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**The role of condensed tannins towards ovine nematodes
and their consequences on host performance**

Spiridoula Georgiou Athanasiadou

A thesis submitted towards the degree of

Doctor of Philosophy at the

University of Edinburgh

2001

Dedication

This work is dedicated to my parents Maria and Georgios for their love and support. Thank you for encouraging me to put my goals high.

Anyone who has never made a mistake has never tried anything new.

Albert Einstein (1879-1955)

Declaration

I hereby declare that this thesis is of my own composition and all assistance has been fully acknowledged. The results presented herein have not previously been submitted for any other degree or qualification.

Spiridoula Georgiou Athanasiadou

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It can be no dishonour to learn from others when they speak good sense.

Sophocles (495-406 BC)

Abstract

The aim of this thesis was to investigate the two hypotheses that have been put forward to account for the reduction in the level of parasitism observed in sheep that consume condensed tannins. For this purpose *in vitro* (larval development/ viability assay) and *in vivo* experiments were performed. In the first two experiments, a model condensed tannin extract (Quebracho) was given as a drench to parasitised sheep to test for the direct effect of condensed tannins towards established adult nematodes. Experiment 1 aimed to provide evidence for a direct anthelmintic effect of condensed tannins towards an established *Trichostrongylus colubriformis* population. For this purpose the extract was administered to sheep four weeks after their initial infection. There was clear evidence for a direct anthelmintic effect of condensed tannins towards adult *T.colubriformis*, as demonstrated by reduced faecal egg counts and worm burdens. As *T.colubriformis* is an intestinal ovine nematode, the susceptibility of other intestinal and abomasal ovine nematodes to condensed tannins was investigated *in vitro* and *in vivo*.

In vitro larval development/viability assays were performed to test for the effects of different concentrations of Quebracho extract on pre-parasitic larval stages of one intestinal (*Trichostrongylus vitrinus*) and two abomasal nematodes (*Teladorsagia circumcincta* and *Haemonchus contortus*) (Experiment 2a). The results from these assays showed that larvae from all species were able to develop to infective larvae, irrespectively the concentration of Quebracho extract. However, the viability of infective larvae of all species decreased, as the concentration of condensed tannins increased. Concurrently, Experiment 2b was designed to test whether condensed tannins had a direct anthelmintic effect towards adult populations of either *T. circumcincta* or *H. contortus*, or towards a mixed intestinal population of *Nematodirus battus* and *T.colubriformis*. Both ovine intestinal species were susceptible to the presence of condensed tannins in the gastrointestinal tract of sheep, as demonstrated from reduced faecal egg counts and worm burdens. On the other hand, neither the fecundity nor the worm burden of abomasal nematodes was affected by the presence of Quebracho extract. The absence of a direct effect against abomasal species could have been due to either species-specific differences in susceptibility or organ-specific physico-chemical differences.

In Experiments 3 and 4, Quebracho extract was incorporated in both low and high protein diets of parasitised sheep, in order to test for direct and indirect effects of condensed tannins during the establishment and the maintenance of an intestinal nematode infection. Experiment 3 tested whether the consumption of high protein foods supplemented with Quebracho extract fed as an allowance of the liveweight would reduce the level of intestinal parasitism in sheep and consequently improve their performance. Although the level of the parasitic infection, as judged by faecal egg counts and worm burdens, was reduced in sheep offered the foods supplemented with Quebracho extract, their performance was not improved. It was proposed that this could have been due to an anti-nutritional effect of condensed tannin on food digestibility, which sheep were not able to overcome because of the restricted feeding regime. The final experiment of the thesis (Experiment 4) aimed to test whether the *ad libitum* consumption of either low or high protein foods supplemented with Quebracho extract would reduce the level of intestinal parasitism and achieve improved performance. It was hypothesised that *ad libitum* intake would enable sheep to eat to the extent required to overcome anti-nutritional effects of condensed tannins upon digestibility. For this purpose, the Quebracho extract was added in the foods of parasitised sheep at the same level as in experiment 3. The level of parasitism was reduced in sheep offered foods supplemented with Quebracho extract only during the initial establishment of the infection, as indicated by reduced faecal egg counts. Although the digestibility of foods was not affected by the addition of Quebracho extract in the foods, the performance of sheep was not improved. It was proposed that sheep fed *ad libitum* might require higher concentrations of Quebracho extract in their foods to reduce the level of parasitism, compared to sheep fed restrictedly, as a result of reduced retention time of condensed tannins in the gastrointestinal tract.

The outcomes of the above experiments are brought together and the potential applications of condensed tannins as an alternative to control gastrointestinal parasitic infections in sheep are considered in the General Discussion.

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Chapter One

General Introduction

1.1. Introduction

Helminth parasitic infections and especially gastrointestinal nematode infections of grazing and browsing ruminants have been recognised as major productivity and welfare challenges in agricultural systems. Subclinical gastrointestinal nematode infections have been considered responsible for reduction in meat, wool and milk production. Clinical infections deteriorate animal health and can even result in livestock death. Many of conventional anthelmintic drugs, which have been traditionally used to control gastrointestinal parasitism, are effective for a period of time, but their efficacy may decrease due to the development of resistant strains of nematodes (Waller, 1997). Pharmaceutical companies are forced to continuously search for new drugs to help farmers maintain high productivity and reduce parasitic infections in their livestock. Resistance of nematodes towards anthelmintic drugs is particularly evident in small ruminants (Coop et al, 1994). The use of low efficacy anthelmintic drugs towards nematodes (90-95% efficacy), the high frequency of treatment, in lower doses and for longer periods of time than necessary are usually responsible for the development of anthelmintic resistance (Hansen and Perry, 1994). Many cases of anthelmintic resistance have been reported world-wide (see review by Jackson and Coop, 2000); resistance of goat and sheep nematodes, such as *Teladorsagia (Ostertagia) spp*, *Trichostrongylus spp*, and *Haemonchus spp* has been reported towards the benzimidazoles, avermectins, tetrahydropyrimidines/imidothiazoles, salicylanilides and organophosphates (see review by Sangster, 1999). It is evident that controlling gastrointestinal nematode infection with frequent anthelmintic drug treatment is problematic and it has become necessary to investigate alternative approaches to control gastrointestinal parasitism for sustainable agricultural systems.

However, it is not only the development of anthelmintic resistance that demands the investigation of alternative ways to control nematode infections. The unrestrained use of anthelmintic drugs, such as their use without prescription, and the introduction of drugs with greater persistence (bolus) in the farm animals, have alarmed both scientists and consumers about the potential presence of drug residues in agricultural products (McKellar, 1997). In addition, effects of anthelmintic drugs or their metabolites on non-target organisms in the environment (see review by Waller, 1992), environmental pollution from drug residues and the development of organic farming systems over the last years (Thamsborg et al, 1999) have all increased the demand for alternative, sustainable approaches to control parasitism.

Various alternatives to control gastrointestinal nematode infections of ruminants are currently being investigated. Breeding parasite resistant hosts by genetic selection (see review by Gray, 1997), developing effective vaccines towards economically important nematodes (see review by Smith, 1999), biological control of nematodes by using nematophagous fungi (see review by Thamsborg et al, 1999), nutrient and especially protein supplementation of the diets of parasitised hosts (see review by Coop and Kyriazakis, 1999) and the use of plants with anthelmintic properties (see review by Hammond et al, 1997) are some of the potential alternatives that are currently being investigated. With regard to the latter, plants with anthelmintic properties have been used in traditional medicine, to prevent and even treat parasitic infections. Tribal people from Africa or Asia have been using extracts from forages or trees to treat gastrointestinal nematode infections in human or animals (Chhabra and Mahunnah, 1994; Akhtar et al, 2000). Extracts from South African plants have shown anthelmintic properties towards free-living nematodes (McGaw et al, 2000). Recent evidence has suggested that the consumption of forages that contain tannins reduces the detrimental effects of parasitism in young grazing sheep (Niezen et al, 1993;

1998b). Tannins, which are secondary plant metabolites, are known as defence mechanisms of plants towards herbivores. High concentration of tannins in plants has been considered responsible for reducing the palatability and hence quality of the forage, and causing anti-nutritional and toxic effects to herbivores (see review by Kumar and Singh, 1984). Reduced food intake, digestibility and growth, renal and liver failure, and even deaths have been reported following the consumption of forages high in tannins (Butler, 1992). However the consumption of condensed tannins, which is a sub-class of tannins, has also been considered responsible for causing beneficial effects to grazing ruminants, when consumed in moderate concentrations (see section 1.4.3.4.). Forages that are considered responsible for reducing the detrimental consequences of parasitism in grazing sheep, as indicated above, contained tannins of the condensed type.

To account for the reduction of the detrimental effects of parasitism in growing sheep following the consumption of forages that contain condensed tannins, two hypotheses have been developed. The first hypothesis concerns a direct anthelmintic effect of condensed tannins on larvae or adult nematodes; the second hypothesis is related to an indirect effect on the parasitised host by increasing protein availability in the small intestine of the ruminant. This thesis focuses on investigating the direct anthelmintic effect of condensed tannins towards larvae and adult gastrointestinal nematodes of sheep and addressing the indirect effect of condensed tannins on the parasitised host.

As young sheep are more susceptible to the detrimental effects of gastrointestinal parasitism than adult sheep, this thesis will focus on the interaction between condensed tannins and gastrointestinal parasitism in growing sheep. This introductory chapter consists of three sections: the first section is an overview of the life-cycle of nematodes and provides

information on their nutritional requirements. This information is considered important to the potential interactions of condensed tannins and nematodes. The second part consists of a review of the properties of condensed tannins, which may play an important role in their effects towards either the nematode or the parasitised host. The last section of this chapter deals with the available evidence concerning the effects of condensed tannins on the parasitised and non-parasitised host and thus prepares the ground for the objectives that will be addressed in the experimental chapters.

1.2. Gastrointestinal nematodes

1.2.1 Life-cycle

Nematodes of ruminants have direct life cycles, i.e. they do not require the presence of an intermediate host to develop (Lyons, 1978). This section will focus on four nematode species used throughout the experiments of this thesis, which belong to the *Trichostrongylidae* family: *Teladorsagia circumcincta* and *Haemonchus contortus* are abomasal ovine nematodes and *Trichostrongylus colubriformis* and *Nematodirus battus* are intestinal ones. These species were chosen for use in the experiments as they are of economic significance and also they have been reported resistant towards anthelmintics in many cases (see review by Jackson and Coop, 2000). Gastrointestinal nematodes spend only half of their life-cycle inside the host as parasites (from infective larvae to adult), and they spend the other half in the environment (from egg to infective larvae; non-parasitic stages). Adult *T. circumcincta* and *H. contortus* inhabit the abomasum of the host and start the production of eggs, which pass through the digestive tract and are excreted in the faeces. Eggs hatch into first stage larvae (L₁), usually within 24h. L₁ develop to second stage larvae (L₂); both these stages feed on bacteria present in the faeces. The infective stage (L₃) can be reached within 7-10 days (Lapage, 1968). Grazing sheep ingest the infective larvae with their food, which at this stage are surrounded by a loose protective sheath (ensheathed larvae). This sheath is the cuticle that remained attached to larvae following the moulting from second to third stage (Urquart et al, 1996). Ensheathed L₃ is the only non-feeding stage of the life-cycle of the nematodes. During this stage, larvae survive from the food reserves they accumulated whilst in the L₁ and L₂ stages. Infective larvae lose the sheath in the rumen, under the influence of CO₂, pH and temperature (exsheathed larvae). Larvae proceed towards the abomasum, moult twice to become adults, and adults are located in the

lumen or in close association with the abomasal glands. Three to four weeks after the initial infection, the sexually mature female worms are able to produce eggs (Georgi and Georgi, 1990).

Gastrointestinal nematodes that are mainly located in the small intestine of sheep as adults, such as *T. colubriformis*, have a similar life-cycle to the one described above; a difference concerns the exsheathment of the L₃, which occurs in the abomasum and not the rumen as mentioned for the abomasal species (Lapage, 1968). Exsheathed L₃ move to the small intestine within 2-3 days after infection, enter the mucosa, moult once and return to the lumen to develop to adult nematodes (Olsen, 1962). The life-cycle of *N.battus* is also similar, with the main difference related to the development of L₃, which occurs inside the egg in the environment. When the infective larvae are formed, and the conditions are suitable in the environment (temperature and moisture) the eggs hatch (Soulsby, 1968). Infection of the host occurs through the mouth and larvae develop to adult nematodes in the intestine about 28 days after initial infection (Lapage, 1968). Adult *N.battus* worms are associated superficially with the surface of the mucosa, whereas *T.colubriformis* adults tend to burrow under the intestinal mucosa.

1.2.2. *The nutrition of the nematode*

It is not well established how larvae and adult gastrointestinal nematodes feed in the alimentary tract of the host. However, it is well known that nematodes have specific nutritional requirements. They need lipids that provide energy for the periods of starvation (ensheathed L₃), purines for the synthesis of DNA and carbohydrates (Matthews, 1998). Although they can synthesise most of the amino acids they require whilst in the host, they still need to obtain some via their food. All nutrients obtained from the food are stored in the

intestinal cells of their alimentary tract, which are especially modified for this purpose (Wharton, 1986a).

Adult nematodes are mainly liquid feeders (Wharton, 1986a). Adult *T.circumcincta* parasites are closely associated with the abomasal mucosa; they cause plasma loss into the abomasum and possibly they either feed from this leakage or from the lumen content in the abomasum. *H.contortus* worms are blood feeders; they ingest blood from the mucosal vessels in the abomasum (Barrett, 1981). For *T.colubriiformis* worms, it has been suggested that they tend to burrow under the intestinal mucosa to obtain the nutrients they require just before their absorption. As *N.battus* adults are located in the lumen of the first half of the small intestine, they obtain the required nutrients from the content of the lumen (Lapage, 1968).

1.2.3. Alternative approaches to control nematode infections

As anthelmintic resistance is becoming an important problem in the control of nematode infections, evaluation of the sustainability of the alternative approaches to control nematode infections is required. Difficulties to identify appropriate vaccine antigens to produce sustainable vaccines towards gastrointestinal nematodes (see review by Smith, 1999), possible long-term environmental effects of the use of nematophagous fungi (see review by Thamsborg et al, 1999), or the time-consuming selection of livestock for natural resistance to nematodes due to genetic variation (Waller, 1999) are problems related to the alternative approaches indicated earlier. Although the consumption of tanniferous forages is not free of adverse effects on animal production (see section 1.4.2.) their incorporation in organic and conventional farming systems is expected to be easy, as they already exist in many temperate and tropical countries (Barry et al, 2001). The following section focuses on the nature and properties of tannins and on the potential ways of tannin interaction with the nematodes and the parasitised hosts.

1.3. Tannins: plant secondary compounds

Tannins are secondary plant polyphenols and they are located inside the vacuole of the plant cell. They may be present in many parts of the plant, but are usually present in higher concentrations in the woody tissues and in lower concentrations in the leafy parts of the plants (see review by Bernays et al, 1989). Various theories have been developed to account for the biological significance of tannins (Mueller-Harvey, 1999). They have been considered waste products, stress products and amongst the main anti-herbivore defence mechanisms of plants. Although tannins are secondary plant metabolites, their production is under strict genetic control and has appreciable energetic requirements (Waterman and Mole, 1994a); thus it is difficult to accept that they are waste by-products (Hagerman and Butler, 1991). The consumption of high concentrations of tannins by insects or mammalian herbivores can result in reduced growth and enzymatic activity, reduced fermentation and even death of the herbivore (see review by Bernays et al, 1989). Tannins have a high affinity towards macromolecules and they can form complexes with proteins, starch, cellulose, fats, nucleic acids, amino acids and digestive enzymes (Mueller-Harvey and McAllan, 1992). Tannins have a great biodiversity: tannins that are produced by different plants, or those produced by the same plants but at different times of the year, or by different parts of the plants, may have different physical and chemical properties (Waterman, 1999).

1.3.1. Classification and properties of tannins

For a compound to be classified as tannin, it must exhibit certain chemical characteristics. It must consist of multiple units that contain free phenolic groups, have a molecular weight above 500 daltons, the ability to precipitate protein and result in astringent taste when consumed by herbivores (Hagerman and Butler, 1991). Tannins can be categorised according to their molecular weight, their structure, their water solubility and their binding

capacity. Due to their variability, their definition and classification is problematic. A widely accepted structural classification of tannins is into two major groups: hydrolysable and condensed tannins, with other smaller groups having been identified (protannins, oxytannins and b-tannins) (Hagerman and Butler, 1991).

Hydrolyzable tannins are polymers of gallic acid (Fig. 1.1a) and consist of a carbohydrate core, in which the hydroxyl groups are esterified to gallic, m-digallic (gallotannins) or hexahydroxydiphenic acid (ellagitannins) (Mangan, 1988). They are polyphenols of low molecular weight and are susceptible to hydrolysis as a result of their intermolecular ester bonds (Field and Lettinga, 1992). When hydrolysable tannins (Fig 1.1b) are consumed by ruminants, they are easily degraded in the digestive tract, due to their intermolecular ester bonds, into gallic acid (Butler and Rogler, 1992). The gallic acid can either be further metabolised into lower molecular weight phenols that can be absorbed in the rumen (Reed, 1995), or be absorbed "as is" in the small intestine and excreted in the urine (Butler and Rogler, 1992). Due to their absorption by the gastrointestinal tract of herbivores, hydrolysable tannins have been considered responsible for causing toxic effects in herbivores (D'Mello, 1992). Necrosis of the intestinal mucosa, liver damage and kidney necrosis were post-mortem findings in chickens (see review by Jansman, 1993), and rats (see review by Mueller-Harvey and McAllan, 1992), following the consumption of diets that contained 1-3% tannic acid, a hydrolysable tannin.

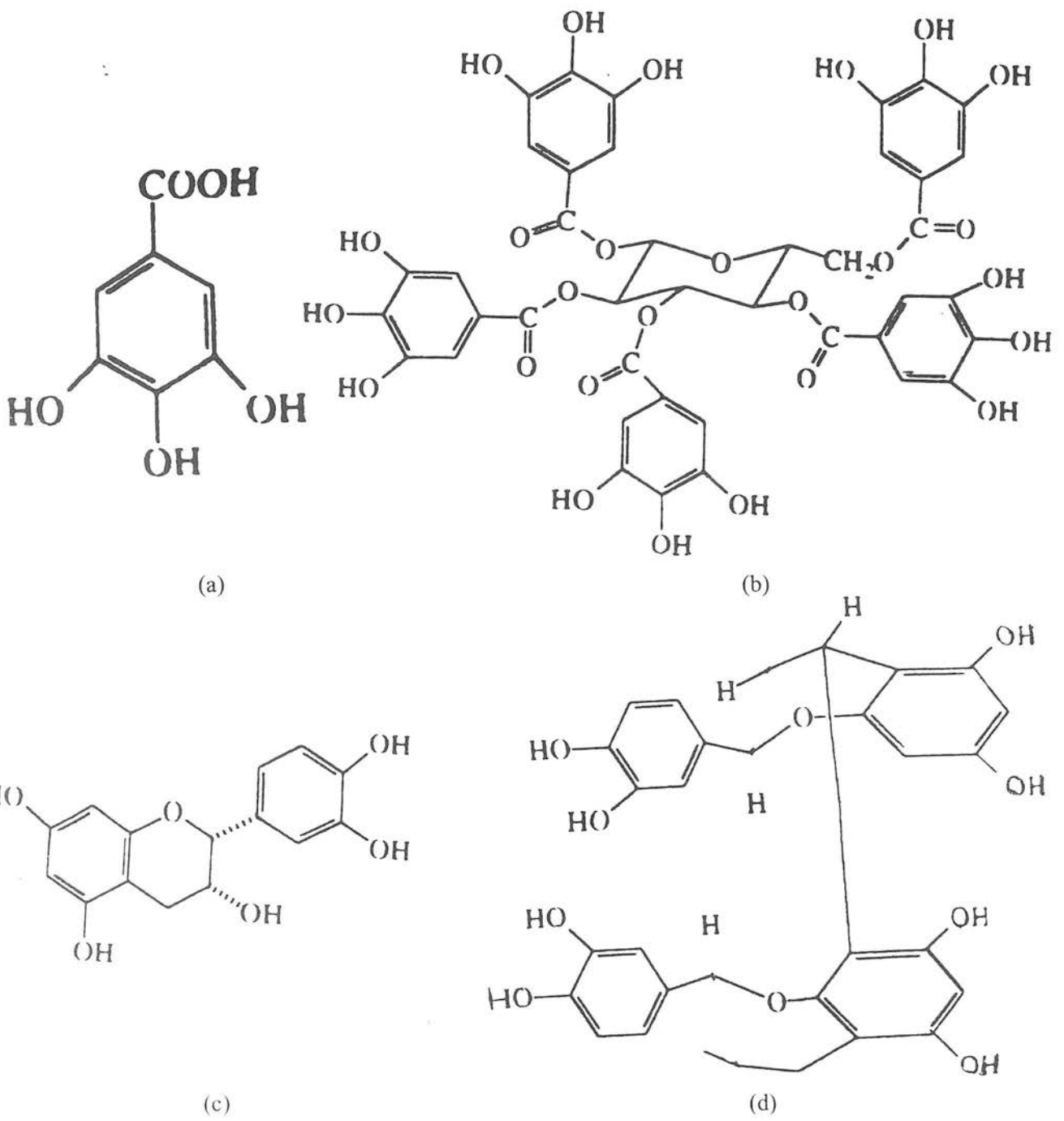


Figure 1.1. Gallic acid (a), a hydrolysable tannin, with esterified bonds between the gallic acid molecules (b), catechin (c), and a dimer of catechin, with a carbon-to-carbon bond between the two molecules (d).

Condensed tannins are polyphenols, usually of higher molecular weight than hydrolysable tannins, and consist of oligomers or polymers of catechin (flavan-3-ols, Fig. 1.1c) and related flavanol residues (See review by Bernays et al, 1989); they do not have a carbohydrate core as hydrolysable tannins have (Mangan, 1988). The oligomers of catechin, or flavanol residues, are linked to each other with carbon-to-carbon bonds (Fig 1.1d), which are not susceptible to hydrolysis (Larrauri et al, 1997). There is a large variation in the structure of condensed tannins, which is related to the number of oligomers that are linked, the positions of the links and their stereochemistry (Waterman and Mole, 1994a). Condensed tannins can be either linear, such as those from sorghum grains, or branched as condensed tannins from quebracho extract. When condensed tannins depolymerise, they produce mainly cyanidin or delphinidin, and therefore have been further classified as procyanidins or prodelfinidins. Other types of condensed tannins are profisetidins and propelargonidins (Hagerman and Butler, 1991).

It remains open to debate whether condensed tannins are degraded and absorbed by the gastrointestinal tract of herbivores, when they are included in their diet. Because of their direct carbon-to-carbon bond and thus stable bonding between the molecules they are not easily degradable (Field and Lettinga, 1992), and because of their high molecular weight they can not be absorbed by the digestive tract of herbivores. Although only 15% of condensed tannin intake was recovered in the faeces of sheep offered a diet that contained condensed tannins, it was proposed that the failure to recover the remaining amount of condensed tannins was due to a weakness in the method used, rather than to the absorption of condensed tannins by the digestive tract of sheep (Terrill et al, 1994). The method used to recover condensed tannins from faeces was a butanol-HCl assay and it was suggested that condensed tannins that were in complexes in the faeces were not extracted. As a result of the

low, if any, absorbency of condensed tannins from the gastrointestinal tract of herbivores, there is little evidence concerning the occurrence of toxic effects in herbivores following the consumption of forages that contain condensed tannins. Some toxic effects observed following the consumption of diets that contained condensed tannins were possibly due to other components of the diet. For instance, high molecular weight condensed tannins from *Sorghum bicolor* were not absorbed from the digestive tract of chickens (Jimenez-Ramsey et al, 1994). It was suggested that the toxic effects observed in chicks following the consumption of sorghum were attributable to lower molecular weight polyphenols that are contained in sorghum.

However, some degree of absorption of condensed tannins by the digestive tract of herbivores has been reported, which can be associated with low molecular weight condensed tannins or with depolymerization of condensed tannins. A 70% acetone solution failed to extract more than 25% of condensed tannin intake from the faeces of sheep, whereas 100% of condensed tannin intake was extracted from the faeces of deer and bears (Robbins et al, 1991). It has also been reported that some types of condensed tannins may get depolymerised and the products absorbed following ingestion (Clausen et al, 1990); the rate of polymerisation and the polymerisation products vary according the structure of condensed tannins. One of the most important chemical properties of condensed tannins is their ability to form soluble and insoluble complexes with macromolecules, such as protein, fibre and starch. As the protein-binding ability of condensed tannins determine the interactions between tannins and herbivores, the following section focuses on this aspect.

1.3.2. Condensed tannins and proteins: mode of action

The condensed tannin-protein complexes are mainly formed with hydrogen bonds between the hydroxyl group of tannins and the carbonyl group of the protein peptide (Waterman and

Mole, 1994a). However, there is evidence that hydrophobic interactions could also be important for the formation of these complexes (Hagerman and Butler, 1991). The affinity of condensed tannin to proteins is determined by both molecular mass and molecular configuration of both the tannin and the protein (see review by Bernays et al, 1989). Proteins of low molecular weight, globular and compact have a low affinity for condensed tannins (Waterman and Mole, 1994a). On the other hand, proteins of high molecular weight and with an open structure have a high affinity to condensed tannins. The ability of tannins to bind strongly to proteins is more effective at pH values close to their isoelectric point (Oh et al, 1985). The protein-binding ability also depends on the form that condensed tannins are available in the plant: for example, condensed tannins contained in forages are in a free form and available to form complexes when ingested by herbivores. In contrast, condensed tannins from cottonseed are in the form of complexes with macromolecules and thus not available to form new complexes (Yu et al, 1995). The tannin-protein binding is usually reversible, unless intermolecular covalent bonds have been established (Hagerman and Butler, 1991). Acid or alkaline pH, treatment with detergents (surfactants), and with phenol or organic solvents result in the disassociation of the complexes (Jones and Mangan, 1977; Hagerman, 1989; Waterman and Mole, 1994b).

Following the formation of condensed tannin-protein complexes, the complexes may or may not aggregate into larger units and precipitate. In cases when protein is present in excess or tannin is present in small amounts, precipitation does not follow the formation of complexes. This is because each molecule of protein is in contact with only a few phenolic molecules (Oh and Hoff, 1987; Hagerman, 1989) and large particles are required for the precipitation of the complexes. Apart from the concentration of tannins or proteins in the solution, the occurrence of precipitation depends also on the ability of the tannin-protein bonds to

overcome possible repulsive electrostatic forces that might exist between the protein molecules (Oh and Hoff, 1987). These forces are at their minimum level at the isoelectric point of the protein, and thus it is more likely to observe protein precipitation at that point. Asquith et al (1987) suggested that sugar molecules might also improve the solubility of tannin-protein complexes. This property of condensed tannins to either bind and precipitate protein or not, may be of great biological value, since it is probably the underlying mechanism responsible for increased protein availability in the small intestine of the ruminant, as will be described in detail in section 1.5.2.

1.3.3. Condensed tannins and other macromolecules

Condensed tannins have a particular affinity to proline-rich protein (PRP). These proteins contain up to 40% carbohydrate by weight (Mehansho et al, 1985). It has been suggested that the strong affinity condensed tannins have for PRP could be due to the presence of the carbohydrates (oligosaccharides) in their molecule (Asquith et al, 1987), since they can maintain the protein in relatively open conformation. A protein in open conformation is more accessible for the formation of bonds with tannins. Gelatin, prolamine, proteins from seed coats and salivary proteins are PRP (Waterman and Mole, 1994a). Salivary PRP are produced in the saliva of certain rodents or mammals (rat, mouse, deer) possibly as a defence mechanism towards the adverse effects of tannins, when they are fed tannin-rich diets (Robbins et al, 1991). Details concerning the biological action of PRP will follow. Condensed tannins can also bind to starch, cellulose and lipids (see review by Mueller-Harvey and McAllan, 1992); there is evidence that they also form complexes with iron (Rao and Prabhavathi, 1982), manganese, copper, aluminium, calcium and other metal ions (Haslam, 1996).

1.4. Condensed tannins and herbivores

The consumption of forages or foods that contain condensed tannins has been associated with various consequences on both invertebrate and vertebrate herbivores; these depend mainly on the type of condensed tannins, and the protein and tannin concentrations in the foods. Studies that have investigated the consequences of consuming condensed tannins are reviewed in the following section. With special reference to ruminants, there are three levels of condensed tannins that have been used to determine the effects of condensed tannins on herbivore nutrition: low (<2% of dry matter (DM)), moderate (between 3 and 6% of DM) and high concentrations of condensed tannins (>7% of DM). Any reference made in the next section, to low, moderate and high concentrations of condensed tannins, corresponds to the concentrations mentioned above.

