Chapter 1. General introduction

Gastrointestinal nematode (GIN) parasitism is a major health cost and welfare issue in the Australian sheep industry. Under favourable conditions, the effects of GIN parasites on grazing sheep can develop rapidly (i.e. in as little as 7-10 days) and are not always detected in a timely manner by routine monitoring. Further, the effects of GIN parasites are not suffered evenly among all sheep and tend to be concentrated in a smaller sub-population of susceptible and non-resilient animals (Sréter *et al.* 1994). Control of GIN in sheep relies heavily on chemotherapy despite the rapid development of anthelmintic resistance in GIN populations.

Integration of GIN management practices has been used to effectively reduce GIN infection and slow anthelmintic resistance development (Kelly *et al.* 2010). These practices include exploiting knowledge of GIN ecology in conjunction with improved use of chemical and non-chemical control methods. In recent years the practice of maintaining unselected GIN populations in refugia (see Section 2.3.1) in integrated parasite management programs has also been recognized as an important component in managing and minimizing anthelmintic resistance development (Besier and Love 2003; Kenyon *et al.* 2009).

Targeted selective treatment (TST) is a GIN management strategy developed with the aim of maintaining unselected GIN in refugia and minimise GIN effects on production. TST is the administration of anthelmintics to animals most severely affected by gastrointestinal nematodes (GIN), while leaving a proportion of animals and their resident GIN untreated (Kenyon *et al.* 2009). A number of selective treatment strategies have been reported which base the decision of animal treatment on a combination of worm egg count (WEC) and condition score (Besier *et al.* 2010) or relative growth rate (Greer *et al.* 2009). These TST strategies require some level of trade-off between maintaining refugia and high levels of animal production (van Wyk *et al.* 2006), and unfortunately, the increased time and labour required for monitoring flocks when carrying out TST management programs, currently makes them impractical in areas such as Australia where flocks are large and labour expensive (Besier 2008).

A technique that provides on-going or strategic control of GIN during periods of high risk while also offering the advantages of TST without the additional labour requirement would be a useful addition for GIN control. The use of medicated feed blocks (MFB) containing anthelmintic, may be a tool that could be used to achieve these aims (Knox and Steel 1996). For example, in contrast to TST strategies, voluntary TST or self-medication may arise if variation in MFB consumption between animals is positively related to the severity of GIN infection and negatively related to host resilience to infection.

Unfortunately the dynamics of block intake (or self-medication) in a grazing environment are not known. The collection of such information is difficult as current techniques for measuring supplement intake are unsuitable for use in grazing animals and/or measuring intake over a prolonged period.

The experiments in this thesis were designed for two purposes. Firstly, to develop a technique that meets the requirements of a marker of MFB consumption in grazing sheep for use over an extended period. Secondly, to use this technique to determine if an MFB could be used to achieve voluntary TST by establishing if grazing sheep display self-medication for GIN infection.

Chapter 2. Literature review

2.1 Introduction

The economic losses caused by gastrointestinal nematode (GIN) parasites make them the most serious disease constraint for the Australian sheep industry. Minimising losses caused by decreased production, mortality and cost of treatment while managing and minimising anthelmintic resistance development in GIN is challenging (Besier and Love 2003; Sackett *et al.* 2006). A range of management techniques can be combined for an integrated approach to reduce GIN infection of sheep and to slow the development of anthelmintic resistance. Integrated approaches rely on a thorough understanding of GIN ecology, improved use of chemical control and integration with non-chemotherapeutic cultural practices. For example the delivery of benzimidazole anthelmintics in low continuous doses potentially increases the efficacy of the anthelmintic but it is also noted that slow release products are considered to decrease the effective life of the anthelmintic (Prichard *et al.* 1978b; Boisvenue *et al.* 1988). Similarly the use of non-persistent anthelmintics with appropriate grazing management, provides paddocks of low GIN infectivity for young sheep (Niven *et al.* 2002) and lambing ewes (Bailey *et al.* 2009a). In addition, maintaining anthelmintic susceptible larvae in refugia has been found to be important in slowing development of anthelmintic resistance (Dobson *et al.* 2011a). A strategy that aims to address the balance between efficacious GIN control and development of anthelmintic resistance is targeted selective treatment (TST). TST is the allocation of anthelmintic to those animals most affected by GIN infection, leaving a proportion of the mob untreated (Greer *et al.* 2009; Kenyon *et al.* 2009). TST is not commonly used in Australia as treatment programs require more frequent flock handling (van Wyk *et al.* 2006; Besier 2008) but the concept of leaving a small proportion of animals untreated, at routine treatment times, may be a useful alternative to reduce treatment frequency when long-acting anthelmintics are used. An example of a preparation based on long acting anthelmintics is the development of medicated feed blocks containing fenbendazole, which in the late 1970s were found to effectively reduce GIN infection (McBeath *et al.* 1979; Bogan and Marriner 1983). It is possible that if animals preferentially ate more of the block (i.e.

self-medicated) when they had a GIN infection, a TST outcome could be achieved in combination with effective control of GIN over an extended period. This possibility has remained untested because methods which allow accurate estimation of an individual animal's intake of block supplements in a grazing mob have not existed. In this literature review, an overview of the main GIN species, their ecology, the cost of GIN parasitism to production and methods of GIN management are given. Then, the evidence for and methods of detecting self-medication behaviour is discussed as are the methods that have been or could be used to estimate individual animal intake, the latter for the purpose of identifying a suitable approach to investigate intake of medicated supplements.

2.2 Gastrointestinal nematodes in sheep

2.2.1 Major gastrointestinal nematode species

In Australia there are four main species of GIN parasites that are responsible for the majority of disease and production loss in sheep. These species are *Haemonchus contortus* (barber's pole worm), *Teladorsagia (Ostertagia) circumcincta* (small brown stomach worm), and *Trichostrongylus* spp. (black scour worm) with *Trichostrongylus colubriformis* and *Trichostrongylus vitrinus* being the post pathogenic of these (Anderson *et al.* 1978). These species are also prominent on a global scale.

H. contortus, *T. circumcincta, T. colubriformis* and *T. vitrinus* are all part of the Trichostrongylid family and share a similar direct lifecycle that is divided between two phases, parasitic in the host animal and free-living on pasture (Figure 2-1) (Urquhart *et al.* 1996). Sexually mature females in the gastrointestinal tract produce eggs which pass out in faeces (Anderson *et al.* 1978; Besier 2004). The speed and success of free living development on pasture then depends on the pasture microclimate, chiefly temperature and available moisture (O'Connor *et al.* 2006). If environmental conditions are favourable, eggs develop to first stage larva (L_1) which feed on bacteria in faeces, grow, and moult to second stage larvae (L_2) which again feed, grow and moult to third stage, infective larvae (L_3). The infective larvae usually migrate to pasture herbage through water films where they become available for ingestion by the grazing animal (Anderson

et al. 1978). Development of eggs to L_3 takes approximately 4-10 days depending on species and conditions (Anderson *et al.* 1978). When infective larvae are ingested they associate with the gut mucosa and undergo the first parasitic moult to fourth stage larvae (L_4). Sexual differentiation then begins and is completed after the final moult to adult male and female nematodes. Mature worms then mate and females begin egg production (Anderson *et al.* 1978).

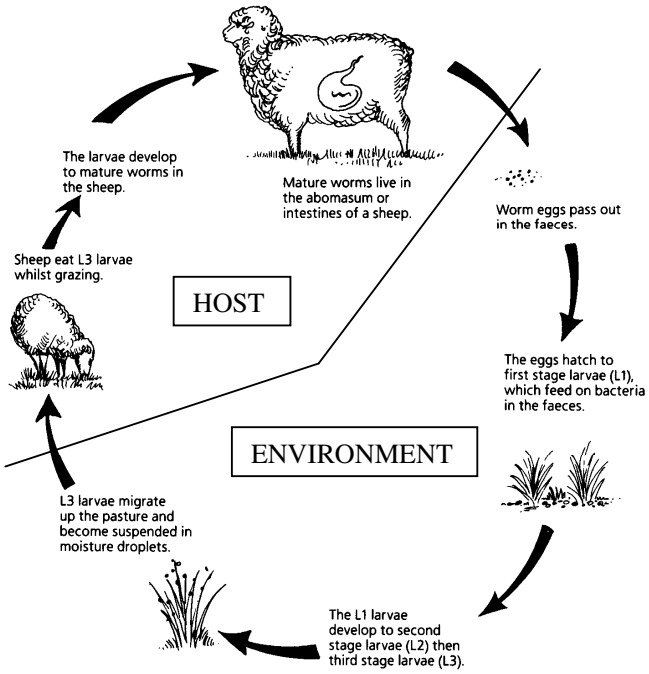

Figure 2-1: Typical lifecycle of the major gastrointestinal parasite species of sheep (Brightling 1994).

2.2.1.1 Haemonchus contortus

Adult *H. contortus* are large (20-30 mm) nematodes that have white ovaries that spiral around blood-filled intestines which gives them a "barber's pole" appearance (Urquhart *et al.* 1996). They are a blood feeding nematode that inhabits the abomasum of the parasitised sheep (Anderson *et al.* 1978). Fourth stage larvae and adults use a piercing lancet to obtain blood from mucosal vessels and a single adult *H. contortus* can remove 0.05 ml of blood each day through ingestion and seepage from lesions (Le Jambre 1995; Urquhart *et al.* 1996). This can result in chronic blood loss and ensuing anaemia, weakness, depression and mortalities, particularly in

weaners (3-7 months of age) which are most susceptible. Mortality is often among the first indications of a chronic infection in a flock (Besier 2004). This extremely pathogenic species can produce up to 10,000 eggs/day (Coyne *et al.* 1991), allowing populations to increase rapidly under favourable conditions (Besier 2004).

H. contortus is a dominant species in tropical and subtropical regions, and temperate regions with summer dominant rainfall such as in northern NSW and southern Queensland. Sporadic occurrences in other areas may also arise during favourable climatic conditions. The parasite can be found in cool temperate zones due to its ability to undergo arrested development at L_4 and through over winter survival of some L_3 (Anderson *et al.* 1978; O'Connor *et al.* 2006). The optimum temperature for egg development to L_3 is 25ºC to 37ºC with high mortality occurring below 10ºC and above 40ºC (O'Connor *et al.* 2006). The seasonal pattern of *H. contortus* faecal egg counts and adult GIN populations is characterised by an increase through spring to reach summer and autumn peaks before declining to low levels during winter. Adequate moisture is an important requirement for survival and development of *H. contortus* to infective L_3 as eggs are highly susceptible to desiccation (O'Connor *et al.* 2007). Rainfall events within four days of egg deposition result in significantly higher recovery of infective L_3 (O'Connor *et al.* 2007).

2.2.1.2 Trichostrongylus spp.

T. colubriformis and *T. vitrinus* are two of the main *Trichostrongylus* species causing disease in Australia. They are small hair like nematodes (approximately 7 mm length) (Urquhart *et al.* 1996) that inhabit the first three meters of the small intestine of sheep (Anderson *et al.* 1978). Following ingestion of L_3 *Trichostrongylus* spp. the larvae penetrate the sub-epithelial tunnels, which rupture after 10 to 12 days to liberate the adult stages resulting in considerable haemorrhage, oedema and plasma proteins lost into the lumen of the gut (Beveridge *et al.* 1989b; Urquhart *et al.* 1996). Chronic infections occur most commonly in lambs and weaners (Anderson *et al.* 1978) with symptoms including reduced voluntary feed intake, scouring, ill-thrift and less commonly death. *Trichostrongylus* spp. are the major cause of scouring in Australia. Because

this symptom may be easily confused with other disease conditions, infection must be confirmed with worm egg count and cultures (Beveridge *et al.* 1989b; Urquhart *et al.* 1996; Besier 2004).

