small intestine. They live in tunnels on the surface of the mucosa, where they feed on mucosal fluid, digested food and cellular debris. These worms are usually less than 7 mm long, and they have a prepatent period of two to three weeks (Urquhart *et al.*, 1996).

The exsheathed L_3 of *T. axei* develop to adults between the gastric glands of the abomasal mucosa, before emerging to live on the surface. The mucosa becomes hyperplastic, and nodules, which may coalesce, form around the larvae. The acid-secreting parietal cells dedifferentiate, increasing the pH of the abomasum, which in turn prevents the conversion of pepsinogen to pepsin. Protein digestion is thus inhibited. Disruption of epithelial tight junctions as part of the inflammatory response leads to loss of protein into the gut. Clinically this manifests as inappetance, weight loss and diarrhoea (Urquhart *et al*., 1996).

The intestinal species of *Trichostrongylus* develop between the epithelium and the lamina propria of the intestinal mucosa, causing significant damage to the mucosa

when they emerge as adults. Inflammation and leakage of blood and plasma proteins into the gut occur, and stunting of the villi causes a reduction in the absorptive capacity of the small intestine. Varying degrees of inappetance, weight-loss and watery diarrhoea are the outcome (Urquhart *et al*., 1996). Myiasis is a common sequel, as blowflies are attracted to the faecal material that accumulates on the fleece (Bowman, 1995).

The eggs and larvae of *Trichostrongylus* species are very drought resistant, as they are able to become anhydrobiotic, rehydrating and resuming development after rain (Urquhart *et al*., 1996).

2.2 Biology of nematode parasites

2.2.1 Structure of the cuticle

The nematode cuticle completely covers the external surface of the worm as well as the luminal surfaces of the oesophagus, rectum, reproductive system, excretory system, and other body openings (Wharton, 1986). The outer layer of the cuticle, the epicuticle, is a three-layered structure consisting of a structural cortical zone, a fluid-filled median zone and a fibrous basal zone. Between the epicuticle and the muscle layers is the cellular epidermis (sometimes called the hypodermis). Both the epidermis and the cuticle appear to synthesise soluble and insoluble proteins which become incorporated in the cuticle (Fetterer & Wasiuta, 1987).

In most infective larvae a glycocalyx is present above the epicuticle (Proudfoot *et al*., 1991). In some species glycoprotein sugars are exposed on the surface, while in others the proteins or potential lectin-binding sites on the proteins are sequestered.

Most surface-associated proteins are synthesised in the epidermis and transported to the surface by an unknown mechanism, but some are thought to be secreted by other structures, including the pharyngeal glands and amphidial glands (Maizels *et al.*, 1984; Fetterer & Rhoads, 1993). It is also possible that some may be derived from the host (Rudin, 1990). Rhoads and Fetterer (1990) used radioiodination to study

H. contortus epicuticle proteins. Four detergent-soluble surface proteins were detected in L_4 larvae and adults but not in free-living stages of the worm. These proteins were shed by the adult worms into the surrounding medium, and three of these were hydrolysed by a cysteine protease secreted by the worms. This phenomenon could enable the parasite to evade the host's immune response. It has also been hypothesised that surface-associated proteins may act as decoys to prevent immune recognition of structural proteins (Pritchard *et al*., 1988) or may be involved in tissue penetration (Maizels & Page, 1990).

The epicuticle consists of lipids, large antigenic glycoproteins that can be disrupted by detergents, and cross-linked non-collagenous structural proteins (cuticlins) that are highly resistant to reducing and denaturing agents (Cox *et al.*, 1989; Rhoads & Fetterer, 1990; Fetterer & Rhoads, 1993). At each stage of the parasite life-cycle, different proteins occur. The structures of cuticlin proteins have proved difficult to elucidate because they are insoluble, but they contain, in addition to proline and tyrosine, large amounts of dityrosine, which is responsible for the formation of the cross-links (Fujimoto, 1975; Fetterer & Rhoads, 1990; 1993).

The nematode cuticle is selectively permeable to water, ions and a number of other compounds (Bird & Bird, 1991). Perfusion experiments in *Ascaris suum* showed that the main route of absorption of cholesterol and glucose appears to be the cuticle rather than the intestine (Fleming & Fetterer, 1984). Lipophilic non-polar solutes were also transported across the cuticle in both *A. suum* epidermis/cuticle preparations and intact *H. contortus* infective larvae (Fetterer, 1986). The epicuticle might be primarily responsible for controlling the entry of substances through the cuticle, as it is thought to be selectively permeable to polar compounds, which are then actively transported through the epidermis (Howells, 1987). The heterogeneous nature of the lipid could relate to this selective permeability. Epicuticular lipids are unusual in that they consist of regions of lipid that are free to diffuse and also regions where the lipid is rigid (Proudfoot *et al*., 1990). The rigid regions may be stabilised by cuticlin in some way.

The cortical zone of the cuticle has an outer layer containing cuticlin and an inner collagen-like layer. The median zone is mainly composed of collagen, but the

structure varies considerably between nematode species. In some species this zone is amorphous, while in others it is fluid filled but contains structural elements such as collagenous struts (Lee, 2002). The basal zone also consists predominantly of collagen, but the arrangement is usually more ordered. In larval stages, collagen fibres usually take the form of vertical rods, linked by lipoprotein filaments. In adult parasitic nematodes, collagen fibres are arranged in a more lattice-like structure, with alternating layers of fibres running in different directions. In *Ascaris*, there are three fibre layers in the basal zone.

In *A. suum*, the quantity of collagen in the cuticle may increase from 20 % in the L_2 to 80 % in the adult, with a concomitant increase in the thickness of the median and basal layers (Fetterer & Urban Jr., 1988). The collagen-like proteins in the *A. suum* cuticle are composed of high percentages of glycine, proline and hydroxyproline (Fetterer & Urban Jr., 1988). However, the composition is not the same at all stages of the life-cycle. More glycine and proline are present in the cuticle of adults and L_4 larvae than in L_2 and L_3 larvae, whereas hydroxyproline is most abundant in the L_2. Specific proteins also differ between stages. The collagen fibres exist in monomeric, dimeric and trimeric forms. Dimers and trimers are cross-linked by both disulfide bridges and by covalent isotrityrosine cross-links (Fujimoto *et al.*, 1981; Betschart & Wyss, 1990; Fetterer & Rhoads, 1990). Collagen synthesis in *A. suum* during development from L_3 to L_4 is interrupted by the inhibition of cross-link formation (Rhoads *et al.*, 2001a)

2.2.2 Moulting and exsheathment

As each larval stage develops, moulting of the cuticle allows continued growth and development of new mouthparts (Bird & Bird, 1991). Immediately prior to moulting, larval activity ceases, a period known as *lethargus*. Moulting begins with *apolysis*, or separation of the cuticle from the epidermis. The new cuticle is secreted by the epidermis and forms beneath the old one (Wharton, 1986). Folds in the new cuticle allow for growth of the next larval stage. The old cuticle is shed in a process known as *ecdysis*. During the second moult, the old cuticle is apolysed but not ecdysed, and thus becomes the sheath of the L_3. Exsheathment is simply delayed ecdysis, but

unlike the first moult, requires a specific trigger stimulus from the host. This stimulus is the presence of dissolved carbon dioxide at physiological temperatures (38 °C) (Rogers & Sommerville, 1960; Rogers, 1966). *Haemonchus* species require neutral pH for exsheathment, whereas intestinal *Trichostrongylus* species require low pH (Rogers, 1966).

The L_3 sheath is composed of non-collagenous protein cross-linked by dityrosine residues (Fetterer & Rhoads, 1996). Prior to exsheathment, a refractile ring forms around the sheath 20 µm from anterior end (Gamble *et al*., 1989a; Gamble *et al*., 1989b). The *H. contortus* refractile ring appears to consist of three different proteins (Gamble *et al*., 1990). Compared with the rest of the cuticle, little proline and glycine are present, but there are higher concentrations of serine and threonine. There are no tyrosine cross-links present at the site of the refractile ring. During ecdysis, it is thought carbon dioxide stimulates the anterior nerve ring, causing associated neurosecretory cells to produce noradrenalin (Goh & Davey, 1985). Noradrenalin may stimulate the release of another messenger (probably a peptide) causing the excretory cells to take up water. The enzymes in exsheathing fluid would thus be activated and released into the space between the old and the new cuticle. The cuticle is hydrolysed at the site of the refractile ring so that the anterior end of the sheath opens like a lid and the larva wriggles out (Bird & Bird, 1991).

Exsheathing fluid acts only on the interior of the sheath, at the site of the refractile ring (Rogers, 1966). The site of release of exsheathing fluid is uncertain, but it may be either the excretory-secretory cells, the oesophagus (Rogers, 1982) or the amphids (Bird & Bird, 1991). Support for this suggestion comes from the observation that the *H. contortus* L_3 aligns its mouth with the refractile ring of the sheath just prior to exsheathment (Gamble *et al*., 1989a).

Moulting can occur within 10 minutes of refractile ring formation, which suggests that the active components of exsheathing fluid are preformed and stored, rather than synthesised as needed (Wharton, 1986). The identity of these active components is unclear. Proteases and chitinases do not appear to be present in hatching fluid from either *H. contortus*, or *T. colubriformis* (Rogers, 1982). Rogers & Brooks (1978b) showed that leucine aminopeptidase (LAP) is produced when exsheathment is

stimulated, and exsheathment is inhibited by LAP inhibitors. On further analysis they concluded that LAP alone could not bring about exsheathment (Rogers & Brooks, 1978a). Some authors refute the involvement of LAP at all (Ozerol & Silverman, 1969; Owen & Slocombe, 1974). Nematode LAP is unstable, which may prevent the development of host antibody responses, but also may explain discrepancies in research results (Rogers & Brooks, 1978b).

The enzyme responsible for the breakdown of the refractile ring is probably a protein, isolated from *H. contortus* exsheathing fluid, that has structural similarities to collagenase but does not possess collagenase activity (Rogers, 1982). This protein is a metalloprotein, mildly catalysed by calcium or magnesium but strongly catalysed by zinc. A similar metalloprotein was isolated by Fetterer and Rhoads (1996).

2.2.3 Gametogenesis

Oogenesis in trichostrongyloid nematodes was studied by MacKinnon (1987), who used a mouse nematode, *Heligmosomoides polygyrus*, as a model. Oogonia form in the germinal zone at the tip of the ovary and are initially connected to a central axis, or *rachis*, by cytoplasmic bridges. The oocytes eventually detach as they mature and migrate down the growth zone of the ovary. The rachis is not present in the distal part of the ovary. As the oocytes mature, granules appear in the cytoplasm. In *H. polygyrus*, three types of granules are present. One type is thought to contain lipoprotein, while a second type contains lipoprotein yolk droplets which probably have a nutritional function. The third type contains lipid and is thought to be involved in formation of the eggshell. The origin of these granules is not clear and it has been postulated that they might be transported across the ovarian wall from the closely opposed intestine. Glycogen is also present in the cytoplasm.

Spermatogenesis in nematodes follows a similar pattern to that of oogenesis. Diploid spermatogonia form in the germinal tip of the testis and undergo further development in the growth zone during movement down the testis to form spermatocytes (Bird & Bird, 1991). The spermatocytes divide to form haploid spermatids. Spermatids develop to spermatozoa either in the testis or in the reproductive tract of the female

worm, depending on the species. Nematode sperm is amoeboid and lacks a flagellum, an acrosome or a nuclear membrane. Numerous membranous vesicles are present in the cytoplasm in most species, but the function of these is not known. A filamentous protein known as major sperm protein facilitates amoeboid movement (Justine, 2002). Mature sperm are stored in the seminal vesicle and must be activated in the female reproductive tract prior to fertilisation of the egg, a process equivalent to sperm capacitation in mammals.

2.2.4 Embryogenesis

Embryogenesis begins with fertilisation either in the seminal receptacle in species where this is present, or in the oviduct, or in the region of the uterus most proximal to the oviduct, as in the case of *H. contortus* (Bird & Bird, 1991). The spermatozoa do not possess flagella and move via pseudopodia. These make contact with oocytes, leading to interdigitation of cell membranes and fusion of the cells, after which the sperm nucleus disintegrates. This process stimulates formation of the eggshell.

The details of embryogenesis have been most closely studied in *C. elegans* and has been summarised by Bird & Bird (1991) and Roberts & Janovy (2005a). After the larva is fully formed, it elongates and larval movement and pharyngeal pumping begin. The cuticle is synthesised after elongation. The cuticle can be seen developing over the surface of the larva within the egg and is thought to be derived from the inner layer (the lipid layer) of the egg shell (Croll, 1976).

The extent to which egg development occurs within the adult is different from species to species. *Haemonchus contortus* eggs usually contain four cells when shed by the female, but develop to the 24 - 26 cell stage by the time they are passed in the faeces (Veglia, 1915). Eggs are obligate aerobes and development beyond the morula stage does not occur in the host due to lack of oxygen (Veglia, 1915; Silverman & Campbell, 1959). Movement of *H. contortus* larvae may be detected from six hours after the eggs are voided in faeces and under optimum conditions, development appears to be complete between 10 and 12 hours after voiding (Veglia, 1915). Hatching begins 14 hours after voiding and is almost complete eight hours later.

The embryonic development rate depends on optimal environmental conditions, which vary from species to species. Temperature is particularly important and for most species, no development will occur below 4 °C. Similarly, development tends to be delayed or inhibited at excessively high temperatures (Bird & Bird, 1991). The optimum range for development of *H. contortus* eggs is 22 – 35 °C and development is impaired when temperatures deviate even 2 °C outside this range (Veglia, 1915).

2.2.5 Structure and formation of eggshells

The three main layers of nematode eggshells are secreted by the egg, although in some species one or two outer layers secreted by the uterus of the adult worm are also present (Foor, 1967; Bird, 1971). Strongyloid eggs do not posses a uterine layer (Wharton, 1980). After fertilisation of the oocyte, a second plasma membrane forms inside the oolemma. The original oolemma separates from the cell, becoming the outer vitelline layer of the eggshell, while the intermediate chitinous layer forms in the space beneath (Foor, 1967; Mansfield *et al*., 1992). The vitelline layer may have carbohydrate residues present on the external surface (Bird & Bird, 1991).

In the chitinous layer, high tensile strength chitin is covalently linked to protein, resulting in a strong, flexible structure that prevents mechanical damage to the lipid layer and contributes to the tremendous resistance of eggshells to chemical attack (Wharton, 1980). The composition of the chitinous layer varies, but consists mainly of protein in strongyloid eggs (Roberts & Janovy, 2005a). The chitin microfibrils in *H. contortus* eggs are surrounded by a protein matrix (Mansfield *et al*., 1992).

Refringent bodies throughout the cytoplasm condense beneath the new oolemma and extrude their granular contents to the cell surface to form the inner lipid layer. Twenty-five percent of the lipid layer in *Ascaris* species is made up of protein, while the rest is composed of ascaroside esthers (sugar moieties linked to monol or diol alcohols) (Fairbairn, 1957). It is not known whether ascarosides occur in the eggs of other nematodes. The lipid layer of *H. contortus* eggshells is thought to contain non-polar hydrocarbon lipids, whereas that of *T. colubriformis* eggshells is more likely to consist of protein or polar unsaturated lipids (Waller, 1971). Species differences in

the lipid layer probably explain differences in the susceptibility of eggs to desiccation and chemical penetration. The lipid layer is permeable only to gases and lipid solvents (Arthur & Sanborn, 1969). Oxygen must be able to diffuse across the egg shell, since larval development is oxygen dependent (LeJambre & Whitlock, 1967). Water vapour can also diffuse across the shell to a limited extent (Wharton 1979).

2.2.6 Mechanism of hatching

If sufficient water is present, hatching proceeds as soon as the L_1 is fully developed (Wharton, 1986). During embryonation of nematode eggs, glycogen is converted to trehalose, which accumulates within the egg to a concentration of 0.2 M, causing a high osmotic pressure. Just before hatching, permeability changes in the lipid layer allow trehalose to leak out of the egg and the larva becomes activated by an influx of water. The larva in turn secretes enzymes that weaken the shell. The egg swells, which in combination with embryonic movement results in the rupture of the shell (Croll, 1974; Weston *et al*., 1984). The stimulus for the initial change in permeability is unknown. It is probably a result of enzymic secretion by the larva. Hatching fluid disrupts the arrangement of the chitin-protein complexes in the medial layer of the shell, removes the lipid layer and damages the vitelline membrane (Mansfield *et al*., 1992). Hatching fluid differs from exsheathing fluid in that, although hatching fluid normally acts on the inside of the eggshell, if released into the medium it will also act on the outside of any shells with which it comes into contact (Rogers, 1966).

Rogers and Brooks (1977) observed *H. contortus* larvae emerging head first from small holes in egg shells, and postulated that the harder outer membrane was broken down in a localised area by enzymes secreted by the embryo, while the inner lipid membrane was broken down mechanically by the embryo. When the eggs were washed in hypochlorite, the shell surface was seemingly altered, resulting in a generalised thinning of the outer shell membranes when they were subsequently exposed to hatching fluid. Hypochlorite therefore seems to increase the surface area available for the enzymes in hatching fluid to act on, by removing the protective outer coat. These workers identified LAP and a lipase in hatching fluid but were unsure of how these enzymes were involved in the hatching process. Purified lipase

or LAP did not break down egg shells. There was no evidence for the presence of chitinase or a peptidase in this study.

Hinck and Ivey (1976) demonstrated that hatching fluid from *A. suum* exhibited strong proteinase activity and further observed that hatching efficiency of 18 – 28 day-old eggs increased as proteinase activity increased. Other possible constituents of the hatching fluid were lipase, chitinase, α-glucosidase and β-glucosidase. However, despite the presence of lipase, hatching fluid did not attack the lipid layer. Possibly, the effects on the lipid membrane were localised.

There is some evidence that calcium bound to lipoprotein membranes in the lipid layer can be displaced by hatching fluid, but the function of calcium in this context and the significance of its displacement are unknown (Wharton, 1986). Eggshells of *Nematodirus battus* have both low and high affinity calcium binding sites and Ash and Atkinson (1984) observed that hatching was inhibited when these were competitively blocked by the dye ruthenium red. The calcium-binding sites are membrane-associated P-glycoproteins (pgp) that appear to be involved in ion transport across the shell membranes. Similar pgps have been identified in all three layers of nematode eggshells (Riou *et al*., 2005).

2.2.7 Excretory-secretory products

Parasitic nematodes secrete or excrete a variety of substances, collectively known as ES products, many of which are stage-specific. Some ES products, particularly those produced by *H. contortus*, have been isolated and characterised. Substances identified have included hyaluronidase (Rhoads *et al*., 2000b; Rhoads *et al*., 2001b), cysteine proteases (Rhoads & Fetterer, 1995), a zinc metalloprotease (Gamble *et al*., 1996), an acid phosphohydrolase, a cathepsin C-like enzyme, a phospholipase C-like enzyme and an *N*-acetyl-β-D-glucoseaminidase (Gamble & Mansfield, 1996), a phosphochlorine hydrolase, an alkaline phosphatase (Fetterer & Rhoads, 1990) and an aminopeptidase (Rhoads *et al*., 1997). The specific functions of these enzymes are unknown, but probably include ecdysis of the cuticle during moults, tissue penetration, anticoagulation and digestion of nutrients prior to uptake by the worm.

Additionally, *T. suis* has been shown to secrete serine protease inhibitors, which may act as a defence mechanism against the host immune response (Rhoads *et al*., 2000a).

2.3 Nutrition/Parasite Interactions

The clinical and subclinical effects of GI parasitism are inextricably linked to nutrition of the host animal. The interaction between nutrition and parasitism is complex. Parasitic infection alters the host nutrient metabolism, but the level and type of nutrients ingested by the host also influences the host's ability to cope with the effects of parasite infection (*resilience*) or to mount an effective immune response (*resistance*). Thus the overall effect of parasitism on a ruminant animal will depend on access to specific nutrients, as well as the degree and type of parasitic challenge.

2.3.1 Feed Intake

Reduced feed intake is one of the most important effects of GI parasitism and may account for 40 – 90 % of lost production (Symons & Jones, 1975; Sykes & Coop, 1976; 1977; Coop *et al.*, 1982; Bown *et al.*, 1989; van Houtert & Sykes, 1996). Symons and Jones (1975) compared feed intake and muscle protein deposition in uninfected control sheep fed *ad libitum*, sheep infected with *T. colubriformis* fed *ad libitum*, and uninfected sheep pair-fed with the infected group. It was evident from that experiment that weight loss in the infected sheep was related to a decrease in intake, since similar changes in body weight were seen in the pair-fed sheep. Similar effects occurred with *O. circumcincta* infections (Sykes & Coop, 1977). In the latter study appetite did not recover in the face of a trickle infection with *O. circumcincta* as it usually does after single large infections. Reduced appetite coincides with the emergence of young adult parasites from the gastric glands. If all emerge at once, appetite may recover afterwards, but continuous dosing causes persistent appetite depression. As trickle infections more closely resemble field situations, depressed intake would be likely to have a profound effect on production in parasitised animals. The extent to which intake is affected may be proportional to the size of the larval

challenge (Steel *et al*., 1980), although this has not been the case in all studies (e.g. see Kyriazakis *et al*., 1998).

The mechanisms by which feed intake is reduced in parasitised animals are not fully known. One possibility is that damage to the mucosa is painful and deters voluntary intake (McKellar, 1993). In abomasal parasitism, plasma gastrin concentrations are usually elevated and are thought to decrease feed intake by inhibiting muscular contractions in the reticulorumen. The rate of emptying of the reticulorumen is impaired and thus appetite is reduced (Fox *et al*., 1989). Appetite depression may also be centrally mediated. Cholecystokinin (CCK), a hormone secreted primarily by cells in the duodenal mucosa, acts both on peripheral receptors and on the satiety centre in the brain. In sheep, the plasma concentration of CCK was observed to rise rapidly after infection of the animal with *T. colubriformis*. This rise was followed by a marked drop in appetite (Symons & Hennessy, 1981). In five out of six animals, the administration of an anthelmintic caused feed intake and CCK concentrations to return to basal levels within four to six days. By administering a CCK antagonist that acts primarily on peripheral CCK receptors, Dynes *et al*. (1998) confirmed that CCK probably acts directly at a central level.

Gastrointestinal parasitism also appears to result in up-regulation of expression of the neuropeptide Y (NPY) gene, which is normally involved in appetite stimulation in animals that are in negative energy balance. However, in parasitised animals, increased NPY gene expression is not accompanied by an increase in feed intake, possibly because other stronger signals override or block the effects of NPY. At least in the rat model, such a possibility may be mediated via alterations in the secretion of hormones such as leptin, insulin and corticosterone, although the evidence for this is not conclusive (Roberts *et al*., 1999). De Jong-Brink *et al*. (1999) postulated that a parasite-induced up-regulation of NPY would benefit the parasite by repartitioning host energy from reproduction and growth to increased glycogen storage, thus making more energy available to the parasite. Although this work used lymnaeid snails as the host, it offers a possible explanation for parasite-induced changes in NPY expression even though feed intake is not affected.

A number of hypotheses to account for parasite-induced inappetance in functional terms were reviewed by Kyriazakis *et al*. (1998). They proposed that reduced feed intake either stimulated the host somehow to mount an effective immune response or increased diet selectivity. Increased selectivity might prevent further infection (by avoidance of contaminated pasture) or increase intake of nutrients and antiparasitic factors to mitigate the infection. However, selectivity usually involves a compromise between nutritional benefits and parasite infection status. Pasture growth around faecal deposits is likely to be improved, thus improving the energy and protein intake of grazing ruminants, but increasing the risk of larval ingestion (Hutchings *et al*., 2003; Hutchings *et al*., 2006). Feeds containing condensed tannins often impair protein nutrition, but this may be of secondary importance in parasitised animals that could suffer severely from further intakes of infective larvae. Parasitised animals actively avoid faecal-contaminated pasture to a greater extent than non-parasitised animals, suggesting that they try to select a diet that balances their nutritional requirements with their specific need to limit GI worm burdens (Hutchings *et al.*, 2003).

Parasitised animals have an increased dietary requirement for protein (see section 2.3.5 for further discussion). There is some evidence that sheep are able to modify their diet selection to meet such a requirement if given the opportunity to do so (Kyriazakis *et al*., 1994). Therefore, although parasitism reduces intake, the degree to which parasitised animals are able to modify their intake to meet the increased protein demands of parasitism determines the severity of clinical signs.

2.3.2 Gastrointestinal function

Several major changes in GI function occur in ruminants parasitised by GI nematodes. These include alterations in morphology, motility, and secretion of hydrochloric acid, gastrin, pepsinogen and mucin.

(a) Morphological changes

Morphological changes that occur in the abomasum in response to resident nematodes include formation of nodules due to invasion of the gastric glands by developing larvae, mucosal erosions, hyperaemia and oedema of the abomasal folds and dedifferentiation of parietal cells (Armour *et al*., 1966; Ross *et al*., 1968). Changes induced by small intestinal *Trichostrongylus* species include flattening and disruption of the mucosa, villous atrophy, elongation of the crypts, and infiltration of the lamina propria by inflammatory cells (Barker, 1975a; Coop *et al*., 1979; Jackson *et al*., 1983). The majority of worms are found in the proximal one third of the small intestine, and most of the damage occurs in this region (Barker, 1975a; Steel & Symons, 1982; Jackson *et al.*, 1983). Parasitic infection of the small intestine is also associated with decreases in the activity of brush-border enzymes, particularly alkaline phosphatase, LAP, maltase, and glycyl-L-leucine dipeptidase (Jones, 1982). Alterations in the concentrations of other digestive enzymes including trypsin, chymotrypsin and acetyl-cholinesterase also occur. Symons and Jones (1970), however, noted that decreases in enzyme activity were not consistent.

(b) Motility

The motility of the GI tract plays a major role in the transit time of digesta, so any factor that alters motility has the potential to disrupt digestive processes. Horak *et al*. (1968) observed stasis of digesta in the rumen and abomasum of lambs with large burdens of *T. colubriformis* and postulated that pain may have resulted in closure of the pyloric sphincter. However, Bueno *et al*. (1982b) used electromyography to measure abomasal and intestinal contractility in lambs infected with *H. contortus* and found that cycles of motility in these organs were shorter than in unparasitised animals. At the same time, duodenal digesta flow rates increased, probably because the rate of gastric emptying increased in response to the higher gastric and duodenal pH induced by the worms. These reductions in abomasal and intestinal motility were accompanied by increased frequencies of migrating myoelectric complexes (MMC), which prevented complete stasis of digesta (Buéno *et al*., 1982b; Gregory *et al*., 1985).

McKellar *et al*. (1990) postulated that the abomasal parasites *O. circumcincta* and *O. ostertagi* secreted a product with muscarinic-like activity, which would stimulate gastric and intestinal contractions and peristalsis, thus reducing transit time of digesta and contributing to the diarrhoea often seen clinically in parasitised animals. However, Fox *et al*. (1989) obtained evidence that motility of the rumen, reticulum and abomasum was inhibited by the hypergastrinaemia that accompanies *Ostertagia* infections. In addition, Poppi *et al*. (1985) noted while collecting digesta from duodenal cannulae in lambs that pH was consistently higher in lambs infected with *T. colubriformis* than in uninfected lambs and the digesta also contained more bile, suggesting that motility in this region was impaired. This apparent contradiction may be explained by differential effects on motility as observed by Buéno and Fioramonti (1979; cited in Buéno *et al*., 1982b). In sheep infected with both *T. axei* and *C. ovina*, motility of the reticulum and abomasum decreased, but jejunal motility increased.

A lapse of 90 - 120 minutes between first exposure to nematode larvae and reductions in abomasal and duodenal motility suggested that these changes occurred in response to the presence of larval antigens (Buéno *et al*., 1982a). It is not known how changes were induced, but Gregory *et al*. (1985) proposed that increased secretion of hormones such as secretin or CCK was involved.

(c) Hydrochloric acid secretion

Abomasal parasites have detrimental effects on the gastric epithelium, due to direct physical damage during attachment and feeding, and to induced biochemical and physiological changes related to the host immune response. In particular, abomasal nematodes inhibit acid secretion by parietal cells, with a consequent rise in gastric luminal pH.

In one study, *H. contortus* larvae caused an immediate increase in abomasal pH by releasing substances that stimulated bicarbonate secretion, while acid secretion was initially unchanged (Buéno *et al*., 1982a). Acid secretion decreased in proportion to the size of the parasite burden about the time the adult parasites emerged from the

gastric glands after the final moult. Although inhibited parietal cells eventually become replaced by undifferentiated cells (Murray *et al*., 1970; Simpson, 2000), the restoration of parietal cell function within two hours of drenching suggests that chemical inhibition and not cell damage reduces acid secretion (Simpson *et al.*, 1997; Hertzberg *et al.*, 2000).

Undefined parasite ES products may be involved in causing parietal cell inhibition (Anderson *et al*., 1985; Simpson, 2000). Parietal cells secrete acid under the stimulus of histamine, which is produced by enterochromaffin-like (ECL) cells in response to high levels of gastrin. Inhibition of acid secretion could therefore be at the level of the ECL cell or the parietal cell (Hertzberg *et al*., 2000) but the exact mechanism is unknown. Inhibition of the ECL cell is most likely because exogenous histamine and carbachol can stimulate acid secretion from apparently inhibited parietal cells, indicating that the proton pump (H^+/K^+-ATPase) at the apical membrane of the parietal cell remains functional.

Reduced acid secretion hinders abomasal function, and may benefit the resident parasites by inhibiting the establishment of other nematode species (Coop *et al*., 1986) or by allowing parasites to survive in abomasal fluid instead of just in the mucus layer (Hertzberg *et al*., 2000). Egg survival may be improved as well. If worm burdens are low, the changes in pH may be localised and abomasal pH as a whole may not be altered (Simpson *et al*., 1997).

Heavy infections with *T. colubriformis* stimulate dedifferentiation of abomasal parietal cells and inhibit gastric acid secretion, despite the fact that the preferred site for this parasite is the small intestine (Barker & Titchen, 1982). This could be due to some form of hormonally-mediated systemic inhibition of gastrin triggered by the worms in the small intestine. However, Gregory *et al*. (1985) found that abomasal pH did not change in response to *T. colubriformis* infection.

The pH in both the abomasum and the duodenum begins to recover 8-10 days after *H. contortus* infection in sheep (Buéno *et al*., 1982b). Similarly, abomasal pH returns to normal around the time *O. circumcincta* infections become patent (Lawton *et al*., 1996).

(d) Gastrin and pepsinogen secretion

Plasma gastrin concentrations increase in animals with GI parasite burdens, stimulated at least in part by decreased abomasal pH (Fox *et al*., 1989). However, Anderson *et al*. (1985) recorded elevated plasma gastrin levels prior to changes in abomasal pH in ewes with *Ostertagia* infections, indicating that some other stimulus was also involved. Hypergastrinaemia occured about the same time as pH began to rise in sheep infected with *O. circumcincta*, eleven hours after elevation of pepsinogen levels (Lawton *et al*., 1996). Physical stimulation of the gastrin-producing G-cells by the worms or chemical stimulation by parasite ES products may be responsible. Gastrin not only stimulates histamine secretion by ECL cells and thus acid secretion by parietal cells, but also promotes proliferation of the parietal cells, leading to fundic hyperplasia. Increased gastrin may initially be due to removal of acid feedback, but soon after infection, gastrin and acid levels become independent of one another, so inflammatory mediators may also be involved (Simpson *et al*., 1997). Gastrin levels in sheep begin to increase approximately four days after infection with *H. contortus*, reach a peak at eight days and begin to decline about ten days after infection. Gastrin concentrations return to normal four days after drenching.

Hyperpepsinoginaemia in GI nematode parasitism is probably due to the leakage of pepsinogen into the blood through disrupted mucosal tight junctions as a result of the inflammatory response to the presence of nematodes (Simpson, 2000). Pepsinogen release into the blood from zymogen cells also increases (Fox *et al*., 1989; McKellar, 1993), partly in response to high circulating gastrin concentrations and partly influenced by parasite ES products (Fox *et al*., 1989; McKellar *et al*., 1990).

(e) Mucin secretion

A substantial increase in mucin production by goblet cells occurs in GI nematode infections (Steel & Symons, 1982). The mucoproteins present in intestinal mucus are particularly rich in cysteine; consequently wool production can be severely impaired in parasitised sheep.

(f) Other factors

Other aspects of GI function affected by GI parasitism have received less attention. Pancreatic polypeptide concentrations decreased after infection of ewes with *Ostertagia* species in one study, but the results were too variable for definite conclusions to be drawn (Anderson *et al*., 1985). Pancreatic polypeptide suppresses pancreatic secretion and may also have effects on GI motility and acid secretion.

Plasma insulin levels declined in sheep infected with *O. circumcincta* around the time of patency, possibly due to reduced protein digestion (Fox *et al*., 1987). Growth hormone levels also declined over the course of the infection. A decrease in total thyroxine concentration in both infected animals and in pair-fed controls was probably caused by reduced feed intake. However, free thyroxine concentrations did not change, indicating that metabolic consequences of changes in thyroid hormones were unlikely.

2.3.3 Energy metabolism

The results of studies investigating the effects of GI parasitism on energy metabolism have been inconsistent. In some experiments using sheep, infection with *T. colubriformis* appeared to decrease the efficiency of utilisation of dietary energy (measured as kg DM consumed per kg bodyweight gained), with little effect on feed digestibility (Symons & Jones, 1970; Coop *et al.*, 1976; Sykes & Coop, 1976; 1977; Bown *et al.*, 1991a). In the latter experiment, designed to investigate the effects of improved protein or energy nutrition on production in parasitised animals, efficiency was restored by post-ruminal infusion of casein. However, glucose infusion into the abomasum had only a small effect, possibly by sparing amino acids from gluconeogenesis and thus making them available for protein synthesis.

On the other hand, MacRae (1993) argued that when energy expenditure was measured in respiration chambers, *T. colubriformis* reduced feed digestibility. Less metabolisable energy (ME) was therefore available to the animal, while the

efficiency of ME utilisation remained unchanged. Steel (1972) demonstrated that acetate production decreased in sheep parasitised by *T. colubriformis* due to reduced appetite and reduced organic matter (OM) digestion. Live weight and wool growth were impaired due to an energy deficit and decreased volatile fatty acid (VFA) production may have decreased microbial protein synthesis. The effect of GI parasitism on energy metabolism is yet to be resolved satisfactorily, but it seems likely that increased protein turnover as a result of intestinal parasitism would increase the maintenance energy requirement of the host animal (van Houtert & Sykes, 1996).

Energy may be more limiting in parasitised goats than sheep because goats have lower fat reserves. Goats browsing tropical forages are particularly likely to have inadequate energy intakes and may require energy supplementation to alleviate the effects of parasites (see review by Hoste *et al*., 2005b).

2.3.4 Minerals

There have been many studies of the effects of GI nematode parasitism on calcium and phosphorus nutrition, due to the incidence of osteoporosis and osteomalacia in growing lambs infected with intestinal parasites (Sykes *et al*., 1975). Infection with *T. colubriformis* decreased dietary phosphorus absorption by 30 % (Wilson & Field, 1983). Concurrently, mucosal damage and increased secretory activity in response to the presence of worms resulted in losses of endogenous phosphorus, causing hypophosphataemia and a reduction in salivary phosphorus. Endogenous calcium loss increased slightly but plasma concentrations remained normal. However, the combined phosphorus and protein deficiency impaired both bone matrix deposition and mineralisation, thus reducing bone growth. Low phosphorus intakes exacerbated these effects (Coop & Field, 1983). Poppi *et al*. (1985) examined calcium and phosphorus absorption in lambs given continuous trickle infections of *T. colubriformis* for 14 weeks and found no improvement in phosphorus metabolism after the development of resistance to the parasite. In contrast, infection with the abomasal parasite, *O. circumcincta*, did not affect either calcium or phosphorus absorption and caused only a small increase in loss of

endogenous calcium (Wilson & Field, 1983). Serum concentrations of calcium and phosphorus remained normal, but skeletal growth was still impaired, the mechanism by which this occurred being unknown (Sykes & Coop, 1977). Abomasal infusion of casein increased calcium retention, possibly in response to increased demand for bone matrix mineralisation. Hypocalcaemia is not generally a feature of GI parasitism, because the large intestine is a major site of calcium absorption (Bown *et al*., 1989). It is also possible that phosphorus deficiency and the consequent decrease in bone matrix deposition could result in a lower demand for calcium in parasitised animals. The main site of phosphorus absorption is the small intestine, so infection may inhibit absorption by direct damage to the proximal small intestine and inability of the distal small intestine to compensate (Bown *et al*., 1989). A further possibility is that rising duodenal pH in intestinal infections causes phosphorus to precipitate in complexes with calcium or magnesium and thus prevents absorption (Poppi *et al*., 1985). However, this does not sufficiently explain why calcium and magnesium absorption are not affected.

There is no evidence that magnesium absorption is affected by GI parasitism (Bown *et al*., 1989), but skeletal deposition may be decreased in severe cases of trichostrongylosis (Reveron *et al*., 1974).

Adequate mineral nutrition is important to compensate for impaired nutrient absorption, but may have other benefits as well. Worm counts and faecal egg counts decreased in lambs infected with *T. colubriformis* and supplemented with molybdenum at rates greater than 0.01 mg/kg live weight, compared with unsupplemented lambs (Suttle *et al*., 1992). This effect was only seen after a second infection, so molybdenum may have been involved in the development of immunity. Similar reductions in worm burdens and faecal egg counts, as well as higher packed cell volume (PCV), occurred in molybdenum-supplemented lambs infected with *H. contortus*. However, no prior exposure to worms was necessary to obtain these results with the abomasal parasite. Adult *H. contortus* exposed to molybdenum in the host diet secreted less protein into culture media than worms derived from unsupplemented hosts, so the impaired secretion or functioning of copper-dependent anti-inflammatory enzymes by the worms may have contributed to an improved host immune response. High levels of supplementation with

molybdenum can impair the immune response. The optimum range seems to be 4 – 8 mg/sheep/day, or 6 – 10 mg/kg dietary DM (McClure *et al.*, 1999; McClure, 2003). Due to dietary interactions between molybdenum and copper, the ratio of copper intake to molybdenum intake may also be important.

Other nutrients have been investigated for potential effects on immunity to GI nematodes. Iron supplementation can accelerate the development of resistance to infection with *H. contortus*, probably because the haemopoietic system is better able to compensate for blood loss. Lambs that receive iron supplementation can survive severe infections without developing clinical signs, despite significant faecal egg counts (Scott *et al*., 1971). The development of resistance may also be improved. Compared to unsupplemented animals, selenium supplementation did not appear to affect the immune response to *H. contortus* in six-month-old Merino lambs (Jelinek *et al*., 1998). However, the unsupplemented animals in this study had high plasma α-tocopherol concentrations, which may have boosted their immunity. Impaired selenium absorption has been shown to induce white muscle disease in lambs with chronic *Trichostrongylus* infections (Horak *et al*., 1968).

2.3.5 Protein metabolism

Damage to the GI tract caused by the presence of nematodes disturbs normal digestive function. Abomasal parasitism with *O. circumcincta* severely reduced protein degradation and therefore nitrogen (N) digestibility due to loss of functional parietal and chief cells (Sykes & Coop, 1977). Nitrogen digestibility returned to normal gradually over the course of the experiment, possibly because intestinal protein degradation increased to compensate.

The abomasal parasite, *H. contortus*, also causes significant leakage of blood from attachment sites on the abomasal mucosa. Rowe *et al*. (1988) found that blood loss in parasitised animals increased the amount of endogenous N entering the duodenum, which was subsequently reabsorbed. However, ammonia was produced in the abomasum, partially by metabolism of blood protein by *H. contortus*, and partially by an unspecified host mechanism. Although the ammonia was reabsorbed, it was

then excreted in the urine instead of being made available for protein synthesis, and so represented a loss of N to the host animal.

An early experiment to determine the effects of intestinal changes on apparent N digestibility found little difference between four-month-old lambs parasitised with *T. vitrinus* and control animals, but whole body protein deposition was markedly decreased in the parasitised group (Sykes *et al*., 1979). This suggested that protein loss and replacement due to plasma leakage into the intestinal lumen or sloughing of intestinal epithelial cells reduced the efficiency of protein utilisation, rather than reduced N digestibility *per se*. This concurred with the findings of others (Symons & Jones, 1970; Roseby, 1973; Poppi *et al.*, 1986).

Prichard *et al*. (1974) demonstrated marked endocrine changes in lambs infected with *T. colubriformis*, including increased corticosteroid secretion and depressed plasma insulin and thyroxine concentrations. Such changes were consistent with the repartitioning of protein synthesis from muscle to liver. In contrast, Sykes and Coop (1977) found no change in protein utilisation apart from the effects of depressed intake in sheep parasitised with the abomasal worm *O. circumcincta*. Parasite location and pathophysiology are probably important in determining the effects of GI nematode parasitism on protein metabolism.

Plasma proteins account for at least part of the endogenous protein loss. The degree of plasma loss is probably related to the degree of epithelial disruption (Beveridge *et al*., 1989). Sheep infected with *O. circumcincta* had high plasma albumen turnover rates and appreciable leakage of plasma into the GI tract and possibly into perivascular tissue spaces (Holmes & MacLean, 1971). However, Poppi *et al* (1986) calculated that most plasma proteins released into the intestinal lumen of lambs parasitised with *T. colubriformis* should have been reabsorbed, and would not account for the total amount of non-ammonia nitrogen (NAN) present at the terminal ileum. They postulated that the remaining NAN must be due to increased mucus production and sloughing of epithelial cells into the intestinal lumen in response to the presence of parasites. Since energy utilisation was not increased, and protein deposition decreased in relation to total liveweight gain (see Sykes & Coop, 1976), it seemed likely that protein synthesis was not increased to meet the extra demand for

protein. Rather, protein synthesis was diverted from normal productive processes to the replacement of protein lost into the intestine. This suggestion was consistent with the finding that *T. colubriformis* infection increased protein synthesis in the liver but decreased protein synthesis in muscle or renal cortex (Symons & Jones, 1975; Jones & Symons, 1982). In another study, Bown *et al*. (1991b) observed that a mixed infection of *O. circumcincta* and *T. colubriformis* increased the loss of endogenous protein (mainly due to mucus production and increased epithelial turnover as part of the host response to the presence of nematodes), but did not impair absorption. Although *T. colubriformis* mainly inhabits the proximal one fifth of the small intestine, the site of maximum absorption of amino acids was seven to fifteen metres from the pylorus (Ben-Ghedalia *et al*., 1974). The increased demand for protein in parasitised ruminants therefore is due mainly to increased loss and replacement of endogenous protein. Any protein that does escape absorption in the small intestine can be fermented by microorganisms in the large intestine to produce ammonia, which is then absorbed and lost in the urine as urea (Steel & Symons, 1982).

Increased oxidation of amino acids by GI tract tissues to meet increased metabolic demand also accounts for some loss of protein. In sheep infected with *T. colubriformis*, blood flow to the GI tract increased, with a concurrent increase in the sequestration and oxidation of leucine (Yu *et al*., 2000). Increased demand for other amino acids by GI tissues has yet to be demonstrated.

The effects of protein supplementation on GI parasitism in sheep have been widely investigated. Liu *et al*. (2003) estimated that sheep required an extra 17 g of protein per day to compensate for the effects of GI parasitism. Abbott *et al*. (1986b) compared the effects of a high or a low protein diet on *H. contortus* infections in lambs. On the low protein diet, clinical signs were more frequent and severe and haematological changes were particularly marked. Both groups had increased rates of erythropoiesis to compensate for gastric haemorrhage, but those on the low protein diet showed a marked degree of inappetance (Abbott *et al*., 1986b). Bown *et al* (1991a) used abomasal infusions of either casein or glucose to demonstrate that increased supply of protein, but not energy, to the post-ruminal GI tract improved DM intake, liveweight gain and ME retention in infected animals. Casein infusion also reduced faecal egg counts and intestinal worm counts 12 weeks post-infection.

Van Houtert *et al*. (1995a) demonstrated that protein-supplemented sheep had increased growth rates and slightly increased wool growth compared with unsupplemented controls, although worm burdens in the two groups were similar. Milk yield and milk fat content of dairy goats were also increased by protein supplementation (Chartier *et al*., 2000). These findings illustrate the improved ability of protein-supplemented parasitised animals to meet the increased demand for amino acids (Steel & Symons, 1982).

2.3.6 Nutritional effects on immunity to parasites

(a) Development of immunity

There are three stages of the ovine immune response to GI parasites (Seaton, 1989; Dobson *et al.*, 1990b; a). Initially, lambs are fully susceptible to infection and larval establishment is unimpeded. During the second stage, larval establishment is reduced and the egg production of female worms is inhibited, and finally, adult worms are expelled.

The effects of protein nutrition on the development of immunity to parasites were investigated by van Houtert *et al*. (1995a). In three-month-old lambs born on pasture, supplementation with either rumen-degradable or rumen-undegradable protein for 36 weeks decreased faecal egg counts but not worm burdens. The lack of effect on worm burdens suggested that protein nutrition did not influence the rate of development of acquired immunity. However, supplementation reduced the need for drenching and increased liveweight gain, wool production and wool fibre diameter, indicating that supplementation had improved resilience.

In a subsequent study (van Houtert *et al*., 1995b) three-month-old lambs were supplemented with fishmeal for 140 days. Serial worm counts throughout the experiment showed that larval establishment of *T. colubriformis* was not affected, but the ability to expel adult worms from Day 70 onwards was enhanced. These two studies suggested that a high-protein diet did not improve the acquisition of

immunity, but did improve the expression of the immune response later in the infection.

In contrast, supplementation with rumen-undegradable protein increased the rate at which lambs developed resistance to *O. circumcincta* when rechallenged after an initial infection (Coop *et al*., 1995). Faecal egg counts and worm burdens decreased in the supplemented lambs and a large proportion of worms failed to develop beyond the L_4 stage. Abbott *et al*. (1988) found that lambs trickle-infected with *H. contortus* larvae resisted reinfection when fed a high protein diet but not a low protein diet. Possibly, differences between the immune responses to abomasal and duodenal nematode species could account for the diverse effects of protein supplementation. Of note, the efficiency of protein utilisation for growth decreased, despite improved immunity to *O. circumcincta* in the study of Coop *et al*. (1995). Possibly protein was diverted away from productive processes to the development of an immune response. This result complemented the finding of van Houtert *et al*. (1995b) that the clinical signs of *T. colubriformis* infection were most severe around day 70 post-infection, when worms were expelled.

More evidence that nutrition can indeed affect the acquisition of immunity comes from a study in which two groups of lambs were trickle-infected with *T. coulubriformis*, then drenched and given a second infection. Lambs exposed to worms before five weeks of age had reduced faecal egg counts and increased plasma immunoglobulin G (IgG) concentrations when rechallenged at seven weeks of age, compared with lambs that were rechallenged after their first exposure to worms at four months of age (Emery *et al*., 1999). This phenomenon was attributed to nutritional stress in the older, newly weaned lambs as opposed to suckling lambs.

(b) Maintenance of immunity

Ongoing exposure to worms may be required for long-term persistence of the immune response, particularly for *H. contortus* (Coyne & Smith, 1992). In sheep that have already mounted an immune response to GI nematodes, worm burdens are usually low. Although minimal mucosal damage and protein loss might be expected in immune animals, significant plasma and protein losses occurred when adult,

immune sheep were exposed to parasitic challenge (Yakoob *et al*., 1983). Such losses may have been caused by increased mucosal permeability as part of the immune response to parasites and represented an ongoing cost to the host animal of maintaining immunity. However, these sheep had been grazing larval-contaminated pastures immediately prior to experimental challenge. Immune sheep that were challenged with *T. colubriformis* after a six-month parasite-free period mounted a rapid immune response following a small, transient period of N leakage into the GI tract (Kimambo *et al*., 1988a). There was no associated decrease in feed intake or growth rates, indicating that maintenance of immunity was not associated with major nutritional costs. This was supported by the failure of protein supplementation to enhance the immune response (measured either in terms of faecal egg counts, worm burdens, or mucosal concentrations of eosinophils and sheep mast cell proteases) in immune animals challenged with *T. colubriformis* (Kyriazakis *et al*., 1996a). These discrepancies may reflect differences in the magnitude of the initial immunity developed or in the time elapsed between primary infections and rechallenge.

The GI mucus of sheep exhibiting resistance to *T. colubriformis* contained a substance that inhibited the migration of larvae *in vitro* (Douch *et al.*, 1983; Kimambo & MacRae, 1988). This substance contained cysteine-rich leukotrienes. Large quantities of this inhibitory factor may be produced to maintain resistance to parasites, limiting the quantity of cysteine available for wool production. Protein supplementation may thus improve the ability of parasitised animals to meet the needs of both parasitism and production. Immunoglobulin production is another important component of the immune response to nematodes in the GI tract (Charley-Poulain *et al*., 1984). However, plasma IgA concentrations were not increased in ewes on high protein diets (Houdijk *et al*., 2001b).

Kimambo *et al*. (1988b) noted that sheep that were trickle-infected with *T. colubriformis* initially grew at a decreased rate compared with uninfected controls, but had developed a degree of resistance to the parasite by Week 13 of dosing and subsequently grew at the same rate as the controls. This suggested that a nutritional penalty would be associated with the development of resistance, since protein would be required for the expression of an immune response and therefore diverted away from growth. A smaller ongoing cost of maintaining resistance was

manifest in the failure of the parasitised animals to undergo a period of catch-up growth despite adequate food intakes. However, Kyriazakis *et al*. (1996b) reasoned that since sheep chose not to select high protein components of their diet, despite previous evidence that they are able to do so (Kyriazakis *et al*., 1994), then protein probably was not limiting growth. In addition, further parasitic challenge of the same immune sheep by Kyriazakis *et al* (1996b) did not affect the growth rates of the animals. Such conflicting results require further clarification.

(c) The partitioning framework

Some of the conflict discussed in Section 2.3.7 (b) may be explained by the partitioning framework proposed by Coop and Kyriazakis (1999). It seems likely that in parasitised animals, priority is given to meeting the protein requirements for the development and maintenance of immunity in preference to meeting the protein requirements for growth and production. If protein intakes are marginal, production suffers. If protein intakes are high enough, satisfactory growth and production can be maintained in parasitised animals. According to the partitioning framework, the maintenance of body protein (including repair and replacement of damaged tissues) is given first priority in young growing animals and the acquisition of immunity is then given second priority. Third priority is given to productive processes such as muscle and wool growth, and the lowest priority is fat deposition. Once the immune response has developed, however, accretion of body protein is prioritised over the expression of immunity. It has been suggested that 20 - 25 % protein above maintenance requirements is required for increased resistance or resilience (Ketzis *et al*., 2006).

Improved protein nutrition can reduce the peri-parturient rise in faecal worm egg counts (PPR) that occurs in ewes (Donaldson *et al*., 1998; Houdijk *et al*., 2000) and goats (Chartier *et al*., 2000). The PPR is probably caused by a drop in immunity in response to lactation-related changes in plasma hormone concentrations (O'Sullivan & Donald, 1970) and disappears if lactation is prevented or stopped. A reduction in the PPR with protein supplementation suggests that pregnancy and lactation take precedence over maintenance of immunity in terms of protein partitioning in

reproductive animals. In other words, maintenance of body protein still has first priority, but reproductive function has second priority, maintenance of immunity third priority and maintenance of body fat last priority.

Kahn *et al* (2000), while agreeing with the overall framework, found that improvements in the expression of immunity took precedence over increased body growth when comparing sheep fed high or moderate levels of protein. These authors suggested that the effects of improved protein supply on immunity might differ, depending on the difference between the protein levels being compared, how close protein intake was to the maintenance requirement of the animal and the physiological demands on the animal. Houdijk *et al*. (2001a) suggested that a proportion of body protein can be classified as essential protein, which must be maintained. However, up to 25 % of protein may be mobilized to meet increased demands and this proportion can be classified as labile protein (McNeill *et al*., 1997). In line with this, ewes that were fed to maintain their body protein during mid-pregnancy had lower periparturient faecal egg counts than ewes fed a low protein diet during mid-pregnancy regardless of the dietary protein levels fed at parturition (Houdijk *et al*., 2001b). It has been estimated that periparturient ewes infected with *T. colubriformis* need an extra 1g metabolisable protein/kg $^{0.75}$ /day to prevent faecal egg counts from rising.

The partitioning framework is based on the assumption that animals have control over nutrient partitioning (Forbes, 1993), giving first priority to metabolic functions necessary for survival and second priority to functions that ensure genetic perpetuation. However, Coop and Kyriazakis (1999) did not offer an explanation as to the mechanism by which control is effected. Such an explanation is necessary for a full understanding of the interaction between parasitism and nutrition.

(d) Persistence of nutritional effects

It may not be necessary to feed high levels of protein continuously to obtain substantial levels of resistance to GI nematodes. Datta *et al*. (1999) fed isoenergetic diets differing in protein content to parasitised, weaned lambs for nine weeks after

weaning, and then put the lambs out to graze for 69 weeks. By the end of the experiment, the lambs that had been fed high protein diets after weaning had higher liveweight gains and wool growth, lower faecal egg counts and higher antibody responses to both *H. contortus* and *T. colubriformis* than the lambs that had been fed low protein diets. It is not known how long this carry-over effect may have lasted had the experiment continued, but it is clear that even short periods of supplementation around weaning can yield long-term benefits. By supplying sufficient protein to allow the development of an immune response to GI parasites without arresting growth, the need for compensatory growth later is avoided. It is likely, however, that prolonged responses to supplementation are only achieved if initial dietary protein levels are significantly limiting (Knox *et al*., 2003).

2.4 Issues in Parasite Control

2.4.1 Anthelmintics

Because the consequences of parasitic infection can be severe and even fatal, it is vital that effective methods for controlling parasites are available. Three broad families of anthelmintic drug commonly used in ruminant livestock are the benzimidazoles, the levamisole/morantel group and the macrocyclic lactones (avermectins and milbemycins). When anthelmintic resistance is not a problem, and when used responsibly, anthelmintics can reduce production losses due to GI parasitism, simply and cost-effectively. Responsible use includes dosing only when necessary to cure clinical disease or prevent the accumulation of infective larvae on pasture, administration of the correct dose rates and use of careful drenching techniques (Waller, 1997b). Whenever possible, narrow-spectrum drugs should be used in preference to broad-spectrum alternatives. Maintaining a proportion of the worm population in refugia (sequestered from drug exposure) may also delay the onset of resistance (Barnes *et al*., 1995). Such practices are essential if the development of anthelmintic resistance is to be prevented.

Anthelmintic resistance is characterised by reduced efficacy of anthelmintic compounds against a population of a particular nematode species compared to a

normal population of the nematode (Sangster & Gill, 1999). Resistance is present in a particular group of livestock if mean faecal egg counts are reduced by less than 95 % after drenching (Australian Agricultural Council, 2003). Resistance of sheep nematodes to the early anthelmintic phenothiazine was first suspected in the early 1950s, less than 20 years after it was first introduced, and was demonstrated experimentally by Drudge *et al*. (1957). The first case of thiabendazole resistance was reported seven years later (Drudge *et al*., 1964). Anthelmintic resistance is now worldwide (see review by Waller, 1997a). Benzimidazole resistance is the most common, but there are increasing reports of resistance to levamasole/morantel and the newer avermectins. Resistance to moxidectin has been described in *O. circumcincta* from sheep in New Zealand (Sutherland *et al*., 1999); in *H. contortus* from sheep in Australia (Love *et al*., 2003); and in *T. colubriformis* and *H. contortus* from goats in Australia (Le Jambre *et al*., 2005). In many cases, resistance is present without clinical signs of parasitism occurring in stock and so remains unsuspected (Australian Agricultural Council, 2003).

There are fewer reports of anthelmintic resistance in cattle than in other ruminant species, which might be related to differences in management. In contrast to sheep and goats, adult cattle are often not treated for GI parasites in many management systems, so less selection pressure is exerted on their parasites to develop resistance (Jackson & Coop, 2000). Nevertheless, a recent survey in New Zealand of 64 beef-producing units identified nematodes resistant to both ivermectin and albendazole on 74 % of farms (Meat & Wool New Zealand, 2006). The failure to identify many cases of anthelmintic resistance in cattle therefore may be due, at least in part, to failure to look for it (Coles, 2002). At the other end of the spectrum, goats have a high prevalence of resistant nematodes due to their need for frequent drenching compared to other species. An innate susceptibility to worms, a high level of rumen by-pass and a short half-life of anthelmintics in this species all contribute to this situation (Jackson & Coop, 2000).

The future of anthelmintic usage remains uncertain. Although widespread resistance problems have made the discovery of new families of anthelmintics highly desirable, the estimated US$100 million minimum required for the discovery and development of a novel antiparasitic compound has made many pharmaceutical companies

understandably reluctant to invest in this area (Witty, 1999). Several potential novel agents have been identified, but at best these are still in the development stage and may not be commercially available for some time (Condor, 1995; Geary *et al*., 1999). Reliance on anthelmintics alone to control GI nematodes is clearly unsustainable.

2.4.2 Alternative control methods

Alternatives to the use of anthelmintics to control GI parasites include rotational grazing strategies (Banks *et al.*, 1990; Barger *et al.*, 1994; Cheah & Rajamanickam, 1997; Barger, 1999), grazing different host species with different parasite susceptibilities on the same pasture (Niezen *et al*., 1996), breeding for resistance to nematodes (Albers *et al*., 1987; Gray, 1997), biological control methods such as predacious micro-fungi or bacteria (Larsen, 2000; Kotze *et al.*, 2005), administration of copper oxide wire particles (Burke *et al.*, 2005; Burke & Miller, 2006) and vaccination (Bain, 1999; Smith, 1999). However, it is rarely possible to avoid the use of anthelmintics altogether. A more common situation is to combine alternative techniques with the strategic use of chemical drenches, thus decreasing but not eliminating the need for anthelmintics. This principle is used in the FARMACHA© system for the control of *H. contortus* in sheep, which allows farmers to assess the degree of anaemia caused by the parasite by comparing the colour of the animal's mucous membranes to a standard chart. The worst affected animals can be identified for treatment, thereby avoiding unnecessary treatment of healthy animals (Van Wyk & Bath, 2002).

One further alternative to anthelmintics is the use of natural products to control parasites. Mannich bases in *Eucalyptus randis* leaves (Bennet-Jenkins & Bryant, 1996), eugenol in the essential oil of *Ocimum gratissimum* (Pessoa *et al*., 2002) and ethanol extracts of the leaves and seeds of *Melia azedarach* all exhibit anthelmintic activity. The seeds of the neem tree (*Azadirachta indica*), were shown to have anthelmintic activity against *H. contortus* in *in vitro* assays (Hördegen *et al.*, 2006). Two plants often used as anthelmintics in tropical regions are wormgrass (*Spigelia anthelmia*) and papaya (*Carica papaya*). Wormgrass contains a toxic alkaloid, spigelline, which was toxic to L_3 and adult *H. contortus* both *in vitro* and when

administered to sheep as a drench (Ademola *et al*., 2007). Papaya latex had anthelmintic activity against *Ascaris suum* in pigs (Satrija *et al*., 1994) and *Heligmosomoides polygyrus* in mice (Satrija *et al*., 1995), possibly due to digestion of the worms by plant proteases. However, the latex may be toxic in ruminants (Satrija *et al.*, 2001). Papaya seeds also have anthelmintic properties, the active compound being benzyl isothiocyanate (Kermanshai *et al*., 2001).

Another group of compounds recently investigated is the condensed tannins. A number of studies have shown that condensed tannins have beneficial effects in parasitised animals, either by reducing worm burdens or by improving production in spite of worm burdens (Niezen *et al.*, 1993; Hoskin *et al.*, 2000; Athanasiadou *et al.*, 2000a). Condensed tannins are present in many different forage species, including tropical shrub legumes.

2.5 Tropical Shrub Legumes

Shrub legumes are used widely throughout the tropical areas of Africa, Latin America, Asia and the Pacific as supplementary feeds for livestock (Devendra, 1995). They are versatile and various species can be used for fences, cover or ley crops, green manure (Skerman *et al*., 1988), firewood, pulp and paper, honey production, erosion control (National Research Council, 1983) and in some cases even provide a source of human food (Maslin & McDonald, 1996). Legumes can be used in cut-and-carry livestock production systems, which are common throughout Indonesia, or can be grown in alleys and fed *in situ* to cattle. The leaves of some species are suitable for preservation by drying. *Gliricidia sepium* has been used in this manner (Panjaitan, 2000).

Legumes have a higher nutritive value than tropical grasses and are useful especially during the dry season in regions where rainfall is unevenly distributed throughout the year. The plants provide a source of green leafy feed at a time when grass quality can be very low (Skerman *et al*., 1988). Although energy content of legumes varies widely and is often similar to that of grasses, protein content is generally much higher than in grasses. In a number of studies, the mean crude

protein content reported was 239 g/kg DM, compared with a mean of 77 g/kg DM for tropical grasses (Devendra, 1995).

It is clear from the discussion in Section 2.3 that the nutrition of the host animal, and in particular protein nutrition, has a profound influence on the outcome of parasitic infections. Shrub legumes, by virtue of their high protein content, may boost livestock nutrition in tropical areas, and in doing so, counteract some of the detrimental effects of GI parasitism. A common feature of many tropical shrub legumes, however, is the presence of condensed tannins in the leaves, bark and stems. The effects of condensed tannins on animal nutrition and production are variable and difficult to predict. There is increasing evidence that condensed tannins can be beneficial in preventing or reducing the effects of GI nematode parasitism through direct effects on the parasites themselves, in addition to the effects of protein supplementation. Conversely, there may be detrimental effects on ruminant protein nutrition due to the ability of condensed tannins to bind proteins. The influence of these properties on ruminant production will be discussed in the following sections.

2.6 Tannins

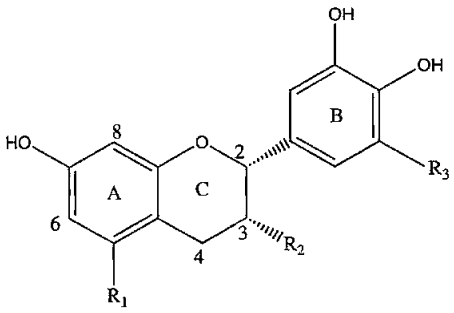
2.6.1 General properties

Tannins can loosely be defined as water-soluble plant polyphenols with a molecular weight of 500-3000, that can precipitate proteins and other macromolecules at an appropriate pH and concentration (Mangan, 1988). The ability to form stable cross-links with proteins is a key feature of tannins in animal nutrition (Swain, 1979). Other related compounds can often associate with proteins by the formation of unstable hydrogen bonds, and may be indistinguishable from tannins using common analytical methods. This, along with the diverse structural nature of tannins, has led to considerable debate as to the exact definition of tannins.

Tannins are toxic to a range of microorganisms including bacteria, yeasts and fungi, and constitute part of the plant defence mechanism against attack by these organisms (Scalbert, 1991). This may be important not only in the intact plant, but in delaying

decomposition of plant litter, thus regulating the supply of nutrients to the soil (Zucker, 1983). Tannins also afford protection against herbivorous insects and animals by complexing with ingested proteins and rendering them unavailable for digestion or by inhibiting digestive enzymes (Swain, 1979). The astringency of tannins may deter ingestion of certain plants.

Four groups of tannins have been identified: hydrolysable, oxytannins, β-tannins and condensed tannins (Swain, 1979). *Hydrolysable tannins*, such as tannic acid, consist of carbohydrates (almost always glucose) esterified to phenolic acids. *Oxytannins* are found only when plants are damaged, as a result of oxidation of other phenolic compounds. The *β-tannins* are a miscellaneous group of low molecular weight compounds (300-500) that are capable of precipitating proteins. The most numerous, however, are the *condensed tannins*, which are polymers of flavan-3-ols. These are also known as proanthocyanidins because the tannins are degraded by acid to anthocyanidins (Santos-Buelga & Scalbert, 2000). The precursors of tannins, such as flavan-3-ols and other phenolics, can be categorised as prototannins. Although they are often considered to be similar to tannins, the compounds generally do not have the same activity. The generalised structures of flavan-3-ols and proanthocyanidins are shown in Figure 2.1 and 2.2 respectively.



Structure	R_1	R_2	R_3
-Epicatechin	OH	OH	H
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH

Figure 2.1 The generalised structure of flavan-3-ols. R_2 = O-galloyl in the catechin gallates. Some examples of common flavan-3-ols are also given. From Schofield (2001).