1.4.1. Invertebrate herbivores

The consequences of consuming diets high in condensed tannins have been studied in many insects and other invertebrate herbivores. They are reported briefly below, as they could prove to be useful in understanding the possible mechanisms of action of condensed tannins towards nematodes, as insects and nematodes have similar biology. Depending mainly on the eating habits of invertebrates, the consumption of tannin diets has been associated with either detrimental or beneficial effects to insects and other invertebrates. Organisms that are not used to including condensed tannins in their diets suffer more from the addition of condensed tannins. Adverse effects on invertebrate herbivores are mainly due to the formation of tannin-protein complexes and hence reduced protein availability for the organism. Condensed tannins were tested against the growth of the insect cotton pest *Heliothis zea* (Klocke and Chan, 1982). It was observed that the higher the concentration of condensed tannin in the diet, the slower was the growth of larvae. Other studies have

reported that insects, which were not regular tannin-consumers, showed many severe pathogenic lesions on the midgut epithelium, whereas insects that were adapted to consuming tannins showed very few or no lesions at all (Steinly and Berenbaum, 1985). Invertebrates that are regularly fed with plants rich in tannins have developed adaptation mechanisms to overcome any adverse effects of condensed tannins. In the insect gut, high pH in combination with the presence of surfactants (lysolecithin) or enzymes (digestive b-glucosidases) can reduce protein precipitation caused by tannins (Martin and Martin, 1984; Martin et al, 1985; Mole and Waterman, 1985). The presence of surfactants in the gut of marine insects allows them to consume tannin-rich brown algae (39%), without showing any signs of detrimental effects of condensed tannins (Tugwell and Branch, 1992). In conclusion, invertebrate herbivores can suffer from reduced growth and viability following condensed tannin consumption; the consumption of condensed tannins may affect similarly nematodes.

1.4.2. Vertebrate herbivores

1.4.2.1. Anti nutritional effects of tannins on simple-stomached animals

The consumption of condensed tannins has been associated with a number of detrimental effects on domestic animals. Non-ruminant animals are usually more susceptible to the presence of tannins in their diets. Food intake, liveweight gain and food conversion efficiency, of rats, pigs and chickens are often reduced following the consumption of moderate to high concentrations of condensed tannins (Rogler et al, 1985; Mangan 1988; van Leeuwen et al, 1993). Reduced digestibility in monogastrics that consume condensed tannins is also commonly observed. The activity of digestive enzymes (trypsin, a-amylase, aminopeptidase) was reduced following the addition of condensed tannin extracts both *in vitro* and in the intestines of rats (Horigome et al, 1988), young chickens (Yuste et al, 1992)

and weaned pigs, although no morphological changes on the gut mucosa could have been attributed to condensed tannins (van Leeuwen et al, 1995). The effects of condensed tannins consumption on the performance of simple-stomached livestock depends on the type and the concentration of tannin, the species and the age of the animals tested, the production level of the animals and the diet composition (see review by Jansman, 1993).

1.4.2.2. Anti nutritional effects of tannins on ruminants

The reduction in voluntary intake of ruminants fed diets that contain condensed tannin is one of the first adverse effects of condensed tannins observed. This can be due to the astringent taste produced when tannins bind to proline-rich salivary protein in the buccal cavity of the animal, which may result in reduced palatability of the food (Hagerman and Butler, 1991). Reduced palatability of a food that contains condensed tannins, however, has also been attributable to post-ingestive adverse effects of condensed tannins, such as reduced rumen function (Waghorn et al, 1994). More specific, *in vitro* studies indicated that condensed tannins inhibited the microbial attachment on the food particles in the rumen, formed complexes with microbial enzymes, and inhibited the digestion of fibre by bacteria (McAllister et al, 1994b). There is also evidence that condensed tannins can inhibit the growth, interfere with the morphology and the proteolytic activity of microbes in the rumen (McAllister et al, 1994a; Acamovic et al, 1999), as well as reduce the fungal population in the rumen (Mcsweeney et al, 1999). Excessive intake of condensed tannins can also result in reduced production of microbial protein due to reduced availability of dietary protein. Most dietary protein passes through the rumen in complexes with condensed tannins and thus there might be not enough available for microbial degradation (McNeill et al, 1999).

Although there is evidence for a reduction in dry matter and nutrient digestibility following the consumption of condensed tannins (see below), the reason for this reduction is not clear.

It has been suggested that condensed tannins may form complexes with the digestive enzymes in ruminants and reduce the digestibility of the food (Kumar and Singh, 1984), as has been mentioned for monogastrics. Haslam (1989) suggested that the possible mechanisms for reduced digestibility were either through the precipitation of the enzymes, or through the formation of soluble but inactive enzyme-tannin complexes, or through the formation of complexes with the substrates of the enzymes. Blytt et al (1988) supported the view that the adverse effects of tannins upon dry matter digestibility are due to the formation of less digestible tannin-protein complexes and not due to direct enzyme inhibition by tannins.

The consumption of diets high in condensed tannins has been considered responsible for increased faecal nitrogen excretion (Waghorn et al, 1994; Woodward and Reed, 1997). This increase could be due to reduced nitrogen digestibility, as has been observed in sheep offered *Lotus pedunculatus* with either moderate or low concentration of condensed tannins (Waghorn et al, 1994; Waghorn and Shelton, 1995). However, investigation concerning the origin of the nitrogen detected in the faeces of sheep offered condensed tannins suggested that it could have been endogenous (see review by Bernays et al, 1989; Acamovic et al, 1999). It is possible that condensed tannins bind to the intestinal wall or mucus present in the intestine of the host and these complexes could be excreted in the environment. There is further evidence supporting the hypothesis about the endogenous origin of faecal nitrogen. Faecal protein was high in proline, in rats that were fed diets that contained 7% crude condensed tannin extract (Mole et al, 1990). This suggests that PRP-tannin complexes, which are formed in the buccal cavity of the rat, may pass intact through the digestive tract and get excreted in the faeces.

Although condensed tannins are highly non-metabolised and undegradable compounds, as indicated previously, the consumption of forages high in condensed tannins has been considered responsible for causing death in grazing livestock. The consumption of *Acacia* spp. leaves, which are high in condensed tannins, resulted in increased mortality rates in ruminants (Rittner and Reed, 1992). Although there is no evidence for altered gut morphology following the consumption of foods high in condensed tannins, it is possible that condensed tannins affect the mucosa of the digestive tract and decrease the absorption of other nutrients (Reed, 1995). Condensed tannins may also be responsible for increasing the toxicity of other plant compounds, through their affinity to bind to essential amino acids. For instance, methionine, an essential amino acid, is involved in detoxifying cyanide; reduced concentration of methionine due to complex formation with condensed tannin will increase the toxicity of cyanide (Reed, 1995).

In conclusion, the anti-nutritional effects that follow the consumption of condensed tannins by ruminants are related to food intake, nutrient digestibility, performance and mortality in extreme cases. It has been proposed that the adverse effects of condensed tannins on ruminant nutrition are particularly evident when the concentration of condensed tannins in the food exceeds 5-6% of dry matter of the food, and they become more severe as the concentration increases (see review by Barry and McNabb, 1999). Apart from the concentration of condensed tannins in the food or forage, the occurrence of adverse effects of condensed tannins also depends on the development of adaptation mechanisms of animals towards tannins; these mechanisms are reported below.

1.4.3.3. Adaptation to condensed tannins

Herbivores that usually consume forages high in condensed tannins have developed various mechanisms to inactivate condensed tannins and overcome possible adverse effects of

condensed tannins on their health. The excretion of proline-rich protein has been considered the major physiological adaptation mechanism that neutralises dietary tannin (see review by Bernays et al, 1989). Deer, rats and black bears have the ability to produce these proteins from their salivary glands; PRP have higher affinity to tannins than most other protein (Robbins et al, 1991). As condensed tannins form complexes with PRP, which can pass through the digestive tract and can be excreted in the faeces of herbivores (Mole et al, 1990), condensed tannins do not have the possibility to bind to dietary protein. Salivary PRP typically contain small amounts of essential amino acids; thus their potential loss in the faeces as a complex with tannin, is a beneficial trade off that spares dietary protein richer in amino acids (Butler, 1992). Pigs do not produce salivary PRP, but they produce mucin, a mucopolysaccharide contained in the mucus of their digestive tract (van Leeuwen et al, 1993). Condensed tannins can form complexes with mucin and thus the function of digestive enzymes and the absorption of dietary protein are not influenced by the presence of condensed tannins in the foods. It has been reported that rats and chickens offered a high condensed tannin diet increased their intestinal mucin excretion, although it was not clear if this was due only to the presence of tannin (Sell et al, 1985). Other species, like sheep, which are of particular interest to the objectives of this thesis, do not produce PRP (Robbins et al, 1991) and they do not have other defence mechanisms towards condensed tannins; thus they are more susceptible to the anti-nutritional effects of tannins.

1.4.3.4. Beneficial effects of condensed tannins on ruminants

The consumption of condensed tannins by ruminants has been associated not only with detrimental but also with beneficial effects. It is generally accepted that the beneficial effects observed following the consumption of condensed tannins are mainly due to their protein-binding ability, which protects dietary protein from rumen degradation and thus increases protein availability in the lower digestive tract (see review by Barry et al, 2001).

Interestingly, the protein-binding capacity of tannins has been also considered responsible for causing adverse effects on livestock production (see above). However, beneficial effects on ruminant production are usually observed following the consumption of moderate concentrations of condensed tannins. Although this statement is not always unconditional, it is relatively representative of most studies investigating the effects of condensed tannins on ruminant nutrition.

During mastication of the plant material by ruminants, the vacuole of the plant cell (that contains condensed tannins) breaks and tannins have the opportunity to bind to plant protein. Mangan (1988) suggested that the most suitable pH for the formation of the complexes range between 4-7 and outside this range the complexes disassociate. This pH range was reported for the soluble leaf protein (F1), and although it is usually accepted as a general rule, it may explain the variation related to tannin-protein interactions. In the abomasum (pH 1-3) and the small intestine (pH 7-9), pH is suitable for the disassociation of the complexes. It has been suggested, however, that the presence of surfactants is also necessary for the disassociation of the complexes (Hagerman and Butler, 1991). Surfactants, like bile salts, are present in the small intestine, and thus it is possible that the disassociation of tannin-protein complexes mainly occurs in the small intestine. Although the mechanisms are not clear, it has been suggested that as tannins have a higher affinity to surfactants than to protein, they release the latter and form new complexes with the surfactants.

Although free condensed tannins in the small intestine can form new complexes with dietary protein, it is highly likely that the complexes are formed with endogenous protein rather than dietary protein (see review by Mole and Waterman, 1987). This was proposed following observations concerning the amino acid absorption from the intestine of ruminants and also

from evidence indicating a higher nutrient efficiency of a food that contains condensed tannins. The absorption of non-ammonia nitrogen post-ruminally in sheep grazing a forage that contained condensed tannins, was increasing as the total condensed tannin concentration in the plant increased up to almost 5% of the DM of the forage (Barry and Manley, 1986). Amino acid flow through the abomasum and absorption of essential amino acids was increased in sheep grazing *Lotus corniculatus* (2.2% of the DM) compared to control sheep (Waghorn et al, 1987). In addition, wool growth increased by 12% in sheep grazing forages high in condensed tannins, without any detectable changes on food intake (Wang et al, 1996) and lactating ewes and cattle increased their milk yield following the consumption of forages high in condensed tannins (McNabb et al, 1999).

Another beneficial consequence of the protein-binding capacity of condensed tannins is the anti-bloat activity of tanniniferous forages. Condensed tannins have been considered responsible for preventing bloat in ruminants, by inhibiting the cell-degrading action of enzymes produced by rumen bacteria. It is well established that the rapid release of soluble leaf proteins from the cells a few minutes after mastication, is the principal cause of the occurrence of bloat in grazing cattle (Lees, 1992). The affinity of condensed tannins towards the soluble leaf protein (F1 leaf protein) is high (Mangan, 1988); thus they bind to these proteins, precipitate them and reduce the possibility of bloat. In addition condensed tannins also diminish the production of gas by reducing rumen fermentation (Haslam, 1989). *Onobrychus viciifolia* (sainfoin) and *Lotus corniculatus* (birdsfoot trefoil) are two common non-bloating grazing legumes and they contain condensed tannins.

Currently, research has focused on the beneficial effect of condensed tannins on livestock production by reducing the detrimental effects of parasitism in grazing livestock. This ability

of condensed tannins may also be attributable to their ability to bind to protein, as it will be discussed in the following section. Most research has focused on ruminant parasites, since the problem of anthelmintic resistance is more evident in small ruminants. A detailed report follows on the possible interactions between condensed tannin and parasites.

1.5. Condensed tannins and parasites

The consumption of forages that contain condensed tannins has resulted in the reduction of the detrimental consequences of gastrointestinal parasitism with or without the level of parasitism being affected. Sheep grazing forages high in condensed tannins maintained high levels of productivity while carrying a substantial burden of *O.circumcincta* and *T.colubriformis* (Niezen et al, 1995), compared to sheep carrying a similar worm burden but offered a high quality tannin-free legume. In another study, faecal egg counts and worm burdens recovered from parasitised sheep grazing *Hedysarum coronarium*, a condensed tannin high forage, were significantly lower compared to sheep on tannin-free legumes (Robertson et al, 1995). Recently, red deer grazing legume forages high in condensed tannins had reduced lung and gastrointestinal worm burdens and maintained their productivity, compared to deer grazing tannin-free forages (Hoskin et al, 1999; 2000). In addition, when a condensed tannin extract was included in the diets of sheep parasitised with an intestinal nematode, faecal egg counts and worm burden of those sheep were lower, compared to those of sheep offered the tannin-free diet (Butter et al, 2000). The addition of condensed tannin extracts in the foods of ruminants has recently been used as an alternative method to investigate the effects of condensed tannins on gastrointestinal parasitism, since it allows the study of the effects of condensed tannins *per se*, in contrast with grazing studies.

The two hypotheses that have been developed to account for the reduction of the adverse effects of parasitism in growing ruminants are reported in detail below and concern a direct anthelmintic effect on the larvae or adult parasites, and an indirect effect of condensed tannins on the resilience and the resistance of the parasitised host.

1.5.1. Direct anthelmintic effect of condensed tannins

A direct anthelmintic effect could be one of the mechanisms via which the consumption of condensed tannins has been considered responsible for reducing the level of parasitism in grazing ruminants. As was indicated above, the disassociation of tannin-protein complexes takes place in the abomasum and small intestine. Consequently, in the lower gastrointestinal tract tannins are in a free form and are available to form new complexes with either endogenous or dietary protein. When condensed tannins are in a free form, they may be capable of causing detrimental effects on either the larvae or the adult nematodes or they may affect the fecundity of the worms. These detrimental effects towards nematodes may be related to the chemical properties of condensed tannins with special reference to their protein-binding ability, as the tissues of nematodes contain proline (Thompson and Geary 1995). There is evidence concerning a possible nematocidal activity of plant extracts high in condensed tannins towards plant nematodes, which was demonstrated *in vitro* (Taylor and Murant, 1966). Further *in vitro* studies have indicated that the addition of cotton condensed tannins to the artificial diets of cotton pests, resulted in a reduction of the population of the pest (Reese et al, 1982). Larvae and adult insects are also detrimentally affected by the presence of condensed tannins in artificial diets, mainly because of feeding inhibition (see review by Bernays et al, 1989). One of the objectives of the present thesis is to investigate the direct anthelmintic effect of condensed tannins towards ovine nematodes, as it will be outlined in section 1.7.

1.5.2. The indirect effect of condensed tannins

In general, the major consequence of subclinical gastrointestinal parasitism is production losses of infected livestock. The depression of food intake, and subsequently protein and energy intake, in combination with reduced nutrient utilisation of the host are the main reasons for the reduced production observed during gastrointestinal parasitism in ruminants

(see reviews by van Houtert and Sykes, 1996; Coop and Kyriazakis, 1999). In addition, gastrointestinal parasitism induces an increased loss of endogenous protein in the alimentary tract of the parasitised host (see review by MacRae, 1993). All the changes observed in protein metabolism during a gastrointestinal parasitic infection indicate that parasitised animals have an increased protein demand. It has been suggested that protein supplementation can improve the resilience of parasitised hosts (see review by Coop and Kyriazakis, 1999); resilience is the ability of the host to maintain a relatively undepressed level of production during the parasitic infection (see review by van Houtert and Sykes, 1996). The effect of improved nutrition on host resilience is more evident in young growing sheep, as they suffer more from the pathophysiological consequences of parasitism. Indeed, increased protein intake or protein supplementation of ruminants grazing poor quality pasture increased their liveweight gain, food conversion efficiency and plasma protein compared to unsupplemented controls (see review by Coop and Kyriazakis, 1999). In the above review it was also suggested that the response of protein supplementation was more effective when protein was not degradable in the rumen. The supply of rumen undegradable protein to the parasitised host may be the first mechanism via which condensed tannins may demonstrate a possible indirect nutritional effect on the host. Via their ability to bind to dietary protein and protect it from rumen degradation, it may be possible to improve the resilience of parasitised hosts. An indirect nutritional effect of condensed tannins on the resilience of parasitised sheep was possibly responsible for increased liveweight gain in sheep grazing forages that contained condensed tannins, compared to those grazing tannin-free forages, whereas neither food intake nor the level of parasitism were altered (Niezen et al, 1998a).

Condensed tannins may also contribute to the enhancement of the resistance of the parasitised host. Resistance is the ability of the host to prevent the establishment and/or the development of a parasitic infection (see review by van Houtert and Sykes, 1996). The mechanism through which resistance is expressed is immunity. Faecal egg counts and worm burden recovery from parasitised ruminants are indirect measurements for monitoring the development of the resistance in parasitised ruminants, and are relatively reliable and are widely used. As indicated above, the population that suffers most from the consequences of gastrointestinal parasitism is young ruminants, as it takes a longer period of exposure to acquire their immune response towards gastrointestinal parasitism, compared to adult ruminants. The development of immunity in young ruminants occurs in two phases, which are a continuum: the acquisition and the expression of immunity. During the acquisition of immunity the larval establishment and the fecundity of worms is high and immunological changes occur in the host, to produce an effective immune response (see review by Coop and Kyriazakis, 1999). It has been suggested that protein supplementation is not expected to affect the rate of acquisition of immunity to nematodes in young ruminants. Indeed, faecal egg counts and worm burdens were not reduced in growing ruminants supplemented with dietary protein during the phase of acquisition of immunity (Bown et al, 1991; van Houtert et al, 1995). It is thus evident that the consumption of condensed tannins during the phase of acquisition of immunity in growing ruminants would not be expected to affect the resistance of parasitised ruminants.

During the phase of expression of acquired immunity, infected hosts limit the establishment of incoming larvae, reduce the fecundity of adult worms and eventually promote worm expulsion (Dobson et al, 1990a). During this period, protein supplementation is considered responsible for enhancing the reduction in faecal egg counts and worm burden in parasitised

sheep (Bown et al 1991; van Houtert et al. 1995). There was also evidence that post-ruminal infusion of extra protein in sheep infected with *T.circumcincta* enhanced the expression of immunity, as faecal egg counts and worm burdens were reduced, and mucosal mast cells were increased (cells associated with the expulsion of GI nematodes) (Coop et al, 1995). Supplementation with rumen undegradable protein caused a more pronounced immune response towards *H.contortus* or *T.colubriformis* nematodes (Abbott and Holmes, 1990; van Houtert et al, 1995). It is evident from the above that condensed tannins could enhance the expression of the acquired immunity in parasitised ruminants. Via their ability to protect dietary protein from rumen degradation, condensed tannins can increase protein availability in the small intestine of the host and thus improve the resistance to gastrointestinal nematode infections. The investigation of the indirect effect of condensed tannins, both on the resilience and the resistance of parasitised young sheep is an objective of the thesis.

1.6. Condensed tannins in animal experimentation

Until now most research that aimed to investigate the anthelmintic properties of condensed tannins towards ovine nematodes has been performed either under grazing conditions, or by supplementing the foods of parasitised sheep with commercially available condensed tannin extracts, or *in vitro*, through the use of pure condensed tannin extracts. Grazing experiments (Niezen et al, 1995; 1998b; Robertson et al, 1995) are undoubtedly a very appropriate method to test the properties of condensed tannins. However, it is difficult to quantify the anthelmintic properties of condensed tannins *per se* in grazing animals, since the effects observed during grazing experiments may be attributable to other nutrients contained in the forage. On the other hand, although pure condensed tannin extract might be a useful alternative to quantify the anthelmintic properties of condensed tannins (Molan et al, 2000a; b), the extraction of condensed tannins from plants is problematic. The whole procedure, from the collection of the plant material to the production of the pure extract, a wide variety of solvents, methods of extraction and purification of tannins is involved (Waterman and Mole, 1994b). Hence the procedure is time consuming, expensive, and not particularly accurate, which result in having difficulties to obtain large amounts of purified extracts.

The use of commercially available condensed tannin extracts has been used to investigate not only the anthelmintic properties of condensed tannins, but also to test for the effects of condensed tannins upon animal nutrition. From the commercially available condensed tannin extracts, Quebracho extract is the one most commonly used as a tannin model in animal studies, since it combines high purity in condensed tannins, availability in large amounts and is relatively inexpensive. It is the extract from the heartwood of *Schinopsis spp*, native trees of south America. The heartwood contains 25% of condensed tannins and following the extraction procedure it yields a crude extract of about 80% condensed tannin (Roux, 1992).

It also contains simple phenolics. Quebracho extract has been used to investigate the effects of condensed tannins on food intake (Landau et al, 2000), on the performance and digestibility in ruminants (Salawu et al, 1999), on the PRP-condensed tannin interactions (McArthur et al, 1995), on enzyme activity (Mole and Waterman, 1985), but also to test the effects of condensed tannins on insect growth (Mallampalli et al, 1996). Condensed tannin extracts from other plants include wattle bark extract (*Acacia* spp), faba-beans extract (*Vicia faba*) and pine extract (*Pinus radiata*); however neither of the above has been used as extensively in animal experiments as Quebracho extract. There are two types of Quebracho extract commercially available, the warm and the cold soluble. Although both types are water soluble, warm soluble Quebracho extract requires higher temperature to be dissolved (100°C), compared to cold soluble one (40°C). The latter has been treated with metabisulphite, oxalic acid and EDTA, to improve its solubility (Roux, 1992) and thus was chosen for use in all the experiments of this thesis. Comparisons between the sulphitised and non-sulphitised Quebracho extract did not show any differences in the properties of condensed tannins (N.L. Butter, “The effect of condensed tannins and dietary protein on ruminant intestinal nematode infections”, The University of Nottingham, PhD thesis, 1999).

During a recent study, an attempt was made to determine the structure of Quebracho extract (Benzie, N “Analysis of tannins in Quebracho extract and shea meal”, The University of Aberdeen, MSc Thesis, 1998). In this study, purification of Quebracho extract was performed by gel filtration (Sephadex-20), qualitative analysis with high performance liquid chromatography (HPLC), structural analysis of the purified extract with nuclear magnetic resonance (NMR) and determination of the molecular weight with mass spectrometry. It was found that Quebracho extract contains catechin and profisetidin units, which form oligomers (hexamers, pentamers, tetramers, trimers and dimers), whereas there

were no polymers detected. The above results indicate that Quebracho extract is a mixture of condensed tannins, each with different molecular weight. Although low molecular weight condensed tannins could be more easily degraded in the gastrointestinal tract of sheep and absorbed, previous work has suggested that Quebracho extract (from the same suppliers) survives well the passage through the rumen (Makkar and Becker, 1995), i.e. it is not rumen degradable.

1.7. Objectives of the thesis

The aim of this thesis was to investigate the two hypotheses that have been developed to account for the ability of condensed tannins to reduce the detrimental consequences of gastrointestinal parasitism in sheep. So far, grazing experiments have attempted to test the effects of condensed tannins on gastrointestinal parasitism, but none of the previous methodologies used allowed the distinction between direct and indirect effect to be made. It was hypothesised that offering a condensed tannin extract as a drench, in a similar way that anthelmintic drugs are administered, would enable us to identify a direct anthelmintic effect of condensed tannins towards adult nematodes. The short-term drenching with Quebracho extract during the first two experiments of this thesis, in combination with *in vitro* assays performed with pre-parasitic larval stages of ovine nematodes, was expected to enable the differentiation of the direct from the indirect effect of condensed tannins. Subsequently, the consumption of high protein foods supplemented with a condensed tannin extract by sheep infected with an intestinal nematode was expected to provide evidence for a direct anthelmintic effect of condensed tannins during the development and on the maintenance of an intestinal nematode infection. This was expected since during the initial period of worm establishment in an intestinal nematode infection, protein supplementation is not expected to enhance the immune response of parasitised sheep. The long-term consumption of low and high protein foods supplemented with Quebracho extract in such experiments was also expected to provide evidence for the potential indirect effect of condensed tannins on the resilience and the resistance of parasitised sheep.

The objectives of the thesis were:

1. To test for a direct anthelmintic effect of condensed tannins towards an established *T.colubriformis* population in growing sheep (Chapter Two).

2. To test for a direct anthelmintic effect of condensed tannins towards either an established *T.circumcincta* or *H.contortus* population or on a mixed *T.colubriformis* and *N.battus* adult population in growing sheep, *in vivo* (Chapter Three).
3. To evaluate the anthelmintic effect of condensed tannins towards larval stages of *T.circumcincta*, *H.contortus* or *T.vitrinus*, *in vitro* (Chapter Three).
4. To test for a direct anthelmintic effect of condensed tannins during the establishment and on the established *T.colubriformis* infection, and for an indirect effect of condensed tannins on the resilience of parasitised sheep offered restricted amounts of high protein foods supplemented with condensed tannins (Chapter Four).
5. To investigate whether *ad libitum* food intake of low and high protein foods supplemented with condensed tannins in sheep infected with *T.colubriformis* would result in improved resilience and enhanced resistance of parasitised sheep (Chapter Five).

Chapter Two

Effects of short-term exposure to condensed tannins on adult *Trichostrongylus colubriformis*

2.1. Abstract

Twelve parasite naive sheep were used to study the possible direct anthelmintic effect of a condensed tannin extract (Quebracho) on the worm population and fecundity of the intestinal nematode *Trichostrongylus colubriformis*. Sheep were infected with a single dose of 20,000 L₃ of *T.colubriformis* on day 1. They were fed restrictedly at 3.5% of their liveweight, throughout the experiment. On day 28 post infection six of the sheep were orally drenched daily for a week with solutions of the tannin extract; the amount of tannin was 8% w/w of food intake. On day 35 all sheep were slaughtered and their small intestines removed to estimate the worm burdens and *in utero* fecundity. Faecal egg counts (FEC) were reduced by approximately 50% in treated compared to control sheep ($P<0.01$) three days after the first drench with tannin extract. Continuation of drenching did not result to further FEC reduction. Worm burden and *per capita* fecundity were reduced by 30% in treated sheep compared to controls ($P< 0.05$). The sex ratios, *in utero* fecundity and length of worms were not affected by drenching with tannin. The experiment gives strong support to the view that condensed tannins may have a direct anthelmintic effect towards the adult stages of *T.colubriformis*. The possible mechanisms of their action are further considered.

2.2. Introduction

Gastrointestinal parasitism contributes to reduced efficiency of production and reduced welfare in ruminants. Traditionally parasitic infections are controlled by anthelmintic drugs, integrated where practical with grazing management strategies. Frequent treatment with anthelmintic drugs has led to widespread development of anthelmintic resistant strains of nematodes, particularly in sheep and goats both in the Northern and Southern hemisphere (Jackson et al, 1993; Waller, 1997). There is also increasing public concern over drug residues in meat and milk products and the potential of environmental contamination (McKellar, 1997).

Alternative control strategies are being investigated in order to reduce the impact of parasitism and the extent of anthelmintic resistance. These include the breeding of genetically resistant hosts (Gray, 1997), vaccination (Emery, 1996), biological control (Gronvold et al, 1996), nutrient supplementation (Coop and Holmes, 1996) and use of forages or plants with anthelmintic properties. In traditional veterinary medicine, still practised in many areas of the world (Chhabra and Mahunnah, 1994), the use of plants with anthelmintic properties has been considered as an alternative to anthelmintic drugs. A systematic examination of the active plant compounds has not yet been performed, but plants high in condensed tannins have been assessed for their detrimental effects toward plant parasites (Klocke and Chan, 1982; Reese et al, 1982).

Condensed tannins, which are secondary plant metabolites, are the most common tannin fraction (Mangan, 1988). They are present in various concentrations in different plant species and it is possible that they have a defensive role against herbivores (Hagerman and Butler, 1991). Condensed tannins have a high affinity to macromolecules and form stable

complexes with proteins under suitable pH values and in the presence of surfactants (Mole and Waterman, 1985; Mueller-Harvey and McAllan, 1992).

Recently it has been shown that sheep trickle-infected with *T.colubriformis* larvae and given access to forages with a high concentration of condensed tannins, show a reduction in their worm burdens, compared to sheep given access to forages low in condensed tannins (Niezen et al, 1995; 1998a). Similar observations have been made in red deer infected with gastrointestinal nematodes and lungworms (Hoskin et al, 1999). Butter et al. (2000) observed a reduction in the *T.colubriformis* burden in trickle infected sheep, which consumed a tannin extract incorporated into their food, compared to infected controls fed a standard ration. These studies suggest that condensed tannins have an effect on the population of larvae and/or adult worms of gastrointestinal nematodes in ruminants. The question is whether this effect is a direct anthelmintic effect or an indirect one or a combination of both. The indirect effect could be through the ability of tannins to bind to dietary protein in the buccal cavity and rumen, and thus increase the amount of undegradable protein passing to the small intestine for absorption (Woodward and Reed, 1996). The latter has been shown to affect the ability of ruminants to mount an effective immune response towards gastrointestinal parasites (see review by Coop and Kyriazakis, 1999). The objective of this experiment was to investigate the possible direct anthelmintic effect of condensed tannins on the viability and fecundity of an established intestinal nematode infection in sheep, when tannins were offered as an oral drench for a relatively short period of time. The duration of the tannin treatment was too short to allow indirect effects on parasites to be expressed.