T. colubriformis is the most widely distributed *Trichostrongylus* spp. and is found across Australia but especially in winter and uniform rainfall regions, though it is also found in summer rainfall areas (Anderson *et al.* 1978; Besier and Love 2003; O'Connor *et al.* 2006). *T. vitrinus* occurs more frequently in winter rainfall regions and is the most dominant *Trichostrongylus* spp. in western Victoria (Beveridge *et al.* 1989b). Free-living stages of *Trichostrongylus* spp. have a greater resistance to desiccation than *H. contortus* and are able to develop at lower temperatures with hatching and larval development (albeit at a low rate) occurring at a minimum of 5ºC (O'Connor *et al.* 2006). Out of the two *Trichostrongylus* spp., *T. colubriformis* eggs are the more resistant to desiccation while *T. vitrinus* is more successful at hatching and larval development at temperatures below 10ºC (Beveridge *et al.* 1989a). Adequate moisture (at least 25 mm of rainfall per month) is also required for *T. colubriformis* to develop to infective L_3 (Levine and Anderson 1973). Adult females of *Trichostrongylus* spp. lay up to 300 eggs/day (Anderson *et al.* 1978). *T. vitrinus* is more pathogenic than *T. colubriformis*, and as such needs to be treated at lower egg counts (Beveridge *et al.* 1989b). Southcott *et al.* (1976) found that *T. colubriformis* showed a pattern of development similar to *H. contortus* in summer, but autumn contamination of pastures could produce infection peaks during spring in summer rainfall regions of Australia.

2.2.1.3 Teladorsagia (Ostertagia) circumcincta

T. circumcincta is a small (10 mm) red-brown worm that is just visible on the lining of the abomasum (Anderson *et al.* 1978). While it is less pathogenic than *Trichostrongylus* spp. it is a significant contributor to disease and production loss in Australia and it causes symptoms that are similar to *Trichostrongylus* spp. (Besier 2004). *T. circumcincta* is abundant in cooler climates and most prominent in winter rainfall zones (Anderson *et al.* 1978; Besier and Love 2003) making spring-born lambs most susceptible. *T. circumcincta* are capable of reaching the infective L_3 under colder conditions than *T. colubriformis* with 20% of *T. circumcincta* eggs hatching at 5˚C as opposed to 5% of *T. colubriformis* (Rossanigo and Gruner 1995). Adult females can lay

from 50 to 100 eggs/day and infective larvae take between 4 and 27 days to develop after hatching, with an average of 14 days in favourable conditions (Callinan 1978). Infective larvae are most abundant when there is sufficient moisture and for regions where *T. circumcincta* is most prevalent, this is winter and spring. Larvae in spring include arrested larvae that were deposited in the previous summer and autumn (Southcott *et al.* 1976; Anderson *et al.* 1978). Like *T. colubriformis* and *T. vitrinus*, *T. circumcincta* eggs are able to develop at lower faecal moisture content then *H. contortus* (Rossangio and Gruner 1995).

The pathogenic nature of these GIN species and their ability to survive in a broad range of climatic conditions make them a significant problem for sheep producers. Despite this, the differences in ecology between species of GIN can be used to target management practices for different regions. A multi-faceted approach is required to control all GIN throughout the year and minimise costs to production caused by this disease.

2.2.2 Cost of gastrointestinal nematodes to the Australian sheep industry

Gastrointestinal nematode (GIN) parasites cause economic losses through decreased meat and wool production, mortality and cost of prevention and treatment (Sackett *et al.* 2006). It is difficult to determine the precise figures on losses incurred in production as they differ depending on the production system, management practices, seasonal variation and regional location. Sackett *et al.* (2006) estimated the annual national cost of GIN parasitism at $369 million, based on the 2001 national flock size. The report estimated GIN to cost 30% more than flystrike and over 200% more than any other sheep disease in Australia (Figure 2-2).

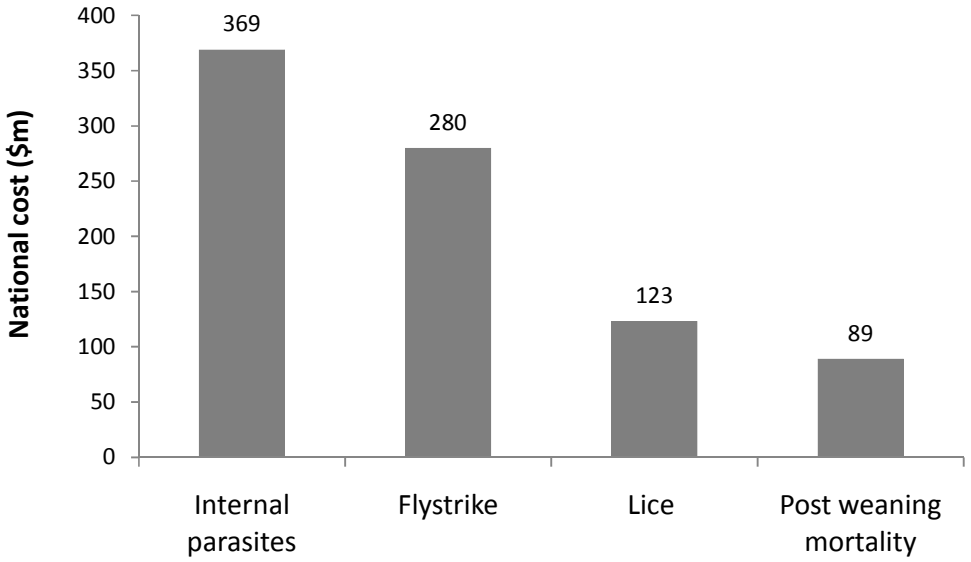

Figure 2-2: Annual national cost of disease to the sheep industry (Sackett *et al.* 2006).

Kelly (2011) revised the national cost of GIN parasitism using 2010 flock data and commodity prices to $267 million using the cost-benefit model from McLeod (1995). This decrease in cost can be attributed mainly to the drop in the national flock size from 111 million in 2001 to 68 million in 2010. When the economic impact of GIN is considered on a per head basis, the cost increased from $3.32/head in 2001 to $3.93/head in 2010 (Kelly 2011). The increase in commodity prices of meat and wool in the last ten years has contributed to this rise.

The majority of economic loss in the national model has consistently been attributed to reduced production rather than the cost associated with treatment and prevention. Barger (1982) attributed 83% to production loss, McLeod (1995) 64%, Sackett *et al.* (2006) 87% and Kelly (2011) 81%. While there have been significant improvements in understanding GIN parasitism, production losses have not been substantially reduced. An increased understanding of the broader effects of GIN infection has contributed to keeping this figure high. Additionally, while experimental evidence shows production loss may be reduced with strategic anthelmintic use and improved management techniques (McLeod 1995; Bailey *et al.* 2009b; Kelly *et al.* 2010)

adoption of these strategies by industry has been variable (Sackett *et al.* 2006; Walkden-Brown *et al.* 2006).

2.2.3 Cost to production

Sheep production and profitability are affected by GIN parasitism through reduced feed intake, changes to nutrient (protein) metabolism and scouring. This in turn reduces liveweight, growth and wool production and increases mortality, the most serve form of production loss, in the flock.

Depression of voluntary feed consumption in sheep caused by GIN infection (particularly *Trichostrongylus* spp. and *T. circumcincta*) varies depending on severity of infection and the GIN species involved. While feed intake is not always affected, up to 40-90% reduction in feed intake has been reported, though 10-30% is a more common estimate (Parkins and Holmes 1989; van Houtert and Skyes 1996). Reduced feed intake has been linked to the host's immune response. Greer *et al.* (2005) observed a typical reduced feed intake response to *T. colubriformis* or *T. circumcincta* infection in immunologically naive lambs but not in similar lambs given immunosuppressants. This suggests the low feed intake of GIN infected animals is related to the development of an immune response to the presence of the parasites in the gut. Once animals have developed immunity, appetite recovers (Kyriazakis *et al.* 1998; Greer *et al.* 2005). For example, Greer *et al.* (2005) reported that immunologically naive lambs recovered their appetite after approximately 64 days of infection.

The effect of GIN infection on nutrient metabolism is largely due to host induced pathology in the gut and increased competition between the gut and other tissues for nutrients, especially amino acids (Yu *et al.* 2000). Host pathology leads to an increased loss of endogenous protein into the gastrointestinal tract (van Houtert and Skyes 1996) in the form of leakage of plasma proteins, increased sloughing of epithelial cells and increased secretion of mucoproteins (Coop and Kyriazakis 2001). There is considerable protein re-absorption in distal sections of the small intestine but inefficiencies due to recycling lower protein availability for growth of muscle and wool (Coop and Kyriazakis 2001).

Considering the effect of GIN on voluntary feed consumption and nutrient metabolism, it is not surprising that reduced growth and liveweight is widely observed in infected animals (Anderson *et al.* 1978; Parkins and Holmes 1989). It is estimated that 40 to 90% of lost production can be attributed to reduced feed intake alone (Sykes 1983). The degree to which weight and growth in sheep is affected is determined by the host's physiology, the severity of the infection and GIN species. More severe infections of GIN generally result in a greater effect on growth. However, in mixed species infections, growth is depressed to a greater extent than the additive effects of equivalent single species infections (Parkins and Holmes 1989; Knox and Steel 1999). The negative effect of GIN on animal growth can reduce survival and reproduction and inhibit animals reaching optimum sale weight. The consequence is either lower sale values or increased costs associated with keeping animals for longer.

Reduced fleece weight, fibre length and diameter are also widely reported effects of GIN infection (Anderson *et al.* 1978; Parkins and Holmes 1989), again resulting primarily from reduced feed intake and reallocation of nutrients. In particular, competition for sulphur amino acids which are required in relatively high amounts for wool growth may be higher due to inflammation in the gastrointestinal tract (Adams and Liu 2003). Mixed species infections have a similar disproportionate effect on wool production, as occurs with liveweight and growth, in comparison with single species infections. For example, wool growth of weaner lambs given a mixed infection of *T. colubriformis* and *T. circumcincta* was reduced by up to 66% compared to a 25% reduction in lambs infected with *T. circumcincta* alone, and there was no effect on wool growth in lambs infected with *T. colubriformis* (Steel *et al.* 1982). Reductions in fleece weight of 7% (Albers *et al.* 1989) to 15% (Barger and Southcott 1975) in response to larval infection have been reported and Kelly (2011) equated a loss of $1.20 in wool income from ewes in the Tablelands of NSW using a dynamic model (GrassGro).

Scouring (diarrhoea) results in faecal soiling of the fleece around the breech area and is a common symptom of GIN infections and ingestion of infective larvae (Larsen *et al.* 1999; Jacobson *et al.* 2009). Scouring is an important economic and welfare issue, particularly because

of the strong links between scouring and incidence of flystrike (cutaneous blowfly myiasis) (Morley *et al.* 1976). Scouring is associated with the ingestion of trichostrongylid larvae and host susceptibility. While there are not always differences in protective immune responses of sheep with or without scouring, there is a hypersensitive inflammatory response in animals with this condition (Larsen *et al.* 1999). Animals affected by scouring due to GIN may require more frequent crutching to reduce flystrike susceptibility and to assist shearing (Larsen *et al.* 1999). The cost of crutching and reduced wool yield due to removal of larger stain areas around the breech area was estimated at $0.86 to $1.45 per head by Larsen *et al.* (1995) in south-west Victoria.