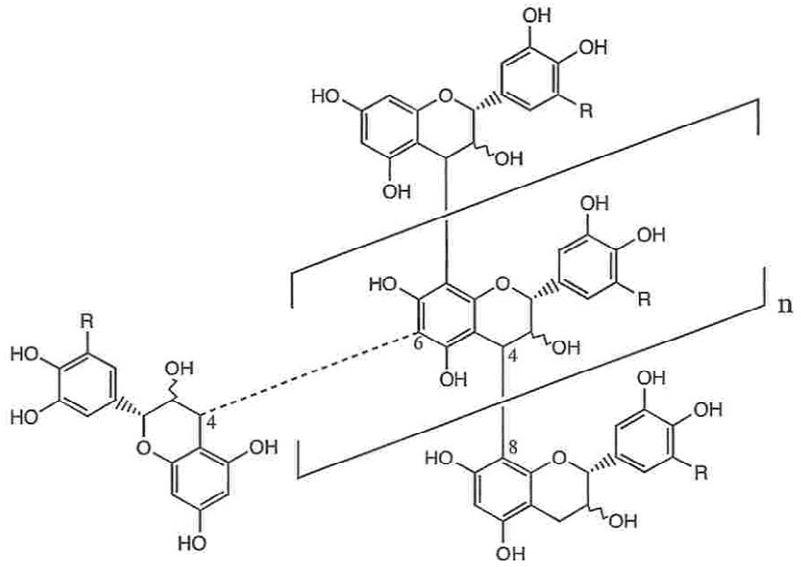


Figure 2.2 The generalised structure of proanthocyanidins. R = H in procyanidin or OH in prodelphinidin. The 4 → 8 linkage is most common, but the 4 → 6 linkage also occurs. The number of repeating units (n) is usually between 3 and 30. From Schofield (2001).

After considering the likely effects of the structures of hydrolysable and condensed tannins on the way they could be expected to interact with proteins, Zucker (1983) hypothesised that the two tannins had different ecological roles. Condensed tannins were more likely to be bound to structural components of the plant and be involved in protection from microbial attack. On the other hand, hydrolysable tannins were more likely to be contained in vacuoles within the cell, where they would be rapidly released after ingestion by a herbivore and bind to digestive enzymes. However, this idea has not been sufficiently tested. Condensed tannins are by far the most common and are the only tannins that will be considered further here.

2.6.2 Protein-binding properties

(a) Effects of tannin structure

Protein-tannin interactions are determined by both tannin structure (the number and position of reactive phenol groups) and by the properties of specific proteins (Asquith & Butler, 1986). Some proteins have a greater propensity to bind tannins than others, and each tannin will have a particular group of proteins to which it binds most readily. In general, tannins interact non-specifically with globular proteins such

as bovine serum albumin (Frazier *et al*., 2003) but have an affinity for proline-rich proteins, which form open random coils. Hydrogen bonds form between proline carbonyl groups and tannin phenolic groups, forming stable structures (Haslam, 1996). Hydrophobic regions on condensed tannin molecules tend to aggregate in water. Thus highly soluble tannins, which have few hydrophobic regions, usually have a lower affinity for proteins than less soluble tannins (Haslam, 1996).

Low molecular weight condensed tannins are too small to form effective cross links with proteins, whereas large molecules do not fit well within protein structures (Goldstein & Swain, 1963). Thus tannins that are intermediate in size are more likely to form tannin-protein complexes. Highly polymerised molecules also have fewer binding sites available to form C-C substitution compounds; however, reactivity of the hydroxyl groups is not affected.

(b) Effects of tannin concentration

Tannins do not bind to proteins in a 1:1 ratio (Jones & Mangan, 1977). A minimum amount of a particular tannin is required to precipitate a given protein, but the further addition of tannin results in an increased ratio of tannin to protein in the complex. The solubility of tannin-protein complexes depends on the size and concentration of both molecules. If tannin is present in excess, complexes are usually insoluble, whereas if protein is present in excess, complexes are usually soluble (Cannas, 2001). Solubility differences are due to the formation of extra bonds between tannin and protein molecules at higher tannin concentrations, forming more stable structures (Molan *et al*., 2001).

The stage of growth of the plant and the geographic and climatic conditions under which it is grown appear to have a strong influence on condensed tannin concentrations. The tannin content of *L. pedunculatus* grown on acid, poorly fertile soils is higher than that grown on high fertility soils or low fertility soils to which superphosphate fertiliser (9 % P, 11 % S) has been added (Barry & Forss, 1983). Considerable seasonal variation also occurs, with peak tannin concentrations seen in the dry season (Max *et al.*, 2004; Alam *et al.*, 2005; Vitti *et al.*, 2005).

In addition to the total condensed tannins (TCT) in a plant, it is also useful to consider the amounts that are extractable (ECT: also known as free condensed tannins), protein-bound (PCT) or fibre-bound (FCT). Only ECT seem to reduce digestibility (Balogun *et al*., 1998).

(c) Effects of pH

The pH of the medium is important in determining tannin-protein interactions. At pH values from 1 – 3, Jones and Mangan (1977) found that 95 % of Fraction 1 leaf protein was released from complexes with sainfoin condensed tannins, but at pH 4 - 6.5, stable complexes formed. Although complexes began to dissociate when the pH exceeded 7, about 70 % of protein was still bound to tannin at pH 8.5. Osborne & McNeill (2001) found that maximum precipitation occurred at the iso-electric point of the protein in the complex, but a change to a more acidic pH caused a greater reduction in precipitation capacity than a change to a more alkaline pH equidistant from the iso-electric point. The presence of some cations, particularly calcium and magnesium, increases the protein-precipitating capacity of condensed tannins (Martin *et al*., 1985).

(d) Effects of heat and drying

Optimum temperatures are required for tannin-protein complexes to form (Jones & Mangan, 1977). Sainfoin condensed tannins did not form complexes with bovine submaxillary mucoprotein at 37 °C and complexes formed below 25 ºC dissociated readily. Exposure to heat, UV light or certain enzymes (polyphenol oxidase) cause polymerisation of tannins and the formation of covalent bonds with protein (Silanikove *et al.*, 2001; Rakhmani *et al.*, 2005).

There have been contradictory reports on the effects that drying has on the condensed tannin content of plants, and on the subsequent nutritive value of the plants for animals. Drying decreased the digestibility and voluntary intake of dried *Calliandra* by sheep (Palmer & Schlink, 1992). Others found that the digestibility

of some species (notably *Calliandra*) was reduced by drying, but the digestibility of other species was not greatly affected (Balogun *et al*., 1998; Norton & Waterfall, 2000). However, increased intake, DM digestibility and N retention in sheep fed a supplement of oven-dried (60 ºC for 48 hours) *Calliandra*, compared with fresh *Calliandra*, have also been reported (Ahn *et al.*, 1997; Norton & Ahn, 1997). Such differences possibly relate to the method of drying. Under aerobic conditions, higher temperatures during the drying process increased PCT and FCT (Palmer *et al*., 2000) but decreased TCT slightly. Under anaerobic conditions, ECT and TCT concentrations increased as temperature increased. Digestibility was maximal at 45 °C for oven-dried samples, and was lower under aerobic conditions than under anaerobic conditions. Freeze drying gave similar results to oven drying at 45 °C. Sun, shade and oven drying techniques had variable effects on plant tannin concentrations depending on the plant species concerned but generally, oven and sun drying reduced extractable tannin concentrations more than shade-drying (Vitti *et al*., 2005).

2.6.3 Tannins and ruminant nutrition

As discussed in Section 2.4.2 (c) condensed tannins form stable complexes with protein between pH 3.5 and pH 7 and dissociate outside this range (Jones & Mangan, 1977). In theory, therefore, bound dietary proteins should be protected from microbial degradation in the rumen and then be released into solution in the abomasum, potentially becoming available for digestion in the small intestine. At the higher pH found in the duodenum, however, tannins could reassociate with dietary protein, digestive enzymes or intestinal wall proteins, with potentially detrimental effects on digestive processes. In practice, tannins may have either adverse or beneficial effects on the nutritive value of plants, depending on the amount and type of tannin present. Tannins have been found to decrease DM intake, DM digestibility, N digestibility (Merkel *et al*., 1999a) and average daily liveweight gain in sheep (Merkel *et al*., 1999b). Animal production is maximised at tannin concentrations of 30 - 40 g/kg, which are just high enough to make plant protein insoluble (Barry, 1985; Barry *et al*., 1986b). Metabolism also plays a role in the relative nutritive value of plants containing tannins. Narjisse *et al*.(1995) found that N balance and

rumen ammonia concentrations were significantly decreased in sheep infused intraruminally with oak tannins, but not in goats. Differences in rumen microbial populations or urea metabolism were postulated to cause this difference.

(a) Feed intake

Tannins are tasteless but astringent; that is, they increase the viscosity of saliva and increase friction in the mouth by binding to salivary proteins (Prinz & Lucas, 2000). This can deter feeding, thus reducing feed intake. Dietz *et al*. (1994) found that high levels of dietary quebracho tannins reduced body weight and survival rate in voles by reducing their feed intake, whereas the digestibility of N, DM and energy was not affected. Astringency may not be solely responsible for reduced feed intakes, however; the ECT content of *Desmodium ovalifolium* and *Flemingia macrophylla* was more closely related to reduced feed intake and digestibility than the relative astringency of the tannins (Barahona *et al*., 1997).

Animals that regularly consume considerable amounts of tannins may produce proline-rich salivary proteins that preferentially bind tannins, enabling the animals to detect and avoid excessive tannin intakes (Prinz & Lucas, 2000). Salivary proteins might also reduce the detrimental effects of tannins on dietary protein digestion (Robbins *et al.*, 1987; Hagerman & Robbins, 1993). In black bears and mule deer, which produce tannin-binding salivary proteins, 98% of quebracho tannin passes through the GI tract unchanged. In sheep, which do not have tannin-binding salivary proteins, 25 % of quebracho tannin is lost in the GI tract (Robbins *et al*., 1991). Salivary proteins vary considerably between species and are usually specific to tannins occurring in the preferred diet of the animal.

(b) Feed degradation in the rumen

In sheep fed *Lotus pedunculatus* with condensed tannin concentrations above 55 g/kg DM, rumen fermentation and transit of digesta through the rumen were slow. Volatile fatty acid production was reduced, but overall digestibility of fibre did not change, since longer fermentation resulted in more complete digestion (Waghorn *et*

al., 1994a; Waghorn *et al*., 1994b). In contrast, Barry *et al*. (1986b) found that the rate of carbohydrate digestion did not change, but the rate of outflow of undegraded carbohydrate increased, resulting in an overall decrease in digestibility. Min *et al*. (1998) noted only minor transient changes in VFA ratios. Wang *et al*. (1994a) recorded decreased ammonia concentrations and the production of minor VFAs associated with the deamination of specific essential amino acids (EAA), but concentrations of major VFAs and total VFA concentrations were not altered.

While plants such as *Calliandra* tend to be high in protein (e.g. more than 10 % of DM), their potential as protein supplements for ruminants is limited by the anti-nutritional effects of tannins. Adverse effects in the rumen may be due either to a direct inhibitory effect on rumen microbes, or to decreased microbial protein synthesis in the absence of sufficient available dietary N (Waghorn & Shelton, 1997; Getachew *et al.*, 2000). *In vitro* studies have demonstrated inhibitory effects of phenolic compounds on several rumen bacteria, including both cellulolytic and proteolytic species (Bae *et al*., 1993; Jones *et al*., 1994).

Condensed tannins reduced the growth rates of most strains of rumen bacteria examined, although a few species had only temporary increases in growth rates at low condensed tannin concentrations (Min *et al*., 2005a). The effect of condensed tannins on *Fibrobacter succinogenes* was bacteriostatic rather than bacteriocidal, and involved interference with bacterial adhesion to fibre and inhibition of bacterial enzymes, particularly extracellular enzymes (Bae *et al*., 1993). Fungal polysaccharidases were also inhibited by condensed tannins from a number of tropical shrub legumes (Barahona *et al*., 2006). In studies on the effect of condensed tannins from *Lotus* species on DM digestibility, hemicellulose digestion was impaired in the absence of any effect on cellulose digestion (Barry *et al.*, 1986b; Waghorn *et al.*, 1994b; Waghorn & Shelton, 1995). This might be explained by the binding of condensed tannins to free hemicellulase but not to cellulase, which is not present in a free form in rumen fluid. In other studies, tannins inhibited cell wall synthesis of susceptible proteolytic species (Jones *et al*., 1994) and reduced the attachment of rumen microorganisms to cellulose, thus reducing fibre digestion (Bento *et al*., 2005).

Mc Sweeney *et al*. (2001a; 2001b) investigated the effects of *Calliandra* condensed tannins in a series of *in vivo* experiments using fistulated sheep. They found that while protozoa, fungi and proteolytic bacteria were largely unaffected, the number of cellulolytic bacteria in the rumen decreased when *Calliandra* was added to the diet. This would probably inhibit fibre digestion. However, there was no effect on microbial protein synthesis, suggesting that the reduction in nutritive value associated with tanniniferous diets was due more to binding of the tannins with nutrients than to an effect on the rumen microorganisms. In studies with *L. pedunculatus*, extensive recycling of urea, probably of salivary origin, occurred in the rumen (Barry *et al*., 1986b; Waghorn *et al*., 1994b), so absence of N was unlikely to limit microbial growth and protein synthesis. Nevertheless, effects on the concentrations of other rumen metabolites cannot be discounted. Terrill *et al*. (1992a) found increases, not decreases in rumen microbial numbers in sulla-fed lambs, but this was probably related to the high fermentable carbohydrate content of sulla.

Which of these proposed mechanisms (inhibition of rumen microbes, reduced microbial protein synthesis, binding of tannins to dietary protein or microbial enzymes, reduced attachment of microbes to substrates) has the greatest effects on ruminant nutrition is as yet unclear. Structurally different tannins may have different effects on digestion in the rumen. Jones *et al*. (1976) compared condensed tannins extracted from a number of plants and found that, in general, astringency increased with increasing prodelphinidin content. In *Calliandra* accessions, polymeric proanthocyanidins seemed to increase the digestibility of plant protein, whereas oligomers, flavonols and flavonol glycosides decreased digestibility (Rakhmani *et al*., 2005). Aerts *et al*. (1999) also suggested that molecular weight was more important in determining the ability of condensed tannins to precipitate proteins than prodelphinidin content. However, astringency and ability to precipitate proteins do not necessarily correlate with digestibility *in vivo* (Barahona *et al.*, 1997; Andrabi *et al.*, 2005). Instead, N digestibility might be closely related to the concentration of ECT in the plant. Further research is needed to define the effects of condensed tannin structure on the biological activity of tannins and their effects on microbes.

Certain strains of tannin-tolerant rumen microorganisms may protect themselves against condensed tannins by the secretion of an extracellular polysaccharide matrix (Brooker *et al*., 1999) or by virtue of a glycocalyx that has a high affinity for tannins (Nicholson *et al*., 1986). *Selenomonas* species isolated from sheep, goats and an antelope adapted to tannins were able to grow in high concentrations of tannin in media, equivalent to 15 - 35 % hydrolysable tannin in feed DM, or 4 % condensed tannin (Odenyo & Osuji, 1998). Browsing animals such as goats that consume large amounts of tannin may be protected from the adverse nutritional effects of condensed tannins by the presence of these bacteria (Tjakradidjaja *et al*., 1999).

(c) Amino acid digestion in the small intestine

Inhibition of ruminal protein degradation by condensed tannins has been shown to increase the flow of amino acids to the small intestine (Waghorn *et al*., 1987; Waghorn *et al*., 1994b). This increase was accompanied by a change in the profile of amino acids entering the ileum, so that the proportion of EAA was significantly increased and the proportion of non-essential amino acids (NEAA) decreased slightly. Wang *et al*. (1996b) investigated changes in availability of the amino acids methionine and cysteine in sheep fed *Lotus corniculatus*. By inhibiting degradation of proteins in the rumen, tannins increased the quantities of these amino acids in duodenal digesta, thus improving the supply of EAA available for protein synthesis in the animal. However, Waghorn *et al*. (1994b) found that there was no increase in the amount of protein available to the animals, due to a concomitant decrease in feed intake and in the proportion of ingested amino acids absorbed from the small intestine. McNabb *et al*. (1993) also observed that *L. pedunculatus* condensed tannins fed to sheep prevented the loss of methionine and cysteine across the rumen and increased methionine absorption in the small intestine, but did not alter cysteine absorption. Condensed tannins decreased the digestibility of cysteine but not methionine. The net result was an increased flow of cysteine to body synthetic reactions, due to transulphuration of methionine to cysteine and decreased oxidation of methionine and cysteine. The mechanism by which absorption was inhibited is unknown, but it may be that pH within the small intestine was low enough (less than 7) to favour the formation of tannin-protein complexes (see Section 2.4.2). If so,

condensed tannins might have bound and deactivated enzymes required for amino acid absorption. Alternatively, tannins might have bound to dietary proteins and indirectly prevented absorption by preventing digestion (Waghorn *et al*., 1994b).

Given the contradictory results that have been obtained in studies on the effects of condensed tannins on digestion and absorption of amino acids, it is not surprising that effects on animal production have been equally variable. It is possible that differences in tannin-protein complex formation due to variable pH in the GI tract could explain differences in amino acid availability, which in turn could affect animal production responses. More research is needed to define the relationships between these factors.

(d) Mineral digestion

Tannins can complex iron, vanadium, manganese, aluminium, calcium, copper and cobalt (Haslam, 1996); however, condensed tannins generally do not alter mineral digestion. The main exception is sulphur, the degradation and absorption of which were impaired in the studies by Pritchard *et al*. (1992) and Waghorn *et al*. (1987; 1994a). In a subsequent study, absorption of sulphur remained unchanged, but because there was less degradation in the rumen the total amount of sulphur amino acids absorbed in the small intestine increased by 45 %. This might explain the increased wool growth seen in tannin-fed sheep in some experiments (Wang *et al.*, 1994b; Douglas *et al.*, 1995; Min *et al.*, 1998; Min *et al.*, 1999). Slightly lower absorption of potassium (Waghorn *et al*., 1987; Waghorn *et al*., 1994a) and magnesium (Waghorn *et al*., 1994a) also occurred in tannin-fed sheep. Copper and zinc were less soluble in the presence of tannins, but their absorption was not affected.

2.6.4 Tannins and animal production

(a) Wool and milk production

As discussed in Section 2.4.3 (e), dietary condensed tannins may increase the availability of sulphur-containing amino acids to body synthetic reactions. These amino acids are important for the synthesis of milk and wool, so increased absorption may partly explain increased milk and wool production in tannin-fed ruminants observed in a number of studies (Terrill *et al.*, 1992a; Wang *et al.*, 1994b; Douglas *et al.*, 1995; Niezen *et al.*, 1995; Wang *et al.*, 1996a; Paterson *et al.*, 1999). Tannins may alter the composition of milk, increasing lactose and protein and decreasing fat content (Wang *et al*., 1996a). Increased wool length and fibre diameter and decreased yellowness were also observed (Min *et al*., 1998; Min *et al*., 1999). However, some authors noted that wool growth in sheep that were fed condensed tannins only increased when conditions were particularly favourable for growth: in spring and summer but not winter (Wang *et al*., 1994b) or in growing lambs but not lactating ewes (Douglas *et al*., 1995). This may reflect differences in the demand for cysteine, such that condensed tannins will only increase wool growth rates when cysteine is limiting. Barry (1985) proposed that increased plasma growth hormone concentrations diverted amino acids to body protein deposition in preference to wool growth in tannin-fed lambs. However, in this study, condensed tannins also depressed growth rates, and it seems likely that changes in intake had the greatest effect on production.

(b) Fecundity

Increased EAA absorption may increase fecundity in ewes that are fed forages containing condensed tannins. Min *et al* (1999) recorded a 25 % increase in lambing percentage (lambs born/ewe mated) in ewes that are fed *L. corniculatus*, due to increased ovulation rates (corpora lutea/ewes mated), although fertility (ewes cycling/ewes mated) was not affected. The exact mechanism by which this occurred was unknown. Further work is needed to confirm the role of improved EAA supply in stimulating ovulation.

(c) Body composition

Abomasal infusions of casein (Oldham *et al*., 1977) or arginine and ornithine (Davenport *et al*., 1990) increased plasma growth hormone concentrations in ruminants, possibly as a result of increased amino acid availability. Consequently, increased absorption of EAA, particularly arginine, in tannin-fed animals might stimulate the release of growth hormone, resulting in increased protein synthesis and decreased fat deposition (Waghorn *et al*., 1987). Terrill *et al*. (1992a) also demonstrated a decrease in the body fat of lambs that were fed sulla. Barry *et al*. (1986a) found a positive linear relationship between plasma growth hormone concentrations and reactive condensed tannin concentrations (14 – 95 g/kg dietary DM) in sheep that were fed *L. pedunculatus* with varying amounts of polyethylene glycol (PEG). This was associated with an increase in lipolysis and a decrease in fat deposition. These authors proposed that growth hormone secretion was stimulated when tannins bound to proteins in the gut wall, in an attempt to promote the synthesis of replacement proteins. In contrast, Douglas *et al*. (1995) failed to show any change in body fat composition in lambs grazed on *L. corniculatus*. The reason for this discrepancy is not clear.

(d) Prevention of bloat

Bloat is a common condition of ruminants grazing lush clover pastures, in which soluble leaf protein causes a stable foam to form in the rumen. The gases formed during normal rumen fermentation thus are thus trapped in the rumen instead of being released by eructation. Death occurs rapidly due to pressure of the distended rumen on the heart and lungs, unless the gas is vented via a cannula or stab wound. Tannins prevent the formation of foams by forming insoluble complexes with leaf protein (Mangan, 1988). Tanniferous forages may also reduce methanogenesis, decreasing gas production (Puchala *et al*., 2005; Tavendale *et al*., 2005).

2.6.5 Potential of tanniniferous plants as animal feeds

Tannin-containing plants may be an economical way of increasing animal production under conditions where the beneficial effects can be optimised and the anti-nutritive effects reduced. Much is yet to be learnt about the mechanisms by which tannins influence nutritive value and the best ways to make use of them. The greatest nutritional benefits may be obtained from tropical shrub legumes when these plants are used to supplement low quality forages that have a low N content (Merkel *et al*., 1999b). However, shrub legumes might also be beneficial as a means of parasite control. This aspect of their use is yet to be explored thoroughly.

2.7 Tannin-Parasite Interactions

2.7.1 Effects on production

Recently there has been interest in the potential of forages containing condensed tannins to moderate the effects of GI parasitism in livestock. Grazing trials in New Zealand (Niezen *et al.*, 1995; Robertson *et al.*, 1995; Hodgeson *et al.*, 1996; Niezen *et al.*, 1998b; Niezen *et al.*, 1998c; Niezen *et al.*, 2002a), focused initially on sulla (*Hedysarum coronarium*), lotus major (*L. pedunculatus*) and birdsfoot trefoil (*L. corniculatus*) but many other plants have since shown promise. Indices used to assess anthelmintic responses to tannins in the diet include weight gain, wool production, faecal egg counts, post-mortem worm counts and dag formation (accumulation of faecal material on the wool of the perineal region).

(a) Feed intake and feed conversion efficiency

Gastrointestinal parasitism causes reduced feed intake, which contributes to the poor productivity of parasitised animals. Inappetance can be largely overcome if the animals are maintained on a high protein diet (Abbott *et al*., 1988; Bown *et al*., 1991a). Forages containing condensed tannins might improve protein nutrition by the formation of tannin-protein complexes in the rumen, thus increasing the supply of protein to the small intestine. Consequently, Niezen *et al*. (1995; 1998c)

observed a higher feed intake by parasitised lambs that were grazed on tanniniferous forages compared with parasitised lambs that were grazed on forages without tannins. However, parasitised lambs that were grazed on sulla had lower rates of liveweight gain than non-parasitised lambs on the same feed, despite higher feed intakes, suggesting that feed conversion efficiency was impaired (Niezen *et al*., 1995). Athanasiadou (2000a) also noted a decrease in the feed conversion ratio of sheep consuming quebracho tannin. This was probably due to decreased digestibility of the diet when tannins were included. In contrast, Hoskin *et al*. (2000) observed that deer that were grazed on sulla had higher growth rates but similar voluntary feed intakes compared with deer grazed on lucerne. They suggested that feed conversion ratio was improved in the former group.

(b) Liveweight gain and wool production

In a series of trials, Niezen *et al* (1993; 1995) compared growth rates in parasitised lambs grazing different pasture species, using both naturally acquired and artificially induced mixed infections of GI nematodes (notably *T. colubriformis*). Lambs that were grazed on *L. pedunculatus* or sulla grew faster than lambs that were grazed on ryegrass or lucerne, which contain only trace amounts of condensed tannins. In anthelmintic-treated animals grazed on lucerne or sulla, growth rates were similar between the two feeds in one experiment but unexpectedly higher on sulla in another (Niezen *et al*., 1995). The wool growth of undrenched sulla-fed lambs was comparable with that of drenched sulla-fed lambs, and was significantly higher than that of undrenched lucerne-fed lambs. Wool fibre diameter followed the same trend; however, the effects on wool growth were probably due to the effects of tannins on amino acid supply [Section 2.6.4 (a)], rather than to a reduction in parasitism.

Further trials compared a number of forage species including *L. pedunculatus*, chicory (*Chicorium intybus*), low oestrogen red clover (*Trifolium pratense* cv G27), high oestrogen red clover (*T. pratense* cv Pawera), lucerne , sulla, ryegrass/white clover (*Lolium perenne*/*T. pratense* cv Huia), plantain (*Plantago lanceolata*) and *L. corniculatus* (cv Goldie) (Niezen *et al*., 1994; Robertson *et al*., 1995). Again,

animals grazing on *L. pedunculatus* and sulla had higher growth rates and wool production than animals grazing on ryegrass/white clover or lucerne pastures. Leathwick and Atkinson (1995) and Ramirez-Restrepo *et al*. (2004) also obtained higher growth rates in lambs fed *L. corniculatus* than lambs fed ryegrass. In the latter study, increased fleece weight and staple length in ewes and lambs were also recorded.

Significantly higher growth rates and carcass dressing-out percentage were seen in deer fed sulla compared to deer fed lucerne, while *L. corniculatus* gave intermediate results (Hoskin *et al*., 2000).

(c) Dag formation and flystrike

Forages containing condensed tannins tend to reduce dagginess in parasitised lambs compared with lucerne or ryegrass/white clover (Niezen *et al.*, 1993; Leathwick & Atkinson, 1995; Niezen *et al.*, 1995; Min *et al.*, 1998), but how this occurs is unclear. Reduced dag formation in lambs grazed on *Lotus* or sulla did not appear to correlate with increased faecal DM , and may have been a direct effect of tannins in the diet rather than an indirect effect due to reduced worm burdens (Leathwick & Atkinson, 1996). However, Niezen *et al* (1993) did find an increase in faecal DM in lambs grazed on *Lotus*; in this case, the lambs also had lower faecal egg counts.

In one study, lambs grazed on sulla had high dag weights; however, this became apparent only after a series of frosts, and it was postulated that the frosts may have altered the constituents of the forage (Robertson *et al*., 1995).

Most research into the antiparasitic effects of forages containing tannins has concentrated on GI nematodes, but there is also a potential benefit in preventing flystrike due to decreased dag formation. Decreases in both dagginess and flystrike were observed in parasitised lambs grazed on *L. corniculatus* for at least seven days per fortnight (Leathwick & Atkinson, 1995; 1996).

(d) Milk production

Studies investigating the effects of condensed tannins on milk production in parasitised animals are few. In one study, lactating Angora does and their kids grazed on either sericea lespedeza (*Lespedeza cuneata*) or crabgrass/Kentucky 31 tall fescue (*Digitaria ischaemum/Festuca arundinacea*) pastures for 81 days. The does had naturally acquired mixed parasite infections. At day 60, milk from the does fed sericea lespedeza had higher total milk solids and solids-not-fat and lower milk somatic cell counts than milk from the does on the control pasture (Min *et al*., 2005b). Faecal egg counts decreased and development of L_3 larvae from the faeces of infected goats was reduced. However, the source of parasite infection for these animals was the pasture itself and pasture larval contamination was assessed only by the use of tracer animals killed after the end of the experiment. Since tracer animals on the sericea lespedeza pasture had lower worm burdens, does on this pasture were probably exposed to lower levels of infected larvae. It is unclear whether this was due to condensed tannins in the pasture or simply to lower pasture contamination rates prior to the experiment.

Alternatives to the use of commercial anthelmintic drenches are of particular relevance in milk-producing animals because of the need to withhold milk from drenched animals from sale. The effects of condensed tannins on parasitised milk-producing animals (both cattle and goats) therefore deserve more attention.

2.7.2 Effects on nematode parasites

(a) Faecal egg counts and worm burdens

In young lambs that have not yet developed an immune response to GI parasites, faecal worm egg counts are a reasonably accurate measure of the level of parasitism. A number of studies have shown that parasitised lambs grazing forages high in condensed tannins, including sulla, sainfoin and *L. pedunculatus,* had lower faecal egg counts than lambs grazing lucerne or ryegrass (Niezen *et al.*, 1993; Niezen *et al.*, 1994; Niezen *et al.*, 1995; Robertson *et al.*, 1995; Butter *et al.*, 2000;

Tavendale *et al.*, 2005; Heckendorn *et al.*, 2007). A variety of worm species, particularly *H. contortus*, *T. colubriformis*, *Teladorsagia circumcincta*, *Nematodirus* species and *Cooperia curtecei* were affected. In many cases, the total worm burdens were also reduced (Niezen *et al.*, 1993; Niezen *et al.*, 1995; Robertson *et al.*, 1995). Smaller abomasal worm burdens were apparent in red deer consuming sulla (Hoskin *et al*., 2000). Other plants with reported anthelmintic effects include Yorkshire fog (*Holcus lanatus*) (Hodgeson *et al*., 1996), heather (*Erica* species and *Calluna vulgaris*) (Osoro *et al*., 2007) and chicory (*Chicorium intybus*) (Hoskin *et al*., 1999).

Reductions in faecal egg counts or worm burdens are not consistent, however. In one study, lambs with mixed natural infections grazed on *L. pedunculatus* had similar faecal egg counts and worm burdens to control lambs grazed on ryegrass/white clover pastures, although lamb growth rates were higher on the *L. pedunculatus* pasture (Niezen *et al*., 1998b). Lambs grazed on sulla had lower worm burdens and higher growth rates than the control lambs, but also had higher faecal egg counts. Pomroy and Adlington (2006) found no reduction in established mixed worm burdens of parasitised young goats after 10 days of feeding on sulla. Leathwick and Atkinson (1995) found no difference in faecal egg counts between lambs fed *L. corniculatus* and lambs fed ryegrass.

In a two-year experiment in which ewes with lambs were grazed on either ryegrass/white clover pastures or *L. corniculatus*, faecal egg counts were lower in the ewes that grazed on lotus in both years (Ramirez-Restrepo *et al.*, 2004). In particular, there was a reduction in the PPR. Early in the grazing season, the lambs grazed on the *L. corniculatus* pasture had lower faecal egg counts, but by Day 70, faecal egg counts in these lambs were equal to or higher than those of the lambs on the control pasture. It was suggested that chicory or sulla should be fed to lambs after weaning, as *L. corniculatus* did not keep faecal egg counts under control in the lambs. Worm burdens were similar in ewes and lambs on both pastures.

When decreased worm burdens were recorded, the nematode species affected were variable. There are reports of decreases in *Ostertagia* and *Trichostrongylus* (Niezen *et al*., 1994); *Trichostrongylus* (Niezen *et al*., 1995); *Ostertagia* but not

Trichostrongylus (Niezen *et al*., 1998c); *Trichostrongylus* and *Ostertagia* but with a greater effect on the former (Hoskin *et al*., 2000); or *Haemonchus, Trichostrongylus, Ostertagia, Cooperia* and possibly *Nematodirus* (Niezen *et al*., 1998b).

In some cases, the proportions of species in worm populations have changed completely. In one study involving both ewes and lambs, numbers of *T. colubriformis* and *Ostertagia* decreased but *Cooperia*, *Chabertia* and *Oesophagostamum* species all increased in animals grazed on *L. corniculatus* compared with animals grazed on ryegrass/white clover pastures. This was mainly of significance in the lambs (Ramirez-Restrepo *et al.*, 2004). In a second study using only lambs, burdens of *H. contortus*, *Teladorsagia* species, *Nematodirus* species and *Cooperia* species were reduced, but burdens of *Trichuris ovis*, *Trichostrongylus*, *Oesophagostamum* and *Chabertia* species increased (Ramírez-Restrepo *et al.*, 2005). However, fewer *Trichostrongylus* developed on incubation of faeces from lambs on the *L. corniculatus* pasture than from those on ryegrass/white clover. In another study, pasture-fed goats supplemented with sainfoin hay 10 days per month had *T. colubriformis* burdens that were 50 % lower than those of goats supplemented with lucerne hay (Paolini *et al*., 2005a). Concurrently, *H. contortus* and *T. circumcincta* burdens increased in the goats fed sainfoin, so that overall worm burdens were not different between the groups.

Such inconsistent effects have yet to be explained but may be due to differences in feeding habits or specific habitats within the GI tract. In Wistar rats infected with *N. brasiliensis* or *T. spiralis* and fed quebracho tannins, only burdens of *N. basiliensis* were reduced, even though *T. spiralis* mortality during *in vitro* exposure to quebracho tannins was very high (Butter *et al*., 2001). In the rat, *T. spiralis* lies embedded in the mucosa and may not have sufficient contact with tannins to be affected. Similarly, in ruminants, nematodes found on the surface of the mucosa, such as *H. contortus*, may be more susceptible to tannins than worms such as *T. colubriformis* that inhabit tunnels in the mucosa. It is clear that the factors determining the effects of condensed tannins on GI nematode need further elucidation.

(b) Larval stages

Tanniniferous forages may affect the free-living stages of GI nematodes, as well as the parasitic stages of the life cycle. Infective larval populations in pot and plot experiments were lower on both birdsfoot trefoil and chicory than on ryegrass (Marley *et al*., 2006a). This is perhaps not surprising, as condensed tannins act as a defence mechanism in plants (Collingborn *et al*., 2000). In another study, seven different forages with condensed tannin concentrations ranging from 0 - 17.4 % DM were examined for their effects on larval development (Niezen *et al*., 2002b). Condensed tannins reduced the development of larvae both *in vitro* and on pasture, with the plants containing the highest condensed tannin concentrations (*Dorynium pentophyllum* and *D. rectum*) having the greatest effect. However, development inhibition was greater on pasture than *in vitro*, possibly because other factors besides condensed tannin content (e.g. plant structure) influence the number and species of L_3 larvae that develop and migrate up the sward (Niezen *et al.*, 1998a; Marley *et al.*, 2005; Marley *et al.*, 2006b).

Condensed tannins may reduce larval intake in other ways as well. Lactating goats at pasture, that were fed sainfoin hay 10 days per month, had small but significant decreases in faecal egg counts compared with controls fed lucerne hay 10 days per month (Hoste *et al*., 2005a). However, the animals fed sainfoin had higher hay intake than those fed lucerne and thus may have had reduced pasture intake. As the pasture was the source of larvae in this experiment, larval intake may also have been reduced.

Parasite eggs and larvae are also exposed to condensed tannins within the GI tract of the host. Egg hatch and larval development assays have been used to demonstrate the effects of tannins on helminth GI parasites *in vitro*. Condensed tannins from *L. corniculatus*, *L. pedunculatus*, sulla and sainfoin were all able to prevent the development of *T. colubriformis* from eggs to L_3 larvae at a concentration of 400 µg/ml (Molan *et al*., 1999). Both eggs and L_1 larvae were affected, but the inhibition of egg development was more marked. The influence of condensed tannins on L_3 larval migration was less noticeable, suggesting that the L_3 were not particularly sensitive to tannins. However, Molan *et al*. (2000b) performed

larval migration inhibition assays on larvae from deer and found that condensed tannins from the same forages significantly inhibited L_1 lungworm larvae and L_3 GI nematode larvae. Inhibition was greater when the larvae were incubated at 37 °C than at 22 ºC. The effects on L_3 lungworm larvae could not be assessed due to inactivity of both the control larvae and those incubated with tannins, but mortality rates were high in L_3 lungworm larvae exposed to the tannins. Perhaps the differences in potency of condensed tannins against L_3 GI nematodes in these two studies were due to the fact that the deer GI parasites cultured by Molan *et al.* (2000b) were predominantly *Ostertagia* species. Possibly *Ostertagia* are more susceptible to the effects of condensed tannins than *Trichostrongylus*. In both experiments the ranking of the plant extracts, from most effective to least effective, was sainfoin > *L. pedunculatus* > sulla > *L. corniculatus*. These differences in potency were attributed to possible differences in the condensed tannin structures in the plants.

Both condensed tannins and sesquiterpene lactones extracted from chicory inhibited larval migration of GI nematode L_3 and *Dictyocaulus viviparus* L_1 and L_3 larvae derived from red deer (Schreurs *et al.*, 2002; Molan *et al.*, 2003a). Condensed tannins inhibited larvae more effectively when incubated in rumen fluid than in abomasal fluid, probably because the pH of rumen fluid was more appropriate for binding of tannins to proteins. The anthelmintic activity of sesquiterpene lactones was not affected by pH.

Sainfoin condensed tannins inhibited migration of *H. contortus* larvae (Barrau *et al.*, 2005).

Reduced egg hatch and larval development rates after exposure to condensed tannins on pasture or in ruminant digesta could reduce pasture contamination with infective larvae, thus decreasing host infection rates. However, the results of *in vitro* studies do not always apply to field situations. Research is necessary to confirm that infection rates are reduced when ruminants consume condensed tannins.

(c) Nutritional and toxic effects

Improved protein nutrition may boost the ability of an animal to mount an immune response to parasites (Coop & Kyriazakis, 1999). If the anthelmintic effects of tannins on parasitised animals were mediated solely through an increased supply of protein (resulting in an improved immune response), similar worm burdens and faecal egg counts would be expected in tannin-fed and control animals when the protein content of the diet was not limiting. Athanasiadou *et al*. (2000a) administered a trickle-infection of *T. colubriformis* to eight-week-old lambs for ten weeks. The lambs were fed a high-protein diet incorporating 0, 30 or 60 g/kg DM quebracho tannin (a commercial extract obtained from trees of the genus *Schinopsis*). Two groups of lambs received quebracho only during the first five weeks, to examine the effects of tannins on the establishment of infection, while two groups received tannin only during the second five weeks to examine effects on the expression of acquired immunity. Two groups received tannins throughout the experiment, one received no tannin and one group served as an uninfected control group. Faecal egg counts were reduced in the lambs receiving quebracho in either period, compared with parasitised sheep that were not given tannins. The faecal egg count reduction was 25 % during the first period and 40 % in the second. Worm burdens were also reduced in lambs that had received quebracho in the second period. The reduced faecal egg counts during the first period, when immunity was not established, supported the suggestion of a direct effect of tannins on the parasites. The greater reduction in the second period was attributed to greater effectiveness of the tannins when parasites were first exposed as adults than when they were exposed mainly at the larval stage, since adults inhabit the intestinal lumen, while larvae penetrate beneath the epithelium. However, the possibility that improved protein nutrition also contributed to an improved immune response during the second period cannot be ruled out.

The likelihood that a direct toxic effect occurs was supported by the results of a second, short-term study. In sheep infected with *T. colubriformis* and drenched with quebracho tannin for a period of one week before slaughter, Athanasiadou *et al*. (2000a) found a 50 % reduction in faecal egg counts and a 30 % reduction in worm burdens compared with controls. The number of eggs *in utero* was similar for female worms from both groups, but the number of eggs produced per female worm was less in the treatment group. However, as faecal production was not measured, it was not

possible to ascertain whether this was due to a decrease in egg laying or to the treatment group producing more faeces (Athanasiadou *et al*., 2000a). The lambs used in this study had not previously been exposed to parasites, and would need four to five weeks to develop an effective immune response, indicating a direct action of tannins upon the worms.

Further evidence that the effects of tannins on worm burdens are not due to protein-induced improvements in immunity comes from the work of Butter *et al* (2000) who fed lambs either a high or low protein diet, with or without quebracho tannin extract. Faecal egg counts were reduced by the high protein diet without tannins and by the low protein diet with tannins. However, the high protein diet with tannins resulted in higher faecal egg counts, similar to those seen in lambs on a low protein diet without tannins. The excess of dietary protein might have resulted in the formation of tannin-protein complexes that were unable to dissociate in the abomasum or that re-formed in the small intestine in conditions of neutral pH. Tannins might thus have been unavailable to exert an effect on intestinal nematodes.

2.7.3 Factors determining efficacy

(a) Timing of tannin administration

It is likely that the timing of tannin administration during nematode infection is important. When adult goats infected with *H. contortus* were drenched with quebracho tannin for eight days during the patent period, faecal egg counts declined by the fifth day of drenching and remained low at least one week after the cessation of drenching. Worm numbers were not affected (Paolini *et al*., 2003a). Similarly, in kids with established infections of *T. colubriformis* and *T. circumcincta*, quebracho tannins decreased faecal egg counts by 50 % compared to control animals that did not receive tannins. The difference was maintained until slaughter, 18 days after tannin administration ceased (Paolini *et al*., 2003b). There was no reduction in total worm burdens, but there was a reduction in fecundity of *T. colubriformis*. In contrast, tannins administered during the period of larval establishment only (three days before and after dosing with larvae) decreased worm burdens but not

fecundity. The anthelmintic effects observed may depend on the stage of the worm lifecycle exposed to condensed tannins.

Effects on developing and established worm populations have not been consistent, however. In another trial by Paolini *et al*. (2005b) neither quebracho extract nor sainfoin hay caused significant reductions in the establishment of *H. contortus* L_3 in 5-month-old kids. Conversely, a diet of sericea lespedeza (*Lespedeza cuneata*) reduced lamb faecal egg counts in both established and developing infections, but reduced worm burdens only in animals with established infections (Lange *et al*., 2006). Athanasiadou *et al*. (2005) found that six tanniniferous forages failed to affect *T. colubriformis* worm burdens or egg production in lambs, regardless of whether the forages were fed during the period of larval establishment or when the worms had developed to the adult stage. Lambs infected with 20,000 *T. colubriformis* and drenched daily with quebracho tannins from days 28 – 35 post-infection, had reduced faecal egg counts and worm burdens, as well as *per capita* egg production by female worms (Coop & Jackson, 2001).

In a further study, sheep infected with *H. contortus* were fed *A. pintoi*, *M. esculenta* or *Gliricidia sepium* forage beginning either on the day of infection or on the day infections became patent (Rojas *et al*., 2006). Faecal egg counts were not monitored, but all three forages reduced worm burdens. However, while *A. pintoi* and *M. esculenta* were effective when fed only after patency, they did not affect worm burdens when fed throughout both phases of the infection. This could indicate that the worms developed some form of resistance to the condensed tannin when exposed for a longer period. In contrast, *G. sepium* only affected worm burdens when fed throughout both phases of the infection. The reason for these discrepancies is unknown, but possibly reflects variations in affinity of condensed tannins in the three plant species for nematode proteins at each stage of the worm life cycle. More information is needed on the effects of condensed tannins at different stages of infection.

(b) Physical form and concentration

Another important determinant of the efficacy of condensed tannins is the form in which they are administered. A pelleted diet containing quebracho tannins at concentrations of 25, 50 or 80 g/kg DM had no significant effect on faecal egg counts when fed for 65 days to sheep trickle-infected with *H. contortus* (Max *et al*., 2005). However, drenching sheep for only three days at a dose rate equivalent to 80 g/kg DM reduced both faecal egg counts and worm burdens. One suggested explanation was that the tannins formed complexes with feed proteins during the pelleting process, reducing the concentration of tannins available to interact with worms in the digesta. Drenching with quebracho also caused increased mucus secretion and diarrhoea, which might help to clear worms from the digestive tract. However, the most likely explanation was that higher concentrations of condensed tannins were reached in the GI tract after drenching than when tannins were incorporated in the feed.

In goats with naturally acquired nematode infections, pelleted sericea lespedeza (*Lespedeza cuneata*) hay reduced faecal egg counts and decreased populations of abomasal (but not intestinal) worms more than ground hay (Terrill *et al*., 2007), despite an increase in the proportion of PCT during the pelleting process. The authors suggested that structural differences in the condensed tannins might explain the superior effect of pelleted hay. However, consumption of the pelleted feed was stated to be higher than consumption of the ground feed, although actual values were not reported. Thus, the improved anthelmintic effect might simply have been due to increased condensed tannin dosage.

Sainfoin proved to be equally effective at reducing *H. contortus* and *C. curticei* faecal egg counts and burdens of *H. contortus* when fed as either hay or silage (Heckendorn *et al*., 2006). Whether or not there is an added anthelmintic benefit of feeding hay or silage over fresh forage, the management advantages of being able to obtain anthelmintic effects from conserved forages should not be overlooked. Athanasiadou (2001b) suggested that sheep fed *ad libitum* were less likely to experience the anthelmintic effects of tannins than sheep that were fed restrictively. These authors suggested that during restricted feeding, the entire ration would be

consumed rapidly, resulting in high concentrations of condensed tannins in the GI tract, whereas the same dose of tannins spread throughout the day might not reach high enough concentrations in the GI tract to affect worms. *Ad libitum* feeding would also increase digesta flow rates and reduce contact time between tannins and worms. Achieving a high concentration of condensed tannins in the GI tract of the host is probably a key factor in obtaining anthelmintic benefits from tannin-rich forages.

(c) Host species differences

Different effects of condensed tannins due to nematode species differences have already been described [Section 5.2.2 (a)]. The efficacy of condensed tannins against GI nematode parasites may be affected by host species also. A wattle (*Acacia mearnsii*) tannin drench administered to goats on days 30 – 32 post-infection failed to decrease faecal egg counts or worm burdens by day 42 (Max *et al*., 2003). When a similar trial was conducted using sheep, faecal egg counts were reduced by 75 %, *Haemonchus* burdens by 87 % and *Oesophagostomum* burdens by 28 % (Max *et al*., 2004). These workers speculated that defence mechanisms enabling goats to tolerate the nutritional effects of tannins (such as tannin-binding salivary proteins, tannin-tolerant rumen bacteria, and microbial tanninase in the rumen) might also reduce the anthelmintic effects of tannins. This possibility has yet to be investigated.

(d) Tannin structure

It is likely that the physical structures of condensed tannins within a plant affect the anthelmintic efficacy of the plant. Scalbert (1991) noted that inhibition of bacterial growth was related to B-ring hydroxylation and postulated that prodelphinidins should be more inhibitory against bacteria than procyanidins. The same is apparently true for nematodes. In two *in vitro* studies, prodelphinidin monomers inhibited egg hatching, larval development and the larval viability of *T. colubriformis*, as well as the exsheathment of *H. contortus* and *T. colubriformis* more effectively than PC monomers (Molan *et al.*, 2003b; Brunet & Hoste, 2006). It appeared that molecules with greater numbers of free hydroxy groups were more potent in interacting with nematodes. Nematode species differences in susceptibility could be due to

differences in the structure of the sheath or the enzymes involved in exsheathment (Brunet & Hoste, 2006).

2.7.4 Persistence of effects

Studies to date on the persistence of the effects of condensed tannins have produced variable results. In some studies reductions in faecal egg counts have been maintained after return to a non-tannin diet (Paolini *et al*., 2003b) whereas others have reported that faecal egg counts rose again almost immediately after tannin feeding ceased (Lange *et al*., 2006). Min *et al*. (2004) used a cross-over design to examine the effects of sericea lespedeza pasture on faecal egg counts in wethers with established nematode infections. Animals that grazed the control forage (*Digitaria sanguinalis*) for the first fifteen days of the trial had a decrease in faecal egg counts when moved to sericea lespedeza pasture for the second fifteen days of the trial. Animals that grazed sericea lespedeza first had lower faecal egg counts in the first half of the trial, but these increased slowly after changing to the control forage for the second fifteen days. The persistence of anthelmintic effects probably depends on whether worm numbers were reduced or whether effects were confined to the suppression of egg output.

2.7.5 Tropical plants

Although early research into the anthelmintic effects of condensed tannins focussed on temperate forage species, many researchers have recently turned their attention to tannin-containing tropical plants. In Uganda, goats feeding on tropical browse had lower faecal egg counts and higher weight gains than goats on the same feed that were drenched twice daily with PEG to bind condensed tannins (Kabasa *et al*., 2000). The main source of tannin in this study was possibly *Acacia* species, but this was unclear. Browsing might reduce parasitism in goats due to the effects of condensed tannins in the diet, as well as by the previously accepted mechanism of preventing uptake of larvae from pasture. Recently, the effects of *A. nilotica* and *A. karoo* on GI nematodes of goats were examined more specifically (Kahiya *et al*., 2003). Significant reductions in both worm burdens and faecal egg counts were achieved in

goats fed a basal diet consisting of 40 % *A. karoo* and 60 % concentrates and offered *Chloris guyana* (Rhodes grass) hay *ad libitum*. Similar effects did not occur when *A. karoo* was replaced by *A. nilotica*, or when concentrates were fed as 100 % of the basal diet. Of the two legume species, *A. nilotica* had a much higher total phenolic content, but contained very little condensed tannin, whereas the condensed tannin content of *A. karoo* (expressed as absorbance units) was approximately 12 times greater. However, lambs on both the *Acacia* diets, but particularly the *A. karoo* diet, had lower weight gains compared to controls, probably due to effects on protein nutrition. *Acacia* meal fed to parasitised sheep for 30 days did not significantly reduce faecal egg counts. However worm burdens in these animals were higher than in sheep on a non-taniniferous diet, so *per capita* egg production by the worms may actually have reduced (Max *et al*., 2004). *Acacia mearnsii* bark reduced both faecal egg counts and worm counts in sheep when administered as a weekly drench over an 84 day period (Cenci *et al*., 2007).

Extracts of four tropical plants (*Zanthoxylum zanthoxyloides*, *Newbouldia laevis*, *C. papaya* and *Morinda lucida*) were found to inhibit egg hatching and larval migration *in vitro* (Hounzangbe-Adote *et al*., 2005a). Adult motility was also reduced. However, responses were not dose dependent. With the exception of papaya seed extract (see Section 2.2.2) the active compounds in the extracts were not identified, but were suggested to be condensed tannins. Fagara (*Z. zanthoxyloides*) was also fed to sheep on a basal diet of guinea grass and cassava (*Manihot esculenta*) peelings and given a single dose of 2500 *H. contortus* larvae (Hounzangbe-Adote *et al*., 2005b). When fed at 4 g/kg bodyweight from days 21 - 23 post-infection, a 41 % reduction in faecal egg counts was observed by day 35 and the number of eggs *in utero* of female worms was markedly reduced. When naturally infected animals were fed 500 g fagara leaves three times weekly for 45 days, faecal egg counts were significantly lower than the control group by day 45. When fagara was fed daily from days 28 to 30 post-infection, a smaller reduction in faecal egg counts occurred, indicating that sustained treatment was more effective.

Cassava foliage (sometimes fed as hay) may also have anthelmintic effects. Some authors reported the active compound to be cyanogenic glycosides (Sokerya & Rodriguez, 2001) while others contended that condensed tannins caused anthelmintic

effects (Netpana *et al.*, 2001; Lin *et al.*, 2003). Goats fed cassava foliage had reduced faecal egg counts, reduced shedding of coccidial oocysts and higher growth rates compared with goats fed various grass species (Sokerya & Rodriguez, 2001; Lin *et al.*, 2003; Sokerya & Preston, 2003). Similar results occurred for goats fed *Leucaena* or jackfruit (*Artocarpus heterophyllus*) foliage (Lin *et al*., 2003) and *Flemingea macrophylla* or banana foliage (Sokerya & Rodriguez, 2001). However, the main source of infection in at least one of these studies was larvae on the cut foliage, so smaller worm burdens would be expected in animals fed tree foliage compared with those fed grasses.

One group of tropical plants with considerable anthelmintic potential is the genus *Calliandra*. *Calliandra portoricensis* is frequently used in Nigeria as a herbal anthelmintic and in laboratory studies an ethanol extract of the ground root had limited success in clearing helminth infections in animals (Hammond *et al*., 1997). However, the best known member of the genus is *C. calothyrsus*. A preliminary trial carried out in Australia using *C. calothyrsus* took the form of a faecal egg count reduction test performed on four naturally infected eight-month old Merino wethers that were fed lucerne pellets and *Calliandra* for seven days (Parker & Palmer, 1991). There were no differences in faecal egg counts between the beginning and the end of the trial. However, there were too few animals in this trial to conclusively reject the possibility that *Calliandra* has anthelmintic potential and it would be worthwhile to investigate the effects of *Calliandra* on both faecal egg counts and worm burdens over a longer period of time and at different dose rates.

2.8 Conclusion

From the preceding discussion it can be seen that condensed tannins have a negative impact on GI nematodes of sheep. The fact that tropical shrub legumes such as *Calliandra* have high concentrations of condensed tannins would suggest a potentially useful role for these forages in an integrated management approach to sheep GI parasite control. However, it is also evident from the literature that there are gaps in our understanding of how condensed tannins exert an anthelmintic effect. It is likely that condensed tannins bind to nematode structures but the sites where binding might occur on the cuticle (Section 2.2.1) or eggshell (Section 2.2.5)

remain speculative. Tannins may bind and inhibit enzymes involved in hatching (Section 2.2.6), exsheathment (Section 2.2.2) or other metabolic processes (Section 2.2.7), but this also has not been demonstrated conclusively.

The way in which tannin structure affects the binding of condensed tannins to proteins or carbohydrates *in vivo* (Section 2.6.2) and the effects that tannin complexes have on ruminant nutrition (Section 2.6.3) or plant anthelmintic properties (Section 2.7) need further investigation. The anthelmintic properties of condensed tannins are variable and the effects of timing, duration, concentration and the physical form of tannin administration need clarification. Different effects of tannins on different worm species or in different host species also need explanation.

CHAPTER 3

ANTHELMINTIC EFFECTS OF *CALLIANDRA CALOTHYRSUS* IN LAMBS: A PILOT STUDY

3.1 Introduction

Recent research has identified several species of temperate pasture plants that can reduce the adverse effects of GI parasites in sheep and other species such as deer. In some cases, burdens of *H. contortus*, *Trichostrongylus* species, *Teladorsagia circumcincta*, *Nematodirus* species, *Cooperia* species and *Dictyocaulus* species were reduced by these plants (Niezen *et al.*, 1993; 1994; 1995; 1998b; 1998c; Hoskin *et al.*, 2000), while in others, the animals showed significant improvements in growth rates and reductions in the incidence of diarrhoea, despite the presence of GI parasites (Leathwick & Atkinson, 1995; Niezen *et al.*, 1995). It appears that the high concentrations of condensed tannins in these plants might have been responsible for the antiparasitic effects observed in the studies. The plants potentially offer a low cost alternative to anthelmintic drugs. In addition, such plants could be used in a GI parasite control program in which the development of parasite resistance to anthelmintic drugs and contamination of meat by drug residues are minimised or avoided altogether.

The tannin-containing plants that have been investigated so far in relation to GI parasites are temperate species that do not grow well in tropical areas. However, a number of tropical shrub legumes, particularly *Calliandra calothyrsus*, also contain high levels of condensed tannins, and thus may be effective for controlling GI parasites in livestock in the tropics. From the preceding discussion, it might be hypothesised that *Calliandra*, when fed as sole feed to sheep infected with GI nematodes, will decrease worm burdens and/or faecal egg counts in these animals.

The aim of the current experiment was to undertake a preliminary examination of the potential anthelmintic effects of *Calliandra*, using sheep as a ruminant model.

3.2 Materials and methods

3.2.1 Experimental design

The design of the experiment is presented in Table 3.1. The experiment was undertaken with the approval of the James Cook University (JCU) Animal Experimentation Ethics Committee (Ethics Approval No. A687_01). A minimum number of lambs was used for this preliminary examination of the potential anthelmintic effects of *Calliandra*.

Table 3.1 Experimental design in which Merino ram lambs were infected with either *Haemonchus contortus* or *Trichostrongylus colubriformis* and fed a diet of either Mitchell grass hay (MGH), pelleted lucerne or *Calliandra* leaves.

	Worm Species					
	Haemonchus contortus			*Trichostrongylus colubriformis*		
Diets	MGH	Lucerne	*Calliandra*	MGH	Lucerne	*Calliandra*
No. of lambs	3	3	3	3	3	3

3.2.2 Animals, housing and management

Eighteen three-month-old merino ram lambs, obtained from a commercial farm in Western Queensland, were transferred to the Metabolism Unit in the School of Veterinary and Biomedical Sciences (SVBS) at JCU. On Day -24, the lambs were weighed, ear tagged, treated for external parasites and vaccinated against clostridial diseases (Ultravac 5 in 1, CSL Animal Health, Australia). Faecal samples were taken from the rectum of each lamb for worm egg counts before the animals were drenched with two broad-spectrum anthelmintics: levamisole hydrochloride (1 mL/10 kg of Nilverm LV, Coopers, Australia) and albendazole (1 mL/5 kg of Alben, Virbac Animal Health, Australia) to remove any existing GI parasites. Prior to the start of the experiment, the lambs were kept in groups of six animals per group in concrete-floored pens (each 3.88 m x 3.65 m) and fed a basal diet of calf weaner pellets (Top End-R Weaner Pellets, Supastok, Australia) and Mitchell grass (*Astrebla* spp) hay, to prevent any exposure to worms.

On Day –14 the animals were divided into three equal groups of six based on live weight and transferred to individual slatted-floor pens (each 1.5 m x 0.78 m) in the Metabolism Unit. The groups were then allocated at random to one of three dietary treatments (Table 3.1). The animals were subjected to natural light throughout the experimental period. Ambient temperature and humidity were recorded daily at 0800 and 1500 hours. The minimum daily temperature ranged from 13 – 24 °C, maximum daily temperature ranged from 19 - 28 °C and humidity ranged from 34 - 95 %.

The live weight of the animals was recorded at weekly intervals using a Smartscale 200 weighing machine (Gallagher, Australia). The animals were monitored twice daily for signs of ill health and appropriate veterinary treatment was provided as necessary.

3.2.3 Feeds and feeding

Animals were fed either Mitchell grass hay, lucerne pellets plus rumen-undegradable casein or fresh leaves of *Calliandra* plus rumen-degradable casein. The amounts of casein included in the *Calliandra* and lucerne diets was calculated to provide these two diets with approximately the same amounts of rumen-degradable and rumen-undegradable protein (see Table 3.2). Lucerne was included as a dietary treatment group because improved protein nutrition could influence the effects of GI parasitism in sheep (e.g see Abbott *et al.*, 1986a; 1986b; 1988; Coop *et al.*, 1995; Perez-Maldonado & Norton, 1996a). Thus it was necessary to distinguish between any protein and non-protein effects on GI nematodes.

Table 3.2 The anticipated dry matter (DM), crude protein, rumen-undegradable protein and rumen-degradable protein in Mitchell grass hay, lucerne and *Calliandra* diets.

Composition	Diets		
	Mitchell grass hay	Lucerne	*Calliandra*
Dry matter (%)	88	90	39
Crude protein (g/kg DM)	30	180	200
Rumen-undegradable protein (g/kg DM)	ND*	63**	130
Casein added:			
Rumen-degradable (g/kg DM)	-	-	47
Rumen-undegradable (g/kg DM)	-	67	-

* Not determined
**Estimated using the data of Widiawati (2000)

(a) Casein

Second-grade acid casein was obtained from Malanda Milk in Mareeba, North Queensland. Half the amount was left untreated and the other half was made rumen-undegradable by subjecting it to the low volume method of Hemsley *et al*. (1973). The casein was mixed with formaldehyde (10 % w/v) at a ratio of 100 mL formaldehyde per kg of casein. Mixing was carried out using an industrial cake mixer (Kenwood Major, Kenwood) for ten minutes, and the treated casein was stored in Ziploc plastic bags at room temperature (19-28 °C) for at least twelve days prior to feeding.

(b) *Calliandra*

Fresh leaves of *Calliandra* were harvested daily from established plots in the SVBS precinct. The trees were five years old and had last been harvested approximately five to nine months previously.

(c) General

The lambs were subjected to a dietary adaptation period of 14 days (Day –14 to Day –1) during which time the animals were fed *ad libitum* (120 % of the previous day's intake). Feed was offered twice daily at 10:00 and 17:00 hours. During the experimental period (Day 0 to Day 35) the total dry matter (DM) intake of each animal was restricted to 2.5 % of its live weight. The amounts of feed offered were calculated after each weekly weighing of the animals. All lambs had free access to clean drinking water and to mineral licks (Mineralised Stock Block, Ridley Agriproducts Pty Ltd, Queensland, Australia), the composition of which is presented in Table 3.3.

Table 3.3 Composition of mineral licks offered to eighteen experimental sheep.

Composition	Amount (g/100 g)
Salt	75.0
Calcium	4.9
Phosphorus	1.0
Sulphur	2.0
Copper	0.06
Cobalt	0.006
Iodine	0.006
Zinc	0.1
Iron	0.11
Selenium	0.0005

3.2.4 Samples and sampling

(a) Nitrogen balance

On Day 7 of the experimental period, the lambs were moved to individual metabolism crates (each 0.55 m x 1.20 m) for an adaptation period of one week (see Figure 3.1). On Day 12 the animals were fitted with urine collectors and from Days 14 to 21 total faeces and urine excreted were collected for N balance determination. Urine was collected into 20 mL concentrated hydrochloric acid in a 2.5 L Winchester bottle. The volume was recorded daily and a 10 % sample pooled for each animal and stored at -20 °C. The feed offered and faeces produced were weighed daily and samples, (approximately 20 %), were dried to a constant weight (24 h) at 100 ºC in a fan-forced drying oven (Wessberg and Martin Pty. Ltd., Australia). Feed residues were treated similarly except that all residues collected were dried. The dried samples were pooled for each animal, ground to pass through a 1 mm sieve and stored in airtight jars at room temperature (24 °C). On Day 22 the lambs were moved back to the slatted-floor pens.

Figure 3.1 Lambs in metabolism crates set up for nitrogen balance determination in the Metabolism Unit, James Cook University.

(b) Parasitology samples

Faecal samples were taken from the rectum of each lamb ten days after drenching (Section 3.2.1) and subsequently once weekly for the duration of the experiment, for faecal egg counts.

3.2.5 Laboratory analyses

(a) Dry matter

The DM content of feed, faeces and feed residue samples were calculated using the formula:

DM (%) = (Dry weight of sample/fresh weight of sample) x 100 ..... *Equation 3.1*

(b) Organic matter (OM)

The OM content was determined by weighing 0.5 g of dried sample in duplicate into weighed, oven-dried beakers and ashing the samples in a muffle furnace (BTC 9090, Ceramic Engineering Furnace Manufacturers, Sydney) for six hours at 600 ºC before reweighing. The ash content was calculated as:

$$\text{Ash (\% in DM)} = \frac{[\text{DW of (ash + beaker)} - \text{DW of beaker}] \times 100}{\text{FW of sample} \times \text{\% DM}} \quad \text{.....} \textit{Equation 3.2}$$

where DW is dry weight and FW is fresh weight. The OM content was calculated as:

$$\text{OM (\% in DM)} = 100 - \text{ash (\% in DM)}. \quad \text{.....} \textit{Equation 3.3}$$

(c) Nitrogen

Feed, feed residue, faeces and urine were analysed for N using the Kjeldahl method. Approximately 0.2 g of ground solid samples or 1 mL of urine were measured in duplicate into digestion tubes, to each of which were added a selenium catalyst tablet (Kjeltabs) and 6 mL of concentrated sulphuric acid. Two tubes in each batch were prepared as blanks, containing only a catalyst tablet and acid, but no sample. A further two tubes in each batch were prepared as controls, using glycine in place of the sample. The samples were digested on a Tecator 2000 digestion block fitted with a Tecator 2001 scrubber unit (Tecator Australia). Sodium hydroxide (30 mL of 40 % v/v) was added to each sample in a Kjeltec 2100 distillation unit and the ammonia produced was distilled into a conical receiving flask containing 20 mL of 4 % w/v boric acid-pH indicator solution. The indicator solution (2.5 % of 1 % bromocresol green + 2.5 % of 1 % methyl red) changes from red to green at alkaline pH. The samples were titrated with concentrated sulphuric acid (1.038N) using a 25 mL digital titrator (Digitrate, Jencons) and the volume of acid used to achieve end-point (solution colour change from green to pink) was recorded. Nitrogen concentration was calculated using the formula:

$$N\ (\%) = \frac{NA \times (\text{mL sample titrant} - \text{mL blank titrant}) \times 14.01}{DW\ (g) \times 10} \quad \textit{Equation 3.4}$$

where NA is the normality of the acid and DW is the dry weight of the sample. Crude protein concentration was estimated the formula:

$$\text{Crude protein}\ (\%) = \%\ N \times 6.25 \quad \textit{Equation 3.5}$$

Protein entering the duodenum (PED) was estimated using the equation of Weston and Hogan (1973):

$$PED = [0.36 \times \text{protein intake}\ (g/d)] + [0.16 \times DOMI\ (g/d)] + 6 \quad \textit{Equation 3.6}$$

where DOMI is digestible organic matter intake.