2.3. Materials and methods

2.3.1. *Animals and housing*

Twelve Texel x Scottish Greyface sheep were used. They were reared indoors from birth under conditions which would exclude accidental nematode infection, weaned at 8 weeks of age and moved to individual pens one month later (liveweight, 30.0 (sd 3.12) kg). The floor of the pens was slatted and raised 0.35m above concrete. Each pen measured 1.50 x 2.00m and contained a food trough and a water bowl which provided free access to water. The sheep received a minimum of 16h light/d. Following weaning sheep were offered *ad libitum* a feed (pre-experimental feed) containing 195 g/kg dry matter (DM) crude protein (CP), 12.3 MJ/kg DM metabolisable energy (ME) and sufficient minerals and vitamins to meet their requirements. Once penned, they were offered a liveweight-based allowance of the pre-experimental feed at 3.5% of their liveweight. This allowance was chosen in order to overcome possible problems associated with anorexia caused by parasitism, and to reduce the likelihood of refusals and spillage; this enabled comparisons to be made on an equal basis (Kyriazakis et al, 1996b). One week prior to the start of the experiment, all sheep were treated with a single dose of levamisole (7.5 mg/kg; Levacide 3% Drench, Norbrook Laboratories Ltd, UK).

2.3.2. *Experimental feed and allowance*

The DM of the experimental food was 887g / kg fresh matter. It contained 170 g CP, 75.1 g ash, 257 g acid detergent fibre, 468 g neutral detergent fibre and 11.2 MJ ME per kg DM. It consisted of barley (384 g/kg), oatfeed (150 g/kg), citrus pulp (200 g/kg), high protein soya (190 g/kg), molasses (50 g/kg), salt (10 g/kg) and a mineral and vitamin mix (16 g/kg). The food was pelleted and offered twice a day throughout the experiment. The protein content of the food was formulated to meet the requirements of the growing lambs (AFRC 1993) and

the food was offered at 3.5% of their liveweight allowance for the reasons outlined above. The allowance was adjusted weekly, immediately after weighing.

2.3.3. Condensed tannin extract

The condensed tannin extract used was the commercially available Quebracho extract (Quebracho ATO, Roy Wilson Dickson Ltd, Chester, UK) of the cold soluble type (powder). Quebracho tannins are extracted from the tropical tree *Schinopsis* spp (ordinary Quebracho) and are often used as a model tannin when the effects of condensed tannins are investigated (McArthur and Sanson, 1993). Treating ordinary Quebracho with 10% bisulphite produces the cold soluble type. This contains 920 g DM /kg, and approximately 730 g of the DM are condensed tannins; the remainder is simple phenolics (190 g) and traces of sulphonic acid groups. The tannin solutions were prepared at 8% w/w of the food intake by dissolving Quebracho in warm water (50°C) using a magnetic stirrer and were administered to the sheep via a stomach tube.

Previous studies have shown that the consumption of a diet containing 50 g/kg of the DM Quebracho tannins, has a detrimental effect on the worm population of lambs infected with a trickle dose of *T.colubriformis* (Butter et al, 2000). We opted for a slightly higher dose of tannins, since they were to be administered for just a week. One-week period of administration is sufficient for any kind of direct anthelmintic effect towards adult parasites to be shown. The practice of longer-term administration of anthelmintic drugs, i.e. via intraruminal controlled-release capsule, has been effectively used (Gogolewski et al, 1997). There were no prior studies where the direct anthelmintic effect of tannins on parasites had been investigated.

2.3.4. *Experimental design*

The sheep were acclimatised to the experimental conditions for one month. In the last week of acclimatisation, sheep were randomly allocated to two groups (n=6), taking into account their liveweight (mean liveweight: 36.0 (sd 3.41) kg). The experimental food was offered to sheep for the 35-day experimental period. Sheep were infected orally with a single dose of 20,000 L₃ of *T.colubriformis* on day 1. Faeces from a donor sheep infected with *T.colubriformis* were cultured for 10 days at 20°C and infected larvae harvested using a standard Baermann procedure. The larvae were stored at 4°C and used within two weeks. The dose of 20,000 L₃ exceeds the W.A.A.V.P. recommendation (Wood et al, 1995) for size of infection to evaluate the efficacy of anthelmintics. However, the recommended nematode doses are calculated for mixed infections and it was considered that a larger dose, which does not lead to clinical infection in young sheep, would be a more suitable challenge to investigate the effect of tannins.

From day 28 to day 34 inclusive, six sheep were drenched orally with a condensed tannin solution each morning just prior to receiving their feed. The remaining six sheep were used as negative controls. All sheep were slaughtered on day 35 and their intestines were removed for worm recovery.

2.3.5. *Sampling procedures and analysis*

Sheep were weighed once a week before the morning feed and because of their restricted feeding, refusals were rare. In cases where these occurred, they were weighed before the morning feeding and re-offered to the animals. Blood samples were taken weekly by jugular venepuncture into heparinised vacutainers (Becton Dickinson, UK) and plasma was stored at -20°C until analysis for calcium, phosphorus, total protein, albumin and urea concentrations (Monarch 2000, Instrumentation laboratory, Cheshire, England).

Faecal samples were taken weekly from the rectum and an additional sample was taken on day 31; thus during the week Quebracho extract was administered, faecal samples were taken on three occasions (days 28, 31 and 34). Eggs per gram of fresh faeces were determined using a modified flotation technique (Christie and Jackson, 1982). Faecal egg counts (FEC) were also expressed relative to dry matter, since *T.colubriformis* infection can affect the consistency of the faeces.

At slaughter the small intestine was removed and the worms recovered from the digesta and the intestinal mucosa by incubation in 0.85% saline for 4 hours at 37°C. The total worm burden was estimated from a 10% aliquot of digesta and mucosal digest and the sex ratio was calculated. Twenty-five female worms were taken from each sub-sample at random and stained with 0.01% cotton blue in lactophenol, for determination of their length and the number of eggs *in utero* (*in utero* fecundity). In addition, *per capita* fecundity was calculated by dividing the egg counts recorded on day 34 by the total number of female worms.

2.3.6. Statistical analysis

The worm burdens, sex ratios, length of female worms, number of eggs *in utero* and *per capita* fecundity were analysed by one-way analysis of variance (ANOVA, Genstat 5, release 3.2; Lawes Agricultural Trust, 1993). Analysis of blood sample parameters, faecal egg counts and liveweight data were analysed by one-way ANOVA for repeated measurements. The data from the egg counts and worm burdens were transformed ($\log(x) + 1$) prior to analysis, in order to stabilise the variance. Direct comparisons were made between the faecal egg counts taken on days 28, 31 and 34, by the use of paired t-tests.

2.4. Results

2.4.1. Liveweight gain and food conversion efficiency.

The amount of food offered was consumed in full by all sheep. There was no significant difference detected between the two groups in their liveweight during the first 28 days of the experiment (44.8 (sd 0.57) kg). However, during the last week of the experiment, tannin drenched animals did not gain any weight, whereas untreated controls increased their liveweight by 1.85 kg; final weights were 44.6 and 47.1 (standard error of the difference, sed: 2.24) kg. As a consequence, the liveweight gain (0.186 vs 0.220, (sed 0.013) kg/day for treated and controls respectively) and the food conversion efficiency (0.128 vs 0.147, (sed 0.006) g gain/kg of food, for treated and controls respectively) between the two groups over the whole experimental period was significantly different ($P < 0.01$).

2.4.2. Faecal egg counts

The infections were patent by day 14 and faecal egg counts increased in both groups up to day 28 (Fig 2.1). In the control group their number remained high until the end of the experiment (day 35), whereas there was a decrease from day 31 in the tannin treated group. The egg reduction was about 50% in the treated group compared to the controls (1,404 vs 3,264 eggs/g dry faeces, respectively; $P < 0.05$). The number of eggs remained stable between days 31 and day 34 for both groups. The mean dry matter content of faeces was similar when the infections were patent (520 vs 464 g DM/kg faeces, sed: 32.3 for treated and controls respectively). The day before slaughter the dry matter content decreased significantly in the treated animals, due to time x treatment interaction (313 vs 417 g for treated and controls respectively, sed 46.8; $P < 0.05$).

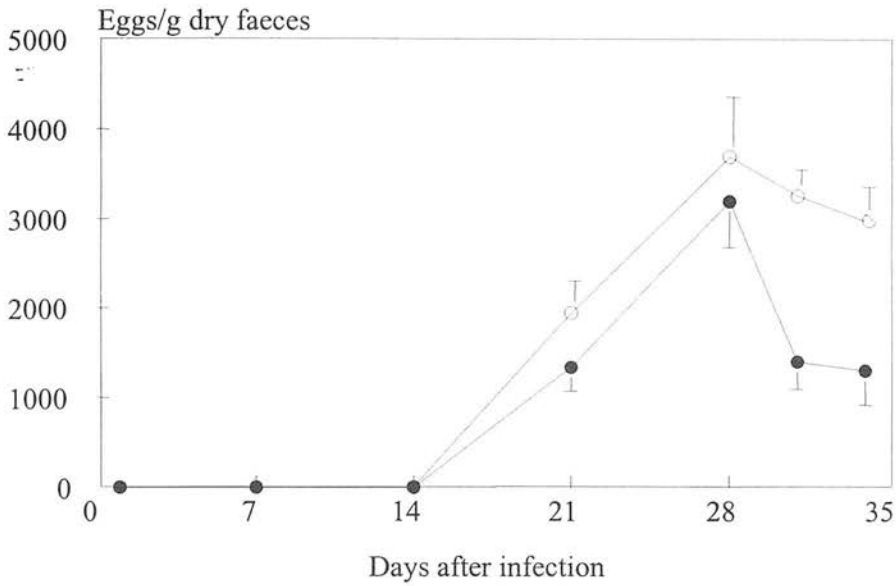


Figure 2.1. Mean faecal egg counts (eggs/ g dry faeces) of lambs infected on day 1 with a single dose of 20,000 L₃ *T.colubriformis*. Lambs were either drenched with condensed tannin solution from day 28 to day 34 (●) or were untreated controls (○). Means were not significant different between the two groups until day 31; the error bars indicate the standard error of the means.

2.4.3. Worm burdens and fecundity

There was approximately a 30% reduction in the total worm burdens of the tannin treated group compared to control group (6,950 vs 10,033 worms, respectively; $P < 0.01$) (Fig. 2.2). The percentage reduction was similar for both male ($P < 0.01$) and female worms ($P < 0.05$) and therefore the sex ratio was not affected by treatment. The number of female worms (3,875 vs 5,375 worms, for treated and controls respectively; $P < 0.05$) was slightly higher than males in both groups (3,075 vs 4,658 worms, for treated and controls respectively; $P < 0.05$).

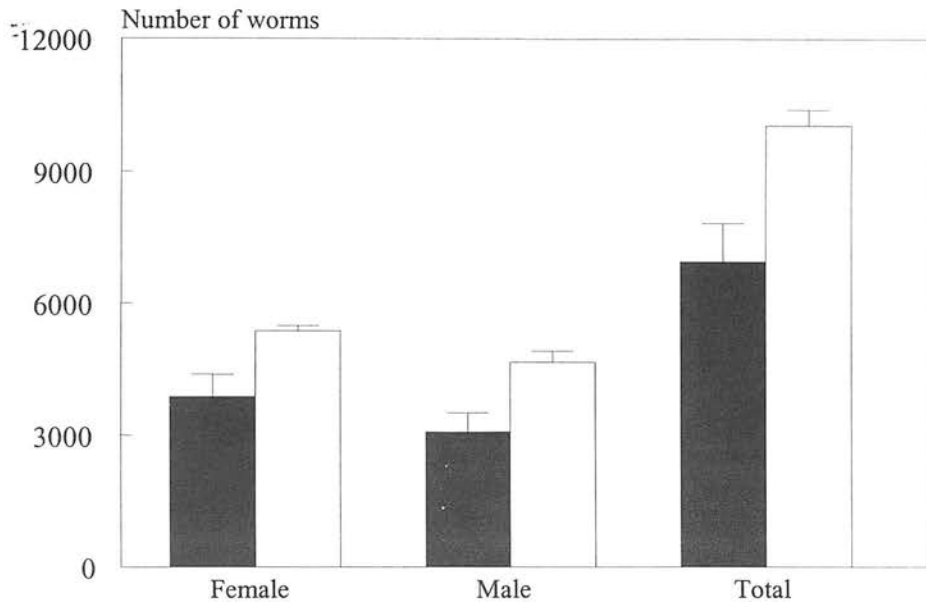


Figure 2.2. Mean number of worms counted in the small intestine of lambs infected with 20,000 L_3 *T. colubriformis*. Lambs were either treated with condensed tannin solutions from day 28 to day 34 (■) or were untreated controls (□). The numbers of male, female and total worms are indicated on the graph.

The numbers of eggs *in utero* were not significantly different between the two groups (treated: 22.2 vs controls: 20.7; sed: 0.55 eggs/female). However, *per capita* fecundity was significantly lower ($P < 0.05$) in the worms of the treated group (0.320 eggs/female), compared to the control sheep (0.560; sed: 0.080 eggs/female). The length of female worms did not differ between the two groups.

2.4.4. Plasma analysis

The only significant difference between the treated and the control sheep in the plasma parameters were in the concentrations of urea ($P < 0.01$). The effect appeared on day 28

(9.56 vs 13.66 mmol/l, for treated and controls respectively; sed: 0.715) and remained evident throughout the experiment (day 34; 5.95 vs 7.86 mmol/l, for treated and controls respectively; sed: 0.715). There were no temporal effects on the plasma concentrations of calcium, albumin or total protein in the blood. However, urea and phosphorus concentrations were affected by time for both groups of sheep. Urea values declined from 11.60 (se 1.250) g/l on day 28, to 6.90 (se 0.596) g/l on day 34 ($P < 0.001$), in both groups. Phosphorus showed a decrease on day 21 (2.37 (se 0.191) mmol/l) in comparison to previous days (3.22 (se 0.001) mmol/l, $P < 0.05$) and remained at this level until the end of the experiment (on day 34, 2.72 (se 0.222) mmol/l), in both groups.

2.5. Discussion

This experiment is the first investigation into the short-term anthelmintic effect of *Quebracho* tannins on the worm population of an intestinal nematode species in sheep. The data show that drenching growing sheep previously infected with a single dose of *T.colubriformis* with *Quebracho* extract, causes a decrease in their total worm burden (TWB), faecal egg counts (FEC) and *per capita* fecundity (PCF). It has been previously reported that sheep infected daily with *T.colubriformis* larvae cope better with the effects of the parasitic infection and show reduced worm burdens when they are fed a diet high in condensed tannins (Niezen et al, 1996; 1998a). In addition, a significant negative relationship has been observed between dietary condensed tannin concentration and the burden of abomasal nematodes in red deer, even though no difference was detected in their faecal egg counts (Hoskins et al, 1999).

The hypothesis tested in this experiment was that reductions in TWB, FEC and PCF might be attributable to a direct anthelmintic effect that is due to either a 'toxic' effect and/or a physiologically mediated effect. A direct 'toxic' effect of condensed tannins has been observed on both larval and adult stages of insects (Martin et al, 1985). Furthermore, *in vitro* development assays have shown that *Quebracho* tannin has a detrimental effect on the survival of early larval stages of various ovine gastrointestinal nematode species (Chapter Three). The mechanism of action of tannins is unknown, but tannins may have the ability to impair vital processes such as feeding and reproduction or they could bind and disrupt the mechanical integrity of the cuticle (Thompson and Geary, 1995). An alternative possibility is that physiological or biochemical changes in the host gut, induced by tannin administration, could decrease the FEC and PCF. It has been shown that condensed tannins can decrease the digestibility of the feed and therefore increase the total faecal output in

animals fed diets high in tannins (Barry and Manley, 1984; Waghorn et al, 1994). Therefore, it is possible that the reduction in the FEC could be partly a dilution effect. Other physiological changes induced by tannins could influence the worm population, as it is known that intestinal nematodes are susceptible to physiological changes in the gastrointestinal tract, such as those mediated by the presence of other parasite species (Mapes and Coop, 1970).

The reduction in FEC occurred within three days of tannin administration, which is similar to the immediate effects following treatment with anthelmintics. However, unlike drugs whose efficacy is generally over 95%, the administration of tannins led to a worm burden reduction of only 30%. This is likely to be due to the low dose of Quebracho extract used, but one can not rule out the possibility that some worms were genetically resistant to the consequences of tannins. Hoekstra et al. (1997) observed similar changes in anthelmintic efficacy in resistant nematode strains. It is possible that an increase in the dose of tannins may lead to a further reduction in worm numbers. However, the tannin dose used in this experiment is comparable to the range found in plants (Audas, 1994) and a higher dose would be unrepresentative of the field situation.

Administration of Quebracho tannins did not have any effect on the number of eggs *in utero* of female worms, when compared to worms of untreated sheep, although the estimated *per capita* fecundity was significantly reduced. This suggests that if the direct effect is not due to faecal dilution but it is a toxic one, then the rate of egg-laying was truly reduced in the Quebracho treated worms. Reduced rates of egg-laying have also been recorded following administration of ivermectin and oxfendazole (Scott and Baxter, 1991).

As expected there was no difference in the food intake and liveweight gain between treated and untreated sheep for the first four weeks of the experiment. However during the last week, a difference was detected in the liveweight of the sheep of the two groups even though daily food intake was identical. Sheep in the treated group failed to gain weight, whereas the control animals continued to grow at the same rate as in the previous weeks. The poor growth of sheep drenched with tannin in this study contradicts previous observations where sheep fed diets rich in condensed tannins had higher liveweight gain than controls (Niezen et al, 1993; 1994; 1996). In these experiments however condensed tannins were a natural part of the diet and were not administered as a single drench. It is likely that the liveweight gain recorded in the former studies was the result of a long-term effect of tannins.

The tannin effect towards *T.colubriformis* reported in the literature could also be accounted for by an alternative mechanism. Increased protein availability in the small intestine could have affected the immune response of the host in the longer term (see review by Coop and Kyriazakis, 1999). Condensed tannins have a high affinity to both endogenous and dietary protein and form stable complexes with it (Butler and Rogler, 1992; see review by Mueller-Harvey and McAllan, 1992). Tannins can protect dietary protein from rumen degradation and thus be responsible for an increased protein flow in the small intestine. It has been shown that supplementation with rumen by-pass protein affects the degree of expression of immunity towards gastrointestinal intestinal nematodes (van Houtert et al, 1995; see reviews by Coop and Holmes, 1996; Coop and Kyriazakis, 1999). The tannin effect observed in our experiment can not be attributed to this hypothesis, due to the short duration of drenching with tannins. This however does not preclude a longer-term effect of tannins on nematodes through this route.

The work reported here demonstrates a decrease in the population of *T.colubriformis* in young growing sheep, when they are drenched with a solution of Quebracho tannins. Further studies are required to elucidate the mechanisms underlying the direct anthelmintic effects attributable to condensed tannins. Such knowledge will assist in evaluating the possible role of tanniniferous plants and their extracts in the effective control of gastrointestinal parasitism of ruminants. This should be accompanied by an agronomic evaluation of tanniniferous plants, since this will determine how they can be incorporated into systems of ruminant production.

Chapter Three

**Direct anthelmintic effects of condensed tannins
towards different gastrointestinal nematodes of
sheep: *in vitro* and *in vivo* studies.**

3.1. Abstract

In vitro and *in vivo* studies were conducted to determine possible direct anthelmintic effects of condensed tannins towards different ovine gastrointestinal nematodes. A larval development/viability assay was used to investigate the effect of a condensed tannin extract (Quebracho) towards larvae of *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus vitrinus*. The development to infective larvae and their viability was assessed in all three species and LD 50 values were calculated. The presence of Quebracho extract in the cultures decreased the viability of L3 in all species; the LD 50 were not significantly different for the different species. Forty-eight sheep were used to investigate the anthelmintic effect of Quebracho extract towards an established adult population of four gastrointestinal nematode species. Sheep were allocated to one of eight groups and were infected with a single dose of either 4,000 L3 *H. contortus* (groups 1 and 2) or 5,000 L3 *T. colubriformis* and 5,000 L3 *Nematodirus battus* simultaneously (groups 3-6) or 10,000 L3 of *T. circumcincta* (groups 7 and 8). From day 28 until day 31 of the experiment, sheep infected with the intestinal species were drenched with Quebracho extract at 4, 8 or 16% w/w of food intake, or remained as undrenched controls. Sheep infected with *H. contortus* or *T. circumcincta* were either drenched for three days with Quebracho extract at 8% w/w of food intake or remained as undrenched controls. All sheep were slaughtered four days after the end of the drenching period. Sheep infected with the intestinal species and drenched with 16% w/w Quebracho had lower FEC compared to sheep drenched with 8% w/w ($P < 0.05$), which in turn were lower than in sheep either drenched with 4% w/w Quebracho or which remained undrenched ($P < 0.05$). The lowest intestinal worm burden was recovered from sheep drenched with 8% w/w Quebracho extract ($P < 0.05$). The administration of Quebracho extract at 8% w/w of food intake for three days did not affect FEC or worm burdens in sheep infected with the abomasal species compared to controls. *In vitro* assays could be a useful

means to study the effects of condensed tannins upon larvae or adult parasites; however, the interpretation of such effects should be considered in combination with *in vivo* studies, as *in vivo* conditions can modify the effects condensed tannins exert upon gastrointestinal parasites.

3.2. Introduction

Tannins are secondary plant metabolites, which have been closely associated with plant defense mechanisms towards insect (see review by Schultz, 1989) and mammalian herbivores (Hagerman and Butler, 1991). They are usually divided according to their chemical structure and properties into two groups: hydrolysable and condensed tannins. The latter is the most widespread group of tannins in nature and has been considered responsible for causing a number of detrimental effects towards monogastric (Vernon, 1999) and ruminant herbivores (Aerts et al, 1999). However, ruminants can also benefit from the presence of condensed tannins in their diets; the consumption of average concentrations of condensed tannins can result in increased weight gain, wool growth, milk secretion (Barry and McNabb, 1999) and decrease the detrimental effects of gastrointestinal parasitism (Aerts et al, 1999). Regarding the latter, reports available from traditional medicine suggest that plants high in condensed tannins have been used or are still being used to combat parasitic infections in both animals (Hammond et al, 1997) and humans (Milliken and Albert, 1996). Due to the increasing problem of the development of parasite resistance towards antiparasitic drugs (see review by Sangster, 1999) and the increased concern over the presence of residues in animal products (McKellar, 1997), the antiparasitic properties of condensed tannins are currently being investigated, amongst other approaches, as alternative strategies to control parasitism in farm animals (Waller, 1999).

There has been evidence for the nematocidal activity of condensed tannins over the last thirty years; plants or plant extracts high in condensed tannins increased the mortality of plant nematodes (Taylor and Murant, 1966). Recent reports from ruminants suggest that parasitised sheep and red deer grazing on forages high in condensed tannins had lower

faecal egg counts and worm burdens compared with animals grazing on forages low in condensed tannins (Niezen et al, 1995; Hoskin et al, 1999).

The reported antiparasitic effect of condensed tannins could be a direct antiparasitic effect or an indirect effect on the parasitised host. Condensed tannins have the ability to bind to dietary protein, thus protecting it from rumen degradation and increase protein availability in the small intestine of the host (see review by Mueller-Harvey and McAllan, 1992). Increased protein availability has been considered responsible for enhanced immunological responses towards parasites (see review by Coop and Kyriazakis, 1999). Recently, a direct anthelmintic effect of a plant extract high in condensed tannins (Quebracho) towards an adult *T.colubriformis* population has been demonstrated (Chapter Two). The methodology described in the above paper was developed to clearly distinguish a direct from an indirect effect of condensed tannins. Three days of Quebracho extract administration at 8% w/w of the sheep food intake, from day 28 post a single infection, reduced the faecal egg counts, worm burden and fecundity of *T.colubriformis*, when compared to parasites of undrenched controls.

Limited research has been undertaken on the effects of condensed tannins towards abomasal parasite species of ruminants; in red deer consumption of forages high in condensed tannins reduced the total gastrointestinal worm burden, including *Teladorsagia (Ostertagia) circumcincta* and *Trichostrongylus axei* (Hoskin et al, 1999). Sheep grazing *Lotus* spp, forages high in condensed tannins, showed reduced establishment of *O.circumcincta* worms (Niezen et al, 1998a). However, no attempt has been made to distinguish between a possible direct from an indirect effect of condensed tannins on abomasal parasites.

The aim of this study was to investigate the possible direct effects of condensed tannins upon different gastrointestinal parasites of sheep. These were investigated by both *in vitro* assays and an *in vivo* experiment. The objective of the former was to investigate a possible anthelmintic effect of Quebracho extract upon the viability of infective larvae of *T.circumcincta*, *Haemonchus contortus* and *T.vitrinus* using a larval development/viability assay. The *in vivo* experiment aimed to study i) the possible direct anthelmintic effect of Quebracho extract on adult parasites of two ovine abomasal species, *T.circumcincta* and *H. contortus* when it was offered as a drench and ii) the effect of the administration of three different levels of Quebracho extract on the adult burden of sheep infected with the intestinal species *T.colubriformis* and *Nematodirus battus*.

3.3. Materials and methods

3.3.1. Condensed tannin extract

The condensed tannin extract used was “cold soluble” Quebracho extract (Quebracho ATO, Roy Wilson Dickson Ltd, Chester, UK). The cold soluble Quebracho extract (originating from the bark of the tree species *Schinopsis*) is a fine powder, soluble in water (40-45°C), which contains 73% tannins of the condensed type, 19% of simple phenolics and 8% water. It is the purest, commercially available condensed tannin extract and has been used in previous studies to investigate the effects of condensed tannins upon immunity of parasitised sheep (Butter et al, 2000) and the short-term effects of tannins towards *T.colubriformis* (Chapter Two).

3.3.2. In vitro assays

The larval development/viability assay (LDVA) used to assess the effect of Quebracho on the development and viability of nematode larvae was a modified version of the method described by Taylor (1990). Eggs were extracted from faeces collected from donor lambs carrying monospecific infections of Moredun ovine anthelmintic susceptible isolates of *T.circumcincta*, *H.contortus* or *T.vitrinus*. Approximately 100 eggs of a given species in 150µl water were added to each well in a 24 well culture plate (Fisher Scientific, Loughborough) containing 150 µl of parasite growth gel supplemented with liver peptone (Oxoid, Unipath Ltd, Hants, UK) together with a mixture of nutrients (20 µl lyophilised *E.coli* 150 µg/ml, 20 µl yeast extract and Earles balanced salt solution) and anti-fungal (10 µl amphotericin B, 5 mg/ml PBS) and antibacterial (20 µl streptomycin, 250 mg/ml and 20 µl penicillin, 250 mg/ml) agents. The gel replaced water or DMSO (dimethyl-sulphoxide) used in previous assays (Taylor, 1990) to maintain all the ingredients in suspension within

wells. In total three 24-well plates were prepared one for each species. The plates were incubated at 22°C at 100% relative humidity (RH) for 24 hours to enable the eggs to hatch to first stage larvae prior to the addition of 150 µl of either distilled water or Quebracho extract solutions to each of 4 replicate wells. Stock solutions containing either 12% (*T.circumcincta*) or 10% (*T.vitrinus* and *H.contortus*) w/v Quebracho extract in distilled water were used to in the preparation of 4 doubling dilutions (1:1) with distilled water. *T.circumcincta* larvae were exposed to 12, 6, 3, 1.5 0.75 and 0% and *T.vitrinus* and *H.contortus* larvae to 10, 5, 2.5, 1.25 and 0% Quebracho extract solutions. Following the addition of the Quebracho solutions, the plates were incubated for a further 6 days at 22°C and 100% RH. After this incubation period, the numbers of live and dead L₁, L₂ and L₃ were counted using an inverted microscope at x 100 (Swift-Microtec, Micro Instruments Oxford Ltd).

3.3.3. *In vivo* experiment

3.3.3.1. *Animals*

Twenty-six female and twenty-two male Suffolk x Finn Dorset sheep were used. They were reared indoors under conditions that excluded nematode infection. Following weaning at 8 weeks of age (mean liveweight: 27.2, standard deviation (sd): 3.45), they were moved into individual pens and allowed to acclimatized to experimental conditions for a week. During this week, they were offered the experimental food (see below) at an allowance of 3.5% of their liveweight. Half of the allowance was offered twice a day, in the morning and in the afternoon, to reduce food spillage (Kyriazakis et al, 1996b) and it was estimated to be just below their *ad libitum* food intake (see Chapter Four).

The experimental unit was concrete floored and contained 48 pens; each pen measured 1.5 x 2.0 m and its slatted floor was raised 0.35m above the concrete. Sheep had 24h access to water and received a minimum of 16h light per day.

3.3.3.2. *Experimental food*

The experimental food was formulated and made into pellets. It consisted of 350 g wheat, 177 g oatfeed, 200 g citrus pulp, 180 g high protein soya, 50 g molasses, 10 g salt, 20 g limestone flour, 5 g ammonium chloride and 8 g of a mineral and vitamin mix per kg fresh matter. It contained 865 g dry matter, 150 g crude protein, 217 g acid detergent fibre, 375 neutral detergent fibre and 73 g ash per kg fresh matter. The food was calculated to provide 10 MJ metabolisable energy and 93 g metabolisable protein per kg fresh matter, which were above the requirements for growing sheep (AFRC, 1993).

The experimental food was offered at the allowance of 3.5% of sheep liveweight throughout the 36-day experimental period, to overcome possible comparison problems between groups arising from anorexia expected to be caused by parasitism (Kyriazakis et al, 1998). The food allowance was adjusted every week, immediately after sheep weighing.