Mortality is the most extreme consequence of GIN infection and an obvious and major economic cost. Kelly (2011) found that a 1% increase in adult mortality rate increased the cost of parasitism by $1.24 per ewe. *H. contortus* is the main species responsible for mortalities by inducing anaemia but other parasites also contribute (Barger 1982; Besier and Love 2003). Mortality rates as high as 36% have been reported in uncontrolled GIN infections (Cohen *et al.* 1972) while Kelly (2011) reported an annual mortality rate of 6% in Merino ewes in the Northern Tablelands of NSW.

2.2.4 Management and control of gastrointestinal nematodes

Managing and controlling GIN effectively and sustainably is difficult especially in the context of other farm activities. Anthelmintics are the mainstay of GIN control despite the problem of anthelmintic resistance (Besier and Love 2003). The recent development of two new anthelmintic classes (i.e. monepantel and derquantel) and advances in refugia-based strategies will help to reduce the impact of anthelmintic resistance and maintain treatment efficacy (Besier and Love 2003). In addition to chemotherapy, there is a range of non-chemical control strategies (see the comprehensive reviews of Waller (1993), Waller (1999), Waller (2006), Besier and Love (2003) and Barger (1997)). Of these non-chemical strategies, grazing management, genetic selection, improved protein nutrition and exploiting an understanding of the ecology of GIN are best placed to contribute to integrated GIN management.

It is not the purpose of this literature review to summarise these non-chemical control strategies or to review all aspects of chemotherapy. Instead focus is placed on those areas of chemotherapy that are relevant to the concept of self-medication using medicated feed blocks.

2.3 Anthelmintics and resistance

Various classes of anthelmintic are available for use in sheep including benzimidazoles, imidazothiazole (levimazole is the dominant member and hereafter will be used to describe this class), macrocyclic lactones (ML), organophosphates, salicylanilides and, most recently, monepantel (an amino-acetonitrile derivative) and derquantel (spiroindole) (see Table 2-1). Benzimidazole (second generation) and levamisole broad spectrum anthelmintics were released in the late 1960s and early 1970s respectively (Besier and Love 2003). There are high levels of resistance to both these classes, with benzimidazole resistance in all major GIN species found on approximately 90% of Australian properties. Levamisole resistance is found in scour worms (*Trichostrongylus* spp. and *T. circumcincta*) and is estimated to occur on 80% of properties in Australia and resistance in *H. contortus* on 20% of properties in summer rainfall regions of Australia (Love 2011). Salicyanilides are narrow spectrum drenches that are most effective against *H. contortus*. Drench resistance to salicylanilides is now widespread in northern NSW and south-east Queensland with approximately 85% of properties in this region affected (Love 2011). Organophosphates are highly effective against *H. contortus* and also control *Trichostrongylus* spp. and *T. circumcincta.* There are two recorded cases of resistance to organophosphates, one in *Trichostrongylus* spp. (Le Jambre *et al.* 2005) and the other in *H. contortus* (Green *et al.* 1981). ML anthelmintics are also broad spectrum drenches. Ivermectin was released in Australia in 1988 followed by other more potent actives within the class. ML resistant *T. circumcincta* are estimated to be found on 70% of farms in Western Australia and 30% of farms in south eastern Australia while 70% of farms in northern NSW and southern Australia have resistant *H. contortus* (Love 2011). Monepantel (Zolvix®, Novartis Animal Health Inc., Switzerland) is the first broad spectrum anthelmintic compound from the new amino-acetonitrile derivative class and was released in Australia in September 2010 (Kaminsky *et al.*

2008; Dobson *et al.* 2011b). As yet, there are no reports of resistance to Monepantel. Derquantel is a mid-spectrum anthelmintic from the new spiroindole class of anthelmintics and on its own is not fully effective against adult and fourth stage *T. circumcincta* larvae or fourth stage *H. contortus* larvae. For this reason derquantel is to be sold in combination with abamectin as the product STARTECT® (Pfizer Animal Health) to provide broad spectrum coverage and is likely to be released in Australia in 2013 (Little *et al.* 2010; Little *et al.* 2011).

Anthelmintics may have activity against susceptible GIN for periods of one day (short-acting), weeks (mid-length acting) or months (long-acting also referred to as persistent or sustained activity). Anthelmintics are available in Australia to be delivered in combinations of two, three or four classes to provide greater efficacy against a broader range of GIN species, strains and life cycle stages. However GIN resistance can and has developed in response to some combinations (Love 2011). Anthelmintics can be delivered in a number of ways (Table 2-1) including oral drenching, subcutaneous injection, intraruminal controlled release devices, feed additives and medicated feed blocks, although in Australia there are no anthelmintics registered to be delivered via medicated feed blocks. The variety of products gives producers a range of options for using anthelmintics and incorporating them with other GIN management strategies.

Table 2-1: Registered drench classes in Australia and their spectrum of activity, route of administration and persistence of activity.

Anthelmintic class	Spectrum	Route of delivery	Persistence (days)
Benzimidazole	Broad; susceptible adult and immature *Haemonchus contortus*, *Teladorsagia* spp., *Trichostrongylus* spp., *Cooperia* spp., *Nematodirus* spp., *Oesophagostomum* spp., *Chabertia ovina, Dictyocaulus filaria* (lungworm) and *Moniezia expansa* (tapeworm).	Oral Feed additive (export sheep only) CRD*	1 100
Levamisole/ Morantel	Broad; susceptible adult and immature *H. contortus*, *Trichostrongylus* spp., *Teladorsagia* spp., *Cooperia* spp., *Nematodirus* spp., *Strongyloids*, *Oesophagostomum* spp., *Chabertia ovina*, *Dictyocaulus filaria* and *Bunastomum trigonocephalum* (hook worm).	Oral CRD Paste	1
Macrocyclic lactone	Broad; susceptible adult, immature and inhibited L4 *H. contortus*, *T. circumcincta* and adult and immature L4 *Haemonchus placei*, *Trichostrongylus* spp., *Cooperia* spp., *Nematodirus* spp., *Oesophagostomum* spp., *Chabertia ovina*, *Trichuris ovis*, *Strongyloides* spp., *Dictyocaulus filaria*, *Psorergates ovis* (itchmite) and *Oestrus ovis* (nasal bot).	Oral Subcutaneous injection CRD	1 14 90 100
Organophosphate	Intermediate; susceptible adult and immature *H. contortus*, *Trichostrongylus* spp., *Teladorsagia* spp. and adult *Nematodirus* spp.	Oral	1
Salicylanilide	Narrow; susceptible adult and immature *H. contortus*, immature stages of *Fasciola hepatica* (liver fluke) and all stages of *Oestrus ovis*.	Oral	28 42 (with abamectin)
Amino-acetonitrile derivative	Broad; adult and immature *H. contortus, Teladorsagia* spp., *Trichostrongylus* spp., *Nematodirus filicollis, Cooperia* spp., *Chabertia ovina,* and adult *Nematodirus spathiger* and immature L4 *Oesophagostomum venulosum.*	Oral	1
Spiroindole	Derquantel + Abermectin Broad; adult, immature and inhibited L4 *Haemonchus* spp., *Teladorsagia* spp. and *Cooperia* spp., adult and immature *Trichostrongylus* spp., *Nematodirus* spp., *Strongyloides* spp., *Dictyocaulus filaria*, *Muellerius capillaries*, and adult *Nematodirus filicollis*, *Strongyloides* spp., *Oesophagostomum* spp., *Chabertia ovina*, *Trichuris ovis* and *Protostrongylus rufescens*.	Oral	1

*Controlled release device (CRD)

2.3.1 Management strategies

During the 1970s and 1980s, regular and suppressive anthelmintic treatments were used to reduce production losses. The high drench frequency and low number of GIN larvae in refugia contributed to a dramatic increase in the development of resistance to benzimidazole and levamisole anthelmintic classes (Besier and Love 2003). Refugia are areas where members of the GIN population can escape exposure to, and consequently selection by, an anthelmintic (Georghiou and Taylor 1976). Later strategic programs such as 'WormKill' assisted in reducing the number of treatments by strategically timing drenches using historical knowledge of parasite epidemiology to minimise disease (Kenyon *et al.* 2009). The pathogenicity of GIN infection was reduced but so was the population of GIN in refugia. Monitoring of worm burdens through worm egg counts has been used to determine time of drenching (when worm burdens exceed a predetermined threshold) again to reduce frequency of anthelmintic treatments and production losses (Kahn *et al.* 2006). The adoption of objectively-based drenching is relatively low in Australia due to the perceived additional costs of monitoring worm burdens (Besier and Love 2003).

When combined with non-chemical strategies, anthelmintics can provide year round protection to animals. However, the long term benefits of grazing and drenching strategies which aim to significantly reduce larval contamination of pasture, such as intensive rotational grazing and strategic drenching, are now being questioned for concern these practices increase selection pressure for anthelmintic resistant nematodes. For example Marley *et al.* (2006) observed gradual increases in anthelmintic resistance in multiple year studies of intensive rotational grazing systems after an initial reduction in egg counts. The development of heritable anthelmintic resistance in GIN is the result of one or more mutations that allow the parasite to survive a therapeutic dose and reproduce, to pass on the resistant trait to their offspring, which may then reinfect the flock (Jabber *et al.* 2006; Papadopoulos 2008). The rate at which anthelmintic resistance develops is dependent on the percentage of GIN surviving anthelmintic treatment and the number of susceptible GIN left in the environment to dilute the frequency of resistant GIN

(Jabber *et al.* 2006). For example Besier (2001) showed that when lambs drenched with a 99% efficacious moxidectin anthelmintic were moved to a GIN free pasture there was a significant increase in moxidectin resistance in *T. circumcincta* compared with a similar flock where 10% of the lambs were left undrenched. Recognition of the importance of unselected GIN in refugia continues to increase but the benefits of maintaining GIN in refugia have to be carefully balanced with the potential loss of animal performance caused by increased larval challenge (Besier and Love 2003; van Wyk *et al.* 2006; Besier 2008).

2.3.2 Targeted selective treatment

Targeted selective treatment (TST) is a drenching strategy that aims to balance production loss with maintenance of GIN in refugia. This is achieved by only treating those animals that have the least tolerance to nematode infection or are the most at risk (Kenyon *et al.* 2009). The FAMACHA system developed with this rationale clinically evaluates anaemia due to *H. contortus* to identify animals to be treated and is known to reduce total drench used and overall losses (van Wyk *et al.* 2002; Besier 2008). The application of TST to non-haematophagous GIN is difficult as the indictors of parasitism are generally not specific to parasitism and severity of symptoms can vary between animals and species of GIN (Besier 2008). However, recent preliminary results using a decision support tool based on animal performance to administer TST was successful at reducing anthelmintic usage and maintaining animal productivity (Greer *et al.* 2009). Despite this, maintaining refugia requires a trade-off against maintenance of higher levels of animal production (van Wyk *et al.* 2006) and unfortunately the increased time and labour required for monitoring flocks when carrying out management programs such as FAMACHA makes these programs impractical in Australia where flocks are large and labour is expensive (Besier 2008). In contrast to TST strategies, which require analytical interpretation, is voluntary TST. Voluntary TST or self-medication would arise if animals' consumption of a medicated feed block (containing an anthelmintic) is positively associated with GIN infection.