3.2.6 Parasitology

Faecal egg counts were performed using a modified McMasters technique (Urquhart *et al*., 1996). Two grams of fresh faeces were mixed thoroughly with 28 mL of saturated magnesium chloride solution (specific gravity 1.2) to float the nematode eggs. The mixture was poured through a 1 mm mesh sieve and the liquid retained in a beaker. One half mL of liquid was extracted during stirring and transferred to one chamber of a Whitlock egg counting slide (Whitlock Universal, J.A. Whitlock and Co., Australia). The number of eggs in the chamber was counted at 63 x magnification under a binocular light microscope (Carl Zeiss, Germany). The number of eggs per gram of faeces (epg) was calculated as:

$$epg = \frac{\text{total mL of solution} \times \text{number of eggs per chamber}}{\text{chamber volume} \times g\ \text{faeces}} \quad \textit{Equation 3.7}$$

$$\text{i.e., } epg = \text{number of eggs per chamber} \times 30 \quad \textit{Equation 3.8}$$

On Day 0, three lambs selected at random from each dietary group were dosed orally with 10,000 *Trichostrongylus colubriformis* larvae and the remaining lambs dosed with 3000 *Haemonchus contortus* larvae. Larvae were anthelmintic-susceptible strains obtained from Mr Peter Bradley at the CSIRO McMaster's Laboratory in New

South Wales. Twenty-eight days post-infection (lambs infected with *T. colubriformis*) or 35 days post-infection (lambs infected with *H. contortus*) the animals were fasted overnight, then each was euthanased by captive bolt and its abomasum and small intestine ligated and removed. The small intestine was divided into proximal small intestine (the first four metres) and distal small intestine. These organs were opened, and their respective contents washed onto a sieve (250 µm aperture) and preserved in 10 % buffered neutral formalin (BNF).

For abomasal samples, digesta was spread out in thin layers in white trays and total numbers of *H. contortus* were counted. Because *H. contortus* from two lambs on the *Calliandra* diet appeared to be bigger than those from the other two diets, 200 worms from each of the nine lambs infected with this species were blotted dry on a piece of filter paper and weighed. Intestinal samples were diluted to one or two litres with water and the total number of worms was extrapolated from counts in two 28 mL aliquots examined at 10x magnification using an Olympus SD-ILK dissecting microscope (Olympus Optical Co. Ltd., Japan). Twenty-five female worms were selected at random from each lamb infected with *T. colubriformis* and the eggs present in the uterus were counted at 40x magnification. This was not possible for *H. contortus* due the large number of eggs present in the uterus of the females of this species.

3.2.7 Statistical analysis

Statistical analyses were carried out using *SPSS for Windows 10.0.7* (SPSS Inc) statistical software. Dietary DM, OM, digestibility, lamb weights, energy requirements, DM intake, energy intake, CP intake, protein entering the small intestine and N balance were compared by two-way ANOVA or ANOVA on ranks, using worm species and diet as fixed factors. The faecal egg counts of sheep on different diets were log-transformed and compared at each time point by one-way ANOVA. Log-transformed worm burdens, *H. contortus* weights, and number of eggs in the uterus of each female *T. colubriformis* were also compared between diets by one-way ANOVA. Where significant differences were detected, pairwise multiple comparisons were performed using the Tukey test, or Dunnet's T3 if the test for equal variance failed.

3.3 Results

3.3.1 Parasitology

No animals showed clinical signs of parasitism at any point during the experiment.

When purchased, none of the 18 lambs had nematode eggs in their faeces, although nine lambs had counts of coccidial oocysts ranging from 15 to 480 oocysts per gram of faeces. No nematode eggs were observed in faeces ten days after drenching and egg counts remained at zero until the artificially induced infections became patent. Coccidial oocysts were found in low numbers in the faeces of each lamb at least once during the experiment but clinical coccidiosis did not occur and no treatment was deemed necessary.

Eggs of *T. colubriformis* were detected in the faeces of all nine infected lambs 21 days post-infection. Three lambs infected with *H. contortus* also had low faecal egg counts by Day 21, but infection did not reach full patency until Day 28, as expected with this particular strain of *H. contortus*. At 21 days post-infection, log-transformed faecal egg counts of *T. colubriformis*-infected lambs fed *Calliandra* were significantly lower than those from lambs fed lucerne (1.79 ± 0.17 *vs* 2.86 ± 0.16; P = 0.005) but not those from lambs fed Mitchell grass hay (2.23 ± 0.10, P = 0.169; Figure 3.2). The difference in egg counts between lambs fed lucerne and hay was very close to significance (P = 0.055). At 28 days post-infection lambs on the *Calliandra* and Mitchell grass hay diets had lower log-transformed faecal egg counts (2.19 ± 0.07; P = 0.001 and 2.65 ± 0.07; P = 0.044 for *Calliandra* and Mitchell grass respectively) than those fed lucerne (3.01 ± 0.10). Lambs fed *Calliandra* also tended to have lower egg counts than those fed Mitchell grass hay (P = 0.061), although this did not quite reach significance. Lambs fed hay had lower egg counts than those fed lucerne (P = 0.044).

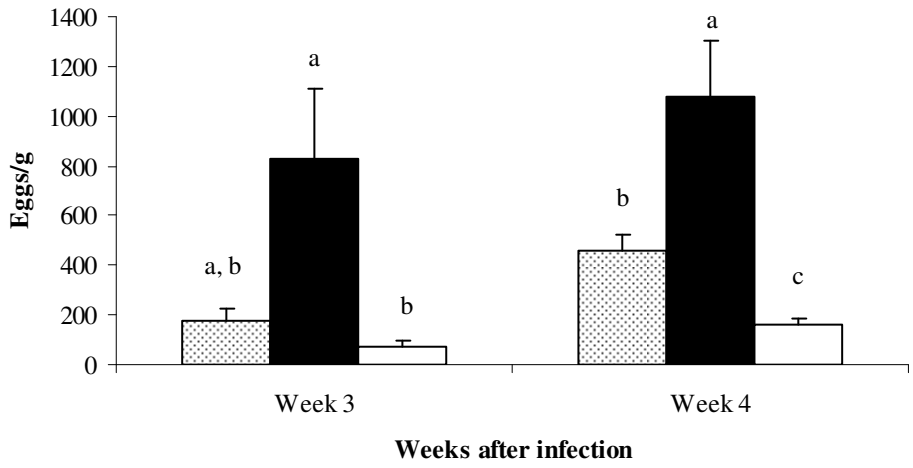


Figure 3.2 Faecal egg counts (mean ± s.e.) of lambs fed Mitchell grass hay (▒), lucerne pellets (■) or *Calliandra* leaves (□) three and four weeks after infection with *Trichostrongylus. colubriformis*. Within each week, treatments with different superscripts are significantly different (P < 0.050).

There were no significant differences in faecal egg counts between any of the diets in lambs infected with *H. contortus* (P = 0.395 on Day 21; P = 0.531 on Day 28 and P = 0.176 on Day 35; Figure 3.3).

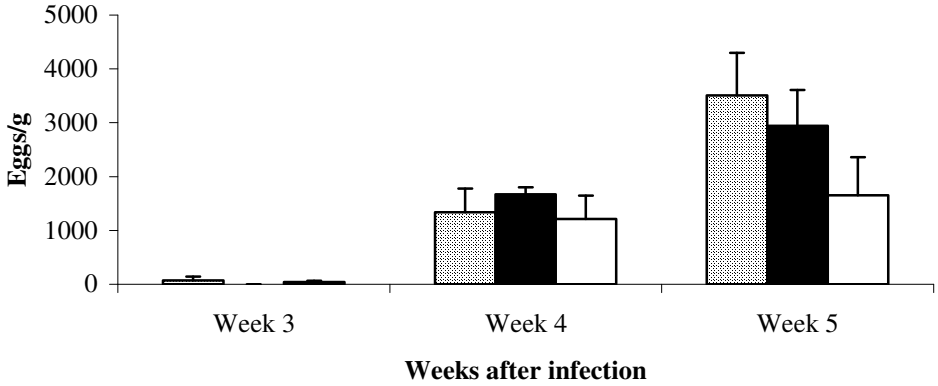


Figure 3.3. Faecal egg counts (mean ± s.e.) of lambs fed Mitchell grass hay (▒), lucerne pellets (■) or *Calliandra* leaves (□), three, four and five weeks after infection with *Haemonchus contortus*. There were no significant differences between dietary treatments within each week (P < 0.100).

Diet did not affect post-mortem worm counts of *T. colubriformis* (P = 0.211) or *H. contortus* (P = 0.149; Figure 3.4). The size difference between adult *H. contortus* derived from lambs fed *Calliandra* and those on the other two diets only just failed to reach significance (P = 0.057; Table 3.4).

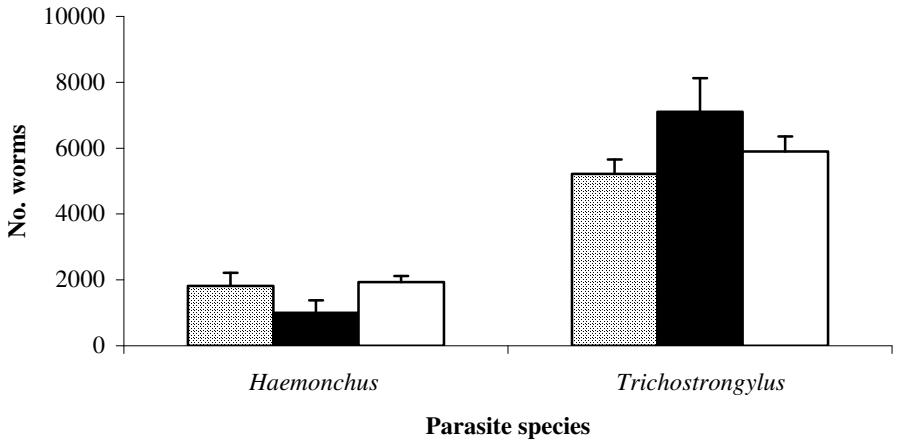


Figure 3.4 Postmortem worm counts (mean ± s.e.) of lambs fed Mitchell grass hay (▒), lucerne pellets (■) or *Calliandra* leaves (□), four or five weeks after infection with *Trichostrongylus colubriformis* or *Haemonchus contortus* respectively. Burdens of each worm species were not significantly different between diets (P > 0.100).

Worms from lambs fed *Calliandra* had a higher number of eggs *in utero* per female *T. colubriformis* than those fed either hay (P < 0.001) or lucerne (P = 0.028; Table 3.4). There was also a trend for worms from lambs fed lucerne to have more eggs *in utero* than those from lambs fed hay, but this did not reach significance (P = 0.066).

Table 3.4 Size of *Haemonchus contortus* and total number of eggs present *in utero* in female *Trichostrongylus colubriformis* harvested immediately after euthanasia of lambs that were infected with either of the two worm species. Values are means ± s.e. Within each row, means with different superscripts are significantly different (P < 0.050).

	Diet		
	Mitchell grass hay	Lucerne	*Calliandra*
Weight of 200 *H. contortus* (g)	0.14^{a} ± 0.010	0.13^{a} ± 0.012	0.24^{a} ± 0.040
Eggs/female *T. colubriformis*	24^{a} ± 0.6	26^{a} ± 0.5	28^{b} ± 0.5

3.3.2 Nutritive value of diets

The analytical results of the feeds offered to the lambs are presented in Table 3.5. Intake and N balance data are presented in Table 3.6. As there was no effect of worm species and no interaction between worm species and diet, the results for the two worm species were pooled within diets.

For lambs infected with either *T. colubriformis* or *H. contortus*, there was no significant difference between the *Calliandra* diet and the lucerne diet in crude protein intake, estimated PED or N retention (P = 0.617, P = 0.560 and P = 0.886, respectively; see Table 3.6). Lambs fed Mitchell grass hay had a low protein intake and a small net N retention. Total N excretion was similar on both the lucerne and *Calliandra* diets. However, on the *Calliandra* and Mitchell grass hay diets, the majority of N excreted was lost through the faeces, whereas in animals on the lucerne diet the predominant route of N excretion was via the urine (see Table 3.6).

Table 3.5 The dry matter (DM), organic matter (OM), crude protein (CP) and metabolisable energy (ME) content and digestibility (D) of the feeds offered to lambs infected with either *Haemonchus contortus* or *Trichostrongylus colubriformis*. Within each row, values with different superscripts are significantly different from one another (P < 0.05).

	Diet		
Diet	Mitchell grass hay	Lucerne	*Calliandra*
DM (%)	$96^{a} \pm 0.4$	$97^{a} \pm 0.4$	$41^{b} \pm 0.3$
OM (%)	$85^{a} \pm 2.6$	$91^{b} \pm 0.1$	$93^{c} \pm 0.2$
CP (%)	$3^{a} \pm 0.3$	$20^{b} \pm 0.4$	$25^{c} \pm 0.7$
D (%)	$40^{a} \pm 1.4$	$56^{b} \pm 0.8$	$54^{b} \pm 0.8$
ME (MJ.kg DM)	$4.90^{a} \pm 0.387$	$8.05^{b} \pm 0.123$	$7.97^{b} \pm 0.067$

Table 3.6 The dry matter (DM), Crude protein (CP) and digestible organic matter (DOM) intakes, estimated protein entering the duodenum (PED) and N analysis of pooled samples from lambs infected with either *Haemonchus contortus* or *Trichostrongylus colubriformis*. Within each row, figures with different superscripts are significantly different from one another (P < 0.05).

	Diet		
	Mitchell grass hay	Lucerne	*Calliandra*
Mean weight of lambs (kg) at start of N balance	$13.5^{a} \pm 0.82$	$15.85^{b} \pm 0.74$	$14.25^{a,b} \pm 0.56$
DM Intake (g/day)	$275^{a} \pm 8.0$	$453^{b} \pm 20.0$	$388^{c} \pm 16.4$
CP intake (g/day)	$12^{a} \pm 1.5$	$93^{b} \pm 4.4$	$98^{b} \pm 4.5$
DOM Intake (g/day)	$50^{a} \pm 2.6$	$59^{b} \pm 0.9$	$57.5^{b} \pm 0.8$
PED (g/day)	$19^{a} \pm 0.6$	$49^{b} \pm 1.6$	$51^{b} \pm 1.6$
N intake (g/day)	$2.0^{a} \pm 0.24$	$14.8^{b} \pm 0.71$	$15.7^{b} \pm 0.73$
Urine N (g/day)	$1^{a} \pm 0.2$	$7^{c} \pm 0.7$	$4^{b} \pm 0.5$
Faecal N (g/day)	$1^{a} \pm 0.2$	$3^{b} \pm 0.3$	$6^{c} \pm 0.5$
N Balance (g/day)	$0.4^{a} \pm 0.33$	$5.1^{b} \pm 1.05$	$5.8^{b} \pm 0.73$
N retained as % N intake	$22^{a} \pm 16.7$	$34^{a} \pm 6.2$	$36^{a} \pm 3.0$
% of excreted N in urine	$36^{a} \pm 7.5$	$67^{b} \pm 0.9$	$40^{a} \pm 4.7$
% of excreted N in faeces	$64^{a} \pm 7.5$	$33^{b} \pm 0.9$	$60^{a} \pm 4.7$

3.4 Discussion

3.4.1 Effects on T. colubriformis

Although this part of the study was a preliminary experiment, involving only three animals per treatment, it showed clearly that *Calliandra*, when fed as a sole feed, was able to reduce the faecal egg counts of lambs infected with *T. colubriformis*. As there was no difference in overall N retention between sheep fed *Calliandra* and lucerne, it is unlikely that *Calliandra* would have caused any increased resistance or resilience of the lambs to GI parasites as a result of improved protein nutrition. The observed effect on egg counts of *T. colubriformis* in faeces (Figure 3.2) might therefore be attributed to a direct toxic or physiological effect of *Calliandra*.

While definitive conclusions could not be drawn as to the constituent(s) of *Calliandra* responsible for such an effect, it might be suggested that condensed tannins are the most likely candidates. This is consistent with the findings of others (e.g., Athanasiadou *et al.*, 2000b; Kabasa *et al.*, 2000). The reported values for TCT content of *Calliandra* foliage range from 6 % (McSweeney *et al*., 1999) to 29 % (Palmer *et al*., 2000). Analysis of *Calliandra* in the Nutritional Physiology & Metabolism (NPM) laboratory at JCU shows values of 11.4 % (D. Martin and E. Teleni, personal communication). Athanasiadou *et al.* (2000b) achieved a 50 % depression in *T. colubriformis* egg counts in faeces by drenching infected sheep with quebracho tannin, a commercial condensed tannin extract, at a rate of 8 % of DM intake. Condensed tannins were also implicated as the cause of reduced faecal egg counts in parasitised goats grazed on tanniniferous forage, compared with control goats that were drenched with PEG to bind tannins (Kabasa *et al*., 2000). However, the actual condensed tannin intake of the goats in the study was not determined.

Total adult *T. colubriformis* burdens were not reduced significantly by the *Calliandra* diet, so for the reduction in faecal egg counts to be so marked there must have been either a reduction in egg output by existing female worms, or a dilution effect caused by higher faecal output. In the current experiment, correction of faecal egg counts on Day 21 for total faecal DM output did not affect the outcome. An interesting

observation was that the number of eggs present *in utero* per female *T. colubriformis* was higher in the *Calliandra* group than in the other two groups (see Table 3.4). It is possible that the rate of egg release was affected, rather than egg production *per se* (see also Athanasiadou *et al.*, 2000c), in which case the higher number of eggs per female in the *Calliandra* group could be due to accumulation of eggs within the uterus.

The reduction in *T. colubriformis* faecal egg counts in sheep fed Mitchell grass hay compared with that of sheep fed lucerne was unexpected and is difficult to explain given the low crude protein content of the hay (see Table 2). It may be that Mitchell grass also contains anthelmintic substances, a possibility that warrants further investigation. Since the number of eggs per female worm in this group was lower than in female worms from lucerne and *Calliandra* diets, the low faecal egg counts were probably due to reduced egg production rather than to reduced egg release.

3.4.2 Effects on H. contortus

There was no apparent effect on worm burdens or faecal egg counts in lambs infected with *H. contortus* in the current experiment. This might be a result of the abomasal habitat of *H. contortus*, compared with the duodenal habitat of *T. colubriformis*. In a previous study, however, Niezen *et al*. (1998c) demonstrated both lower faecal egg counts and lower burdens of *Teladorsagia circumcincta* (formerly *Ostertagia circumcincta*, an abomasal species) in sheep fed *Lotus pedunculatus*. Reductions in the faecal egg counts and abomasal worm burdens of deer were also shown to occur after feeding tanniniferous forages, but the effect was more apparent against *Trichostrongylus axei* than against *Ostertagia*-type species (Hoskin *et al*., 2000). Possibly, the lack of an obvious effect on *H. contortus* in the current experiment was due to the small sample size used. Also, differences in feeding behaviour or physiological processes between worm species may account for the observed differences. Clearly more work is required in this area. The reason for the apparently larger size of *H. contortus* derived from *Calliandra*-fed sheep (Table 3.4) as opposed to those on the other two diets was also unclear. It should be noted that the difference was due to worms from lambs on the lucerne and Mitchell grass hay diets being

unusually small, rather than an increase in the size of the lambs on the *Calliandra* diet.

3.4.3 Protein nutrition

As anticipated, animals fed the lucerne and *Calliandra* diets had similar levels of N intake. The estimated quantity of protein flowing to the duodenum of these lambs and their overall N retention were also similar. However, the patterns of urinary and faecal N excretion observed were different (Table 3.6). Lambs fed *Calliandra* excreted significantly more N in the faeces and less in the urine than lambs fed lucerne, suggesting that less N, proportionate to intake, was digested and absorbed in lambs on the *Calliandra* diet. This concurs with the findings of Merkel *et al*. (1999a; 1999b) and Widiawati *et al*. (2000). Although tannin-protein complexes dissociate at a pH value of less than 3.5 (Jones & Mangan, 1977) such as are normally found in the abomasum, the pH in the small intestine may reach 5.5. Thus there may be insufficient time for digestion and absorption to occur before complexes reform (Waghorn *et al*., 1994b; Wang *et al*., 1996b). It is possible also that highly astringent condensed tannins, such as those found in *Lotus pedunculatus*, may not dissociate properly from proteins in the first place (Waghorn *et al*., 1994b). Perez-Maldonado and Norton (1996b) observed that there was very little PCT in the faeces of sheep fed *Calliandra* or *Desmodium intortum* and concluded that dissociation of protein-tannin complexes had not been inhibited in the intestinal tract of their animals, but the high faecal N of *Calliandra*-fed lambs in the current study would suggest otherwise. A direct effect of condensed tannins on intestinal function also cannot be ruled out. Dawson *et al*. (1999) noted degeneration and ulceration of the jejunal and ileal mucosa in tannin-fed sheep during the first two weeks of feeding and Waghorn *et al*. (1994b) postulated that tannins could bind to the intestinal mucosa, blocking amino acid absorption. Whatever the cause, it is apparent that the increase in faecal N loss that occurs in sheep fed *Calliandra* at least partly obviates the potential benefits that usually occur when feeding diets containing a high proportion of rumen-undegradable protein.

The most likely mechanism by which condensed tannins might exert their effects on GI nematodes is through the binding of tannins to nematode proteins. Since such binding is pH-dependent, it might be postulated that the different results obtained for *H. contortus* and *T. colubriformis* were due to differences in pH between the abomasum and duodenum. As discussed above, the pH in the abomasum is likely to favour dissociation of tannin-protein complexes, and thus tannin may be unable to bind to nematodes in this region of the GI tract. High faecal N losses in the *Calliandra*-fed lambs lend support to the proposition that complexes reform distal to the abomasum, and this could explain the effect of the *Calliandra* diet on the duodenal worm species.

3.5 Conclusion

The results of this study suggest that *C. calothyrsus* is effective in reducing *T. colubriformis* egg counts in faeces, while having little or no impact on worm burdens in sheep fed the legume. *Calliandra* therefore has the potential for a role in parasite control programs in tropical regions, where it could be used to reduce pasture contamination with *T. colubriformis* eggs and thus reduce the transmission of this parasite. Mitchell grass may also be useful in this regard, although more work is required to determine the constituent of Mitchell grass hay responsible for the reduced *T. colubriformis* egg production observed in this experiment. The reasons for the lack of apparent effect on worm burdens or egg production of *H. contortus* by either *Calliandra* or Mitchell grass hay are unclear and also merit further investigation.

CHAPTER 4

ANTHELMINTIC EFFECTS OF *CALLIANDRA CALOTHYRSUS* IN SHEEP INFECTED WITH *H. CONTORTUS* OR *T. COLUBRIFORMIS*

4.1 Introduction

The results of the pilot experiment described in Chapter 3 suggested that *Calliandra* was able to suppress egg output by female *T. colubriformis* in sheep when the latter were fed the legume as a sole diet. However, although there was a trend for egg output by *H. contortus* to be lower, this did not reach statistical significance, possibly due to the small number of lambs used in the experiment.

The apparent differences between the two worm species in egg output might have been due to the different habitat of the two species in the GI tract and the pH-dependent properties of tannins. At pH values of less than 3.5, as would be expected normally in the abomasum, protein-tannin complexes would dissociate (Jones & Mangan, 1977), so *Calliandra* condensed tannin would be unlikely to interact with worm proteins in such an environment. In the duodenum, where the pH would be higher, conditions would favour the formation of tannin-protein complexes, with probable consequent effects on the worms. Although such complexes have maximum stability between pH 3.5 and pH 7, and duodenal pH may exceed 7, significant dissociation probably does not occur until pH exceeds 8.5. Thus the extent to which worms might be affected by tannin could be determined by the location of their habitat in the GI tract.

As discussed in Chapter 3, part of the mechanism of action of *Calliandra* could be an improvement in host immune responses to nematode parasites mediated by improvements in host protein nutrition. Although the results of the pilot experiment do not preclude this possibility, especially in the long term, they do suggest that there is a more immediate direct toxic effect of the legume on worms. In the current experiment, protein intake was manipulated again to minimise any differences, between diets, in their effects on worms based on host protein nutrition.

Generally, lambs do not mount an effective immune response to *H. contortus* or *T. colubriformis* until at least six months of age (see review by Douch, 1990) but they can do so if exposed to worms when very young. Circulating antibody levels are usually higher than normal in resistant animals. The lambs used in the current experiment were acquired from drought-affected areas one day after weaning at about three months of age, and so were unlikely to have any prior exposure to nematode parasites. Nevertheless, it was decided to monitor a number of plasma constituents as well as nutritional parameters in order to assess the ability of *Calliandra*-fed lambs to cope with the effects of GI nematode parasite infections.

The current experiment was designed to confirm the results of the pilot experiment (Chapter 3) and to expand on these by investigating the possible effects of pH on nematode-tannin interactions in different segments of the GI tract. It was hypothesised that:

a. worm burdens in lambs would not be affected by feeding *Calliandra* to the lambs;
b. fecundity of female *T. colubriformis* and possibly female *H. contortus* would be reduced by feeding *Calliandra* to lambs;
c. reductions in fecundity would be seen when worms were exposed to pH values at which tannin-protein binding is maximal, i.e. between pH 3.5 and pH 7, but not to values outside this range.

4.2 Materials and Methods

4.2.1 Experimental design

The experiment was undertaken with the approval of the JCU Animal Experimentation Ethics Committee (Ethics Approval No. A826_03). Due to constraints on legume production, observations were replicated over two periods. In each period, three-month-old merino ram lambs were obtained from a farm in Western Queensland and were weaned the day prior to arrival at JCU. The lambs were stratified by weight and randomly allocated to treatment groups within each stratum. In Period 1, 16 lambs were assigned to four treatment groups (see Table

4.1). In Period 2, another 16 lambs were assigned to the same four treatment groups. In addition, eight lambs (non-infected) were assigned to the two diets in Period 2.

Table 4.1 Lambs infected or not infected with either of two worm species, *Haemonchus contortus* (Hc) or *Trichostrongylus colubriformis* (Tc) and fed a diet of either *Calliandra** or lucerne** over two observation periods.

	Period 1				Period 2					
Diets	*Calliandra*		Lucerne		*Calliandra*			Lucerne		
Worm species	Hc	Tc	Hc	Tc	Hc	Tc	-	Hc	Tc	-
No. lambs	4	4	4	4	4	4	4	4	4	4

*Includes a calculated amount of rumen-degradable casein protein (see Section 4.2.3)
**Includes a calculated amount of rumen-undegradable protein (see Section 4.2.3)

4.2.2 Animal management

Prior to the start of the experiment, the lambs were treated and housed as described previously (Section 3.2.2). The management of the animals in the two periods was similar except for the procedures relating to the non-infected group in Period 2. One week after arrival of the experimental lambs in Period 2, the assigned non-infected group was transferred to individual slatted-floor pens in the Metabolism Unit at JCU for a one-week dietary adaptation period. The group was then moved to individual metabolism crates for a further week before N balance determination. The assigned infected group was moved into the slatted floor pens after the assigned non-infected group had been moved into metabolism crates. The non-infected lambs remained in the metabolism crates until slaughter, two days after completion of N balance determination, whereas the infected group of lambs was returned to the slatted floor pens for two weeks until time for the second N balance determination.

The animals were subjected to natural light throughout the experiment. Ambient temperature and humidity were recorded three times daily at 0800, 1300 and 1700 hours. Throughout the experimental periods, minimum daily temperature ranged from 21 – 30 °C in Period 1 and 13 - 30 °C in Period 2, maximum daily temperature ranged from 29 - 38 °C in Period 1 and 21 - 24 °C in Period 2, and humidity ranged from 39 - 85 % in Period 1 and 32 - 95 % in Period 2.

4.2.3 Feeds and feeding

All animals were initially fed lucerne pellets *ad libitum* for seven days until DM intake exceeded 3.5 % of live weight, and then DM intake was restricted to 2.8 % live weight to ensure that all feed offered was consumed. The lambs remained on lucerne pellets until Day –14, when they were gradually introduced to the experimental diets. The lucerne diet included rumen-undegradable casein protein. The amount of the rumen-undegradable casein included was calculated to give the diet a total rumen-undegradable protein content equal to that estimated to be in the *Calliandra* diet. The *Calliandra* diet consisted of fresh leaves of the legume and rumen-degradable casein protein. The amount of casein included was calculated to give the diet a total protein content equal to that of the lucerne diet. The methods used to protect the casein from degradation in the rumen and to determine the protein and DM contents of the diets are described in Sections 3.2.3 and 3.2.4. Casein was not introduced until the second week of the adaptation period, when the lambs were eating the *Calliandra* and lucerne feeds well. Feed was offered twice daily at 08:30 and 17:00 hours. Lambs were weighed weekly before the morning feed and the amounts of the feeds to be offered for the week were adjusted accordingly. All lambs were supplied with a mineral lick (see Table 3.3) and fresh water was available at all times. The animals were monitored twice daily for signs of ill health, and veterinary treatment was provided as necessary.

Fresh leaves of *Calliandra* were harvested once daily from the plots used in the pilot experiment [see Section 3.2.3.(b)]. The trees had last been cut back approximately five to nine months prior to feeding in Periods 1 and 2.

4.2.4 Samples and sampling

(a) Feed, faeces and urine

During the seven-day N balance determination, feed, feed residue, faeces and urine samples were collected and analysed as described in Section 3.2.4, with the exception that the digestion of samples for Kjeldahl analysis was carried out on a

Tecator 2040 digestion block fitted with a Tecator 2015 lift system and a Tecator 2001 scrubber unit. Lambs in the two uninfected groups underwent a single period of N balance, whereas the infected lambs (four treatment groups; see Table 4.1) were subjected to one period of N balance beginning on the first day of infection with nematode larvae (Post-infection 1), and a second period beginning on the day infections were expected to become patent (Day 21 post-infection for *T. colubriformis* and Day 28 post-infection for *H. contortus*; Post-infection 2).

(b) Blood

Blood samples from the lambs were collected weekly (during both the adaptation and experimental periods) by jugular venepuncture into 10 mL lithium heparin Vacutainer tubes. The samples were placed on ice and processed as soon as collections were completed. Duplicate blood samples were collected from each Vacutainer tube into microhaematocrit tubes and centrifuged at 10,000 rpm for five minutes in a Sigma 1-15 microcentrifuge (Sigma Australia) to determine PCV. The remainder of samples in Vacutainer tubes were centrifuged at 3000 g for 10 minutes at 4 ºC in an Eppendorf 5702 refrigerated centrifuge. The resultant plasma samples were transferred into 5 mL plastic tubes and stored at –20 ºC, pending analyses.

4.2.5 Biochemical analyses

(a) Urea

On the day of analysis, the plasma was defrosted and urea concentration measured in duplicate on a Technicon Autoanalyser II (Bran and Leubbe Analyzing Technologies, Germany) using the Technicon method 339-01 (Technicon, 1977). This method relies on the hydrolysis of diacetyl monoxime in an acid solution to produce free diacetyl, which then reacts with urea in the presence of thiosemicarbazide and ferric ions to form a yellow chromagen. Colour intensity was measured with a spectrophotometer at 460 nm and urea concentration determined accordingly. Urea concentration in urine samples was also determined using this

method, following dilution of the urine with distilled water. The remaining plasma was analysed for inorganic phosphate, glucose, total protein and albumin concentrations using a Cobas Mira Autoanalyser (Roche), with reagents from Trace Scientific Ltd., Australia.

(b) Plasma Total Protein

Total protein was measured by the Trace biuret method, in which the sample is added to an alkaline solution of copper II sulphate (Trace Scientific, 2001). Any peptide bonds present form complexes with the copper ions, resulting in the formation of a blue-violet solution. Protein concentration is proportional to the absorbance of the solution at 540 nm at 37 °C. Potassium iodide and potassium sodium tartrate were added to promote stability of the complexes formed.

(c) Albumin

Albumin was measured by the Trace Bromocresol Green (BCG) method (Trace Scientific, 1999). Albumin in the sample binds to the BCG dye, resulting in an alteration in absorbance. Absorbance of the complex, which is proportional to the concentration of albumin in the sample, is measured at 625 nm at 37 °C.

(d) Phosphorus

Inorganic phosphorus was measured by the Trace direct UV method without reduction (Trace Scientific, 1997). Ammonium molybdate reacts with inorganic phosphorus in the sample to form blue phosphomolybdate. Absorbance is measured at 340 nm at 37 °C.

(e) Glucose

Glucose was measured by the Trace glucose oxidase method (Trace Scientific, 2000). In this method, glucose is converted to gluconic acid and hydrogen peroxide

by the action of glucose oxidase. The peroxide so formed then reacts with 4-hydroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase to produce a red quinoneimine dye. Absorbance, which is proportional to the concentration of glucose in the sample, is read at 460-560 nm at 37 °C.

4.2.6 Parasitology

One group of lambs on each diet was dosed orally with 10,000 *T. colubriformis* larvae (prepatent period 21days) and the remaining lambs were dosed with 3200 *H. contortus* larvae (prepatent period 28 days). The larvae were anthelmintic-susceptible strains obtained from the CSIRO McMaster's Laboratory in New South Wales. Faecal samples were collected weekly throughout the experiment for faecal egg counts as described in Section 3.2.6.

At 29 days post-infection (lambs infected with *T. colubriformis*) or at 36 days post-infection (lambs infected with *H. contortus*) the animals were euthanased by captive bolt. The digestive tract was ligated at the junction of the oesophagus with the rumen and at the rectum and removed. The intestines were further ligated at the omaso-abomasal junction, the pylorus, 4 m and 5 m distal to the pylorus, at the ileocaecal junction, and at the junction of the caecum and colon. These segments were designated Rumen (R), Abomasum (A), Duodenum (D), Jejunum (J), Ileum (I), Caecum (Cae) and Colon (Co). After pH and volume measurements (see below), the abomasum and small intestine were opened, washed and the contents collected on a sieve (250 µm aperture) and preserved in 10 % BNF for counting. For abomasal samples, washings were placed in white trays and the total numbers of *H. contortus* were counted. Intestinal samples were diluted to one or two litres with water and the total numbers of worms extrapolated from an appropriate number of aliquots examined at 10x magnification under an Olympus SD-ILK dissecting microscope (Olympus Optical Co. Ltd., Japan). The number of aliquots required was determined by the method of Clark *et al*. (1971). Samples that obviously contained worms were assumed to have total counts greater than 2000, so four aliquots each comprising 1 % of the diluted volume were counted and the number of additional 1 % aliquots to count was determined by the number of worms in the first 4 %. Samples with no

visible worms were assumed to have total counts less than 2000, so four 5 % aliquots were counted and the number of additional 5 % aliquots determined by the number of worms in the first 20 %. Since 20 mL of digesta were removed from each segment during volume measurements (see Section 4.2.7), the number of worms in the missing 20 mL was estimated and added to the total obtained during counting. The sex of the first 100 worms counted in each lamb was recorded to determine the ratio of females to males.

Twenty-five female worms were selected at random from each lamb infected with *T. colubriformis* and the eggs present in the uterus were counted at 40 x magnification.

4.2.7 pH values

Immediately after the slaughter of each lamb and removal and ligation of its GI tract (Section 4.2.6) an incision was made in each segment of the tract, and the pH of the digesta content determined using an Intermediate Junction IJ44 pH meter (Ionide Pty Ltd., Australia) at 37 ºC and calibrated in pH 4 and pH 7 buffers. The pH values were recorded for the dorsal sac of the rumen, the fundus of the abomasum, the proximal end of the first duodenal segment, the distal end of the second duodenal segment, in the distal ileum 2 cm from the caecum, and in Period 2 only, the middle of the caecum and at the sigmoid flexure of the spiral colon. The digesta in each segment was transferred to an appropriate sized beaker, and after thorough mixing, 20 mL digesta were removed from each segment before the remaining volume was processed for worm counts as described in Section 4.2.6. Of the 20 mL, 15 mL were analysed for condensed tannin by another researcher (see results in Section 4.3.5) and 5 mL were retained to estimate the number of worms lost in the 15 mL sample. Tissue samples were collected from each GI segment for histopathology and tannin staining (Chapter 7).

4.2.8 Statistical analysis

Statistical analyses were carried out using *SPSS for Windows 10.0.7* (SPSS Inc) statistical software.

Due to an unintended age difference in some of the lambs obtained (up to two months difference), it became necessary to introduce age as a factor in the experimental design. This resulted in insufficient degrees of freedom for the statistical analysis. Consequently, preliminary one-way analysis of variance was used to examine the importance of each factor (worm species, diet, period and age) and those that were not significant were excluded from the subsequent analyses.

Nutrition data for the four treatment groups were compared by repeat measures analysis of variance, with diet, age and period as factors. Worm burdens, percent female worms and number of eggs produced per female worm were compared by multivariate analysis of variance on ranks, with diet, species and period as factors. Faecal egg counts failed the test for equal variance and were compared by the Mann Whitney test. Number of eggs *in utero* of female *T. colubriformis* was analysed by univariate analysis of variance, with diet and period as factors.

The pH data were analysed by multivariate analysis of variance. For R, A, D, J and I pH data, species, diet and period were included as factors in the model. For the volume data and pH of caecum and colon, which were recorded only in Period 2, species and diet were included as factors.

Haematological data (total protein, albumin, glucose, urea, inorganic phosphate and PCV) were analysed by repeated measures analysis of variance. In cases where the Mauchly's test for sphericity failed, the Huynh-Feldt correction was used. Baseline values for all four treatment groups and the two uninfected groups were compared for the three weeks prior to infection, with period and diet as factors in the model. Values for the four treatment groups only were compared during Post-infection 1 and Post-infection 2, with species, diet and period as factors in the model.

4.3 Results

4.3.1 Parasitology

No clinical signs of GI nematode parasitism were observed in any of the animals during the course of the experiment. There were no worm eggs present in the faeces of any of the lambs on arrival at JCU, or during the prepatent period. Lambs infected with *T. colubriformis* had positive egg counts at three and four weeks after infection, whereas only half of the lambs infected with *H. contortus* had low positive egg counts three weeks after infection, and most did not reach patency until the fourth week. Consequently, the first faecal egg count after patency for each species was designated FEC1 (at Week 3 for *T. colubriformis* and Week 4 for *H. contortus*) and the second was designated FEC2 (at Week 4 for *T. colubriformis* and Week 5 for *H. contortus*). Faecal egg counts were significantly higher in lambs infected with *H. contortus* than in lambs infected with *T. colubriformis* ($P = 0.010$ and $P < 0.001$ for FEC1 and FEC 2 respectively), and also higher in lambs fed lucerne than lambs fed *Calliandra* ($P < 0.001$ and $P = 0.003$ for FEC1 and FEC2 respectively) - see Figure 4.1.

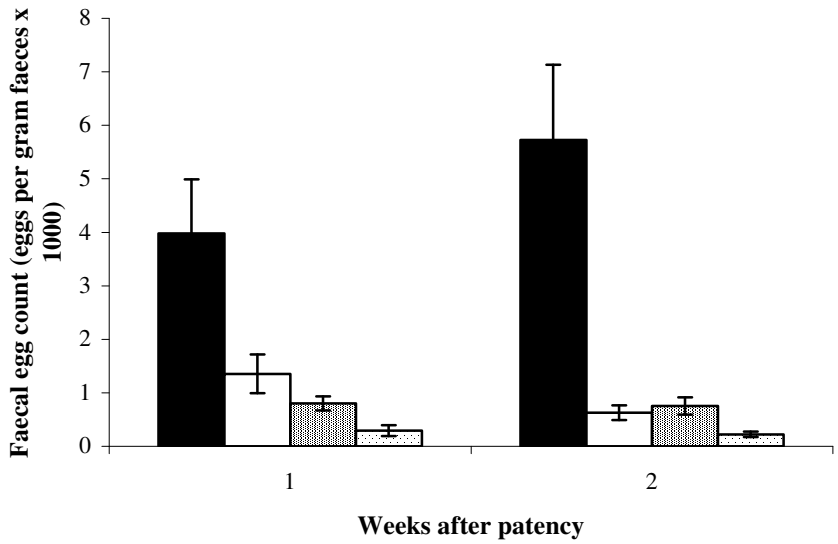


Figure 4.1 Weekly faecal egg counts for lambs infected with *Haemonchus contortus* or *Trichostrongylus colubriformis* and fed either the lucerne or *Calliandra* diets. (■ *Haemonchus contortus*, lucerne diet; □ *Haemonchus contortus*, *Calliandra* diet; ▒ *Trichostrongylus colubriformis*,

lucerne diet; ▒ *Trichostrongylus colubriformis*, *Calliandra* diet). Values are means ± standard errors. Differences in faecal egg count were significant for both diet and species (P < 0.050).

Worm burdens of *T. colubriformis* were higher than burdens of *H. contortus* (P < 0.001). Although there was an interaction between diet and species (P = 0.042), due to slightly higher burdens of *H. contortus* on the *Calliandra* diet, and slightly higher burdens of *T. colubriformis* on the lucerne diet, worm burdens on the two diets were not different (P = 0.532, Table 4.2). Worm burdens were higher in Period 1 (4030 ± 1980 worms) than in Period 2 (3351 ± 1912 worms; P = 0.007).
As shown in Table 4.2, the percentage of female worms was the same for all groups (P > 0.100), but female *H. contortus* produced more eggs than female *T. colubriformis* (P < 0.001) and females exposed to the lucerne diet produced more eggs than females exposed to the *Calliandra* diet (P < 0.001). The number of eggs present in the uterus of female *T. colubriformis* was not affected by diet (P = 0.878), but was higher in Period 2 (26 ± 1.6 eggs) than in Period 1 (21 ± 2.9 eggs; P = 0.002).

Table 4.2 Total number of worms, percentage of female worms, estimated egg output and eggs present *in utero* in *Haemonchus contortus* and *Trichostrongylus colubriformis* harvested immediately after euthanasia of lambs that were infected with either of the two worm species and fed either lucerne or *Calliandra*. Values are means ± standard errors.

	H. contortus		*T. colubriformis*	
	Lucerne	*Calliandra*	Lucerne	*Calliandra*
Total worms (no.lamb)[§]	1861 ± 230	2237 ± 395	5718 ± 339	4862 ± 452
Female worms (%)	55 ± 2	51 ± 3	51± 1	51± 2
Eggs (no./female worm/24 h)* [§†]	3299 ± 560	274 ± 65	134 ± 15	57 ± 21
Eggs (no. *in utero*)/female worm	ND**	ND**	24 ± 3	24 ± 4

*Estimated from faecal egg count and 24 hour faecal output per lamb eight days after patency.
**Not determined due to the high fecundity of female *Haemonchus contortus.*
[§]Values (pooled for diet) are significantly different for each worm species (P < 0.050).
[†] Values (pooled for worm species) are significantly different for each diet (P < 0.050).

4.3.2 Nutritional analysis

(a) Feeds

Data were examined for differences between the two periods of N balance determination (Post-infection 1 and Post-infection 2) and between the two dietary

treatments. Data on the composition of the lucerne and *Calliandra* diets are presented in Table 4.3.

Table 4.3 The dry matter (DM), organic matter (OM) and crude protein (CP) content of the lucerne and *Calliandra* diets fed to lambs infected with *Haemonchus contortus* or *Trichostrongylus colubriformis* immediately after infection and during the patent period. Values are means ± standard error.

	Post-infection 1		Post-infection 2		Within lambs	Effect of diet
Diet	Lucerne	*Calliandra*	Lucerne	*Calliandra*	P	P
DM (%)	90 ± 0.1	41 ± 0.4	91 ± 0.1	41 ± 0.4	0.055	<0.001
OM (%)	91 ± 0.1	92 ± 0.1	91 ± 0.0	93 ± 0.2	> 0.100	<0.001
CP (%)	24 ± 0.3	25 ± 0.2	24 ± 0.2	24 ± 0.1	>0.100	0.006

The DM values for lucerne were higher than those for *Calliandra* during the two N balance periods. The OM values of the *Calliandra* diet were higher than the lucerne diet during Post-infection 1 and Post-infection 2 and so was the CP content during Post-infection 1. The dietary CP was higher in Period 1 than Period 2 ($P < 0.001$); the difference was due to a higher CP value in lucerne in Period 1.

(b) Energy and nitrogen

Data relating to energy and N transactions in lambs fed either the lucerne or the *Calliandra* diets during Post-infection 1 and Post-infection 2 are presented in Table 4.4. Data for the uninfected lambs, which only underwent a single period of N balance, are presented separately in Table 4.5.

Table 4.4 Live weight (LW), maintenance energy requirement (Mm), intakes of dry matter (DM), digestible organic matter (DOM) and metabolisable energy (ME), nitrogen (N) intake, excretion and retention in lambs infected with either *Trichostrongylus colubriformis* or *Haemonchus contortus* and fed either the lucerne or *Calliandra* diet during the immediate post-infection period or during the patent period. Values are means ± standard errors.

	Post-infection 1		Post-infection 2		Within lambs	Effect of diet
Diet	Lucerne	*Calliandra*	Lucerne	*Calliandra*	P	P
Energy transactions						
LW of lambs (kg)	19.1 ± 0.55	17.9 ± 0.80	21.6 ± 0.52	18.9 ± 0.83	<0.001	0.867
Mm (MJ/d)	3.6 ± 0.06	3.5 ± 0.09	3.9 ± 0.07	3.6 ± 0.11	<0.001	0.870
DM intake	553 ± 14.6	475 ± 35.1	626 ± 17.6	540 ± 29.3	<0.001	0.003
DM digestibility (%)	57 ± 0.5	46 ± 1.1	58 ± 0.5	50 ± 0.7	0.029	<0.001
DOM intake	293 ± 7.8	223 ± 16.2	339 ± 11.2	261 ± 15.3	<0.001	<0.001
ME diet (MJ/kg DM)*	8.0 ± 0.07	6.8 ± 0.14	8.1 ± 0.06	7.2 ± 0.09	0.009	<0.001
ME intake (MJ/d)	4.4 ± 0.12	3.3 ± 0.27	5.1 ± 0.18	3.9 ± 0.23	<0.001	<0.001
Nitrogen transactions						
N intake (g/d)	20.6 ± 0.68	18.6 ± 1.07	23.6 ± 0.94	21.0 ± 1.15	<0.001	0.010
N excreted (g/d)						
Faeces	5.7 ± 0.22	10.8 ± 0.58	6.2 ± 0.27	11.1 ± 0.45	0.010	<0.001
Urine	12.5 ± 0.49	6.7 ± 0.28	14.6 ± 0.98	6.9 ± 0.57	0.016	<0.001
Urine urea N	8.6 ± 0.73	4.6 ± 0.25	11.7 ± 1.04	4.6 ± 0.53	0.017	<0.001
N retained (g/d)	2.6 ± 0.53	1.7 ± 0.68	2.7 ± 0.70	2.9 ± 0.35	0.245	0.606
N excreted via faeces (%)	31.6 ± 1.15	61.2 ± 1.22	30.6 ± 1.64	62.6 ± 1.45	0.795	<0.001
N excreted via urine (%)	68.4 ± 1.15	38.8 ± 1.22	69.4 ± 1.64	37.4 ± 1.45	0.795	<0.001
Proportion of N intake retained (%)	12.1 ± 2.13	6.2 ± 4.15	11.1 ± 2.86	13.3 ± 1.39	0.038	0.614
PED (g/day)**	61.7 ± 1.59	55.2 ± 2.64	68.5 ± 2.22	61.4 ± 2.61	<0.001	0.003

*Estimated using the equation: ME (MJ/kg DM) = 0.15 x DOMD, where DOMD is digestible organic matter expressed as percent.
**Protein entering the duodenum estimated using the equation described by Weston & Hogan (1973): Equation 3.6.

Table 4.5 Live weight (LW), maintenance energy requirement (Mm), intakes of dry matter (DM), digestible organic matter (DOM) and metabolisable energy (ME), nitrogen (N) intake, excretion and retention in nematode-free lambs fed either the lucerne or *Calliandra* diet. Values are means ± standard errors.

Diet	Lucerne	*Calliandra*	P
Energy transactions			
LW of lambs (kg)	18.6 ± 1.2	18.1 ± 1.3	0.781
Mm (MJ/d)	3.6 ± 0.2	3.5 ± 0.2	0.781
DM intake	495 ± 32	469 ± 36	0.599
DM digestibility (%)	50.8 ± 1.2	43.3 ± 2.3	0.027
DOM intake	240 ± 20	204 ± 21	0.255
ME diet (MJ/kg DM)*	7.2 ± 0.2	6.5 ± 0.3	0.054
ME intake (MJ/d)	3.6 ± 0.3	3.0 ± 0.3	0.236
Nitrogen transactions			
N intake (g/d)	16.4 ± 1.2	17.8 ± 1.4	0.530
N excreted (g/d)			
Faeces	5.2 ± 0.3	10.8 ± 0.7	<0.001
Urine	10.4 ± 0.8	6.6 ± 0.9	0.019
Urine urea N	18.2 ± 1.0	10.4 ± 2.0	0.012
N retained (g/d)	0.7 ± 0.4	0.4 ± 0.2	0.487
N excreted via faeces (%)	33.4 ± 0.8	62.6 ± 2.0	<0.001
N excreted via urine (%)	66.6 ± 0.8	37.4 ± 2.0	<0.001
Proportion of N intake retained (%)	4.2 ± 2.6	2.5 ± 1.0	0.561
PED (g/day)**	102.5 ± 7.7	111.2 ± 9.0	0.636

*Estimated using the equation: ME (MJ/kg DM) = 0.15 x DOMD, where DOMD is digestible organic matter expressed as percent.
**Protein entering the duodenum estimated using the equation described by Weston & Hogan (1973): Equation 3.6.

Dry matter intake was higher in the groups of lambs fed the lucerne diet than those fed the *Calliandra* diet (P = 0.003). The digestibility value of lucerne was also higher than that of *Calliandra* (P < 0.001), and these dietary differences were evident in both Post-infection periods (Table 4.4).

Consistent with the DM intake and digestibility values, the estimated ME intake values were higher for lambs on the lucerne diet than for those on the *Calliandra* diet (P < 0.001) during both Post-infection periods. Overall, the mean ME intake was higher in Period 2 (4.5 ± 0.18 MJ/d) than in Period 1 (3.8 ± 0.18 MJ/d; P = 0.002).

Nitrogen intake of the lambs was higher during Post-infection 2 than Post-infection 1 (P < 0.001). Overall N intake was higher in animals on the lucerne diet than in those on the *Calliandra* diet (P = 0.010).

Urinary N excretion was greater in the groups on the lucerne diet than in the groups on the *Calliandra* diet (P < 0.001). In animals fed the lucerne diet urinary N excretion was higher in Period 1 (15 ± 0.8 g/d) than in Period 2 (11 ± 0.5 g/d), whereas in lambs on the *Calliandra* diet, urinary N excretion was higher in Period 2 (8 ± 0.4 g/d) than in Period 1 (6 ± 0.3 g/d; P < 0.001).

The proportion of excreted N that was present in the urine was higher in animals on the lucerne diet than in those on the *Calliandra* diet (P < 0.001) and was higher in Period 1 (55 ± 1.1 %) than in Period 2 (52 ± 0.9 %; P = 0.001). The Period difference was mainly due to lambs on the lucerne diet (P < 0.001).

Total faecal N excretion was higher in animals on the *Calliandra* diet than in those on the lucerne diet (< 0.001) and higher in Period 2 (9 ± 0.4 g/d) than in Period 1 (8 ± 0.3 g/d; P = 0.002).

The percentage of total excreted N that was present in the faeces remained constant over both Post-infection periods (P > 0.100; see Table 4.4). Between dietary treatment groups, the percentage was higher in lambs on the *Calliandra* diet (P < 0.001). The percent faecal N was also higher in Period 2 (47.9 ± 0.88 %) than in Period 1 (44.6 ± 1.13 %; P = 0.001).

Despite differences in N intake between lambs on the *Calliandra* and lucerne diets, there was no difference in net N retention (P = 0.614; Table 4.4). The N retention of lambs expressed as a percentage of N intake increased between Post-infection 1 and Post-infection 2 in lambs on the *Calliandra* diet (P = 0.038), but overall N retention as a percentage of intake remained constant across the diets. However, there was a significant iinteraction of diet and year (P = 0.010). Percent N retention of lambs on the *Calliandra* diet was higher in Period 2 than in Period 1, but was similar for both Periods in lambs on the lucerne diet.

The estimated amount of PED was greater in lambs fed the lucerne diet than in those fed the *Calliandra* diet (P = 0.003).

4.3.3 Blood biochemistry

Data on a number of biochemical parameters in the plasma of lambs fed the lucerne and *Calliandra* diets are presented in Table 4.6. The plasma concentration profiles of the metabolites presented in Table 4.6 during the pre- and post-infection periods are presented in Figures 4.2-4.10.

Table 4.6 Plasma total protein, albumin, urea, glucose and inorganic phosphate concentrations and packed cell volume (PCV) in lambs fed either the lucerne or *Calliandra* diets immediately after infection (Post-infection 1) or during the patent period (Post-infection 2). Values are means ± standard errors.

	Post-infection 1		Post-infection 2		Within lambs	Species effect	Diet effect
Diet	Lucerne	*Calliandra*	Lucerne	*Calliandra*	P	P	P
Total protein (g/L)	58.7 ± 1.38	58.9 ± 1.59	61.0 ± 0.82	61.8 ± 1.18	0.372	0.647	0.194
Albumin (g/L)	36.9 ± 0.58	38.8 ± 0.43	36.7 ± 0.70	37.4 ± 0.64	0.089	0.051	0.085
Urea (mM)	26.2 ± 0.92	16.2 ± 0.95	23.2 ± 1.67	14.8 ± 1.00	<0.001	0.562	<0.001
Glucose (mM)	3.9 ± 0.12	3.9 ± 0.09	3.9 ± 0.14	4.1 ± 0.11	0.085	0.226	0.646
Inorganic phosphorus (mM)	2.5 ± 0.07	2.0 ± 0.14	2.3 ± 0.17	1.8 ± 0.12	0.187	0.017	0.009
PCV (%)	33 ± 0.8	37 ± 1.0	31 ± 1.0	31 ± 2.2	< 0.001	0.0003	0.090

(a) Total protein

The mean plasma protein concentrations were similar in sheep that were fed the two diets during both Post-infection periods (P = 0.372). Despite this similarity, the concentration appeared to diverge with increasing weeks post-infection (Figure 4.2). The plasma protein concentration decreased in lambs on the lucerne diet, but appeared to increase slightly on the *Calliandra* diet. However, these changes did not reach significance during the course of the experiment (P = 0.194). Plasma protein concentrations were higher in Period 1 (62.9 ± 0.91 g/L) than in Period 2 (57.7 ± 1.33 g/L; P = 0.012).

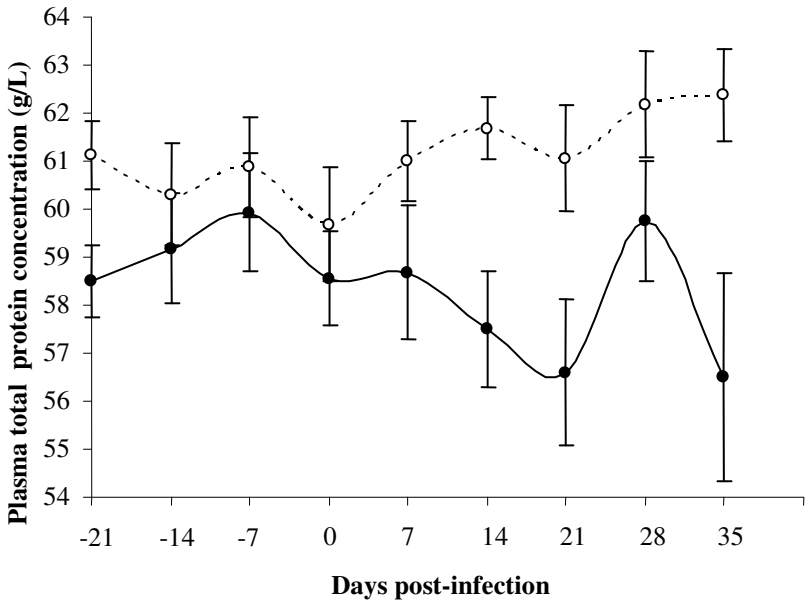


Figure 4.2 Concentration of total protein in plasma of lambs fed the lucerne (–●–) or *Calliandra* (···○···) diets pre- and post-infection with *Haemonchus contortus* or *Trichostrongylus colubriformis*. Data are pooled for worm species. Values are means ± standard errors.

(b) Albumin

There was a tendency for the mean albumin concentration in plasma of lambs fed the *Calliandra* diet to be higher than that of lambs fed lucerne. This was significant during the pre-infection period (P = 0.001) but did not quite reach statistical significance during Post-infection 1 and 2 (P = 0.085). In the post-infection periods, there appeared to be a general decline in plasma albumin concentrations in the animals on both diets (Figure 4.3), but this was not significant (P = 0.089). This trend was more apparent in *Calliandra*-fed lambs than in lucerne-fed lambs (P = 0.085).

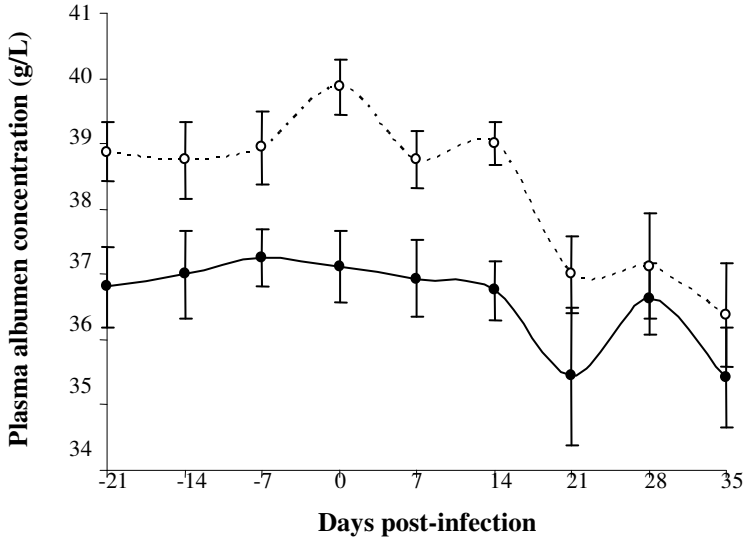


Figure 4.3 Concentration of albumin in plasma of lambs fed the lucerne (–●–) or *Calliandra* (···○···) diets pre- and post-infection with *Haemonchus contortus* or *Trichostrongylus colubriformis*. Data are pooled for worm species. Values are means ± standard errors.

The mean plasma albumin concentration was higher in lambs infected with *T. colubriformis* than in those infected with *H. contortus* during Post-infection 1 and 2 (P = 0.051). The observed decrease in plasma albumin concentrations was also more pronounced in lambs infected with *H. contortus* than in those infected with *T. colubriformis* (P = 0.034; Figure 4.4).

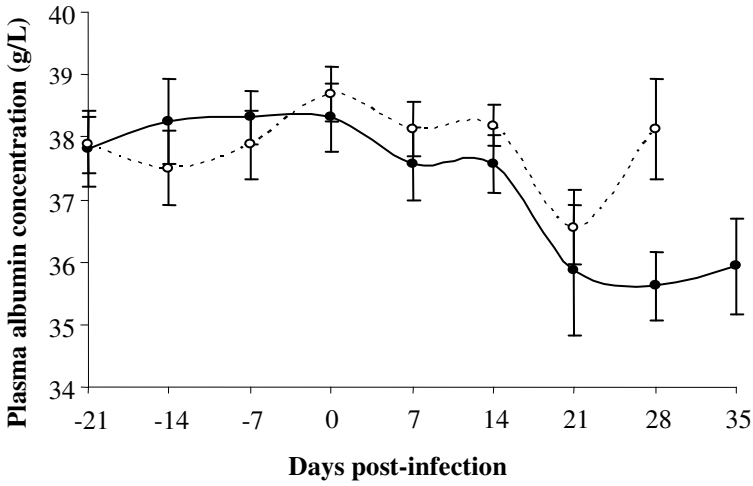


Figure 4.4 Concentration of albumin in plasma of lambs infected with *Haemonchus contortus* (–●–) or *Trichostrongylus colubriformis* (···○···) and fed either the *Calliandra* or lucerne diets pre- and post-infection. Data are pooled for diet. Values are means ± standard errors.

(c) Urea

During the pre-infection period, plasma urea concentration decreased over time in lambs on the *Calliandra* diet but increased with time in lambs on the lucerne diet (P = 0.013; see Figure 4.5). This resulted in higher plasma urea concentrations in the lucerne-fed lambs than in the *Calliandra*-fed lambs (P < 0.001). The pre-infection plasma urea concentration of the lambs was higher in Period 1 (23.4 ± 0.88 g/L) than in Period 2 (20.7 ± 0.51 g/L).

Plasma urea concentration decreased slightly between Post-infection 1 and Post-infection 2 (P < 0.001), and this decrease was more marked in lambs on the *Calliandra* diet than in lambs on the lucerne diet (P < 0.001). Overall plasma urea concentration was higher in lambs on the lucerne diet (P < 0.001).

Plasma urea concentrations were not affected by infecting nematode species (P > 0.100).

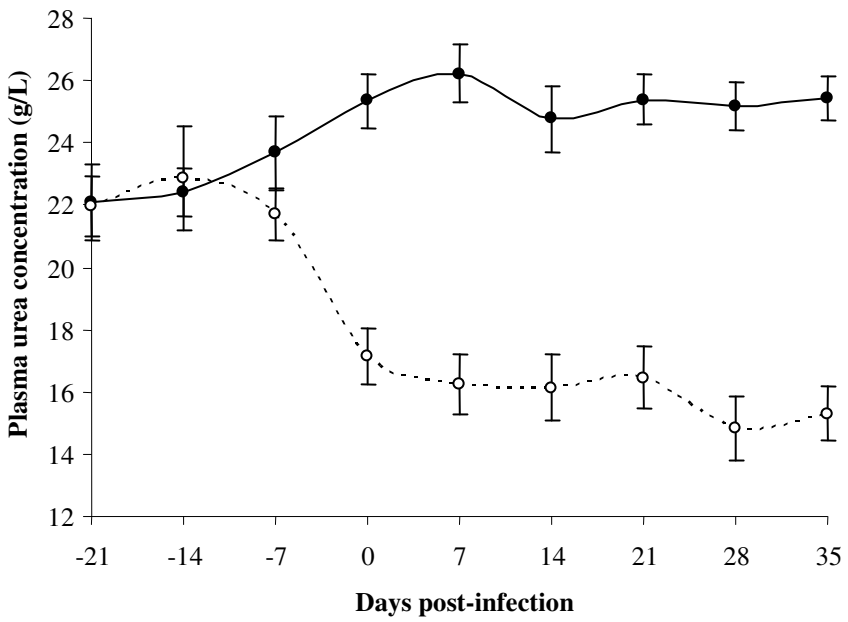


Figure 4.5 Concentration of urea in plasma of lambs fed the lucerne (–●–) or *Calliandra* (···○···) diets pre- and post-infection with *Haemonchus contortus* or *Trichostrongylus colubriformis*. Data are pooled for worm species. Values are means ± standard errors.

(d) Glucose

Plasma glucose concentrations were similar in all sheep during the pre-infection period (P = 0.560) and remained constant until the end of the experiment (P = 0.085). There was a small increase in plasma glucose concentration in the lambs infected with *H. contortus* but not in those infected with *T. colubriformis* between Post-infection 1 and Post-infection 2 (Figure 4.6). Despite this, there were no significant differences between groups (P > 0.100), either during the pre-infection period or after infection. Diet did not affect plasma glucose concentrations.