3.3.3.3. *Infective larvae*

Faeces from monospecifically infected lambs with Moredun ovine anthelmintic susceptible isolates of either *T.circumcincta*, *H.contortus* or *T.colubriformis*, were cultured at 22°C for 10 days to provide infective larvae. Infective larvae were harvested by using a standard Baerman procedure. A Moredun ovine anthelmintic susceptible isolate of *N.battus* was used to infect a donor lamb; *N.battus* larvae were prepared according to the procedure described by Mapes and Coop (1970). Larval suspensions were prepared and administered to each animal in a total volume of 20-ml tap water.

3.3.3.4. Experimental design

One day prior the start of the experiment, sheep were randomly allocated into eight groups, taking into account their liveweight (mean liveweight: 28.8, sd: 3.62). On day 1 of the experiment, two groups were dosed with a single dose of 4,000 L₃ *H. contortus* (groups 1 and 2) and four groups were dosed with a single dose of 5,000 L₃ *T.colubriformis* together with 5,000 L₃ *N.battus* (groups 3-6). The two remaining groups were dosed with a single dose of 10,000 L₃ *T.circumcincta* one week later (see below, groups 7 and 8). The dose of L₃ for each parasite species followed the WAAVP guidelines for evaluating the efficacy of anthelmintics against single or combined infections (Wood et al, 1995). As *T.colubriformis* and *N.battus* are easy to distinguish during egg counting and worm recovery, sheep were dosed simultaneously with both species. The short duration of the experiment was expected to reduce the likelihood of any interactions between the two species.

In ruminants, twenty-eight days after a single infection with *H.contortus*, *T.colubriformis* and *N.battus* have been reported as optimal to test the efficacy of an anthelmintic drug towards adult parasites (Wood et al, 1995). There is evidence that worm burdens may begin to decline after 21 days of a single infection with *T.circumcincta* (Armour et al, 1966) and therefore groups 7 and 8 were infected with *T.circumcincta* larvae on day 7 of the experiment.

From day 28 until day 30 of the experiment inclusive, sheep infected with the intestinal species were drenched with Quebracho extract at either 4, 8, or 16% w/w of the food intake (groups 3, 4 and 5) and the remaining group was undrenched controls (group 6). One group of sheep infected with *H.contortus* (group 1) and one group infected with *T.circumcincta* (group 7) were drenched with Quebracho extract at 8% w/w of the food intake; the other two

groups infected with the abomasal species were undrenched controls (groups 2 and 8). Quebracho extract solutions were prepared daily for each sheep individually, by weighing amounts of the powder equivalent to 4, 8, or 16% w/w of the food intake of each sheep. This amount was then dissolved in distilled warm water (300-400 ml) and the solutions were then administered to sheep via a stomach tube before the morning feeding. Three days of drenching with Quebracho solutions were considered sufficient to test the direct anthelmintic effect of Quebracho extract, since previously sheep infected with *T.colubriformis* reduced their FEC within 3 days from the first drench of Quebracho extract (Chapter Two). Following the three day of Quebracho administration, sheep were monitored until day 35 and were all slaughtered on day 36 of the experiment.

3.3.3.5. *Measurements*

Food refusals were recorded daily early in the morning. They were rare because of the restricted feeding regime, but in cases where these were observed, they were weighed and an equal proportion of fresh food was offered to sheep the following day. Sheep liveweight was recorded once a week before the morning feed. Blood samples were collected weekly by jugular venepuncture into heparinised vacutainers (Becton Dickinson, UK); they were centrifuged at 2060 g for 20 min. and plasma was stored at -20°C until analyzed for calcium, phosphorus, total protein, albumin and urea concentrations. Plasma analysis was performed using a microcentrifugal analyser (Monarch 2000, Instrumentation laboratory, Warrington, Cheshire, England).

Faecal samples were collected directly from the rectum once a week during the first four weeks of the experiment and every afternoon between days 28-31 and on days 34 and 36. Faecal samples were collected more frequently during the period of Quebracho administration, to determine its efficacy over time. Samples were either processed for

determination of faecal egg counts immediately or stored at 4°C and processed within two days. Numbers of nematode eggs per gram were determined by a modified flotation technique (Christie and Jackson, 1982), where polyallomer centrifuge tubes were used to separate the meniscus containing the eggs. Dry matter of faeces was determined every week and number of eggs was expressed as eggs per g dry matter of faeces.

At slaughter the abomasum or the small intestine was removed and worms were recovered from digesta and either the abomasal or intestinal mucosa after a 4-hour incubation in saline at 37°C. Total worm burdens were estimated from 2% aliquots of digesta and mucosal digest for each sheep. *Per capita* fecundity was calculated by dividing the FEC recorded at the last day of the experiment by the total number of female worms recovered.

3.3.3.6. Statistical analysis

Data from LDVA were transformed by probit transformation and plotted against the logarithm of Quebracho concentrations (Minitab, release 11.1) (Hubert and Kerboef, 1992). Probit transformation was performed to transform a typical sigmoid curve dose response to a linear function. The Quebracho extract concentration required to kill 50% of L₃ (LD 50) was calculated from these linear regressions (for $y = 0$ on the probit scale). LD 50 values were backtransformed and backtransformed means with 95% confidence intervals are presented in mg Quebracho/ml. Confidence intervals were calculated by the Delta method (Morgan, 1992).

The effects of Quebracho administration on blood parameters and FEC were analyzed by one-way analysis of variance (ANOVA) for repeated measurements (Genstat 5, release 3.2; Lawes Agricultural Trust, 1993). The effect of sheep sex on blood parameters and FEC was determined by analyzing the above variates separately at each time point by performing two-

way ANOVA, using Quebracho administration and sex as factors. The effect of sex was not significant on any of the time points and neither was the interaction between Quebracho administration and sex; therefore, these effects are not reported further in the results. Food conversion efficiency, liveweight gain, worm burdens and *per capita* fecundity of the worms were analyzed by two-way ANOVA, using Quebracho administration and sex of the sheep as factors. The interaction between Quebracho administration and sheep sex was not significant on any of the above variables; therefore, only the effects of Quebracho administration and sex are reported in the results. Worm burdens, fecundity and FEC data were transformed ($\log(x+1)$) prior to analysis, to stabilize the variance. Backtransformed means with 95% confidence intervals are reported for transformed data.

3.4. Results

3.4.1. Larval development/viability assays

There was no evidence for a Quebracho effect on larval development following the incubation period; more than 98% of the larvae in either wells containing Quebracho extract or control wells were infective third stage. However, for all parasite species a linear relationship was observed between larval viability and Quebracho concentration (Fig. 3.1).

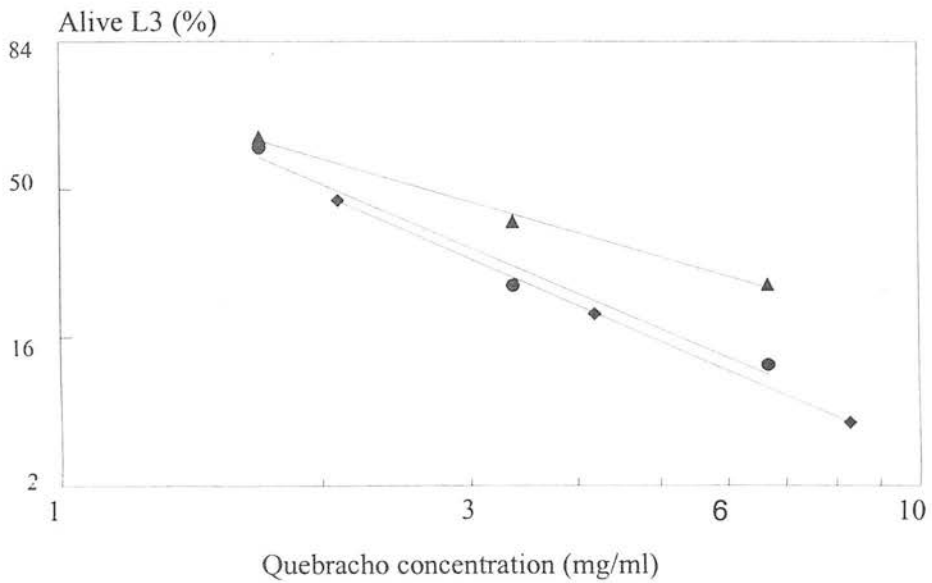


Figure 3.1. Linear relationships between mean values of alive L3 (on a probit scale, %) of *T. vitrinus* (●), *H. contortus* (▲) and *T. circumcineta* (◆) following a seven days incubation period in Quebracho solutions and Quebracho concentration (on a logarithmic scale, mg/ml). Each point represents the mean of four replicates.

The R-square values for the linear regressions were 1.00, 0.992 and 0.974 for *T. circumcineta*, *H. contortus* and *T. vitrinus* respectively. The calculated LD 50 of Quebracho

extract for *T.circumcincta* was 1.95 (0.93-2.96), for *H.contortus* 2.66 (1.34-3.98) and for *T.vitrinus* 2.07 (0.81-3.32) mg /ml solution; these values did not differ significantly.

3.4.2. *In vivo studies*

3.4.2.1. *Sheep health*

On day 28 of the experiment sheep infected with *T.colubriformis* and *N.battus* and drenched with the 16% w/w Quebracho extract, reduced dramatically their food intake and five out of six sheep stopped eating by day 30. As food intake had not recovered after the 3-day administration period of Quebracho, all sheep were removed from the experiment and for welfare reasons were killed on day 32. Consequently, analysis of liveweight gain and food conversion efficiency during the last week of the experiment does not include data from these animals. FEC of these sheep were recorded and are reported until day 32 of the experiment.

3.4.2.2. *Food intake, liveweight and food conversion efficiency*

In most cases sheep consumed all the food offered throughout the 35-day experimental period. A significant effect of sheep sex on both liveweight gain and food conversion efficiency was present until day 28 of the experiment. Male sheep increased their liveweight by 225g /day, whereas female by 170 g/day (sed: 10.2, $P<0.001$). This was the result of more efficient conversion of food in males (196 g gain/kg food intake) compared to females (150 g gain/ kg food intake, sed: 10.9, $P<0.001$).

From day 28 until the end of the experiment, all parasitised sheep drenched with the 8% w/w Quebracho reduced their liveweight gain significantly compared to undrenched parasitised sheep (289 g gain/day vs 378g gain/day respectively; sed: 43.4, $P<0.05$). Food conversion

efficiency was lower for sheep drenched with 8% w/w Quebracho extract compared to undrenched controls (223 vs 288 g gain/kg food intake respectively; sed: 30.1, $P < 0.05$). Sheep drenched with the 4% w/w Quebracho had a liveweight gain of 331 g gain/day and food conversion efficiency of 256 g gain/ kg food intake; neither of the above was different from control sheep. Male sheep were still gaining more weight during the last week of the experiment compared to females (359 vs 307 g gain/day respectively; sed: 26.9) and were converting food more efficiently, but the differences were not significant (272 vs 240 g gain/kg food intake respectively, sed: 18.6).

3.4.2.3. Faecal egg counts

Sheep infected with *T.colubriformis* and *N.battus* reduced their FEC from day 28 ($P < 0.05$), which was the first day of Quebracho extract administration. The reduction in egg numbers was higher as Quebracho concentration increased. From day 28 until day 31 after infection, *T.colubriformis* eggs from sheep drenched with 16% w/w Quebracho were lower compared to sheep drenched with 8% w/w Quebracho ($P < 0.001$, Fig. 3.2). FEC of sheep drenched with 4 or 8% w/w Quebracho extract were generally similar. Control sheep had the highest FEC ($P < 0.001$) throughout the experiment and were significantly higher than FEC of sheep drenched with 4% w/w Quebracho extract.

FEC of sheep infected with *N.battus* followed a similar direction as for *T.colubriformis* (Fig. 3.3). However, FEC from controls and sheep drenched with 4% w/w Quebracho extract were not significantly different. During the drenching period (days 28-31) sheep that were offered 16% w/w Quebracho extract had lower FEC than sheep offered 8% w/w Quebracho extract ($P < 0.05$) and both these were significantly lower than FEC of sheep drenched with 4% w/w Quebracho or controls ($P < 0.05$).

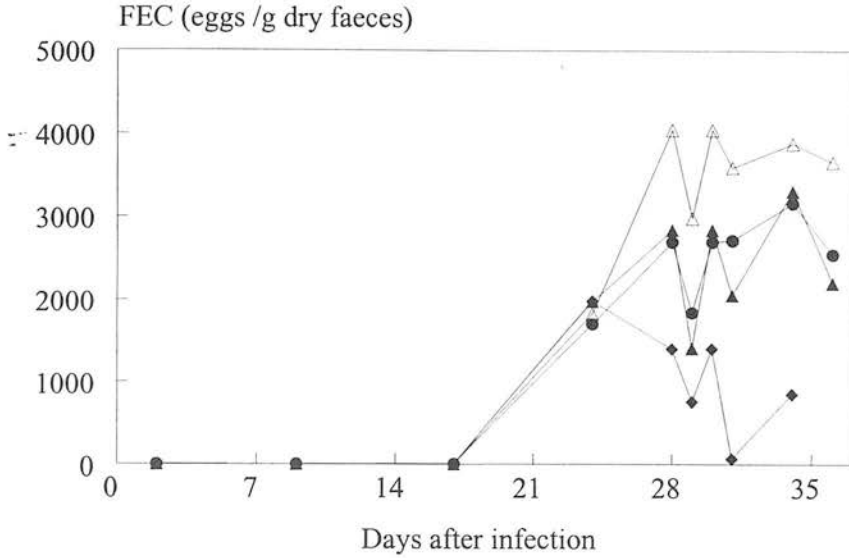


Figure 3.2. Backtransformed means of *T.colubriformis* faecal egg counts (eggs/g dry faeces) of sheep infected with 5,000 *L3 T.colubriformis* and 5,000 *L3 N.battus* on day 1 of the experiment. On day 28 sheep were drenched with either 4% (●), 8%(▲) or 16% (◆) w/w of food intake *Quebracho* extract. Undrenched controls are also shown (△).

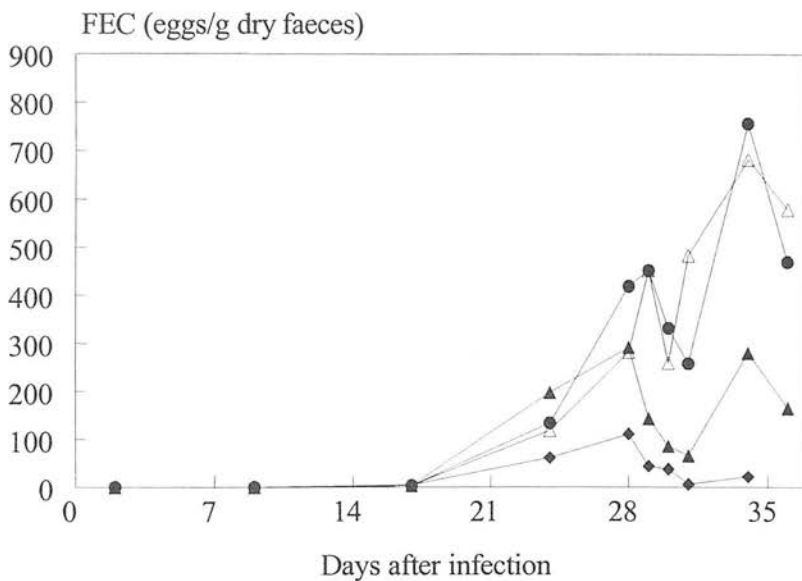


Figure 3.3. Backtransformed means of *N.battus* faecal egg counts (eggs/g dry faeces) of sheep infected with 5,000 *L3 T.colubriformis* and 5,000 *L3 N.battus* on day 1 of the experiment. On day 28 sheep were drenched with either 4% (●), 8%(▲) or 16% (◆) w/w of food intake *Quebracho* extract. Undrenched controls are also shown (△).

FEC of sheep infected with *T.circumcincta* were not affected by Quebracho administration (8% w/w). FEC of sheep offered the 8% w/w Quebracho extract was 241 (178-325) and for control sheep was 339 (251-459) eggs/g dry faeces on day 35 of the experiment (backtransformed data (95% confidence intervals)). Similarly the FEC of sheep infected with *H.contortus* were not affected by Quebracho administration at 8% w/w (backtransformed means (95% confidence intervals): 6284 (3898-10128) vs 9851 (6112-15878) eggs/g dry faeces on day 35, for control and drenched sheep respectively).

3.4.2.4. Worm burden and per capita fecundity

Total worm burden of *T.colubriformis* was significantly reduced in sheep drenched with 8% w/w Quebracho extract compared to control sheep ($P<0.05$, Fig. 3.4). Sheep drenched with the 4% w/w Quebracho extract had worm burdens between controls and sheep offered the 8% Quebracho, but they did not differ significantly from either. However, worm burdens of sheep drenched with 16% w/w Quebracho extract were similar to sheep drenched with 4% w/w Quebracho extract, which was not different from worm burdens of control sheep. Backtransformed means of worm burdens were considerably higher than respective arithmetic means in all groups (backtransformed and arithmetic mean worm burden of control group was 7,000 and 4,400 worms respectively). This was the result of the skewed distributions of worm burdens (one sheep had a much lower worm burden compared to the rest).

Total *N.battus* burden from sheep drenched with 8% w/w Quebracho extract was significantly reduced compared to those drenched with 4% w/w Quebracho extract or control sheep ($P<0.05$, Fig. 3.5). Sheep offered the 16% Quebracho extract had lower worm burdens than controls but the difference was not significant.

Worm burdens of sheep infected with *H. contortus* were unaffected by Quebracho extract administration (backtransformed means (95% CI): 1193 (740-1922) vs 1177 (730-1897) worms for sheep offered 8% w/w Quebracho and control sheep respectively).

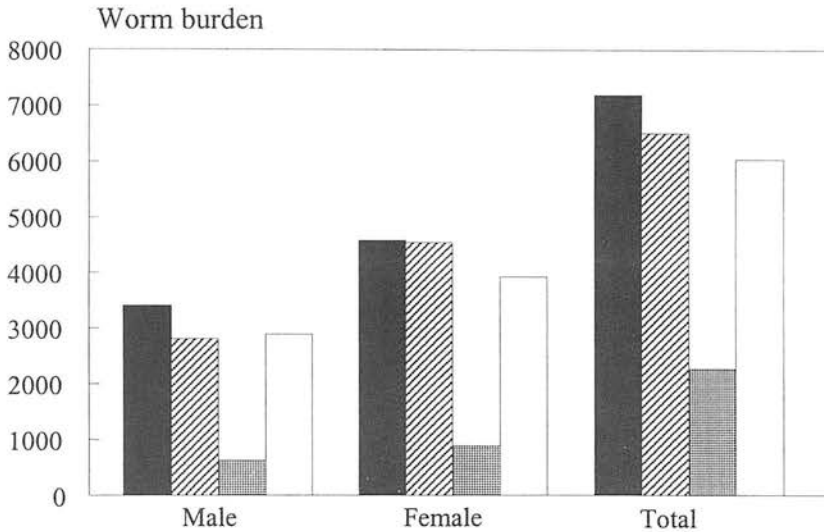


Figure 3.4. Backtransformed means of *T. colubriformis* male, female and total worm population in sheep infected with 5,000 L₃ *T. colubriformis* and 5,000 L₃ *N. battus* on day 1 of experiment. On day 28 sheep were drenched with either 4% (▨), 8% (▩) or 16% (□) w/w of food intake Quebracho extract. Undrenched controls are also shown (■).

Quebracho administration did not affect the worm burdens in the *T. circumcincta* groups (backtransformed means (95% CI): 1270 (788-2047) vs 2414 (1497-3892) worms, for sheep offered 8% w/w Quebracho and control sheep respectively). Numbers of male and female worms were similar amongst drenched and undrenched parasitised sheep

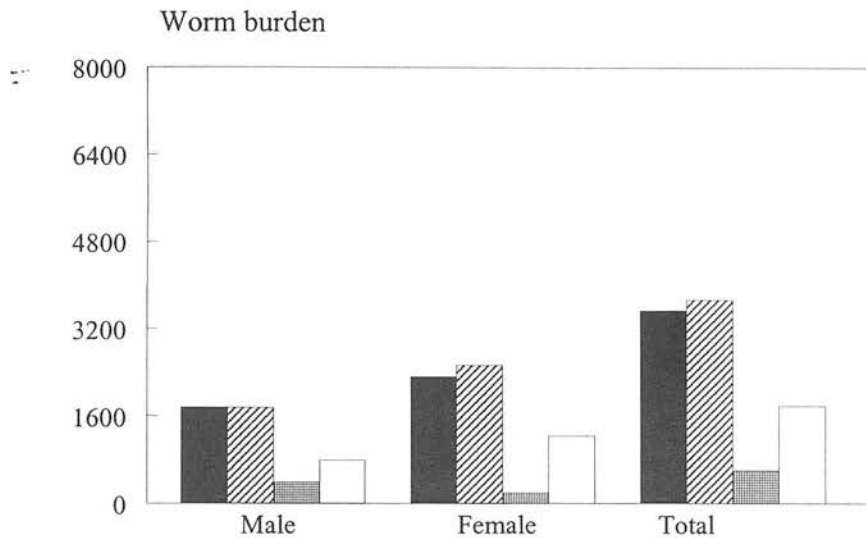


Figure 3.5. Backtransformed means of *N.battus* male, female and total worm population in sheep infected with 5,000 *L3 T.colubriformis* and 5,000 *L3 N.battus* on day 1 of experiment. On day 28 sheep were drenched with either 4% (▨), 8% (▩) or 16% (□) w/w of food intake Quebracho extract. Undrenched controls are also shown (■).

Per capita fecundity of female worms was not affected by sheep sex in any of the cases; however female worms of *T.circumcincta* parasitising male sheep showed lower fecundity compared to worms parasitising female sheep (0.064 vs 0.148 eggs/female worm, $P < 0.01$). The fecundity of *T.colubriformis* worms was reduced in sheep drenched with 16% w/w Quebracho extract compared to worm fecundity in control sheep (0.055 vs 0.181 eggs/female worm, $P < 0.01$, respectively). Similarly, the fecundity of *N.battus* worms was reduced in sheep drenched with 16% w/w Quebracho extract compared to controls (0.07 vs 0.012 eggs/female worm, $P < 0.05$). *Per capita* fecundity was not affected by Quebracho administration at 4 or 8% w/w. *Per capita* fecundity was not affected in *T.circumcincta* and *H.contortus* worms as a consequence of Quebracho administration.

3.4.2.5 *Blood parameters*

Quebracho administration did not affect the concentrations of any plasma parameters from day 28 of the experiment onwards. Total protein, albumin and calcium concentrations remained at the same levels throughout the experimental period. Plasma phosphorus concentration however decreased in all groups from 2.97 mmol/l on day 30 to 2.69 mmol/l on day 36 (sed: 0.11, $P < 0.05$). Urea concentrations increased in all groups from 5.72 mmol/l on day 1 to 8.25 mmol/l on day 7 (sed: 0.21, $P < 0.001$) and remained at this level until day 30. On day 35 of the experiment urea concentration had increased to 11.1 mmol/l in all groups.

3.5. Discussion

The larval development/viability assay provided no evidence that Quebracho affected larval development *per se*, but clear evidence that it affected the viability of infective larvae that had developed under exposure to Quebracho. The *in vitro* studies showed no species differences in susceptibility in contrast to the *in vivo* studies, which clearly demonstrated interspecific differences in susceptibility to Quebracho.

The observed anthelmintic activity of Quebracho extract *in vitro* could be attributable to tannin capacity to bind to proteins and could operate via several mechanisms. Tannins could bind to the free protein available in the wells for larvae nutrition; reduced nutrient availability in the wells could have resulted in larvae starvation and death. Tannins have a similar action towards microbes (McAllister et al, 1994a). Insects and insect larvae can ingest condensed tannins; as a result condensed tannins bind to the intestinal mucosa and cause autolysis (see review by Schultz, 1989). However the above could apply only for developing nematode larvae since infective larvae are a non-feeding stage of the life-cycle of nematodes. Condensed tannins may also bind to the cuticle of larvae, which is high in glycoprotein (Thompson and Geary, 1995) and cause their death. Recently it has been reported that some condensed tannin extracts inhibit the *in vitro* migration capacity of several gastrointestinal nematode larvae from deer as well as lungworm larvae, which could imply that certain types of condensed tannins might also cause neurological damage to parasites (Molan et al, 2000a).

Reduction of FEC and worm burden has been observed after a three-day administration of 8% of food intake Quebracho extract, to sheep infected with a single dose of *T.colubriformis* (Chapter Two). Despite continuing the administration of Quebracho for a week, FEC did not

decrease any further. The present study indicates that the reduction of FEC of sheep parasitised with *T.colubriformis* can occur within the first day of Quebracho administration and can be maintained at the same level for at least one week. Sheep receiving the 16% w/w Quebracho extract reduced their FEC more than sheep on 8% w/w Quebracho; however the former sheep reduced their food intake from day 28 of the experiment and showed severe diarrhoea from the second day of Quebracho administration. It is possible that the excessive reduction in FEC observed in sheep infected with both *T.colubriformis* and *N.battus* and drenched with 16% w/w Quebracho extract, could be attributable to a possible toxic effect on sheep due to a high dose of Quebracho extract. High concentrations of certain types of condensed tannins have been considered responsible for causing toxic effects in ruminants (Reed, 1995). Even though condensed tannins are polyphenols of high molecular weight and therefore not easily absorbed from the digestive tract, reports indicate that some types of condensed tannins may depolymerize and can be absorbed by herbivores (Clausen et al, 1990). Condensed tannins may also affect the abomasal or intestinal mucosa and therefore decrease the absorption of other nutrients (Reed, 1995). In addition, an increased flow of digesta caused by the induced diarrhoea might have created a hostile gut environment for the intestinal parasites, thus reducing their fecundity and consequently their FEC.

FEC of sheep infected with both intestinal nematode species showed a dose effect of Quebracho extract towards parasites; however worm burdens did not follow a similar direction. It is appreciated that as sheep drenched with 16% w/w Quebracho were killed four days earlier than the rest, a comparison between them and the rest could only provide an indication for a possible dose effect of Quebracho extract upon worm burdens. However, these sheep showed relatively high worm burdens compared to those which received 8% w/w extract. Firstly this could be attributable to the four-day difference amongst the two

slaughter points. It has been suggested that 4-7 days after an anthelmintic drench is the optimum time to check the gastrointestinal tract of sheep for the presence of worms, since by then worm expulsion is expected to be accomplished (Wood et al, 1995). Thus, it is possible worms were still present in the small intestine of sheep drenched with 16% w/w Quebracho, but were not alive. The high number of worms could also be a result of physiological changes in the intestine of those sheep, as discussed above. The presence of Quebracho extract in the faeces was observed eight hours after the first drench; the subsequent increase in digesta flow rate would have decreased the period of time that condensed tannins remained in the small intestine and in contact with the parasites. Evidence from early literature indicates that the nematocidal activity condensed tannins towards plant nematodes could be related to the duration of tannin presence (Taylor and Murant, 1966).

The two intestinal parasite species behaved similarly to the administration of Quebracho extract. The main difference between them was observed in the number of worms recovered from sheep drenched with 16% w/w Quebracho extract. In these sheep *T.colubriformis* burden was similar to that of control sheep, but *N.battus* burden was significantly lower than in controls. This difference could be due to physiological differences between the two species. *N.battus* worms are evenly located in the first half of the small intestine and the adults are associated superficially with the surface of the mucosa. On the other hand, *T.colubriformis* worms are located in the duodenum and anterior third of the small intestine and tend to burrow under the surface epithelium. Therefore it is possible that more *N.battus* worms were mechanically expelled than *T.colubriformis* as a result of the induced diarrhoea. Alternatively the degree of exposure to condensed tannins was probably higher in *N.battus* worms compared to *T.colubriformis* worms due to its location in the gut lumen (Lapage, 1968).

Per capita fecundity of female intestinal worms in sheep drenched with Quebracho extract at 16 % of food intake was the lowest of all treatments. Sheep drenched at 8% w/w of food intake Quebracho extract did not reduce their fecundity compared to controls, which contradicts previous studies (Chapter Two). However in this study the administration period of Quebracho extract was for a week, whereas in the present study it was given for three days. It is possible that the anthelmintic activity of Quebracho extract increases as the administration period is extended. Evidence for such effect has been obtained for the anthelmintic activity of raspberry extracts towards plant nematodes (Taylor and Murant, 1966).

T.circumcincta and *H.contortus* were not affected by Quebracho administration in our experiment. In previous studies, the consumption of *Lotus pedunculatus*, a forage high in condensed tannins, resulted in reduced total worm burden of *T.circumcincta* in sheep that were slaughtered 35 days after a single infection (Niezen et al, 1998b). On the other hand, total abomasal worm burden in sheep, consisting of *T.circumcincta* and *H.contortus* was not affected by the consumption of six different forages which contained condensed tannins (Niezen et al, 1998a). These contradictory results probably occur because of the complicated nature of tannins (see review by Mueller-Harvey and McAllan, 1992), of the tannin-protein complexes (Mueller-Harvey, 1999) and the different factors responsible for the formation of the complexes (Hagerman, 1989; Hagerman and Butler, 1991). Therefore different types of condensed tannins will be expected to have different consequences upon parasites. In addition, the amount of condensed tannins used in the present study or the three-day period of Quebracho administration might have been insufficient to reduce abomasal worm burdens.