Reducing GIN control expenditure, production loss, labour requirements and minimising anthelmintic resistance pressure is important for the economic viability of the Australian sheep

industry. Medicated feed blocks are a useful part of GIN control methods in many areas of the world but have not been adopted in Australia (Salem and Nefzaoui 2003). The focus of the remainder of this review will be on the advantages and disadvantages of the use of medicated feed blocks and on the ways medicated feed blocks might be used to control GIN. The areas which need further work in order for such products to be used in Australia will also be discussed.

2.4 Medicated feed block

2.4.1 Prolonged administration of benzimidazoles

Medicated feed blocks allow anthelmintics to be delivered over an extended period. In the case of benzimidazole anthelmintics, this is beneficial because they are absorbed to a greater extent and have a potentially greater impact on the GIN population when delivered in small doses over a prolonged period (Prichard *et al.* 1978a; Prichard *et al.* 1978b). The prolonged low doses have the effect of causing accumulation of unabsorbed anthelmintic in the rumen, prolonging the period of high circulating levels of FBZ. This subjects drug-tolerant GIN to longer periods of exposure to the FBZ treatments (Boisvenue *et al.* 1988).

All benzimidazoles have a similar mode of action against GIN by binding to tubulin a structural protein in the parasite causing it to be immobilized, eventually resulting in death (McKellar and Scott 1990; Lanusse *et al.* 1995). In Australia, the most efficacious benzimidazoles used to control GIN in sheep are sulphide benzimidazoles and include albendazole and fenbendazole. These compounds are reversibly metabolised in the liver of sheep to their sulphoxide derivative (albendazole oxide and oxfendazole respectively) which are also produced as commercial anthelmintics. The sulphoxides undergo further metabolism to sulphones by irreversible oxidation in the liver and are removed from the body in urine (Marriner and Bogan 1981a; McKellar and Scott 1990; Hennessy 1993). Other benzimidazole anthelmintics used in Australia include mebendazole and triclabendazole (activity restricted to liver fluke).

Benzimidazole anthelmintics range in efficacy and activity against different GIN species. This is the result of differences in bioavailability of the drug within the host animal. The chemical

structure of each benzimidazole determines its rate of absorption, metabolism and excretion which in turn determines the period of exposure of GIN to the anthelmintic. The more efficacious the anthelmintic, the slower the absorption and elimination rates (Prichard *et al.* 1978b). For example oxfendazole removed more benzimidazole resistant *T. colubriformis* than fenbendazole and took longer to reach half maximum concentration in sheep plasma (Prichard *et al.* 1978b).

Delivering a benzimidazole anthelmintic in small doses over a prolonged period favours total plasma concentration (area under the curve) at the expense of peak plasma concentration. Thus there is a maximum period over which a dose of benzimidazole can be divided and administered and still be effective. Providing a dose over too long a period can reduce the average concentration and fail to deliver a full therapeutic effect and may lead to anthelmintic resistance in the GIN (Bogan and Marriner 1983). This was observed by Thomas (1978) when a 3.5 mg/kg dose of fenbendazole was split equally over 14 days and was less effective than the same dose split over 4 or 7 days.

The effectiveness of prolonged administration of fenbendazole in medicated feed blocks was confirmed by Thomas (1978) who reported a reduction in mean worm egg count from 2,700 eggs/g faeces (epg) to 0 epg in sheep with a naturally acquired infection (*T. circumcincta* dominant; presumable benzimidazole sensitive) after access to a medicated feed block (0.83 g fenbendazole/kg) for 4 and 7 days with sheep consuming 1.4 and 0.8 mg fenbendazole/kg liveweight per day respectively. Similarly McBeath *et al.* (1979) provided sheep, with a naturally acquired GIN infection, access to a medicated feed block (0.45g fenbendazole/kg) and observed average worm egg counts declined from 180 epg to less than 10 epg in two weeks in which time animals had consumed a therapeutic dose of 5 mg fenbendazole/kg liveweight. The control groups' average worm egg count rose from 180 epg to 260 epg in the same period.

Intraruminal infusion of fenbendazole over a prolonged period is also effective against benzimidazole resistant nematodes (Prichard *et al.* 1978a; Boisvenue *et al.* 1988). Infections of benzimidazole resistant *H. contortus* (isolate survived 66 mg/kg oral dose of thiabendazole) in

lambs were reduced by 98% when fenbendazole was infused (0.2 mg/kg bodyweight per day) into rumen cannulated lambs over 7 or more days (Boisvenue *et al.* 1988). A realization of this increased efficacy led to the development of albendazole intraruminal controlled-release capsules (0.5 mg/kg/day) in the 1990s when benzimidazole resistance in Australia began to impair the drug's efficacy (from single oral dose) in the field (Knox *et al.* 1995).

As stated before, medicated feed blocks containing anthelmintic are available in the world market but currently not in Australia. Because benzimidazoles are more stable chemically than most anthelmintics and have low mammalian toxicity over a wide range of effective dosages, they lend themselves to deployment in medicated feed blocks (Duwel 1977; Lanusse *et al.* 1995).

2.4.2 Advantages and disadvantages of medicated feed blocks

2.4.2.1 Advantages

Medicated feed blocks that contain an anthelmintic have a number of advantages in addition to the increased efficacy of benzimidazoles resulting from prolonged administration discussed in the previous section (1.4.1). For example, a medicated feed block can provide a simple and labour saving means of administering anthelmintics to sheep, avoiding the need to handle stock (Thomas 1978). This may be particularly important during lambing when preparturient rises in worm egg count may require ewes to be re-treated or during periods where drench treatments are aimed at reducing infective larvae on pasture rather than combating parasitism in the sheep. The use of medicated feed blocks at this time would reduce ewe handling which in turn would reduce abortion, mismothering and metabolic disorders (McBeath *et al.* 1979).

Another advantage of medicated feed blocks, as an effective method of controlling GIN (both susceptible and anthelmintic resistant strains) in sheep is that the period of control is flexible. That is, the medicated feed block can be placed in the paddock with sheep for varying periods of time to provide short term or extended anthelmintic treatment. This increases the options in which the medicated feed block could be incorporated with other GIN management strategies.

An additional advantage would be accrued if sheep adjust their intake of a medicated feed block according to their individual needs for anthelmintic treatment i.e. if they self-medicate. For self-medication to be effective, variation in medicated feed block consumption between sheep would need to be positively related to the severity of the pathophysiology of GIN infection and negatively related to their resilience to infection. If this were the case voluntary TST might be achieved and this would bring with it the advantages of TST (reduced selection pressure for anthelmintic resistance while maintaining efficacious GIN control) without the need for additional labour to monitor animal performance (Besier 2008).

2.4.2.2 Disadvantages

The major disadvantage of use of medicated feed blocks as an alternate form of chemical control is the potential to increased selection of anthelmintic resistance and failure to adequately control worms in some individuals. The lack of information on the reasons for between-animal variability in intake is one of the reasons why we cannot use medicated feed blocks with confidence at present. In a mob of grazing sheep some animals may not ingest blocks at all, while others exhibit a range of intakes; furthermore, this intake variation is greater when animals are offered feed block supplements rather than supplements in troughs (Kendall *et al.* 1980; Lobato *et al.* 1980; Kendall *et al.* 1983; Bowman and Sowell 1997). For example intake of molasses-urea blocks (estimated using chromic oxide as an intake marker) gave a mean intake of 117 g dry matter/animal per week and co-efficient of variation (CV) of 140% in three groups of grazing sheep. The mean intake and CV over the same periods for feed oats was 1851 g/animal per week and 23% CV and for milled hay was 3326 g/animal per week and 30% CV (Lobato *et al.* 1980). In another study (using four groups of 16 ewes and chromic oxide as a means of determining intake by individuals) block supplement intake was also more variable (CV 56%) than trough supplement (feed block meal with equivalent dry matter to block supplement) intake (CV 39%) ($p<0.05$) (Kendall *et al.* 1983).

Neophobia, literally ‘fear of new’, exhibited by sheep in response to novel foods contributes to variation in supplement intake and the proportion of the mob that ingests the supplement. Factors

such as trough space, supplement allowance, supplement form, supplement formulation and animal familiarity with supplement, have all been shown to influence variation in intake in animals individually and within mobs (Bowman and Sowell 1997). For example in a study by Lobato and Pearce (1980) 1188 sheep in seven grazing flocks were offered molasses-urea blocks over three weeks. The average percentage of animals not consuming the blocks in each flock was 50% but there was large variability between flocks with a CV of 55%. The sheep that did not lick the blocks while grazing were confined in yards and again offered the blocks. After a further three weeks 81% of the sheep had licked the blocks and the CV between mobs was reduced to 26%.

Owing to the variation in supplement intake between animals, the possibility exists that some animals will receive less than a therapeutic dose. This underpins the potential for medicated feed blocks to worsen anthelmintic resistance (Ranjan *et al.* 2002). In addition, the prolonged exposure of GIN to the anthelmintic when using medicated feed blocks, while increasing the efficacy of the anthelmintic (Prichard *et al.* 1978b), will also increase the selection pressure for nematodes resistant to anthelmintics (Dobson *et al.* 1996). This key concern of using medicated feed blocks however, could potentially be offset through reduced drench frequency and maintaining unselected GIN in refugia (Dobson *et al.* 1996; Besier and Love 2003).

It is important to know more about self-medication by sheep consuming medicated feed blocks, because of the potential for hastening anthelmintic resistance and the possibility that some animals may remain GIN-infected and suffer production loss due to inadequate intake. In particular, the effect of GIN infection on block consumption needs to be determined. If sheep do self-medicate when offered medicated feed blocks then voluntary TST could be achieved, thereby reducing the labour and technical challenges of TST.

2.4.3 Animal self-medication

Self-medication in animals or zoopharmacognosy is a new field of study which describes the process by which animals select and use plant secondary metabolites and other non-nutritional

substances for treating and preventing disease (Villalba and Provenza 2007). Until recently, research on self-medication in animals had almost exclusively been based on observation of animals, mainly primates, in the wild and in zoos. Much of this has been subjective and anecdotal (Villalba and Provenza 2007; Gradé *et al.* 2009). However, there is growing evidence of self-medication in a number of wild animal species including chimpanzees, starlings, lemurs and elephants (Villalba and Provenza 2007; Jain *et al.* 2008; Gradé *et al.* 2009). Animals have been observed to use plants, soils, insects and fungi as curative or therapeutic medicines to relieve unpleasant symptoms (Jain *et al.* 2008). Other evidence of self-medication includes matching active compounds in non-nutritional plants that animals have been observed to eat in the wild with the pharmacological effects it produces in the animal. For example, leaves and pith of non-nutritional plants that are chewed and or swallowed by chimpanzees have been shown to contain compounds that have negative effects on internal parasites *in-vitro* (Huffman *et al.* 1993; Jain *et al.* 2008; Gradé *et al.* 2009). However, many compounds that have effective *in-vitro* activity against parasites have lower *in-vivo* activity; thus there is still little definitive evidence of self-medication.

Self-medication in domesticated livestock has only recently been considered. On the one hand, there is little information that suggests that animals can directly sense nutritional components in foods or that they only like foods that are nutritious (Provenza 1995; Keunen *et al.* 2003). Animals occasionally die from over-ingesting plants that contain toxins; and in studies by Pamp *et al.* (1977) and Burghardi *et al.* (1982) lambs did not ingest appropriate amounts of minerals when given multiple options. On the other hand, ruminants do select diets that contain more nutrients and less toxins than the average food available (Provenza and Balph 1990). That is, domesticated ruminants are often able to meet their nutritional requirements despite having a diverse array of plant species to choose from that vary in nutrient and toxin type and quantity and having changing nutritional requirements due to age, physiology and environmental factors. There is also increasing evidence that interactions between the senses (taste, sight, smell and touch) and signals from the internal organs, which are mediated by the nervous system and

hypothalamus, are what enables ruminants to sense consequences of food ingestion (Provenza 1995). Considering this, animals should be able to select different foods and alter the amount consumed based on previous experience.