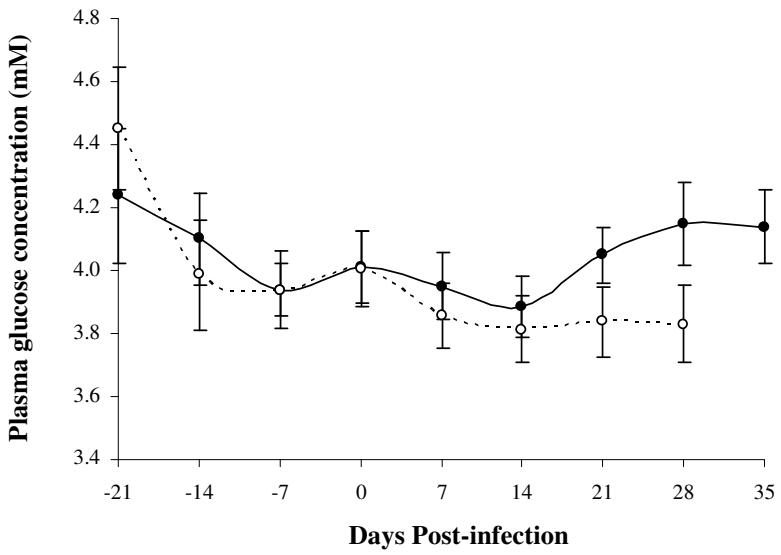


Figure 4.6 Concentration of glucose in plasma of lambs infected with *Haemonchus contortus* (–●–) or *Trichostrongylus colubriformis* (···○···) and fed either the *Calliandra* or lucerne diets pre- and post-infection. Data are pooled for diet. Values are means ± standard errors.

(e) Phosphorus

Plasma phosphorus concentrations were higher on the lucerne diet than the *Calliandra* diet throughout the course of the experiment (P = 0.010; see Figure 4.7). There was a tendency for phosphorus concentration to decrease irrespective of diet in the pre-infection period (P = 0.089) but not between Post-infection 1 and Post-infection 2 (P = 0.187). Plasma phosphorus concentrations were similar for lambs infected with either worm species during the pre-infection period (P = 0.080), but were higher for lambs infected with *H. contortus* than for those infected with *T. colubriformis* during Post-infection 1 and 2 (P = 0.017; Figure 4.8).

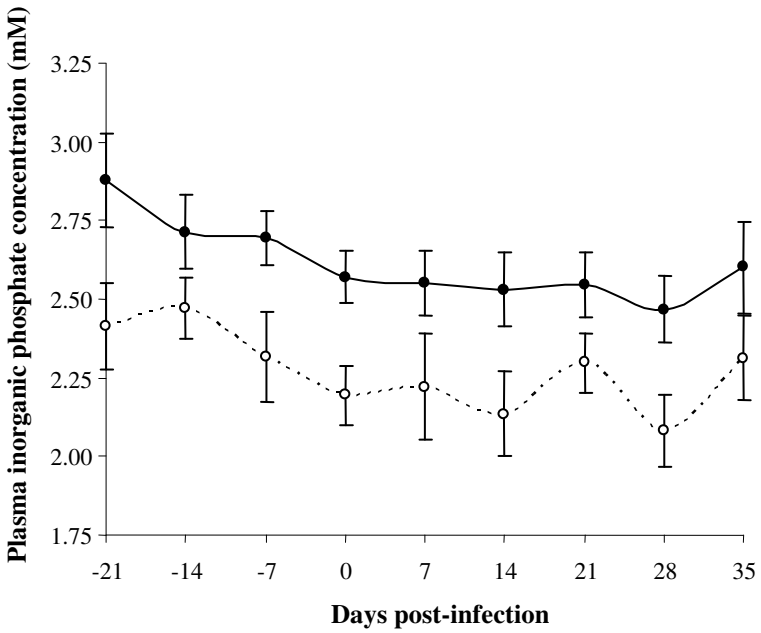


Figure 4.7 Concentration of inorganic phosphate in plasma of lambs fed the lucerne (–●–) or *Calliandra* (···○···) diets pre- and post-infection with *Haemonchus contortus* or *Trichostrongylus colubriformis*. Data are pooled for worm species. Values are means ± standard errors.

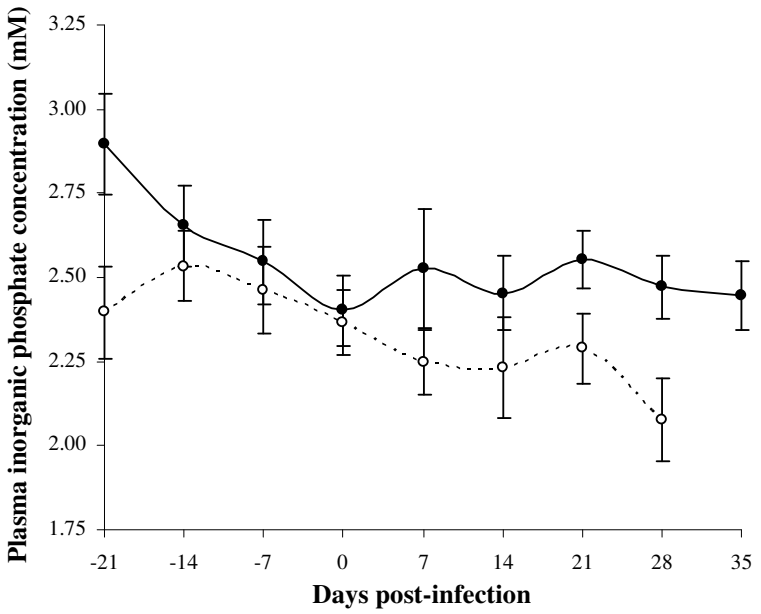


Figure 4.8 Concentration of inorganic phosphate in plasma of lambs infected with *Haemonchus contortus* (–●–) or *Trichostrongylus colubriformis* (···○···) and fed either the *Calliandra* or lucerne diets pre- and post-infection. Data are pooled for diet. Values are means ± standard errors.

(f) Packed cell volume

During the pre-infection period, PCV remained constant in all the lambs (P =0.825). However, PCV was higher in Period 2 (38 ± 0.5 %) than in Period 1 (37 ± 0.4 %; P = 0.027)

During Post-infection 1 and 2, PCV decreased in the lambs on both diets (P < 0.001). However, as can be seen in Figure 4.9, the PCV declined further in lambs on the *Calliandra* diet than in lambs on the lucerne diet (P < 0.001). There was an overall trend for PCV in *Calliandra*-fed lambs to be higher than that of lucerne-fed lambs, but this was not significant (P = 0.090).

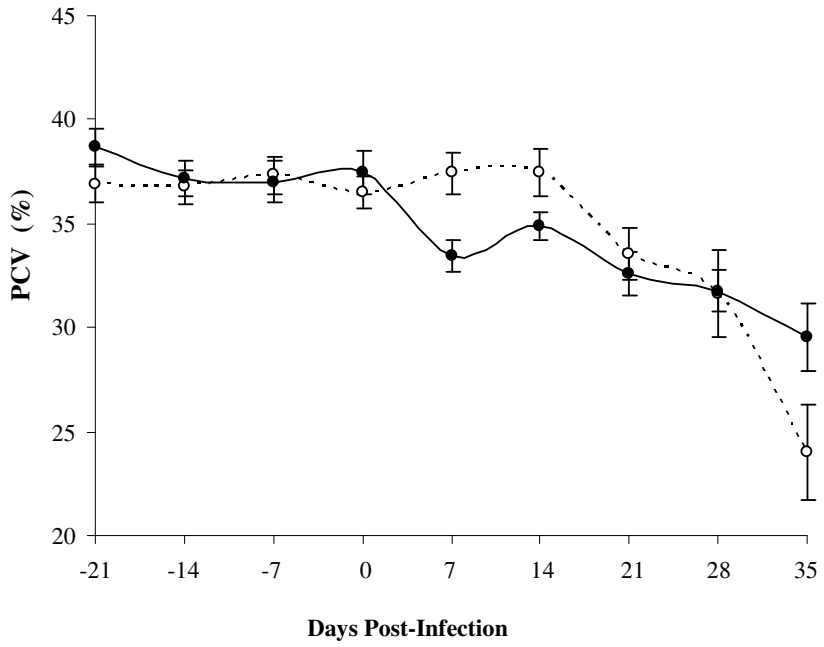


Figure 4.9 Packed cell volume (PCV) blood of lambs fed the lucerne (–●–) or *Calliandra* (···○···) diets pre- and post-infection with *Haemonchus contortus* or *Trichostrongylus colubriformis*. Data are pooled for worm species. Values are means ± standard errors.

During Post-infection 1 and Post-infection 2, PCV remained constant in lambs infected with *T. colubriformis* but decreased markedly in lambs infected with *H. contortus* (P < 0.001; Figure 4.10). This resulted in higher PCV values in the lambs infected with *T. colubriformis* (P = 0.003). The decrease in PCV between Post-infection 1 and Post-infection 2 was more marked in Period 1 than in Period 2

(P = 0.001), but the absolute values were not significantly different between Periods (P = 0.475).

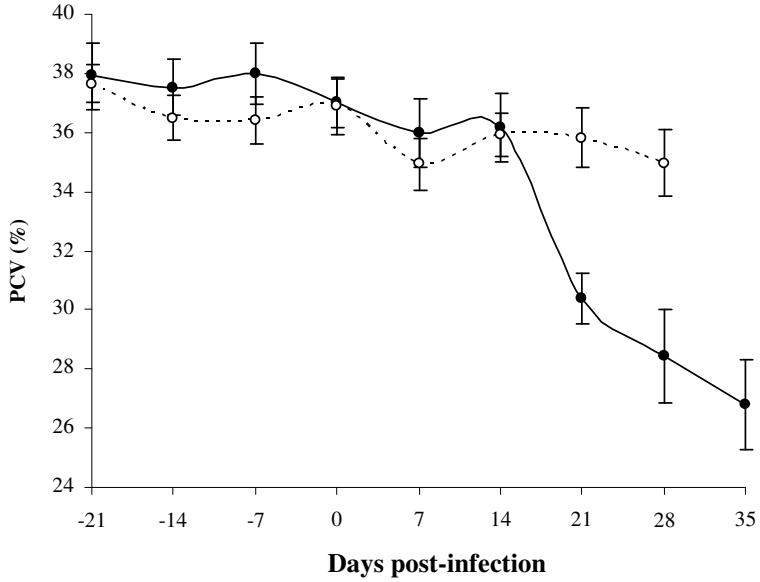


Figure 4.10 Packed cell volume (PCV) in the blood of lambs infected with *Haemonchus contortus* (–●–) or *Trichostrongylus colubriformis* (···○···) and fed either the *Calliandra* or lucerne diets pre- and post-infection. Data are pooled for diet. Values are means ± standard errors.

4.3.4 pH

The pH in each GI segment is presented in Table 4.7.

Table 4.7 The pH of digesta in different segments of the gastrointestinal (GI) tract of uninfected lambs or lambs infected with either *Trichostrongylus colubriformis* or *Haemonchus contortus* and fed a diet of either lucerne or *Calliandra*. Values are means ± standard error. R = rumen, A= abomasum, D = duodenum, J = jejunum, I = ileum, Cae = caecum, Co = colon.

	GI nematode species						
	Uninfected control		*H. contortus*		*T. colubriformis*		Diet
Diet	Lucerne	*Calliandra*	Lucerne	*Calliandra*	Lucerne	*Calliandra*	effect
Digesta pH							P
R	6.9 ± 0.03	6.7 ± 0.05	6.8 ± 0.05	6.8 ± 0.06	6.8 ± 0.04	6.8 ± 0.07	0.005
A	3.0 ± 0.02	3.9 ± 0.35	3.2 ± 0.16	3.5 ± 0.34	3.4 ± 0.18	3.4 ± 0.25	0.060
D*	5.8 ± 0.27	4.6 ± 0.35	5.7 ± 0.21	5.5 ± 0.29	5.2 ± 0.39	4.7 ± 0.50	0.047
J	6.3 ± 0.07	6.3 ± 0.17	6.2 ± 0.10	6.6 ± 0.16	6.3 ± 0.06	6.3 ± 0.10	0.221
I	7.3 ± 0.11	7.5 ± 0.08	7.4 ± 0.05	7.7 ± 0.06	7.4 ± 0.04	7.6 ± 0.03	< 0.001
Cae	6.8 ± 0.03	7.2 ± 0.18	6.7 ± 0.04	7.0 ± 0.04	6.9 ± 0.06	7.2 ± 0.09	< 0.001
Co	7.1 ± 0.04	7.2 ± 0.05	6.9 ± 0.03	7.2 ± 0.07	7.1 ± 0.04	7.3 ± 0.06	< 0.001

*pH values were significantly affected by worm species (P = 0.023)

There was an interaction between species and diet in the abomasum (P = 0.031). In animals infected with *H. contortus*, the pH was slightly higher in *Calliandra*-fed lambs than in lucerne-fed lambs, while in animals infected with *T. colubriformis*, the pH was highest on the lucerne diet. In uninfected animals, the abomasal pH was the same on both diets.

The pH was affected by nematode species only in the duodenum and colon (Table 4.8 and Figure 4.11). In the duodenum the pH was higher in *T. colubriformis*-infected lambs than in controls (P = 0.036) or *H. contortus*-infected lambs (P = 0.091), although the latter did not reach significance. There was no difference in duodenal pH between lambs infected with *H. contortus* and uninfected controls (P =0.680). A similar trend was observed in the colon, where pH was lower in lambs infected with *T. colubriformis* than in uninfected lambs (P = 0.002) or those infected with *H. contortus* (P = 0.094).

Table 4.8 The pH of digesta in different segments of the gastrointestinal (GI) tract of uninfected lambs or lambs infected with either *Trichostrongylus colubriformis* or *Haemonchus contortus* across both lucerne and *Calliandra* diets. Values are means ± standard error.

	GI nematode species			
Digesta pH	Uninfected control	*H. contortus*	*T. colubriformis*	P
R	6.8 ± 0.04	6.8 ± 0.04	6.8 ± 0.03	0.850
A	3.4 ± 0.14	3.4 ± 0.23	3.3 ± 0.18	0.875
D	5.0 ± 0.31	5.2 ± 0.26	5.6 ± 0.18	0.023
J	6.3 ± 0.05	6.3 ± 0.09	6.4 ± 0.10	0.441
I	7.5 ± 0.04	7.4 ± 0.02	7.5 ± 0.01	0.111
Cae	7.1 ± 0.07	7.0 ± 0.12	6.9 ± 0.07	> 0.100
Co	7.2 ± 0.07	7.2 ± 0.03	7.0 ± 0.06	0.005

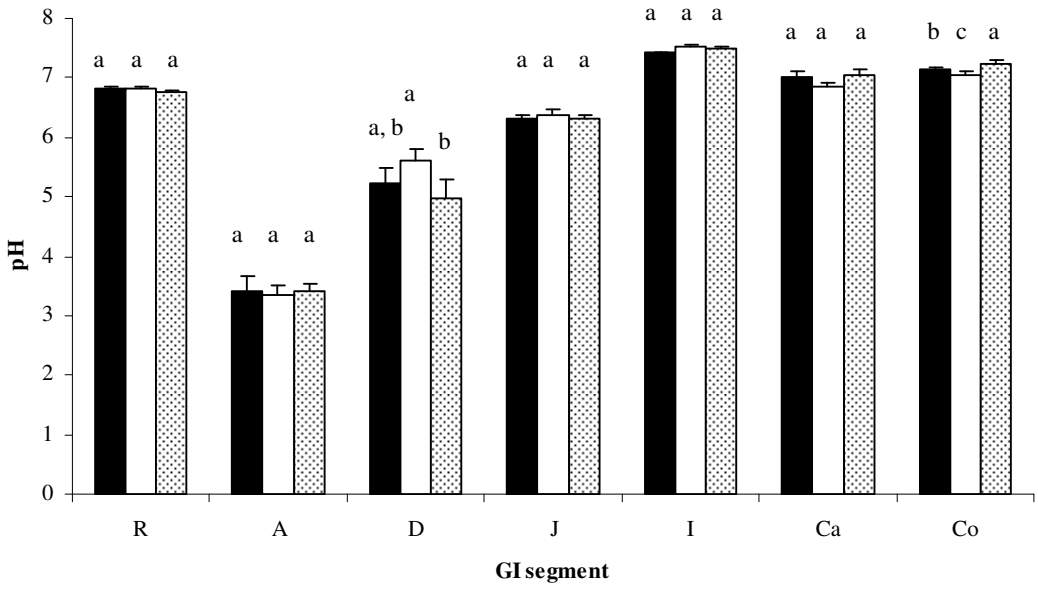


Figure 4.11 The pH in the digestive tract of uninfected lambs (▒) and lambs infected with either *Haemonchus contortus* (■) or *Trichostrongylus colubriformis* (□) across both lucerne and *Calliandra* diets. Values are means ± standard errors. Within each GI segment, columns with different superscripts are significantly different (P < 0.050). R = rumen, A= abomasum, D = duodenum, J = jejunum, I = ileum, Cae = caecum, Co = colon.

The effect of diet on pH of digesta is presented in Table 4.9 and Figure 4.12. The pH was higher in the ileum, caecum and colon in *Calliandra*-fed lambs than in lucerne-fed lambs, but in the rumen and duodenum pH was lower in the *Calliandra*-fed lambs.

Table 4.9 The pH of digesta in different segments of the gastrointestinal (GI) tract of lambs fed a diet of either lucerne or *Calliandra* and either uninfected or infected with either *Haemonchus contortus* or *Trichostrongylus colubriformis*. Data are pooled for worm species. Values are means ± standard error.

	Diet		
Digesta pH	Lucerne	*Calliandra*	P
R	6.9 ± 0.02	6.8 ± 0.03	0.005
A	3.1 ± 0.11	3.6 ± 0.20	0.060
D	5.7 ± 0.16	5.0 ± 0.21	0.047
J	6.3 ± 0.05	6.4 ± 0.09	0.216
I	7.3 ± 0.05	7.6 ± 0.04	0.000
Cae	6.8 ± 0.04	7.1 ± 0.07	0.000
Co	7.0 ± 0.04	7.2 ± 0.04	0.000

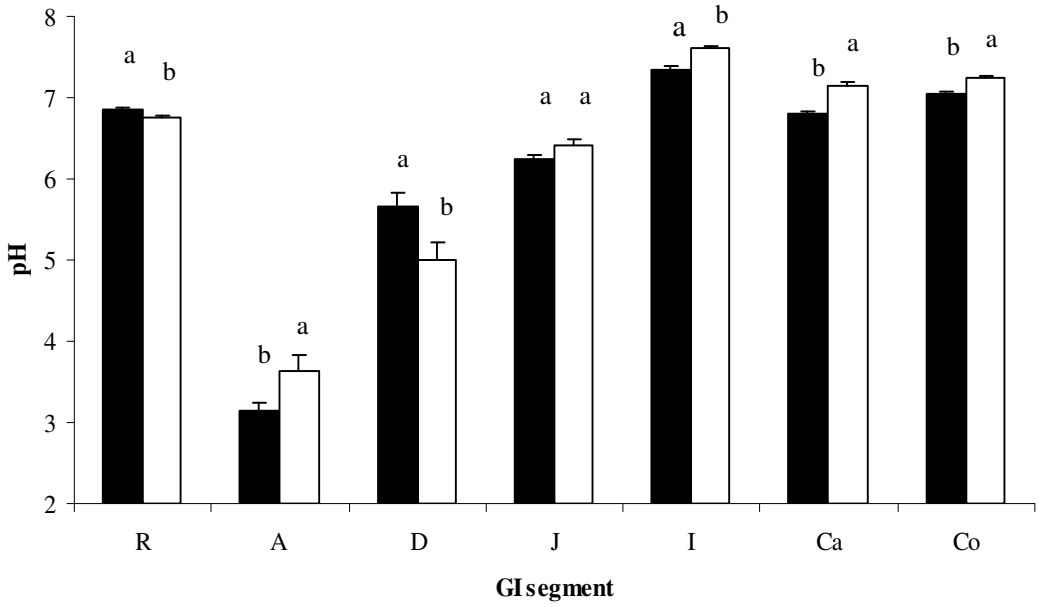


Figure 4.12 The pH in the digestive tract of lambs fed either the lucerne (■) or *Calliandra* (□) diet and either uninfected or infected with either *Haemonchus contortus* or *Trichostrongylus colubriformis*. Data are pooled for worm species. Values are means ± standard errors. Within each GI segment, columns with different superscripts are significantly different (P < 0.050). R = rumen, A= abomasum, D = duodenum, J = jejunum, I = ileum, Cae = caecum, Co = colon.

The pH in the ileum was higher in Period 1 (7.6 ± 0.05) than in Period 2 (7.4 ± 0.05; P = 0.005).

4.3.5 Total condensed tannin concentration

Total condensed tannin concentrations in each segment of the GI tract of the uninfected *Calliandra*-fed lambs were determined as part of a separate study and are presented in Table 4.10.

Table 4.10 Condensed tannin (CT) concentration in different GI segments of uninfected *Calliandra*-fed lambs. Data from D. Martin, personal communication. Condensed tannin analysis was carried out using a modification of the method of Terrill *et al.* (1992b).

GI segment	R	A	D	I	Ca	Co
CT concentration (g/L digesta)	19.1 ± 0.9	52.7 ± 2.0	9.0 ± 1.3	26.0 ± 0.8	29.6 ± 0.8	31.4 ± 1.2

4.3.6 Gastrointestinal pathology

No gross pathological changes were evident in the GI tract of lambs infected with *T. colubriformis* in this experiment. Lambs infected with *H. contortus* had pinpoint haemorrhages in the abomasal mucosa indicating sites of worm attachment, but no other obvious macroscopic changes were evident. Histological findings are described in Section 6.3.1.

4.4 Discussion

4.4.1 Effects of *Calliandra* on GI nematodes

Generally, the parasitology results obtained in the current experiment confirmed those observed in the pilot experiment (Section 3.3.1). Faecal egg counts (either calculated as eggs per gram of faeces or as eggs per female worm per day) were reduced markedly in animals fed *Calliandra* compared to those fed lucerne. However, in the current experiment, not only was the reduction in faecal egg counts significant for both worm species, rather than for *T. colubriformis* only as was observed in the pilot experiment, but it was greater for *H. contortus* than for *T. colubriformis*. This suggests that the lack of a significant effect of *Calliandra* on *H. contortus* faecal egg counts in the pilot experiment was due largely to the small number of lambs used in the experiment. The faecal egg counts of *Calliandra*-fed lambs infected with *H. contortus* in the pilot study were decreased only by approximately 28 % for FEC1 and 44 % for FEC2, compared with the corresponding values of 66 % and 89 % in the current experiment. It is not clear why the reduction in *H. contortus* egg output should almost double in the current experiment, particularly since the reverse was observed with *T. colubriformis*. For the latter species, reductions in faecal egg counts of approximately 92 % and 85 % for FEC1 and FEC2 respectively were observed in the pilot experiment, but reductions were somewhat lower at 64 % and 70 % in the current experiment. It is possible that there were differences in environmental conditions in the GI tract of animals in the two experiments. For example, subtle pH differences could have affected interactions

between nematodes and the active constituent of *Calliandra* (presumably condensed tannins).

An observation that was not consistent with that in the pilot experiment was the apparent ineffectiveness of diet in causing egg retention in female *T. colubriformis*. This may imply a slower rate of oogenesis in the worms in the current experiment, rather than reduced egg shedding as suggested in Chapter 3. Alternatively, the discrepancy may be due to the smaller reduction in faecal egg counts for this nematode species observed during the current experiment. The number of eggs *in utero* in the pilot experiment was only 7 % more in *Calliandra*-fed lambs than in the control animals, so a smaller degree of egg retention in the current experiment may have been too small to detect. More research is needed to elucidate the effects of *Calliandra* on nematode egg production.

Worm burdens were similar in lambs on the two diets, and there was no evidence to suggest that feeding *Calliandra* had any effect on worm establishment or survival during the course of the experiment. There was a statistically significant interaction between diet and worm species, suggesting a reduction in *T. colubriformis* establishment on the *Calliandra* diet or a reduction in *H. contortus* establishment on the lucerne diet. However, the effect of diet alone was not significant (see Table 4.2), and it seems likely the interaction was a result of the inherent variability of establishment rates, as reported by others (Waller & Thomas, 1981). However, the work of Paolini *et al*. (2003b; 2005b) should be noted in this context. These authors examined the effects of feeding quebracho condensed tannin to goats over the period of larval establishment. For *Trichostrongylus* species, worm burdens were decreased although fecundity of the resulting adult female population was not affected, but for *H. contortus* there was no effect on either worm burdens or fecundity. In a similar experiment, Athanasiadou *et al*. (2001a) also found reductions in burdens of *Trichostrongylus* species but not *H. contortus*, whereas Niezen *et al*. (1998c) reported reductions in establishment of the abomasal species *O. circumcincta* but not *T. colubriformis* in lambs fed *L. pedunculatus*. These differences could be due to the type of condensed tannin in the feed, rather than to the location of the parasites in the digestive tract, as has been suggested (Paolini *et al*., 2005b). It is possible that *Calliandra* had a small differential effect on the establishment of the two species in

the current experiment, but if so, the magnitude was too small to affect overall worm burdens.

There was no difference in the ratio of female to male worms observed in lambs fed the two diets in the current experiment. This is in contrast to the observation reported by Shaik *et al*. (2006), who recorded lower female to male ratios in sheep fed *Lespedeza cuneata* hay than in control sheep fed Bermuda grass hay (*Cynodon dactylon*). This difference was apparently due to greater reductions in the establishment of females relative to males, even though both male and female numbers were reduced. Since there was no effect of diet on parasite establishment in the current experiment, differences in sex ratios due to diet were not to be expected.

4.4.2 Development of immunity

One of the mechanisms by which condensed tannins bring about reductions in worm burdens or faecal egg counts is through enhanced development of an immune response to GI nematodes due to improved protein nutrition (Molan *et al.*, 1999; 2003a). Three stages of immunity to parasites occur (Seaton, 1989; Dobson *et al.*, 1990b): in the first, animals are susceptible to parasites; in the second, larval establishment is inhibited and/or egg production of females decreases; and the in third adults are expelled. The onset of the second stage may begin from eight to thirteen weeks after primary infection (Chiejina and Sewell 1974; Kimambo *et al* 1988). Kyriazakis *et al* (1986) found that faecal egg counts fell from the eleventh week of infection, and remained low when sheep were re-challenged after drenching.

To minimise the effects of an acquired immune response in this study, lambs were sourced from a drought-affected area immediately after weaning and had never grazed pasture. It was thus deemed unlikely that they had ever been exposed to GI nematode parasites, and the negative faecal egg counts obtained on arrival at JCU added support for this assumption. Young, weaned lambs do not tend to develop resistance to burdens of *T. colubriformis* or *H. contortus* between three and six months of age (Manton *et al.*, 1962; Dineen *et al.*, 1978). Younger lambs (less than

three months old) are better able to mount an effective immune response (Emery *et al*., 1999), but the reason for this is unknown, since maternal immunity does not seem to be the cause (Dineen *et al*., 1978). Lambs with a live weight greater than 22 kg have lower faecal egg counts and worm burdens than lighter lambs (McClure *et al*., 1992; McClure *et al*., 1999), possibly indicating enhanced development of resistance in heavier animals. In the present study, most lambs did not reach this weight until the end of the experiment. The lambs were also given a single dose of worms at the beginning of the experimental period rather than a trickle infection, as the former was likely to trigger a smaller immune response (Dobson *et al*., 1990a). It is recognised that the lambs may still have been able to mount an innate immune response, and that this may have been quite variable between individual lambs. However, acquired immune responses should have been minimal.

The experiment was concluded at five weeks post-infection because the earliest onset of immunity was likely to be six weeks after exposure to worms (Coop & Kyriazakis, 1999). This limited the study to examination of the effects of *Calliandra* on worms during the establishment phase of infection and the early patent period. However, manipulation of the protein content of the lucerne diet (as previously discussed) to eliminate any nutritional advantage conferred by the higher protected protein content of *Calliandra* suggests that the reduction in faecal egg counts caused by feeding *Calliandra* was due to immune suppression of egg-laying by the female worms. The direct anthelmintic effects of *Calliandra* were thus separated from possible immunological effects.

4.4.3 Energy and protein nutrition

The diets were fed at a DM allowance equivalent to 2.8 % of live weight. This was calculated to be less than *ad libitum* intake, so that the lambs would consume all the feed offered to them. The dietary allowance was designed to provide enough ME for maintenance and moderate growth and to maintain a relatively steady level of intakes of energy and protein across the different groups of animals. This was not completely successful, as DM and N (or protein) intakes were lower in lambs on the *Calliandra* diet (Table 4.4). The mean ME intake of the lambs on the *Calliandra* diet was also lower, reflecting the lower digestibility of *Calliandra* due to its high tannin content.

While N excretion was slightly higher in lambs on the lucerne diet, consistent with the higher N intake, the routes of N excretion were markedly different on the two diets. The major route of N excretion in lambs on the *Calliandra* diet was via the faeces, whereas most was excreted in the urine of lambs on the lucerne diet. This observation is consistent with that recorded in the pilot experiment and indicates a probable reduced rate of N absorption in the GI tract of animals on the *Calliandra* diet. Presumably the loss of N in the faeces was due to the binding of dietary protein to *Calliandra* condensed tannin. It might be suggested therefore that feeding *Calliandra* to lambs did not improve the protein nutrition of these animals compared with those fed the lucerne diet. This is also consistent with the observation that the N retention of lambs that were fed the *Calliandra* and lucerne diets was similar. However, the amount of protein entering the small intestine was higher in animals fed the lucerne diet. Although plant protein (rubisco) and casein form similar numbers of complexes with *Calliandra* tannins, casein is precipitated to a much larger extent (Rakhmani *et al*., 2005). This may reflect the higher proline content and higher binding affinity of casein. The implication of this in the current experiment was that the extra rumen-degradable casein included in the *Calliandra* diet could have formed complexes with free condensed tannin in the rumen, thus increasing the proportion of rumen-undegradable protein in the *Calliandra* diet. However, this should have resulted in extra PED, which apparently was not the case.

4.4.4 Effects of diet and GI parasitism on plasma constituents

(a) Albumin

As expected, plasma albumin concentrations were stable prior to infection with GI nematodes, but gradually decreased after infection due to probable losses of the protein into the GI tract. Albumin concentration declined at a higher rate in lambs infected with *H. contortus* (Figure 4.4), illustrating the relatively greater pathogenicity of this species. Overall concentrations throughout the experiment were higher in the *Calliandra*-fed lambs than in lambs on the lucerne diet, but concentrations were higher in these lambs even before the introduction of the *Calliandra* diet at Day -14 (see Figure 4.3), so this probably was a random factor,

rather than a dietary one. Although it was not statistically significant, albumin concentration declined more rapidly in lambs on the *Calliandra* diet, and from Figure 4.3 it would appear that albumin concentrations on both diets may have reached similar levels if the experiment had continued. Despite this decline, albumin concentrations consistently were above the normal reference range of 24-30 g/L for sheep (Blood & Radostits, 1989). This probably reflected the high dietary protein intake by the lambs and thus adequate albumin synthesis in the liver to compensate for losses due to sub-clinical parasitism. Plasma albumin concentrations reflect protein intakes over the long term (Blood & Radostits, 1989) but may decline due to increased secretion or leakage of the protein into the gut in parasitised animals (Reveron *et al*., 1974). If a clinical protein-losing enteropathy (PLE) or severe haemorrhage were present due to parasitism, the globulin fraction of the plasma protein also would be expected to decrease. This probably did not occur in the current experiment judging from total plasma protein concentrations (see below).

(b) Total protein

The normal range for total plasma protein concentration in young sheep is 60 - 79 g/L (Blood & Radostits, 1989). Pre-infection values for all lambs were within this range, and there were no differences between groups. Infection with *H. contortus* or *T. colubriformis* could be expected to reduce plasma total protein concentrations, due to blood loss in the case of the former, or due to PLE caused by villus atrophy and disruption of the mucosa in the case of the latter. In the current experiment, worm burdens were moderate and did not result in clinical disease. Nevertheless, the presence of haemorrhagic foci in the abomasa of lambs infected with *H. contortus* and the histological changes in the small intestine of lambs infected with *T. colubriformis* (see Section 6.3.1) would suggest that sufficient damage had been done to these organs to result in protein losses. However, the high protein diets fed to lambs in the current experiment would likely improve the ability of the lambs to cope with subclinical parasite infections, by facilitating the synthesis of plasma proteins to replace those lost through the GI tract. Also, the moderate size of the infections would probably allow reabsorption of the protein lost into the GI tract. Comparing values for PED with those for faecal N in Table 4.4, a net absorption of protein did

indeed occur in the small intestine of lambs on the lucerne diet, but small net losses occurred in *Calliandra*-fed lambs. This could be due to formation of tannin-protein complexes, which would prevent the absorption of protein and is consistent with the greater rate of albumin loss in these lambs.

Higher levels of crude protein intake and PED in the lucerne-fed lambs combined with protein losses in the *Calliandra*-fed lambs should have resulted in higher plasma protein concentrations in the former, since there was no difference attributable to worm species. However, plasma protein concentrations were not different in lambs on the two diets, and there was a tendency for plasma protein concentration of lambs on the lucerne diet to decrease with time, while concentration remained fairly stable on the *Calliandra* diet (see Figure 4.2). These changes were very small and non-significant and may have been due in part to the higher initial albumin levels in *Calliandra*-fed lambs [see Section 4.4.4(a)]. Increased globulin production would also cause protein concentrations to remain high in the *Calliandra*-fed lambs, but as discussed previously, an acquired immune response to the nematodes is unlikely to have occurred only four to five weeks after first exposure to GI nematodes (Section 4.4.2). Sykes *et al*. (1979) observed that globulin concentrations did not increase until 11 weeks post-infection with *T. vitrinus*.

(c) Urea

Blood urea concentration is closely associated with total protein intake and may be used as an acute measure of sufficiency of protein intake (Blood & Radostits, 1989). In the current experiment, plasma urea concentrations were consistently above the reference range of values for sheep (3.0-7.0 mM) as might be expected from the high protein content of the diets. If energy for microbial protein synthesis was limiting, excess rumen-degradable protein in the diet would be deaminated to form ammonia. The latter would be absorbed from the rumen, transported to the liver and converted to urea, causing plasma urea concentrations to increase (McDonald *et al*., 1981). Although some urea might be recycled to the rumen, most would be excreted in urine and thus would represent wastage of protein. Similarly, rumen-undegradable protein could supply amino acids that could be used for protein synthesis by the ruminant

animal. Amino acids in excess of requirement would be catabolised to form urea that is subsequently excreted. The protein intake of animals in this study would thus seem to be well in excess of requirements.

Plasma urea concentration decreased significantly on introduction of the *Calliandra* diet at Day -14, compared with an equally significant increase in plasma urea concentration in lucerne-fed lambs as a result of the addition of rumen-undegradable casein to the basal diet (see Figure 4.5). As discussed previously, the increase in plasma urea concentration in lambs on the lucerne diet might be expected, since the extra protein (in the form of rumen-undegradable casein) would have been in excess of requirements, and thus the excess amino acids would be catabolised in the liver. The high rate of urinary excretion of urea in lucerne-fed lambs bears this out (Table 4.4). In contrast, the transition from the lucerne diet to the *Calliandra* diet resulted in a decrease in urea concentration. This may have been due in part to the lower protein intake of the animals on the *Calliandra* diet, but it is also likely that much of the tannin-bound protein escaping degradation in the rumen was not released in the small intestine and passed out of the animal in the faeces.

Urea concentrations reached plateau as the lambs adapted to the diets. Infection with worms on Day 0 did not appear to have any effect, similar to the findings reported by Sykes *et al*. (1979) in lambs given trickle-infections of *T. vitrinus*. In contrast, Roseby *et al*. (1973; 1974) found that plasma urea concentrations increased in parasitised lambs relative to uninfected controls, presumably because amino acid absorption in the small intestine was impaired by parasitic damage to the mucosa. Consequently fermentation occurred in the caecum, and the ammonia produced was converted to urea (Roseby, 1977). However, the lambs in the latter study had worm burdens three times larger than the *T. colubriformis*-infected lambs in the current experiment. The intestinal absorptive capacity of the lambs in the current experiment probably was not severely impaired, so further increases in urea production beyond those due to dietary effects did not occur.

(d) Glucose

Plasma glucose concentrations in the experimental lambs remained within the reference range of 1.7 - 3.6 mM (Blood & Radostits, 1989) and there were no differences between the groups. Worm species did not have a significant effect on plasma glucose concentrations. Results from other work suggest that GI nematode parasitism has negligible effects on glucose metabolism in lambs (Horak *et al.*, 1968; Coop *et al.*, 1976; Bown *et al.*, 1991a).

(e) Phosphorus

Prior to infection, plasma inorganic phosphus concentrations were constant within the groups. Lambs on the lucerne diet had higher concentrations than lambs on the *Calliandra* diet, but these differences were present before introduction of the experimental diets (Figure 4.7). Phosphorus concentrations were also lower in lambs infected with *T. colubriformis* than those infected with *H. contortus*. This might have been expected in lambs parasitised with *T. colubriformis*, since a number of workers (Reveron *et al.*, 1974; Wilson & Field, 1983; Poppi *et al.*, 1985) have observed that the uptake of dietary phosphorus would be impaired and secretion or leakage of phosphorus into the GI tract would increase as a result of damage to the intestinal mucosa. Infection with *H. contortus* could also impair phosphorus absorption (Blood & Radostits, 1989), but not to the same extent.

A study carried out on New Zealand sheep suggested that the absorption of phosphorus in the small intestine could be enhanced slightly by feeding *Lotus corniculatus* condensed tannins (Waghorn *et al*., 1994a), although this finding was not discussed further and no mechanism of action was proposed. Worms such as *T. colubriformis* damage the major site of phosphorus absorption in the proximal small intestine, so if able to offset, even partially, the phosphorus-depleting effects of these worms, enhanced absorption could have major significance in parasitised lambs. In the current study, plasma phosphorus concentrations were lower in lambs on the *Calliandra* diet than in those on the control diet, which does not support an improvement in uptake. However, the phosphorus content of the diets was unknown,

so it is possible that intake was higher on the lucerne diet, although reported values for *Calliandra* (0.24 % DM; McDonald *et al*., 2001) and lucerne pellets (0.12-0.28 % DM; Muhikambele *et al*., 1994) are similar. The phosphorus content of casein is approximately 0.08 % (United States Biochemical Corporation, 1991), and the supplied mineral licks also contained phosphorus (Table 3.3). It seems likely that the phosphorus intakes of the lambs on both diets were adequate, given that the recommended phosphorus density in feeds for growing lambs is approximately 0.23 % DM (Agricultural Research Council, 1980). This is also supported by the fact that plasma phosphorus concentrations were within or above the reference range for sheep (1.3-2.25 mM; Blood & Radostits, 1989) throughout the experiment (see Figure 4.7). Other workers have also failed to detect improvements in plasma phosphorus levels when condensed tannins were fed to parasitised goats, (Paolini *et al*., 2005a) or sheep (Athanasiadou *et al.*, 2000c; 2001a). It would appear that any improvements in phosphorus absorption are insufficient to affect plasma levels in animals fed balanced diets, but the effect on animals with marginal intakes is unknown.

(f) Packed cell volume

As expected, blood PCV decreased further in lambs infected with *H. contortus* than in lambs infected with *T. colubriformis*, due to the greater blood losses in the GI tract in lambs infected with the former species. The mean PCV of the lambs infected with *H. contortus* was at the lower end of the reference range (27 - 45 %) for sheep (Blood & Radostits, 1989) at the time of slaughter. It is interesting that the PCV value in the *Calliandra*-fed lambs infected with *T. colubriformis* remained high while the PCV value in the lucerne-fed lambs decreased, whereas the reverse, although not statistically significant, was observed in lambs infected with *H. contortus*. Also, the *Calliandra*-fed lambs maintained PCV at higher values for longer than lambs on the lucerne diet, although no difference between the two diets was evident by the time the infections became patent (Figure 4.9). In another study, goats infected with *H. contortus* and fed sericea lespedza hay were able to maintain PCV above that of the control group for six weeks after infection (Shaik *et al*., 2006). A positive effect

of tanniniferous feeds on PCV would be of considerable benefit in improving the resilience of parasitised lambs, but was not confirmed by the results of this study.

4.4.5 Gastrointestinal pathology

Heavy burdens of *H. contortus* usually cause haemorrhagic foci in the abomasum, as seen in the current experiment. Other clinical signs include weakness, lethargy, weight loss, pallor of the mucous membranes, ventral oedema, and darkening of digesta due to partially digested blood (Urquhart *et al*., 1996). None of these, however, were noticeable in the current experiment. Gross pathological findings in intestinal trichostrongylosis generally consist of an erosive duodenal enteritis, accompanied by clinical signs of inappetance, diarrhea and weight loss, which again were not evident in the current experiment. This is consistent with the moderate worm burdens administered and the short duration of the infections before the host lambs were euthanased.

Histological examination of the GI tracts from these lambs is discussed in Section 7.4.2.

4.4.6 pH

The pH values of digesta along the GI tract were determined because of the importance of pH in the formation of condensed tannin-protein complexes. Complexing of nematode proteins with condensed tannin could be a mechanism by which *Calliandra* would affect GI nematodes. The effects that the different diets and the different worm species had on pH were also of interest, since pH in itself may affect egg-shedding by ruminant parasitic nematodes (Hondé & Buéno, 1982).

The nematode species examined in the current experiment had little effect on GI digesta pH, except in the duodenum. Duodenal pH was significantly higher in lambs infected with *T. colubriformis* than in lambs infected with *H. contortus* or in the uninfected control groups. Infection with *T. colubriformis* has been shown to increase duodenal pH from a mean of pH 2.5 in control sheep, to a mean of pH 4.0,

13 weeks after infection (Poppi *et al.*, 1985; Poppi *et al.*, 1986). The increase in pH value was attributed to reduced motility in the duodenum, but it could also be due to an increased rate of bile and/or pancreatic juice release into the duodenum. Increased duodenal pH values from 3.7 to 5.3, 10 - 12 days after infection have also been reported for sheep infected with *H. contortus* (Buéno *et al*., 1982b). In the current experiment, duodenal pH values were appreciably higher than these reported values in all groups, particularly in the lambs infected with *T. colubriformis*. The animals used by Poppi *et al.*, (1986) were fed a pelleted diet formulated from barley and barley straw, while those used by Bueno *et al*., (1982b) were fed an unspecified type of hay. Diet is likely to influence duodenal pH values in parasitised lambs.

Most reports in the literature indicate that abomasal pH values would increase in response to both *H. contortus* and *T. colubriformis* infections (Barker & Titchen, 1982; Buéno *et al.*, 1982a; Buéno *et al.*, 1982b; Simpson *et al.*, 1997). In the current experiment, however, there was no difference in abomasal pH values between infected and uninfected lambs. Buéno *et al*. (1982b) found that both abomasal and duodenal pH values reached a peak 8 - 10 days after *H. contortus* infection in sheep, then gradually declined. Thus it is possible that corresponding pH values observed in the current experiment did increased in the parasitised lambs, but had returned to basal levels by the time of slaughter. The pH values in the uninfected lambs were relatively high in comparison with other studies, probably due to an effect of diet.

The pH was higher in lambs fed the *Calliandra* diet in all segments except the rumen and the duodenum, where pH was lower on the *Calliandra* diet (Figure 4.12). The reason for this is not clear. Rumen pH may have been higher on the lucerne diet as result of increased secretion of saliva, due to the high DM content of the diet. Although saliva has a buffering action, the bicarbonate content does tend to increase rumen pH when present in copious amounts (Beghelli *et al*., 1969). The higher energy content of the lucerne diet would suggest a higher rate of VFA production than on the *Calliandra* diet after feeding, causing a drop in pH. However, at the time of sampling (16-20 hours after the last feed), both salivary secretion and VFA concentration in the rumen would have been low on either diet.

As discussed in Section 4.1, protein-tannin complexes form between pH 3.5 and pH 7, and dissociate outside this range, although only limited dissociation occurs in alkaline conditions until the pH exceeds 8. A relationship between abomasal and/or duodenal pH and faecal egg counts would thus be expected if pH determines the interaction between condensed tannins and parasitic nematodes. *Calliandra* condensed tannin complexes with both casein and rubisco proteins have been shown to be stable (less than 20 % dissociation) at pH 2.5, pH 4.5 and pH 8 (Rakhmani *et al*., 2005). With regard to pH values in the current experiment (Figure 4.12), tannin-protein complexes would be expected to be stable in the rumen, duodenum and jejunum, and only slight dissociation would have been expected in the ileum, caecum and colon. Even in the abomasum of lambs on the *Calliandra* diet, the mean pH value tended to be greater than 3.4, suggesting that in most cases, complexes would not have dissociated. It therefore might be postulated that complexes between condensed tannins and dietary or microbial protein would form in the rumen of *Calliandra*-fed lambs, and would remain intact throughout the length of the GI tract. This would account for the loss of a high proportion of excreted protein via the faeces of *Calliandra*-fed lambs. More difficult to explain is the effect of condensed tannins on nematodes in the GI tract. Any free condensed tannin available would be likely to bind to exposed nematode proteins (see Section 2.2) at the pH values found in this study. However, most of the tannin could be expected to have formed complexes with protein in the rumen, prior to contact with nematodes. Despite this, there were significant effects on faecal egg counts of both abomasal and small-intestinal worms, suggesting that either condensed tannins were able to affect nematodes even in the bound form, or there was sufficient free condensed tannin present to allow interactions between the tannin and the worms. Condensed tannins have multiple binding sites and do not bind proteins in a 1:1 ratio (Hagerman & Butler, 1981). It is therefore feasible that bound tannin could still interact with worms. However, Athanasiadou & Kyriazakis (2004) considered that complexed condensed tannins were unavailable to act against parasites. The alternative explanation, for substantiation, would require an analysis of free condensed tannin in the digesta of the experimental animals. From preliminary data in our NPM laboratory (D. Martin and E. Teleni, personal communication) there are indications of significant dissociation of condensed tannin in the abomasum. Free condensed

tannin, although less than in the abomasal digesta, was also evident in the rumen and duodenal digesta (and in the ileum, caecum and colon).

Environmental conditions other than pH could have an effect on tannin binding in the GI tract. For example, surfactants (such as bile acids) would decrease the protein-precipitating capacity of tannins (Martin *et al*., 1985), and probably play a role in determining the ultimate fate of tannin-protein complexes. Single pH measurements made post mortem, most likely, would not adequately reflect the dynamic conditions in the GI tract during the course of the infective period, and fluctuations in abomasal and duodenal pH could allow tannin-protein complexes to dissociate and reform.

Because pH values in the GI tract of *Calliandra*-fed lambs were generally above 3.5 in the current experiment, the effects of condensed tannin on nematode egg production under conditions where tannin-protein complexes are unable to form could not be determined. Thus, it was not possible to assess fully the nature of the relationship between pH, tannin-binding and egg production.

4.4.7 Total condensed tannin concentrations

In the current experiment, the highest concentrations of condensed tannin in the GI tract of lambs were found in the abomasum, and a large decrease in concentration occurred in the small intestine. This might partly explain the smaller reduction seen in *T. colubriformis* egg counts in comparison with *H. contortus* egg counts in this experiment. Concentrations in the caecum and colon were higher than those in the small intestine. This could have implications for the viability of nematode eggs, which would be exposed continuously to significant concentrations of condensed tannins during passage down the GI tract. These effects were examined in a subsequent experiment (Chapter 5).

In a review of published studies on anthelmintic effects of forages containing condensed tannins, Min and Hart (2003) estimated that maximum reduction in faecal egg counts was usually obtained when TCT concentrations in the forage were between 4.5 - 5.5 % DM. Forages with TCT concentrations above this range did not

reduce faecal egg counts to the same extent. A condensed tannin content of 3 – 4 % is generally recommended for maximal animal production (Barry *et al*., 1986b). The mean TCT concentration in *Calliandra* grown at JCU considerably exceeds these levels, at 11.4 % DM (D. Martin, personal communication). This comprises 85 g/kg DM free condensed tannin, 15 g/kg DM PCT and 13 g/kg DM FCT. It is interesting to speculate, then, that the efficacy of *Calliandra* condensed tannins in reducing faecal egg counts could be maintained by feeding it as part of a mixed ration, instead of as a sole diet. This is a much more practical proposition in a field situation. To bring the condensed tannin content of the total ration down to 5 % of the DM, *Calliandra* would need to comprise about 44 % of the ration. This is not far from the 30 % feeding level that is usually employed to maximise production benefits (Norton & Ahn, 1997; Norton & Waterfall, 2000). As discussed previously, it is not known whether bound condensed tannin is equally effective, or whether only free condensed tannin is available to interact with nematodes, but even if only free condensed tannin is active, the high levels of free condensed tannin in *Calliandra* should be sufficient theoretically to allow the legume to be fed as less than 100 % of the ration. Of course, other factors determine tannin reactions besides concentration; for example, polymer size, stereochemistry and procyanidin:prodelphinidin ratios (Hagerman & Butler, 1981; Clausen *et al.*, 1990; Kraus *et al.*, 2003) and simply diluting the tannin by mixing the legume with another feedstuff could reduce rather than increase efficacy. This is certainly an area that deserves further investigation.

4.5 Conclusion

The results of the current experiment were in general agreement with the results of the pilot experiment, and confirmed that *Calliandra*, when fed to parasitised lambs over the course of an infection, reduced egg production by female *H. contortus* and *T. colubriformis*, but had no effect on worm burdens. The sex ratios of the worms were also unaffected. Because there were no improvements in the protein nutrition of the *Calliandra*-fed lambs relative to that of the lucerne-fed lambs, improvements in resilience or resistance would not have occurred, indicating that *Calliandra* had a direct physiological or toxic effect on the worms. The active component of

Calliandra could not be identified from this experiment, but it is probably condensed tannin.

There was no indication in this study that *Calliandra*, when fed as a sole diet, can improve the ability of ruminant animals to cope with the effects of GI nematode parasitism during the establishment and early patent stages of infection. The extra PED as a result of tannin binding was lost in the faeces and therefore could not be of any benefit to the animal. Also, the diet did not promote improvements in phosphorus absorption, PCV or glucose metabolism.

The relationship between diet, GI nematode infections and pH along the GI tract is complex, and examination of the effects of pH on the ability of *Calliandra* to reduce faecal egg counts in parasitised lambs was inconclusive. This was due to the insufficient variations in pH in any of the GI segments to determine a relationship between pH and egg production by nematodes. Most of the worms were exposed to pH values between 3.5 and 7, and as hypothesised, egg production by the female worms was reduced within this pH range. This is consistent with the suggestion that the effects of *Calliandra* on worm egg production could be due to condensed tannins binding to worm proteins. However, there were not enough worms exposed to pH values outside this range to be sure that egg production would not still have been reduced at pH values below 3.5 or above 7, at which condensed tannins would not be expected to bind to proteins. This situation needs to be clarified.

The concentration of condensed tannins throughout the GI tract was estimated for comparison with future *in vitro* work. The concentration of condensed tannins in *Calliandra* is possibly higher than required for optimal effects on GI nematode parasites, and further work is necessary to determine whether *Calliandra* could be used in conjunction with other feeds to maximise both production benefits and anthelmintic effects.

CHAPTER 5

THE EFFECT OF *CALLIANDRA* CONDENSED TANNINS ON FREE-LIVING STAGES OF OVINE *H. CONTORTUS AND T. CIRCUMCINCTA*

5.1 Introduction

The results of the *in vivo* studies described in Chapters 3 and 4 indicated that in lambs, egg production of resident GI nematodes could be suppressed by feeding the animals with *Calliandra*. It was not clear from those experiments what component of the legume was responsible for the observed effect, but it might be suggested that the relatively high concentration of condensed tannins in the plant was the most likely cause. It is possible that *Calliandra* (and thus condensed tannins) could have effects on the hatchability of eggs and the viability of the resultant larvae, as well as on egg production.

In the feeding studies in Chapters 3 and 4, *Calliandra* was fed as 100 % of the diet. However, from a nutritional viewpoint *Calliandra* normally should constitute only about 30 % of the daily feed (Norton & Ahn, 1997; Norton & Waterfall, 2000). To be able to exploit both the nutritional and medicinal potential of the legume, it would thus be desirable to quantify the amount of *Calliandra* that should be fed to animals during the establishment phase of infection to gain any anthelmintic benefit.

This study was undertaken to test the hypothesis that the active component in *Calliandra* against GI nematodes is condensed tannin, and to examine in quantitative assays:

a. the hatchability of *H. contortus* and *T. colubriformis* eggs exposed to either
   i. purified *Calliandra* condensed tannin extract, or
   ii. *Calliandra* diet *in vivo*; and

b. the development of *H. contortus* and *T. colubriformis* larvae
   i. derived from eggs exposed to *Calliandra* condensed tannins, or
   ii. exposed to *Calliandra* condensed tannins from the L_1 stage.

5.2 Materials and Methods

5.2.1 Experiment 1: Egg hatch assays (EHA)

(a) Reagents for extraction of condensed tannins

All chemicals used were of analytical grade.

Reagent 1 (70 % acetone) was prepared by adding seven volumes of acetone to three volumes of distilled water, followed by the addition of 0.1 % ascorbic acid (w/v).

Reagent 2 [acetone saturated with sodium chloride (NaCl)] was prepared by mixing 200 mL Reagent 1 and 8 g NaCl in a 250 mL separating funnel. As the NaCl dissolved, further small amounts of NaCl were added until the solution separated into two phases. The lower aqueous phase and undissolved NaCl were discarded and the upper acetone phase retained.

(b) Crude *Calliandra* condensed tannins

Crude extracts of condensed tannins were prepared from *Calliandra* leaves using a method based on that of Broadhurst & Jones (1978).

Fresh *Calliandra* leaves were harvested from an established plot at JCU. The leaves were either frozen whole in Ziploc plastic bags and cut into 2 cm lengths before use or cut into 2 cm lengths before freezing. Fifty grams of frozen, chopped leaves were macerated in a Waring blender. To this were added 150 mL of Reagent 1 and blending continued for a further minute. The blend was transferred to 50 mL Falcon tubes and centrifuged for 10 minutes at 2000 g. The supernatants were transferred to a 250 mL separating funnel and the residues returned to the blender, to which a further 75 mL of Reagent 1 was added. After blending for five minutes, centrifugation was repeated and the supernatants added to the separating funnel. The resulting residue was blended again, but this time with 50 mL of Reagent 1. After

centrifugation the supernatant was added to the separating funnel and the residue discarded.

The combined supernatant in the separating funnel was saturated with NaCl by adding approximately 4 g of salt per 100 mL of plant extract. Extra NaCl was added as necessary until the solution separated into a lower aqueous phase and an upper acetone phase containing the condensed tannins. The aqueous phase was transferred into a measuring cylinder and the acetone phase retained in a 500 mL Schott bottle. The aqueous phase was returned to the separating funnel with an equal volume of Reagent 2. The separating funnel was agitated until the solution again separated into two phases. The lower aqueous phase was discarded and the upper acetone phase retained.

The acetone in this extract was removed by rotary evaporation at 45 ºC for approximately 10 minutes. The remaining solution was washed into a 100 mL separating funnel using a minimum volume of water (approximately 20 mL) and mixed with an equal volume of diethyl ether to remove pigments. The diethyl ether then was discarded and the process repeated three to five times, until the upper ether phase remained clear. This was followed by three similar extractions with ethyl acetate.

Solvents were removed from the aqueous solution by rotary evaporation for 10 minutes at 45 ºC and the remaining extract washed into 50 mL Falcon tubes. These were centrifuged for 15 minutes at 2000 g and the supernatants transferred to 100 mL plastic beakers in 15 mL aliquots. These were frozen at –80 ºC for a minimum of two hours, and then freeze-dried overnight. The aliquots of freeze-dried crude extract were transferred to 15 mL glass vials and stored at –20 ºC.

(c) Purified *Calliandra* condensed tannins

Calliandra crude condensed tannin extracts were purified according to the method of Molan *et al.* (2002). A glass chromatography column (35 mm x 400mm) was packed under gravity with 50 g Sephadex LH-20 (Sigma-Aldrich) primed in distilled water.

The column was equilibrated with 50 % methanol (v/v; methanol/water). Ten g of *Calliandra* crude condensed tannin, dissolved in 150 mL of 50 % methanol was transferred to the column and washed with 2 L of 50 % methanol to remove impurities. The condensed tannin was eluted with acetone and the eluent collected into 20 mL glass vials. The absorbance of each vial was observed at 350 nm using a Novaspec II spectrophotometer (Pharmacia Biotech). Fractions containing condensed tannins were combined and rotary evaporated for 10 minutes at 45 °C, frozen in 15 mL aliquots for two hours at –80 ºC and freeze-dried, before storage at –20 ºC in 20 mL glass vials. Ten g of crude condensed tannin extract yielded approximately 3 g purified condensed tannin.

(d) Extraction of nematode eggs

Approximately 200 g of fresh faeces were collected from each of four five-month-old Merino ram lambs that had patent infections of either *T. colubriformis* (two lambs) or *H. contortus* (two lambs). Two lambs, each infected with one of the two worm species were fed a diet of lucerne pellets throughout the period of infection; the remaining two lambs were fed only *Calliandra* during the same period. Each batch of faeces was placed in a Waring blender with water and blended on low power until the faeces were just homogenized. The slurry was washed through a 1 mm mesh sieve, and the resulting eluent washed successively through 150 µm and 20 µm meshes. Nematode eggs were washed off the 20 µm mesh into 50 mL Falcon tubes with a minimal amount of water. After centrifugation at 1785 *g* for seven minutes, the supernatant was discarded and replaced with egg flotation solution (magnesium sulphate solution with a specific gravity of 1.2). The tubes were inverted gently and centrifuged for a further seven minutes at 1785 *g*, after which the supernatant was washed through 75 µm and 20 µm meshes to retain eggs. The eggs were washed back into clean Falcon tubes and allowed to settle, then the supernatant volume was reduced to 20 mL and the number of eggs in ten 10 µL aliquots counted. The volume was readjusted to give a final concentration of 1600 eggs/mL.

(e) Condensed tannin solutions

A stock solution of 10 mg/mL purified *Calliandra* condensed tannins in distilled water was used to prepare working solutions of 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 9 mg/mL for the egg hatch assays (EHA). One mL of each EHA working solution was diluted 1:4 with distilled water to prepare a series of larval development assay (LDA) working solutions (see Section 5.2.2). Distilled water was used as a control.

(f) Experimental design

Egg hatch assays were carried out in four sets of 24-well cell culture plates, set up as indicated in Table 5.1. In each well, 0.15 mL of *Calliandra* condensed tannin working solution was added to 0.1 mL of either *T. colubriformis* or *H. contortus* egg suspension and 1.25 mL distilled water, giving final concentrations of 0, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800 and 900 µg/mL. Five replicates were prepared for each tannin concentration.

Table 5.1 Design of egg hatch assays, showing diet of sheep from which eggs were obtained and worm species used in each assay. (CT = condensed tannins.)

Worm species	*H. contortus*		*T. colubriformis*	
Diet	Lucerne	Calliandra	Lucerne	*Calliandra*
CT concentrations	0-900 µg/mL	0-900 µg/mL	0-900 µg/mL	0-900 µg/mL
Treatment group	HL	HC	TL	TC

The prepared plates were placed into plastic containers and incubated at 27 ºC. At this temperature the majority of eggs would be expected to hatch within 24 hours (Veglia, 1915). After 26 hours, a drop of Lugol's iodine was added to each well to stop further development. The contents of each well were transferred to a 2.5 mL Eppendorf tube containing 1 mL concentrated formalin solution for storage. When ready for counting, the contents of the Eppendorf tube were transferred to the chambers of a Whitlock egg counting slide, examined at 63x magnification and the numbers of larvae and unhatched eggs recorded. Larvae in the process of hatching were counted as eggs if less than 50 % of the larva was protruding from the shell.

5.2.2 Experiment 2: larval development assays (LDA)

(a) Condensed tannins and nematode eggs

Calliandra condensed tannins were extracted and purified as detailed in Sections 5.2.1 (b) and (c), and working solutions prepared as described in Section 5.2.1 (e). Nematode eggs were extracted as described in Section 5.1.2(d).

(b) Parasite growth medium

A parasite growth medium was prepared according to the method of Molan *et al*. (2002). Fifteen mg of sterile, lyophilised *Escherichia coli* cells were suspended in 100 mL of distilled water, and a nutritive medium was made by dissolving 0.5 g of yeast extract (Sigma) in 45 mL of 0.9 % saline solution and 5 mL of Earle's Balanced salt solution (Sigma). The growth medium was prepared by mixing equal volumes of the *E. coli* suspension and the nutritive medium and then adding 5.8 µg of amphotericin B (Fungizone, Squibb) per mL of mixture.

(c) Experimental design

Larval development assays were set up in 96-well cell culture plates, using a similar design as in Experiment 1, but two identical sets of plates were prepared for each assay (see Table 5.2). In each well, 100 µL of LDA *Calliandra* condensed tannin working solution was added to 60 µL egg suspension and 40 µL parasite growth medium to give the same final concentrations of extract as for the EHA [see Section 5.2.1 (e)]. However, in the first set of plates the tannin solutions were added on Day 0, whereas in the second set of plates, the tannin solution was not added until after the first 24 hours of incubation. All plates were incubated at 27 ºC for seven days. The contents of the plates were transferred to Eppendorf tubes with 200 µL concentrated formalin and stored. When ready for counting, the contents of the Eppendorf tubes were transferred to the chambers of a Whitlock egg-counting slide, the preparation was examined at 63x magnification and the numbers of first, second

and third larval stages (L_1, L_2 and L_3) and unhatched eggs were recorded. Degenerating worms were also recorded.

Table 5.2 Design of larval development assays, showing diet of sheep from which eggs were obtained and worm species used in each assay. Two sets of plates (Day 0 and Day 1) were used (CT = condensed tannin; L = lucerne; C = *Calliandra*.)

	Day 0				Day 1			
Worm species	*H. contortus*		*T. colubriformis*		*H. contortus*		*T. colubriformis*	
Diet	L	C	L	C	L	C	L	C
CT (µg/mL)	0 - 900	0 - 900	0 - 900	0 - 900	0 - 900	0 - 900	0 - 900	0 - 900
Treatment group	HLD0	HCD0	TLD0	TCD0	HLD1	HCD1	TLD1	TCD1

For *T. colubriformis*, larval stages were distinguished by measuring each larva with an eyepiece micrometer. Published results for *T. retortaeformis* in rabbits (Audebert *et al*., 2000) indicated that the L_1 were 309 - 313 µm long, L_2 were 381 - 509 µm long and L_3 were 449 - 531 µm long. The L_3 could also be distinguished by the presence of the sheath (incompletely shed cuticle of the L_2 stage). Once all larvae had been counted it was apparent that the measurements for *T. colubriformis* were slightly different, so the criteria used to differentiate the stages in the current experiment were 164 - 410 µm for L_1, 426 - 557 µm for L_2 and greater than 574 µm and possessing a sheath for L_3.

Stages of *H. contortus* were determined according to the work of Veglia (1915). First stage larvae (340 - 350 µm in length) were recognised by their distinctive shape, being shorter than the L_2 or L_3 (500 - 756 µm and 614 - 820 µm respectively) and tapering from the oesophagus to the tail, whereas the L_2 and L_3 are cylindrical, tapering only at the tail. Since there is a considerable overlap in length between the L_2 and the L_3, these were distinguished by the presence or absence of the sheath.

5.2.3 Calculations

(a) EHA

The fraction of eggs hatched in control wells (CH) was calculated by the equation:

CH = No. larvae in well/(No. eggs + No. larvae in well) ..... . *Equation 5.1*

Effective number of eggs treated in test wells (ET) was calculated by the equation:

ET = (No. eggs + No. larvae in test well) x CH..... . *Equation 5.2*

Egg hatch inhibition (EHI) was calculated by the formula:

$$EHI = 100 \times \frac{(ET - \text{No. larvae in test well})}{ET} \quad \text{..... Equation 5.3}$$

The effective number treated was used rather than absolute values because the actual number of eggs placed in each well was extremely variable.

(b) LDA

Egg hatch rates were determined by Equation 5.1. Larval development inhibition (LDI) was determined by Equation 5.4.

$$LDI = 100 \times \frac{(ET - \text{No. } L_3 \text{ in test well})}{ET} \quad \text{..... Equation 5.4}$$

5.2.4 Statistical analysis

All analyses were carried out using Genstat statistical software (VSN International Ltd, Oxford, UK).

(a) EHA

The data were analysed by fitting a generalised linear mixed model with species, diet and *Calliandra* condensed tannin concentration as fixed effects and assuming that the error distribution was a binomial distribution with a logit link function (Genstat, VSN International Ltd, Oxford, UK). From this analysis the least square mean values for the species-diet combination at each tannin concentration were determined. Splines were fitted to these means. Separate logistic curves were fitted for each species-diet combination, using the equation A + C/(1 + EXP(-B*(X-M))). The

concentration of tannins required to prevent 50 % of the eggs from hatching (ED_{50}) was calculated by probit analysis. A logit model (log(p/(1-p)) with a log transformation on the tannin concentration was used.