All concentrations of Quebracho extract used in the experiment were consistent with concentrations used in previous studies and those present in nature (see review by Mueller-Harvey and McAllan, 1992). The concentrations of Quebracho extract that were chosen to be used in the *in vivo* experiment were comparable to concentrations used in the *in vitro* assays. The stock solutions of 12% and 10% w/v used in LDVA resulted in concentrations of 33 and 27 mg/ml Quebracho respectively in total volume of each well. Assuming that the rumen volume of growing sheep is about 5l and knowing that mean food intake was about 1200 g/day, the concentration of Quebracho in the rumen could be approximately estimated as 38 mg/ml, when the 16% w/w Quebracho was offered.

It is appreciated that *in vitro* conditions are *per se* different to conditions in the gastrointestinal tract of sheep; however larvae from both abomasal species were susceptible to the presence of Quebracho extract in LDVA but adults were unaffected by Quebracho administration *in vivo*. One possible explanation would be that condensed tannins are effective towards larvae but not towards adult parasites. However infective larvae are considered as a highly resilient stage of the life cycle of helminth parasites (Wharton, 1986b). The different effects observed between *in vitro* and *in vivo* may be attributable to the different conditions present in each case. The main factor that contributes to the formation and disassociation of complexes between proteins and tannins is the pH. *In vitro*, pH was not manipulated and therefore it was approximately neutral. It has been reported that complexes between condensed tannins and protein remain stable in pH between 5-7, but they disassociate in pH higher or lower than the above (see review by Mueller-Harvey and McAllan, 1992). In LDVA, condensed tannins from the Quebracho extract could bind to any macromolecule available in the growth medium, form stable complexes and via the mechanisms that were discussed above, they could damage the parasite larvae. When

Quebracho was administered to sheep via a stomach tube, condensed tannins arrived in the rumen where pH was suitable for the formation of complexes with microbial and/ or dietary protein (Jones and Mangan, 1977). Under normal conditions, abomasal pH is suitable for the disassociation of the complexes; however, abomasal pH increases during a parasitic infection of *T.circumcincta* or *H.contortus* and could possibly become inhibitive for the disassociation of the complexes. Moreover, the presence of surfactants, such as bile acids, has been reported to be important for the disassociation of tannin-protein complexes (Tebib et al, 1994; Martin et al, 1985) and these are not available in the abomasum. Therefore, it is possible that the abomasal nematodes were not exposed sufficiently to free condensed tannins during the three-day administration period and thus were not affected by their potential nematocidal action. The effects of condensed tannins on different gastrointestinal parasites of sheep is an area of research that merits further investigation.

Liveweight gain and food conversion efficiency were reduced in sheep drenched with 8% w/w Quebracho extract compared to sheep drenched with 4% w/w and undrenched controls, during the last week of the experiment. Similar observations were made following a week of administration of Quebracho extract at 8% w/w of food intake to sheep parasitised with a single dose of *T.colubriformis* (Chapter Two). Additionally, the digestibility of a food containing 50 g per kg DM Quebracho has been found to be reduced in sheep due to the presence of Quebracho and consequently their growth was impaired (Dawson et al, 1999). In the present study reduced growth of sheep was probably attributable to reduced digestibility, since food intake did not change. A dose effect of Quebracho is also indicated by the results, since sheep offered 4% w/w Quebracho showed liveweight gain and food conversion efficiency between that of control sheep and sheep drenched with 8% w/w Quebracho. However, it has been reported that sheep fed *ad libitum* with forages high in condensed

tannins had increased food intake and liveweight gain compared to sheep fed on forages low in condensed tannins (Niezen et al, 1998a). Therefore *ad libitum* food intake might be one way to overcome the adverse effects of condensed tannins upon productivity and maintain their detrimental effects upon parasitism.

In conclusion, condensed tannins from Quebracho extract reduced the level of intestinal parasitism in the present study, when they were administered as a drench for a three day period. Abomasal infections were not affected by Quebracho administration at the level of 8% w/w of food intake over three days. Further research is required to establish the rational use of condensed tannins as a supplement or alternative control strategy to reduce the use of anthelmintic drugs and at the same time diminish the detrimental effects they can cause on the ruminant host. Although *in vitro* assays, such as LDVA, could provide a useful means of screening different condensed tannins and/ or characterizing specific larval responses against them, the results from the present study indicate that they may provide a relatively poor indicator of parasite susceptibility in the short term *in vivo*.

Chapter Four

**Consequences of long-term feeding with condensed
tannins on sheep parasitised with *Trichostrongylus*
colubriformis.**

4.1. Abstract

Naive wethers were used to investigate the long-term effects of dietary condensed tannins from Quebracho extract, during an intestinal parasitic infection in sheep. Sheep were allocated to eight groups; seven groups were daily infected with 3,000 L₃ *Trichostrongylus colubriformis* for 10 weeks and the eighth group was the uninfected control. The 10-week experiment was divided into two periods; Period 1 (P₁, week 1-5) corresponded to high worm establishment and acquisition of immunity, whereas Period 2 (P₂, week 6-10) to the established worm population and expression of host immunity. Three experimental foods with similar composition were formulated: Q0, Q3 and Q6. Their difference was in the content of Quebracho extract, which was 0, 30 and 60 g per kg fresh matter, respectively. All foods were offered at an allowance of 3.5% of sheep liveweight. During P₁, parasitised sheep were offered one of the three experimental foods and during P₂ they either remained on the same food or changed food according to the design (P₁-P₂): Q0-Q0, Q0-Q3, Q0-Q6, Q3-Q0, Q3-Q3, Q6-Q0, Q6-Q6. Control sheep were offered the allowance of Q0 throughout. Sheep that consumed Q3 and Q6 reduced their faecal egg counts (FEC) compared to sheep offered Q0, during both periods (P<0.05). No differences were observed in the FEC between sheep offered Q3 and Q6. The changeover from Q0 in P₁ to either Q3 or Q6 during P₂, was accompanied by a reduction in FEC (P<0.05), whereas an increase in FEC was observed when food changed from Q3 or Q6 to Q0 (P<0.05). Worm burdens and fecundity at the end of the experiment were reduced in sheep offered foods Q3 and Q6 compared to sheep offered Q0. A significant decrease in liveweight gain and in food conversion efficiency of parasitised sheep offered Q3 and Q6 compared to sheep offered Q0, was observed in P₁ (P<0.05) but not in P₂. By the end of the experiment control sheep had achieved higher liveweight and converted food more efficiently than parasitised sheep (P<0.05). In conclusion, evidence for a long-term effect of Quebracho extract, during both

the initial establishment and on the established *T.colubriformis* population in sheep, was provided by the present study. It is suggested that the effect observed was a direct anthelmintic effect of the condensed tannins included in sheep diets.

4.2. Introduction

Since the 1960s, when thiabendazole was introduced as the first broad-spectrum anthelmintic drug against helminth infections, chemoprophylaxis has been used as the main strategy to control gastrointestinal parasitism in ruminants (Waller, 1997). However, the development of resistant strains of nematodes to anthelmintics (Jackson, 1993) and the move towards organic farming systems over the past few years, have increased the demand for alternatives to chemoprophylaxis, in order to reduce or even exclude the use of anthelmintic drugs to control parasites. The alternatives that are currently being investigated include immunisation (Smith, 1999), the breeding of hosts resistant to parasites (Gray, 1997), biological control of nematodes (Larsen, 1999), nutrient supplementation of the diet (see review by van Houtert and Sykes, 1996) and use of plants with anthelmintic properties (Chhabra and Mahannah, 1994). Amongst the latter, consumption of forages high in condensed tannins has been considered responsible for reducing the level of parasitism in young ruminants (Niezen et al, 1998a; b; Hoskin et al, 2000).

Condensed tannins are secondary plant metabolites that comprise the most widespread class of tannins in nature (Mangan, 1988). They have been associated with plant defences against insects and herbivores (Hagerman and Butler, 1991) and have been considered responsible for a number of both beneficial and detrimental effects when they are included in ruminant diets (Aerts et al, 1999). Recent reports from New Zealand have showed that the consumption of forages high in condensed tannins by sheep infected with gastrointestinal parasites resulted in reduction of their faecal egg counts (FEC) and total worm burden (Niezen et al, 1995; 1998b). Similar observations have been made in red deer infected with round- and lung-worms when they were offered freshly cut legumes high in condensed tannins (Hoskin et al, 2000).

In order to account for the effects of condensed tannins towards gastrointestinal parasites, two hypotheses could be developed. The first hypothesis concerns a direct effect of condensed tannins on parasite larvae, adult parasites and/or fecundity of the worms. Condensed tannins of Quebracho extract have been shown to have a direct anthelmintic effect on an established *T.colubriformis* population when they were given as a drench to parasitised sheep for 1 week (Chapter Two). The effect observed was a reduction of the adult worm population, their FEC and the *per capita* fecundity of the female worms; these effects were considered direct, since immunity was not expected to be expressed against an adult *T.colubriformis* population over the experimental period.

The second hypothesis suggests an indirect effect of condensed tannins towards gastrointestinal parasites evoked through protein nutrition of the host. Condensed tannins have the ability to bind to dietary protein and protect it from rumen degradation. Data from Martin et al (1985) indicate that the presence of surfactants could be very important for the disassociation of complexes; surfactants as bile acids are excreted in the small intestine and thus it is possible that tannin-protein complexes disassociate mainly in the small intestine (Jones, 1992). Protein is thus released and protein availability to the host is increased (Waghorn et al, 1987; Woodward and Reed, 1997). It has been suggested that protein supplementation can be responsible for enhanced immunological responses towards gastrointestinal parasites in ruminants (see review by Coop and Kyriazakis, 1999). However, if the food provides levels of protein that greatly exceed the maintenance requirements of the animals, such beneficial effect of protein supplementation in parasitised sheep might not be observed (Kahn et al, 2000).

The objective of the present study was to investigate i) the direct effect of intake of a condensed tannin extract (Quebracho), which was added to a high protein food, on the establishment of an intestinal nematode infection and also on the established parasitic population in sheep; ii) the effect of two levels of condensed tannins offered throughout the parasitic infection; both levels of Quebracho extract used in this experiment were comparable to condensed tannin concentrations found in plants included in ruminants diets, and iii) whether the detrimental effects of Quebracho extract towards parasites generate an improved performance of parasitised sheep.

4.3. Materials and methods

4.3.1. *Animals and housing*

Forty-eight Texel x Scottish Greyface wethers were used. They were reared indoors, from birth to weaning, under conditions that excluded nematode infection. Following weaning at 8 weeks of age, they were moved into individual pens (mean liveweight 31.2, se: 0.46 kg) and offered a high quality pelleted food *ad libitum* for 2 weeks. The food contained 180 g crude protein per kg fresh matter and was calculated to provide 12.3 MJ metabolisable energy per kg fresh matter. During the third week, the feeding protocol changed; sheep were offered a liveweight-based allowance of the food at 4% of their liveweight. This allowance was expected to be just below their *ad libitum* food intake (Kyriazakis et al, 1996b). Sheep were fed twice daily to reduce spillage. During the following week, which was the last of the acclimatisation period, sheep were introduced to the basal experimental food (Q0, see below) offered at the same allowance.

4.3.2. *Condensed tannin extract*

Cold soluble Quebracho extract (Quebracho ATO, Roy Wilson Dickson Ltd) was the source of condensed tannins used. This type of Quebracho extract is commercially available and contains 73% of condensed tannins, 19% of simple phenolics and 8% water. It is produced by treating the natural Quebracho extract (from the bark of the tropical dicotyledon *Schinopsis* spp.) with metabisulphite, oxalic acid and EDTA, to improve its solubility; this treatment does not appear to have an effect on its properties towards parasites (N.L. Butter, “The effect of condensed tannins and dietary protein on ruminant intestinal nematode infections”, The University of Nottingham, PhD thesis, 1999). The cold soluble Quebracho extract is of the same type used to investigate the short-term anthelmintic effect of

condensed tannins towards intestinal nematodes (Chapter Two), as well as their possible beneficial consequences on the immune system of parasitised sheep (Butter et al, 2000).

Quebracho extract was mixed in the food prior to pelleting, which took place at the lowest possible temperature (about 80°C). This was because it has been suggested that condensed tannins are sensitive to oxidation at temperatures around 100°C and can become inactive, i.e. they could lose their physical and possibly their chemical properties when treated at high temperatures (Larrauri et al, 1997).

4.3.3. Experimental foods and allowance

Three experimental foods were formulated and pelleted. A basal food (Q0) consisted of 384 g barley, 150 g oatfeed, 200 g citrus pulp, 190 g high protein soya, 50 g molasses, 10 g salt and 16 g of a mineral and vitamin mix per kg fresh matter. It contained 887 g dry matter, 151 g crude protein, 66.6 g ash, 228 g acid detergent fibre, 415 g neutral detergent fibre per kg fresh matter. It was calculated to provide 11.2 MJ metabolisable energy and 97 g metabolisable protein per kg fresh matter. The two other foods were made by diluting food Q0 with 30 (food Q3) and 60g (food Q6) Quebracho extract per kg fresh matter. Foods Q3 and Q6 were calculated to provide 11.1 and 10.7 MJ metabolisable energy and 95 and 92 g metabolisable protein per kg fresh matter respectively. The protein content of all foods was formulated to be above the requirements of growing lambs (AFRC, 1993). The foods were offered twice a day at 3.5% of their liveweight allowance throughout the experimental period. The 4% allowance offered during the acclimatisation period was adjusted to 3.5% for the 10-week experimental period, to overcome possible comparison problems between the groups arising from anorexia expected to be caused by parasitism (Kyriazakis et al, 1996b). The allowance was adjusted weekly, immediately after weighing.

4.3.4. Experimental design

One day prior to the start of the experiment, sheep (n=6) were randomly allocated into one of eight groups taking into account their liveweight; their mean liveweight was 37.4 kg (se: 0.5). Sheep of seven groups (group 2-8) were trickle infected with 3,000 infective larvae (L₃) of *Trichostrongylus colubriformis* 5 days weekly, from day 1 of the experiment; the remaining group (group 1) was the uninfected control. L₃ were harvested by a standard Baermann procedure, following a 10-day culture of faeces at 22°C, from donor sheep infected with *T.colubriformis*.

The 10-week experiment was divided into two periods: Period 1 (P₁) from week 1 to 5 and Period 2 (P₂) from week 6 to 10. The first 5 weeks of the infection have been associated with the phase of acquisition of immunity in naive hosts; during this period the rate of worm establishment is high in *T.colubriformis* infections and many immunological changes are initiated in the host (Dobson et al, 1990a). After week 6 of the infection acquired immunity is expressed, which enables hosts to limit incoming larvae and to eventually reject the adult worm population (Dobson et al, 1990b; see review by Coop and Kyriazakis, 1999). By dividing the experiment into two periods and offering to sheep one of the three foods according to the design presented on Table 1, we were able to investigate the effects of condensed tannins during the initial establishment of the parasitic infection (P₁) and also once the infection was established and immunity was being expressed (P₂). Nevertheless we appreciate that acquisition and expression of immunity is a continuum and there will be overlap between the two periods (see review by Coop and Kyriazakis, 1999).

Sheep offered the same food (Q0, Q3 and Q6) for the 10-week experimental period would provide information on the possible dose effect of condensed tannins throughout the

parasitic infection (Table 4.1). The change of food from Q0 in P₁ to either Q3 or Q6 in P₂ (groups 7 and 8 respectively) and *vice versa* (groups 5 and 6 respectively), would provide information on the separate effects of condensed tannins during the two different phases of the development of the infection. At the end of P₂ all sheep were slaughtered and their intestines were removed for worm recovery using procedures described later.

Table 4.1. Groups and corresponding feeding treatments (Quebracho supplementation) of sheep daily infected with 3,000 L₃ T.colubriformis (P, Groups 2-8) over two periods (Period 1 (P₁): week 1-5 and Period 2 (P₂): week 6-10) and the uninfected controls (C, Group 1). Foods Q0, Q3 and Q6 contained 0, 30 and 60 g Quebracho per kg of the fresh matter respectively.

Groups	Parasitic Status	Feeding treatments	
		Period 1 (P ₁)	Period 2 (P ₂)
1	C	Q0	Q0
2	P	Q0	Q0
3	P	Q3	Q3
4	P	Q6	Q6
5	P	Q3	Q0
6	P	Q6	Q0
7	P	Q0	Q3
8	P	Q0	Q6

4.3.5. Measurements and sampling procedures

Sheep were weighed on the first day of each week, before morning feeding. Feed refusals were rare, because of the restricted feeding. When observed, they were recorded and an equivalent amount of fresh food was offered back to the animals. Blood samples were taken on the first day of each week by jugular venepuncture into heparinised vacutainers (Becton Dickinson, UK). Plasma was separated by centrifugation at 2060 g for 20 min and stored at -20°C until analysed for total protein, albumin, urea, phosphorus and calcium concentrations. The analyses were performed using a microcentrifugal analyser (Monarch 2000, Instrumentation Laboratory, Warrington, Cheshire, England) and standard commercial kits.

Faecal samples were collected on the second day of each week directly from the rectum and were processed either immediately or stored at 4°C and processed within 2 days. Numbers of nematode eggs per g fresh faeces were determined using a modified flotation technique (Christie and Jackson, 1992), in which polyallomer centrifuge tubes (Beckman) were used to separate the meniscus containing the eggs. The dry matter of the faecal samples was estimated every week and faecal egg counts (FEC) were expressed relative to dry matter (eggs per g dry faeces). Final measurements of liveweight, blood and faecal samples were taken on the last day of week 10 (measurement 11).

At slaughter the small intestines were ligated, removed, washed and the worms were recovered from the digesta and the intestinal mucosa following incubation in physiological saline at 37°C for 4 h. The total worm burden and the sex ratios were calculated from a 2% aliquot of digesta and mucosal digest. For determination of the number of eggs in the *uterus* of female worms (*in utero* fecundity), 25 females were separated at random from each aliquot and stained with 0.01% cotton blue in lactophenol. *Per capita* fecundity was

calculated by dividing the FEC recorded the day before slaughter by the total number of female worms recovered.

4.3.6. Statistical analysis

Total worm burdens, *in utero* and *per capita* fecundity were analysed by two-way analysis of variance (ANOVA) with the feeding treatments during the two periods as factors (Genstat 5, release 3.2; Lawes Agricultural Trust, 1993). Since the interaction between the feeding treatments over the two periods was not significant, only the effects from the feeding treatments are reported for P₂. Liveweight gain and food conversion efficiency were analysed by one-way ANOVA for P₁ and by two-way ANOVA for P₂, using the feeding treatments during the two periods as factors. Again, the interaction between the feeding treatments over the two periods was not significant; therefore, only the feeding treatment effects are reported for P₂. Comparisons on the liveweight gain and food conversion efficiency were made between non-parasitised (group 1) and parasitised sheep offered the Q0 food (group 2) during P₁ and P₂ by one-way ANOVA. Blood parameters and FEC during P₁ were analysed by one-way ANOVA for repeated measurements (split plot model) with the feeding treatments as factor. Groups offered the same food were combined for the purposes of the analysis, e.g. groups 2, 7, 8 offered Q0, groups 3 and 5 offered Q3 and groups 4 and 6 offered Q6 in P₁, were analysed together. Comparisons of blood variables between non-parasitised and parasitised animals (groups 1 and 2) were performed using the same model.

Blood parameters and FEC during P₂ were analysed for each feeding treatment separately, with an ante-dependence model for repeated measurements. The interaction between the feeding treatments over the two periods was analysed by including a two-way ANOVA in the model, using the feeding treatments for P₁ and P₂ as factors. However as this interaction

was not significant, only treatment effects are reported for P₂. The ante-dependence model was considered as the most appropriate for data analysis during P₂, since it provided a means of making comparisons between the groups at each time point, taking into consideration differences at previous time points (Kenward, 1987). Data from FEC and total worm burden were transformed ($\log(x + 1)$) prior to analysis, to stabilise the variance. Back-transformed means with 95% confidence intervals (CI) are reported for transformed data.

4.4. Results

4.4.1. Food intake, liveweight gain and food conversion efficiency

The amount of food offered was consumed in full by most animals. However, three of the control sheep reduced severely their food intake at week 10 of the experiment; they were diagnosed as having obstructed urolithiasis and were immediately removed from the experiment. Their data during the final week of the experiment were treated as missing values.

The daily liveweight gain and the food conversion efficiency (g gain / g food) during P₁ and P₂ for all groups are shown in Table 4.2. During P₁, liveweight gain and food conversion efficiency were not affected by the parasitic status of the animals (comparison between groups 1 and 2). Significant differences were observed between parasitised sheep offered the Q0 (groups 2,7,8,) and sheep offered Q3 and Q6 (groups 3,5 and 4,6 respectively). There were no differences detected in liveweight gain or food conversion efficiency between sheep offered Q3 and Q6 during P₁ (groups 3 and 4).

During P₂, control sheep had higher liveweight gain and food conversion efficiency than parasitised sheep offered Q0 ($P < 0.05$) (Table 4.2). Feeding treatment during P₁ did not have any effect on the liveweight gain or the food conversion efficiency in any of the groups in P₂. Parasitised sheep offered Q0 had similar liveweight gain and food conversion efficiency to those offered Q3 and Q6.

Table 4.2. Mean liveweight gain (g/day) and food conversion efficiency (g gain/ g food) of sheep daily infected with 3,000 L₃ *T.colubriformis* (P, Groups 2-8) and the uninfected controls (C, Group 1). Details of the feeding treatments are given in Table 4.1.

		Period			Period		
		1			2		
Status	Feeding treatment	Group	Live weight gain	Food conversion efficiency	Group	Live weight gain	Food conversion efficiency
C	Q0	1	203	0.145	1	206	0.130
P	Q0	2,7,8	216	0.153	2,5,6	167	0.106
P	Q3	3,5	173	0.123	3,7	162	0.101
P	Q6	4,6	177	0.129	4,8	155	0.097
sed			21.1	0.014	12.0		0.005
Effects of:							
Feeding treatment			*	*	NS		NS
Status ¹			NS	NS	*		*

¹: Comparison between C and P sheep offered Q0.

sed: standard error of the difference of the means

NS: non significant difference

*: P< 0.05.

4.4.2. Faecal egg counts

Eggs were first observed in the faeces of parasitised sheep on week 4 from the start of the trickle infection. During P₁, FEC were significantly lower ($P < 0.05$) in sheep offered foods Q3 (groups 3 and 5) and Q6 (groups 4 and 6) compared to sheep offered Q0 (groups 2, 7 and 8) (Fig. 4.1). No differences were observed on the FEC between sheep offered Q3 and Q6. At the end of P₁ FEC were (backtransformed means (95% CI)): 1730 (1343-2227), 1133 (831-1543) and 989 (725-1348) for sheep offered Q0, Q3 and Q6 respectively.

During P₂, FEC of sheep offered Q3 and Q6 were lower compared to sheep offered Q0; this difference was significant on weeks 7, 8 and 9 ($P < 0.05$). FEC of sheep offered Q3 and Q6 did not differ during P₂ (Fig. 4.1). FEC values converged for all sheep by week 10 of the experiment (backtransformed means (95% CI): 2238 (434-11535), 1892 (367-9754) and 1338 (260-6895) for sheep offered Q0, Q3 and Q6 respectively). Observations on the change over of the foods at week 5 from Q0 to Q6 and *vice versa* (Fig. 4.2a), showed that sheep which changed their food from Q0 during P₁ to Q6 during P₂ had significantly lower FEC, compared to sheep that remained on Q0 during P₂ ($P < 0.05$). Sheep that changed their food from Q6 to Q0 in P₂, had significantly higher FEC compared to sheep that remained on Q6 for P₂ ($P < 0.05$). This difference was present up to week 9 of the experiment. The results of the change of the foods from Q0 to Q3 and *vice versa* followed the same direction (Fig. 4.2b); however the statistical analysis did not show any significant differences between these treatments.

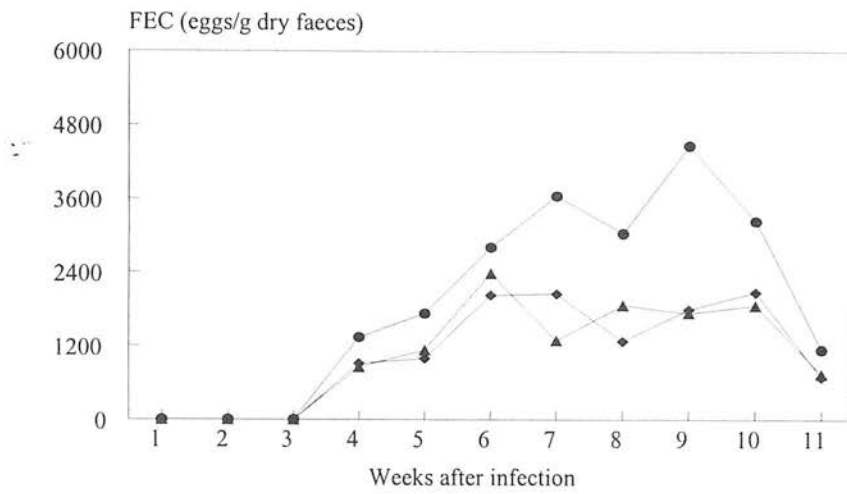


Figure 4.1. Backtransformed means of faecal egg counts (FEC, eggs/ g dry faeces) of sheep daily infected with 3,000 L₃ *T. colubriformis* and offered either a basal food (Q0, ●) or food Q0 supplemented with 30 g (▲) and 60 g (◆) Quebracho extract per kg fresh matter.

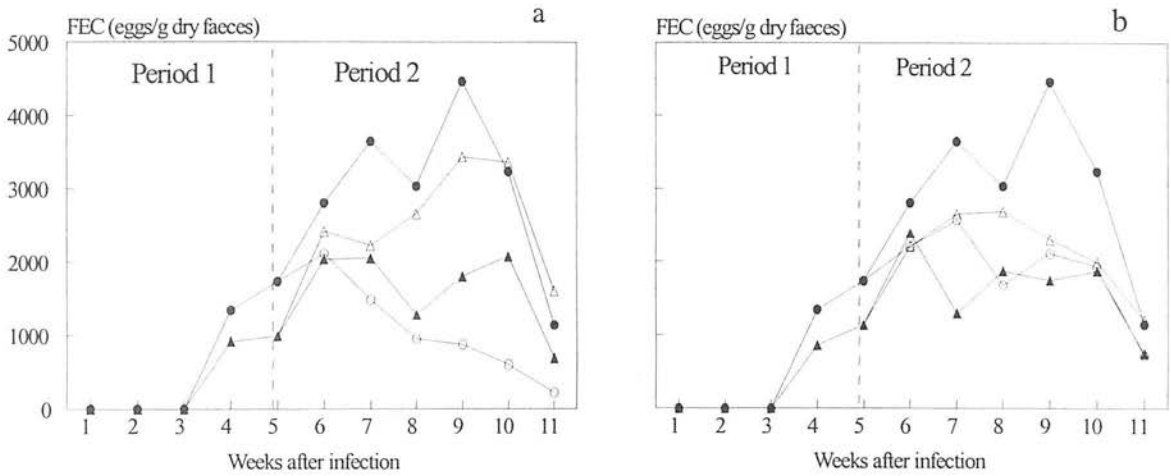


Figure 4.2. Backtransformed means of faecal egg counts (eggs/g dry faeces) of sheep daily infected with 3,000 L₃ *T.colubriformis* over two periods. During Period 1 sheep were offered either a basal food (Q0, ●), or (a) food Q0 supplemented with 60 g (Q6, ▲) Quebracho extract per kg fresh matter. During Period 2 sheep either remained on the same food as in Period 1 or changed from Q0 to Q6 (○) and from Q6 to Q0 (Δ), (b) food Q0 supplemented with 30 g (Q3, ▲) Quebracho extract per kg fresh matter. During Period 2, sheep either remained on the same food as in Period 1 or changed from Q0 to Q3 food (○) and from Q3 to Q0 (Δ).

4.4.3. Plasma analysis

The statistical analysis of phosphorus during P₁ did not show any significant differences among sheep in different feeding treatments (3.08, se: 0.07 mmol/l). However during the first 2 weeks of P₁, a significant time effect ($P < 0.05$) was observed, as phosphorus concentrations declined for sheep in all groups; after week 4, phosphorus values increased (data not shown). During P₂, a significant effect of the parasitic status of sheep was observed from week 7 to 9 ($P < 0.05$); non-parasitised sheep maintained their phosphorus levels whereas, infected sheep showed a decrease (3.78 vs 2.51, se of difference: 0.34 mmol/l). There were no differences in plasma phosphorus between sheep in parasitised groups.

Urea concentrations decreased with time during week 2 and 3 for sheep in all groups ($P < 0.05$) but they recovered from week 4 onwards (Fig. 4.3). During P₁, sheep offered Q6 showed a significantly lower urea concentration compared to sheep offered Q0 ($P < 0.05$). No differences were detected between control and parasitised sheep offered Q0. During P₂, urea values in sheep offered Q3 and Q6 were lower than in sheep offered Q0 food but the difference was not significant. The observed difference was present until week 10 of the experiment. Control sheep showed higher urea concentrations than parasitised sheep offered Q0, until week 8 of the experiment ($P < 0.05$). No differences were present between sheep offered Q3 and Q6 throughout the 10-week experimental period.

Plasma calcium, total protein and albumin concentrations did not show any differences between all treatments throughout the 10-week experimental period. However during weeks 2 and 3 of P₁, a significant time effect was present for all variables ($P < 0.05$); values from

sheep in all groups declined over this period, but they returned to normal levels from week 4 onwards. (data not shown).