2.4.3.1 Affective and cognitive learning processes.

The interactions between senses and internal organs that dictate feeding in ruminants have been illustrated by Garcia (1989) (Figure 2-3) as two interrelated systems. The first is the affective system which links taste with positive and negative post-ingestive feedback and the second is the cognitive system which links taste with sight and smell. While these two systems are depicted in Figure 2-3 as separate processes, this is somewhat artificial, the two are actually tightly linked when it comes to foraging (Provenza *et al.* 1992).

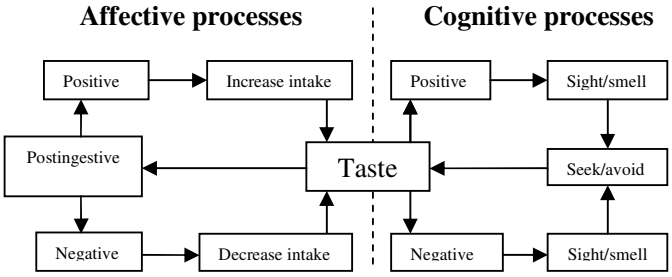

Figure 2-3: Schematic representation of affective and cognitive processes in diet selection, modified from Provenza *et al.* (1992).

In the affective system, consumption of a particular dietary component may be increased as a result of positive post-ingestive feedback where the animal benefits from the nutritional consequences. Alternatively, a negative post-ingestive feedback such as malaise will reduce intake of a feed. This feedback is integrated with taste (Provenza *et al.* 1992). The cognitive system then integrates the subtle differences in sight and smell of foods with taste so the animal can differentiate between foods and select or avoid foods based on post-ingestive feedback (Provenza *et al.* 1992; Provenza 1995). Evidence of post-ingestive feedback and its links with sight, taste and smell is given by Provenza *et al.* (1994). Lambs were given a novel food with either a toxicant, lithium chloride (LiCl; toxicity dependent on concentration), or antiemetic

drugs which would have attenuated the development of aversion caused by the LiCl or the novel food with both the toxicant and antiemetic drug or the novel food with neither. They found that the lambs that had received only LiCl had a lower intake of the novel feed than the lambs in the other groups.

The cognitive system can be broken down further into three subcategories; 1) learning from mother, this starts in utero and can continue after weaning (Hepper 1988; Mirza and Provenza 1990); 2) learning from peers (Provenza and Burritt 1991); and 3) learning through trial and error.

There are inherent risks of learning through trial and error. Accordingly, animals display neophobia in response to new feeds and will sample them cautiously and initially in small amounts: however, they may use generalisation of flavours of familiar safe foods to minimise risks (Burritt and Provenza 1989; Villalba and Provenza 2000). For example, lambs fed daily for seven weeks with coconut flavoured sorghum grain subsequently ate more coconut flavoured straw than plain straw when offered both (Villalba and Provenza 2000). After animals sample a new feed they will continue to eat it, or not, based on post-ingestive feedback. For example, when the lambs from the above experiment were given intraruminal infusions of energy (starch), post-consumption of the novel food, and the amount infused was proportional to the quantity consumed, they ate more in the subsequent offering. If they had infusions with a toxin (LiCl) they ate less at the subsequent offering of the novel food (Villalba and Provenza 2000). If offered both novel and familiar foods, sheep will attribute any negative post-ingestive experience to the novel food rather than the familiar one, regardless of whether or not the novel food was the cause of malaise (Burritt and Provenza 1989). The reason for this is that the animal has already associated the positive post-ingestive experiences with the familiar food and it would be more likely that a novel food would be harmful (Provenza and Balph 1988; Provenza 1996). The ability to discriminate between novel and familiar foods and associate the magnitude of new post-ingestive events with the amount of novel food eaten can be considered an important factor in animals choosing a safe diet (Villalba and Provenza 2000).

The duration and degree of aversion caused by negative post-ingestive feedback are influenced by both severity of illness and the delay between digestion and illness (Provenza 1995). For example, aversion in sheep given a novel food along with 3 g or more of LiCl was greater than in animals that received the novel food and 2 g LiCl and animals that had 0 or 1 g LiCl with the novel food did not display any aversion (du Toit *et al.* 1991). The relationship between degree of aversion and severity of post-ingestive feedback has been demonstrated in both sheep and cattle of varying ages (Burritt and Provenza 1989, 1990; du Toit *et al.* 1991; Ralphs and Cheney 1993). Persistence of learned preferences or aversion can therefore vary. Green et al (1984) reported preferences to feeds persisting for up to three years while Squibb *et al.* (1990) claimed feed preferences were retained for nine months without re-exposure. Burritt *et al.* (1989) reported feed aversions lasting for one year.

Even though aversions are persistent, animals and especially younger animals, will continue to sample small quantities of food to which they are repeatedly exposed after being adversely conditioned (Burritt and Provenza 1989). This is logical, because foods, especially plant foods, will vary in composition over time for a variety of reasons (season, soil type, moisture conditions) and animals stand to benefit if they modify their feeding behaviours to take advantage of these changes. Sheep also tend to graze a range of foods and change food preferences within meals. For example, ruminants generally prefer alternatives to forages they have consumed for several days or even hours regardless of the nutrient value of the alternative (Parsons *et al.* 1994; Provenza *et al.* 1996). This may be the result of the senses and post-ingestive feedback interacting during foraging to constantly make food ingestion satisfying (Provenza 1995; Provenza *et al.* 1996). A varied diet has several benefits including a more balanced energy and nutrient supply, reduced chance of over ingesting toxins, sampling of new foods and maintaining a diverse microflora in the rumen (Provenza 1995).

While there is substantial evidence of the ways in which animals learn how to associate taste and smell (and possible tactile properties) with post-ingestive feedback and how they retain these lessons, there are still gaps in our understanding. This leads to the question: can animals learn

self-medication and can they the use ability to learn from feed consequences to treat themselves for diseases, particularly infectious diseases such as GIN parasitism.

2.4.3.2 Self-medication in sheep

There is a limited amount of evidence in support of self-medication by sheep. Sheep have been shown to avoid foods containing toxins and eat foods that alleviate malaise caused by toxins. Villalba *et al.* (2006) challenged lambs with illness-inducing foods. The first group of lambs had previously been fed illness-inducing foods followed by compounds known to rectify each state of malaise over three periods of eight days. When these animals were fed the illness-inducing foods again they were able to identify the medicinal feeds from a selection of feeds to offset the illness. The second group of lambs ate the same illness-inducing foods and medicines but disassociated temporally so they did not recover from illness. Subsequently, this second group did not change their eating habits when offered the medicinal feed that could have rectified the malaise caused by the illness-inducing food (Villalba *et al.* 2006). This not only demonstrated that self-medication is a learned behaviour but also that sheep are able to form multiple malaise-medicine associations (Villalba *et al.* 2006).

There is also evidence that sheep self-medicate by adjusting their diet in response to GIN to meet the increased protein requirements resulting from infection. Kyriazakis *et al.* (1994) showed that lambs infected with GIN (2500, *T. colubriformis*, third stage larvae) and then given unrestricted access to two pelleted feeds with different crude protein contents (90 and 214 g/kg feed), chose a diet significantly higher in crude protein than uninfected lambs.

In another example of self-medication (Lisonbee *et al.* 2009), lambs naturally infected with mixed species of GIN (mean WEC 134 epg) ate more low-quality food (grape pomace) containing 30% quebracho tannins (a natural antiparasitic agent) than non-parasitised lambs in the same mob for the first 12 days of the study ($p<0.001$). Differences in feed intake between infected and uninfected lambs declined as WEC decreased, suggesting the sheep detected the presence of GIN or associated symptoms and modified their ingestion of the antiparasitic agent

as a function of need (Lisonbee *et al.* 2009). This study was followed by Villalba *et al.* (2010) who compared intake of feed containing tannins in parasitized and un-parasitized lambs. After a period of training, the lambs with GIN were exposed to the ameliorating effects of food containing tannin. A trend was again seen in which parasitised lambs showed a greater preference to the feed containing tannins ($p=0.07$), suggesting self-medication with tannins against parasites (Villalba *et al.* 2010).

2.4.4 Block consumption

Block consumption by individual grazing animals is difficult to determine. Previous studies of medicated blocks have often avoided this issue by treating the flock as the experimental unit and weighing the block before and after animal access (McBeath *et al.* 1979). Alternatively, intake has been determined in animals in individual pens (Knox *et al.* 1995; Sanyal and Singh 1995). A combination of these methods has also been used by Blagburn *et al.* (1987). Calves were grazed together in weight stratified groups and then each calf was moved into an individual pen with a medicated block where consumption was determined by the change in weight of the block. The problem with these methods is they do not take into consideration the effect that grazing in a flock may have on block consumption or the effects of social interaction between animals.

Other researchers have used the concentration of fenbendazole (the active constituent in the medicated blocks) in the animal's plasma, to measure intake (Bogan and Marriner 1983; Sanyal and Singh 1995). However, in none of these cases had plasma concentrations of fenbendazole been shown to be dependent on dose rate.

A number of studies have incorporated other markers into the feed blocks such as tritiated water, chromic oxide and lithium chloride; concentrations of these markers in blood or faecal samples can then be used to estimate block intake (Graham *et al.* 1977; Lobato *et al.* 1980; Ducker *et al.* 1981). For example, Lobato *et al.* (1980) used chromic oxide to determine individual animal intake of feed blocks in both penned and grazing experiments and Graham *et al.* (1977) used tritiated water to determine individual intake by cattle of a medicated bloat block. However for a

marker to be useful for measuring block intake in a grazing situation, over an extended period, it needs to meet a number of criteria. Unfortunately current markers have various disadvantages which limit their use. Thus determining block intake in grazing sheep is still an area worthy of further research.

2.5 Markers

2.5.1 Marker characteristics

The purpose of a marker is to quantify intake of a particular feed or supplement of interest by individual animals. Most markers follow a pattern of ingestion, absorption from the gastrointestinal tract and then, ideally, they are retained in the body at measurable levels for several days before excretion from the body. Markers may be measured during the early phase of increasing concentration in the animal's system and/or as they are removed, usually in faeces. For example, both chromic oxide and ytterbium have been used to calculate intake by measuring their recovery in faeces (Dove and Mayes 1991; Curtis *et al.* 1994). Markers that are measured as they increase in concentration within the animal may be point marker or cumulative markers. A point marker gives a single measure of the concentration of the marker (often in blood samples) at a single time point. A number of samples must therefore be taken to determine concentration changes of the marker over time in the system and rate of removal or dilution. Cumulative markers differ as they do not get removed from the system (quickly) and continue to accumulate, usually in fat. This means they can be used to determine total intake of the marker over a period of time and or, depending on how the marker is deposited in the body, a profile of intake over time.