(b) LDA

The data were analysed by fitting a generalised linear mixed model with day, species, diet and *Calliandra* condensed tannin concentration as fixed effects and assuming that the error distribution was a binomial distribution with a logit link function. From this analysis the mean values for the day-species-diet combination at each condensed tannin concentration was determined. Separate exponential curves were fitted using the equation A + B*(R**X). The ED_{50} and the LD_{50} (the concentration of tannins required to prevent 50 % of eggs developing to the L_3) was calculated by probit analysis. For each parameter examined, six models were investigated. These were probit, logit (log(p/(1-p))) and complementary log (log(-log(1-p))) with and without a log transformation on the concentration of the extract, with either parallel or separate lines fitted. The most appropriate model for each parameter is presented in Section 5.4.2. Due to the use of species-diet-day combinations in the analysis, the individual effects of each main factor could not be assessed statistically. Where there was an apparent effect, means and standard errors are presented in the results.

Because different patterns of development were observed for each species-diet-day combination, probit analyses were carried out to determine the differences in development to the L_1, L_2 and L_3 stages for each combination. For each parameter examined, probit, logit (log(p/(1-p))) and complementary log (log(-log(1-p))) with and without a log transformation on the concentration of the extract models were investigated. The most appropriate model for each parameter is presented in the results.

5.3 Results

5.3.1 Experiment 1: EHA

In the control wells, eggs from lucerne-fed sheep (Groups HL and TL) hatched readily, producing normal L_1 larvae (Plate 5.1 A). Mean egg hatch rates in these wells were 78 % and 69 % respectively (Figure 5.1). The mean hatch rates in the control wells for eggs that were obtained from *Calliandra*-fed lambs were significantly lower, at 45 % for *H. contortus* eggs and 28 % for *T. colubriformis* eggs, and many poorly developed unhatched eggs were present (Plate 5.1 B).

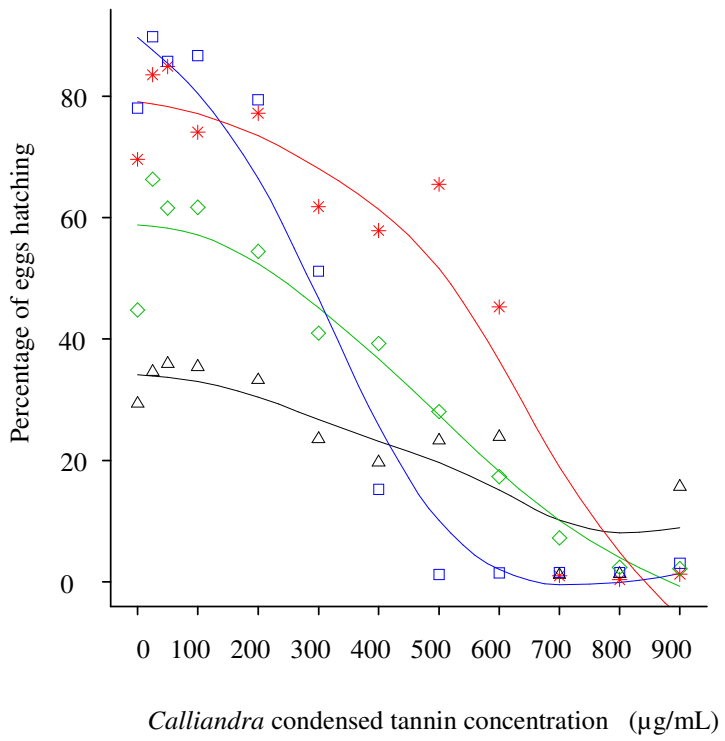


Figure 5.1 Effects of *Calliandra* condensed tannins on hatchability of eggs of *H. contortus* or *Trichostrongylus colubriformis* from lambs fed *Calliandra* or lucerne diets. (—✳— *H. contortus*, lucerne diet; —△— *Haemonchus contortus*, *Calliandra* diet; —□— *Trichostrongylus colubriformis*, lucerne diet; —◇— *Trichostrongylus colubriformis*, *Calliandra* diet).

The probit model for EHI is shown in Figure 5.2. Parallel lines were fitted to the model and it can be seen that EHI was higher for *T. colubriformis* eggs derived from lucerne-fed sheep than for the other three treatments. This is reflected in the ED_{50} for

each treatment, which is presented in Table 5.3. The ED_{50} for *T. colubriformis* eggs without previous exposure to condensed tannin was lower than those for the other three treatments.

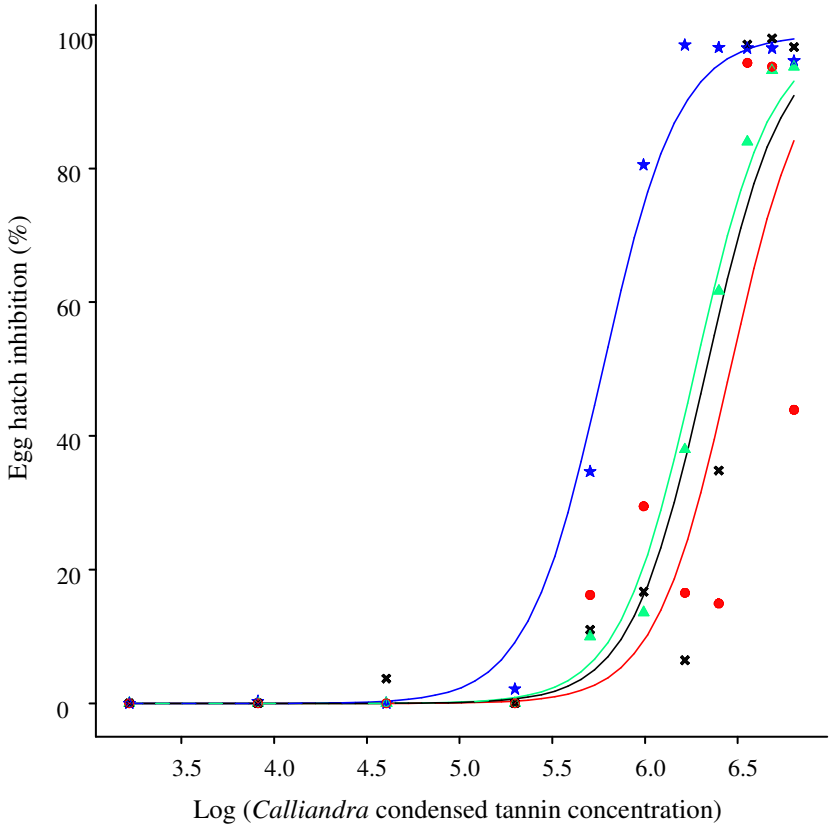


Figure 5.2 Logit model of the effects of *Calliandra* condensed tannins on hatchability of eggs of *H. contortus* or *Trichostrongylus colubriformis* from lambs fed *Calliandra* or lucerne diets. (—×— *H. contortus*, lucerne diet; —•— *Haemonchus contortus*, *Calliandra* diet; —★— *Trichostrongylus colubriformis*, lucerne diet; —▲— *Trichostrongylus colubriformis*, *Calliandra* diet).

Table 5.3 Dose of *Calliandra* condensed tannins required to inhibit hatching in 50 % of eggs (ED_{50}) of *Haemonchus contortus* or *Trichostrongylus colubriformis* from lambs fed either *Calliandra* or lucerne diets. Values are means and 95 % fiducial limits (FL). Means with different superscripts are significantly different ($P < 0.050$).

	Nematode species			
	H. contortus		*T. colubriformis*	
Diet of host lamb	Lucerne	*Calliandra*	Lucerne	*Calliandra*
ED_{50} (95% FL)	562^{a} (487 - 645)	660^{a} (512 – 1019)	328^{b} (266 – 382)	531^{a} (446 – 618)

At very low tannin concentrations, the few unhatched eggs present were all at the morula stage. However, as the number of unhatched eggs increased with increasing tannin concentration, a variety of embryonic stages were seen. At tannin concentrations between 50 and 600 µg/mL, many *T. colubriformis* eggs from the lucerne-fed lambs were enlarged up to twice normal size and contained larvae that appeared to be fully developed and ready to hatch (Plate 5.1 F). Some larvae were seen half in and half out of the eggs (Plate 5.1 D). Both *T. colubriformis* and *H. contortus* larvae appeared to hatch head first from the shells, although some larvae were seen with both ends protruding at once. At concentrations above 600 µg/mL, eggs were normal in size and contained embryos rather than larvae. A few of the larvae that hatched were degenerating, but most had a normal appearance. Enlarged and half-hatched eggs were more prevalent when eggs were pre-exposed to condensed tannins (Group TC), and seen at all concentrations except the control. Maximum numbers of these were seen at 400 and 500 µg/mL of added tannins. In addition, a few eggs at all tannin concentrations had shrunken contents (Plate 5.1 E), or the contents appeared to be extruding through a pore in the shell (similar to the *Haemonchus* egg in Plate 5.1 C). No such degenerating eggs were seen in the control wells. A few larvae also appeared to be degenerating. The degenerating larvae were almost normal in appearance but had an unusually granular appearance or had 'vacuoles' or spaces in the pseudocoelom where normal anatomy could not be identified. Some had loops of what appeared to be the female reproductive tract extruding from the developing vulva. Often an empty space was visible between the cuticle and the body structures of the worms, and in these larvae, there was usually an annular dilation of the cuticle near the caudal end of the larva. The degenerating *H. contortus* larvae had unusually blunt heads (Plate 5.2 A).

Very few enlarged eggs were seen in the *H. contortus* assay using eggs from lucerne-fed sheep, but half-hatched eggs began to appear at 50 µg/mL and low numbers of degenerating larvae began to appear at 25 µg/mL. Enlarged and half-hatched eggs began to appear in the assays using the eggs from *Calliandra*-fed sheep at 25 µg/mL. Some degenerating eggs and larvae were present at all tannin concentrations and also in the control wells.

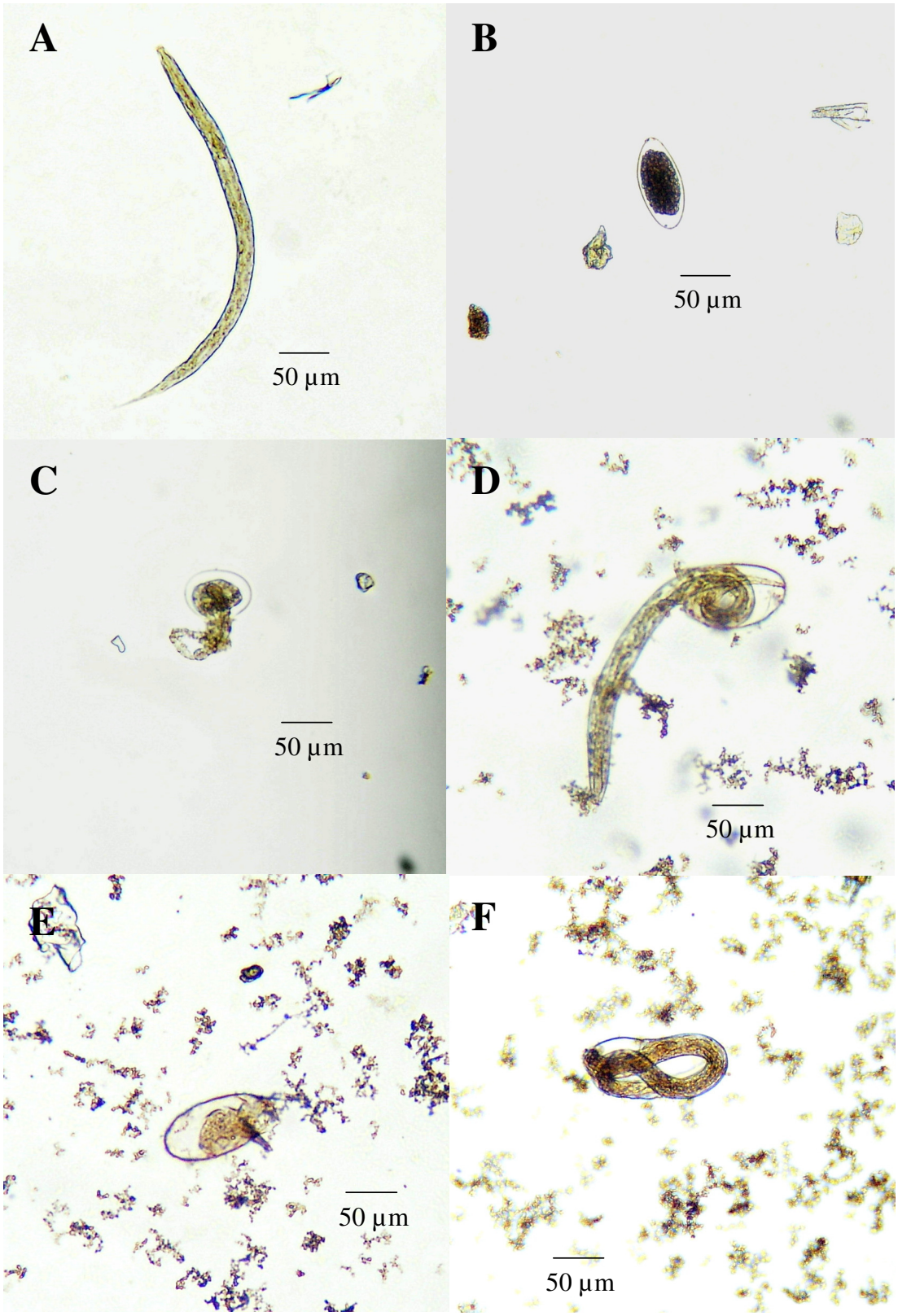


Plate 5.1 Results of egg hatch assays using **s**trongyle eggs after 26 hours incubation at 27°C. **A.** Normal *Trichostrongylus colubriformis* larva, TL control. **B**. Unhatched *T. colubriformis* egg, TC control. **C.** Degenerating egg with contents extruding, HC control. **D.** Larva half hatched, TL 400 µg/ml. **E.** Egg with degenerating contents, TL 400 µg/ml. **F.** Enlarged egg with fully developed larva, TL 400 µg/ml.

5.3.2 Experiment 2: LDA

(a) Hatch rates

The hatch rates obtained in the LDA are shown in Figure 5.3. Hatch rates for all groups decreased as tannin concentrations increased ($P < 0.001$). Hatch rates were also significantly different between treatment groups ($P < 0.001$).

Complementary log (log(-log(1-p)))with a log transformation on the concentration of the extract with parallel lines was the model used for probit analysis. However, the ED_{50} values were beyond the limits of the data.

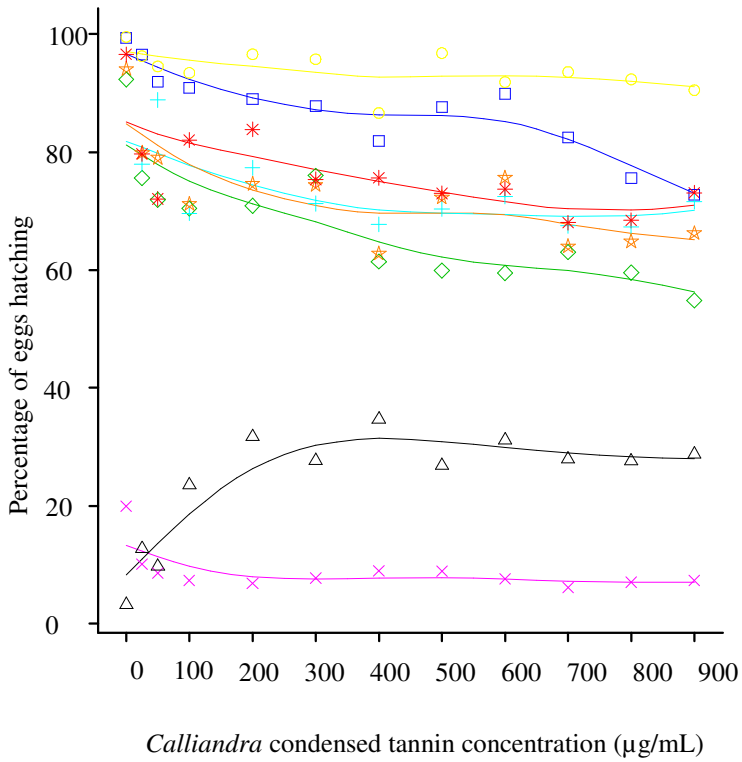


Figure 5.3 Hatch rates of *Haemonchus contortus* (H) or *Trichostrongylus colubriformis* (T) eggs after seven days incubation with increasing concentrations of *Calliandra* condensed tannins. Eggs were obtained from lambs fed either a *Calliandra* (C) or lucerne (L) diet and tannin was added either before (0) or after the eggs hatched (1).
HL0 —✳—; HL1 —+—; TL0 —□—; TL1 —○—; HC0 —△—; HC1 —x—; TC0 —◇—; TC1—☆— .

From Figure 5.3, it appears that hatch rates for *T. colubriformis* eggs were greater than those for *H. contortus* eggs. The hatch rates for eggs from lucerne-fed sheep were also higher than the hatch rates of eggs from *Calliandra*-fed sheep. Adding tannins on Day 0 affected the hatching of *T. colubriformis* eggs more than adding tannins on Day 1, but the opposite was true for *H. contortus* eggs.

At high tannin concentrations, many unhatched eggs were seen to be degenerating. Unhatched *T. colubriformis* eggs maintained their normal shape, although they were often enlarged (Plate 5.1 B and F). However, those of *H. contortus* became spherical (Plate 5.2 B) and often larvae were seen half in and half out of the shells. This effect was only seen when eggs were pre-exposed to tannins by feeding a *Calliandra* diet to the host sheep (HC D1 and D0).

(b) Development to the L_1

The percentage of eggs developing to the L_1 stage is shown in Figure 5.4. Tannin concentrations did not affect the number of larvae developing to the L_1 stage ($P = 0.250$). However, the number of L_1 developing differed significantly between treatment groups ($P < 0.001$) and there was an interaction between treatment group and tannin concentrations ($P < 0.001$).

Logit link with a log transformation on the concentration of the extract with separate lines was the model used for probit analysis.

From Figure 5.4 it appears that there was no difference between *T. colubriformis* (39.1 ± 2.0) and *H. contortus* (42.1 ± 1.9) in the percentage of eggs that had reached the L_1 stage at the end of seven days. However, for *T. colubriformis* greater numbers of L_1 developed when condensed tannins were added on Day 1 (25.1 ± 8.9 vs 53.1 ± 2.6 for Day 0 and Day 1 respectively), whereas greater numbers of L_1 *H. contortus* developed when the tannins were added on Day 0 (45.8 ± 2.5 vs 38.4 ± 2.0 for Day 0 and Day 1 respectively). A greater percentage of eggs from lucerne-fed sheep developed to the L_1 stage (56.1 ± 2.0) than of those from *Calliandra*-fed sheep (25.2 ± 1.3).

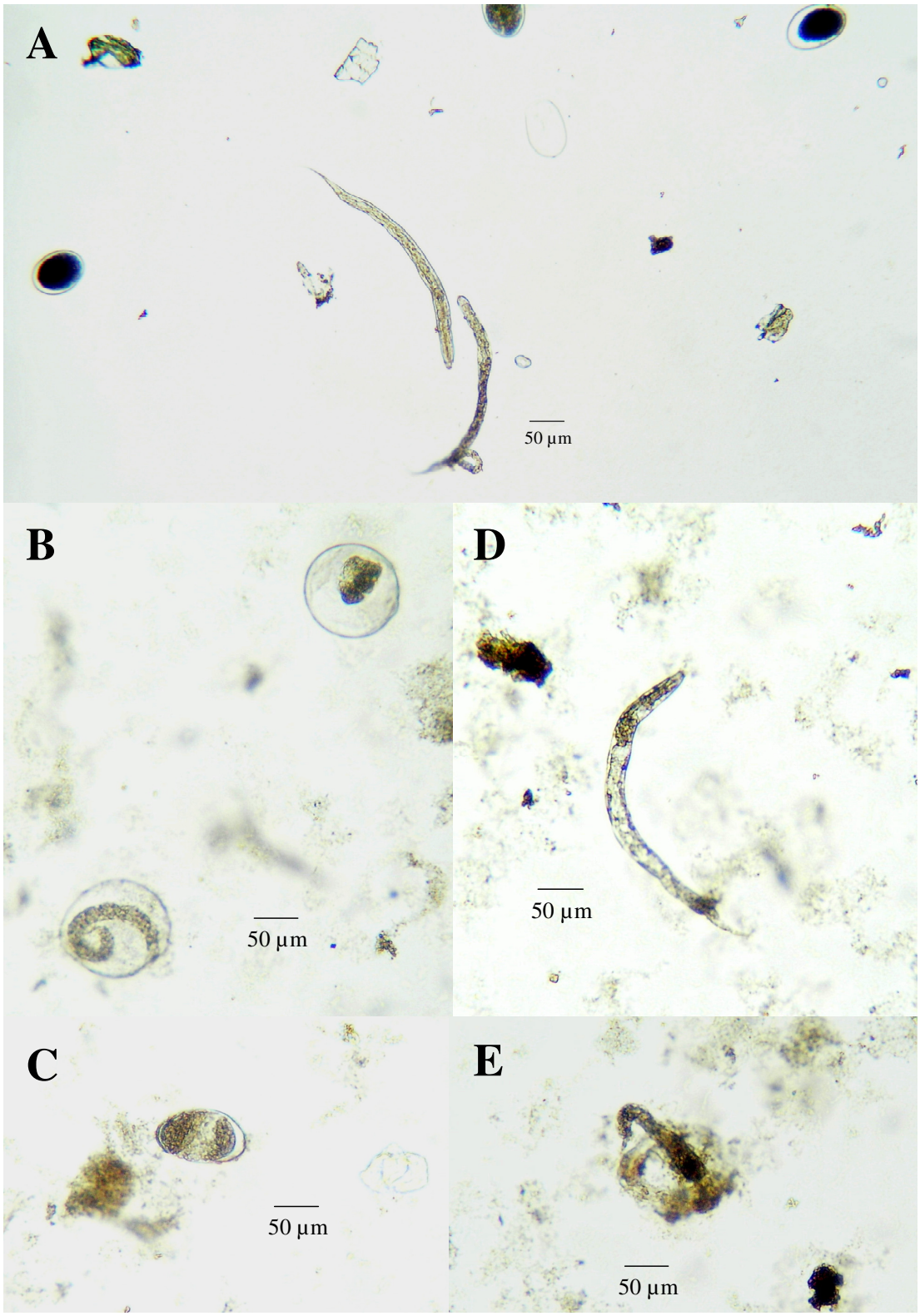


Plate 5.2 A. *H. contortus* L1 larvae from *Calliandra*-fed sheep after 26 hours incubation of eggs at 27°C in tap water without added tannins. While the larva on the left looks normal, the one on the right has a shrivelled appearance and has a loop of reproductive tract extruding from the vulval opening. Three undeveloped eggs and an empty shell are present in the background. **B.** Enlarged *H. contortus* eggs with degenerating contents after seven days incubation in larval development assay. HC 900µg/ml. **C**. *T. colubriformis* egg with degenerating contents. TC 900µg/ml **D.** Degenerating larvae from *Calliandra*-fed lamb in larval development assay. HC control. **E.** Degenerating larvae from *Calliandra*-fed lamb in larval development assay. TC 900µg/ml.

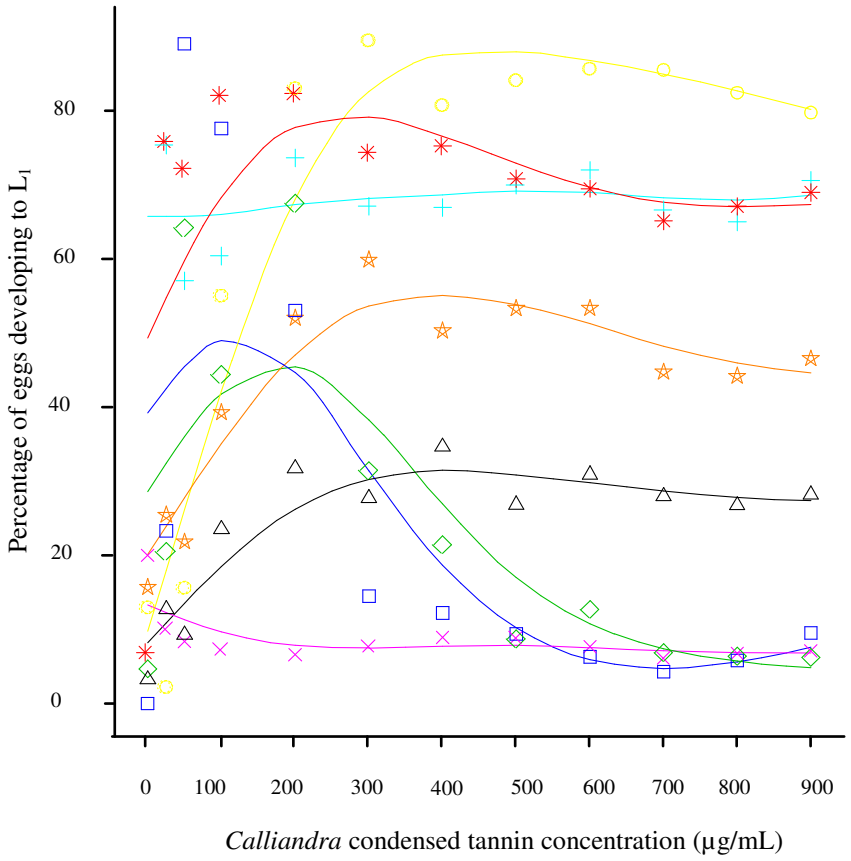


Figure 5.4 Percentage of eggs of *Haemonchus contortus* (H) or *Trichostrongylus colubriformis* (T) developing to the L_1 after seven days incubation with increasing concentrations of *Calliandra* condensed tannins. Eggs were obtained from lambs fed either a *Calliandra* (C) or lucerne (L) diet and tannins were added either before (0) or after the eggs hatched (1).
HL0 —✳—; HL1 —+—; TL0 —□—; TL1 —○—; HC0 —△—; HC1 —X—; TC0 —◇—; TC1—☆— .

(c) Development to the L_2

The percentage of eggs developing to the L_2 is shown in Figure 5.5. Both tannin concentrations ($P < 0.001$) and treatment group ($P < 0.001$) significantly affected the number of larvae developing to the L_2 stage. There was also an interaction between tannin concentrations and treatment group ($P = 0.011$).

Logit link without a log transformation on the concentration of the extract with separate lines was the model used for probit analysis. The HC D0 and HC D1 treatments were excluded from the analysis because no L_2 developed from eggs exposed to these treatments.

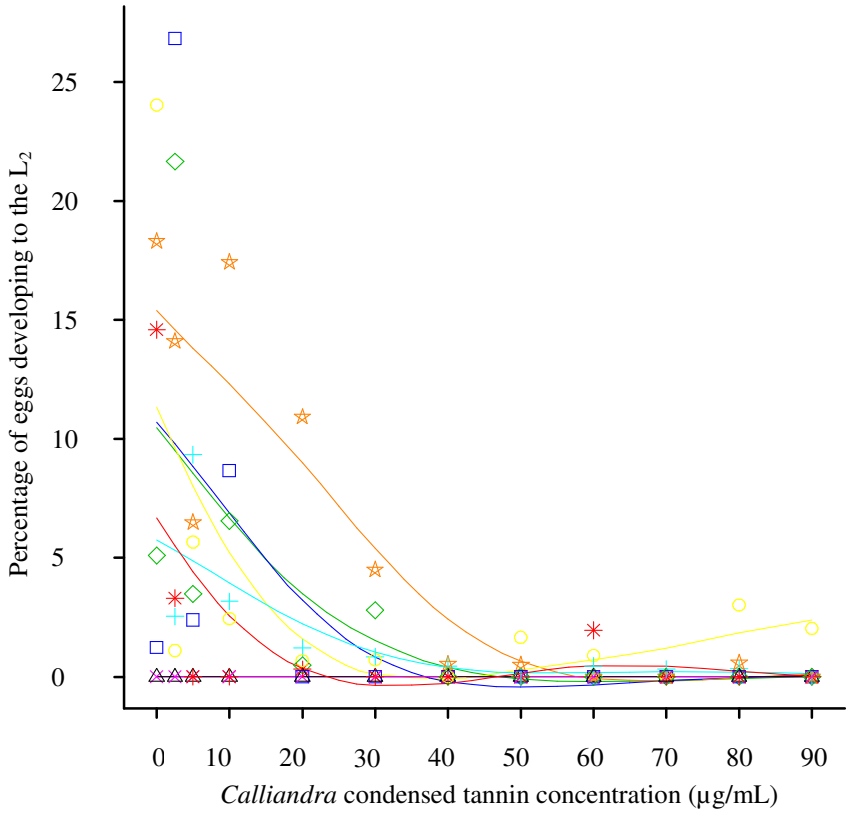


Figure 5.5 Percentage of eggs of *Haemonchus contortus* (H) or *Trichostrongylus colubriformis* (T) developing to the L_2 after seven days incubation with increasing concentrations of *Calliandra* condensed tannins. Eggs were obtained from lambs fed either a *Calliandra* (C) or lucerne (L) diet and tannins were added either before (0) or after the eggs hatched (1).
HL0 —✳—; HL1 —+—; TL0 —□—; TL1 —○—; HC0 —△—; HC1 —x—; TC0 —◇—; TC1—☆— .

From Figure 5.5, there were no obvious differences between individual worm species, diets or days in the percentage of larvae developing to the L_2.

(d) Development to the L_3

The percentage of eggs developing to the L_3 is shown in Figure 5.6. Both tannin concentrations ($P < 0.001$) and treatment group ($P < 0.001$) affected the number of larvae developing to the L_3 stage. There was also an interaction between tannin concentrations and treatment group ($P = 0.011$).

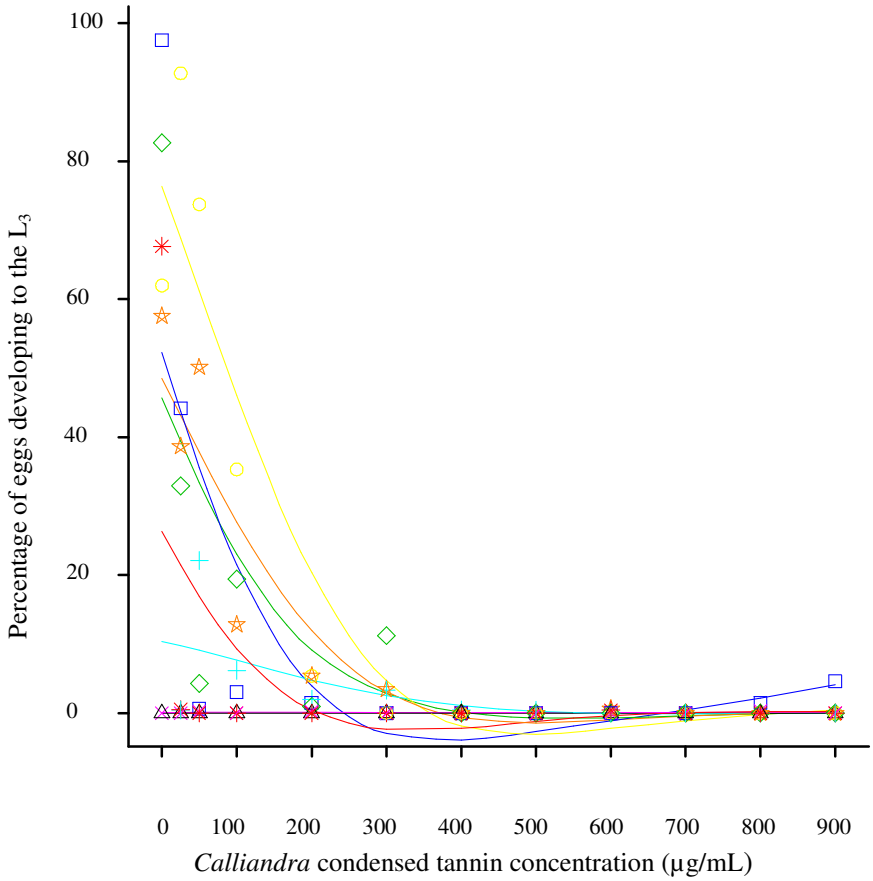


Figure 5.6 Percentage of eggs of *Haemonchus contortus* (H) or *Trichostrongylus colubriformis* (T) developing to the L_3 after seven days incubation with increasing concentrations of *Calliandra* condensed tannins. Eggs were obtained from lambs fed either a *Calliandra* (C) or lucerne (L) diet and tannins were added either before (0) or after the eggs hatched (1).
HL0 —✳—; HL1 —+—; TL0 —□—; TL1 —○—; HC0 —△—; HC1 —x—; TC0 —◇—; TC1—☆— .

From Figure 5.6 it can be seen that more *T. colubriformis* eggs developed to the L_3 (15.4 ± 1.8) than *H. contortus* eggs (1.8 ± 0.6). Adding tannins on Day 1 or Day 0 made little difference to the number of larvae reaching the L_3 stage. A greater percentage of eggs from lucerne-fed sheep developed to the L_3 (10.7 ± 1.6) than eggs from *Calliandra*-fed sheep (6.7 ± 1.1).

Logit link with a log transformation on the concentration of the extract with separate lines was the model used for probit analysis. The HC D0 and HC D1 treatments were excluded from the analysis because no L_3 developed from eggs exposed to these treatments. The LD_{50} values for development to the L_3 are presented in Table 5.4.

Table 5.4 Dose of *Calliandra* condensed tannins required to inhibit development to the L_3 of 50 % of eggs (LD_{50}) of *Haemonchus contortus* or *Trichostrongylus colubriformis* from lambs fed either *Calliandra* or lucerne diets and exposed to condensed tannins either before (Day 0) or after hatching (Day 1). Values are means and 95 % fiducial limits. Means with different superscripts are significantly different (P < 0.050).

	Nematode species			
	H. contortus		*T. colubriformis*	
Diet of host lamb	Lucerne	*Calliandra*	Lucerne	*Calliandra*
LD_{50} (95% FL)				
Day 0	0^a	N.d.*	21^a (8 – 31)	23^a (9 – 37)
Day 1	8^a (0 – 25)	N.d.*	123^b (96 – 179)	78^b (55 – 108)

*Not determined because all values were 0.

(e) Degenerating worms

A probit model could not be fitted to the data for degenerating worms.

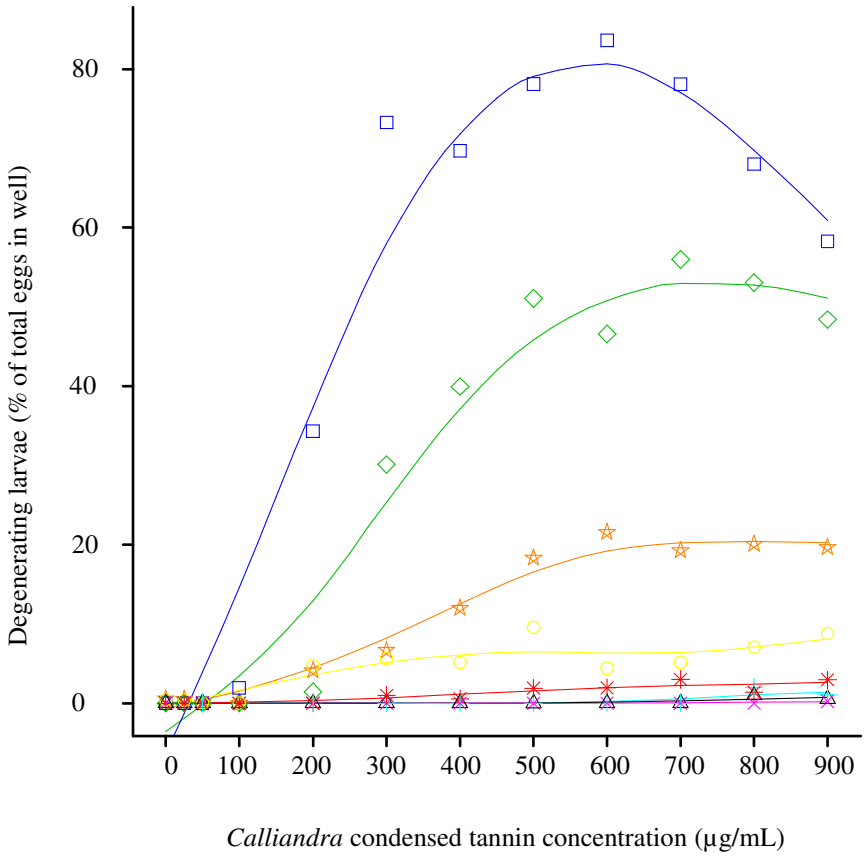


Figure 5.7 Percentage of eggs of *Haemonchus contortus* (H) or *Trichostrongylus colubriformis* (T) resulting in degenerating larvae after seven days incubation with increasing concentrations of *Calliandra* condensed tannins. Eggs were obtained from lambs fed either a *Calliandra* (C) or lucerne (L) diet and tannins were added either before (0) or after the eggs hatched (1).
HL0 —✳—; HL1 —+—; TL0 —□—; TL1 —○—; HC0 —△—; HC1 —x—; TC0 —◇—; TC1—☆— .

The percentage of eggs resulting in degenerating larvae is shown in Figure 5.7. Only a few *H. contortus* eggs resulted in degenerating larvae, regardless of diet or the day the tannins were added. However, a large percentage of *T. colubriformis* eggs resulted in degenerating larvae, and this was particularly marked when tannins were added on Day 0. The diet of the sheep from which the eggs were obtained did not seem to affect the percentage of degenerating larvae.

Mildly degenerating larvae were similar in appearance to those seen in the EHA (Plate 5.1 D). Larvae in advanced stages of degeneration had a coiled or knotted appearance and anatomical features could not be distinguished (Plate 5.1 E). Larval stages usually could not be identified.

5.4 Discussion

5.4.1 Identity of the plant extract

When a crude acetone plant extract is applied to a Sephadex LH-20 column in 50 % methanol, proanthocyanidin oligomers and polymers adsorb to the column. Low molecular weight phenolics including hydrolysable tannins do not adsorb and can be eluted with 50 % methanol (Porter, 1989). In the case of procyanidin, tetramers and above are retained on the column. These can be eluted with 70 % acetone. The *Calliandra* extracts used in the EHA and LDA in the current study would therefore consist of a pure mixture of *Calliandra* condensed tannins, although some condensed tannins with very low molecular weights may have been lost. Thus the observed effects of the extract on egg hatching and larval development can be attributed to the action of condensed tannins.

5.4.2 Egg hatching

In the EHA, the hatch rates in all treatment groups were decreased by increasing concentrations of condensed tannins, with ED_{50} values between 500 and 700 µg/mL for three of the treatments. The lower ED_{50} for the TL treatment suggested that *T. colubriformis* eggs without exposure to condensed tannins in the sheep were more

susceptible to the effects of added tannin than *H. contortus* eggs or *T. colubriformis* eggs from tannin-fed sheep. Very few eggs hatched for any of the treatments at concentrations above 700 µg/mL.

However, when *T. colubriformis* eggs were incubated with *Calliandra* condensed tannins for a period of seven days in the LDA, the decline in hatching was not as marked, and hatch rates above 50 % were achieved even at the highest tannin concentrations. The same was true of *H. contortus* eggs from lucerne-fed sheep. Hatch rates of *H. contortus* eggs from *Calliandra*-fed sheep were much lower, but were still higher than those obtained in the EHA. These results suggest that condensed tannins delayed hatching in some of the eggs instead of causing complete inhibition. The presence of enlarged eggs containing fully developed larvae and of eggs in various stages of hatching at medium to high tannin concentrations would tend to support this. However, the presence of degenerating eggs indicated that some were sufficiently damaged by the tannins to prevent hatching altogether.

Silverman and Campbell (1959) found that unembryonated *H. contortus* eggs were more vulnerable to adverse conditions such as low temperatures than eggs that had reached the gastrula stage. Similarly, eggs containing vermiform embryos were more resistant to dessication than eggs at an earlier embryonic stage. Exposure to condensed tannins might therefore inhibit hatching to a greater degree when it occurs at an earlier stage of development. In the current experiment, exposure to tannins on Day 0 depressed hatch rates of *T. colubriformis* eggs to a greater extent than exposure on Day 1, as might be expected, since the majority of eggs should already have hatched before the tannins were added. However *H. contortus* egg hatch rates were slightly higher when tannins were added earlier. This suggests that the few late-hatching eggs exposed to tannins when they were added on Day 1 were more vulnerable than eggs exposed at an earlier stage. The reason for this is not clear. Exposure of the eggs to condensed tannins at an even earlier stage, within the host sheep, reduced EHA hatch rates at low tannin concentrations, but improved hatch rates at high tannin concentrations. It may be that exposure to condensed tannins during the early stages of egg development had some kind of protective effect against later exposure to tannins. One possibility is that pre-exposure to condensed tannins allowed the larvae to develop some kind of incomplete resistance to the effects of

tannins. Another possibility is that "tanning" of the eggshell occurred within the digestive tract of the lambs on *Calliandra* diets, so that the eggs were more impervious to the effects of added tannins later. This is not a new idea. In some species of nematode, egg shells appear to become quinine-tanned as they pass down the host GI tract, although this has not been conclusively proven (Monné & Hönig, cited by Anya, 1964; Wharton, 1980; Wharton, 1983). This protective effect was more pronounced for *T. colubriformis* than for *H. contortus*. A similar protective effect was not observed in the LDA, in which hatch rates of eggs from *Calliandra*-fed sheep were lower at all tannin concentrations. As discussed earlier, this was probably due to delayed hatching of the eggs from lucerne-fed sheep instead of complete inhibition. However, the much smaller effect of diet on *T. colubriformis* hatch rates than on *H. contortus* hatch rates in the LDA would suggest that the former species was much more resistant to the additive effects of *in vivo* and *in vitro* exposure to tannins. In addition, the TL treatment, which had the lowest ED_{50} in the EHA, resulted in the highest hatch rates in the LDA. It would appear that *T. colubriformis* eggs were more likely to undergo delayed hatching when exposed to condensed tannins, whereas the hatching of *H. contortus* eggs was more likely to be permanently inhibited. Bahuaud *et al*. (2006), examining the effects of condensed tannin extracts from five plants on exsheathment of *H. contortus* and *T. colubriformis* L_3 found that in general, exsheathment was delayed by the extracts rather than completely inhibited. In addition, incomplete restoration of exsheathment could be obtained by addition of PEG for *T. colubriformis* but not *H. contortus*. There are a number of similarities between exsheathment and egg hatching, so it would not be surprising if condensed tannins had similar effects on both processes. It would appear also that *H. contortus* is the more susceptible species in both cases.

The lower overall hatch rates for eggs from *Calliandra*-fed sheep suggest that the eggs were affected within the GI tract of the host and possibly even in the uterus of the female worm. In one study, *H. contortus*, *T. colubriformis* or *O. circumcincta* from sheep treated with sub-therapeutic doses of fenbendazole produced eggs that were normal in size but contained morphologically abnormal blastomeres (Kirsch & Schleich, 1982). Eggs retrieved from the uteri of adult *O. circumcincta* also showed morphological changes and appeared to be affected by the drench in the oviduct, before formation of the eggshell. Possibly the abnormalities seen in eggs and larvae

exposed to condensed tannins in the current experiment were also due to exposure to an anthelmintic substance *in utero*, although it is unlikely that condensed tannins have the same mode of action as fenbendazole. (Fenbendazole binds to nematode β-tubulin, which prevents microtubule formation, thus disrupting intracellular transport processes).

The mechanism by which condensed tannins inhibit egg hatching is unknown. One possibility is that tannin binds to the eggshell, so that it is more difficult for the larva to rupture the shell mechanically. This could explain both delayed hatching and the presence of degenerating eggs in the assays, as larvae that failed to escape would use up their nutrient reserves and die. The egg shells of *Nematodirus battus* are known to possess membrane-associated pgps, which bind calcium and are probably involved in ion transport across the shell membranes. Hatching can be inhibited when these binding sites are competitively blocked (Ash & Atkinson, 1984). P-glycoproteins have been identified in all three layers of *H. contortus* eggshells and cuticles (Riou *et al*., 2005). Condensed tannins could conceivably exert inhibitory effects on hatching by either binding to and blocking these sites, or by sequestering calcium and thus inactivating ion transport.

The presence of enlarged eggs containing apparently normal fully developed larvae suggests that normal permeability changes and water influx occured in eggs that were ready to hatch, but that for some reason the shell did not rupture. It is possible that tannins bind to and inactivate the hatching fluid enzymes responsible for breaking down or weakening the shell, so the larva is unable to escape. Alternatively, the larva itself might be affected, resulting in reduced motility or viability. Such larvae might either die within the shell, or have difficulty in escaping. This would explain the presence of incompletely hatched eggs in the assays, as well as the numbers of degenerating larvae. Molan *et al*. (2000a) studied the effects of sulla condensed tannins on migration and motility of third stage larvae and found that the inhibitory effect was much less on *T. colubriformis* than on larvae of other species (*H. contortus* and *O. circumcincta*). If a similar effect occurred in L_1 larvae prior to hatching in the current experiment, the higher eventual hatch rates of *T. colubriformis* eggs would also be explained.

The effects on hatching fluid enzymes or larvae as possible mechanisms of action of condensed tannins would require the entry of tannins into the shell. In *Calliandra*-fed lambs, tannins might be incorporated in the egg prior to shell formation as discussed previously. However, it is more difficult to explain how tannins could enter the egg after shell formation. The lipid layer of the eggshell is permeable only to gases and lipid solvents and constitutes the main barrier to the entry of substances into the egg (Arthur & Sanborn, 1969). Condensed tannins, being large hydrophilic molecules, would be unlikely to penetrate the lipid layer of nematode eggshells under normal circumstances. However, the changes in permeability of the shell just prior to hatching could possibly allow entry of condensed tannins along with the influx of water. Species differences in the lipid layer are probably responsible for differences in the susceptibility of eggs to dessication and chemical penetration (Waller, 1971). The xylene soluble lipid layer of *H. contortus* eggs is thought to contain non-polar hydrocarbon lipids, whereas the inner layer of the *T. colubriformis* eggshell is more likely to consist of protein or polar unsaturated lipids (Wharton, 1980). Whether these differences affect the entry of tannins is unknown.

5.4.3 Larval development

(a) *T. colubriformis*

Condensed tannins appeared to inhibit development of *T. colubriformis* larvae in a dose-dependent manner. Eggs from lucerne-fed sheep (Group TL D1) developed readily to the L_3 stage when tannins were added at low concentrations after hatching, but development rapidly decreased as tannin concentrations increased, until no L_3 developed once tannin concentrations reached 300 µg/mL (Figure 5.6). This effect was more marked when tannins were added before hatching (TL D0), since development to the L_3 was negligible even at a low concentration of 50 µg/mL.

The number of L_1 larvae was low in control wells, where development proceeded to the L_3 stage. As tannin concentrations increased, the number of L_1 also began to increase as development to the L_3 was inhibited. When tannins were added after hatching, the maximum number of L_1 was reached at approximately 300 µg/mL and

was relatively constant beyond this point. However, when tannins were added before hatching, maximum development to the L_1 occurred at a much lower dose, at about 50 µg/mL, and then the number of L_1 decreased (Figure 5.4), at the same time as the number of degenerating larvae present in the wells sharply increased (see Figure 5.7). This would suggest that the viability of the hatched larvae was more severely affected by exposure to condensed tannins during development within the egg than by exposure after hatching. Degeneration began to be noticeable at 200-300 µg/mL. The same trends were seen whether the eggs had been pre-exposed to condensed tannin in the sheep or not; however, pre-exposure decreased hatch rates and therefore overall development. Feeding *Calliandra* to the host sheep did not cause higher rates of degeneration, and in fact the highest rate of degeneration was seen in the TL D0 group. Possibly, exposure to condensed tannins in the sheep had conferred some degree of resistance to the effects of added condensed tannins on larvae in the TC groups. The TL D1 group had the lowest rate of degeneration. This was probably due to complete lack of exposure to condensed tannins, either *in vitro* or *in vivo*, while the larvae were still in the eggs.

Very few L_2 were present in any of the assays. Development either proceeded completely to the L_3 stage or was inhibited at the L_1 stage, indicating that the L_1 was more susceptible to the effects of condensed tannins. However, because the stage of development reached by degenerating larvae in the LDA could not be determined, the possibility that these were L_2 or L_3 larvae could not be ruled out.

(b) *H. contortus*

Development of *H. contortus* to the L_3 was somewhat lower than expected, even in the control wells. It was shown in culture experiments with *H. contortus* that larval growth was not as good when heat-killed bacteria instead of live bacteria were used as a food source (Wang, 1971). Presumably replication of live bacteria maintains a higher concentration in the medium, thus promoting larval growth. In the current study, which used killed bacteria, food may have been a limiting factor. Nevertheless, development rates in the control wells were above 70 % and were deemed to be sufficient for the purposes of the assay.

Eggs derived from lucerne-fed sheep had low development to the L_3 at low tannin concentrations and none beyond tannin concentrations of 300 µg/mL. This was approximately the same concentration at which development of *T. colubriformis* to the L_3 was inhibited. However, in contrast to *T. colubriformis* eggs, there was no development to the L_2 or L_3 of *H. contortus* eggs derived from *Calliandra*-fed sheep, even at low tannin concentrations. This suggests that there was an additive effect of *in vitro* and *in vivo* exposure to condensed tannins. Since there was little development beyond the L_1 no matter when the tannins were added, it would appear that the *H. contortus* L_1 was particularly susceptible to the effects of added tannins.

In the eggs from lucerne-fed lambs, development to the L_2 and L_3 decreased more rapidly with increasing condensed tannin concentration when the tannins were added on Day 0 than when they were added on Day 1, but there was little overall difference. In contrast to the *T. colubriformis* incubations, few degenerating larvae were seen in the *H. contortus* incubations. The reason for this is not known, but it may indicate that the mechanism of action of condensed tannins on the two species is different.

(c) Possible mechanisms of action

As with inhibition of egg hatching, the mechanism by which condensed tannins inhibit larval development is unknown. It is evident from the results of this study that exposure of L_1 larvae to *Calliandra* condensed tannin both before or after hatching can reduce development to the L_3. It is not clear, however, whether the larvae were affected by toxic effects from ingested condensed tannins during normal feeding activity or whether the tannins were able to bind to the larvae in some way.

First and second stage trichostrogylid larvae normally feed on bacteria and it is possible that ingestion of tannins in the medium could occur during this process. The most likely sequel to this would be that tannins could bind to and deactivate larval digestive enzymes, resulting in starvation. Various gut membrane-associated proteins with a digestive function have been characterised for adult worms, including *H. contortus* (Cox *et al.*, 1990; Jasmer *et al.*, 1996; Smith *et al.*, 1997; Munn & Munn, 2002). Less is known about proteins occurring in the gut of non-parasitic stages, but these would be possible binding sites for tannins. Transport proteins such

as pgps are also known to occur in the digestive tract of nematodes. In adult *H. contortus*, these are found in the pharyngeal glands and the first part of the intestine, mainly on the luminal surface of cells (Smith & Prichard, 2002). Tannins could bind to these and interfere with essential transport processes.

Perfusion experiments in *Ascaris suum* showed that the main route of absorption of cholesterol and glucose appears to be the cuticle rather than the intestine (Fleming & Fetterer, 1984). *Brugia pahangi*, a filarial parasite, is also able to take up glucose, amino acids and adenosine through the cuticle (Howells & Chen, 1981). This occurs partly by diffusion and partly by carrier transport. The epicuticle is selectively permeable to polar compounds, which are then transported through the hypodermis (Howells 1987). Although it is unlikely that condensed tannins would be able to penetrate the cuticle, it is possible that they could bind to transport proteins, thus depriving the nematode of essential nutrients. However, transcuticular transport of nutrients is probably less important in trichostrongylids than in filarids. Other proteins present on the nematode surface have been discussed in Section 2.2.1. Although these could represent possible binding sites for condensed tannins, it is unclear what effect (if any) such binding would have on the worm.

A further possibility is suggested by research that shows that cuticular collagen synthesis in *A. suum* during development from L_3 to L_4 can be interrupted by the inhibition of cross-link formation (Rhoads *et al*., 2001a). Cross-linking of tyrosine residues is thought to be enzymatically mediated (Fetterer *et al*., 1993) and could therefore be interrupted by the binding of condensed tannins to the catalytic enzyme (thought to be a peroxidase) or to the tyrosine residues themselves. Such a mechanism of action has been shown for some ellagitannins, which block the phosphorylation and activation of enzymes (Nomara *et al*., 2005). More likely however, is that condensed tannins bind to enzymes secreted by the larvae, which may play a role in larval development. Cysteine protease and aminopeptidase inhibitors have been shown to inhibit development of *A. suum* by interference with moulting (Rhoads *et al*., 1998).

For any of these proposed mechanisms, the proteins involved would be likely to differ between larval stages and also between species, which would account for stage

and species differences in the effects of condensed tannins. The cross-linking of cuticular proteins has mainly been studied in infective and parasitic stages, but is also likely to be important in the free-living stages. Again, most studies involving characterisation of secreted proteins have been carried out on infective stages and adult worms, so little is known of the nature and function of ES products of L_1 and L_2 larvae. The LD_{50} results presented in Table 5.5 suggest for the most part that the later larval stages were more susceptible to the effects of condensed tannins than the L_1, possibly due to a greater abundance or variety of target proteins in the more developed stages. This is consistent with studies that have shown that cuticular proteins increase in both quantity and diversity at each stage of development (e.g. Fetterer *et al*., 1990).

5.4.4 Effects of condensed tannin concentrations

The ED_{50} values obtained in the EHA would suggest that *Calliandra* condensed tannin concentrations between 300 and 700 µg/mL would have an appreciable affect on egg hatch rates of *H. contortus* and *T. colubriformis*. Lower concentrations, below 180 µg/mL, significantly inhibited development to the infective stage. These concentrations are far lower than those obtained in the GI tract of *Calliandra*-fed lambs, reported in Section 4.3.5. Of particular interest was the concentration in the colon, since this would be expected to be most similar to concentrations found in faecal pellets, and therefore to concentrations encountered by larvae under field conditions. At approximately 30 mg/mL, the tannin concentrations in the distal GI tract were at least 50 times greater than that required to reduce hatching and larval development *in vitro*. Reduction of egg hatching and larval development might thus be expected to occur in free-living stages of parasitic nematodes developing in faecal pellets on pasture. Although the *Calliandra*-fed donor sheep in this study had been fed the legume as 100 % of the diet, significant reductions in larval development could probably be achieved by much lower levels of intake. In order to confirm this, analysis of the condensed tannin concentrations in the faeces of sheep fed *Calliandra* at a lower level of intake is required. These results complement the findings presented in Chapter 4 and offer further support for the suggestion that optimum

anthelmintic and nutritional benefits might be achieved by feeding *Calliandra* as part of a mixed ration.

5.5 Conclusion

The results of this study confirm that purified *Calliandra* condensed tannins have anthelmintic effects on the eggs and larvae of *H. contortus* and *T. colubriformis*. Exposure to tannins caused a delay in hatching and a small reduction in the hatch rates of the eggs of both species. The larval development of both nematode species was also reduced in a dose-dependent manner. It is possible that larval development was also delayed, and may have proceeded further if the incubation time was extended. However, this seems unlikely, given the large number of degenerating larvae in the *T. colubriformis* incubations and the very low numbers of L_3 that occurred in the *H. contortus* incubations, even at low tannin concentrations.

Some differences in the effects of *Calliandra* condensed tannins on the two nematode species were apparent. Hatching of *H. contortus* eggs was permanently inhibited and larval development rarely proceeded beyond the L_1 stage. In *T. colubriformis*, hatching and larval development was inhibited less than *H. contortus*, but more L_1 tended to degenerate. However, in both species, development to the L_3 was almost completely inhibited at tannin concentrations above 300 µg/mL.

The main effect of exposure of nematode eggs to *Calliandra* condensed tannins *in vivo* was a reduction in hatch rates, with a smaller reduction in larval development. Conversely, exposure to condensed tannins *in vitro* reduced larval development to a greater extent than hatch rates. The effects of exposure to condensed tannins both *in vivo* and *in vitro* was additive for *H. contortus* eggs and larvae. For *T. colubriformis* eggs, exposure to tannins both *in vivo* and *in vitro* caused a marked delay in hatching but did not delay hatch rates or larval development compared with exposure *in vitro* only.

There was a reduction in development to the L_3 of *T. colubriformis* larvae that were first exposed to condensed tannins in the egg compared with those that were first

exposed as L_1 larvae, but only at low condensed tannin concentrations. However, exposure while still in the egg increased the number of larvae that degenerated, suggesting that this was the most vulnerable stage. The time of exposure to condensed tannin had little effect on the development of *H. contortus* to the L_3, but hatch rates were unexpectedly reduced when tannin was added later. The reason for this is not clear, but it suggests that late-hatching *H. contortus* eggs were more susceptible to the effects of condensed tannin.

Condensed tannin concentrations above 300 µg/mL caused significant inhibition of larval development *in vitro* and might be expected to have a similar effect in a field situation. It is likely that concentrations well above this level could be achieved in faecal pellets by feeding *Calliandra* to the host sheep at a recommended level of 30 % of dietary DM. This would result in lower numbers of infective larvae on pasture and thus lower infection rates of grazing ruminants.

CHAPTER 6

SCREENING OF TROPICAL SHRUB LEGUMES FOR ANTHELMINTIC ACTIVITY AGAINST *H. CONTORTUS* AND *T. COLUBRIFORMIS* IN SHEEP

6.1 Introduction

In Chapter 5, the condensed tannins extracted from *Calliandra* were shown to inhibit the development of ovine GI nematode eggs and larvae. It is not known, however, whether different fractions of the extract would exhibit the same inhibitory effect, as previously observed. Definition of the anthelmintic effectiveness of the different fractions of the extract should facilitate future study into the anthelmintic effects of *Calliandra* condensed tannins.

The gold standard for the assessment of any proposed treatment to control GI worm parasites is a clinical trial where the treatment is applied to a group of infected animals and the efficacy of the treatment is measured in terms of faecal egg counts or post-mortem worm counts. Such a trial is time-consuming, laborious and relatively expensive to run. Also, in consideration of animal welfare, it is desirable to minimise or avoid the use of experimental animals. For these reasons there would be merit in the use of *in vitro* screening assays for the evaluation of the potential anthelmintic properties of a given plant species.

In the current study, it was hypothesised that different fractions of *Calliandra* condensed tannins would have different effects on free-living stages of parasitic nematodes, and that crude tannin extracts of other tannin-containing plants would also exhibit anthelmintic effects when used in EHA and LDA.

The aims of this study were to:

a. determine the hatchability of two species of nematode eggs exposed to 11 different plant extracts; and
b. examine the development of two species of nematode larvae
    i. derived from eggs exposed to eleven different plant extracts, or
    ii. exposed to plant extracts from the L1 stage.

6.2 Materials and Methods

In vitro assays were used to test the effects of *Calliandra* fractions and identify tropical legumes that may have potential as anthelmintic agents. Egg hatch assays and LDA as described in Chapter 5 were chosen for this purpose.

The plants chosen for screening were tropical legumes commonly used as animal feeds in tropical regions that would have potential for inclusion in a practical feeding regime if they proved to have anthelmintic activity. Some of the plants (including *Calliandra*) were chosen because they have been reported to contain significant quantities of condensed tannins (Norton, 1999) although some non-tanniniferous plants and some with an unknown tannin content were also included for comparison. Mitchell grass hay was included because it appeared in the pilot study to have some ability to suppress nematode egg production. Lucerne pellets were used as a non-tannin control. Crude extracts were used instead of purified extracts, to increase the rapidity and ease of screening.

6.2.1 Experiment 1: Screening EHA

(a) Reagents for condensed tannin extraction

Reagents were prepared as described in Section 5.2.1 (a).

(b) Crude condensed tannins for screening assay

Leaves and small stems from a number of tropical legume species growing in demonstration plots at the SVBS precinct, JCU, were collected and stored in Ziploc bags at –20 ºC until use. The legumes included *Calliandra*, *Leucaena leucocephala*, *Gliricidia sepium, Albizia lebec, Sesbania sesban, Desmanthus virgatus* and *Stylosanthes scabra*. Samples of lucerne pellets and Mitchell grass hay (*Astrebla* sp.) were also included. Tannin extraction from the above samples was carried out as detailed in Section 5.2.1 (b). The plant specimens and hay were cut into 2 cm lengths while still frozen, whereas the lucerne pellets were added to the blender whole.

(c) Fractionation of *Calliandra* condensed tannins

Fractionation of *Calliandra* condensed tannins was carried out according to the method of Horigome *et al*.(1988). Ten grams of freeze-dried purified condensed tannin was dissolved in 100 mL of 70 % acetone and applied to a Sephdex LH-20 column equilibrated with 70 % acetone. The liquid flow rate through the column was adjusted to 45 mL/min and the eluent collected in 3 mL fractions. The absorbance of each fraction was observed using a Novaspec II spectrophotometer (Pharmacia Biotech) at 350 nm and the fractions grouped as each peak formed. Each group (representing a single absorbance peak) was rotary evaporated at 45 ºC, freeze-dried and stored at –20 ºC.

(d) Preparation of condensed tannin solutions

Because the concentrations of condensed tannins in crude extracts were unknown, a solution of each crude plant extract, and of each fraction of the purified *Calliandra* extract, was prepared for use in the screening EHA by dissolving 0.035 g of freeze-dried extract in 3.5 mL distilled water, resulting in a concentration of crude extract of approximately 9.90 mg/mL. For the crude *Calliandra* extract, this would give an actual condensed tannin concentration of approximately 300 µg/mL. The same concentration was used for the other extracts to determine their potency relative to *Calliandra*. Solutions for the screening LDA were prepared by diluting the screening EHA solutions 1:4 with distilled water.

(e) Extraction of nematode eggs

Nematode eggs were extracted as described in Section 5.2.1 (d). All eggs were obtained from sheep fed a diet of lucerne pellets.

(f) Experimental design

Egg hatch assays were carried out in two sets of 24-well cell culture plates, one set for each nematode species. In each well, 0.15 mL of crude plant extract solution was

added to 0.1 mL of either *T. colubriformis* or *H. contortus* egg suspension and 1.25 mL distilled water, giving a final concentration of 990 µg/mL. Five replicates were prepared for each extract.

Incubation and counting procedures were as described for the *Calliandra* condensed tannin EHA described in Section 5.2.1 (f).

6.2.2 Experiment 2: Screening LDA

(a) Reagents for condensed tannin extraction

Reagents were prepared as described in Section 5.2.1 (a).

(b) Condensed tannins for screening assay

Condensed tannin solutions were prepared as described in Section 5.2.1 (e). *Calliandra* condensed tannins were fractionated by the procedure described in Section 6.2.1 (c).

(c) Preparation of plant extract solutions

Solutions of plant extracts for use in the screening LDA were prepared as described in Section 6.2.1 (d).

(d) Extraction of nematode eggs

Nematode eggs were extracted as described in Section 5.2.1 (d). All eggs were obtained from sheep fed a diet of lucerne pellets.

(e) Preparation of parasite growth medium

The parasite growth medium was prepared as outlined in Section 5.2.2 (b).

(f) Experimental design

The design of the experiment was similar to that described for the *Calliandra* condensed tannin LDA described in Section 5.2.2 (c) and is summarised in Table 6.1. However, nematode eggs from sheep fed *Calliandra* were not included. Instead of increasing concentrations of *Calliandra* condensed tannins, a single concentration of each of the 11 plant extracts listed in Section 6.2.1 (b) was added to the wells. Five replicates were used for each extract. Incubation, counting and identification procedures were as described in Section 5.2.2 (c).

Table 6.1 Design of larval development assays, showing the day of tannin addition and worm species used in each assay. Two sets of plates (Day 0 and Day 1) were used.

Worm species	*H. contortus*		*T. colubriformis*	
Day	0	1	0	1
Plant extracts	1 - 11	1 - 11	1 - 11	1 - 11

6.2.3 Calculations

The calculations for the screening EHA and LDA were as described in Sections 5.2.3 (a) and (b). The lucerne extract was used as the control to calculate EHI.

6.2.4 Statistical analysis

All analyses were carried out using Genstat.

Data from the screening EHA were analysed by fitting a generalised linear mixed model with species and extract as fixed effects. It was assumed that the error distribution was a binomial distribution with a logit link function. The interaction of species and extract were used to estimate the residual because the replicates were more like sampling. Egg hatch inhibition (over 26 hours) was analysed by analysis of variance.

The larval development screening assay was analysed by fitting a generalised linear mixed model with day, species and extract as fixed effects. It was assumed that the

error distribution was a binomial distribution with a logit link function. The interaction of day, species and extract were used to estimate the residual. Inhibition of egg hatching over seven days was analysed by analysis of variance.