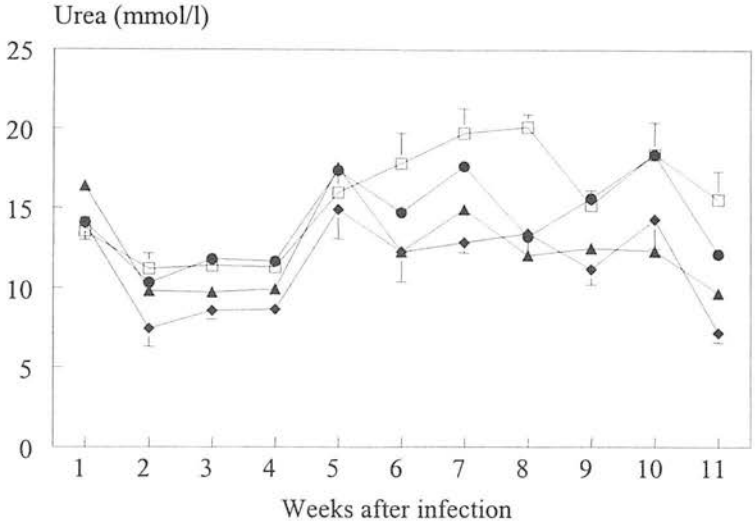


Figure 4.3. Plasma urea concentrations (mmol/l) of sheep daily infected with 3,000 L₃ *T. colubriformis* and offered a basal food (Q0, ●) or food Q0 supplemented with 30 g (▲) and 60 g (◆) *Quebracho* extract per kg fresh matter for 10 weeks; the uninfected controls (□) that were offered food Q0 are also shown. The bars indicate the standard error of the mean.

4.4.4. Worm burden and fecundity

Feeding treatments during P₁ did not have any effects upon worm counts or *per capita* fecundity at the end of the experiment. The total worm burden recovered from sheep offered Q3 during P₂ was significantly reduced (P<0.05) compared to sheep offered Q0 during the same period (backtransformed means (95% CI): 10,465 (4,813-22,750); 29,836 (13,725-64,858) for Q3 and Q0 foods respectively). Sheep that were offered Q6 during P₂, showed a tendency for lower worm burden than sheep offered Q0 (backtransformed means (95% CI): 12,542 (5,769-27,264); 29,836 (13,725-64,858) for Q6 and Q0 foods respectively). *Per capita* fecundity of worms in sheep offered Q6 during P₂ was significantly (P<0.05) reduced

compared to sheep offered Q0 (backtransformed means (95% CI): 0.107 (0.040-0.147); 0.121 (0.040-0.161) 0.042 (0.032-0.079); for Q0, Q3 and Q6 respectively). *In utero* fecundity of worms was not significantly different amongst sheep from different feeding treatments (backtransformed means (95% CI): 9.31 (5.27-15.9); 9.12 (5.15-15.6); 7.49 (4.16-12.9) for Q0, Q3 and Q6 respectively).

4.5. Discussion

Sheep trickle infected with *T.colubriformis* larvae had reduced FEC when they were offered a food containing condensed tannins from Quebracho extract for 10 weeks, compared to sheep offered a Quebracho-free diet. This reduction was observed during the period of the initial establishment of the parasitic infection (weeks 1-5), as well as during the established infection (weeks 6-10). The effect of condensed tannins during P₁ was most likely due to a direct anthelmintic effect towards *T.colubriformis* and not due to an indirect nutritional effect. In naive hosts, the first 4-5 weeks of *T.colubriformis* infection constitute the period of the acquisition of immunity, which is not expected to be affected by protein supplementation (van Houtert et al, 1995; see reviews by Coop and Kyriazakis, 1999; 2000). A direct anthelmintic effect of Quebracho extract has recently been reported towards a *T.colubriformis* adult population in infected sheep. A seven-day administration of Quebracho extract at 8% of their food intake caused an immediate reduction on the FEC, the worm burden and the fecundity of female worms compared to the parasite population in control sheep (Chapter Two). The consequences of feeding Quebracho for a longer period of time in this experiment are consistent with that effect. However, changes on the intestinal environment caused by condensed tannins of Quebracho extract should not be ignored. Reduced microflora in the gastrointestinal tract due to antimicrobial properties of tannins (Scalbert, 1991), increased mucin production in the small intestine (Dawson et al, 1999), or low availability of nutrients for parasite nutrition due to tannin binding abilities, could have a negative effect upon worm population.

The reduction in the FEC during P₂ could be attributed to either a direct effect, similar to that observed during P₁, or an indirect nutritional effect. During this period (after week 5 of the infection), naive hosts would start expressing their acquired immunity (see review by

Coop and Kyriazakis, 1999). When expression of immunity occurs, parasitised sheep have been shown to benefit from dietary protein supplementation, through the development of an effective immune response (Bown et al, 1991; van Houtert et al, 1995). Condensed tannins could affect intestinal parasites via this route, since by binding to dietary protein, they protect it from rumen degradation and make it available for the host in the small intestine (see review by Mueller-Harvey and McAllan, 1992). However, the beneficial effects of protein supplementation have been observed in cases where the host had access to a food inadequate in protein (see review by Coop and Kyriazakis, 1999). In the present study, the protein content of all three foods was calculated to be well above the protein requirements for maintenance and potential growth of sheep, thus an indirect effect of Quebracho should perhaps be discounted. Therefore, the reduction in the FEC observed during the 10-week experimental period could be attributable to a direct anthelmintic effect of condensed tannins towards *T.colubriformis*.

The change over of the foods during P₂ indicated that the anthelmintic effect of Quebracho extract is rapid and reversible, since it appeared from the first week of administration of the tannin containing food and it disappeared when it was replaced with food Q0. A rapid effect of condensed tannins has also been reported by Niezen et al (1995); sheep grazing a forage high in condensed tannins (*Hedysarium coronarum*) reduced their FEC as early as day 14 after a single dose of *T.colubriformis*, compared to sheep that grazed a forage low in condensed tannins (*Medicago sativa*). The observed reversible effect of condensed tannins towards *T.colubriformis* supports the view discussed above, that the reduction in the FEC during P₂ is unlikely to be mediated via acquired immunity. It has been suggested that a breakdown of immunity is not expected, unless the nutrition of the host deteriorates (see

review by Coop and Kyriazakis, 1999); in our case all foods were of high quality and calculated to cover the requirements of growing sheep.

The reduction in the FEC could also be the outcome of physiological changes occurring in the host, due to the presence of Quebracho extract in the food. Quebracho extract increased the water content of faeces when it was offered as a drench in parasitised sheep (Chapters two and Three). However in the present study, FEC of sheep were expressed as eggs per g dry matter of faeces in order to account for this condensed tannin effect. On the other hand, addition of Quebracho extract in the diets of sheep has been shown to reduce the digestibility of the food offered to sheep and increase their total faecal output, compared to sheep offered tannin free food (Dawson et al, 1999). Recently, Quebracho extract has been considered responsible for causing an increase in the total faecal output of parasitised sheep when it was included in a high protein diet (220 g per kg fresh matter), compared to control sheep; however no difference was observed when it was included in a low protein diet (97 g per kg fresh matter) (Butter et al, 2000). In our study, a dilution effect of the faeces caused by an increase in faecal output could be considered an additional contributing factor to the observed reduction of the FEC of sheep offered the Q3 and Q6 foods. However, this reduction could only partly be due to the reduction in digestibility of the food, since the differences observed in worm burdens support the anthelmintic effect of Quebracho tannins. In addition, FEC among all groups during the last two measurements of the experiment were similar, thus indicating that the detrimental effect on digestibility of the food caused by condensed tannins could not have been severe.

The reduction of the parasite burden was anticipated to be followed by improved performance of parasitised sheep. However the liveweight gain and the food conversion

efficiency during P₁ were higher for sheep offered Q0 compared to sheep offered Q3 or Q6. The lack of beneficial effects on sheep performance could be attributable to the reduced digestibility caused by the addition of Quebracho in the foods, or the artificial dilution caused by the formulation of the foods. A dilution effect would be expected to be smaller in sheep offered Q3 and larger in sheep offered Q6; as this was not observed, reduced liveweight gain and food conversion efficiency in sheep offered Q3 and Q6 is more likely to be due to a reduction in digestibility. In grazing conditions, sheep that grazed forages high in condensed tannins (sulla) showed higher liveweight gain than controls (Niezen et al, 1995). However, in that experiment, sheep had free access to the forages throughout the day and therefore they could eat until they met their requirements.

During P₂, the difference in the liveweight gain and the food conversion efficiency among sheep from different feeding treatments was reduced. This occurred mainly because parasitised sheep offered food Q0 reduced their liveweight gain and food conversion efficiency as a result of parasitism; the comparison with the control sheep supports this. The performance of parasitised sheep, which consumed foods Q3 and Q6, did not decline to the same extent as those offered Q0. This beneficial effect could be attributable to reduced level of parasitism caused by the consumption of condensed tannins by parasitised sheep.

The reduction in the FEC of sheep offered foods Q3 and Q6 during P₁ was about 25% compared to parasitised sheep offered Q0; during P₂ this difference increased to 40%. It is possible that during P₂ condensed tannins of Quebracho extract were more effective against the parasites, either due to a cumulative effect of tannins or to a higher efficacy of tannins on the adult parasites rather than the larval stages. However, a cumulative effect of condensed tannins can not be justified since there is evidence for a reversible effect from the

changeover of the foods in P₂. A higher efficacy of condensed tannins against adult parasites than against nematode larvae is possible, because of the location of the larvae and the adults in the gastrointestinal tract of sheep. *T.colubriformis* larvae penetrate between the epithelial cells of the small intestine mucosa and stay under the epithelium until they develop to adult parasites (Urquhart et al, 1996). Adult parasites on the other hand can be embedded in the mucosa, with parts of their bodies in the lumen (Coop et al, 1979). Since condensed tannins do not seem to be absorbed by the gastrointestinal tract of sheep (Reed, 1995) hence are present in high concentrations in the lumen, they could have been able to affect more the adult worms than the larvae.

There was no significant dose dependent effect of Quebracho tannins towards *T. colubriformis* during the 10-week experimental period. FEC from sheep that changed their food from Q3 to Q0 and *vice versa* in P₂, followed a similar direction, which however was not significant, as for sheep that changed from Q6 to Q0 and *vice versa*. These data imply that Q6 could have a higher effect on parasites than Q3, but this effect was not strong. Dose dependent effect of condensed tannins has been observed in sheep and red deer. Grazing ruminants that consumed forages high in condensed tannins (about 12% of the dry matter) showed a lower FEC compared to those given access to forages with lower concentration (about 5%) of condensed tannins (Niezen et al, 1998a; Hoskin et al, 2000). However, high concentrations of condensed tannins (over 70 g per kg dry matter of the food) have been considered responsible for causing detrimental effects on ruminants (Barry, 1985), and thus they were not included in the present study.

The consumption of Q3 and Q6 resulted in a reduction of the total worm burden and *per capita* fecundity of female worms, even though the differences observed were not always

significant. This could be attributable to the individual variation amongst sheep, which was observed towards the end of the experiment, due to the occurrence of expression of host immunity. Reduction in total worm burden of intestinal and abomasal species has been reported in sheep grazing forages containing condensed tannins (Niezen et al, 1998b) but these differences were observed before immunity was expressed. Reduction of *per capita* fecundity of female worms could be attributable to reduced digestibility caused by Quebracho extract and thus dilution of eggs in the faeces as discussed above.

The decline in all blood variables during weeks 2 and 3 of the experimental period was probably due to the dietary change of the food allowance from 4% of the liveweight during acclimatisation period to 3.5% during the experimental period. Their increase after week 4 and maintenance at high levels supports this view. Despite reduced parasitic burdens in sheep offered Quebracho containing foods, the level of hypophosphataemia was similar in all parasitised sheep. Hypophosphataemia is due to intestinal damage caused by parasites and is consistent with findings from trickle challenged sheep with *T.colubriformis* (Kyriazakis et al, 1996a). The low plasma urea concentrations observed in sheep offered food Q6 may be attributable to the presence of condensed tannins; it has been reported that reduced protein degradation in the rumen, due to the tannin-protein complexes, can decrease urea production and consequently the urea absorbed into the blood circulation is lower (Waghorn and Shelton, 1995). On the other hand low plasma urea concentration could be a physiologically regulated effect caused by lower food intake, due to a reduced growth rate of sheep offered food Q6 compared to sheep offered Q0.

In conclusion, the present study investigated the direct long-term anthelmintic effect of Quebracho tannins on sheep infected with *T.colubriformis*. It provided evidence that

Quebracho extract reduced the rate of establishment and the development of the parasitic infection, when it was included in the foods of infected sheep. However, Quebracho extract possibly reduced the digestibility of the food it was included in and therefore the performance of sheep did not improve. It is suggested that *ad libitum* intake of foods supplemented with Quebracho extract could reduce the level of parasitism and enhance the performance of parasitised sheep, since sheep would be able to eat according to their requirements. Different types of condensed tannins could have different properties on ruminant physiology and digestion as these depend on their chemical structure (see review by Mueller-Harvey and McAllan, 1992). However, as Quebracho extract is commercially available in large amounts, its addition to the food provided the opportunity to study the effects of condensed tannins *per se* over a relatively long period of time.

Chapter Five

The effects of condensed tannin supplementation of foods with different protein content on parasitism, food intake and performance of sheep infected with *Trichostrongylus colubriformis*

5.1. Abstract

The aim of the study was to investigate the consequences of offering *ad libitum* either a high or a low protein food supplemented with condensed tannins on parasitised sheep. As the consumption of condensed tannin has been related to reduced food digestibility, it was hypothesised that *ad libitum* intake would enable sheep to cope with this effect, by eating according to their requirements. Hence, following a reduction of sheep worm burden, attributable to the anthelmintic properties of condensed tannins, the consumption of supplemented foods was expected to improve sheep performance to different extent in the low and high protein foods. For this purpose, 48 previously parasite naive sheep (n=12) were infected with 2,000 *T.colubriformis* larvae per day for a 67-day experimental period. Two experimental foods were made: L, formulated to be inadequate in meeting the requirements of growing sheep for metabolisable protein (MP) and H expected to be above the requirements of growing sheep for MP. Two additional foods were made by adding 60 g Quebracho (a condensed tannin extract) per kg fresh matter to foods L and H (foods LQ and HQ respectively). This level of Quebracho supplementation has been shown to reduce the level of parasitism in restrictedly fed sheep. The experiment was divided into two periods: period 1 (P₁, day 1-38) which has been associated with worm establishment and acquisition of immunity and period 2 (P₂, day 39-67) which corresponded to the established worm population and expression of immunity. Six sheep from each group were slaughtered at the end of P₁, and the remaining sheep were slaughtered at the end of P₂ (day 67). Although faecal egg counts (FEC, number of eggs/g faeces) and total egg output were reduced in sheep offered the supplemented foods during P₁ (P<0.05), worm burdens on day 38 were unaffected by the consumption of the supplemented foods. FEC and worm burdens were not affected by either Quebracho supplementation or food protein content during P₂. Food intake was higher in sheep offered food HQ compared to sheep offered food H (P<0.05); it

was similar in sheep offered foods LQ and L throughout the experiment. Sheep offered foods L_Q and HQ did not increase their liveweight gain compared to sheep offered foods L and H respectively, and dry matter digestibility of the supplemented foods was not affected by Quebracho supplementation. As the anthelmintic properties of condensed tannins were not demonstrated following *ad libitum* intake of foods supplemented with Quebracho, the performance of parasitised sheep was unaltered. Higher levels of condensed tannin supplementation may be required to lessen parasitism and improve the performance of parasitised sheep fed *ad libitum*, as has been observed for grazing sheep.

5.2. Introduction

The control of helminth infections in both ruminant and non-ruminant farm animals has traditionally been anthelmintic drug-dependent. However, the development of anthelmintic resistant strains of parasites (see review by Jackson and Coop, 2000), which is particularly prevalent for small ruminant nematodes, the increasing concern for drug residues in animal products and the growth of organic farming systems have increased the need for alternative sustainable approaches to control parasitism. Amongst the alternatives under consideration, the use of plants with anthelmintic properties appears to be a promising approach for controlling gastrointestinal parasitism in both temperate and tropic climates (Niezen et al, 1996). Although, many of the active plant metabolites involved have not yet been identified, there is evidence that the consumption of forages high in condensed tannins can reduce the level of parasitism in grazing and browsing ruminants (Niezen et al, 1998b; Hoskin et al, 1999). Recent *in vitro* and *in vivo* studies suggest that condensed tannin extracts can affect directly different nematode parasites of sheep and red deer (Chapters Two and Three; Molan et al, 2000a; b),

Condensed tannins are the most widespread fraction of tannins, which are phenolic secondary plant metabolites. The presence of condensed tannins, mainly in the woody and to a lesser extent in the leafy parts of the plants, has been thought to act as a defence towards insect and mammalian herbivores (McArthur et al, 1991). Nevertheless, the biological significance of condensed tannins has not been clearly defined, since the consumption of condensed tannins can lead to both detrimental and beneficial consequences in ruminants. High concentrations of condensed tannins in forages have been considered responsible for reducing food digestibility and intake (Barry and Duncan, 1984) of grazing ruminants; on the other hand, moderate concentrations appear to increase wool growth (Wang et al, 1996)

In the present study we aimed to investigate whether *ad libitum* food intake of a low and a high protein food supplemented with condensed tannins (Quebracho extract) would reduce the level of parasitism and improve the performance of parasitised sheep, during the initial worm establishment and when an established parasitic infection was present. We hypothesised that when tannin supplemented foods were offered *ad libitum*, sheep would have the opportunity to eat to the extent required to overcome any adverse consequences of condensed tannins upon digestibility. Once the above was achieved, the performance of sheep on the low protein food supplemented with Quebracho extract would be expected to improve, both by the extra protein available for absorption in their small intestine and the reduced level of parasitism caused by condensed tannins. Sheep on the high protein food supplemented with Quebracho extract were expected to benefit only from the anthelmintic effect of condensed tannins. Thus the benefits of tannin supplementation were expected to be higher in sheep on the low than on high protein food.

5.3. Materials and methods

5.3.1. Animals

Fifty-four Texel x Greyface female sheep were used. They were reared indoors under conditions that excluded the likelihood of nematode infections. After weaning at 8 weeks of age (mean liveweight: 29.5 kg, standard deviation (sd): 4.60), they were transferred to individual pens. The experimental unit was a concrete floored animal shed and contained two rooms of 48 and 16 pens respectively. Sheep were randomly allocated to 40 and 14 pens of each room respectively. All pens contained a food trough and either a water bowl or an automatic drinker, which gave free and continuous access to water. Sheep also received a minimum of 16h artificial light per day. The experiment took place from July to September 1999.

Once penned, sheep were injected with a coccidiostat (Bimalong 25%, 2 ml subcutaneously, Cross Vetpharm Group Ltd, Dublin) and a clostridia/ *Pastereulla* vaccine (Heptavac, 2 ml subcutaneously, Hoechst Roussel Vet Ltd, Milton Keynes). During a two-week acclimatisation period they were offered a high quality pre-experimental food *ad libitum*, with 876 g dry matter (DM) and a calculated yield of 102 g metabolisable protein (MP) and 11.7 MJ metabolisable energy per kg DM. One week prior to the start of the experiment, sheep were drenched with benzamidazole (Panacur 10%, Hoechst Roussel Vet Ltd, Milton Keynes) at 0.2 ml per kg liveweight.

5.3.2. Condensed tannins

Cold soluble Quebracho extract was used as the source of condensed tannins (Quebracho ATO, Roy Wilson Dickson Ltd, Chester, UK). This type of Quebracho extract contains 793

g condensed tannins per kg DM Quebracho and a small amount of simple phenolics. Quebracho is a fine powder, which in the present experiment was added in the foods of sheep at 60g per kg fresh matter, before pelleting. The above concentration was comparable with concentrations of condensed tannins found in plants and has been considered responsible for reducing the parasitic burden of infected sheep, when included in their foods (Chapter Four). As certain types of condensed tannins are sensitive to oxidation at temperatures around 100°C (Larrauri *et al*, 1997), the pelleting of the foods was performed at the lowest possible temperature (approximately 80°C).

5.3.3. *Experimental foods*

Three basal foods were formulated (Table 5.1). Foods L, H and VH were calculated to yield 49, 115 and 148 g MP per kg DM respectively and were formulated to be isoenergetic. Food L was formulated to be inadequate in meeting the MP requirements of growing sheep, when offered *ad libitum*. Foods H and VH were formulated to be above the MP requirements in growing sheep. Food VH was included to ensure that food H was indeed not limiting for growth. In addition, foods L and H were supplemented with 60 g Quebracho extract per kg FM to form foods LQ and HQ respectively.

Prior to the pelleting of the foods, a quantity from each food was supplemented with 19.6 g acid insoluble ash (Fine, BDH, Lutterworth, Leics) per kg fresh matter, as a marker to estimate dry matter digestibility of the foods (see below).

Table 5.1. *Ingredients and composition of low (L), high (H) and very high (VH) protein foods offered ad libitum to 10-week-old sheep trickle infected with 2,000 L₃ T.colubriformis per day.*

<i>Ingredients (g per kg fresh matter)</i>	L	H	VH
Wheat	350	276	170
Oatfeed	194	207	207
Citrus pulp	347	210	160
Soypass (protected soya)	0	100	200
Hi-pro soya	0	98	155
50% fat premix	25	25	25
Molasses (CMS20) ^a	50	50	50
Salt	10	10	10
Dicalcium phoshate	9	4.2	1.1
Limestone flour	13	18	20
Mineral and vitamins mix ^b	2.5	2.5	2.5
<i>Composition (g per kg dry matter)</i>			
Dry matter (DM)	874	877	879
Crude protein	80	188	228
Metabolisable energy (MJ per kg DM)	11.4	11.4	11.4
Fermentable metabolisable energy (MJ per kg DM)	10.3	10.3	10.3
Effective rumen degradable protein (eRDP)	59	104	133
Digestible undegraded protein (DUP)	11.4	49	82

^a CMS 20 soluble condensed molasses supplied by Internal, Cobham, Surrey

^b Mineral and Vitamins mix supplied by Scotmin Ltd, Ayr, Scotland

5.3.4. Infective larvae

Sheep were trickle dosed with infective larvae (L₃) of *T.colubriformis*, a common intestinal nematode of sheep in temperate climates. L₃ were harvested using a standard Baerman procedure after a 10-day incubation period of faeces collected from monospecifically infected donor sheep (20-21°C). The strain used to infect the donor sheep was the Moredun ovine anthelmintic susceptible strain. Following the harvesting of L₃, larvae were maintained in tap water (1000 L₃ per ml) at 4°C, until oral administration to sheep.

5.3.5. Experimental design

One day prior the start of the experiment sheep were allocated into one of five groups, taking into account their liveweight (mean liveweight 32.2 kg, sd: 5.23). From day 1 of the experiment, sheep from each group were offered one of the five experimental foods *ad libitum* (n=12 for L, LQ, H, HQ and n=6 for VH group). Sheep were infected with 2,000 L₃ *T.colubriformis* per day, five days per week, throughout a 67-day experimental period. This intake of larvae is within the range that young sheep may ingest through grazing moderately contaminated pasture, and is capable of establishing a subclinical infection in growing sheep (Coop et al, 1979; Kyriazakis et al, 1994).

The experiment was divided into two periods: P₁ (days 1-38) and P₂ (days 39-67). In *T.colubriformis* infections the first period has been associated with high worm establishment (acquisition of immunity) in previously parasite naive sheep (Dobson et al, 1990a). At the end of this period (day 38) six sheep from groups L, LQ, H and HQ were slaughtered and their intestines removed for worm recovery. This was expected to provide evidence for a reduced level of parasitism attributable to a direct anthelmintic effect of condensed tannins. During P₂, the acquired immunity of the host is expected to be expressed (Dobson et al,

1990b); hosts reduce the numbers of incoming larvae that establish, the fecundity of adult worms and eventually reject the adult worm population. On the last day of the experiment (day 67) all remaining sheep were slaughtered and their small intestines removed for worm recovery (see below). Information about possible reduction in level of parasitism and improved performance was expected to be obtained during the second experimental period.

In order to estimate the dry matter digestibility of the foods, sheep were introduced to the foods that contained the marker on days 15 and 45 of the experiment. The first week was considered a period of acclimatisation and faecal samples were collected during five-day periods of P₁ (days 22-27) and P₂ (days 52-57) of the experiment (see below).

5.3.6. Measurements

Food intake was recorded daily. Food refusals were weighed early in the morning and then the food troughs were refilled with fresh food. Sheep were weighed on the first day of each week before the morning feeding; last liveweight recording was conducted on day 64 of the experiment. Faecal samples were collected on the third day of each week directly from the rectum; they were then either processed immediately or stored at 4°C and processed within two days. Nematode eggs per gram fresh faeces were estimated using a modified flotation technique (Christie and Jackson, 1982); polyallomer centrifuge tubes (Beckman) were used to separate the meniscus containing the eggs.

For determination of dry matter digestibility faecal samples of 10-15 grams were collected daily from each sheep, during collection periods (days 22-27 and 52-57). At the end of the collection periods, faeces were stored at -20°C until analysis for acid insoluble ash. Dry matter of the faeces was determined for each sheep for every day of the collection periods to determine total egg output (see below). Samples from each collection period were mixed

thoroughly for each sheep and subsamples were used for analysis. Food digestibility was estimated by calculating the amount of acid insoluble ash contained in the foods and faeces during the two faecal collection periods of the experiment. The ash residue of the samples was estimated by incineration at 500°C. Acid insoluble ash was determined after washing the ash residue of the foods or faeces by hydrochloric acid (25 ml hydrochloric acid per 100 ml water) (Official methods of analysis of the Association of Official Analytical Chemists, 1990).

Following the estimates of dry matter digestibility, total faecal output and total egg output were calculated. Total faecal output was expressed in grams dry faeces per day. Total egg output represented the total amount of *T.colubriformis* eggs excreted daily (eggs in dry matter faeces per day). This is considered a more reliable indicator for monitoring the parasitic infection than faecal egg counts, which is a concentration number. Total egg output was calculated by the formula:

$$\text{Total egg output} = \text{eggs per gram dry faeces} \times \text{total dry faecal output}$$

At slaughter the small intestines of sheep were ligated, removed and the worms were recovered from digesta and the intestinal mucosa following incubation in physiological saline at 37°C for 4h. The total worm burden and sex ratios were estimated from 2% aliquots of digesta and mucosal digest from each sample. *Per capita* fecundity was calculated by dividing the faecal egg counts recorded the day before slaughter by the number of female worms recovered on the slaughter day; this is widely used as an indicator of the fecundity of female worms.

5.3.7. Statistical analysis

All data collected were analysed by Genstat 5, release 3.2 (Lawes Agricultural Trust, 1993). Prior to any further analysis, comparisons were made between sheep offered foods H and VH over the 67-day experimental period (n=6) to ensure food H was indeed not limiting for the growth of parasitised sheep. Following this, statistical analysis focused on data from L, LQ, H and HQ groups.

Liveweight gain (g gain per day) was determined by linear regression for each sheep. During days 15-27 and 45-57, when sheep were offered the foods that contained the marker, daily food intake was corrected for the consumption of acid insoluble ash. Food conversion efficiency (g gain per g food) was calculated by dividing the liveweight gain by the mean food intake for each sheep.

Liveweight gain, mean food intake and food conversion efficiency for groups L, LQ, H and HQ were analysed: i) for P₁-P₂ with 6 sheep per treatment (sheep that remained in the experiment throughout), by a two-way ANOVA within a repeated measurement analysis (split plot), using protein level and Quebracho extract as factors and ii) for P₁ only, with 12 sheep per treatment by a two-way ANOVA using the protein level and Quebracho extract as factors. The latter analysis was performed to benefit from increased statistical power during this period (n=12). Digestibility and total faecal and egg output were analysed as above: for the two faecal collection periods (n=6, days 22-27 and 52-57 of the experiment) by a repeated measurement structure, or for the first collection period only (n=12, days 22-27).

Faecal egg counts were analysed separately for P₁ (n=12) and for the whole experimental period (P₁-P₂, n=6 for sheep that remained in the experiment throughout), by an

antependence model for repeated measurements (Kenward, 1987) with protein level and Quebraço extract in the foods as factors. Worm burden and *per capita* fecundity were analysed by two-way ANOVA with protein level and Quebracho as factors for each slaughter point separately. FEC, worm burden, *per capita* fecundity and total faecal and egg output were transformed before analysis ($\log x+1$) to stabilise the variance. For transformed data backtransformed means with 95% confidence intervals (lower and higher limit) are reported.

5.4. Results

In most cases, the results obtained by the two-way ANOVA for P₁ (n=12) were not different from those obtained by the repeated measurement analysis (P₁+P₂). Unless otherwise stated results from P₁ will not be reported separately.

5.4.1. Food intake

Daily food intake of sheep offered L, LQ, H and HQ during P₁+P₂ (n=6) is presented in Fig.

5.1.