The desired marker characteristics vary depending on how the marker is to be used. To determine differences in intake of a feed block by grazing sheep over time several characteristics are desirable. For example, the marker needs to be incorporated easily and uniformly into a block, stable in a range of temperatures and relatively insoluble in water so that it does not leach when exposed to rain or dew. A non-hazardous marker will ensure personnel and animal safety and allow animals to be sold in the future. The marker should also be a compound that is not

encountered in the animal's normal diet and should not affect metabolism or feeding behaviour of the animal such as through feed aversion. Preferably feeding behaviour should not be affected by the way the marker is sampled from the animal. A marker that requires samples to be collected from animals only once every few days, or less, is highly desirable. The marker should pass through the rumen relatively unchanged or with few, easily identified, metabolites to ensure accurate measurements of intake. It should be easily measured by standard analytical procedures and be inexpensive both to purchase and analyse (Suharyono 1992). Most importantly, the marker needs to be useful over a prolonged period, which is not the case with most currently available markers of intake. The marker and or its metabolites would need to remain in the animal at measurable and relatively stable levels for several days.

2.5.2 Potential markers

A number of substances have been used to estimate intake of a supplement by grazing livestock. These include tritiated water, deuterium oxide, ytterbium, chromic oxide, plant wax alkanes and lithium chloride. Tritiated water, deuterium and lithium chloride are point markers which measure the build up or plateau level of a marker in the body, while ytterbium, chromium oxide and plant wax alkanes are measured by the excretion of the marker in faeces. Cumulative markers, those that are deposited in the wool fibre and adipose tissue are also considered here.

2.5.2.1 Tritiated water

Tritiated water is water containing the radioactive isotope tritium (^3H) instead of hydrogen (^1H) and is represented as T_2O or ^3H_2O. Tritiated water was used by Nolan *et al.* (1974; 1975) to measure intake of a urea molasses supplement by grazing cattle and sheep respectively. The methodology used by (Leng *et al.* 1975; Nolan *et al.* 1975) assumed that tritiated water will become distributed evenly throughout all body water spaces in living tissue. Its removal from the body was well described by a constant concentration-dependent process (that can be determined in any study if two or more blood samples are taken from each animal after the labelled supplements are replaced by similar unlabelled supplements). With an estimate of total body water volume, the amount of tritiated water in the body at the end of the period of supplement

access was determined from the radioactivity in a single blood sample (Leng *et al.* 1975; Nolan *et al.* 1975).

In order to determine the total quantity of radioactivity ingested and thus supplement consumed over the period it is necessary to allow for the loss of tritiated water from the body in the period between ingestion of the labelled supplement and the time of blood sampling. Fortunately tritiated water has a long retention time in ruminants which reduces errors associated with losses occurring prior to blood sampling. Different and undetermined patterns of ingestion while the block is accessible and before blood samples are collected means that only an approximation of the true supplement intake is given using this method (Leng *et al.* 1975; Nolan *et al.* 1975). Tritiated water is liquid at room temperature so, to prevent evaporation after incorporation into supplements such as feed blocks, it can first be combined with gypsum in the water of crystallisation (Hedges and Rocks 1980; Dove 1984). With this approach, tritiated water has been used to determine intake of salt and bloat blocks and pelleted supplements by housed and grazing sheep and grazing steers (Graham *et al.* 1977; Hedges and Rocks 1980; Dove 1984). Nevertheless, use of tritiated water as a supplement marker has the major disadvantage that its radioactive nature makes it hazardous for operators and prevents sale of experimental animals (Suharyono 1992; Dixon *et al.* 2003). Errors in estimates of supplement intake can be caused by the assumptions concerning patterns of intake, especially when animals have access to the supplement for more than 4-7 days. For example, if an animal only consumed the supplement at the earliest or latest point in a one-week period, the estimate of intake would be approximately 30% higher or lower than the actual intake (Nolan *et al.* 1974; Nolan *et al.* 1975). The radioactive nature of tritiated water and the potential errors arising from time-dependency mean that it does not lend itself to routine use with grazing animal experiments.

2.5.2.2 Deuterium oxide

Deuterium oxide (D_2O) (also known as deuterated water or heavy water) is water containing the stable hydrogen isotope deuterium (^2H) (Rogers *et al.* 1985). D_2O can be used to measure supplement intake in a similar way to tritiated water using body water dilution techniques.

However, unlike tritiated water, D_2O is not radioactive (Lary *et al.* 1988; Bocquier *et al.* 1991; Butte *et al.* 1991). The concentration of D_2O in a blood sample is determined by infrared spectrometry (Rogers *et al.* 1985; Bocquier *et al.* 1991). The amount of D_2O consumed by the animal is then determined using similar calculations as those used for tritiated water (Rogers *et al.* 1985). Due to the cost and difficulty of estimation there has been less work conducted using D_2O as a marker than with many other substances (Bocquier *et al.* 1991). However D_2O has been used to determine milk intake in lambs as it is detected in milk from lactating ewes that have ingested D_2O (Dove 1988; Bocquier *et al.* 1991). D_2O has also been used to trace animals through abattoirs in the USA. In this context, it was found to slowly metabolise, meet product safety standards, and be quantifiable and easily identified (Lary *et al.* 1988). D_2O has not been used to measure feed block intake. It is likely that problems experienced with incorporating tritiated water into feed blocks would be similar with deuterium oxide. Similarly, errors associated with calculation assumptions when using tritiated water are the same with D_2O (Hedges and Rocks 1980; Dove 1984). The difficulties with incorporation into feed blocks, assumption errors and cost make D_2O, like tritiated water an unsatisfactory marker for measuring supplement intake in grazing animals.

2.5.2.3 Ytterbium

Ytterbium (Yb) is a rare earth element that is absorbed onto binding sites of particulate matter within feed stuffs (Teeter *et al.* 1984). It has been used in nutritional studies as a marker of particulate matter (Teeter *et al.* 1984) as well as to estimate rumen particulate turnover rates (Siddons *et al.* 1985) and faecal output (Krysl *et al.* 1985; Krysl *et al.* 1988; Hatfield *et al.* 1990). Yb has also been used to measure intake of lupin seed supplement in grazing animals (Curtis *et al.* 1994; Holst *et al.* 1994). Yb is added to feed using an adhesive solution that is sprayed onto seed and allowed to dry (Curtis *et al.* 1994; Holst *et al.* 1994). Lupin consumption over a 24 h period was estimated by collecting all faeces from sheep for seven hours following their access to the lupin seed. The faeces were weighed and the Yb content in a 10% subsample was determined (Curtis *et al.* 1994; Holst *et al.* 1994). The low levels of Yb in the environment and

its characteristic of not being absorbed in the gastrointestinal tract make it a relatively accurate marker. It does not affect palatability of feed and is non-radioactive (Curtis *et al.* 1994).

Yb has the disadvantage of requiring total collection of faeces using a faecal collection harness which can affect normal feeding behaviour of animals and is labour intensive (Hatfield *et al.* 1990; Curtis *et al.* 1994; Holst *et al.* 1994). Incomplete recovery of Yb in faeces (85-97%) also reduce the accuracy of Yb as a marker of feed intake (Curtis *et al.* 1994; Holst *et al.* 1994). Chromic oxide could be used in combination with Yb to estimate total faecal output so that all faeces wouldn't have to be collected. However relatively large and frequent faecal samples would still be required and assumptions made while estimating total faecal output would reduce accuracy (Curtis *et al.* 1994; Holst *et al.* 1994). Yb is a simple method that is relatively accurate; however the large scale faecal collection required makes it unsuitable for grazing animal experiments and particularly those with a focus on feeding behaviour.

2.5.2.4 Chromic oxide

Chromic oxide (Cr_2O_3) is a digestibility marker like Yb which is used to measure faecal output in ruminants (Prigge *et al.* 1981; Brandyberry *et al.* 1991; Dove and Mayes 1991). Chromic oxide has also been used to measure dry matter intake of feeds and supplements through techniques based on dosing, faecal collection times and or mathematical models (Prigge *et al.* 1981). This means total or partial faecal collection and digestibility assumptions are required (Lobato *et al.* 1980; Prigge *et al.* 1981; Dove and Mayes 1991). As with Yb, the disadvantage of collecting faeces in the field is that it is laborious and inconvenient and can disrupt normal grazing behaviour (Dove and Mayes 1991).

Chromic oxide has been used to measure intake of supplements including feed blocks, oats and hay in grazing sheep (Lobato *et al.* 1980). Estimating intake of feed blocks was difficult however due to the uneven distribution of the marker throughout the block which led to lower and more variable estimates of recovery of chromic oxide than with oats or hay (Lobato *et al.* 1980). In the feed block the chromic oxide was added as an extra ingredient during mixing before manufacture

while in the oats and hay supplements chromic oxide was first mixed with wheat flour and gradually mixed into the feed with a suspension of starch based glue (Lobato *et al.* 1980). Another concern of using chromic oxide, particularly as a marker of faecal output, is diurnal variation in faecal chromium concentration, which increases the risk that faecal samples are not representative of the mean chromium concentration. The issue of diurnal variation has been addressed for measuring faecal output by using controlled-release devices to administer chromic oxide (Prigge *et al.* 1981; Brandyberry *et al.* 1991; Dove and Mayes 1991).

2.5.2.5 Plant wax alkanes

Plant wax alkanes have been used to measure forage and forage based supplement intake (Dove and Mayes 1991). Alkanes are saturated, straight, carbon chains with no cyclic structures and are a key component in plant cuticular wax. The majority of the alkanes in plant wax are odd-chain alkanes with a relatively small number of even-chain alkanes which can be produced artificially (Dove and Mayes 1991). Alkanes are favoured for digestibility studies as they are widespread, easily analysed and present in large quantities permitting intake estimation (Dove and Mayes 1991; Duncan *et al.* 1999). However, it is the relatively inexpensive even-chain alkanes that may potentially be used as markers of food or supplement intake. The relative recovery of odd-chain (plant wax) and even-chain (alkane markers) are compared using ratios within formulae giving intake estimates (Berry *et al.* 2000). Small amounts of naturally occurring even-chain alkanes may be found in the supplement or forage but this can be accounted for by sampling pasture to determine alkane fingerprint and making adjustments in intake calculations (Dove and Mayes 1991). The disadvantage of using synthetic alkanes is the necessary extensive faecal collection, which is labour intensive and liable to disrupt normal feeding behaviour of animals. In addition a significant amount of even-chain alkanes would need to be incorporated into a feed block and as yet alkanes have not been used in non-forage based supplement intake or in supplements with multiple ingredients (Dove and Mayes 1991). Finally, as the botanical diversity in the animal's diet increases, the accuracy and predictive capacity of this approach diminishes making this

method difficult to use with grazing animals (Lee and Nolan 2003). For these reasons plant wax alkanes have not been used to measure feed block consumption in grazing animals.

2.5.2.6 Lithium

Suharyono *et al.* (1992) suggested the use of lithium chloride as a marker of feed intake. Lithium (Li) is a trace element which is present in very low levels in the environment but is an essential micronutrient due to its role in enzyme activities in mammals (Suharyono 1992; Dixon *et al.* 2003). While lithium can cause feed aversion in ruminants, this only occurs at levels above 50 mg/kg (liveweight/day) (Suharyono 1992; Kahn 1994; Dixon *et al.* 2003). Once lithium is ingested, most is absorbed across the rumen wall or lower gut wall into the blood pool (Suharyono 1992; Kahn 1994). Suharyono *et al.* (1992) found that plasma lithium concentrations in sheep are linearly related to amount of lithium salts ingested. The profile of lithium in plasma shows that concentrations peak at 4 h post ingestion and remain near this level for another 10 h in grazing animals (Kahn 1994). Plasma lithium concentrations can be readily obtained using a flame photometer which is accurate and economical (Suharyono 1992; Kahn 1994; Dixon *et al.* 2003). Due to the short plateau of lithium concentrations in plasma, it is useful for measuring feed intake during a single feeding event rather than estimating feed intake over a continuous week long period (Suharyono 1992; Kahn 1994). This is the main disadvantage for using lithium to measure supplement intake, as changes of intake over a prolonged period are of particular interest in determining whether sheep self-medicate or not.