6.3 Results

6.3.1 Screening EHA

The percentages of eggs hatching when incubated for 26 hours with each plant extract in the screening EHA are shown in Figure 6.1. The degree to which egg hatching was inhibited by each extract tended to be different for the two worm species (P = 0.067). Fraction A of the *Calliandra* extract had a slightly greater effect on *H. contortus* eggs than on *T. colubriformis* eggs, but Fraction B had a similar effect on both species. *Stylosanthes*, Mitchell grass hay, *Desmanthus* and to a lesser extent, *Calliandra* inhibited hatching of *T. colubriformis* eggs more than *H. contortus* eggs. The remaining extracts appeared to affect both species similarly. However, when the effects of both nematode species were pooled, none of the extracts had a significant effect on egg hatch rates (P = 0.675).

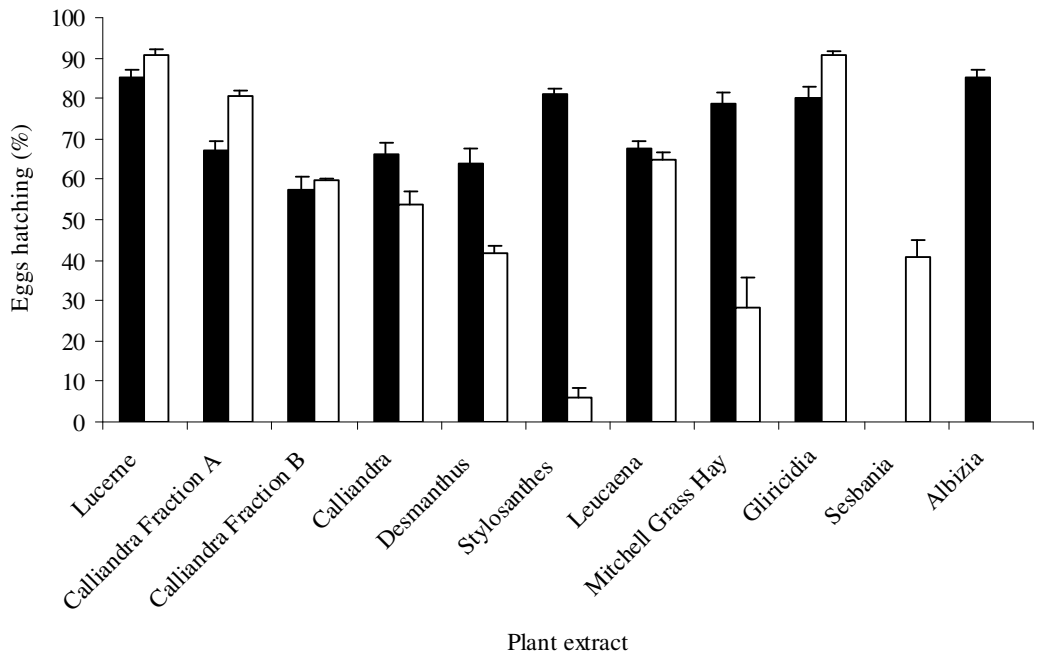


Figure 6.1 Percentage of *Haemonchus contortus* (■) or *Trichostrongylus colubriformis* (□) eggs hatching when incubated for 26 hours with 990 µg/ml of acetone extracts from a variety of tropical

plant species. There was no difference between the extracts (P > 0.100). Data for *H. contortus* exposed to the *Sesbania* extract and *T. colubriformis* exposed to the *Albizia* extract are missing.

In the wells containing the two *Calliandra* fractions, there were high numbers of half-hatched larvae present for both species and high numbers of degenerating *T. colubriformis* larvae. The eggs of both species were normal in appearance.

There were moderate numbers of half-hatched *H. contortus* larvae in wells containing the *Calliandra* and *Desmanthus* extracts and a few in the wells containing the *Stylosanthes* and *Albizia* extracts. High numbers of half-hatched *T. colubriformis* larvae were present in the wells containing the *Calliandra* and *Desmanthus* extracts, moderate numbers in the wells containing the lucerne extract and a few in the wells containing the *Leucaena* extract. Most *H. contortus* eggs and larvae were normal in appearance. In contrast, (with the exception of the wells containing the *Gliricidia* extract), enlarged *T. colubriformis* eggs were abundant. The highest numbers were present in the wells containing the *Stylosanthes* and *Sesbania* extracts. High numbers of degenerating *T. colubriformis* larvae were present in wells containing the *Leucaena*, *Calliandra* and *Desmanthus* extracts.

6.3.2 Screening LDA

(a) Hatch rates at seven days

The effects of different plant extracts on the egg hatch rates of the two species of nematode after seven days incubation are shown in Figure 6.2. There were no significant interactions between species, day and extract. The hatch rates were higher (P < 0.001) for *T. colubriformis* (92 ± 1 %) than for *H. contortus* (52 ± 7 %). Hatching of *T. colubriformis* eggs was only inhibited by 0.8 %, whereas inhibition of *H. contortus* eggs was significantly greater at 33 % (P < 0.050).

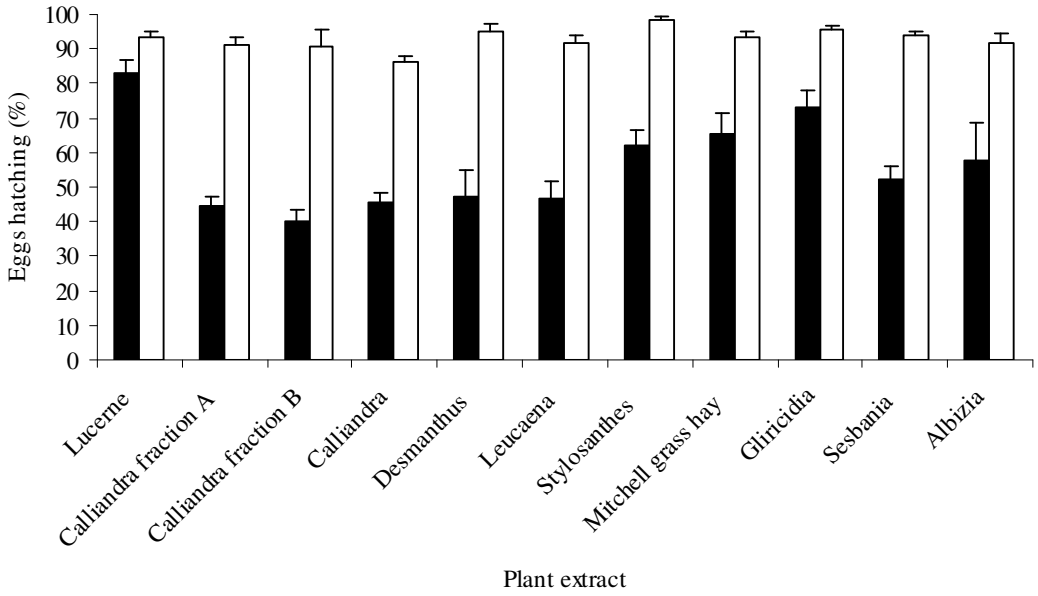


Figure 6.2 Percentage of *Haemonchus contortus* (■) or *Trichostrongylus colubriformis* (□) eggs hatching when incubated for seven days with 990 µg/ml of crude acetone extracts from a variety of tropical plant species. *T. colubriformis* eggs had higher hatch rates than *H. contortus* eggs ($P < 0.050$).

Hatch rates were significantly higher ($P = 0.012$) when tannins were added on Day 1 (70 ± 1 %) than when tannins were added on Day 0 (67 ± 1 %). The *Leucaena* and *Desmanthus* extracts were mainly responsible for this difference (Figure 6.3).

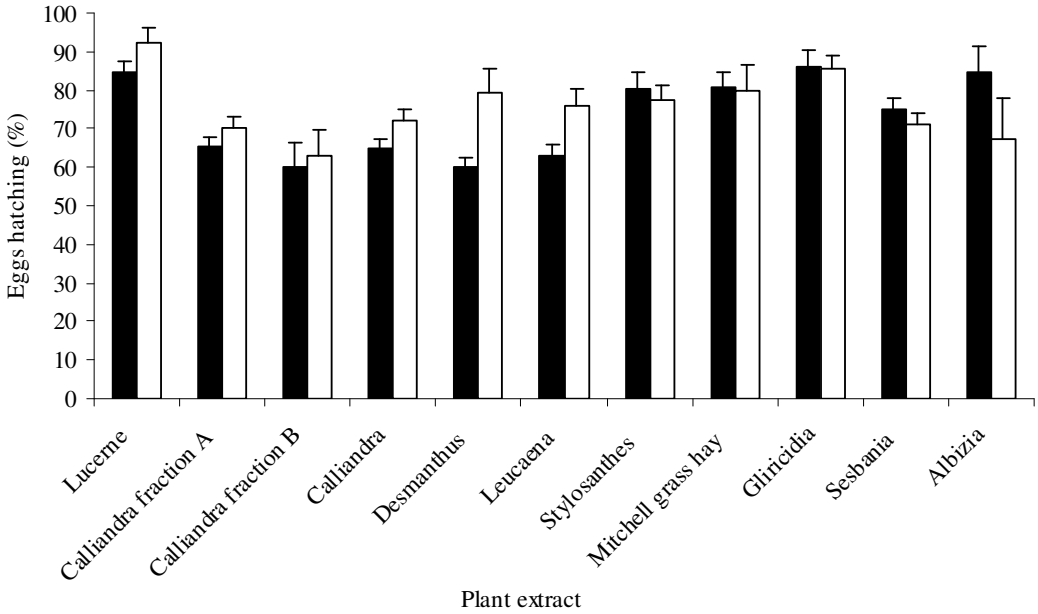


Figure 6.3 Percentage of nematode eggs hatching when incubated for seven days with 990 µg/ml of crude acetone extracts from a variety of tropical plant species. Extracts were added either on Day 0 (■) or Day 1 (□). Hatch rates were higher on Day 1 than on Day 0 ($P < 0.050$).

The effects of the different extracts on hatch rates (pooled for worm species) are presented in Figure 6.4.

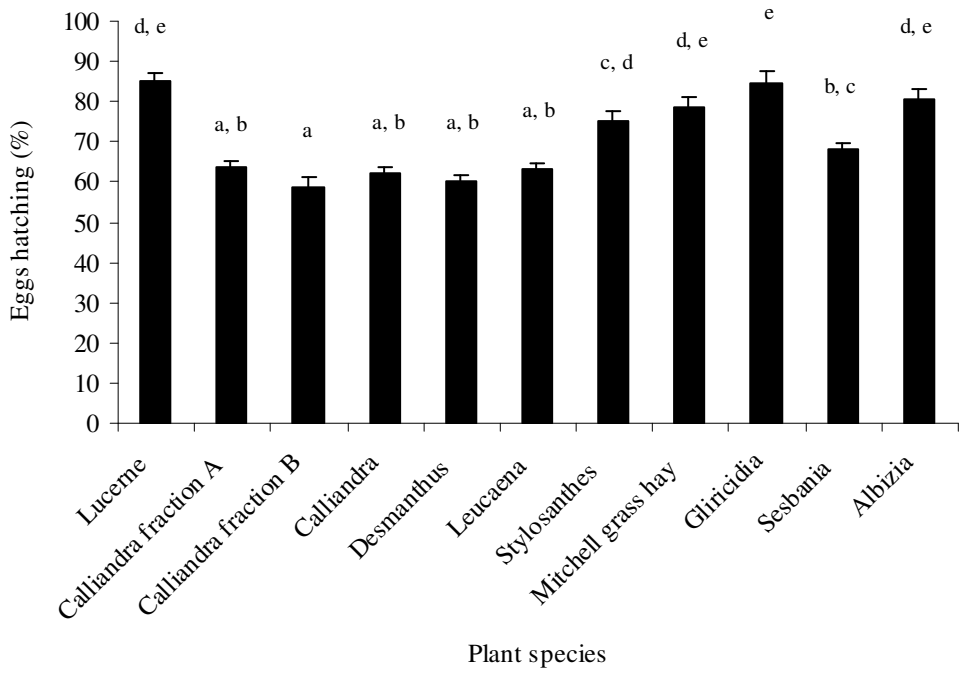


Figure 6.4 Hatch rates of nematode eggs (pooled for worm species) exposed to 9.09 µg/mL of crude acetone extracts from a number of tropical plant species. Columns with different letters are significantly different ($P < 0.05$).

Hatch rates of eggs exposed to the lucerne extract were greater than 80 %. The *Calliandra* A and B fractions inhibited hatch rates compared to lucerne by 25 % and 29 % respectively. The values for the two *Calliandra* fractions were not significantly different. The *Calliandra*, *Leucaena*, and *Desmanthus* extracts inhibited hatch rates to a similar extent as the two *Calliandra* fractions (23 – 24 % inhibition), whereas the Mitchell grass hay, *Gliricidia*, and *Albizia* did not inhibit hatch rates compared with lucerne ($P > 0.050$). The *Sesbania* and *Stylosanthes* extracts were intermediate in effect, inhibiting hatch rates by 18 % and 11 % respectively.

(b) Development to L_1

There was a significant interaction between species and day ($P = 0.003$). The number of larvae at the L_1 stage was higher for *T. colubriformis* Day 1 (74 ± 4 %) than for

T. colubriformis Day 0 (40 ± 4 %), *H. contortus* Day 1 (49 ± 4 %) or *H. contortus* Day 0 (50 ± 3 %).

The mean percentage of eggs developing to the L_1 when exposed to the various plant extracts ranged from 44 – 82 % and was not significantly different between fractions or extracts (P = 0.097). The means for the different extracts are shown in Figure 6.5 for each species-day combination.

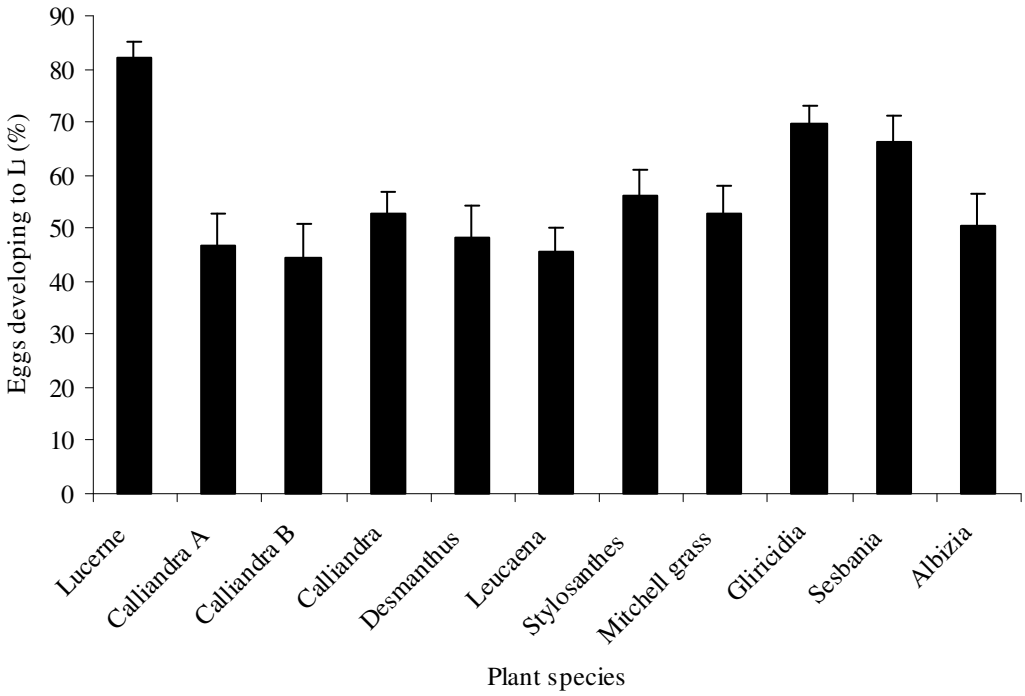


Figure 6.5 The effect of exposure to different plant extracts for seven days on the percentage of nematode eggs that developed to the L_1 stage. Development to the L_1 was not affected by extract (P > 0.100).

(c) Development to L_2

There were no significant interactions between species, day and extract. After incubation for seven days, the percentage of L_2 that had developed from *T. colubriformis* eggs (6 ± 1 %) was higher than the percentage that had developed from *H. contortus* eggs (0.5 ± 0.1 %).

The effect of plant extract on the number of eggs developing to the L_2 stage is shown in Figure 6.6. At the end of seven days, there were very few larvae at the L_2 stage in any of the wells.

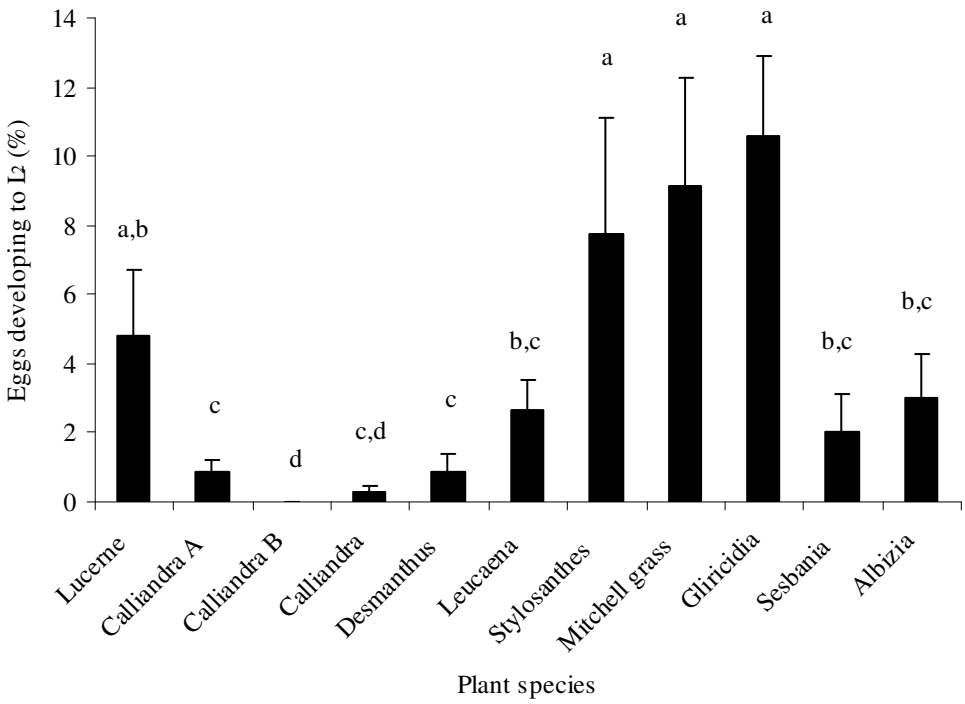


Figure 6.6 The effect of exposure to different plant extracts for seven days on the percentage of nematode eggs that developed to the L_2 stage. Columns with different letters are significantly different ($P < 0.05$).

(d) Development to L_3

The interaction between species and day affected the number of larvae developing to the L_3 stage ($P = 0.003$). The percentage of eggs that developed to the L_3 stage for each species-day combination is presented in Table 6.2.

Table 6.2 The percentage of *Haemonchus contortus* or *Trichostrongylus colubriformis* eggs developing to the L_3 stage over seven days when crude plant extracts were added on Day 0 or Day 1. Values are means ± standard error. Values with different superscripts are significantly different ($P < 0.05$).

	Nematode species	
Extract added	*H. contortus*	*T. colubriformis*
Day 0	0.2 ± 0.2^{a}	12 ± 2^{c}
Day 1	4 ± 1^{b}	$9 \pm 2^{b, c}$

The effect of plant extract on the number of eggs developing to the L_3 stage is shown in Figure 6.7 for each species-day combination. Larval development to the L_3 was relatively low for all extracts. No larvae developed to the L_3 after exposure to the two *Calliandra* fractions. Exposure to the *Calliandra* and lucerne extracts resulted in poor development to the L_3 stage, while most eggs developed to the L_3 when exposed to the *Albizia*, Mitchell grass and *Stylosanthes* extracts. The *Leucaena*, *Gliricidia*, *Sesbania* and *Desmanthus* extracts had an intermediate effect.

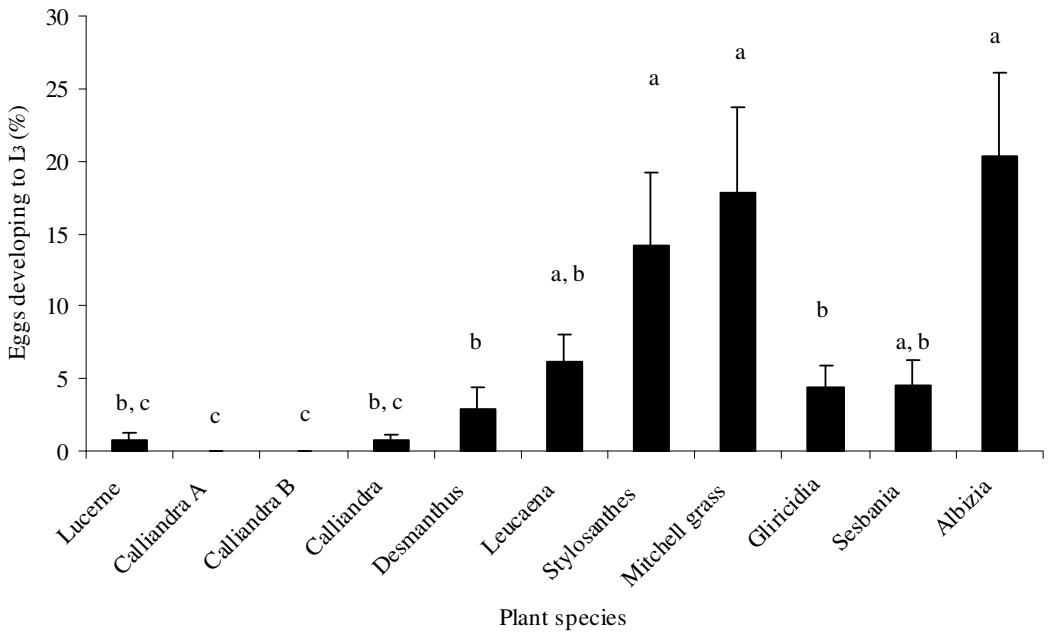


Figure 6.7 The effect of exposure to different plant extracts for seven days on the percentage of nematode eggs that developed to the L_3 stage. Columns with different letters are significantly different ($P < 0.05$).

(e) Degenerating larvae

The interaction between species and day significantly affected the percentage of eggs that resulted in degenerating larvae ($P = 0.001$). The percentage of eggs that resulted in degenerating larvae for each species-day combination is presented in Table 6.3.

Table 6.3 The percentage of *Haemonchus contortus* or *Trichostrongylus colubriformis* eggs resulting in degenerating larvae over seven days when crude plant extracts were added on Day 0 or Day 1. Values are means ± standard error. Values with different superscripts are significantly different (P < 0.050).

	Nematode species	
Extract added	*H. contortus*	*T. colubriformis*
Day 0	0.2 ± 0.1^{a}	35 ± 1^{c}
Day 1	0.3 ± 0.1^{a}	4 ± 1^{b}

The effect of plant extract on the number of eggs resulting in degenerating larvae is shown in Figure 6.8. Both *Calliandra* fractions resulted in significant numbers of degenerating larvae, as did the *Calliandra*, *Desmanthus* and *Leucaena* extracts. Few degenerating larvae resulted from eggs exposed to the remaining extracts.

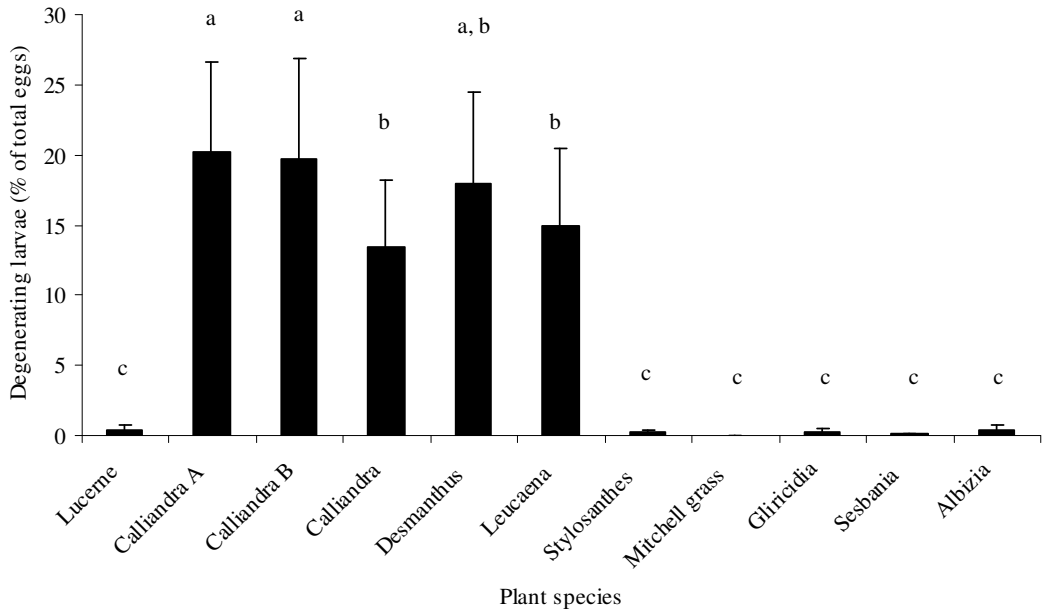


Figure 6.8 The effect of exposure to different plant extracts for seven days on the percentage of nematode eggs that resulted in degenerating larvae. Columns with different letters are significantly different (P < 0.05).

6.4 Discussion

6.4.1 *Calliandra* condensed tannin fractions

Kumar and Horigome (1986) used Sephadex LH-20 to fractionate condensed tannins from four plants on the basis of molecular weight by dissolving and eluting the

tannin in 70% acetone. This procedure yielded three to four tannin fractions for each of the plants studied. Only two fractions were obtained using the same procedure in the current experiment, possibly due to insufficient separation. However, Rakhmani *et al*. (2005) used SepPak column chromatography to separate *Calliandra* condensed tannins into phenolic acids, monomers, oligomers/flavonols and polymeric proanthocyanidins. When subjected to high-performance liquid chromatography, multiple peaks were obtained for each of the first three fractions, but only a single wide peak was obtained for the much larger polymeric fraction. Thus, although the proanthocyanidin content of *Calliandra* may be high, it seems likely that only one or two tannin polymers are present, in addition to a diversity of low molecular weight phenolics. Low molecular weight phenolics would have been removed in the current study during the purification process prior to fractionation.

6.4.2 Extract concentrations

At the time the screening assays were set up, the effective concentration of purified *Calliandra* condensed tannins against nematode eggs and larvae *in vitro* was unknown. Based on *in vitro* work by Molan *et al*. (2002) 300 µg/mL was chosen as a concentration at which *Calliandra* condensed tannins might be expected to cause a high reduction in larval development (equivalent to approximately 1000 µg/mL of crude *Calliandra* extract). This estimate proved to be very similar to the effective concentrations (300 µg/mL and above) obtained in the experiments with purified *Calliandra* condensed tannin reported in Chapter 5.

Because they had been purified prior to fractionation, the concentration of the condensed tannin fractions used in the current study should have been 300 µg/mL, instead of 990 µg/mL as for the crude plant extracts. This was an oversight that invalidates direct comparisons between the *Calliandra* fractions and the other extracts. Nevertheless, the relative efficacy of the two fractions can still be determined.

6.4.3 Effects of plant extracts on egg hatching

When exposed to *Calliandra* fractions for 26 hours, hatch rates of *H. contortus* were 67 % for Fraction A and 57 % for fraction B. Hatch rates for *T. colubriformis* were 81 % and 59 % for these fractions. After seven days of incubation in the LDA, hatch rates for *H. contortus* were 44 % and 40 % for Fractions A and B respectively, and hatch rates of *T. colubriformis* were 94 % and 95 % for these fractions. Possibly, Fraction B delayed hatching longer than Fraction A, but the overall effects on hatch rates appeared to be similar.

For the remaining plant extracts, *H. contortus* hatch rates in the EHA ranged from 65 % for the *Desmanthus* extract to 85 % for the lucerne and *Albizia* extracts. Hatch rates for *T. colubriformis* eggs ranged from 6 % for the *Stylosanthes* extract to 91 % for the lucerne and *Gliricidia* extracts. Again, these differences were not statistically significant.

When the incubation period was extended to seven days, differences in hatch rates between the extracts were more apparent. The hatch rates of *T. colubriformis* ranged from 86 – 98 %, suggesting that none of the extracts had a significant effect on hatch rates long-term. Had the incubation time been extended, the great numbers of enlarged *T. colubriformis* eggs that were present in the screening EHA for most of the extracts would probably have hatched. In contrast, *H. contortus* hatch rates were reduced for at least seven days after exposure to some of the extracts. After seven days hatch rates ranged from 46 % for the *Calliandra* extract to 83 % for the lucerne extract. Lucerne was the only extract to result in a hatch rate above 80 %. The *Calliandra*, *Leucaena* and *Desmanthus* extracts significantly reduced hatch rates by the end of seven days and the *Sesbania* and *Stylosanthes* extracts had a smaller but still significant effect. These results are similar to those obtained with the purified *Calliandra* condensed tannin in the experiment described in Chapter 5. However, in the *Calliandra* assays, the *H. contortus* hatch rates were higher after seven days incubation than after 26 hours incubation, whereas in the current study, hatch rates were lower after seven days. The reason for this is unclear.

It was expected that hatch rates would be higher when the plant extracts were added to the eggs on Day 1, as the majority of eggs should have hatched in the first 24 hours and thus should not have been affected by any inhibitory substances present in the extracts. Overall, this proved to be the case but the difference was very small. The small significant difference between days can be explained for *T. colubriformis* by the fact that tannin merely delays but does not prevent hatching, so that by the end of seven days incubation, Day 0 hatch rates had caught up with Day 1 hatch rates. The situation is more confused for *H. contortus*, since hatching was clearly inhibited by many of the extracts even when they were added after 24 hours incubation. Although this is consistent with the results obtained for *H. contortus* in the *Calliandra* LDA [Section 5.5.3 (b)] the degree of hatch inhibition in the current study was much greater. It is possible that *H. contortus* hatch rates in the current study were maximal between 24 and 26 hours after the start of the incubation, in which case the extracts added on Day 1 of the screening LDA would still have had a major effect on egg hatching. Normally 24 hours would be sufficient for *H. contortus* eggs to hatch at 27 °C in liquid medium (Veglia, 1915) but it is possible some unidentified aspect of the culture conditions slowed the hatching process.

6.4.4 Effects of plant extracts on larval development

Lucerne is often used as the control forage in experiments investigating the effects of condensed tannins in ruminants because it has a negligible condensed tannin content. Jackson *et al*. (1996b) reported that the condensed tannin content of lucerne when analysed using the vanillin assay was 0.42 %, but no condensed tannin was detected when the butanol-HCl assay was used for analysis. This was thought to be due to the lower specificity of the vanillin reagent, which reacts with low molecular weight phenolics as well as tannins.

Based on the results of the preceding experiments (Chapters 3 to 5) it was thought that the lucerne extract could be used as a suitable negative control, as it was assumed that this extract would have no adverse effects on larval development. A distilled water control was also intended to have been included, but this was omitted by mistake. In the *Calliandra* LDA described in Chapter 5, 73 – 98 % development

to the L_3 occurred in the control wells, but there was limited development to the L_3 in the lucerne extract wells of the current experiment. It is difficult to know whether the lack of development in the current study was due to a problem in the lucerne wells of the assay or due to an unexpected effect of the lucerne extract. The screening assays in the present study were set up and incubated concurrently with the *Calliandra* assays in Chapter 5, using the same culture medium and conditions. When eggs derived from lucerne-fed sheep were used, larval development rates in the control wells of the *Calliandra* assays were acceptable. It thus seems likely that it was the lucerne extract itself that inhibited development. The reason for this is unknown. Lucerne showed no anthelmintic effects *in vivo* (see Chapters 3 and 4). Hatch rates in the current experiment were unaffected as previously discussed in Section 6.3.3. The effects of the lucerne extract in the present experiment therefore remain unexplained.

Consistent with the results obtained for this species in the *Calliandra* assays in Chapter 5, there was very little development of *H. contortus* beyond the L_1 stage after exposure to any of the extracts. This may have been due to inadequate larval nutrition [see Section 5.5.3 (b)] or to delayed development. Larval degeneration was not a feature of the exposure of *H. contortus* to the extracts or to *Calliandra* tannins. The few degenerating *H. contortus* that did occur were exposed to either the two *Calliandra* fractions or the *Calliandra* and *Desmanthus* extracts. These, along with *Leucaena*, were the same extracts that caused significant reductions in *H. contortus* hatch rates. However, the *Leucaena* and *Desmanthus* extracts also resulted in the highest rates of development to the L_3 for this species, although this was below 20 % of the hatched eggs.

The development rates of *T. colubriformis* were higher than those of *H. contortus*. *Stylosanthes*, Mitchell grass hay, *Albizia* and *Gliricidia* extracts inhibited development the least, followed by *Sesbania* and lucerne. The greatest inhibition of larval development for this species was caused by the two *Calliandra* fractions and the *Calliandra*, *Leucaena* and *Desmanthus* extracts. These also caused 30 – 45 % of hatched larvae to degenerate. This is consistent with the results obtained for *H. contortus*, and with the degree of larval degeneration observed for *T. colubriformis* in the screening EHA (Section 6.3.3) but not with hatch rates in the EHA. The EHA therefore seems to have value as a screening test for the efficacy of

plant extracts against *T. colubriformis* only in terms of predicting larval degeneration.

Development rates for *H. contortus* were higher when tannin was added on Day 1 than on Day 0, suggesting that the extracts had a greater effect on the eggs than on the L_1. Development rates for *T. colubriformis* were higher when extracts were added on Day 0, suggesting that the L_1 larvae were more susceptible than the eggs. However, this is at odds with the fact that 35 % of larvae degenerated when the extracts were added on Day 0, but only 4 % when the extracts were added on Day 1. This might indicate that a proportion of *T. colubriformis* larvae exposed to tannins within the egg subsequently degenerated after hatching, but the remaining larvae were less susceptible to the effects of the extracts. This would have serious implications for the development of strains of worms that are resistant to the effects of condensed tannins.

Apart from a slightly higher proportion (< 1 %) of larvae at the L_2 stage after exposure to the A fraction, there was no difference in effect on larval development between the two *Calliandra* fractions. There was also little difference in effect between the fractions and the *Calliandra* crude extract, except for slightly lower degeneration of *T. colubriformis* larvae when exposed to the crude extract. This is consistent with the results of the experiments reported in Chapter 5, which indicated that *Calliandra* condensed tannins had near maximal effects at a concentration of 300 µg/mL. As this was the approximate concentration of tannin in the crude extract used in the current experiment, the higher concentrations of tannin in the A and B fractions were unlikely to have much additional effect.

Although overall larval development in the screening LDA was disappointing, the observed trends were consistent with estimated tannin levels in the plant extracts. *Albizia lebec* and *S. sesban* do not contain condensed tannin (Ahn *et al*., 1989) and although *G. sepium* has been reported to contain 35 g/kg DM bound condensed tannin (Jackson *et al*., 1996a) the bound fraction would not have been extracted from the plant by the techniques used in the current experiment. The free condensed tannin in *Gliricidia* is negligible. The tannin content of the Mitchell grass hay was unknown, but grasses usually have condensed tannin concentrations of less than

20 g/kg DM (López *et al*., 2004). The extracts from these plants resulted in the highest overall larval development rates and *H. contortus* hatch rates, and failed to cause degeneration of *T. colubriformis* larvae. There is therefore little evidence to suggest that these plants have anthelmintic properties. However, it should be noted that anthelmintic effects due to compounds that are not extractable with acetone cannot be ruled out.

Stylosanthes scabra has reported condensed tannin concentrations of about 80 g/kg DM. This extract had minimal effects on *T. colubriformis* larvae and *H. contortus* hatch rates, but caused complete inhibition of the development of *H. contortus* to the L_3. This result is difficult to interpret given the generally poor development rates of *H. contortus* in the assay. It may suggest that the tannins in *Stylosanthes* have differential effects on the two parasite species, but this needs to be clarified. The lack of an effect on *T. colubriformis* despite relatively high tannin concentrations might be related to the particular structure of the tannins in *Stylosanthes*.

The tannin content of *Leucaena* species ranges from 14-170 g/kg DM; tannin concentrations in *L. leucocephala* are usually 14-67 g/kg DM (Norton *et al*., 1994). The results of the current experiment suggest that *L. leucocephala* may have anthelmintic effects. Other species such as *L. pallida* and *L. trichandra*, which have condensed tannin concentrations of about 47 and 100 g/kg DM respectively (Osborne & McNeill, 2001), may have lower nutritional value than *L. leucocephala* (Norton *et al*., 1994) but are likely to present other advantages such as cold tolerance or psillid resistance (Shelton & Jones, 1994). The anthelmintic properties of *L. leucocephala* and other species with high tannin levels are therefore worth investigating in further detail.

The plant identified in the screening assays as having the most promise as an anthelmintic was *Desmanthus*, which produced results very similar to those of the *Calliandra* extract. Although development of *H. contortus* larvae exposed to the *Desmanthus* extract was relatively high, *H. contortus* hatch rates were less than 50 % and over 35 % of *T. colubriformis* larvae degenerated. Tannin concentrations in *D. virgatus* have been reported to be about 15-31 g/kg DM (Adjei *et al*., 1993; Singh

et al., 2000). This is somewhat lower than the concentration in *Calliandra*, suggesting that the structure, as well as the concentration, of condensed tannin in the plant may help to determine plant anthelmintic properties. Alternatively, the *Desmanthus* grown at JCU may have a higher tannin concentration than the values cited above. There is at least one report of *Desmanthus* with a condensed tannin concentration of 89 g/kg (Ramírez *et al*., 2000). Factors such as soil fertility, rainfall, light intensity, genetics and stage of growth all influence the condensed tannin content in plants (Barry & Forss, 1983; Salawu *et al.*, 1999; Paolocci *et al.*, 2005). Although *Desmanthus* has low biomass production and is deciduous in the dry season (Djogo *et al*., 1994), it has many valuable agronomic characteristics, including: the ability to grow in acid or clay soils, high seed production, vigorous growth enabling it to survive competition, tolerance of heavy grazing, reasonable drought tolerance and ready self-propagation (Gardiner & Burt, 1995). The CP content of *Desmanthus* leaf is high, although total CP content of the plant decreases somewhat if the leaf/stem ratio is low (Adjei & Pitman, 1993). *In vitro* organic matter digestibility (IVOMD) tends to be low compared with other legumes, probably due to the tannin content of the plant, but research on the *in vivo* nutritive value of *Desmanthus* is lacking. *Desmanthus* may not offer a nutritional advantage over *Calliandra*, but might be useful for its vigour, particularly in drier regions where *Calliandra* does not grow well.

Although *Desmanthus* and *Leucaena* may have anthelmintic potential, the *Calliandra* extract still had the greatest efficacy against both the eggs and larvae of the two parasite species examined. *Calliandra* thus remains the plant of choice for further investigation.

6.5 Conclusions

Calliandra condensed tannins were separated into two unidentified fractions which probably represented two different polymeric proanthocyanidins. When the anthelmintic effects of these fractions were investigated, there was no statistically significant difference between the A and B fractions in hatch rates or larval development. When differences in predicted condensed tannin concentration were

taken into account, there was also no difference between the two fractions and the crude extract. Thus the *in vitro* anthelmintic effects of the two fractions were very similar to each other and did not appear to offer any advantage over the crude extract for use in future *in vitro* studies.

Although the results were not as definitive as envisaged at the outset of the experiment, the EHA proved to be a useful technique for rapidly screening tropical shrub legumes for potential anthelmintic activity against *H. contortus* and *T. colubriformis*. Plant extracts known to contain condensed tannins had effects on the two nematode species qualitatively similar to those obtained using *Calliandra* condensed tannins in the experiment described in Chapter 5. Because the hatch rates of *H. contortus* were permanently reduced, the screening EHA effectively identified extracts with anthelmintic activity against this species. The same was not true for *T. colubriformis*, because hatching of *T. colubriformis* eggs was only delayed. In addition, there was no apparent relationship between *T. colubriformis* EHA hatch rates and either estimated tannin content of the extract or development to the L_3 in the LDA. However, the hatched *T. colubriformis* larvae exposed to extracts assumed to contain tannins had significant rates of degeneration in the screening assays, whereas larvae exposed to non-tannin extracts did not. The rate of degeneration of *T. colubriformis* larvae in the EHA could therefore be a useful indicator of possible anthelmintic activity.

The results of the LDA were more difficult to interpret due to overall poor development, particularly for *H. contortus*. This may have been due to experimental error, but there was no indication where this error might have occurred. However, for *T. colubriformis*, development to the L_3 was much lower for extracts expected to be high in tannin than in extracts with low tannin concentrations. Degeneration of larvae also only occurred after exposure to extracts thought to be high in tannin. The LDA may be necessary to confirm EHA results for *T. colubriformis*, as hatch rates were not reliable.

The eggs of *H. contortus* appeared to be more susceptible than the L_1 to the effects of the plant extracts, whereas the development of *T. colubriformis* was reduced to a greater extent by the exposure of the L_1 to the extracts. Of concern was evidence that

the exposure of *T. colubriformis* eggs to the extracts might exert a selection pressure on the eggs, resulting in a population of larvae that were more resistant to subsequent exposure to the extracts. This situation must be clarified in future work before plants containing condensed tannins are used for parasite control.

In addition to *Calliandra*, the *Leucaena* and *Desmanthus* extracts were identified as having anthelmintic effects *in vitro*. These plants are both valued in tropical regions for their nutritional and agronomic attributes and thus have potential for inclusion in parasite control programs. The anthelmintic effects of these plants *in vivo* should therefore be investigated.

CHAPTER 7

HISTOLOGICAL DEMONSTRATION OF CONDENSED TANNINS *IN SITU* IN PLANT AND ANIMAL TISSUES INCLUDING NEMATODES

7.1 Introduction

It is evident from the results of the feeding experiments presented in Chapters 3 - 5 that *Calliandra* condensed tannins decrease fecundity of adult GI nematodes and affect the viability of eggs and larvae. The mode of action of condensed tannins against nematode parasites has not been established, but it seems likely that these could bind to worm proteins in the GI tract of the host in a way that would inhibit worm egg production. Condensed tannins also have anti-nutritive effects in the GI tract of ruminant animals where, it has been postulated, tannins may bind to and inactivate digestive enzymes.

There is some evidence that dietary condensed tannins are visible histologically in ovine intestinal tissue (Dawson *et al*., 1999). Although the fate of condensed tannins in the GI tract of ruminant animals has yet to be fully defined, some tannins may be absorbed (Perez-Maldonado & Norton, 1996b). However, the results of experiments presented in Chapters 3 and 4 suggest that, at least in the case of *Calliandra* tannins, a significant proportion would pass through the ruminant GI tract and be excreted in the faeces. Possibly some tannins would bind to GI tissue or to proteinaceous materials in the GI tract lumen. To examine such a possibility, a technique that would identify the presence of condensed tannins in non-plant tissues needs to be developed. The formation of coloured products by phenolic compounds in certain chemical reactions may be exploited for this purpose.

The aim of the current experiment was to develop a staining technique that would define the presence of condensed tannins in ruminant GI tissue, or in nematode adults, eggs or larvae, to identify structures that may be affected by dietary condensed tannins.

7.2 Materials and Methods

Condensed tannins occur naturally in many plants but not in mammalian tissues. It is not surprising therefore that published techniques for staining condensed tannins were developed to define the presence of these polyphenols in plants. In developing a procedure that would be appropriate for use in staining condensed tannins in mammalian and nematode tissues, the following approach was adopted. First, relevant published staining techniques were examined first using tissues from selected plants known to contain (or not contain) condensed tannins. These techniques were then further examined using ovine GI tissues that were incubated with purified condensed tannins extracted from *Calliandra* leaves. The techniques were modified at each phase of examination as described. The most appropriate technique was finally selected to define the presence of condensed tannins in the GI and worm tissues obtained from the sheep used in the experiment described in Chapter 4.

7.2.1 Staining techniques examined

(a) Haematoxylin and Eosin (H & E)

The H & E technique is a common staining technique in histology used to highlight basophilic and eosinophilic structures. Haematoxylin is a basic dye that colours basophilic structures blue-purple. Eosin, a counterstain to haematoxylin, is an acidic dye that colours eosinophilic structures pink-red.

Mayer's Haematoxylin was prepared by adding 2 g Haematoxylin, 0.4 g sodium iodate and 100 g aluminium ammonium sulphate to 2 L distilled water. After dissolving overnight, 100 g chloral hydrate and 2 g citric acid were added. The solution was boiled for five minutes, cooled and filtered before use. Eosin Y was prepared by dissolving 15 g yellowish Eosin, 5 g erythrosine and 5 g calcium chloride in 2L distilled water.

The H & E staining procedure followed was that routinely used in the Pathology Laboratory of the SVBS, JCU. Fixed tissue sections were taken to water through xylene and ethanol and stained in Meyer's Haematoxylin (Bancroft & Cook, 1984) for eight minutes. Scott's tap water substitute was used to blue the sections, before counterstaining with Young's Eosin (15 g) for four minutes prior to differentiation in water. After dehydration in ethanol and clearing in xylene, the sections were permanently mounted in DPX synthetic mounting medium.

(b) Ferric chloride

Ferric chloride (or ferric sulphate) has been used to demonstrate tannins in fresh or formalin-fixed plant sections (Reeve, 1959). Although ferric chloride forms complexes with phenolic compounds non-specifically, it may be useful as a means of identifying the presence of condensed tannins *in situ* in mammalian tissue.

Fresh plant sections can be stained with 0.5 - 1 % aqueous ferric chloride containing 0.1 N hydrochloric acid. When the excess ferric chloride is washed from the sections, a green or blue-black precipitate indicates the presence of tannins. Acidification of the solution prevents the formation of brown ferric hydroxide precipitates.

(c) Butanol H_2SO_4

Whereas ferric chloride forms complexes with phenolic compounds non-specifically, the acid-butanol reaction is specific for proanthocyanidins (Mole & Waterman, 1987; Makkar, 2003). Acidified butanol is often used in quantitative assays for condensed tannins. Acid hydrolysis of the polymer cleaves inter-flavan bonds, resulting in red anthocyanidin products (Mole & Waterman, 1987; Porter, 1989; Makkar, 2003). The butanol reagent will react with some of the protein and fibre bound condensed tannins in plant tissues, as well with as the extractable tannin fraction (Makkar, 2003). Gutmann (1993) adapted the butanol-H_2SO_4 technique to demonstrate condensed tannins in plant material fixed in glutaraldehyde and embedded in glycol methacrylate, and it was this method that was adapted for use in the current study.

According to Gutmann (1993), the staining solution consists of 3 M sulphuric acid in 1-butanol. Prepared tissue sections are flooded with the staining solution and heated in a 400 W microwave oven for 10 seconds or until the solution boils. After agitating the preparation, heat is applied for two or more shorter periods until colour develops. The solution should be kept near boiling point (117 °C), and the total heating time should be less than 30 seconds. Stained sections are rinsed in 1-butanol and mounted in Canada balsam.

Monomeric phenols present in the tissues stain dark brown with this technique, rather than red. Heating at lower temperatures results in the formation of brown phlobaphenes rather than diagnostic red anthocyanidins. However, overheating causes tissue destruction.

(d) Vanillin-HCl

The vanillin-HCl technique is based on the reaction of the acidified aromatic aldehyde, vanillin, with flavanoid compounds that have a single bond between C2 and C3 and have free meta-hydroxy groups on the A ring (see Section 2.6 for structures of condensed tannins). Colour development is due to the formation of a red chromium ion at the C6 position (Pew, 1951; Mole & Waterman, 1987). This reaction was used by Dehon *et al*. (2002) to demonstrate condensed tannins in wood.

The vanillin reagent used by Dehon *et al*. consisted of "5 % (w/v) vanillin dissolved in HCl plus alcohol 100 % (v/v)"(Dehon *et al.*, 2002; p304). Freshly cut sections of wood 25 µm thick were incubated with this solution for ten minutes at room temperature, before examination.

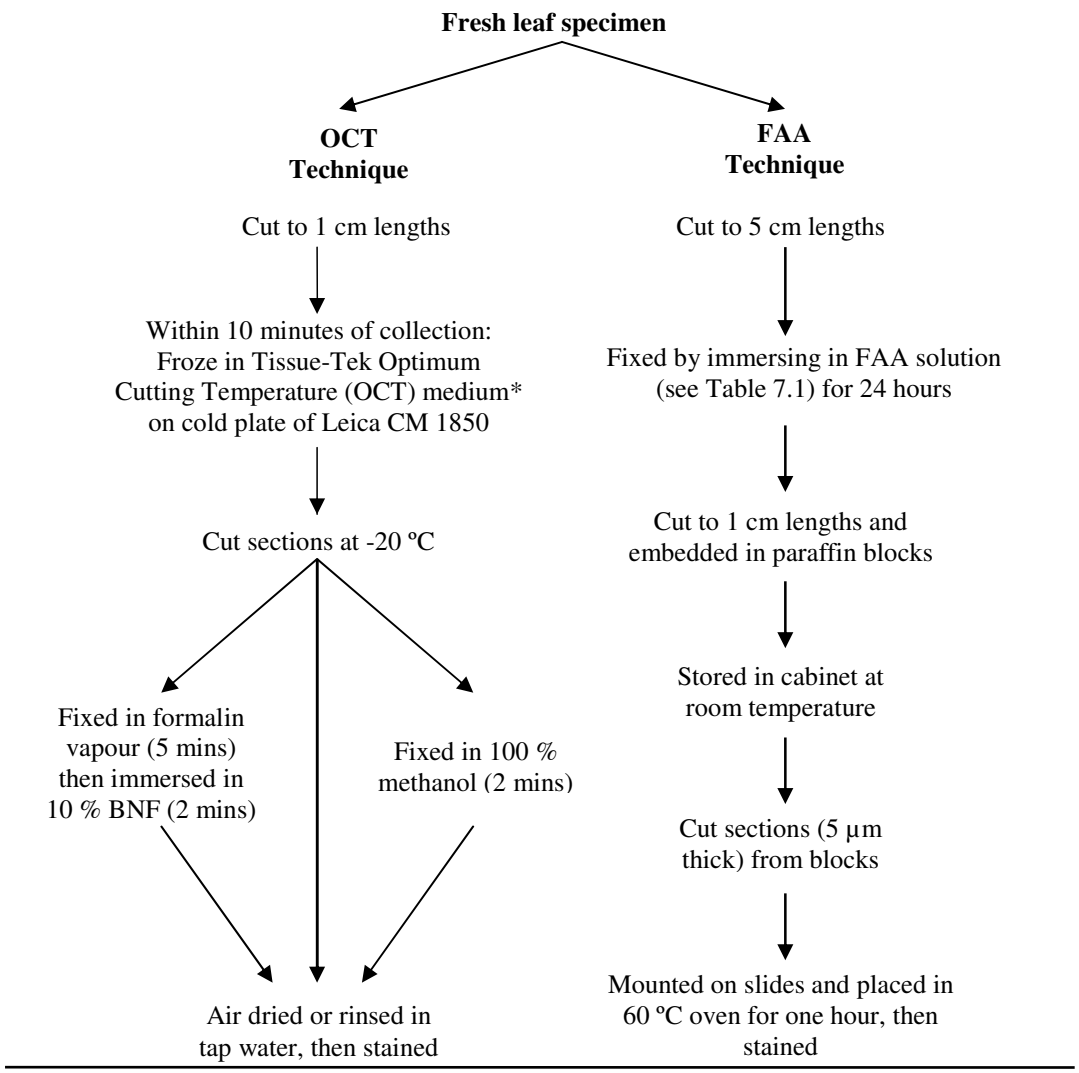
7.2.2 Preparation of tissues for staining

(a) Plant tissues

Plants were selected based on their predicted condensed tannin content. The selected high condensed tannin plants included *Calliandra* and *D. virgatus* and those with

negligible condensed tannin content included *S. sesban*, *A. lebbek* and *L. collinsii* (Ahn *et al.*, 1989; Adjei *et al.*, 1993; Osborne & McNeill, 2001). Specimens from these selected plants were prepared for staining by either of the procedures outlined in Figure 7.1.

Figure 7.1 Procedures for the preparation of plant tissues for staining.



*Sakura Finetek, USA

Table 7.1 Composition of FAA solution used for fixation of fresh plant material prior to embedding and staining. From Sass (1958).

Constituent	Quantity (mL)
Ethyl alcohol (95 %)	50
Glacial acetic acid	5
Formaldehyde (37 – 40 %)	10
Deionised water	35

(b) Ovine GI tissues (positive control)

Sections of the proximal duodenum and of the distal ileum (approximately 2 cm lengths) were collected from a freshly killed ewe and placed immediately into Kreb's solution (Table 7.2) at 37 °C. The sections were opened along the anti-mesenteric border, and pinned (mucosal surface up) to a piece of sterilised balsa wood cut to fit tightly into the bottom of a sterilised 500 mL glass beaker. In order to submerge the tissue completely, the beaker was filled with 250 mL of the Kreb's solution.
One gram of purified *Calliandra* condensed tannin extract, as prepared in Section 5.2.1, was added.

Table 7.2 Composition of Kreb's Solution used to incubate sections of GI tissue from a freshly killed ewe.

Chemical	M.W.	mM	g/L
NaCl	58.44	118.0	6.9
KCl	74.5	4.8	0.36
NaH_2PO_4	156.01	1.0	0.15
$NaHCO_3$	84.01	25.0	2.1
$MgSO_4$	246.47	1.2	0.3
Glucose	180.16	11.0	2.0
$CaCl_2$	147.02	2.5	1.0

The beaker was placed in a water bath at 37 °C for 24 hours with carbogen (95 % oxygen, 5 % carbon dioxide, BOC Gases, Townsville) bubbling into the solution continuously. At the end of the incubation period, the tissue sections were removed and placed in 10 % BNF in separate labelled containers. After 48 hours the tissues were trimmed, dehydrated in graded ethanols, cleared in xylene and embedded in paraffin. Sections (5 µm thick) were cut, dried at 60 °C for one hour and stained according to the procedures described in Sections 7.2.1 (a) – (c).

A number of modifications to the procedure described above were undertaken in the preparation of a second batch of slides. Sections of rumen and abomasum were also included and the incubation time was reduced to six hours. The concentration of condensed tannins used was reduced (0.25 g in 250 mL) and 1 g/L ascorbic acid was added to the Kreb's solution to prevent oxidation of the tannins. Each tissue section was divided into two, one half being incubated in the tannin solution and the other in

a separate beaker of Kreb's solution and ascorbic acid only. During subsequent processing, both halves of each section were mounted together on the same slide, pending staining.

(c) Silanised slides

Silanised slides were prepared to promote the adherence of tissue when making frozen tissue sections. Batches of 180 plain glass slides (SuperFrost, Menzel Glaser, Germany) in racks of thirty were soaked in detergent (Radacon, Fronine Laboratory Supplies, Australia) in tap water for two hours and then rinsed for 30 minutes in tap water in a pipette washer. The slides were then rinsed in two changes of deionised water and two changes of absolute ethanol for five minutes each. After drying at 37 °C for 10 minutes, each rack of slides was placed in a solution of 8 mL Slide Coating Adhesive (3-aminopropyltriethoxysilane in acetone, ProSciTech, Thuringowa Central, Qld, Australia) in 400 mL 100 % acetone for 45 seconds. This was followed by a final rinse in distilled water for 30 seconds, before the slides were returned to the drying oven (37 °C) where they remained overnight. The silinised slides were stored in the original boxes until use.

(d) Ovine GI tissue (experimental samples)

Segments of GI tissue were collected from parasitised lambs in the experiment reported in Chapter 4. Half of the lambs had been fed *Calliandra* and the other half lucerne pellets. At slaughter, six tissue samples approximately 10 mm x 5 mm x 5 mm were taken from each of the rumen (R), abomasum (A), duodenum (D), jejunum (J) and ileum (I) of each lamb after collection of worms (Section 4.2.6). Three samples were fixed in 10 % BNF for a minimum of 48 hours, dehydrated in graded ethanols, cleared in xylene and embedded in paraffin. Sections were cut at 5 µm and dried at 60 °C for one hour prior to staining using the techniques described in Sections 7.2.3 (a) – (c).

The remaining three tissue samples from each GI segment were embedded in Tissue-Tek Optimum Cutting Temperature (OCT) medium (Sakura Finetek, USA Inc.)in

plastic moulds (20 mm x 20 mm x 4 mm) and rapidly frozen by immersion in a beaker of iso-pentane suspended over dry ice for about five minutes. The frozen sections were placed in small Ziploc bags, stored at -20 °C until the end of the day and then transferred to a -80 °C freezer. To cut the sections, frozen blocks of tissue were removed from the freezer and placed on ice. Sections 5 µm thick were cut at -20 °C in a cryostat (Leica CM 1850) and adhered to silanised slides [Section 7.1.1 (e)]. The prepared slides were stored overnight at room temperature. The next day the slides were fixed in either methanol or formalin, as unfixed sections tended to wash off the slides during the staining process.

Both formalin and methanol fixation of the OCT sections was attempted. Formalin fixation involved exposure of the sections to formalin vapour for six minutes, followed by immersion in 10 % BNF for four minutes. Methanol fixation involved immersion of slides in absolute methanol for a specified time (see Table 7.3 for immersion times investigated). Either fixation technique was carried out immediately prior to staining by the vanillin-HCl technique described in Section 7.2.3 (d).

(e) Nematode experimental samples

Adult worms (*T. colubriformis* and *H. contortus*) were collected at necropsy from the four donor lambs described in Chapter 8. Throughout the infection period, one donor infected with each species of worm had been fed a diet of lucerne pellets and the other had been fed a diet of *Calliandra* as described in Section 8.2.3. Adult worms were also collected from four lambs representing each of the recipient treatment groups in the same experiment. Each group of recipient lambs had received a dose of infective larvae derived from one of the four donor lambs, and had been fed a diet of lucerne pellets only. After removal from the GI tract, the worms were rinsed in saline, and either stored in saline for staining intact within 24 hours, or frozen in OCT medium in a plastic mould on the cold-plate of a Leica CM 1850 cryostat. Approximately ten *H. contortus* or 15 - 20 *T. colubriformis* of both sexes were frozen in each block, and the blocks were stored at -20 ºC until use.

Nematode L_3 larvae from the four donor lambs were obtained by the procedures described in Section 8.2.4 (a) and stored in tap water at 4 ºC.
T. colubriformis and *H. contortus* eggs were obtained from the four donor lambs by homogenising approximately 2 g faeces collected from the rectum of each lamb in 28 mL saturated magnesium sulphate solution and passing the resultant suspension through a tea-strainer with a 1 mm aperture mesh. The suspension was poured into a plastic 25 mL specimen vial that was filled to the brim, forming a convex meniscus, and a cover slip was placed carefully over the top. The preparation was allowed to stand for ten minutes, and then the cover slip was lifted off vertically, inverted and placed on a rack. Eggs adhering to the cover slip were stained immediately.

7.2.3 Development of staining techniques using plant and ovine GI tissues

(a) H & E

Formalin-fixed plant and ovine GI tissues were stained according to the standard technique described in Section 7.2.1 (a) for H & E staining.

(b) Ferric chloride

Sections of unfixed, formalin-fixed, methanol-fixed and FAA-fixed *Calliandra* leaf were prepared by the techniques described in Section 7.2.2 (a). The FAA-fixed sections were rehydrated through xylene and ethanol before staining. Fixed ovine GI tissue was prepared by the techniques described in Sections 7.2.2 (b) and (d). All the sections were flooded with 1 % aqueous ferric chloride for two minutes and rinsed in tap water. The fixed sections were dehydrated in ethanol, cleared in xylene and mounted in DPX after staining.

Aqueous toluidine blue (1 %) and Safranin O were investigated as counterstains for plant tissue stained with ferric chloride. Safranin O stain was prepared by dissolving 1 g Alcian blue in 100 mL distilled water and 1 g Safranin separately in a further 100 mL distilled water. When completely dissolved, the solutions were mixed together and filtered through Number 2 Whatman paper. Formalin-fixed sections of

Calliandra leaf were stained for two minutes, and then rinsed with tap water. Sections of ovine tissue stained with ferric chloride were counterstained with H & E as described in Section 7.2.1 (a).

(c) Butanol H_2SO_4

The butanol H_2SO_4 staining solution was prepared from 167 mL 18 M sulphuric acid made up to 1 L with butan-1-ol. Tissue sections prepared for staining according to the procedures described in Section 7.2.2 (b) were placed on a glass rack inside a 650 W microwave oven (National NE-6750, Mitsubishi Electric Industrial Co. Ltd., Japan) in a fumehood. A 100 mL water load was also placed in the oven. Each section was flooded with approximately 1mL of the staining solution and heated on high power (boiling did not occur at a lower setting). The sections were heated two to three times, for five to ten seconds, and then rinsed with butan-1-ol before microscopic examination. Various heating times were examined to determine the optimum time for staining. On one batch of sections, water was used instead of staining solution, to determine whether the sections were being damaged by the sulphuric acid.

Fresh *Calliandra* leaves and *Calliandra*, *Desmanthus*, *Sesbania* and *Leucaena* leaves fixed in FAA (Figure 7.1) were stained using the butanol H_2SO_4 procedure. *Calliandra* sections were stained both with and without removal of the paraffin embedding medium in xylene.

Pieces of ovine GI tissue (R, A, D, J and I) prepared as described in Section 7.2.2 (d) were also stained. Sections from one lamb on each diet were chosen at random and used to try out the butanol-H_2SO_4 technique on mammalian tissue. Methylene blue (1 % in 20 % acetic acid), toluidine blue (2 % solution in 60 % ethanol) and H & E were all tried as counterstains. Methylene blue and toluidine blue were applied to air-dried butanol-H_2SO_4 stained sections for one minute and H & E staining was as described in Section 7.2.1 (a). After staining the sections were rinsed with tap water, dehydrated in ethanol, cleared in xylene and mounted.

(d) Vanillin-HCl staining

The description of the vanillin reagent used by Dehon *et al*. (2002) was confusing and was not used in this experiment. Instead, the reagent that was used was based on that of Broadhurst and Jones (1978) and consisted of a 4 % (w/v) solution of vanillin (Sigma) in absolute methanol, mixed 2:1 with concentrated hydrochloric acid to give a 2.7 % final solution. The reagent was prepared fresh daily and kept protected from light. The staining procedure was that described by Dehon *et al*. (2002). The prepared tissue sections were flooded with the vanillin reagent for 10 minutes at room temperature and the slides examined while wet. However, because other authors have reported the sensitivity of the vanillin reaction to light (e.g. Broadhurst & Jones, 1978) the incubation was carried out in the dark.

Desmanthus was chosen as a tannin-positive subject for initial experiments with vanillin staining, due to the intense staining reaction obtained in the butanol-H_2SO_4 staining procedure. *Sesbania* was used as the negative control. Prior to staining, sections of *Desmanthus* and *Sesbania* fixed in FAA were rehydrated through xylene and ethanol. After staining, the FAA-fixed plant sections were counterstained with either toluidine blue or H & E as described in Sections 7.2.3 (a) and 7.2.1 (a), then dehydrated, cleared and mounted.

Formalin-fixed ovine GI tissue sections were rehydrated through xylene and ethanol prior to staining. Methylene blue (1 % solution in 20 % acetic acid), toluidine blue (2 % solution in 60 % ethanol) methyl green (2 % aqueous) and H & E were all investigated as possible counterstains. Methylene blue, toluidine blue and methyl green were applied to wet vanillin-HCl treated sections for one minute, while staining for H & E sections was as described in Section 7.2.2. The stained sections were dehydrated, cleared and mounted.

Although the initial vanillin staining of FAA-fixed *Desmanthus* leaves successfully demonstrated condensed tannins in the plant tissue, the technique did not work satisfactorily on formalin-fixed ovine GI tissues. Therefore, sections of *Calliandra* and *Albizia* leaf were used for experimenting with fresh leaf material. The leaves

were prepared for staining according to the OCT technique outlined in Figure 7.1 and stained in the dark with the vanillin reagent for ten minutes. The stained sections were examined either wet, or after rinsing with water or absolute methanol, to compare the effectiveness the different fixation techniques.

Following comparison of the effects of fixation techniques on plants, staining was carried out on ovine GI tissue prepared according to the OCT technique described in Section 7.2.2 (c). A number of counterstaining techniques were tried, as described for the formalin-fixed GI tissue. After staining, excess solution was drained off the slides, and the sections were examined immediately at 100x magnification with a cover slip applied directly to the wet section. A binocular light microscope (Zeiss, Germany) was used for this purpose. Where no background staining was evident, the microscope diaphragm was partially closed to increase contrast, improving visibility of the tissue architecture.