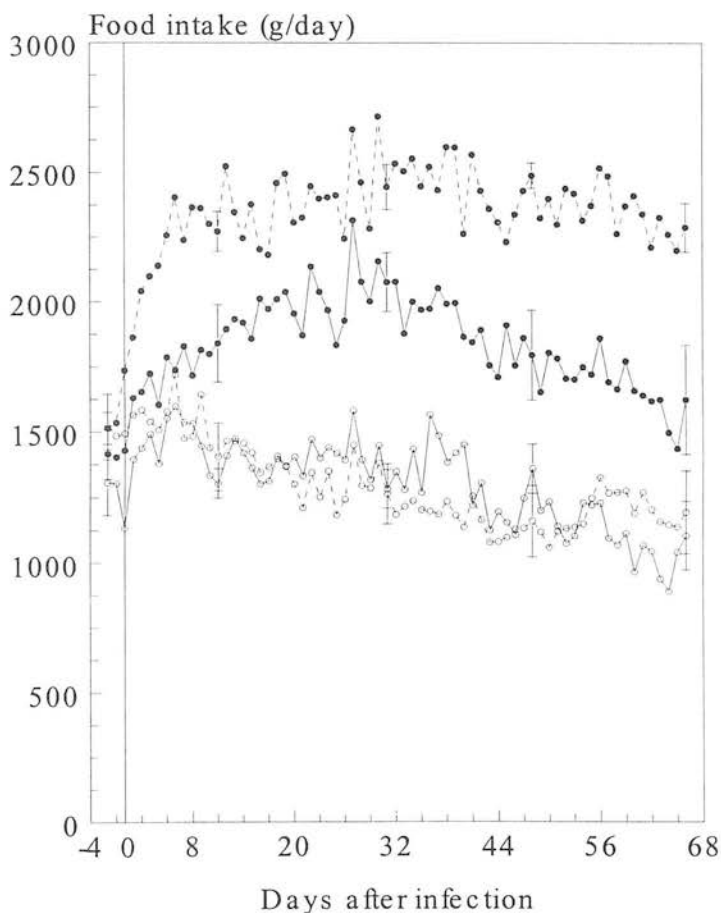


Figure 5.1. Daily voluntary food intake of sheep parasitised with 2,000 L₃ *T.colubriformis* per day, five days per week, and offered either low (○) or high (●) protein food supplemented with either 0 (—) or 60 g per kg fresh matter Quebracho extract (- - -). The bars indicate standard errors at the start, the middle and the end of the experiment.

There were no differences observed between sheep offered foods H and VH (mean over the whole experimental period: 1813 vs 1937, sed: 245 g per kg fresh matter respectively). Sheep offered the H foods (H and HQ foods) had higher mean food intake compared to sheep offered the L foods (L and LQ foods) over the whole experiment (Table 5.2). Sheep offered the Quebracho-containing foods (LQ and HQ foods) had significantly higher food intake than sheep offered the Quebracho-free foods (L and H foods) over the whole experiment. However, a significant protein x Quebracho interaction indicated that sheep offered food HQ had significantly higher intake compared to sheep offered food H, whereas the intakes of sheep offered foods LQ and L were very similar. Mean food intake of all sheep was significantly reduced during P₂ compared to P₁. Interactions with period were not significant.

5.4.2. Liveweight gain and food conversion efficiency

Sheep offered foods H and VH had similar liveweight gain throughout the experimental period (mean liveweight gain: 199 vs 207, sed: 52 g per day respectively). Although sheep (n=12) offered the Quebracho-containing foods showed significantly higher liveweight gain than sheep offered the Quebracho-free foods (P<0.01) during P₁, this effect was not significant over the whole experiment (Table 5.2). Sheep on the H foods showed higher liveweight gain compared to sheep on the L foods over P₁+P₂. A significant protein x Quebracho interaction indicated that sheep offered food HQ had significantly higher liveweight gain compared to sheep offered food H, whereas liveweights of sheep offered foods LQ and L were similar. Liveweight gain during P₂ was significantly reduced in all sheep compared to liveweight gain during P₁. However, the reduction in liveweight gain of sheep offered the H foods was significantly more pronounced compared to the reduction in sheep offered the L foods. In addition, the reduction observed in liveweight gain of sheep

during P₂ was more severe in sheep offered Quebracho-free foods than in sheep offered Quebracho-containing foods.

Food conversion efficiencies of sheep offered L, LQ, H and HQ foods throughout the experiment are also reported on Table 5.2. Although during P₁ (n=12) sheep on Quebracho-free foods converted food significantly more efficiently than sheep on Quebracho-containing foods (P<0.001), this effect only tended to be significant during P₁+P₂ (P=0.093). Sheep offered the H foods converted food significantly more efficiently compared to sheep offered the L foods over P₁+P₂. However, sheep offered food LQ tended to convert food less efficiently than sheep on food L, whereas sheep on H and HQ foods had similar food conversion efficiencies (P=0.074). Although sheep from all feeding treatments converted food less efficiently during P₂ compared to P₁, the reduction in food conversion efficiency during P₂ was significantly more pronounced in sheep offered the H foods compared to those offered the L foods. In addition, the reduction observed in food conversion efficiency of sheep during P₂ was significantly more severe in those offered the Quebracho-free foods compared to sheep offered the Quebracho-containing foods.

Table 5.2. Mean voluntary food intake (VFI, g per day), liveweight gain (LWG, g gain per day) and food conversion efficiency (FCE, g gain per g food) of sheep infected with 2,000 *L3* *T.colubriformis* per day and offered foods low (L) or high (H) in protein, throughout a 67-day experimental period. Details for the foods are included on Table 1 and in the text. *P*₁ has been associated with high worm establishment (days 1-38) and *P*₂ has been associated with the expression of host immunity towards parasites (days 39-67).

Time	Protein	Quebracho	VFI	LWG (g per day)	FCE (g gain per g food)
<i>P</i> ₁ (n=6)	L	-	1410	132	0.094
		+	1353	51	0.037
	H	-	1936	297	0.152
		+	2400	323	0.134
<i>P</i> ₂ (n=6)	L	-	1170	44	0.031
		+	1181	44	0.032
	H	-	1734	100	0.051
		+	2373	168	0.071
	sed		164	32.3	0.015
Effects of:					
Quebracho			*	NS	†
Protein			***	***	***
Period			***	***	***
Quebracho x Protein			*	*	†
Quebracho x Period			NS	*	**
Protein x Period			NS	***	**
3-way interaction			NS	NS	NS

NS: $P > 0.1$; †: $0.05 < P < 0.1$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

sed: standard error of the difference of the means

5.4.3. Digestibility and total faecal output

Food digestibility and backtransformed data of mean faecal output are presented in Table 5.3. Dry matter digestibility and total faecal output of sheep offered foods H and VH were similar over the whole experimental period (digestibility: 0.70 vs 0.76, sed: 0.05 for sheep offered H and VH foods respectively). The addition of Quebracho extract did not alter food digestibility. There was a significant increase in digestibility of all foods on days 52-57, compared to digestibility observed during days 22-27; this increase tended to be higher for L foods than for H foods ($P=0.057$). Period x Quebracho interaction did not affect food digestibility.

Mean faecal output was significantly higher in sheep offered the H foods compared to sheep offered L foods. A significant reduction in total faecal output was observed during days 52-57 compared to days 22-27 of the experiment. This reduction was significantly more pronounced in sheep offered the L foods (days 22-27: 476 (428-528) vs days 52-57: 213 (191-236) g dry faeces per day) compared to sheep offered the H foods (days 22-27: 676 (594-770) vs days 52-57: 508 (445-578) g dry faeces per day). The addition of Quebracho extract in the foods did not affect total faecal output of sheep, throughout the experiment.

Table 5.3. Dry matter digestibility (kg digested food per kg DM food), total faecal output (TFO, backtransformed data, g dry faeces per day) and total egg output (TEO, backtransformed data, number of eggs excreted per day), calculated by the addition of acid insoluble ash in the foods of sheep infected with 2,000 L₃ *T.colubriformis* per day, throughout a 67-day experimental period. Sheep were offered either a low (L) or a high (H) protein food.

Period	Protein	Quebracho	Digestibility	TFO ^a	TEO (x 10 ⁴) ^a
Days 22-27	L	-	0.59	578	5.4
		+	0.58	539	3.6
	H	-	0.63	713	5.6
		+	0.67	765	2.9
Days 52-57	L	-	0.78	239	3.4
		+	0.81	214	3.3
	H	-	0.77	376	7.3
		+	0.72	619	10.6
	sed		0.06	-	-

Effects of:

Quebracho	NS	NS	†
Protein	NS	***	NS
Period	***	***	NS
Quebracho x Protein	NS	NS	NS
Quebracho x Period	NS	NS	NS
Protein x Period	†	**	*
3-way interaction	NS	NS	NS

NS: P>0.05; †: 0.05<P<0.1; *: P<0.05; **: P<0.01; ***: P<0.001

^a Indication of the variance (95% confidence intervals) is provided in the text.

5.4.4. Faecal egg counts and total egg output

Backtransformed faecal egg counts (FEC) of sheep offered L, LQ, H and HQ foods for P₁ (n=12) and P₁ -P₂ (n=6) are presented in Fig 5.2 (a, b). FEC of sheep offered foods H and VH were similar during P₁ or P₂ (i.e. day 59 of the experiment: 503 (320-788) vs 940 (600-1470) eggs per gram faeces respectively). During P₁, sheep offered the Quebracho-containing foods had significantly lower FEC compared to sheep offered the Quebracho-free foods (Fig 5.2a). There were no differences on FEC of sheep offered L or H foods and protein x Quebracho interaction was not significant during P₁. Over P₁+P₂, there were no differences observed on FEC of sheep offered foods that differed in protein level or Quebracho content (Fig 5.2b).

Backtransformed total egg outputs of sheep are presented in Table 5.3. Total egg outputs of sheep offered foods H and VH were similar (for days 52-57: 7.2 (5.2-10.0) vs 10.5 (7.6-14.6) x10⁴ eggs per day, respectively). Sheep offered the Quebracho-containing foods tended to have lower total egg output compared to sheep offered the Quebracho-free foods. A significant protein x period interaction indicated that sheep on the H foods increased their total egg output during days 52-57 (6.3 (5.0-8.1) x10⁴ eggs per day), compared to days 22-27 (3.0 (2.4-3.6) x10⁴ eggs per day); sheep offered the L foods had similar egg output during both periods (3.0 (2.4-3.8) vs 4.2 (3.4-5.1) x10⁴ eggs per day respectively).

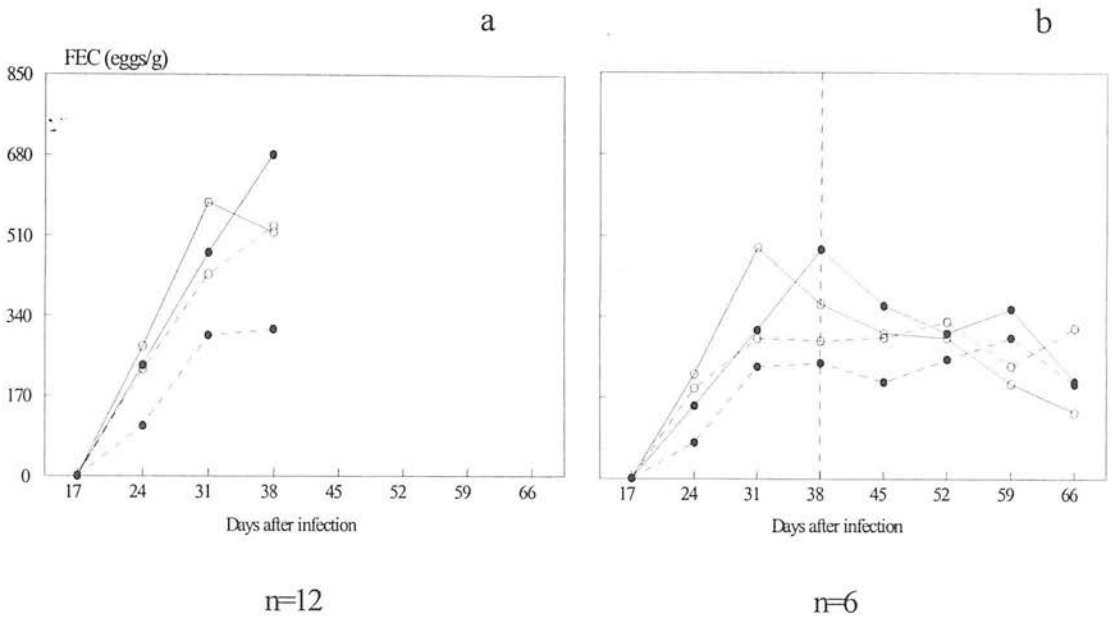


Figure 5.2. Backtransformed means of faecal egg counts of sheep infected with 2,000 L3 *T.colubriformis* per day, five days per week, and offered either low (O) or high (●) protein food supplemented with either 0 (—) or 60 g per kg fresh matter Quebracho extract (---) during a) P₁ (days 1-38, n=12) and b) P₂ (days 39-67, n=6). Indication of the variation within each treatment (95% confidence intervals) is provided in the text.

5.4.5. Worm burdens and per capita fecundity

At the first slaughter point (day 38) worm burdens, sex ratio and immature stages of parasites between sheep on different feeding treatments were very similar (Fig 5.3a). At the end of the experiment (day 67) worm burdens between sheep offered foods H and VH were similar (total worm burden: 13945 (12502-15555) vs 12639 (12331-14098) respectively, Fig 5.3b). Worm numbers recovered from sheep offered the L foods were significantly lower compared to worm numbers recovered from sheep offered the H foods (11,550 (10,492-14,696) vs 15,533 (14,110-17,100) worms respectively, $P < 0.05$). Sheep that consumed

Quebracho-containing foods had higher worm burdens than sheep on Quebracho-free foods (16,030 (14,637-17,553) vs 11,133 (10,166-13,998) worms respectively, $P < 0.01$). The protein x Quebracho interaction was not significant.

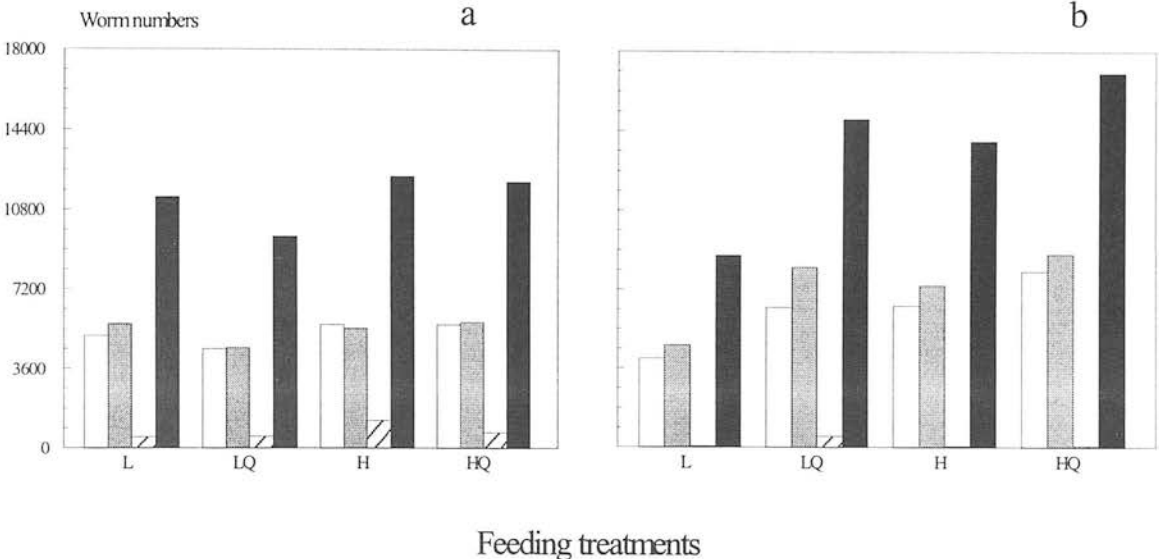


Figure 5.3. Backtransformed means of adult male (□), adult female (▒), immature (▨) and total worm burdens (■) of sheep infected with 2,000 *L3 T.colubriformis* per day, five days per week, and offered either a low (L) or a high (H) protein food or the L and H foods supplemented with 60 g per kg fresh matter Quebracho extract (LQ and HQ respectively) at the end of a) P_1 (day 38, $n=6$) and b) the end of P_2 (day 67, $n=6$). Indication of the variation within each treatment (95% confidence intervals) is provided in the text.

There was no difference observed on the fecundity of worms between sheep offered foods H and VH. *Per capita* fecundity of female worms, at the end of P_1 , was lower in sheep offered the H foods, compared to sheep offered the L foods (0.09 (0.07-0.10) vs 0.12 (0.11-0.14) eggs per female respectively, $P < 0.05$). However, a significant protein x Quebracho interaction was present indicating that fecundity of female worms was lower in sheep offered food HQ than in sheep offered food H (0.05 (0.03-0.06) vs 0.12 (0.11-0.14) eggs per

female respectively, $P < 0.001$); *per capita* fecundity of female worms was similar in sheep offered foods L and LQ. Fecundity of female worms at the end of the experiment was not affected by protein or Quebracho extract in the foods of sheep.

5.5. Discussion

To the best of our knowledge, this is the first experiment where the consequences of *ad libitum* intake of foods supplemented with condensed tannin on parasitism and performance of sheep were investigated. The *ad libitum* intake of parasitised sheep on either high or low protein foods supplemented with Quebracho extract did not cause the expected reduction in the level of intestinal parasitism in sheep. As a result, the performance of sheep offered the supplemented and unsupplemented foods was similar. Although the performance of sheep offered the low protein food supplemented with Quebracho extract was also expected to be improved due to the extra protein flow in their small intestine, the results did not support this hypothesis.

5.5.1. Faecal egg counts and worm burden

An anthelmintic effect of Quebracho extract towards *T.colubriformis* was only evident on FEC of sheep offered the Quebracho-containing foods, during the first experimental period. Since this reduction was observed within the first five weeks of the infection, it is likely that it could be attributed to a direct effect of Quebracho towards the parasites *per se* and was not a consequence of an enhanced immune response, that could result from an increased protein flow. It could be argued, however, that the reduction on FEC could also be attributed to a dilution of the faeces, due to the addition of the “inert” Quebracho extract in foods LQ and HQ. However, since total egg output was also reduced during P₁, the reduction on FEC observed during the same period should be mainly attributable to the anthelmintic effects of condensed tannins. Worm burdens on the other hand, as recovered at day 36 of the experiment, were not affected by the presence of Quebracho extract in the foods of parasitised sheep. This indicates that possibly only the fecundity or the egg-laying ability of female worms was affected by the addition of Quebracho extract in the foods of parasitised

sheep, as has also been proposed in an earlier study (Chapter Four). There was no evidence for reduced fecundity or egg laying ability of female worms during P₂, as FEC of sheep were not affected by Quebracho supplementation. This was probably attributable to reduced food intake observed during P₂, which would have resulted in a lower Quebracho concentration *per se*. At the end of the experiment (day 67) worm numbers were lower in sheep offered the Quebracho-free foods than in those offered the Quebracho-containing foods. It is possible, however, that this reduction was mainly due to the large decrease observed in worm burden of sheep offered food L (see below).

The lack of a direct anthelmintic effect of Quebracho extract on worm burden at the end of P₁ and P₂, and on FEC during P₂ contradicts previous evidence for a direct effect of Quebracho towards a *T.colubriformis* population over a 10-week experimental period (Chapter Four). In the previous study, FEC and worm burdens were reduced in sheep offered foods that contained similar amounts of Quebracho extract to the present study (60 g per kg respectively). In the previous study, however, sheep were offered restricted amounts of foods (about 80% of their expected *ad libitum* food intake), whereas in the present study they were offered the supplemented foods *ad libitum*. It has been reported that animals can consume a high proportion of their intake (80-90% of the expected *ad libitum* intake) within a short period of time when they are fed restricted, whereas they spread their food consumption throughout the day when they are fed *ad libitum* (Bornett et al, 2000). It is possible that condensed tannins need to reach a certain concentration in the gastrointestinal tract of sheep to be effective towards parasites and via *ad libitum* feeding this might not be easily achieved. In addition, *ad libitum* intake can lead to increased flow rates of digesta (Grovmum and Hecker, 1973); hence it is possible that the retention time of condensed tannins in the digestive tract may be reduced, and thus to be less effective in causing detrimental

effects on parasites. Increased food intake has also been considered responsible for reduced efficacy of anthelmintic drugs in sheep due to increased digesta passage rate (Ali and Hennessy, 1995).

The impact of the low protein nutrition on the parasitic burden of sheep during P₂ also merits discussion. The second experimental period (day 39-67) has been associated with the expression of immunity in previously naïve sheep (van Houtert et al, 1995). During this period, high protein nutrition is expected to contribute to a reduction in the level of parasitism, by reducing worm establishment, fecundity and enhancing worm expulsion (Quinell and Keymer, 1990). In the present study worm burdens recovered at the end of the experiment, were lower in sheep offered food L rather than H. This unexpected result was probably due to the very low protein intake of sheep offered food L; they ingested 2 g MP per kg liveweight during P₁ and only 1.2 g MP per kg liveweight during P₂. To the best of our knowledge, the effects of such low protein intake have not been previously tested in parasitised sheep. It is proposed that the very low protein intake could have mediated nutritional deficiencies for the parasites or changes in the natural environment of the worms. Since parasites have specific nutritional demands (Matthews, 1998), which are independent of the host, failure to meet them could cause reduction in survival and/or maturation of worms (Bundy and Golden, 1987). In addition, low protein nutrition may also be responsible for altering the biochemistry and/or the physiology of the gut, which is the natural environment of the worms. Hence, very low protein nutrition might be responsible of creating an unsuitable environment for the establishment and/or the survival of parasites in the gut. It is evident from the above that the relationship between protein host nutrition and parasitism may not be a linear one, as it has been previously suggested (Datta et al, 1998).

5.5.2. Food intake and performance

Voluntary food intake and performance were identical in sheep offered foods H and VH; this met the necessary requirement of the experimental foods, that protein allowance of foods H and HQ should not be limiting for the growth of sheep. However, food intake of sheep offered food HQ was about 20% higher compared to food intake of sheep offered foods H and VH. This percentage was higher than the dilution caused to food H by the addition of Quebracho (6% w/w). According to our initial hypothesis, sheep on foods HQ and LQ were expected to increase their food intake to overcome the possible adverse effects of condensed tannins upon digestibility. Digestibility of foods LQ and HQ was similar to that of foods L and H respectively, although intake of sheep offered food LQ was not increased. It is suggested that the increased intake of sheep offered food HQ, compared to sheep offered food H, was possibly a mechanism to compensate for dietary or endogenous protein loss caused by condensed tannins. Although condensed tannins have the ability to increase protein availability in the small intestine of the host (see review by Barry and McNabb, 1999), recent studies have suggested that the absorption of non-ammonia-nitrogen in the small intestine of sheep may be reduced following consumption of Quebracho extract (Komolong and McNeill, unpublished). This indicates that some of the dietary protein ingested by sheep is not absorbed and excreted possibly still bound to condensed tannins, or that tannins release dietary protein in the small intestine, and form new complexes with endogenous protein. Loss of endogenous protein has been previously associated with the consumption of condensed tannins, and is a possible explanation for the increased faecal nitrogen concentration in sheep consuming condensed tannins (Butler and Rogler, 1992).

If we accept that sheep on food HQ increased their intake to overcome possible losses of endogenous protein, the question arises as to why sheep offered food LQ did not also

increase their intake. It is possible that sheep on food LQ either could not or did not increase their intake, to avoid any adverse effects arising from excess of condensed tannins on rumen microflora (McAllister et al, 1994a). Although the daily amount of Quebracho extract consumed by sheep offered food LQ was lower (70 g per day) compared to that of sheep on food HQ (140 g per day), most of condensed tannins in the rumen of sheep on food HQ would be expected to be present as complexes due to protein excess (see review by Barry and McNabb, 1999). On the other hand, most condensed tannins present in the rumen of sheep on food LQ would be expected to be free, due to the lack of protein. High concentrations of free condensed tannins have been considered responsible for inhibiting and/or reducing the growth of rumen micro-organisms (Jones et al, 1994). The results of a choice-feeding experiment support this hypothesis: sheep offered a choice of foods with similar concentrations of condensed tannins (100-200 g per kg) preferred the food with the highest crude protein content (153 g per kg), whereas they preferred a food with lower crude protein content (128 g per kg) when the same foods were offered as tannin-free (Titus et al, 2000). In the above study, sheep probably aimed to reduce the amount of free condensed tannins in their rumen and thus lessen the detrimental effects of condensed tannins in the rumen microflora.

The addition of Quebracho extract at 60 g per kg FM in the foods did not affect dry matter digestibility. Previous studies both in rats and sheep have indicated that food digestibility was decreased (Dawson et al, 1999) and total faecal output of sheep increased (Butter et al, 2000) by the addition of Quebracho extract in the foods. The differences observed between the latter and the present studies could be due to the different markers used to estimate the digestibility. Different estimates of the dry matter digestibility have been reported when acid insoluble ash or chromic oxide have been used for its estimation (Thonney et al, 1985).

However, although digestibility of foods LQ and L was similar and sheep offered either of the foods had similar intakes, sheep offered food LQ had lower liveweight gain than those did on food L. This could be due to possible loss of endogenous protein, as discussed above. Sheep on HQ food on the other hand, were expected to increase their liveweight gain only as a result of reduced level of parasitism. As this did not occur, it is suggested that the increased liveweight gain observed in sheep offered food HQ compared to sheep on food H might have been due to increased gut fill, because of their increased food intake. There is evidence from grazing experiments suggesting that sheep consuming forages with high protein and condensed tannin content, such as *Hedysarium coronarium*, and *Lotus spp.*, had a reduced level of parasitism and improved liveweight gain, compared to sheep grazing forages with high protein content but tannin-free (Niezen et al, 1998a; b). However, as a result of various structural differences that characterise condensed tannins (see review by Mueller-Harvey and McAllan, 1992), different types of condensed tannins may have different properties when consumed by parasitised hosts. An investigation into the anthelmintic properties of different condensed tannins, including Quebracho, seems to be an area where effort could be usefully diverted.

The above experiment was performed to investigate whether *ad libitum* food intake of foods supplemented with condensed tannins from Quebracho extract can decrease the level of parasitism and improve the performance of sheep infected with *T.colubriformis*. The anthelmintic properties of condensed tannins were expected to be responsible for reduced level of parasitism, whereas *ad libitum* intake was expected to allow sheep to eat as much as they needed to overcome possible adverse effects of condensed tannins upon dry matter digestibility. Both the level of parasitism and the performance of parasitised sheep remained unaltered following *ad libitum* intake of supplemented foods that differed in protein content.

It is suggested that to achieve reduced level of parasitism in sheep offered *ad libitum* Quebracho-containing foods, a higher concentration of Quebracho extract may need to be included in the foods. Increased concentrations of Quebracho extract, however, may have various detrimental effects on the hosts (see review by Barry and McNabb, 1999). As it is likely that different tannins might have different properties towards ruminant physiology and hence consequences on parasites, the option of feeding sheep foods high in condensed tannins to control parasitism, remains a possibility. Indeed, this is supported by experiments on sheep grazing forages high in condensed tannins (Niezen et al, 1993; 1998a; b).

Chapter Six

General Discussion

6.1. Introduction

This thesis has provided novel information on the mechanisms via which the consumption of condensed tannins can contribute to a reduction in the detrimental effects of gastrointestinal parasitism in sheep. The two hypotheses that have been developed to account for the effects of condensed tannins towards parasitised sheep are summarised in Figure 6.1. In brief, the first hypothesis relates to a direct anthelmintic effect of condensed tannins towards ovine nematodes. Condensed tannins may reduce larval establishment or survival, worm fecundity or adult worm burden, and thus decrease the level of parasitism in growing sheep. As a result, the consequences of parasitism are expected to be reduced in parasitised sheep and thus their resilience will be improved (dotted line).

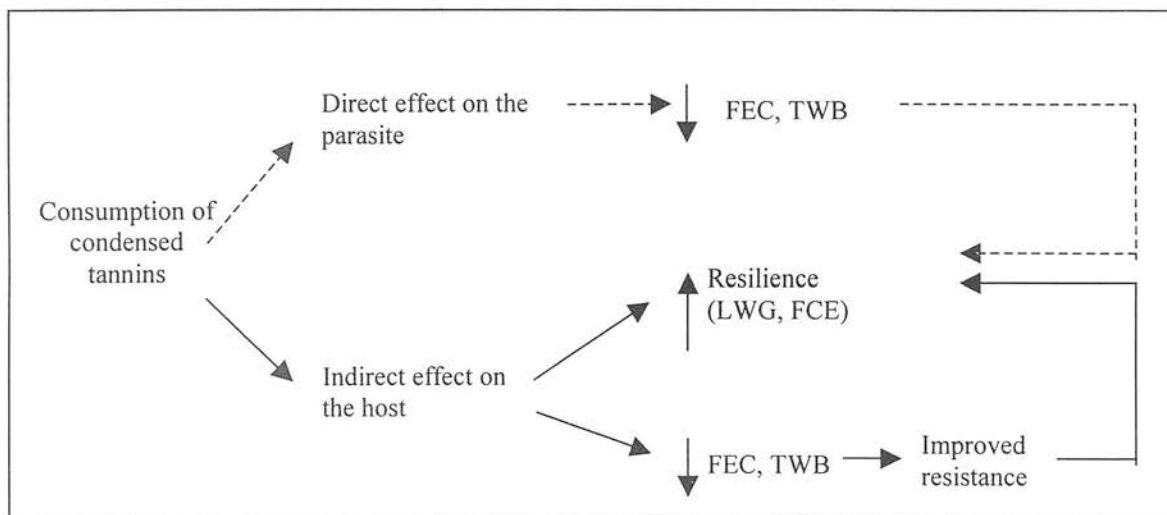


Figure 6.1. The two hypotheses that have been developed to account for the reduction of the detrimental effects of parasitism in sheep consuming foods that contain condensed tannins.

(LWG: liveweight gain; FCE: food conversion efficiency; FEC: faecal egg counts; TWB: total worm burden).