2.5.2.7 Cumulative markers

Cumulative markers are compounds that are deposited over time in tissue or fibre at a rate that reflects ingestion. For example a cumulative marker that is deposited in wool could give an indication of time between feeding events, captured as bands in the emerging fibre. Wool or hair fibre is metabolically dead material after it has left the epidermis but during growth in the root bulb it is very metabolically active and various compounds may be taken up through the root cells and stored in the fibre (Raab *et al.* 2002). Examples of this are heavy metals, such as arsenic that is transported in the blood. However, arsenic incorporation into the fibre is erratic because

uptake, metabolism and the rate of wool growth all vary (Raab *et al.* 2002). Other compounds that are incorporated into the wool fibre include selenium, sulphur and radioactive substances such as [^{35}S]cystine. Selenium and sulphur are naturally occurring compounds and affect the formation and growth of wool fibres which means they would be unsuitable as markers of intake (Demiruren and Slen 1963; Doney and Evans 1970). Radioisotopes on the other hand have been used to determine growth rate of wool but, are hazardous and as such are undesirable (Downs *et al.* 1967). The variability in wool growth rate (and hence concentration of marker) between animals would also diminish the usefulness of a marker deposited in wool but this could potentially be overcome by the simultaneous use of dye bands to record wool growth on each animal (McCloghry 1997). While the idea of a compound that is deposited in wool in a dose dependent fashion is appealing, there are no compounds that would be viable options as cumulative markers of supplement intake.

Cumulative compounds that are deposited in the adipose tissue or fat of an animal could also theoretically be used to quantify supplement intake. Adipose tissue contains triglycerides which are used as an energy store for the body and in the subcutaneous layers, help to shape and protect the body. Stores of adipose tissue reflect the intake of dietary fat and other fat soluble compounds except in cases of fasting, or high nutrient demand when fat stores may be depleted (Arab 2003). This means that a fat soluble compound that is deposited in adipose tissue at a rate reflective of ingestion could be used as a marker of supplement intake. It is known that fat soluble pesticides such as DDT and other organo-phosphates are deposited in fat in this manner (MacLachlan and R.Bhula 2008), as are a number of pesticides commonly used in sheep such as spinosad and macrocyclic lactones (Kirst *et al.* 2002; Pérez *et al.* 2008). However it would be necessary to find a substance with these properties which was not detrimental or hazardous to the health of the animal and did not compromise the sale of the animal for human consumption. Such a marker would require a biopsy of fat to be taken from the animal. This small surgical procedure requires the animal to be given a local anaesthetic before a small incision is made at the preferred site to collect the adipose tissue (Huerta-Leidenz *et al.* 1993). To use this procedure, sufficient

evidence of a dose dependent relationship would be required to justify further research into the compound as a marker of supplement intake.

While some of the above markers have been used to measure intake of a feed block supplement, none meets the criteria of being non hazardous, remaining useful over a prolonged period and minimising disturbance of grazing animals.

2.5.3 Fenbendazole as a marker

Fenbendazole (methyl 5-(phenylthio)-2-benzimidazole-carbamate) is a benzimidazole oral anthelmintic that contains sulphur. It is extensively metabolised in mammals; the parent drug is short lived while the metabolic products predominate in the plasma and tissues of the animal. In the liver of sheep microsomal sulphoxidation converts fenbendazole to oxfendazole; this process is fast and reversible and favours sulphoxidation (Duwel 1977; Marriner and Bogan 1981a, 1981b; Lanusse *et al.* 1995). Oxfendazole also has anthelmintic activity (Marriner and Bogan 1981a, 1981b; Soraci *et al.* 1997). A portion of the oxfendazole undergoes a second, slower, irreversible oxidation reaction to the sulphone metabolite, fenbendazole-sulfone (Lanusse *et al.* 1995) which also has anthelmintic activity but is not as effective as fenbendazole (Marriner and Bogan 1981a, 1981b).

Fenbendazole, oxfendazole and fenbendazole-sulfone have been reported to have a long retention time in plasma (70, 120 and 140 h respectively) following oral administration of 5 mg fenbendazole/kg liveweight (Prichard *et al.* 1978a; Lanusse *et al.* 1995) (see Figure 2-4). Peak plasma concentrations in sheep occur at approximately 24 h for fenbendazole and oxfendazole and 36 to 48 h for fenbendazole-sulfone after a single oral dose of 5 mg fenbendazole/kg liveweight (Prichard *et al.* 1978a; Marriner and Bogan 1981a). The long apparent “plateau” seen in Figure 2-4, suggest that fenbendazole may be a useful marker over an extended period. The longer retention of the metabolites in plasma may indicate that they are likely to be more useful as markers of intake than fenbendazole itself (Prichard *et al.* 1978b; Lanusse *et al.* 1995).

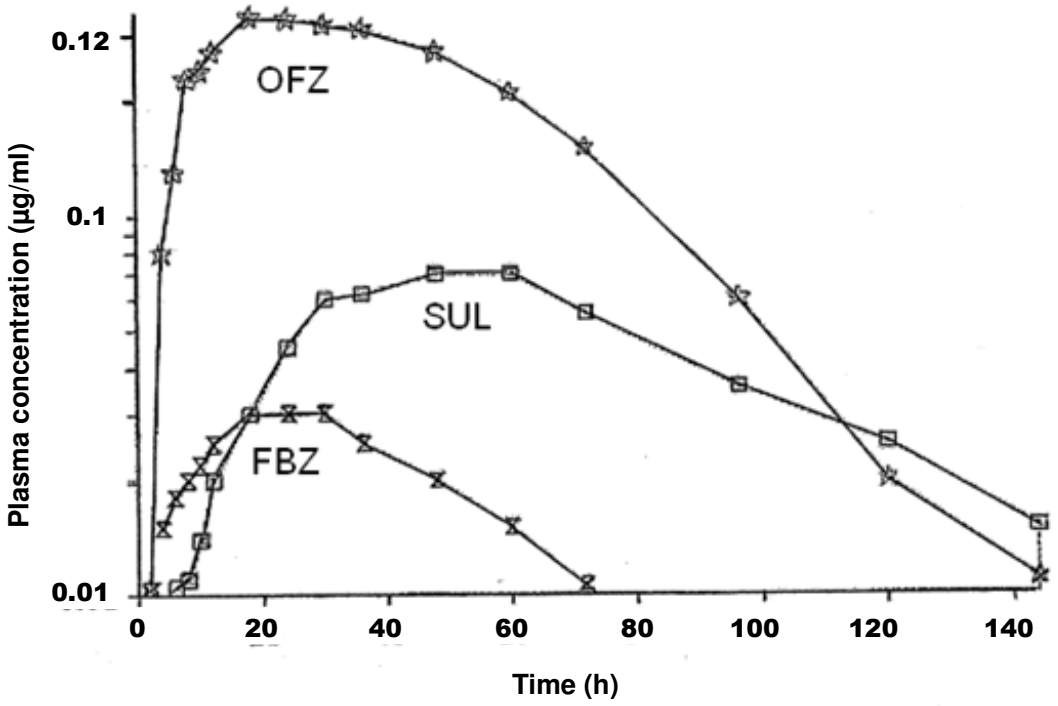

Figure 2-4: Mean plasma concentration of fenbendazole (FBZ), oxfendazole (OFZ) and fenbendazole-sulfone (SUL) obtained following the oral administration of FBZ (5 mg/kg) to sheep (n=6; Lanusse *et al.* 1995).

Bogan and Marriner (1983) used fenbendazole and oxfendazole to estimate medicated feed block intake by grazing ewes and determine the efficacy of feed blocks as a means of administering anthelmintics to grazing sheep. At that time, the form of the relationship between fenbendazole and oxfendazole concentration in sheep plasma and dose rate was not established. Knox *et al.* (1995) demonstrated that fenbendazole and oxfendazole concentrations in plasma of housed sheep increased in a linear manner with increased intake of a molasses supplement block containing fenbendazole, and with intraruminal infusion of fenbendazole (see Figure 2-5).

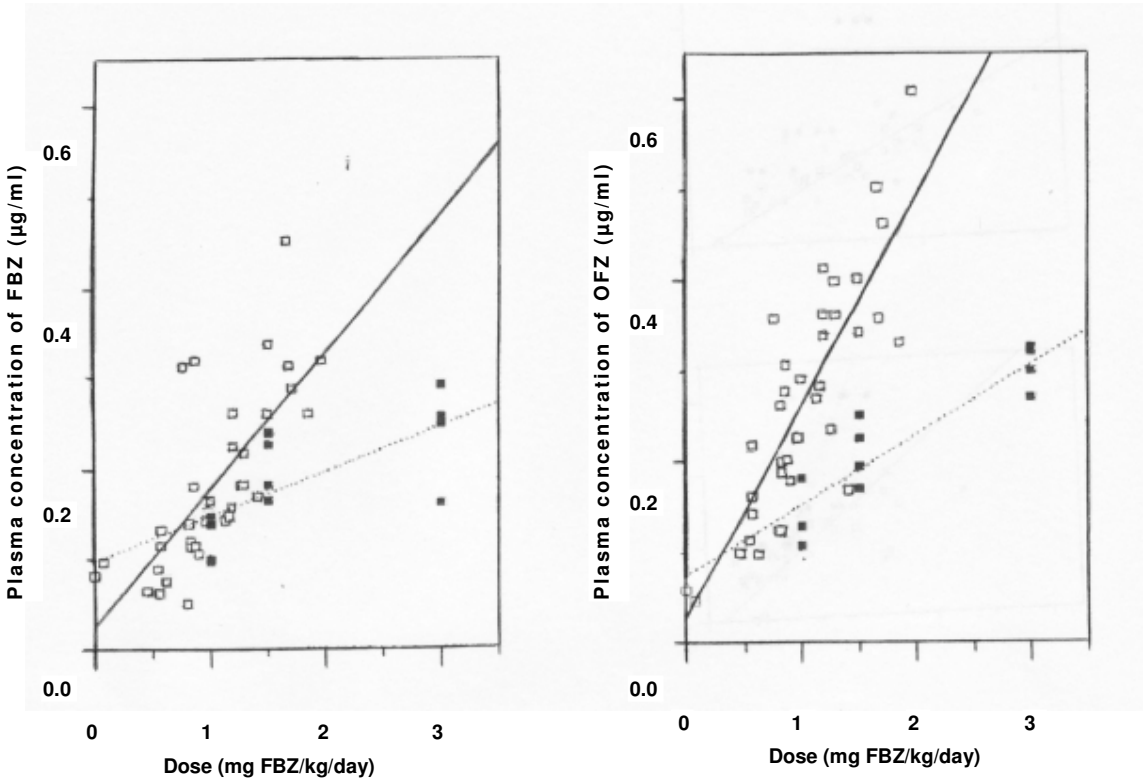

Figure 2-5: Scatter plots (□, block; ■, infusion) and fitted regression lines (—, block; ---, infusion) for dose versus plasma concentration of fenbendazole (FBZ) and oxfendazole (OFZ) 48 h after delivery of FBZ by urea-molasses blocks or 4, 5 or 6 days after intraruminal infusion of FBZ to sheep (Knox *et al.* 1995).

When fenbendazole was delivered by continuous intraruminal infusion, however, the relationship between plasma concentrations and dose administered was not significant (Knox *et al.* 1995). While this evidence suggests plasma concentration of fenbendazole is dose dependent, there is still a need to verify this relationship across a range and frequency of fenbendazole doses. The importance of understanding the plasma concentration resulting from multiple doses is important where it is used to quantify block intake in grazing animals over a number of days.