7.2.4 Staining of experimental ovine GI tissues

(a) Haematoxylin and eosin

The experimental ovine GI tissues were stained by the H & E procedure described in Section 7.2.1 (a). The sections were examined microscopically and assessed for the presence of condensed tannins.

(b) Vanillin-HCl

The technique eventually chosen to demonstrate condensed tannins in ovine GI sections involved fixation in absolute methanol for two minutes. Fixation was followed by incubation with vanillin-HCl for ten minutes, in the dark, at room temperature, application of a cover slip and immediate examination of the section without counterstaining or rinsing. The microscope diaphragm was partly closed to increase contrast. Preliminary trials indicated that 25 – 60 % of ileum sections from *Calliandra*-fed lambs stained positive for tannins, whereas all sections from lucerne-

fed lambs were negative. It was therefore decided that examination of five sections per GI segment for each lamb would be adequate to detect the presence of condensed tannins. The sections examined were not serial sections but were taken approximately 65 µm apart from the block of frozen tissue, that is, every 12th slice. The location of any red staining was noted for each slide, and the number of positive slides for each GI segment per lamb was recorded.

7.2.5 Staining of nematodes

The technique described in Section 7.2.4 was adapted to stain eggs, the external surface of the third-stage larval sheath and external and internal structures of *H. contortus* and *T. colubriformis* adults.

(a) Eggs

Cover slips removed from vials containing saturated magnesium sulphate solution were placed on a rack, wet side uppermost, and a drop of absolute methanol was added to fix the eggs. After two minutes, the cover slips were flooded with vanillin-HCl prepared as described in Section 7.2.3 (d) and the preparation was covered with an inverted specimen tray to exclude light. After ten minutes, a glass slide was lowered onto the cover slip and the adherent eggs were examined microscopically.

(b) Larvae

Before staining, nematode larvae were fixed by adding 0.5 mL absolute methanol to 0.5 mL larval suspension in a 5 mL screw-capped plastic specimen vial covered with aluminium foil. After two minutes, 2 mL vanillin-HCl was added. Ten minutes later, drops of liquid from the vial were transferred to glass slides with a pipette for examination under a binocular light microscope, and the remainder of the solution was placed in a Petri dish for examination under a dissecting microscope.

(c) Intact adults

Adult *H. contortus* were placed in a screw-capped 10 mL specimen container covered in aluminium foil and immersed in methanol for two minutes. The methanol was removed with a pipette and vanillin-HCl solution added for ten minutes. At the end of this time, the worms were removed from the vial and either placed in a Petri dish in a small amount of vanillin-HCl solution for examination under a dissecting microscope or placed on a glass slide in a drop of vanillin-HCl for examination under a light microscope. Adult *T. colubriformis* were stained by the same technique but were more difficult to handle because of their small size, and had to be removed from the solutions with a pipette.

(d) Sectioned adults

It was not known whether the vanillin-HCl reagent would be able to penetrate the cuticle of an intact adult nematode to stain any tannins present in internal structures. For this reason, histological sections of adult nematodes were prepared according to the procedure described in Section 7.2.2 (e). The staining technique was identical to that described in Section 7.2.4.

7.2.6 Photography

Photographs were taken on an Olympus C5050Z digital camera (Olympus Optical Co., Ltd.) fitted with a microscope eyepiece attachment.

7.2.7 Statistical analysis

The number of slides staining positive for tannins by the vanillin-HCl method for each GI segment was analysed by Mann-Whitney nonparametric test (rank sum test) to detect differences due to diet or nematode species. The Mann-Whitney test tests the assumption that the median of two populations is the same

7.3 Results

7.3.1 Haematoxylin and eosin

The results obtained for ovine GI tissue segments from *Calliandra*-fed and lucerne-fed lambs are presented in this Section. The results obtained when H & E was used as a counterstain or comparison for other techniques are reported under the appropriate section for those techniques.

The procedures used to collect nematodes from the lambs at post-mortem resulted in extensive loss of the epithelial tissues for all gut segments except the rumen. This meant that many of the pathological changes expected in parasitised lambs, or lambs fed condensed tannins (for example, degree of villus atrophy, surface erosions) could not be assessed.

(a) Positive controls

Signs of autolysis were present in the positive control sections due to death of the tissue during incubation in the condensed tannin solution. Condensed tannins were present as a layer of golden-brown granular material either adherent to the mucosal and serosal surfaces, or free in the lumen. Sometimes accumulations of macrophages containing golden-brown material were associated with the tannin layer. These features were not observed in the negative controls.

(b) Rumen

There was mild infiltration of lymphocytes in some sections from uninfected lucerne-fed animals, but otherwise the sections appeared normal. The epithelium was intact in almost all the rumen samples. In animals on the *Calliandra* diet, condensed tannins were often present in plant material in the lumen, or as granules adherent to the epithelium. There was no evidence of condensed tannins present within the mucosa, although in occasional sections, granules were present at the cut edge of the

sub-mucosal connective tissue or on the surface of the serosa. No condensed tannins were seen in sections from animals on the lucerne diet.

(c) Abomasum

Very few sections had intact epithelium, so it was not possible to assess surface damage caused by either nematodes or condensed tannins.

Sections from uninfected lucerne-fed lambs were unremarkable, but in those from *Calliandra*-fed lambs there were occasional small focal infiltrates of lymphocytes in the lamina propria. Condensed tannins were present in the form of golden-brown granules in the lumen, and often formed a continuous layer across the surface of the tissue (Plate 7.1 A). There were numerous macrophages in the lamina propria containing golden-brown condensed tannins in sections from all but one lamb on the *Calliandra* diet, but not in sections from any lucerne-fed lambs (Plate 7.1 B). Macrophages were mainly present near the luminal surface but could also be found deep in the mucosa. In some instances, the macrophages had coalesced to form giant cells (Plate 7.2 A).

In *T. colubriformis*-infected lambs on either diet, sections appeared normal. In *H. contortus*-infected lambs, there were increased numbers of eosinophils, lymphocytes and plasma cells in the mucosa, although in most cases these were confined to basal areas of the lamina propria. In many sections the goblet cells were full of large, prominent secretory vacuoles.

(d) Duodenum

Few of the duodenal sections had areas of intact epithelium, but in those that did, the epithelium consisted of tall columnar cells. Virtually all duodenal sections regardless of worm species or diet (including the uninfected controls) were characterised by extensive diffuse infiltrates of eosinophils, plasma cells and lymphocytes, although the former two were more marked in the *T. colubriformis*-infected lambs. Degree of villus atrophy could not be assessed because of the disruption of the mucosal surface.

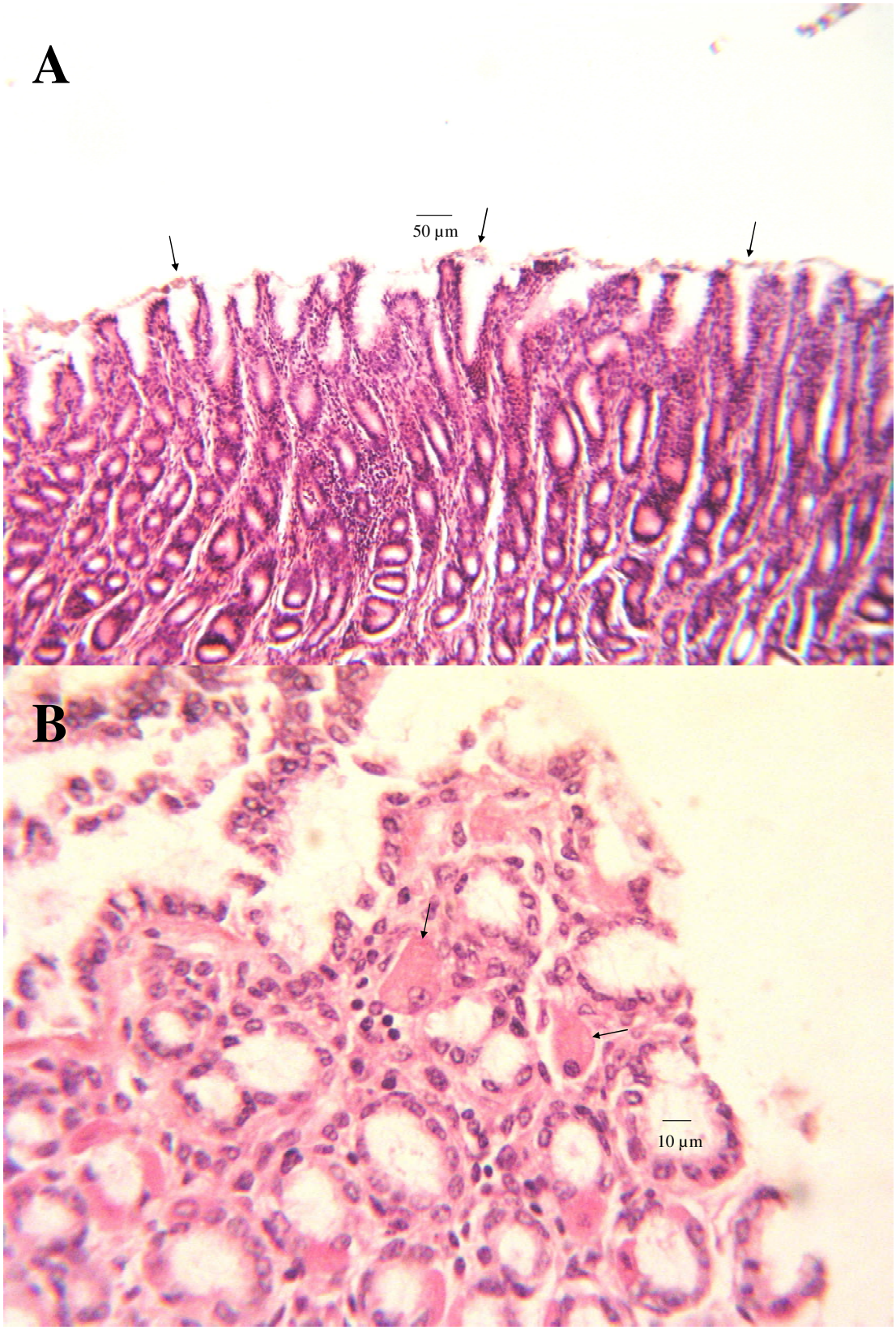


Plate 7.1 Abomasal sections from a *Calliandra*-fed lamb (H & E). **A.** Condensed tannins are present in a layer across the luminal surface. **B.** Macrophages containing condensed tannins in the lamina propria.

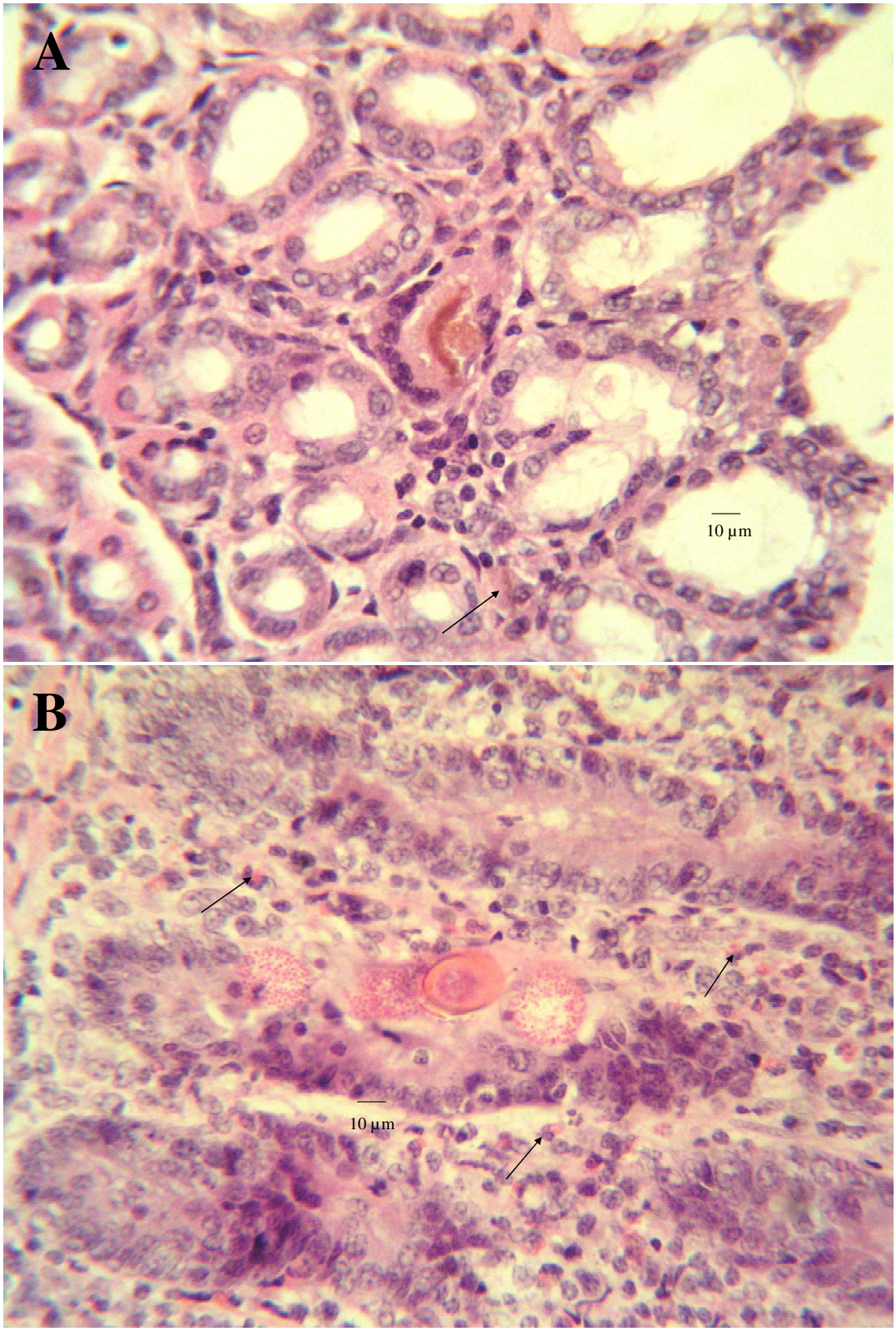


Plate 7.2 Gastrointestinal sections from *Calliandra*-fed lambs (H & E). **A.** Giant cell containing condensed tannins in the lamina propria of the abomasum. A smaller macrophage containing tannin is also present (arrow). **B.** Coccidial tissue-associated stages in the ileum. Eosinophils are abundant (arrows).

Macrophages containing condensed tannins were seen in all but two lambs on the *Calliandra* diet (one uninfected control and one infected with *H. contortus*). In general these were not as abundant as in the abomasal sections. Occasionally coccidial stages were present in the sections (Plate 7.2 B).

(e) Jejunum and Ileum

Jejunal and ileal sections were very similar to duodenal sections, but coccidial tissue-associated stages were abundant, and were often associated with destruction of the surrounding tissues. Mucosal depth appeared to be reduced in comparison with the duodenum, and the crypts were often straight and dilated. Diffuse cellular infiltrates composed mainly of lymphocytes and eosinophils were present in all animals, including the uninfected controls.

(f) Distribution of condensed tannins

The percentage of sections of each GI segment in which condensed tannins were identified is shown in Table 7.3. Worm species did not affect the number of sections in which condensed tannins were identified in any GI segment ($P > 0.100$).

Table 7.3 Percentage of H&E-stained GI sections from lambs fed either *Calliandra* or lucerne in which condensed tannins were identified. Results for lambs infected with *Trichostrongylus colubriformis* and *Haemonchus contortus* and uninfected controls have been pooled within diet. Values are means ± standard error.

Diet	Rumen	Abomasum	Duodenum	Jejunum	Ileum
Lucerne	0.0	0.0	0.0	0.0	0.0
Calliandra	43.8 ± 9.5	90.3 ± 6.0	71.9 ± 7.0	65.3 ± 8.2	76.7 ± 4.9
P	0.002	<0.001	<0.001	<0.001	<0.001

7.3.2 Ferric chloride

(a) Plant tissue

Unfixed sections of *Calliandra* leaf stained for 2 minutes with ferric chloride developed discrete circular areas of black stain within the epidermis. These areas

appeared to be vacuoles within the cells. Other parts of the sections appeared bright green and the cell walls were not easily distinguished. Fixation with methanol or FAA was unsatisfactory, but the formalin-fixed tissue stained well and the cellular definition was better than in the unfixed tissue. Neither toluidine blue nor Safranin O was successful as a counterstain to differentiate background structures.

(b) Ovine GI tissue

Formalin-fixed sections of lamb GI tissue stained with ferric chloride were difficult to interpret due to the presence of a large amount of black precipitate that formed during the staining process. Tannins were visible before staining on the positive control slide as a layer of pale golden brown material on both mucosal and serosal surfaces. After ferric chloride staining there was a small amount of greenish-black colouration in this area but much of the brown material remained unstained. The stained areas were not easily distinguishable after counter-staining with H & E. Ferric chloride thus was considered unsuitable for demonstrating condensed tannins in fixed ovine tissues.

7.3.3 Butanol-H_2SO_4

(a) Plant tissue

The results of attempts to demonstrate condensed tannins in plant tissue with butanol-H_2SO_4 were inconsistent. The first two sections examined appeared to have been either destroyed by the acid or cooked, so the stain was replaced with water and the process repeated. Because the tissue was still destroyed when the acid was replaced with water, fixed sections were used instead of fresh, to prevent cooking. Initial heating times less than 10 seconds (s) did not result in boiling. A heating time of 10 s, then 7 s, then 5 s gave the best results, but sometimes did not result in boiling and other times resulted in the tissue being cooked off the slide. Sections rehydrated through xylene and ethanol before staining gave similar results to those that had paraffin *in situ*, but the rehydrated sections tended to cook off the slide more readily.

Excessive amounts of butanol-HCl had a similar effect, and best results were obtained when just enough butanol-HCl was added to cover the tissue.

Butanol-H_2SO_4 resulted in bright red or maroon staining of vacuoles in the epidermal cells of leaflets of *Calliandra* and *Desmanthus*, but not *Sesbania* or *L. collinsii*. Significant staining also occurred in the petiole of *Desmanthus*. All other parts of the sections remained colourless and tissue architecture was difficult to identify, particularly in sections of *Sesbania* and *Leucaena*. The butanol H_2SO_4 technique was thus shown to be useful for demonstrating the presence of condensed tannins in plant tissue, but a counterstain to show background tissues was needed.

(b) Ovine GI tissue

Although still identifiable, the tissue architecture of the positive control samples was poor, presumably because the tissue died during incubation. In the unstained positive control sections, tannins were visible as a thin pale brown layer on the mucosal and serosal surfaces. The butanol-H_2SO_4 reagent did not stain background tissues, but some pale red or red-brown staining occurred in the positive control slides. The duodenal controls stained all over, although staining was most intense in areas along the serosal surface. The ileal controls stained only in small areas of the mucosal and serosal surfaces.

In tissues from *Calliandra*-fed lambs, a small amount of light-brown staining was present, mainly on the mucosal surface, but occasionally on the serosa. No red colour was visible. Equivalent sections from lucerne-fed lambs exhibited no staining.

Counterstaining of sections treated with butanol-H_2SO_4 was unsuccessful due to poor uptake of the stains by the acid-treated tissues. The problem of tissue sections cooking off the slides during heating in the microwave was exacerbated when the technique was used on ovine tissues, so that it was usually necessary to stain at least five sections to obtain a reasonable one. Due to these technical difficulties and the poor staining observed in GI sections from *Calliandra*-fed lambs, the butanol-H_2SO_4 technique was not considered suitable for use with mammalian tissue.

7.3.4 Vanillin-HCl

(a) Fixed plant tissues

As expected, FAA-fixed *Sesbania* did not stain with the vanillin reagent, whereas there was extensive red staining of *Desmanthus* tissues. The red material appeared to be contained in vacuoles within cells in the epidermis of leaflets, at the base of the petiole, and in parts of the stem, especially the epidermis. The background tissue did not stain.

Toluidine blue and H & E were unsuccessful as counterstains, due to poor uptake of the dye. Tannins turned turquoise with toluidine blue and maroon or red-gold with H & E.

(b) Fixed ovine GI tissue

In unstained ovine GI positive control sections, a pale golden-brown layer of tannins was visible mainly on the serosal surface. Sometimes a layer of tannins was present on the mucosal surface, but was not as prominent. The tannin layer developed a patchy, pale red-brown colour when exposed to the vanillin reagent, but this was not as obvious as in the plant sections. The negative control samples did not stain with the vanillin reagent.

The attempt to find a counterstain for vanillin-HCl was unsuccessful. In ovine tissue, as in plant tissue, dye uptake was poor after vanillin-HCl. Toluidine blue, methyl green and methylene blue stained condensed tannins turquoise or pale green and H & E stained them maroon or red-gold. In each case, there was insufficient contrast between the tannin layer and the background tissues to identify the tannins with certainty. Thus, despite reasonable staining of fixed plant tissues with vanillin-HCl, unsatisfactory results were obtained with the fixed ovine GI tissues.

(c) Fresh plant tissue

Fresh *Albizia* and *Calliandra* sections appeared uniformly black or green before staining. *Calliandra* sections turned red as soon as the vanillin-HCl solution was added, whereas *Albizia* sections did not change colour. After the 10-minute staining period, *Calliandra* was still grossly red, but the colour disappeared when the sections were rinsed in water. *Calliandra* sections examined without rinsing retained a bright red colour, which was particularly apparent along the margins of the leaflets, in the epithelial cells. Rinsing in methanol caused some colour loss, resulting in a dull red hue. *Albizia* sections appeared uniformly yellow after staining, regardless of the rinse solution.

When fresh and formalin-fixed sections of *Calliandra* were compared, fresh sections stained bright red, whereas formalin-fixed sections stained brown, with occasional pale red areas along the margins of the leaflets. Formalin therefore appeared to interfere with the vanillin-HCl staining process.

(d) Fresh ovine GI tissue

Unfixed ovine GI tissue tended to wash off the slides during staining and had poor tissue architecture. Some form of fixation was therefore required prior to vanillin-HCl staining. Fixation in absolute methanol for two minutes before vanillin treatment proved to be the most successful fixation method. The use of silanised slides also improved the quality of stained sections.

Staining of fixed sections with vanillin-HCl alone, resulted in the formation of distinctive red spots in the mucosa of *Calliandra*-fed lambs, but not lucerne-fed lambs. There was no background staining in any of the sections. All solutions used for rinsing sections washed off the red colour, as did counterstains and fixatives applied after the vanillin treatment. When vanillin-HCl was applied after the counterstain, red spots appeared but the background stain was removed.

Due to the difficulties that arose in finding a successful counterstain for vanillin-HCl, a different approach was required. The methanol-fixed sections were treated with vanillin-HCl alone and then examined under a microscope with the diaphragm partly closed to increase contrast. Although it was not possible to see the tissue architecture clearly with this technique, the presence of tannins could be specifically confirmed and the general location of the tannins could be identified (e.g. in the lamina propria, sub-mucosa, muscle, serosa). This technique was therefore adopted to demonstrate the presence of condensed tannins in experimental tissues.

7.3.5 Demonstration of condensed tannins in experimental tissues

(a) Ovine GI tissues

The technique described in Section 7.3.4 (d) was used to determine the presence of condensed tannins in GI tissues from lambs in Period 1 of the experiment described in Chapter 4. The results are presented in Table 7.4.

No slides stained positive for condensed tannins for any of the lucerne-fed lambs. All *Calliandra*-fed lambs had one or more positive slides for every GI segment, except for one lamb infected with *H. contortus* that had no positives in the jejunum, and one lamb infected with *T. colubriformis* that had no positives in the rumen. Tannins were present only on the surface of rumen sections (see Plate 7.3 A) mainly in plant material adherent to the surface, but for all other sections of the GI tract red staining was present within the mucosa. Condensed tannins were sometimes present as a layer of granular material adherent to the surface of the mucosa. This was particularly common in abomasal sections (Plate 7.3 B). Occasionally tannins were also present adhering to the serosal surfaces. Nematode species did not affect the percentage of sections staining positive for tannins in any GI segment ($P > 0.100$).

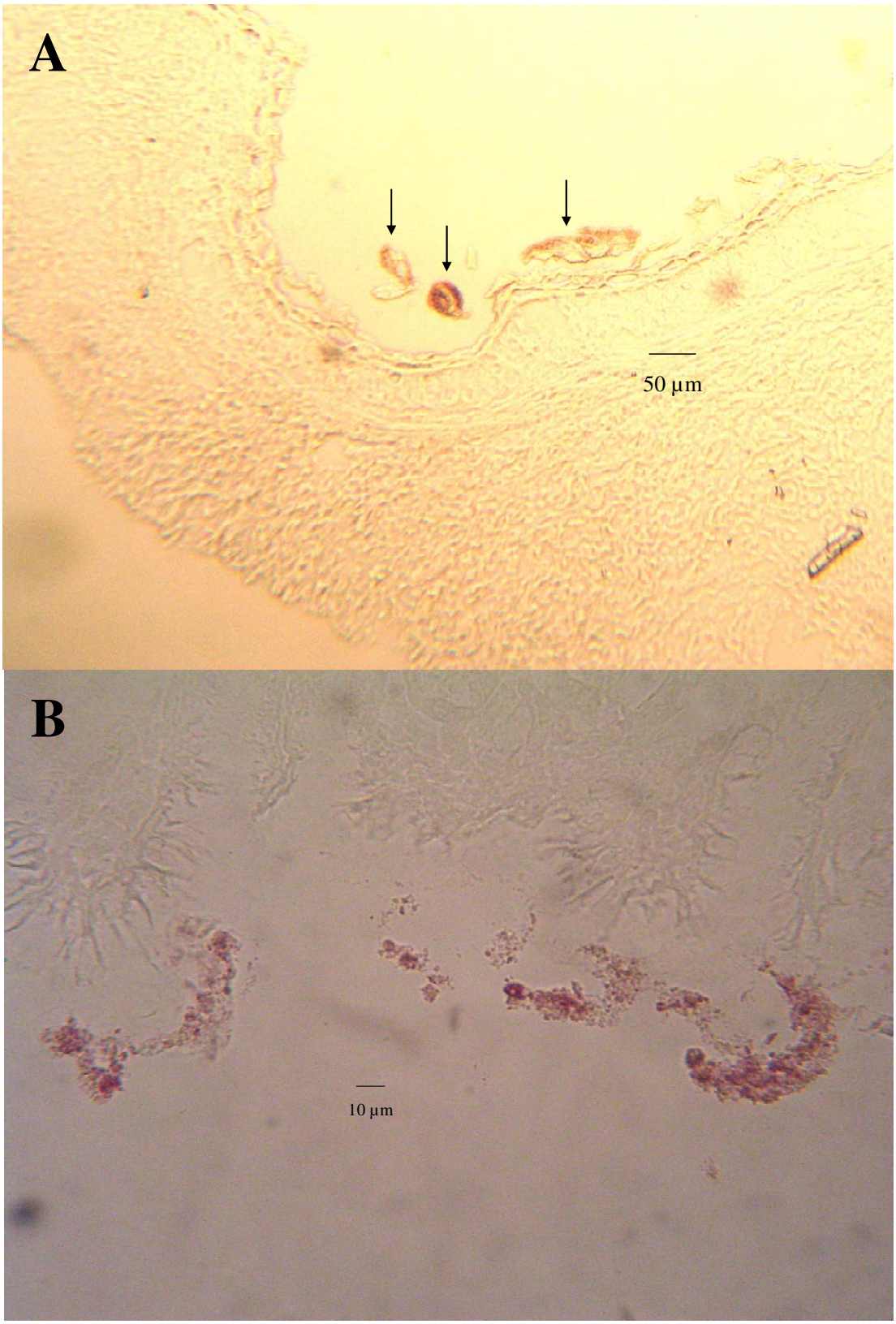


Plate 7.3 Gastrointestinal sections from *Calliandra*-fed lambs (Vanillin-HCl). **A.** Red-staining digesta in the rumen. **B.** Condensed tannins on the luminal surface of the abomasum.

Table 7.4 Percentage of vanillin-HCl-treated GI sections from lambs fed either *Calliandra* or lucerne in which condensed tannins were identified. Results for *Trichostrongylus colubriformis* and *Haemonchus contortus* infected lambs have been pooled within diet. Values are means ± standard error.

Diet	Rumen	Abomasum	Duodenum	Jejunum	Ileum
Lucerne	0	0	0	0	0
Calliandra	78 ± 9.7	95 ± 2.4	53 ± 5.5	30 ± 3.9	55 ± 7.2
P	0.001	<0.001	<0.001	0.005	<0.001

(b) GI nematodes

No *H. contortus* or *T. colubriformis* eggs or larvae from lucerne-fed or *Calliandra*-fed lambs exhibited any positive staining when treated with vanillin-HCl.

Intact adult worms obtained from lucerne-fed lambs did not stain with vanillin-HCl either, but adults from *Calliandra*-fed lambs had varying degrees of external bright red staining. Of the 11 male and 13 female *T. colubriformis* examined, five of the males had red material on the bursa, one female had red staining around the vulval flap, and one female had a red area on the outside of the cuticle. However, despite the fact that the worms were rinsed in saline, there was intense-staining digesta present in the preparations microscopically, and much of the red staining seen on the worms could have been due to adherent digesta. Eighteen of the 20 male *H. contortus* examined had red material caught on the bursa (Plate 7.4 A), and all except three of the 20 females examined had red staining around the vulval flap (Plate 7.4 B). One female also had parallel lines of red staining apparently following the contours of surface striae on the cuticle (Plate 7.5A). None of the intact worms examined appeared to have any red staining internally, except for two female *H. contortus* that had very faint red colouration down the edges of the oesophagus.

One female *T. colubriformis* from a *Calliandra*-fed lamb had three eggs *in utero* containing almost fully developed larvae (Plate 7.5B). Two of the eggs were in the ovijector (Plate 7.6 A), while the third was just anterior to the ovijector, interspersed with the unembryonated eggs usually seen in gravid females (Plate 7.6 B). There was no staining associated with any of these eggs.

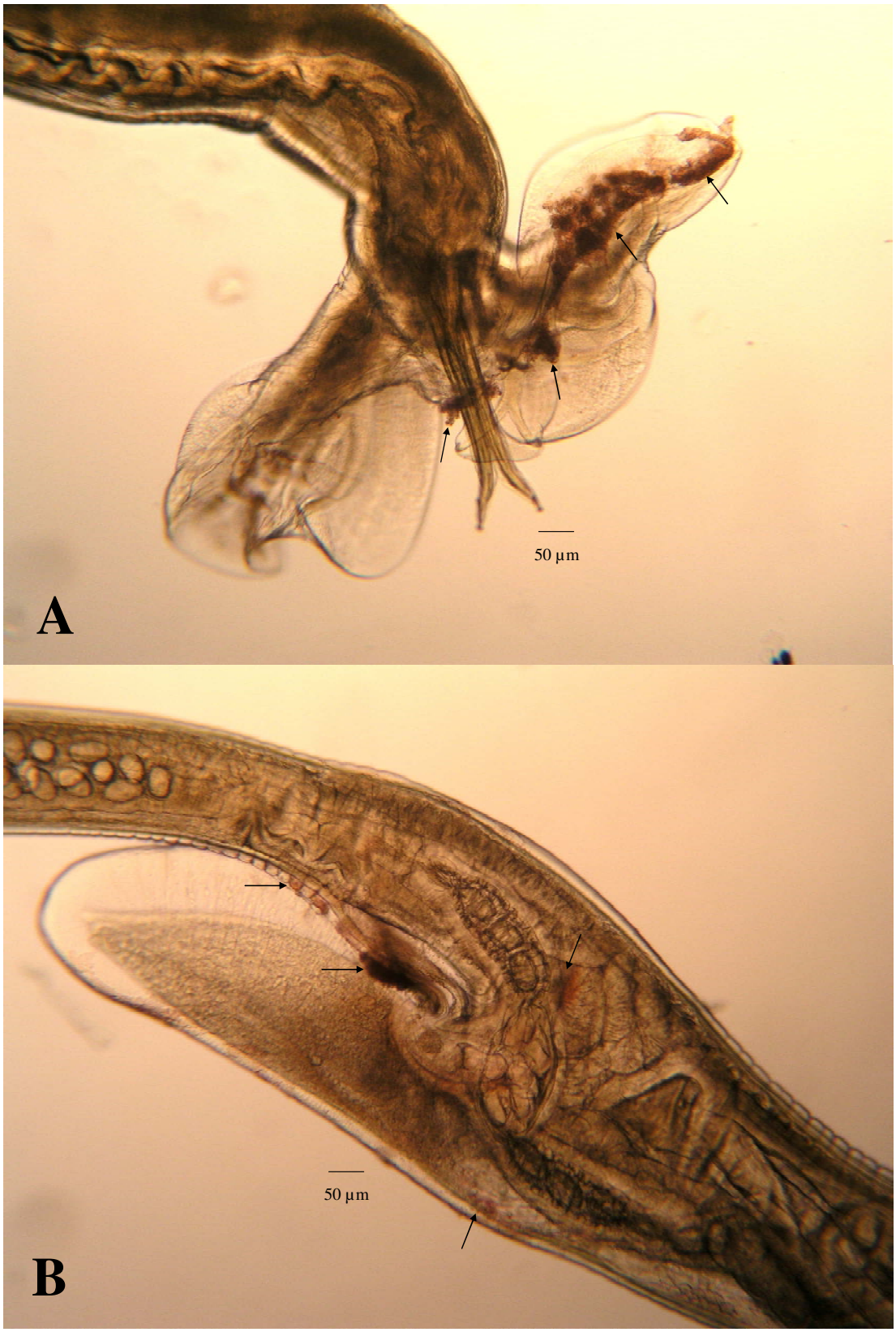


Plate 7.4 Intact *H. contortus* stained with vanillin-HCl. **A.** Bursa of male worm showing adherent red-staining digesta. **B.** Vulval flap of female showing red staining of the cuticle.

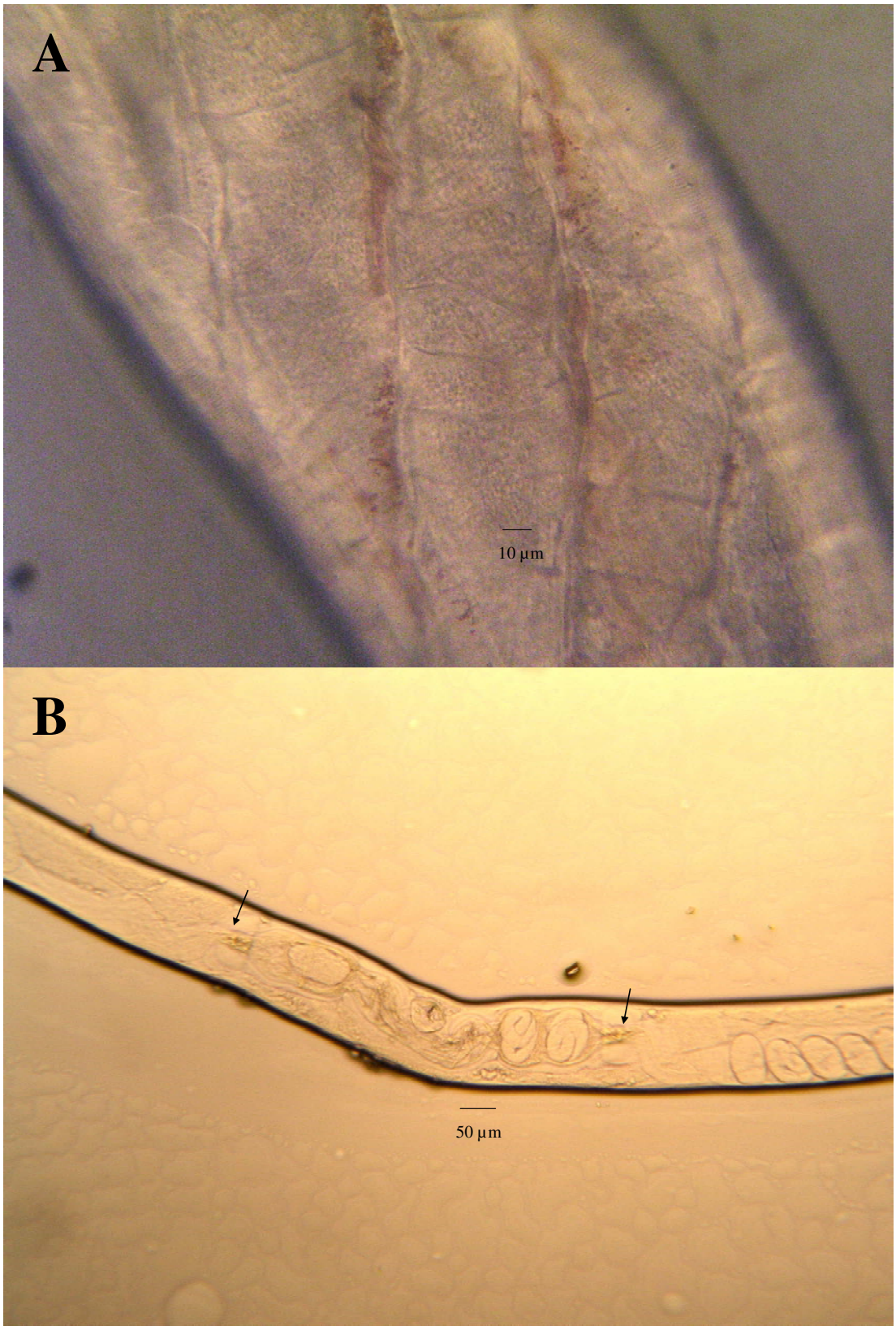


Plate 7.5 Intact adult worms stained with vanillin-HCl. **A.** Cuticle of female *H. contortus* showing red staining following the striae of the cuticle. **B.** Ovijector of Female *Trichostrongylus colubriformis* containing larvated eggs. Arrows define the ends of the ovijector.

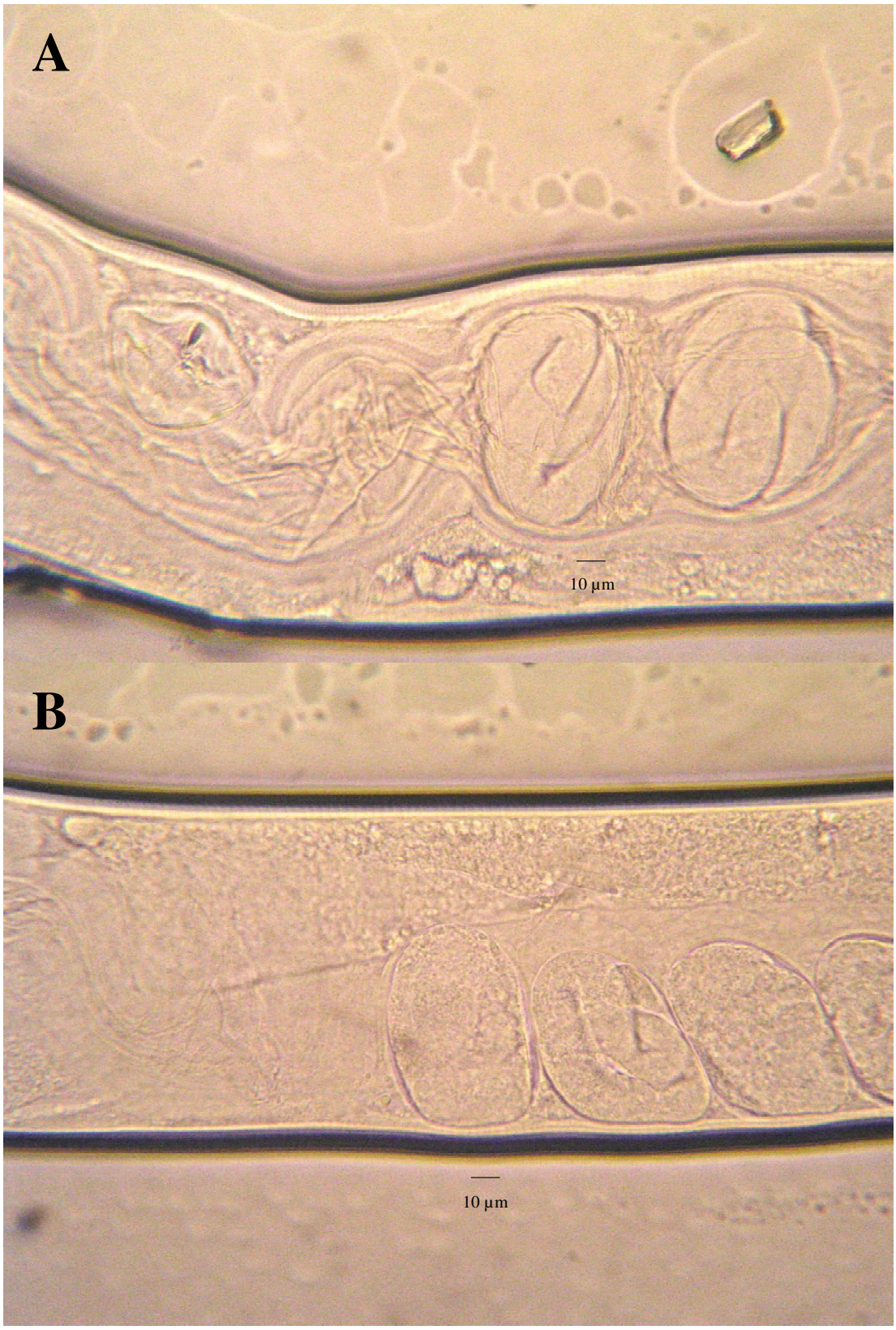


Plate 7.6 Larvated eggs in the uterus of a female *T.colubriformis* from a *Calliandra*-fed lamb (Vanillin-HCl). **A.** Eggs in the ovijector. **B.** Eggs in the uterus just anterior to the ovijector.

Adult nematodes sectioned in OCT had to be examined within 10 minutes of staining, since crystals of OCT medium began to form on the slides after this time, obscuring the sections. The nematodes were not killed before being placed in the OCT medium, as it was possible that substances used to kill them would interfere with the vanillin reaction. Most of the sections were therefore obliquely cut, due to movement of the worms after they were placed in the moulds. In most cases, better sections were obtained for *H. contortus* than for *T. colubriformis*, since the worms were larger and easier to handle.

No red staining was seen in any of the sections from lucerne-fed lambs. In contrast, bright red staining digesta was always present in the sections from *Calliandra*-fed lambs (Plate 7.7 A). Red staining was often present on the cuticle of the nematodes in these sections, but generally appeared to be due to adherent digesta rather than bound tannins (Plate 7.7 B).

In male nematodes, there was no staining of the bursa, spicules or reproductive tract. In female worms there was generally no internal staining, although in female *H. contortus* there was usually red staining digesta trapped between the body wall and the vulval flap, down to the opening of the vulva (Plate 7.4 B). In one cross-section from a female *H. contortus*, a single egg in the uterus stained positive for tannins, but only in a small area (Plate 7.9 A). In another female, there was prominent red staining of the cells lining a muscular organ in the region of the vulva (Plate 7.8 A & B). However, it was difficult to identify the organ involved, and although it appeared to have been the intestine, it may have been the reproductive tract. In a number of worms, a similar staining pattern was seen in the cells lining the pharynx, particularly in the region of the junction of the pharynx and intestine (Plate 7.9 B; Plate 7.10 A & B).

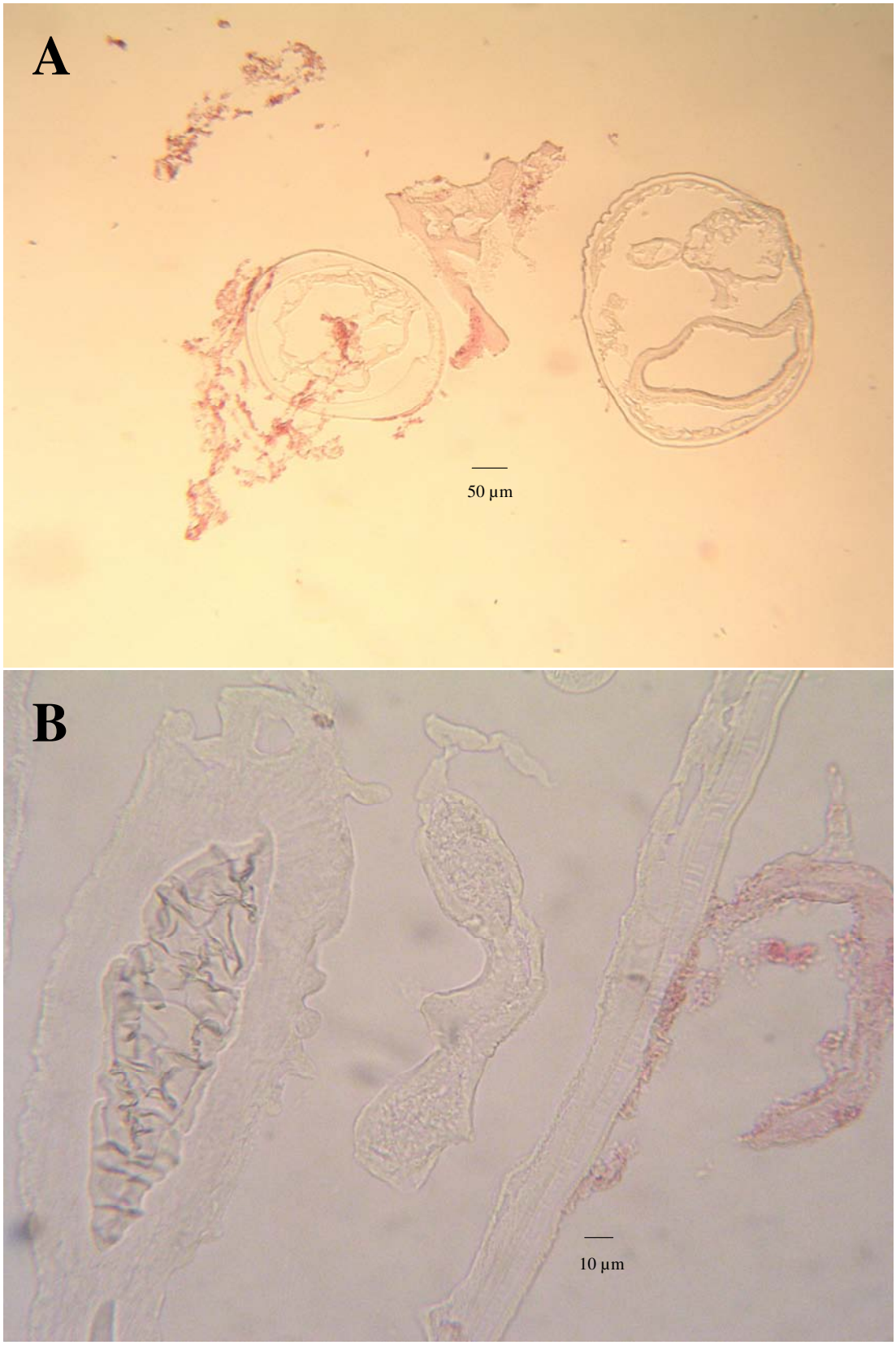


Plate 7.7 Condensed tannins in OCT sections of adult *Haemonchus contortus* (Vanillin-HCl). **A.** Red-stained digesta overlying a transverse section through a worm. **B.** Condensed tannins (possibly in digesta) adhering to the cuticle of a worm.

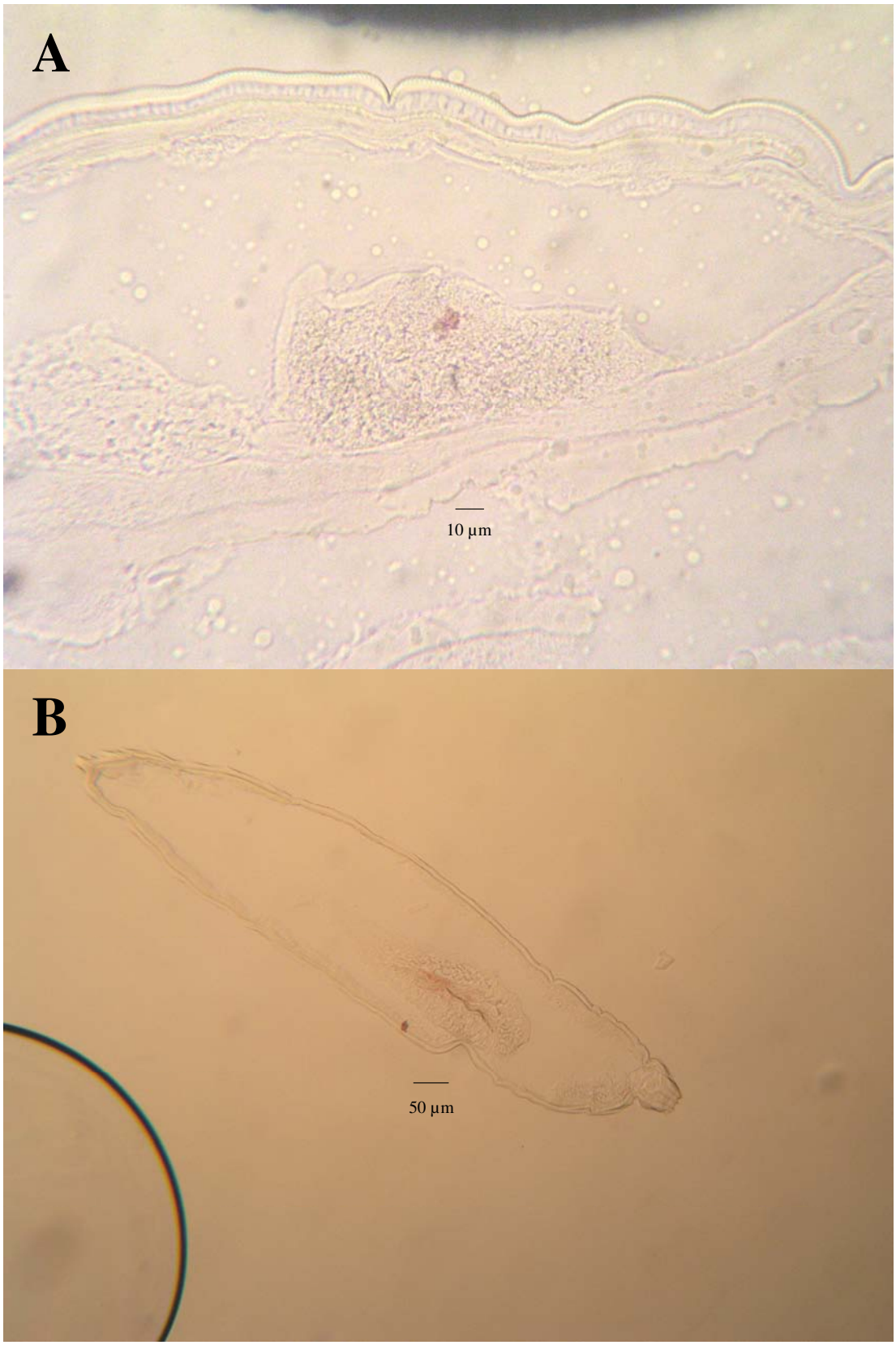


Plate 7.8 Condensed tannins in OCT sections of adult *H. contortus* (Vanillin-HCl). **A.** In the centre of an egg in the uterus of a female worm. **B.** In the cells lining the oesophagus.

Plate 7.9 Condensed tannins in an OCT section from an adult *Haemonchus contortus* (Vanillin-HCl). **A.** Tannins in cells lining a hollow organ (probably the microvilli of the gut) at the level of the vulval flap. **B.** Higher power view of the same section.

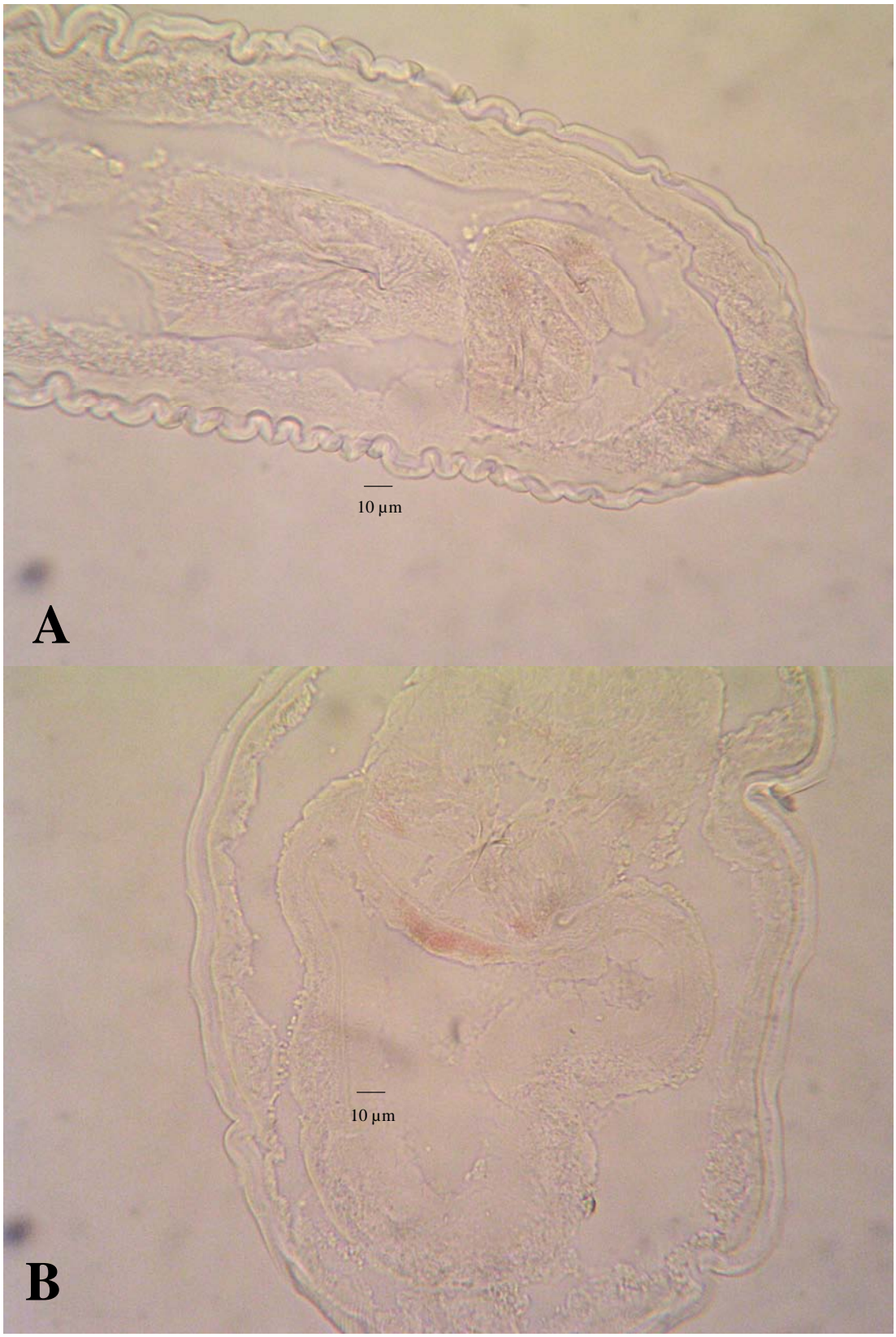


Plate 7.10 Condensed tannins in OCT sections from adult *Haemonchus contortus* (Vanillin-HCl). **A.** Condensed tannins in cells lining the oesophagus, longitudinal section. **B.** Condensed tannins in cells lining the oesophagus, oblique section.

7.4 Discussion

7.4.1 Positive controls

Histological staining techniques designed to demonstrate specific chemical compounds require positive control sections to enable differentiation of negative results that are due to a lack of the compound of interest from negative results that are due to failure of the technique. One of the main difficulties encountered in developing a technique for demonstrating condensed tannins in mammalian and nematode tissues was the lack of appropriate positive controls, since condensed tannins are not a natural part of animal tissues. Although plant tissues with known tannin content were used to develop the staining techniques, the cellular distribution of condensed tannins in the plants was not accurately known, and was unlikely to be similar in animal tissues. Thus a mammalian positive control tissue was required.

During the preparation of ovine positive control sections, condensed tannins showed a predilection for the serosal surface of the gut, a surface that is not normally exposed to dietary condensed tannins. This is not surprising, since condensed tannins bind particularly well to proline-rich proteins (Hagerman & Butler, 1981), such as collagen, which is found in abundance in the serosa. However, little was found on the mucosal side of the sections. A further problem was that the tissues did not remain alive for the entire incubation period, even when it was reduced to six hours. Improvements in the gas perfusion technique would probably remedy this problem. There were two problems associated with the early death of the tissue: poor tissue preservation with subsequent poor uptake of H & E in the prepared sections, and more importantly, failure of the tissue to respond to condensed tannins in the normal manner. All the condensed tannins were present on the surface of the sections, with only a few small clusters of macrophages present in the lumen near clumps of tannins. In contrast, sections from *Calliandra*-fed lambs clearly showed condensed tannins in macrophages and giant cells throughout the GI tract. Better sections may have been obtained if the viability of the tissues could have been sustained for a longer period.

Despite these difficulties, the incubated sections made serviceable positive controls, and were at least as useful as plant controls. The ovine GI controls were fixed in formalin because they were prepared for use with the butanol H_2SO_4 technique, which is too harsh to use with fresh tissues. Unfortunately, there was no appropriate tissue available later to make new controls by the OCT technique for vanillin staining. The only controls available for use during vanillin staining were therefore plant sections. The difficulty of finding adequate controls was compounded further when staining nematode adults, eggs and larvae, and no attempt was made to prepare spiked samples of nematode tissues. These problems need to be addressed if any extensive work in this area is to be carried out.

When used on the experimental ovine GI sections, the vanillin technique gave positive results for all of the *Calliandra*-fed lambs and negative results for all of the lucerne-fed lambs. These results were corroborated by the plain H & E sections and were in accordance with the plant controls, so it would seem that the vanillin-HCl technique was suitable for detecting the presence of condensed tannins in frozen tissue sections.

7.4.2 Haematoxylin and Eosin

(a) General histology

For the most part, the histological changes seen in the GI tracts of parasitised lambs (as far as they could be assessed) concurred with the findings of others (Barker, 1975a; Al-Zubaidy *et al.*, 1987). The only major difference observed between the treatment groups was the presence or absence of condensed tannins in the sections examined. This is discussed in Section 7.4.2 (b).

No significant changes were noted in the rumen of any parasitised lambs. In those infected with *T. colubriformis*, abomasal sections appeared normal, consistent with the absence of worms in these areas. The inflammatory infiltrates in the abomasal mucosa of lambs infected with *H. contortus* reflected the host immune response to the presence of the worms. The lambs were killed at Day 36 post-infection (see

Section 4.2.6), which probably explains the localisation of the cellular infiltrates to the basal areas of the lamina propria. Hunter & Mackenzie (1982) found that infiltrates of eosinophils and mononuclear cells reached a peak at Day 18 post-infection in young lambs infected with *H. contortus*, but by Day 35 cellular infiltrates had become focal in the deep lamina propria and haemorrhage had largely disappeared. The low magnitude of the inflammatory response was probably due to the age of the lambs at the time of infection (eight weeks). Salman and Duncan (1984) found that 21 days after primary infection with *H. contortus* and 6 days after secondary infection, there was no increase in mast cell numbers and only slight increases in eosinophils and plasma cells in the abomasal lamina propria of lambs infected at 6-8 weeks of age. In contrast, adult ewes had marked increases in all three cell types.

Trichostrongylus colubriformis has a predilection for the first three metres of the small intestine, and over 90 % of the adult population is usually found in the first six metres (Barker, 1974; 1975a). Typically, infection with *T. colubriformis* is associated with proliferation of lymphocytes, plasma cells and eosinophils in the small intestine, particularly in the duodenum, but increased numbers of mast cells and macrophages do not tend to occur (Barker, 1975a; c). In the current experiment, cellular infiltrates in the duodenum were more marked in the *T. colubriformis*-infected lambs, but distally, lesions were observed throughout the small intestine in all animals regardless of the infective worm species. This may have been related to the presence of coccidial tissue-associated stages. Where present, the epithelium consisted of tall columnar cells, rather than the shorter, more cuboidal cells that are usually a feature of heavy *T. colubriformis* infections, but this was not surprising, considering the infective dose of 10,000 larvae was reasonably low. Adult nematodes, which can often be found in superficial tunnels in the mucosal surface (Barker, 1973; Coop & Angus, 1975), were not observed.

One of the prominent features of *T. colubriformis* infection is usually villus atrophy, with a compensatory hyperplasia of the intestinal crypts (Barker, 1975a; Coop & Angus, 1975). These changes tend to be seen only at the site of the infection, and do not affect distal regions of the small intestine (Barker, 1975b; c), although Hoste and Mallet (1990) and Roy *et al*. (1996) reported compensatory mucosal hyperplasia

distal to the site of infection in rabbits and sheep with low burdens of *T. colubriformis*. Villus atrophy tends to occur with population densities greater than 4000 worms/m (Barker, 1975c). In the current experiment, villus atrophy was difficult to assess, but would be unlikely because the worm density would have been much lower than 4000 worms/m. The crypts were normal in appearance in the duodenum, but became broader and less convoluted in the jejunum and ileum, regardless of the nematode species present. A marked feature of the jejunal and ileal samples was the presence of coccidial stages. It seems likely that the coccidial lesions and associated immune response in the distal small intestine were sufficient to obscure the histological changes (or lack of changes) due to the nematodes. Dilation of the crypts occurs in sheep fed quebracho tannins (Hervás *et al.*, 2003), but in the current study this change occurred in both *Calliandra*-fed and lucerne-fed animals.

(b) Condensed tannins

The visibility of the condensed tannins was very variable in H & E sections. Sometimes they were distinctive as obvious large, golden-brown accumulations and sometimes they were a subdued maroon colour, which was difficult to distinguish from normal eosinophilic staining. In some of the rumen sections, condensed tannins were visible in the connective tissue at the edge of the section, or on the serosal surface, but this was probably due to the transfer of tannins from the lumen to these areas during sectioning. In abomasal sections in which the epithelium was missing, tannins were often present as a complete layer along the luminal surface of the lamina propria (Plate 7.1 A). This did not occur in sections with intact epithelium, as might be expected if the tannins were binding to digestive enzymes in the brush border. Macrophages containing phagocytosed golden-brown material were present in large numbers in both the lumen and the lamina propria, but only in lambs fed the *Calliandra* diet (Plate 7.1 B).

In a previous study, administration of quebracho to sheep resulted in severe mucosal ulceration in the abomasum (Hervás *et al*., 2003) and similar findings were noted by Dawson *et al*. (1999). In the latter study, six sheep were killed in pairs after two, five

or seven weeks on a diet containing 50 g/kg DM quebracho tannins. Two of the animals (which of the animals these were, was not stated) showed mucosal ulceration in the jejunum and ileum, and all six had increased numbers of macrophages in the same areas. Only two animals showed any changes in the duodenum, and rumen and abomasal tissues were not examined. These authors felt that such observations represented an early response to dietary tannins, as the pathology was less pronounced in the animals slaughtered at seven weeks. This is in contrast to the present study, in which large numbers of macrophages and giant cells were found in all sixteen lambs slaughtered after approximately eight weeks on the *Calliandra* diet. Condensed tannins were also far more evident in the abomasum, and to a lesser extent, the duodenum, than in the distal small intestine. The greater extent of the pathological changes in the current study probably reflects higher condensed tannin intakes.

7.4.3 Ferric chloride

Tannins (especially tannic acid) have long been known to complex with iron to produce a blue-black colour, and tannic acid has been used histologically as a mordant dye in conjunction with ferric ions to stain complex carbohydrates in animal tissues (Chaplin, 1985). The reaction is not specific for condensed tannins, but will include hydrolysable tannins and monomeric flavanols (the precursors of condensed tannins), hence its use as a quantitative test for total phenolics (Hagerman & Butler, 1978). It was unlikely that hydrolysable tannins and flavanols would be present in the GI tract of lambs fed *Calliandra* or lucerne in sufficient quantities to give positive reactions with ferric chloride, but the possibility was considered one of the drawbacks of this technique. On the other hand, some phenolics will not react with ferric chloride (Reeve, 1951).

The attractive feature of ferric chloride staining was that it could be easily performed on large batches of formalin-fixed tissues, in conjunction with the processing of H & E sections for routine histology. However, it was found to be rather unreliable, as some sections barely stained, and others were difficult to interpret because there was

so much black precipitate present. This unreliability combined with the non-specificity of the technique dictated the need to find a more dependable technique.