The second hypothesis relates to an indirect nutritional or immune effect of condensed tannins on the parasitised host. Condensed tannins, via their ability to bind dietary protein, can increase protein flow in the small intestine of the host. Increased protein availability may

contribute to improved resilience of parasitised sheep by improving their performance (nutritional effect), and can also enhance the resistance of parasitised hosts (immune effect) towards gastrointestinal nematodes (solid line). It is evident from Figure 6.1 that both the direct and the indirect effect of condensed tannins are expected to improve the resilience of parasitised hosts.

To test for the direct and indirect effect of condensed tannins in this thesis, two lines of research have been developed. The first focused on testing the anthelmintic ability of condensed tannins on an established, largely adult population, by drenching parasitised sheep for a short period of time with condensed tannin solutions. The first experiment aimed to test the direct anthelmintic effect of condensed tannins, when administered as a drench to sheep with an established adult *T.colubriformis* population in their small intestine (Chapter Two). A subsequent experiment broadened this approach and investigated the anthelmintic activity of condensed tannins, when administered as a drench to sheep with established abomasal or intestinal populations (Chapter Three, *in vivo*). A series of *in vitro* larval development/viability assays were also performed to investigate a direct anthelmintic effect of condensed tannins towards the pre-parasitic larval stages of two abomasal and one intestinal nematodes (Chapter Three, *in vitro*).

The second line of research focused on testing the anthelmintic ability of condensed tannins during the establishment and on an established intestinal infection, when condensed tannins were added in the foods of parasitised sheep over a 10-week experimental period (Chapters Four and Five). The long-term consumption of condensed tannins also enabled us to test: i) the indirect nutritional effect of condensed tannins on the resilience of parasitised hosts offered restricted amounts of high protein foods, supplemented with condensed tannins (Chapter Four); and ii) the indirect effect of condensed tannins on the resilience and

resistance of parasitised hosts offered *ad libitum* low and high protein foods, supplemented with condensed tannins (Chapter Five).

The principal issues raised by the experimental studies, which warrant further discussion in this Chapter are:

- The use of Quebracho extract to test the anthelmintic properties of condensed tannins.
- Effects of condensed tannin administration during the establishment of an intestinal nematode infection.
- Effects of condensed tannin administration on an established intestinal nematode infection.
- The efficacy of condensed tannin from Quebracho extract as anthelmintic.

Subsequently, special reference is given to problems that may follow the consumption of condensed tannins and future research lines are proposed to further investigate the properties of condensed tannins as an alternative to chemoprophylaxis to control gastrointestinal parasitism in sheep.

6.2. The use of Quebracho extract to test the anthelmintic properties of condensed tannins.

Investigating the effects of condensed tannins by feeding tanniniferous forages, although closer to farming conditions, does not offer the opportunity to investigate the effects of tannins *per se*. One of the main reasons that Quebracho extract was chosen to study the effects of condensed tannins towards ovine nematodes, was that it is a relatively pure, commercially available condensed tannin extract and has been extensively used in animal experimentation (see section 1.6.). However, it could be hypothesised that the effects observed towards nematodes following the administration of Quebracho extract in this thesis were due to either effects caused by condensed tannin *per se* on the physiology of the host or other active compounds of the Quebracho extract. The first hypothesis will be discussed in sections 6.3. and 6.4. With regard to the latter, it could indeed be argued that the detrimental effects observed on the parasitic burden of sheep, following the consumption or administration of Quebracho extract were not due to condensed tannins, but to the simple phenolics that Quebracho contains. Although this could be a valid point, recent *in vitro* studies performed with pure condensed tannin extracts clearly show that condensed tannins have anthelmintic properties (Molan et al, 2000a; b). In general, simple phenolics have low affinity to proteins and other macromolecules (Field and Lettinga, 1992). Since it is likely that both the direct and the indirect effect of condensed tannins are related to their protein-binding ability, as indicated earlier in this Chapter, it is unlikely that the simple phenolics of Quebracho extract are responsible for the effects observed in the present studies. The issue of whether simple phenolics *per se* have direct effects that could have contributed to the effects of condensed tannins, is beyond the scope of this thesis.

6.3. Condensed tannin mode of action against different nematode species

A reduction in the FEC of sheep with an established adult *T.colubriformis* (Chapter Two) or *T.colubriformis* and *N.battus* (Chapter Three) infection was observed within three days or one day respectively, following the drenching with Quebracho at 8% w/w of their food intake. Although a reduction in FEC of sheep drenched with Quebracho extract could be due to a dilution effect on the faeces, as discussed in the experimental Chapters, the reduction of the worm burden following tannin administration suggests that condensed tannins from Quebracho extract had indeed a direct anthelmintic effect towards intestinal nematodes. In contrast, adult *T.circumcincta* or *H.contortus* were not affected by the three-day administration of Quebracho extract at 8% w/w of food intake. This raises the important question of why condensed tannin had different effects upon intestinal and abomasal adult worms in the experiments performed (Chapter Three). Few studies have compared the effects of condensed tannins on abomasal and intestinal parasitic burdens of sheep in the literature, and none has attempted to explain the different susceptibilities of abomasal and intestinal nematodes to condensed tannins. The consumption of the same forage has been reported to result in reduced abomasal and/or intestinal worm burden in one study, but not in another. Grazing studies have suggested that in a mixed naturally acquired infection, abomasal worm burdens (*T.circumcincta* and *H.contortus*) were not reduced following 6 weeks of consumption of either *Lotus* spp or *Hedysarum coronarium*, forages that contained 4 to 12% condensed tannins by dry matter (Niezen et al, 1998a). In the same study intestinal worm burdens were reduced following the consumption of *Hedysarum coronarium* only. In further study the consumption of *Lotus pedunculatus* resulted in reduced worm burden of *T.circumcincta* in artificially infected sheep 35 days post-infection; intestinal worm burdens were not affected following the consumption of the same forage (Niezen et al, 1998b). There

is no systematic effort to account for such contradictions, although it has been reported that the same forages may have different concentration of condensed tannins according to the time of the year, the fertility of the soil and environmental conditions (Hagerman and Butler, 1991; Waterman et al, 1999).

The differences observed between abomasal and intestinal adult nematodes might be attributable either to species differences or to different parasite-tannin interactions that exist in the gastrointestinal tract of parasitised sheep. It has already been mentioned that the pH, as well as the presence of surfactants, is important for the disassociation of protein-tannin complexes (Martin et al, 1985; Mole and Waterman, 1985). It is possible that condensed tannins that exist as complexes with protein or other macromolecules may not exert a direct anthelmintic effect, since this anthelmintic effect may be related to their binding capacity. Since surfactants, like bile salts, are present mainly in the small intestine of ruminants (Miller et al, 1997), it is likely that the disassociation of the complexes mainly occurs in this site rather than in the abomasum. As a result, condensed tannins could pass through the abomasum in the form of complexes, and thus less likely to have a direct anthelmintic effect towards abomasal nematodes, but when they are free in the small intestine they are capable of affecting intestinal nematodes.

Although adult abomasal nematodes were not affected by the presence of Quebracho extract in the digestive tract of sheep, it was evident from *in vitro* assays that the larvae of abomasal species were susceptible to the presence of Quebracho extract (Chapter Three, *in vitro*). It is essential to appreciate that although *in vitro* assays have been considered reliable for testing the efficacy of anthelmintic drugs (Coles et al, 1988; Amarante et al, 1997), *in vitro* conditions are markedly different from *in vivo* conditions. During the *in vitro* assays (Chapter Three), pH was not manipulated and was approximately neutral. Since neutral pH advances the formation of tannin-protein complexes, free condensed tannins were probably

able to affect both abomasal and intestinal larvae, once they were added in the *in vitro* assays. Various mechanisms may be responsible for the demonstration of an anthelmintic effect of condensed tannins towards abomasal and intestinal larvae. The cuticle of the nematodes consists of carbohydrates and proteins (elastin-like protein, collagen and collagen-like protein) which are rich in proline (Thompson and Geary, 1995). The high affinity of condensed tannins towards proline-rich proteins is well-established (Asquith et al, 1987), thus condensed tannins could bind to the cuticle of the nematodes. This binding could affect the cuticle and lead to impairment of normal function and even death of nematodes. Nematode pre-parasitic stages could also ingest condensed tannins with their food, as has been reported for insects (see review by Schultz, 1989). *In vitro* studies have suggested that the consumption of condensed tannins by insects resulted in reduced growth and increased mortality of insect and insect larvae (Klocke and Chan, 1982; Reese et al, 1982). In the gut of these insects, condensed tannins can bind to the gut wall and can either cause autolysis or mucosal lesions, thus disrupting growth and development and finally causing the death of the insect (see review by Schultz, 1989). As abomasal and intestinal nematode larvae have the opportunity to ingest *in vitro* condensed tannins with their food, they may suffer the same consequences as insects do. Apart from their potential effect on the gut when ingested by nematodes, condensed tannins can also impair the motility of nematodes. *In vitro* larval migration/inhibition assays performed recently, have showed that both intestinal and abomasal infective larvae of sheep and deer reduced their motility and thus migration ability following incubation in condensed tannin extracts (Molan et al, 2000a; b). Although the mechanism of action is not known, it might be a result of adverse effects of condensed tannins on the cuticle of nematodes, as indicated above, or a systematic effect on the neuro-muscular tissues of nematodes. Many anthelmintic drugs, like levamisole, ivermectin, piperazine, are neuro-muscular agents and cause reduction in motility and paralysis of the pharynx, both of which will limit the capacity on nematodes to ingest nutrients (Geary et al, 1993; Martin et al, 1997). The capacity of condensed tannins to bind to proteinaceous

surfaces might also affect excretory and secretory functions of nematodes, if excretory and secretory pores of nematodes are exposed to free condensed tannins.

The above possible mechanisms of action of condensed tannins can also apply to adult nematodes *in vivo*. As condensed tannins were probably free in the small intestine, only intestinal nematodes were affected by the presence of condensed tannins in Chapter Three. Both intestinal nematodes used in Chapter Three were exposed to direct contact with condensed tannins in the lumen of the small intestine. Condensed tannins could either damage their cuticle and thus nematodes could lose their defence towards the digestive enzymes of the host (Matthews, 1998), or tannins may be ingested by nematodes and affect normal feeding, locomotion, secretory and excretory functions. As conventional microscopy (x 20) of both larval and adult nematodes provided no evidence of gross morphological differences between the cuticles of nematodes that were exposed or not to condensed tannins, one could probably discount severe cuticular effects as the principal cause of action of condensed tannins.

6.4. Effect of condensed tannin administration during the establishment of an intestinal nematode infection

During the initial establishment of an intestinal nematode infection, when a growing animal acquires immunity towards infection, there are two potential beneficial effects of condensed tannins on the parasitised host: a direct anthelmintic effect of condensed tannins towards nematodes and an indirect nutritional effect on the resilience of parasitised sheep. During the establishment of an intestinal nematode infection additional protein availability is not expected to enhance the development of immunity (see review by Coop and Kyriazakis, 1999) and thus an indirect effect on the resistance of parasitised hosts, as presented in Figure 6.1, was not anticipated. Offering foods supplemented with Quebracho extract resulted in reduced FEC during the establishment of the parasitic infection, irrespectively of the feeding regime of parasitised sheep (Chapters Four and Five). Although this reduction could be due to a dilution effect on the faeces, the expression of FEC on dry matter basis and the reduction of total egg output in sheep offered the supplemented foods in Chapter Five, indicate that it was probably due to a direct anthelmintic effect of condensed tannins. More specifically this reduction on FEC could be due to a direct anthelmintic effect of condensed tannins on either larval establishment, worm development, worm expulsion or fecundity of *T.colubriformis*. However, worm burdens as recovered at the end of the initial period of worm establishment (Chapter Five) were not affected by Quebracho administration. This suggests that condensed tannins are not responsible for a reduction in worm establishment but they perhaps affect the fecundity of female worms over this period. This view is in agreement with a previous study, where *T.colubriformis* worm burdens were not reduced following four weeks grazing of *Lotus pedunculatus* (Niezen et al, 1998b). Although there is contradictory evidence from sheep that reduced their *T.colubriformis* worm burdens following the consumption of forages high in condensed tannins for six weeks (Niezen et al,

1995; 1998a), the sheep used in the above studies were not parasite naive, but had some previous exposure and thus had an enhanced naturally acquired immunity. It is argued that the consumption of condensed tannins by parasitised sheep during the phase of initial worm establishment and acquisition of immunity, initially only affects the fecundity of worms and/or their growth but not larval establishment or worm expulsion.

Irrespective of the feeding regime of sheep (Chapters Four and Five), the resilience of parasitised sheep offered the supplemented foods was not improved, during the period of initial worm establishment. On the contrary, sheep offered the supplemented foods in restricted amounts (Chapter Four) had poorer performance compared to sheep offered the unsupplemented foods at the end of the phase of acquisition of immunity. Although this was contrary to expectations, as the resilience of parasitised sheep was expected to be improved due to the reduced level of parasitism and the extra protein availability in the small intestine of the hosts, it was in agreement with a previous study (Butter et al, 2000). It is possible that a reduction in food digestibility observed following Quebracho administration in mice and sheep (Dawson et al, 1999) may have been responsible for the lack of a beneficial effect observed on sheep resilience. As *ad libitum* intake of forages that contain moderate concentrations of condensed tannins have resulted in improved resilience of parasitised sheep during the initial establishment phase of a nematode infection (Niezen et al, 1995; 1998a; b), it was expected that *ad libitum* consumption of foods supplemented with Quebracho extract would have a similar outcome. However, it was evident from the results of Chapter Five that the effects of condensed tannin on the resilience of parasitised sheep offered foods supplemented with Quebracho extract *ad libitum*, depended on the amount of protein contained in the food. Food intake and liveweight were improved in sheep offered the high protein supplemented food, but they deteriorated in sheep offered a low protein supplemented food. This could be attributable to possible adverse effects of condensed tannins on the rumen environment. High levels of condensed tannin or low levels of dietary

protein in a food or forage can result in a high ratio of free condensed tannin in the rumen. Free condensed tannins are able to bind to rumen bacteria and fungi (McSweeney et al, 1999) and thus reduce carbohydrate digestion (Barry and McNabb, 1999), protein digestion (Acamovic et al, 1999) and microbial protein synthesis (McNeill et al, 1999). Under grazing conditions, the consumption of forages that contain moderate to high concentrations of condensed tannins does not usually result in the demonstration of adverse effects on the grazing ruminant (Niezen et al, 1994; 1998a; b), possibly of the high dietary protein content that is often present in tanniniferous forages. It seems reasonable to suggest that the high protein content of a food or a forage that contains condensed tannins can contribute to overcoming the possible adverse effects of condensed tannins on the rumen environment and could improve the performance of parasitised sheep.

6.5. Effect of condensed tannin administration on an established intestinal nematode infection

Once an intestinal nematode infection is established and the host starts expressing immunity towards nematodes, there are three potential beneficial effects of condensed tannins on the parasitised host: a direct anthelmintic effect towards the nematodes (Chapters Two, Three, Four and Five), an indirect nutritional effect on the resilience of hosts offered high protein supplemented foods (Chapters Four and Five) and an indirect immune effect on the resistance of the host offered low protein supplemented foods (Chapter Five). Although the direct anthelmintic effect of condensed tannins was clearly evident when they were administered to parasitised sheep as a drench (Chapters Two and Three), or when added in foods offered restrictedly (Chapter Four), it was not evident when the supplemented foods were offered *ad libitum* (Chapter Five). The feeding regime of parasitised sheep was probable responsible for the differences observed in the anthelmintic activity of condensed tannins on the established intestinal infection in Chapters Four and Five. When animals are fed *ad libitum* they tend to spread their food intake throughout the day (Bornett et al, 2000) and consequently the condensed tannin intake is similarly affected. This could result in lower concentrations of condensed tannins in the small intestine over time, possibly lower than the concentration that is required to cause adverse effects to nematodes. This suggests that a threshold concentration of condensed tannins may be required in the digestive tract of sheep to detrimentally affect nematodes. Results from grazing experiments have shown that *ad libitum* intake of condensed tannin containing forages reduced the level of intestinal parasitism in grazing ruminants (Niezen et al, 1995; 1998a); however in these studies the concentration of condensed tannins in the forages was higher than the one used for Chapter Five. In the grazing experiments, the concentration of condensed tannins in the forages was about 100 g per kg dry matter of the leaf material, whereas in Chapter Five, condensed

tannins from Quebracho extract were included at about 50 g per kg dry matter of the food. There is evidence from other grazing experiments that low to medium concentrations of condensed tannins, as used in Chapter Five, may not reduce the level of parasitism in grazing sheep (Niezen et al, 1998b). These results suggest that in order to demonstrate an effect of condensed tannins on an established intestinal nematode infection, sheep fed *ad libitum* require a higher intake of condensed tannins to achieve an effective threshold level of condensed tannin bioavailability.

Although the increased liveweight of sheep offered the high protein supplemented food in Chapter Five may be considered responsible for providing some evidence of an indirect nutritional effect of condensed tannins on the resilience of parasitised sheep, it could also be due to increased food intake and thus gut fill (see 5.5.2). On the other hand, the resistance of parasitised sheep was not affected following Quebracho supplementation of low protein foods offered *ad libitum* (Chapter Five). An indirect immune effect of condensed tannins on parasitised sheep offered the low protein supplemented foods was expected to be observed, as a result of increased protein availability in the small intestine of sheep (Mueller-Harvey and McAllan, 1992). As neither the resilience nor the resistance of parasitised sheep were improved following the consumption of Quebracho supplemented foods, it is argued that maybe there was inadequate additional protein released in the small intestine. This was contrary to the initial expectations. Although, there is *in vivo* and *in vitro* evidence demonstrating that condensed tannins release dietary protein in the lower digestive tract (see review by Asquith and Butler, 1986; Hedqvist et al, 2000), recent preliminary studies with Quebracho extract have suggested that the consumption of condensed tannins does not always result in increased protein absorption in the small intestine of sheep (McNeill, personal communication). This view is also supported by increased faecal nitrogen concentration observed in sheep offered foods high in condensed tannins (Waghorn et al, 1994), which in some cases was of endogenous origin (Barry and McNabb, 1999). It is

argued that condensed tannins from Quebracho extract may not release sufficient dietary protein in the small intestine to cause beneficial effects on the resilience and the resistance of hosts. Most studies performed in parasitised sheep with Quebracho extract are in agreement with the above (Butter et al, 2000; McNeill, personal communication; Chapters 4 and 5). It is appreciated that this might be a property of condensed tannins from Quebracho extract, since condensed tannins from other sources may increase protein availability for the host, as they have been considered responsible for higher performance compared to sheep grazing tannin-free forages (Niezen et al, 1998a; b).

6.6. The efficacy of condensed tannin from Quebracho extract as anthelmintic.

It was evident from the results presented in this thesis that condensed tannins from Quebracho extract were more effective when offered as a drench for a short period of time against an established infection, than when included in the diets of parasitised sheep, during the establishment or against an established infection. When condensed tannins were offered as a drench, the digestive tract of the sheep was relatively empty, as Quebracho was administered to sheep before the morning feeding. In addition, the sheep were fed restricted amount of foods. Consequently, the higher efficacy of condensed tannins when administered as a drench could be attributable to increased bioavailability of condensed tannins in the small intestine of the host. Increased bioavailability and hence efficacy has also been reported for anthelmintic drugs, as a consequence of reduced level of food intake in parasitised sheep (Ali and Hennessy, 1995; Hennessy et al, 1995).

In order to attempt an evaluation of the anthelmintic activity of condensed tannins contained in Quebracho extract towards *T.colubriformis*, the results obtained from the two drenching experiments are combined in Figure 6.2. Whilst it is appreciated that the two experiments are not directly comparable, sheep from both experiments (Chapters Two and Three) were approximately of the same age and liveweight. Figure 6.2 was constructed to provide some indication of whether the anthelmintic efficiency of condensed tannins is dose dependent or not. All points on Figure 6.2. represent the mean daily intake of Quebracho extract per sheep and the equivalent worm burden recovered from each sheep at the end of the drenching period. It is evident that the anthelmintic effect of condensed tannins, in the two drenching experiments, was not dose-dependent, but probably related to a specific threshold. This hypothesis is also supported by the results from long-term administration of condensed

tannins, as indicated in section 6.4. The bioavailability of condensed tannins probably varies according to the type and the structure of condensed tannins and dietary protein. This, influenced by gut flow rates may account for the differences in the anthelmintic properties of condensed tannins observed under grazing conditions (Niezen et al, 1994; 1998a), or when condensed tannins are given as a supplement in the foods of parasitised sheep (Butter et al, 2000; Chapters Four and Five).

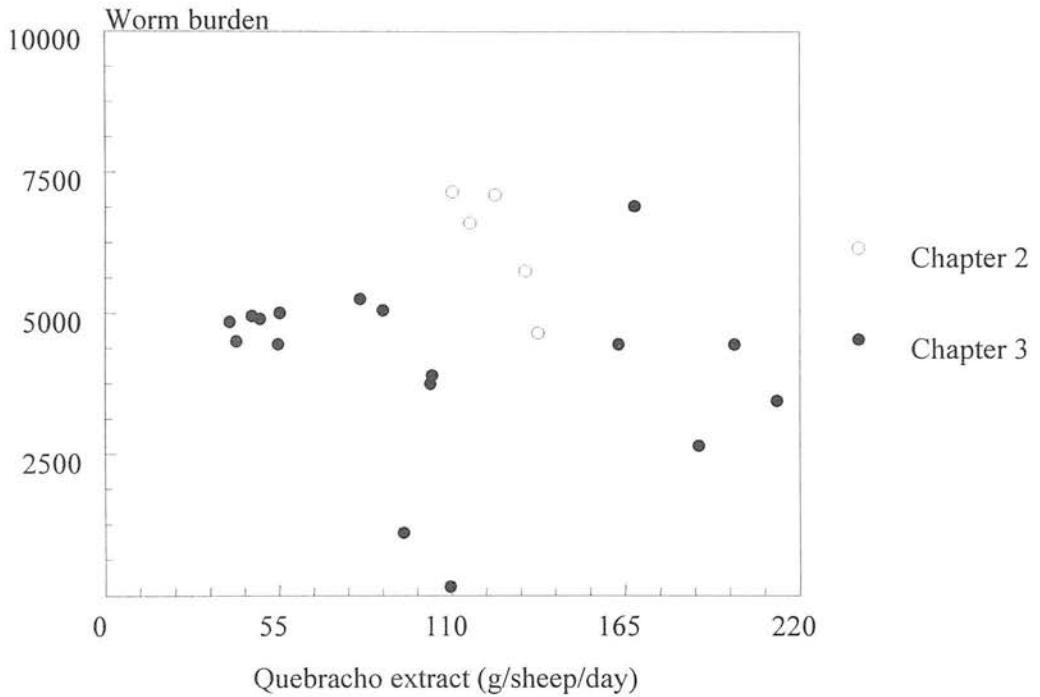


Figure 6.2. *T.colubriformis* worm burden of parasitised sheep and the amount of Quebracho extract administered. The data are taken from Chapters Two and Three.

The maximum efficacy of condensed tannins in the studies of the thesis never exceeded 50-60% and this is consistent with results from grazing studies (Niezen et al, 1994; 1998a). This efficacy of condensed tannins is much lower than that of anthelmintics, which is considered low when it is below 90-95%. Given this level of efficacy, condensed tannins would have a prophylactic rather than a therapeutic role and could be valuable within organic production systems or as a natural resource of anthelmintics for subsistence farmers. Condensed tannins

as natural polyphenols, are present in forages and legumes of temperate and tropical areas, in concentrations that vary from 0.1% up to even 50% of dry matter in some tropical forages (Barry et al, 2001). Farmers in many areas of the world could benefit from the anthelmintic properties of condensed tannins, at a lower cost than anthelmintics. In tropical countries, forages that contain condensed tannins are widespread and could play an important role in livestock production. In developing countries, where the costs of controlling parasitism can sometimes be prohibitive, grazing on tanniniferous forages might be an alternative control strategy.

6.7. Problems associated with the use of condensed tannins to control nematode infections

6.7.1. Condensed tannins and the parasitised host.

One of the major drawbacks affecting the use of condensed tannins as a potential way of controlling parasitism is their adverse effects on the parasitised host. In the experiments in this thesis, only on two occasions has the administration of Quebracho extract resulted in adverse effects on the host: in Chapter Three, in sheep administered with 16% of food intake Quebracho extract and in Chapter Five, in sheep offered the low protein food supplemented with Quebracho extract (section 6.3.). Although there is little information in the literature reporting toxic effects of condensed tannins, there was evidence for a possible toxic effect in sheep drenched with 16% w/w Quebracho extract (Chapter Three). Pale kidneys, thickened pylorus mucosa, ulceration of abomasal and colon were evident at post mortem in sheep offered the 16% w/w Quebracho extract. Although condensed tannins are polyphenols of high molecular weight and it is difficult for them to be absorbed “as is”, some types of them can get depolymerised and the products of the depolymerisation could possibly get absorbed by hosts (Clausen et al, 1990). On the other hand, according to the commercial suppliers, Quebracho extract contains a small amount of simple phenolics, which have low molecular weight and thus can be absorbed by the digestive tract of sheep. Toxic effects have been recorded in chickens following the consumption of sorghum tannins, which is a mixture of condensed tannin and simple phenolics (Jiménez-Ramsey et al, 1994). From the distribution of the radiolabelled compounds in the tissues, it was suggested that only the simple phenolics were absorbed and probably they were responsible for the toxic effects observed in chickens. Given these findings it is possible that the toxic effects observed following the administration of high concentrations of Quebracho extract in Chapter Three could be almost entirely attributable to the simple phenolics contained in Quebracho.

Due to the high structural variability, there is considerable polymorphism in the chemical properties of condensed tannins (see review by Mueller-Harvey and McAllan, 1992), and it is difficult to generalise. Quebracho extract was used in the present thesis only as a model, to provide some indication of the potential of condensed tannins to cause adverse effects on gastrointestinal nematodes of sheep. Since different results have been reported following the use of various types of condensed tannins towards both the parasites and the hosts (Niezen et al, 1994; 1998b), the potential of using condensed tannins as a means to control gastrointestinal parasitism in ruminants merits further investigation.

6.7.2. Condensed tannins and nematodes

Another problem that might occur following the use of condensed tannins to control parasitism is the development of adaptation/resistance mechanisms of nematodes towards tannins. Insects that consume diets high in condensed tannins have developed defence mechanisms to overcome possible adverse effects on their growth and viability; the presence of surfactants in their gut and high alkaline gut pH are some of them (Ayres et al, 1997). Mammals, which consume condensed tannins as a part of their diets, have also developed defence mechanisms, such as the excretion of salivary proline-rich proteins and mucin from their gut mucosa (Mehansho et al, 1987), to cope with the detrimental effects of condensed tannins. Thus if nematodes are exposed to condensed tannins they may develop adaptation mechanisms to cope with the adverse effects of condensed tannins. However, if we assume that there is a gene or genes responsible for the development of nematode resistance towards tannins, as there is for resistance to anthelmintic drugs, the low efficacy of condensed tannins against nematodes may delay the development of resistant strains of nematodes to tannins. It has been suggested that treating part of the parasitic population or treating to achieve reduced efficacy (<90%) are steps that may help in reducing the genetic selection to anthelmintics (Sangster, 1999). Other alternative approaches to control nematode infections,

such as vaccination and biological control of nematodes, have similar efficacy to condensed tannins towards nematodes (Barnes et al, 1995). It has been suggested that efficacy of 50-70% probably allows the survival of susceptible as well as resistant worms, sustains relatively good worm control and probably delays the occurrence of resistance (Barnes et al, 1995). Therefore the use of plants that contain condensed tannins as potential alternatives to control gastrointestinal parasitism, either alone or in combination with other methods, is a promising approach.

6.8. Future directions

This thesis has provided some of the first *in vivo* evidence for a direct anthelmintic effect of condensed tannins contained in Quebracho extract, through a series of four experiments. In addition, the *in vitro* larval development/viability assays have supported the evidence for an anthelmintic effect. This evidence could be the basis for investigating the anthelmintic properties of condensed tannins extracted from grazing forages, as their incorporation in conventional and organic farming systems could provide a complementary method of controlling nematode infections. As the activity of condensed tannins is probably related to their structure, which varies according to the distribution of tanniniferous forages, it seems possible that it will be necessary to test the efficacy of condensed tannins regionally. *In vitro* screening, using purified extracts against a range of species may provide some indication of their efficacy. However, *in vivo* activity of condensed tannins is affected by the physico-chemical environment, as reported in Chapter Three. Hence, the use of freshly cut forage in pen trials and extensive grazing trials will also be required.

Since many cases of anthelmintic resistance have also been reported for goats, the investigation of the anthelmintic properties of condensed tannins towards caprine nematodes would be challenging. Preliminary studies performed in parasitised goats have suggested that the administration of Quebracho extract may reduce abomasal worm burdens (Hoste H, personal communication), which contradicts with the findings from the present thesis. This difference maybe a result of different tannin-parasite interaction, as goats have developed adaptation mechanisms to cope with the consumption of high concentration of condensed tannins; thus it would be interesting to study the different and similar effects of condensed tannins towards goats and sheep.

Most of the research performed, including studies from the present thesis, has focused on young growing lambs, which are more susceptible to nematode infections because of their slow development of immunity towards gastrointestinal nematodes. However, studying the potential use of condensed tannins towards nematodes in adult ruminants, could be important since in intensive production systems ewes are often responsible for initiating the infection of growing animals. If pasture contamination can be reduced by feeding adult sheep with condensed tannins and knowing that non-parasitic larval stages of nematodes are affected when exposed to tannin-rich environment, then tanniniferous plants may be used to control gastrointestinal parasitism in conventional and organic farming systems. The results from this thesis provide a starting point for future investigation on the anthelmintic properties of condensed tannins.

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