7.4.4 Butanol-H_2SO_4

The butanol H_2SO_4 reagent was investigated as a possible stain for mammalian tissues because it reacts specifically with condensed tannins. The different absorbances of the anthocyanidin end-products can cause problems with the quantitative use of this assay (Makkar, 2003), but this was unlikely to matter for qualitative work.

Facilities were not available for glycol methacrylate embedding as performed by Gutmann (1993), so FAA or formalin-fixed sections were embedded in paraffin instead. Because colour yield with acidified butanol is decreased by water (Makkar, 2003), staining was compared in sections that had been taken to water to remove paraffin and in dehydrated sections with the paraffin *in situ*. Little difference was observed in the intensity of staining, but the quality of the dehydrated sections was better. The maintenance of adequate temperatures and short staining times were the main difficulties when treating animal tissues with butanol H_2SO_4. Although the method successfully identified condensed tannins in the control tissues, sections from *Calliandra*-fed lambs only stained brown and not red. It was difficult to obtain intact sections due to the destructiveness of the hot acid, and repeatability was a problem. Two batches of slides with the same amount of acid added, and heated by the same regime, resulted in sections of very different quality, with the difference between underheating and overheating often less than one second. Similar difficulties pertained to the staining of plant sections, but these were more robust and survived the process much better, presumably due to the presence of cell walls.

7.4.5 Vanillin-HCl

Vanillin-HCl is not as specific as the butanol reagent, as it will react with monomeric flavanols, anthocyanidins and dihydrochalcones in addition to condensed tannins (Sarkar & Howarth, 1976). Monomeric flavans, in particular, result in intense colour

formation, and some of the less common condensed tannins, with a high deoxyflavanol content, do not react with vanillin (Makkar, 2003). The composition of the phenolic compounds in *Calliandra* is unknown, so it is difficult to say whether non-tannin compounds are likely to interfere in the use of vanillin-HCl in animal tissues exposed to *Calliandra*. In one study, phenolic monomers comprised between 0.3 % and 2.6 % of the total phenolics in different *Calliandra* accessions, while the proanthocyanidin fraction ranged from 56.0 % to 68.7 % (Rakhmani *et al*., 2005). It seems likely that positive reactions with vanillin would be mainly due to the proanthocyanidin fraction.

The chromophore produced in the vanillin reaction has a maximum absorbance at 500 nm, but peak absorbance is reduced by exposure to light (Broadhurst & Jones, 1978). The optimum temperature range for the reaction was 15 - 25 °C. Variations on the composition of the vanillin reagent used in quantitative assays have been reported (Swain & Hillis, 1959; Burns, 1971; Broadhurst & Jones, 1978). For use qualitatively as described in the current experiment, the concentration of vanillin in solution did not appear to be critical. A 2.7 % solution of vanillin was convenient, but the solution was equally effective when made up at about one eighth strength.

Swain and Hillis (1959) recommended that the absorbance of solutions be measured as soon as possible in condensed tannin assays, because of fading, and suggested the use of sulphuric instead of hydrochloric acid to improve colour retention. However, when staining OCT-embedded frozen sections, fading was not the limiting factor. Colour development increased with time, up to about 10 minutes after staining, but after 10 minutes crystals of OCT began to form on the slides, obliterating the sections.

A successful background stain for *in situ* vanillin staining was not identified. The acid in the solution prevented uptake of dyes by the treated tissue, regardless of whether the dye was applied before or after the vanillin [Section 7.3.4 (b)]. The technique, while useful for confirming the presence of flavanols in animal tissues, should therefore be used in conjunction with H & E staining to determine the precise cellular location of compounds in the tissues. Acetone and water are both incompatible with the vanillin reaction (Makkar, 2003), hence the need to examine

the slides without rinsing [Section 7.3.4 (d)]. Methanol washed out most of the colour; Swain and Hillis (1959) also reported colour alterations with excess methanol in vanillin-H_2SO_4 solutions. Consequently, the sections were examined with the acidic vanillin reagent still on the slide, and great care had to be taken in wiping off the excess reagent around the cover slip to avoid corrosion of the microscope.

7.4.6 Condensed tannins in GI tissue

Treatment of GI tissue sections with vanillin-HCl confirmed the presence of condensed tannins in all segments of the GI tract as seen in the H & E sections. Condensed tannins were present only in the lumen in rumen sections, but were seen in the lamina propria in all the other GI segments, particularly the abomasum. In some abomasal sections where the epithelium was missing, condensed tannins were present as a continuous layer along the luminal surface, possibly entrapped in mucus. These findings were also in agreement with the observations made on the H & E sections. In the small intestine, tannins were identified in the H & E sections with a greater frequency than in the vanillin-HCl sections (compare Tables 7.4 and 7.9). The development of a readily visible red colour with vanillin-HCl may require a certain minimum concentration of condensed tannins in the tissue, in which case giant cells would probably be distinguished more easily than individual cells. The larger red patches seen in vanillin-HCl sections and the presence of areas with very faint staining would tend to confirm this. In the rumen sections, tannins were identified more often in the vanillin-HCl sections, probably because the bright red staining caused it to stand out more from other debris in the lumen than in the H & E sections. With the exception of the rumen sections, the overall trends were similar in the H & E and vanillin-HCl sections, with intracellular condensed tannins most abundant in the abomasum, declining in the proximal small intestine, and rising again in the ileum. This is the same as the trend observed for concentration of condensed tannins in digesta (Section 4.3.5).

7.4.7 Condensed tannins in nematodes

There was no convincing evidence that condensed tannins bind to the cuticle of GI nematodes. Despite the fact that the worms were rinsed in saline, there was a lot of red-stained digesta present in the preparations microscopically, and some of the red staining seen on the worms was clearly due to adherent digesta containing tannins (see Plate 7.4 A). Positive staining was seen in depressions in the surface of the cuticle and was particularly common around the bursa and vulval flap, possibly because digesta was more prone to catch on these protuberances. This is consistent with the lack of staining of intact eggs and larvae from *Calliandra*-fed lambs, which do not have large surface projections. Having said this however, the nematode cuticle possesses surface-associated proteins (Fetterer & Rhoads, 1993) that would possibly be able to bind to condensed tannins, and may have been responsible for the red staining. Some surface proteins can be removed with detergents without damage to the nematode, and are shed into the environment on a regular basis, so it is unlikely that tannins binding to these proteins would have much effect on the worm. Cuticular proteins vary at different stages of the nematode lifecycle (Cox *et al*., 1989; Fetterer, 1989; Fetterer & Rhoads, 1993) which could explain the absence of staining in larvae. The L_3 sheath, which is derived from the second moult cuticle, may not possess surface proteins compatible with tannin-binding.

Eggs and larvae might also fail to stain if condensed tannins normally interact, not with eggshells or larval cuticles, but with enzymes involved in hatching and exsheathment (see Sections 2.2.1 and 2.2.6) thus preventing development without actually binding to eggs or larvae. However, it is also possible that the vanillin staining method used in the current experiment may be unsuitable for use with eggs and larvae. These specimens were suspended in tap water prior to staining, which might have interfered with colour development. Without similar structures of known tannin content to use as positive controls, it is not possible to determine whether these structures failed to stain due to lack of bound tannins or due to failure of the staining technique.

Internal staining of the pharynx was seen in several adult *H. contortus* (Plate 7.10) but not in *T. colubriformis*, but this may have been due more to the difficulty of obtaining reasonable sections of the latter than to actual absence of condensed tannins. Staining of the oesophagus suggests that the worms ingest condensed tannins. Butter *et al*. (2001) observed condensed tannins grossly in the gut lumen of *N. brasiliensis* incubated in media containing quebracho tannins *in vitro*. As *H. contortus* is a haematophagous worm, it might be suggested that the source of ingested condensed tannins was the blood of the host. However, Terrill *et al*. (1994) failed to detect ^{14}C in the blood of sheep after the infusion of ^{14}C-labelled condensed tannins into the abomasum. In a similar experiment, Perez-Maldonado and Norton (1996a) found that about 10 % of the infused ^{14}C was recovered in the urine, but these authors did not measure specific radioactivity in the blood. If only small quantities of condensed tannins enter the bloodstream of the host animal, it seems unlikely that worms would be exposed to significant amounts of condensed tannins by this route. It is more likely that worms ingest tannins from the surface of the mucosa or from the digesta, either during feeding or while relocating to new feeding sites on the mucosa. If so, it is likely that *T. colubriformis* would also ingest significant quantities of condensed tannins, although this was not demonstrated in the current experiment.

The fate of the tannins after ingestion is not clear. In the current study, internal structures were often difficult to identify because the nematodes tended to became distorted during sectioning, and in many cases, the level at which they occurred within the worm was also unclear. Internal red staining was seen distal to the oesophagus in a female *H. contortus*, at the level of the vulval flap (Plate 7.8). In this worm, the tannins appeared to be coating the microvilli of part of the gut but the identification of the stained structure was uncertain. Numerous membrane-associated enzymes and other proteins, to which condensed tannins could bind, are present on the surface of the gut (Haslam *et al.*, 1996; Munn & Munn, 2002). Athanasiadou *et al*. (2001a) suggested that tannins might be ingested by larvae and bind to the surface of their intestine, causing local damage or forming a barrier to absorption. Although the apparent location of the tannins in adult worms in the current experiment was consistent with this suggestion, the viability of the adult worms was not affected, as would be expected if absorption was impaired. However, condensed tannins in the

nematode gut might inhibit the absorption or utilisation of a specific nutrient required for adequate reproductive function. In addition to proteins, tannins may form complexes with polysaccharides and transition metals such as iron, vanadium, manganese, aluminium, calcium, copper, and cobalt (Haslam, 1996). The nutrient requirements of nematodes, particularly parasitic nematodes, are not well defined and need further investigation.

Condensed tannins were visible in one developing egg in the uterus of a worm (Plate 7.9 A), but were not visible in any other eggs, either in the same worm or in other worms. Tannins sometimes appeared as artefacts in worm tissue caused by overlying digesta (see Plate 7.7 A) but in the case of the stained egg, the tannin did not seem to be artefactual. Possibly, this was the only egg cut at the appropriate angle to show the staining, but this is unlikely, given the large number of eggs present in the uterus of *H. contortus* females. Eggs in these sections appeared as an amorphous mass, and nothing distinguished the part of the egg that stained red from the parts that did not. The significance of the observed staining in the egg is therefore a matter for speculation.

When the eggs of trichostrongyle nematodes are laid, they normally contain a morula (Roberts & Janovy, 2005b). Therefore the presence of larvated eggs in the uterus of a female *Trichostrongylus* (Plates &.7 B & 7.8) is intriguing. This phenomenon is known to occur under unfavourable conditions in the free-living nematode, *Caenorhabditis elegans* (Hope, 2002) and can cause death when the eggs hatch inside the female (Barnes & Hekimi, 1997) but does not appear to have been previously reported in *T. colubriformis*. Veglia (1915), after extensive observation of *H. contortus* eggs, noted that eggs were normally released by the female at the four cell stage, and never developed beyond the morula stage in the intestines of the host, presumably due to lack of oxygen. No larvated eggs were seen in any other stained worms, and a brief examination of fixed female *T. colubriformis* from other *Calliandra*-fed lambs did not reveal any more. This particular worm would seem to be an exception rather than the norm, but it does add weight to the theory that eggs are retained in the uterus beyond the normal time of oviposition. No red staining was observed anywhere on this worm.

The knowledge of the control of oviposition in nematodes comes mainly from work on either *C. elegans*, because it is easy to maintain in culture, or *Ascaris suum*, which lends itself to study due to its large size. The ovijector is composed mainly of circular muscle fibres, innervated externally by a large nerve plexus (Fellowes *et al*., 1999). Acetyl choline (ACh) and L-glutamate (Fellowes *et al*., 2000b) and numerous neuropeptides are all thought to play a role in egg-laying by female worms (Fellowes *et al.*, 1998; Fellowes *et al.*, 1999; Fellowes *et al.*, 2000a). It is possible that condensed tannins could act by binding to and inactivating one or more of these compounds, or by binding to and blocking their receptors. Since ACh and other neurotransmitters are also present in other areas such as the dorsal and ventral nerve cords and the pharynx, antagonism by condensed tannins should result in generalised paralysis or starvation of the worm. However, *Calliandra* condensed tannins do not appear to affect nematode viability. Those peptides studied in detail all differ in their actions, and some have been shown to have direct inhibitory or excitatory effects on the vagina vera (the section of the vagina immediately adjacent to the vulva, which is lined by a continuation of the external cuticle). The actions of these peptides on the vagina vera are often quite different to their actions on the somatic muscle of the nematode (Fellowes *et al*., 1998) so it is possible that condensed tannins could inhibit oviposition, without necessarily having adverse effects on locomotion or pharyngeal pumping. However, condensed tannins have not so far been identified in the reproductive organs and it is not known how they might get there. Whether or not nematode neuropeptides could be possible targets for condensed tannins requires further research.

7.5 Conclusion

The most appropriate technique developed during the current experiment to identify condensed tannins in mammalian and nematode tissues was the vanillin-HCl technique in conjunction with routine H & E staining. While H & E staining was the most sensitive technique for pinpointing the exact location of tannins within tissues and visualising the accompanying pathology, it did not define the chemical nature of the substance in the tissue. The vanillin-HCl technique was used successfully to definitively identify condensed tannins in both mammalian and adult nematode tissues and was certainly the most appropriate technique for this purpose.

A number of modifications were made to the vanillin-HCl technique to adapt it for use with ovine tissues. In contrast to the plant tissues used in the original technique, animal tissues required support during cutting and staining. This was achieved by embedding the tissues in OCT prior to cutting, adhering the cut sections to silanised slides and fixing the sections in methanol for two minutes prior to staining. The vanillin reagent used was that described by Broadhurst and Jones (1978), but the concentration of vanillin in the solution did not appear to be critical to the end result. In plant sections, the presence or absence of stained tissues can be determined grossly, but in animal tissues microscopic examination is necessary and can be achieved by light microscopy with the diaphragm partly closed.

The main difficulty with the vanillin technique as used in the current experiment was the lack of appropriate positive controls. For mammalian tissue this could be overcome by incubating tissues with condensed tannins as described in Section 7.2.2 (c), but the procedure needs to be refined to improve the viability of the tissues during incubation. A technique has not yet been devised to prepare positive controls for nematode tissues. Additional problems with the vanillin technique were the lack of background staining and the short-lived nature of the stained specimens. Ideally methods to counterstain and preserve the sections should be developed.

Of the other staining techniques investigated, ferric chloride stained the tissues too indiscriminately and the stained sections were difficult to interpret. Butanol-H_2SO_4 was unsuited to use in animal sections because of the fragility of the tissues, which often did not survive treatment with the hot acid.

Although the ultimate fate of condensed tannins entering the mammalian GI tract remains unknown, it is clear that they are recognised as potentially harmful and are extensively taken up by macrophages. The extent to which this occurs appears to be a function of condensed tannin concentration in the digesta. In this study it was not possible to assess whether tannins had caused mucosal ulceration, but there was no evidence that they bound to surface proteins. Condensed tannins within tissue macrophages were present in greater abundance in the abomasum than in more distal regions of the GI tract. This probably reflects the greater concentration of free condensed tannins present in abomasal digesta (see Section 4.3.5).

Adult *H. contortus* were shown to ingest tannins, which appeared to bind to the microvilli on the surface of the nematode intestine. It was not clear whether the same was true for *T. colubriformis*, although this is possible. The significance of condensed tannin ingestion for the worm is unknown. There was no clear evidence that tannins were able to bind to the cuticle of adult worms or larvae or to the exterior of nematode eggs, but this could not be ruled out. The means by which condensed tannins might affect egg production remains unknown, but it is possible that they bind and inhibit neurotransmitters or neuropeptides involved in the control of oviposition.

CHAPTER 8

SECOND GENERATION ANTHELMINTIC EFFECTS OF *CALLIANDRA CALOTHYRSUS* IN LAMBS

8.1 Introduction

Anthelmintic effects of condensed tannins against GI nematode eggs and larvae *in vitro* have been shown by the experiments reported in Chapters 5 and 6, which also confirmed the work of others (Molan *et al*., 2002). The number of larvae developing to the infective stage was reduced, both through failure of the eggs to hatch and through reduced development of larvae. However, conditions under which *in vitro* observations are made may not mimic accurately those normally encountered *in vivo*, where nematode eggs and larvae may be exposed to widely varying conditions and changing concentrations of condensed tannins in the reproductive tract of the female worm, during passage through the GI tract of the host animal and in faeces on pasture. Niezen *et al*. (2002b) found that condensed tannins in *Dorycnium* species caused significantly greater reductions in egg hatch and larval development rates *in vivo* than *in vitro*. Therefore it is necessary to study the development of these free-living stages under conditions more closely resembling those encountered in the field in order to be able to assess the impact of *Calliandra* condensed tannins on the contamination of pasture by nematode eggs and larvae.

The work described in Chapter 5 indicated that despite significant reductions in the development of nematode eggs to larvae, a small percentage of eggs exposed to condensed tannins did develop normally. It would be of interest to examine whether L_3 larvae that were exposed to condensed tannins during early development would continue to mature normally into reproductive adults. The mode of action of condensed tannins against GI nematodes is unknown, so any long-term effects of these on nematode juvenile stages would be merely speculative. It is possible that the prior exposure of larvae to condensed tannins might harm their ability to establish in a new host. Alternatively, their development to the adult stage and subsequently their ability to lay eggs might be impaired.

The working hypotheses of the current experiment were:

a. Nematode eggs developing in the faeces of *Calliandra*-fed lambs would have impaired development compared with eggs developing in the faeces of lambs fed a non-tanniniferous diet.

b. Without further exposure to condensed tannins, *H. contortus* and *T. colubriformis* derived from *Calliandra*-fed lambs would either have reduced rates of establishment in a new host and/or would produce fewer eggs as adults, than would nematodes of the same species derived from lambs fed a non-tanniniferous diet.

The purpose of the current experiment, therefore, was to investigate the effects of feeding *Calliandra* to host lambs on the subsequent development of *H. contortus* and *T. colubriformis* larvae in faecal cultures, and to examine the establishment and fecundity of the resulting second generation adults *in vivo*.

8.2 Materials and Methods

8.2.1 Experimental design

Twenty-four lambs, designated as recipients, were stratified for live weight and divided into four balanced groups of six animals. The groups then were allocated at random to the four treatments: HL, HC, TL and TC (Table 8.1). The experiment was undertaken with the approval of the JCU Animal Experimentation Ethics Committee (Ethics Approval No. A826_03).

Table 8.1 Four donor lambs infected with either *H. contortus* or *Trichostrongylus colubriformis* larvae and fed a diet of either lucerne or *Calliandra*, supplied the four groups of recipient lambs with respective nematode species.

	Nematode species in donor lambs			
	H. contortus		*T. colubriformis*	
Diets of donor lambs	Lucerne	*Calliandra*	Lucerne	*Calliandra*
No. of recipient lambs	6	6	6	6
Name of treatment group	HL	HC	TL	TC

8.2.2 Animals, housing and management

Thirty Australian tropical Merino-cross ram lambs were transferred from a commercial sheep farm in Western Queensland to the Metabolism Unit at JCU. The lambs were approximately seven months old and had been weaned from their dams the day before arrival. The animals were housed and treated on arrival as described for animals in previous experiments (see Section 3.2.2). The lightest and heaviest animals were culled and the next four heaviest lambs were chosen as donor animals. The number of donors was restricted to four due to a limited supply of *Calliandra*, as only a single donor per treatment (Table 8.1) was needed to supply enough nematode eggs for the examination of the effects of *Calliandra* on the development of eggs to larvae. Due to the decreased fecundity of female worms observed in every one of the *Calliandra*-fed lambs involved in experiments reported in Chapters 3 and 4, a single donor per treatment group was deemed sufficient. However, it was acknowledged that differences between individual lambs in faecal egg counts and worm burdens could affect the results.

The remaining 24 lambs were used as recipients (Table 8.1).

The animals were fed twice daily at 0900 and 1600 hours at a DM allowance equivalent to 2.8 % of live weight per day. Feed allowances were adjusted weekly after the animals were weighed. All animals were started on a basal diet of lucerne pellets on entering the Metabolism Unit. Experimental diets for the four donor lambs were begun one week after arrival (two weeks prior to infection) and were gradually increased over the course of a week. Two donor animals were fed 100 % *Calliandra* harvested from the same plots used in previous experiments. The legume had last been harvested about five months earlier. Rumen-degradable casein was added to the *Calliandra* fed to the two donor animals, while rumen-undegradable casein was added to the lucerne pellets fed to the remaining two donor lambs (see Sections 3.2.3 for details). The donor animals were supplied with blocks of mineral lick (see Table 3.3).

The 24 recipient lambs were fed lucerne pellets only, throughout the course of the experiment.

All animals had free access to clean drinking water.

8.2.3 Donor lambs

Infective larvae of drench-susceptible *T. colubriformis* (McMasters) and *H. contortus* (Kirby) were obtained from the CSIRO McMasters Laboratory in New South Wales and stored in tap water at 4 °C. Viability was checked on arrival and again four weeks later, just prior to use. Two donor lambs (one per diet) were infected with 10,000 *T. colubriformis* larvae and the remaining two lambs were each infected with 3200 *H. contortus*. The larvae were administered using a syringe and the syringe was refilled with water that was also administered to the lamb. Faeces were collected weekly from the rectum of each lamb and faecal egg counts performed using a modified McMaster technique (Section 3.2.6).

Two weeks after infection, the two lambs on the *Calliandra* diet had low positive faecal egg counts, seven days before the experimental infections should have become patent. It was thought that they had become infected by exposure to sheep from another experiment unavoidably housed in the same facility at the same time. These sheep had been drenched with ivermectin on arrival and were presumed free of parasites, but subsequent faecal egg counts revealed significant worm burdens. The infected sheep were removed from the animal house and the lambs on the *Calliandra* diet were drenched and exchanged with two recipient lambs of similar weight that had not been exposed to the infected sheep. The replacement lambs were introduced to the the *Calliandra* diet and infected as soon as they were eating 100 % of the ration. This took one week for the *H. contortus* donor and two weeks for the *T. colubriformis* donor. The experiment was continued without interruption for the lucerne-fed donors, as they had not been near the infected sheep and had no nematode eggs in their faeces.

(a) Larval cultures

In order to collect faeces for larval cultures, the four donor lambs were placed in metabolism crates as soon as each infection became patent. Faecal diverters attached

to the bottom of the metabolism crate channelled the faeces into a labelled plastic bag which was changed every 24 hours. The lambs were fitted with urine collectors to prevent contamination of the excreted faeces with urine. The collection period for each lamb lasted two to three weeks.

At the end of each 24-hour period, the total amount of faeces excreted by each lamb was weighed and a 100 g sub-sample collected for DM determination. A further 2 g was removed for faecal egg count. The remainder of the faeces was mixed with an approximately equal volume of vermiculite and enough water to ensure that the mixture was kept moist but not too wet. The mixture was transferred into 4 L plastic containers, each to a maximum depth of 8 cm. In most cases, two containers per day were required for lambs fed lucerne but only one for the lambs fed *Calliandra*. The lids were placed on the containers but not sealed, and the mixture incubated (Qualtex Thermstat, Watson Victor Ltd., Australia) at 27 °C for eight days. The temperatures of both the incubator and the mixture were recorded daily. The mixture was stirred daily to ensure adequate aeration, and extra water was added as required. Faeces from *Calliandra*-fed lambs were crushed daily with a stainless-steel coffee tamper as these were hard and did not break up as easily as faeces from the lucerne-fed lambs.

After eight days, the faeces were removed from the incubator and placed in a Baermann apparatus consisting of a brass sieve (≥ 295 µm aperture) sitting in the top of a large plastic funnel (approximately 3 L), to the outlet of which was attached a 20-cm length of rubber tubing. The end of the tubing was closed with a clamp. The funnel was filled with water (room temperature) until the water just covered the faeces but did not overflow the top of the sieve. The preparation was left to stand overnight for larvae to migrate out of the faeces and settle to the bottom of the tubing. In the morning approximately 400 mL of water were collected by opening the clamp of the tubing. Two Baermann funnels were set up each night for the faeces from *Calliandra*-fed lambs. Because the lucerne-fed lambs produced a greater mass of faeces, half of the faeces were placed in the two funnels overnight, while the other half was placed in them the following morning and the larvae collected late in the afternoon.

The faeces from the lucerne-fed lambs were very crumbly and tended to break up extensively during culture, resulting in a large amount of sludge in the bottom of the Baermann funnels. To clean and concentrate the larvae, the liquid removed from the Baermann apparatus was first filtered through a 250 µm nylon mesh to remove as much of the sludge as possible. The filtrate was transferred to a measuring cylinder and diluted to 2 L with tap water. After several hours, most of the water was removed by suction, leaving 400 mL undisturbed in the bottom of the cylinder. This was filtered through the mesh into a 1 L measuring cylinder and the process repeated. Faeces from lucerne-fed lambs required passage through 2 L, 1 L, 1 L, 250 mL and 100 mL measuring cylinders to obtain a clear supernatant, but a significant amount of debris still remained in the bottom of the last cylinder. The final volume at each step was 400 mL, 150 mL, 150 mL, 50 mL and 30 mL respectively. After the last cylinder in the series, the solution was filtered again and the number of larvae in ten 10 µL aliquots was counted. After the calculation of larval yields, the solution was shaken, allowed to stand for 30 seconds and then filtered again, taking care not to pour any remaining debris through the mesh. Cleaned larvae were pooled for each donor sheep and stored at 4 °C until sufficient clean larvae were obtained to infect one group of six recipient lambs.

The faeces from the *Calliandra*-fed lambs required less cleaning, so the second 1 L cylinder and the 100 mL cylinder were omitted from the washing process. This was done to maximise the number of larvae available to infect the recipient lambs, although it was recognised that this would affect the calculations of larval yield. The viability of the larvae from the lucerne-fed lambs may also have been reduced in comparison to larvae from the *Calliandra*-fed lambs due to the extra handling.

(b) Worm collection and culture

The donor lambs remained in individual metabolism crates until the end of the faecal collection period, when they were euthanased by captive bolt and the abomasum and small intestine of each lamb were removed for worm counts. As soon as the abomasum (lambs infected with *H. contortus*) or duodenum (lambs infected with *T. colubriformis*) was opened, a number of female worms were transferred to 0.9 %

saline at 39 °C. Female *H. contortus* were easily identified grossly, and sixteen females were picked (using fine forceps) from the surface of the emptied abomasum and placed in saline in a vial for rinsing. The worms were transferred to clean saline in sixteen wells of a 24-well cell culture plate and maintained at 39 °C in a water bath (Shaking Bath SB4, Techne, USA) for one hour. The *T. colubriformis* were too difficult to detect grossly in digesta, so aliquots of the duodenal contents were diluted with saline and examined in a Petri dish under a dissecting microscope (Olympus SD-ILK, Olympus Optical Co. Ltd., Japan). Twenty-four female worms were removed with forceps, rinsed in warm saline and placed into the wells of a cell-culture plate as described for *H. contortus*. The *T. colubriformis* were maintained at 39 °C for two hours to allow adequate egg production, since this species is much less fecund than *H. contortus*. After the allocated time had elapsed, worms were removed from the cell culture plates and placed in 5 mL plastic screw-capped tubes of saline at 39 °C for a further two hours to monitor their viability. The cell culture plates were retained and examined under a dissecting microscope within 24 hours to determine the number of eggs produced by each female during the allotted time. The worms from the cell culture plates and ten male worms removed from each lamb at the same time were frozen in OCT for tannin staining as described in Chapter 7.

Procedures for the collection and storage of samples for total worm counts were as described in Section 3.2.6 and counting techniques were as described in Section 4.2.6.

8.2.4 Recipient lambs

The recipient lambs were housed and managed similarly to the donor lambs, so all had a minimum of six weeks adaptation to the animal facility and the lucerne pellet diet. Each of the four groups of recipient lambs was designated to receive a dose of parasite larvae from one of the four donor lambs (Table 8.1). An infective dose of either 10,000 *T. colubriformis* or 3200 *H. contortus* was administered via a syringe to the lambs. All lambs were weighed weekly (Section 3.2.2) before the morning feed. At the same time a faecal sample was taken from the rectum of each lamb for faecal egg count.

On Days 21 and 28 after infection, each group of lambs was transferred from individual pens to individual metabolism crates fitted with faecal diverters and faecal collection bags. A urine collector was fitted to each lamb in order to avoid contamination of faeces with urine. At the end of the 24-hour period, the total mass of faeces excreted was recorded and a 100 g sub-sample was taken for DM determination. The sub-samples were dried in paper bags [Section 3.2.4 (a)] at 100 °C for 24 hours. The DM of the samples was calculated using *Equation 3.1* [Section 3.2.5 (a)].

On Day 29 post-infection, the lambs in each group were euthanased with a captive bolt and the abomasum and small intestine of each lamb were removed for worm counts. Female worms from one lamb, chosen randomly from each recipient group, were incubated in cell culture plates as described for the donors in Section 8.2.4 (b). However, *H. contortus* females were incubated for thirty minutes only, as previously, the large number of eggs produced by this species in one hour made counting difficult.

8.2.5 Statistics

The larval culture data from the donor lambs (faecal egg counts, eggs produced per female worm per day, larvae cultured per gram of faeces, ratio of eggs to larvae and larvae cultured per female worm) were compared for each worm species by the non-parametric Mann-Whitney test. The number of eggs produced by female worms incubated in cell culture plates (for both donors and recipients) was also compared for each worm species by Mann-Whitney test. The number of eggs *in utero* for female *T. colubriformis* (both donors and recipients) on the different diets was log-transformed ($\log_{10}(n+1)$) and compared by either Student's t-test or Mann-Whitney test. Faecal egg counts, worm counts and percent female worms for the recipient lambs were log-transformed ($\log_{10}(n+1)$) and analysed by two-way ANOVA. The data for eggs per female worm failed the test for equal variance and were analysed separately for each species using the Mann-Whitney test. All tests were carried out using SPSS 13.0.1 for Windows (SPSS Inc., Chicago, USA).

8.3 Results

8.3.1 Animal health

No lambs showed clinical signs of GI parasitism at any time during the experiment. Most had low to moderate numbers of coccidial oocysts in their faeces at some point during the study, but this was not associated with any signs of ill-health and the lambs were not treated for coccidia. One lamb had a mild, intermittent nasal discharge. This lamb and two others from the same group (TL) were diagnosed with mild nasal bot (*Oestrus ovis*) infections.

8.3.2 Feed intake

There were no feed refusals throughout the experiment except for the *Calliandra*-fed *H. contortus* donor, which refused between 3 g and 23 g of rumen-undegradable casein per day but ate all the *Calliandra* offered.

8.3.3 Calculations

Three of the four groups of recipient lambs (HC, TL and TC) were found to be infected with low numbers of nematodes of the wrong species in addition to the intended infective species (37 – 75 *T. colubriformis* per lamb in the HC group and 4 – 24 *H. contortus* per lamb in the TL and TC groups); presumably due to cross-contamination of the larval cultures. In order to correct for this, the mean number of eggs produced per female worm in lambs in the uncontaminated HL group was calculated by Equation 8.1:

$$\text{Eggs}/HC_{f\,HL} = (\text{FEC} \times \text{AF})/HC_{fHL} \quad \text{.....} \textit{Equation 8.1}$$

where HC_{fHL} is the total number of female *H. contortus* in the lambs in the HL group, FEC is faecal egg count (eggs/g wet faeces) and MF is the mass of wet faeces excreted by the host lamb in 24 hours (g faeces/24 hours).

The number of eggs produced by the *H. contortus* females present in the TL lambs was calculated using Equation 8.2:

$$Eggs/HC_{f\,TL} = Eggs/HC_{f\,HL} \times HC_{fTL} \quad \text{..... } \textit{Equation 8.2}$$

where HC_{fTL} is the number of female *H. contortus* found in the TL lambs.

The egg production by the female *T. colubriformis* in the TL lambs was calculated using Equation 8.3:

$$Eggs/TC_{fTL} = \frac{\text{Total eggs} - (Eggs/HC_{fTL} \times HC_{fTL})}{TC_{fTL}} \quad \text{..... } \textit{Equation 8.3}$$

where TC_{fTL} is the number of *T. colubriformis* females in the TL lambs and total eggs is the total number of eggs produced by both species present in the lambs.

The number of eggs produced per female *H. contortus* in the HC group was calculated using Equation 8.4:

$$Eggs/HC_{fHC} = \frac{\text{Total eggs} - (Eggs/TC_{fTL} \times TC_{fHC})}{HC_{fHC}} \quad \text{.....} \textit{Equation 8.4}$$

where HC_{fHC} is the number of female *H. contortus* present in the HC lambs, and TC_{fHC} is the number of *T. colubriformis* females present in the HC lambs.

The number of eggs per female *T. colubriformis* in the TC group was calculated using Equation 8.5:

$$Eggs/TC_{fTC} = \frac{\text{Total eggs} - (Eggs/HC_{fHC} \times HC_{fTC})}{TC_{fTC}} \quad \text{.....} \textit{Equation 8.5}$$

where TC_{fTC} is the number of female *T. colubriformis* present in the TC lambs and HC_{fTC} is the number of female *H. contortus* present in the TC lambs.

Larval establishment rates were calculated using the following equation:

$$\text{Larval establishment (\%)} = \frac{\text{No. adult worms at necropsy x 100}}{\text{Infective dose}} \quad \text{.....} \textit{Equation 8.6}$$

8.3.4 Parasitology

(a) Donor lambs

Data for worm counts in donor lambs are presented in Table 8.2.

Table 8.2 Total worm numbers and percent female worms for four lambs infected with either *Haemonchus contortus* or *Trichostrongylus colubriformis* and fed either lucerne or *Calliandra*.

	Nematode species			
	H. contortus		*T. colubriformis*	
Diet	Lucerne	*Calliandra*	Lucerne	*Calliandra*
Total worm count	1240	977	1840	4983
% female worms	49	48	50	56

Data obtained from the larval cultures are presented in Tables 8.3 and 8.4. All larvae appeared to have reached the L_3 stage. Both the number of eggs and the number of larvae produced daily by female nematodes were significantly higher for both species of nematode when the host was fed lucerne. Donor animals on the lucerne diet also produced significantly more faeces in each 24 hour period than those on the *Calliandra* diet. For *H. contortus*, more larvae per gram of faeces were cultured on the lucerne diet than on the *Calliandra* diet, but for *T. colubriformis* the difference between diets was not statistically significant. The ratio of eggs to larvae was greater on the lucerne diet than the *Calliandra* diet for *T. colubriformis* but not *H. contortus*.

Table 8.3 Numbers of eggs and larvae cultured from the faeces of four donor sheep infected with either *Haemonchus contortus* or *Trichostrongylus colubriformis* and fed either lucerne or *Calliandra*. Values are means ± standard errors.

	Nematode Species					
	H. contortus			*T. colubriformis*		
	Lucerne	*Calliandra*	Sig. (P)	Lucerne	*Calliandra*	Sig. (P)
Eggs/g faeces	2817 ± 320	139 ± 26	<0.001	570 ± 59	92 ± 14	<0.001
Larvae/g faeces	131 ± 93	11 ± 9	<0.001	79 ± 145	29 ± 13	0.817
Faecal DM (g)	452 ± 24	328 ± 32	<0.001	395 ± 34	328 ± 26	<0.001

Table 8.4 Eggs shed per female worm, L_3 larvae developed per female worm and ratio of eggs to larvae from larval cultures of four donor lambs infected with either *Haemonchus contortus* or *Trichostrongylus colubriformis* and fed either lucerne or *Calliandra*. Values are means ± standard errors.

	Nematode Species					
	H. contortus			*T. colubriformis*		
	Lucerne	*Calliandra*	Sig. (P)	Lucerne	*Calliandra*	Sig. (P)
Eggs/female worm	6546 ± 2314	198 ± 136	0.000	670 ± 190	25 ± 12	0.000
Larvae/female worm	287 ± 76	14 ± 3	0.000	90 ± 60	7 ± 1	0.002
Ratio eggs: larvae	32 ± 11	23 ± 7	0.377	36 ± 11	5 ± 2	0.010

Data on other measures of worm fecundity are presented in Table 8.5. The number of eggs present in the uterus of each female *T. colubriformis* counted post-mortem was not affected by host diet, and neither was the number of eggs produced by each female *H. contortus* incubated in cell culture plates for one hour. However, female *T. colubriformis* from the *Calliandra*-fed donor produced significantly fewer eggs in two hours than those from the lucerne-fed donor.

Table 8.5 Number of eggs laid by female worms in the wells of cell culture plates in one hour (*Haemonchus contortus*) or two hours (*Trichostrongylus colubriformis*), and number of eggs *in utero* of 25 female *Trichostrongylus colubriformis* from lambs fed different diets Values are means ± standard errors.

	Nematode Species					
	H. contortus			*T. colubriformis*		
	Lucerne	*Calliandra*	Sig. (P)	Lucerne	*Calliandra*	Sig. (P)
Eggs/well	201 ± 32	163 ± 23	1.000	20 ± 2	8 ± 1	<0.001
Eggs *in utero*	N.D.*	N.D.*		22 ± 1	25 ± 1	0.114

*Not determined due to the very high number of eggs *in utero*.

(b) Recipient lambs

Parasitological data from observations on recipient lambs are presented in Table 8.6. Across nematode species, faecal egg counts on Days 21 and 28 and total worm counts were significantly higher for lambs on the *Calliandra* diet than lambs on the lucerne diet ($P = 0.042$, $P < 0.001$ and $P < 0.001$ respectively). Faecal egg counts were higher and worm counts were lower for lambs infected with *H. contortus* than

for lambs infected with *T. colubriformis* (P < 0.001 for all three parameters). There was a diet-species interaction for worm count (P = 0.023), as the mean worm count for the HC group was similar to that of the TL group. Species (P = 0.001) but not diet (P = 0.179) influenced the percentage of female worms, with *T. colubriformis* having a higher percentage of females and *H. contortus* a higher percentage of males.

Table 8.6 Faecal egg counts on Day 21 (FEC1) and Day 28 (FEC2), number of worms and percentage of female worms in recipient lambs fed lucerne and infected with either *T. colubriformis* (T) or *Haemonchus contortus* (H) larvae obtained from donor lambs infected with either of the nematode species and fed either a lucerne (L) or a *Calliandra* (C) diet. Data are means and standard errors. Means with different superscripts within rows are significantly different (P<0.050).

	Group of recipient lambs			
	HL	HC	TL	TC
FEC1 (eggs/g faeces)	$695^{a} \pm 129$	$1050^{b} \pm 335$	$105^{c} \pm 25$	$295^{d} \pm 61$
FEC 2 (eggs/g faeces)	$1525^{a} \pm 187$	$3065^{b} \pm 405$	$140^{c} \pm 34$	$400^{d} \pm 64$
No. of worms	$897^{a} \pm 90$	$1393^{b} \pm 204$	$1445^{b} \pm 177$	$3901^{c} \pm 254$
Female worms (%)	$49^{a} \pm 1$	$49^{a} \pm 1$	$56^{b} \pm 2$	$52^{b} \pm 1$

Data on fecundity of adult female worms derived from one recipient lamb per treatment is presented in Table 8.7. Diet did not affect the number of eggs produced per female worm during the two faecal collection periods for either *H. contortus* or *T. colubriformis*. However, in contrast to worms from the donor lambs, female *T. colubriformis* from the recipient lambs on the *Calliandra* diet had significantly more eggs *in utero* than those on the lucerne diet. The number of eggs shed in culture plates within a specified time was significantly less for both *H. contortus* and *T. colubriformis* on the *Calliandra* diet.

Table 8.7 Eggs per female worm shed on Day 21 (D21) and Day 28 (D28), number eggs produced in each well during incubation of female worms in cell culture plates and number of eggs *in utero* per female worm in recipient lambs fed lucerne and infected with either *Trichostrongylus colubriformis* (T) or *Haemonchus contortus* (H) larvae obtained from donor lambs infected with either of the nematode species and fed either a lucerne (L) or a *Calliandra* (C) diet. Data are means ± standard errors.

	Nematode Species					
	H. contortus			*T. colubriformis*		
	Lucerne	*Calliandra*	Sig. (P)	Lucerne	*Calliandra*	Sig. (P)
Eggs/female worm D21	1710 ± 347	1130 ± 274	0.126	138 ± 49	158 ± 44	0.792
Eggs/female worm D28	4122 ± 583	4689 ± 402	0.931	129 ± 50	192 ± 35	0.177
Eggs/well	340 ± 39	96 ± 23	0.000	13 ± 2	5 ± 1	0.001
Eggs *in utero*	N.d	N.d.	-	24 ± 0.4	26 ± 1	0.004

8.4 Discussion

8.4.1 Donor lambs

(a) Data considerations

Because there was only one lamb per treatment, variables such as faecal output, faecal egg counts and measures of larval development could not be analysed by repeated measures analysis of variance. Instead, these were treated as separate independent observations for each donor. All observations were directly related to either dietary intake or worm burden. Dietary intake was known to be constant over the course of the observation period, and worm burden was assumed to be constant, based on the lack of effect of *Calliandra* on worm burdens recorded in Chapters 3 and 4. Previous work by Barker (1974) also suggested that once larvae are established in the GI tract of naive lambs, they are not expelled during the pre-patent period. Thus variation of the parameters measured within each donor would be minimal. However, the possibility of individual variation between donors must be kept in mind when considering the results.

A difficulty encountered in the current experiment involved the determination of larval yields, where the removal of all the debris from the lucerne diet cultures proved difficult even after five washes. Larval yields were determined after the fifth

wash, as the final step in the cleaning process was found to involve significant loss of larval numbers. Smaller numbers probably would have been lost during the washing process, before yields were determined. The *Calliandra* diet resulted in far less sludge in the Baermann funnels than there was in the control cultures and only three washes were required. Due to the very low faecal egg counts of the HC and TC donors and the necessity of obtaining sufficient larvae to infect the recipients, further washes were deemed undesirable, as too many larvae would be lost. In retrospect, the larval yields for the HL and TL lambs should have been determined after the third wash, but it was not apparent that the HC and TC cultures were going to be cleaner until the TL and HL cultures were completed. For this reason, the larval yields determined for all groups were probably lower than the actual yield. Yields for the HL and TL groups were likely to have been underestimated in comparison with the HC and TC groups.

(b) Fecundity of adult worms

The lucerne-fed lambs produced slightly more faecal DM daily than the *Calliandra*-fed lambs, an observation consistent with those in previous experiments. Worm burdens also appeared to be higher in the lucerne-fed lambs. The egg output (calculated per unit of female worm) in the HC and TC lambs was reduced by 97 - 98 % compared with the HL and TL lambs (Table 8.4). This is consistent with the results of the *in vivo* work discussed in Chapters 3 and 4. It is surprising, therefore, that the incubation of female worms in cell culture plates confirmed this result for *T. colubriformis* but not *H. contortus* (Table 8.5). It is possible that an unsuspected minor variation in the procedure involving the incubation of the *H. contortus* was the cause of this inconsistency.

The number of eggs present in the uterus of female *T. colubriformis* was higher on the *Calliandra* diet, but not significantly different from controls, so there is no conclusive evidence in this lamb that the reduction in egg output by female worms was due to inhibition of oviposition. However, the values obtained in the current experiment were consistent with values obtained for lambs in the pilot experiment (Chapter 3) and also with those obtained in Period 2 of the experiment described in Chapter 4.

(c) Development of free-living stages in faecal cultures

As might be expected, lower egg output was associated with decreased larval production, since there were fewer eggs available to develop. However, it is also possible that exposure to *Calliandra* condensed tannins prevented egg hatching, or killed or inhibited development of the larvae that did hatch out. In hindsight, egg counts should have been performed on the faecal residues in the Baermann apparatus to determine the number of un-hatched eggs. Larval development appeared to be unaffected, as the larvae counted were all L_3. However, it is possible that dead or small larvae were removed from the cultures before the yields were assessed. The Baermann technique relies on larvae migrating out of the faecal mass through a mesh screen. They then sink to the bottom of the funnel, where they can easily be tapped off (Urquhart *et al*., 1996). Larvae that die during the culture process may be passively washed through the sieve, but these are less likely than actively swimming larvae to be released from the faeces. Also, larvae that died early on in the culture process would probably undergo some degree of degeneration and may not remain intact to be counted. Infective L_3 larvae are adapted to actively migrate out of faeces and seek a host, whereas the L_1 and L_2 stages are less active, particularly the former. During the pre-moult lethargus they barely move at all (Veglia, 1915). The pre-infective stages also seem to prefer the middle of the faecal mass when not feeding. It is possible, then, that the L_1 and L_2 stages could remain largely un-recovered by the Baermann technique used here, since the faecal mass in the sieve was large.

The ratio of eggs to larvae was calculated to give an indication of larval viability. If the ratio of eggs to larvae had been larger in the *Calliandra*-fed lambs compared with the lucerne-fed lambs, it would have been an indication that hatch rates and/or larval survival were impaired. However, the ratios of eggs to larvae from *Calliandra*-fed and from lucerne-fed lambs infected with *H. contortus* were similar, suggesting that there were no differences in hatch rates and larval development between the two diets. The unexpectedly smaller ratio for *T. colubriformis* probably indicates that eggs exposed to *Calliandra* were more likely to hatch and complete development to the L_3 stage. This observation may reflect the experimental technique used, where the two extra washes adopted for the HL and TL cultures before larval yields were calculated [Section 8.2.4 (a)] probably resulted in larval losses and possibly reduced

larval viability. This would have affected both nematode species, but the effect would have been more pronounced for *T. colubriformis* due to the lower fecundity of this species.

Despite the difficulties with the experimental procedure, it is possible that the enhanced larval development on the *Calliandra* diet is not entirely artifactual. A certain amount of natural attrition would be expected on any diet, so that only a proportion of the eggs excreted in host faeces would develop into infective larvae. Under conditions favourable for development, it might be suggested that larvae failing to reach the L_3 stage after exposure to the lucerne diet do so because of some inherent defect. Furthermore, *Calliandra* has been shown to reduce the number of eggs appearing in the faeces. In this case it is possible that embryonic larvae with questionable viability die in the host when exposed to tannins; possibly even before the eggs are shed by the female, and thus do not appear in the faeces. On the other hand, weaker larvae exposed to the lucerne diet may survive longer and die after the eggs hatch. However, the above would not account for the full magnitude of the reduction in egg numbers seen in *Calliandra*-fed lambs (up to 97 % in this study), so healthy larvae were probably also affected.

Support for the above suggestion can be found in the work of Marley *et al*. (2003a), who cultured the faeces of naturally parasitised lambs grazing four different pastures, including birdsfoot trefoil (containing condensed tannins) and two ryegrass/clover combinations (without condensed tannins). The hatch rates of nematode eggs were unaffected by host diets, but a higher percentage of the eggs that hatched developed to the L_3 stage when the host lambs were grazing birdsfoot trefoil. Because faeces were homogenized before culture and adjusted to 37 % DM to eliminate differences in consistency and DM as factors affecting parasite development, the effect was apparently due to faecal composition. Differences in egg production by the adult worms were not assessed in this study, but birdsfoot trefoil has been reported to reduce faecal egg counts in grazing livestock in some studies (Marley *et al.*, 2003b; Ramirez-Restrepo *et al.*, 2004). Marley *et al*., (2003a) did not attempt to explain the observed reduction in larval development. In contrast, Shaik *et al*., (2006) found that the percentage of larvae developing from eggs in the faeces of goats fed tannin-rich *Sericea lespedeza* hay was decreased compared to the controls. These discrepancies

may reflect differences in the condensed tannin characteristics of the different plant species, or they may be due to differences in experimental techniques. The implications of these observations will be discussed further in Chapter 9.

8.4.2 Recipient lambs

(a) Larval establishment

The higher worm burdens in the HC and TC recipient groups compared with the controls are difficult to interpret. Infection with high numbers of *O. ovis* concurrently with *T. colubriformis* or *H. contortus* has been shown to result in both lower faecal egg counts and lower worm burdens (Dorchies *et al.*, 1997; Yacob *et al.*, 2002; Yacob *et al.*, 2004), and the three lambs known to have been infected with *O. ovis* in the current experiment were all in the *T. colubriformis* control group. The infection status of the other lambs in the study was unknown, as was the total nasal bot burden in the affected lambs, but lambs in the other groups had an equal chance of being infected, and the absence of clinical signs suggests that any burdens would have been low. In addition, the worm burdens and faecal egg counts of the three infected lambs were similar to those of the other three lambs in the same group. Thus, it is unlikely that the presence of nasal bot would explain the higher worm burdens in the *Calliandra*-fed lambs, although the possibility cannot be discounted.

Unlike the donor lambs, the two recipient groups for each nematode species were infected from separate larval cultures, so it is possible that any errors in calculating larval concentrations in the cultures could have resulted in different infective doses. Reduced viability of the larvae derived from lucerne-fed sheep due to the extra washing steps is also possible. However, the possibility that the larvae from the *Calliandra*-fed donors were better able to establish an infection in a new host than the larvae from the lucerne-fed donors cannot be ruled out. This would be consistent with a possible increase in larval development to the L_3, and would support the suggestion that feeding *Calliandra* to the host sheep may select for more robust larvae. This possibility is rather disturbing and needs to be further investigated.

(b) Fecundity of second generation nematodes

The lower faecal egg counts in the control groups may simply reflect the lower worm burdens, as the number of eggs produced per female worm, calculated on the basis of 24-hour faecal collections, was similar regardless of diet. However, this is at odds with the finding that, when incubated in culture plates for the same length of time, worms from the HC and TC hosts produced far fewer eggs than worms from the HL and TL hosts. This is especially interesting in light of the almost opposite results obtained for the donor lambs from the same procedures. In the *Calliandra*-fed donor lambs, female worms were exposed directly to condensed tannins as the larvae developed from the L_3 stage to the mature, egg-laying adult, and during the period of egg production. Worms from the HC and TC recipient lambs were exposed to condensed tannins in the digestive tract of the host (see Section 4.3.5), during development in the faeces and possibly *in utero* before the eggs were shed by the female, but not during the parasitic stages of their life-cycle. Thus it might be suggested that *Calliandra* would have different effects on worms from the donor and recipient lambs. However, apparently inconsistent expressions of fecundity in both donors and recipients as observed in the current experiment are difficult to explain.

In theory, 24-hour faecal collection was a less sensitive technique for detecting differences in egg output than maintaining female worms in culture plates, since egg counts were only performed on a small sub-sample of faeces for each 24-hour period, and the Mc Masters technique used had a sensitivity of only 30 epg. In addition, any variance in the parameters measured (faecal egg counts, faecal output, faecal DM content) would become multiplied in the final calculation of eggs per female worm. Incubating female worms in culture plates allowed direct measurement of total egg output for individual worms over a specified period of time, giving a much more reliable result as long as the culture conditions remained constant. This being the case, large differences in egg output between diets would be detected by either technique, but small differences may not be evident from a 24-hour faecal collection.

For *T. colubriformis* it is reasonable to postulate that differences in donor egg output between the diets were large and easily detected by either technique, whereas in the recipients there was a much smaller reduction in egg output, detectable only by the

more sensitive technique. This could also apply to *H. contortus*, but only if the probable reasons given in Section 8.5.1 (b) to explain the different results for donor egg production were correct. If not, the reason for these discrepancies remains speculative.

The larger number of eggs *in utero* for female *T. colubriformis* in the TC group compared with the TL group suggests an inhibition of egg shedding similar to that seen in the pilot experiment (Section 3.3.1) since egg output was not increased regardless of how it was measured. This lends support to the idea that there was in fact a small decrease in egg output by female *T. colubriformis* in the second generation.

In contrast to the observation of Shaik *et al*. (2006) who noted reduced percentages of females in three species of GI nematodes in tannin-fed goats, there was no effect of diet on male: female ratios in the current experiment. Although there was a slight species difference in the ratio in both donor and recipient lambs, the biological significance of this difference is doubtful, since the ratio was nearly 1:1 in all groups. Generally a 1:1 ratio is considered normal. Although an increase in the percentage of male worms is known to occur in unfavourable environmental conditions (e.g. overcrowding of worms within the host; Wharton, 1986) there is no reason to believe that this occurred in the current experiment.

8.5 Conclusion

Feeding *Calliandra* condensed tannins to parasitised lambs effectively reduced GI parasite egg output for *T. colubriformis* and probably for *H. contortus*, despite the conflicting results obtained for the latter from the different techniques used to measure egg output. The number of infective larvae resulting from the infections also decreased for both species of worm, but whether this was solely due to the reduced egg output or whether there was a concomitant reduction in hatch rates and/or larval development remains unclear. The possibility that eggs exposed to tannins are more likely than controls to develop to the infective stage cannot be discounted, and

deserves further investigation as it could pose a serious drawback to the use of *Calliandra* for GI parasite control.

Exposure of the parent worms to condensed tannins, instead of reducing establishment rates in the second generation, actually seemed to improve establishment. The effects on egg production by this second generation of worms were more equivocal, but there is evidence that, at least for *T. colubriformis*, some degree of inhibition of egg shedding occurred. This is probably also true for *H. contortus*, but the results were confused by problems with the experimental technique.

CHAPTER 9

GENERAL DISCUSSION

Gastrointestinal nematode parasites cause significant economic losses to livestock farmers through loss of production, clinical illness and the death of severely affected stock. Unfortunately the usefulness of chemical drenches to control GI nematodes is becoming increasingly curtailed by the development of anthelmintic resistance. In the search for viable alternatives to commercial drenches, forage legumes containing high concentrations of condensed tannins have emerged as possible contenders. These plants may improve both the resistance and resilience of ruminants to parasite infections by improving protein nutrition. More importantly, tannin-rich forages may also have some anthelmintic action against common GI nematodes in ruminants.

The main purpose of the current study was to investigate the possible role of selected tropical legumes, particularly *C. calothyrsus*, on two important tropical parasites of sheep. *In vivo* experiments were used to determine the effects of *Calliandra* on the establishment, viability and reproductive capacity of parasitic stages of the worms. *In vitro* techniques were used to determine the effects of *Calliandra* on free-living stages and to identify other legumes with anthelmintic activity in a rapid screening test. An attempt was also made to develop a staining technique that could be used to examine the interaction of condensed tannins with nematode and animal tissues.

The predominant finding of this work was that *Calliandra* condensed tannins reduced the reproductive capacity of adult *H. contortus* and *T. colubriformis*. The experiments described in Chapters 3 and 4 clearly demonstrated that *Calliandra* reduced the egg production of these two worms when fed as the sole diet to the host lambs. Protein nutrition and the responses of the host animals to the worms were not improved by feeding *Calliandra*, indicating that the reduced egg production was due to a direct effect of the tannins and not to improvements in the resistance or resilence of the host. The worms in these experiments were exposed to condensed tannins for the entire parasitic phase of the life cycle and it remains to be seen whether *Calliandra* tannins inhibit worm egg production if the worms are first exposed to tannins as adults.

The mechanism by which *Calliandra* tannins reduce egg production by female worms is still not clear, but there is some evidence, at least in *T. colubriformis*, that eggs are retained in the uterus. *Calliandra*-fed lambs had lower faecal egg counts than lucerne-fed lambs, despite increased numbers of eggs *in utero* in the female worms from the *Calliandra*-fed lambs (Sections 3.3.1 and 4.4.1). The presence of larvated eggs *in utero* [Section 7.3.5 (b)] might also suggest that eggs were retained longer than usual, although such a conclusion should be drawn cautiously as retained larvated eggs were observed only once. The results of the staining experiment (Chapter 7) suggested that worms ingest condensed tannins. The tannin appeared to be bound to the microvilli of the gut, rather than free in the gut lumen. However, the link between the ingestion of tannins and reduced egg production (if any) remains unknown.

In addition to reducing egg production, *Calliandra* also reduced the egg hatch rates of *H. contortus* and *T. colubriformis* (Chapters 5 and 8). There was no evidence from the staining experiment (Chapter 7) that condensed tannins were able to bind to the outside of eggshells, but this could have been due to the limitations of the technique rather than to a lack of binding. The staining technique used may need modification for use with microscopic structures such as eggs and larvae.

The lambs in the *in vivo* experiments consumed *Calliandra* continuously for at least a week prior to infection with worms and throughout the period of infection. *Calliandra* condensed tannins thus did not appear to affect worm establishment in any way. Infective larvae usually exsheath in the GI segment immediately anterior to the adult habitat. Therefore, *T. colubriformis* would be exposed to abomasal concentrations of tannin at exsheathment, and *H. contortus* to ruminal concentrations. Tannin concentrations in both segments were well above the range required to prevent the development of eggs and early free-living larval stages (Chapter 5) or to reduce egg production in adults (Chapters 3, 4 and 8). It would thus appear that the L_3 and L_4 stages were more resistant to the effects of *Calliandra* tannins than other life-cycle stages.

Calliandra did not affect the viability of adult worms. In the staining experiment (Chapter 7), condensed tannins were present on the external surface of the cuticle in

some instances, but it was not possible to determine whether this was bound tannin or adherent digesta containing tannins. Adult worms in the three *in vivo* experiments survived exposure to tannins throughout the pre-patent period and eight days after patency. It thus seems unlikely that *Calliandra* tannins exert any adverse effects on GI nematodes by binding to the cuticle, even if such binding does occur.

The failure of *Calliandra* condensed tannins to eliminate worm burdens is not necessarily a disadvantage. Continued antigenic challenge may be required to stimulate the host immune system to maintain an effective immune response against GI nematodes such as *H. contortus*. While this inevitably entails some loss of productivity, it is preferable to the consequences of clinical parasitism that may occur in the absence of any host resistance (see Chapter 2). Complete removal of nematode burdens could leave the host animal susceptible to re-infection, and to severe production losses or clinical disease when challenged. Anthelmintic plants such as *Calliandra* may be beneficial by reducing worm burdens to a level that stimulates the host immune response without eliciting pathogenic effects. Longer-term studies of the effects of feeding *Calliandra* are required to confirm this possibility.

The primary use for forages containing condensed tannins is likely to be as a means of decreasing pasture contamination with nematode eggs, either as a result of reduced egg output as in the case of *Calliandra*, or as a result of reduced numbers of egg-producing females. Ultimately such reductions would result in lower re-infection rates, but there could be short-term benefits as well. Exposure to in-coming larvae results in production losses because it represents a nutritional cost to the host due to the need to mount an immune response [Section 2.3.7 (b)]. Reductions in pasture larval contamination could thus mitigate the effects of larval challenge. Feeding of tanniniferous plants alone is unlikely to reduce worm populations enough to eliminate the need for chemical drenches, but they could be used in conjunction with other alternative control methods to maximise the effects of strategic drenching and reduce the number of drenches required.

For *Calliandra* to be beneficial to small-holder farmers as an anthelmintic, practical guidelines for its use need to be developed. As discussed in Chapter 4, feeding *Calliandra* as a sole diet is not tenable long-term, due to the antinutritive properties

of the legume. However, digesta tannin concentrations sufficient to reduce faecal egg counts and egg hatch rates could probably be obtained by feeding *Calliandra* at dose rates closer to 30 % of dietary DM to maximise both the anthelmintic and nutritive benefits of the legume. This needs to be confirmed in future studies. Because the concentration of condensed tannins in the GI tract is likely to be critical to achieving anthelmintic effects [Section 2.7.3 (b)] other factors affecting tannin concentration also require investigation. The lambs used in the current study were restrictively fed but in farming situations animals are more likely to spread their intake throughout the day. This could result in lower but more sustained levels of condensed tannins in the GI tract. It would be desirable to see if faecal egg counts would still be reduced if the same quantity of *Calliandra* was fed in divided doses. The effects of the rate of feed intake may need to be taken into account when designing feeding strategies.

The main objective of the experiment described in Chapter 8 was to determine the effects of exposure to *Calliandra* tannins on a second generation of worms. Although there was a reduction in the number of infective larvae due to the previously documented effects on egg production and hatching, larval development to the adult stage was not impaired and worm burdens were actually increased. Although the capacity of the resulting adults to reproduce normally appeared to be slightly reduced, in practical terms, *Calliandra* would most effectively control GI nematode parasites in lambs when fed to the lambs directly, rather than relying on a carry-over effect to future generations of parasites.

The anthelmintic effects of some tanninferous forages are retained and possibly even improved by preservation of the forage as hay, pellets or silage [Section 2.7.3 (b)]. Condensed tannin concentrations in tropical forages tend to be highest during the dry season [2.6.2 (b)]. This would suggest that the antinutritive effects of tannins in tropical forages would be greatest during the dry season, the time of year when the nutritive value of grasses is lowest. Assuming the nutritive value of the forage is not compromised by the preservation process, there may be some nutritional benefit in preserving forages containing condensed tannins during the wet season, when yield is high and tannin content is low. Current research in the NPM unit at JCU has shown that the nutritive value of dried *Calliandra* is comparable to that of fresh *Calliandra* (Machanja, 2007). The anthelmintic effects

of dried tropical forages deserve further attention in order to increase management options for farmers.

Expansion of the range of plants known to have anthelmintic properties would benefit farmers by enabling them to choose forages to suit particular climatic and geographical conditions. In addition to *Calliandra*, *Desmanthus* and *Leucaena* were identified in egg hatch and larval development assays (Chapter 6) as having anthelmintic potential. These *in vitro* results need confirmation *in vivo*, but offer the prospect of alternative plants for use in varying agronomic conditions. *Calliandra* is not very resistant to drought, and as discussed in Section 6.4.4, *Desmanthus* might be a better option in dry conditions. *Desmanthus* also lends itself to grazing *in situ*, whereas the taller shrubs would be more convenient in cut and carry feeding systems.

The main advantage of *Leucaena* is its high nutritive value. The tannins in *L. leucocephala* have low affinity for protein at pH 2.5 and pH 7.5 (McNeill *et al*., 1998). Tannin-protein complexes formed in the rumen readily dissociate in the abomasum and duodenum, releasing the protein for use by the animal. Thus although *L. leucocephala* appears to be less potent than *Calliandra* in terms of anthelmintic effects, the higher nutritive value of the former would make it a viable alternative to *Calliandra* in parasite control programs.

In a review of alternative control methods, Barger (1997) pointed out that any successful parasite control strategy would ultimately exert a degree of selection pressure on the exposed nematode population to develop resistance to that particular strategy. Brooker (2005), in a discussion on the bioactive effects of terpenoids suggested that, because terpenoids interacted with a number of structurally different cellular targets, it would be difficult for microorganisms to develop resistance. It could be argued that condensed tannins, which can bind to numerous proteins, as well as carbohydrates and minerals, might also present a challenge to the development of resistance. However, such an argument would be premature while the exact nature of the interaction between tannins and worms remains unknown. In the experiment reported in Chapter 8 of this study, the development of larvae from eggs exposed to *Calliandra* condensed tannins in the host diet may have been higher than the development of larvae from unexposed eggs, despite lower hatch rates in the

former. The larvae exposed to *Calliandra* in the egg were also better able to establish an infection in a new host. The logical conclusion is that *Calliandra* condensed tannins exerted a selection pressure on nematode eggs, resulting in the survival of the more robust larvae. These robust larvae may have been the ones most resistant to the effects of tannins. It is sobering to reflect that this occurred after only one exposure to *Calliandra* tannins. The *in vitro* work reported in Chapter 5 did not support these observations, but instead suggested that *Calliandra* tannins reduced larval development. In light of this, it would be interesting to follow larval development through several generations of nematodes and determine whether larval development increased after repeated exposure to condensed tannins.

Because *Calliandra* does not appear to kill the egg-producing females, any selection pressure exerted on the larval stages favouring the development of resitance to *Calliandra* tannins could possibly be overcome by a dilution effect. It is possible that if exposure to condensed tannins was interrupted, the continued production of eggs containing larvae of both susceptible and resistant genotypes could slow the development of resistance by diluting the resistant population with susceptible larvae. Although purely speculative, this might be an argument in favour of intermittent feeding of *Calliandra* to livestock, rather than offering it on a continuous basis. Currently, the duration of reduced faecal egg counts after cessation of *Calliandra* feeding is unknown. Elucidation of the persistence of the anthelmintic effects of *Calliandra*, and the potential for development of resistance, should be given high priority if *Calliandra* is to be considered seriously as a means to control parasites.

Shrub legumes such as *Calliandra* already have a vital role in tropical ruminant nutrition due to their value as high protein feeds. Some of these plants, particularly *Calliandra*, now show additional promise as herbal anthelmintics to reduce GI nematode larval contamination of tropical pastures and the subsequent reinfection of livestock. Although there remains a question about the prospects for larvae to develop resistance to the tannins in these plants, they have the potential to be a valuable tool, used in combination with other alternative control methods, to reduce the need for the use of chemical drenches.

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