As discussed in Section 2.4.1, fenbendazole and its metabolites are non-hazardous and the stable nature of fenbendazole lends itself to deployment in feed blocks. Plasma levels of fenbendazole, oxfendazole and fenbendazole-sulfone are also easily and accurately determined with high

performance liquid chromatography. These reasons favour fenbendazole as a potential marker of feed block intake in a way that is independent of its anthelmintic activity.

The promising evidence for dose dependence and relatively long retention time in plasma makes fenbendazole and its metabolites potential candidates as markers of supplement intake. Further research into the relationship between plasma levels and the effect of different dose amounts and frequencies of fenbendazole on plasma concentrations of metabolites is required before it could be used with confidence to determine block intake in grazing animals.

2.6 Conclusions

GIN parasitism poses a significant cost to the Australian sheep industry. With drench resistance threatening the effectiveness of anthelmintics, strategies that reduce and delay the development of anthelmintic resistance are necessary. One such strategy is the concept of refugia and TST but this can be difficult to incorporate into Australian sheep farming systems that require high labour efficiency. The possibility of achieving TST through animal self-medication with medicated feed blocks would mean the benefits of TST could be achieved in a labour efficient manner and open up avenues for alternative management practices.

While self-medication in animals is a relatively new area of research, there is growing evidence of how grazing ruminants use post-ingestive feedback to choose safe and satisfying diets. However, evidence that ruminants can medicate themselves to control infectious diseases and GIN infections is limited to only a handful of studies. While it is clear the animals can be treated successfully for susceptible GIN through medicated feed blocks containing anthelmintic, there have been no studies to determine to what extent this is due to self-medication. That is whether medicated feed block consumption is positively related to severity of GIN infection negatively related to host resilience to infection

As this literature review has shown, until the body of work for this thesis was undertaken, there were no entirely satisfactory methods for measuring supplement intake accurately over a prolonged period in grazing livestock. However, the characteristics of fenbendazole suggest that

it could potentially have the desirable attributes of a marker for determining supplement intake. It is emphasized that its use as a potential marker would be independent of its anthelmintic activity. To determine if medicated feed blocks can be used for TST through self-medication, block intake in infected and uninfected grazing animals needs to be examined, but first, a technique that satisfies the requirements of a marker of intake over a prolonged period needs to be developed.

The review of the literature has identified the potential of self-medication in sheep and the lack of a suitable technique for measuring supplement consumption. The experimental section of this thesis consists of two separate but linked approaches. Firstly, to determine the usefulness of fenbendazole and its metabolites as a marker of supplement intake in grazing sheep over a prolonged period. Secondly, to utilise the measurement technique to investigate self-medication by sheep for gastrointestinal parasites using a medicated feed block.

Chapter 6. General discussion

6.1 Introduction

Medicated feed blocks (MFB, containing an anthelmintic) could be used to control gastrointestinal nematodes (GIN) in sheep and achieve voluntary targeted selective treatment (TST) with the benefits of reduced selection pressure for anthelmintic resistance and reduced labour associated with providing treatment. For this to happen, MFB consumption by sheep should be positively associated with GIN infection and negatively associated to host resilience to infection. Prior to the experiments described in this thesis, the dynamics of MFB intake by sheep in a grazing environment were largely unknown. The collection of such information was hampered by the lack of suitability of current techniques for measuring supplement intake in grazing animals and/or measuring intake over a prolonged period.

The experiments in this thesis were designed for two purposes. Firstly, to develop a technique that met the requirements of a marker of MFB intake for use over an extended period in grazing livestock. Secondly, to use this technique to determine if an MFB could be used to achieve voluntary TST by establishing if grazing sheep display self-medication in response to GIN infection.

The data collected in these experiments provide support for fenbendazole (in a role completely separate to its anthelmintic activity) as a useful and accurate marker of feed block intake in grazing sheep over (at least) a six day period. The results presented in Chapters 4 and 5 provide valuable information on self-medication with MFB and the response of sheep to the effects of GIN infection.

6.2 Fenbendazole as a marker of feed block intake

Fenbendazole metabolite concentrations in plasma increase with increasing oral dose rates of fenbendazole, delivered as single or multiple doses and at varying time intervals (see Chapter 3). This dose-dependent relationship existed in housed and grazing sheep and when fenbendazole

was provided as an oral dose or when incorporated into a molasses based feed block. Importantly, the relationship between dose and blood metabolite levels was unaffected by GIN infection.

Fenbendazole offers a number of advantages over other methods of measuring supplement intake. It requires no faecal collection or pasture composition analysis and the method of analysis is inexpensive. Fenbendazole is non-toxic and stable in a range of temperatures even when incorporated into a feed block. It provides the ability to measure intake at a single point or for an extended period through composite samples or as a series of point samples over time. While intervention to collect blood samples is still required this can be minimised to a 48 h sampling frequency and animals do not need to be kept in separate pens or paddocks.

The main constraint of the fenbendazole method is the limit of detection of oxfendazole and fenbendazole-sulfone in plasma (0.03 µg/ml) which may reduce the ability to discriminate between very low intake levels. This could be improved as Lanusse *et al.* (1995) claims a detection limit of 0.01 µg/ml for oxfendazole and fenbendazole-sulfone in plasma using a different method of sample extraction and HPLC mobile phase and gradient.

It is important to note that the reason for developing this method of estimating intake over a prolonged period was to address the key aim of this thesis (i.e. do grazing sheep self-medicate in response to GIN infection). Subsequent experiments used fenbendazole successfully as a marker to examine the effects of GIN infection on MFB consumption in grazing sheep.

6.3 MFB intake

6.3.1 MFB intake with susceptible GIN

MFB intake of wethers infected with anthelmintic susceptible *T. colubriformis* and *H. contortus* was investigated in Chapter 4. The hypothesis, that GIN infection coupled with positive post-ingestive consequences of consuming the MFB would provide the stimulus for MFB consumption (Figure 6-1), was supported. The hypothesis involved the expectation that MFB

intake of sheep with a GIN infection would initially be higher than for uninfected sheep and self-medication would then lead to a reduction in faecal worm egg count (WEC). In response to the curative benefits of medication, the MFB intake of infected sheep was expected to decline as the animals ceased to experience positive post-ingestive consequences from eating the MFB (See Chapter 2, Section 2.4.3.1). When MFB intake of the infected sheep had declined to a level similar to the uninfected sheep it was then expected to remain at that level (Figure 6-1).

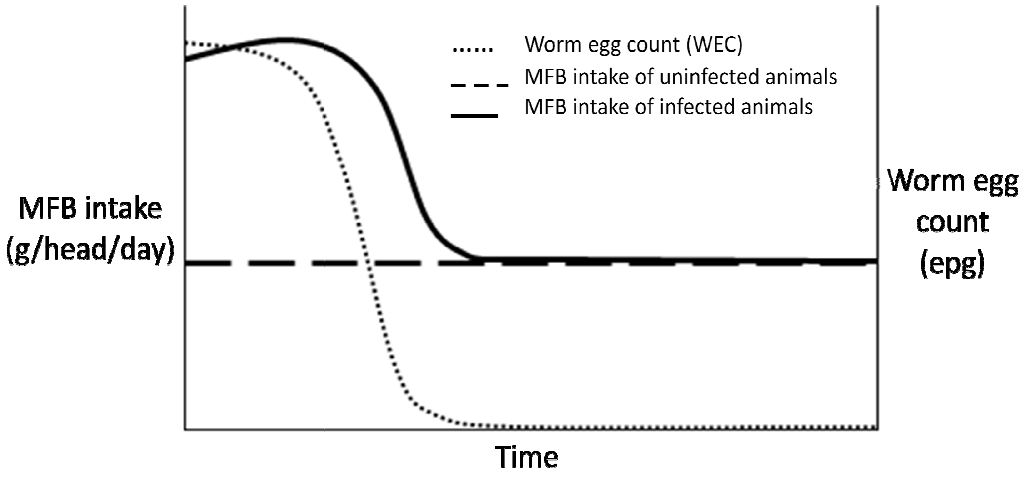

Figure 6-1: Diagrammatic representation of the hypothesized medicated feed block (MFB; containing an anthelmintic) consumption by sheep infected or uninfected with anthelmintic susceptible gastrointestinal nematodes and associated change in faecal worm egg count if the pathophysiology of GIN infection provided the stimulus for MFB consumption. Note: assumption in this figure is that animals are already familiar with feed blocks.

In support of the hypothesis, infected sheep did initially consume the MFB (30 g/day; 45mg fenbendazole) at a higher daily rate than uninfected sheep (22 g/day) (see Chapter 4) and this declined in association with a reduction in WEC, presumably because of the therapeutic effect of the MFB against sensitive GIN. Unfortunately the frequency of blood (every 48 h) and faecal sampling (weekly) was insufficient to record the precise timing in the reduction in MFB intake and WEC.

In addition to effects on daily MFB intake, GIN infection reduced the number of non-eaters to zero with all animals consuming a therapeutic dose. This confirms the assertion that infected animals are more likely to eat the MFB and as a result get a therapeutic dose. It should be noted that those animals that did not eat the MFB during the initial phase of experiments reported in Chapter 4 were excluded from this experiment. This allowed a closer examination of the effects of GIN on MFB intake by animals known to have overcome phobia of the MFB. It is possible that in an unselected group of animals there may still be animals with a GIN infection that do not consume the MFB.

For voluntary TST to be achieved through self-medication, consumption of an MFB should be positively associated with the severity of GIN infection pathophysiology, so that like other approaches to TST only those animals that are affected by GIN are treated and a proportion of the mob is left untreated. This ensures that some GIN are unselected (from the latest treatment) for anthelmintic resistance and are deemed in refugia and effects of GIN on production are minimised. While the results presented in Chapter 4 demonstrate self-medication in response to GIN infection, there was no relationship between WEC (proxy of severity of GIN infection) and MFB consumption. It is likely that the frequency of WEC measurements was inadequate to identify a relationship or that none existed.

6.3.2 MFB intake with resistant GIN

The aim of the experiments reported in Chapter 5 was to confirm the relationship between GIN infection and MFB intake and to extend our understanding of the relationship by using different rates of GIN infection and classes of unselected sheep (i.e. lactating ewes and one year old wethers). The discovery of fenbendazole resistant GIN in these field experiments forced a re-evaluation of the initial hypothesis, because resistant GIN removed the positive post-ingestive consequence (i.e. curative therapy) of MFB. An alternate explanation developed with the support of results from the earlier experiment (i.e. Chapter 4) was that the pathophysiology of GIN infection stimulates the exploration by sheep for food resources (whilst remaining consistent with the typical GIN symptom of anorexia) but the curative benefits of MFB stimulate actual MFB

intake. The implication of this explanation is that the existence of resistant GIN, while not affecting exploratory effects of GIN infection, would remove the curative benefits of MFB consumption resulting in no differences in MFB intake as a result of GIN infection.

Indeed a greater proportion of the GIN infected ewes ate the MFB and there was no difference in the amount of MFB consumed between infected and uninfected ewes. In contrast to the ewes, infection with anthelmintic resistant GIN did not affect the proportion of wethers consuming the MFB, but as observed for the ewes, there was no difference in the amount of MFB consumed between infected and uninfected wethers. The lower WEC and lack of apparent symptoms of GIN infection in the wethers suggests the possibility that a threshold level of GIN infection (and associated pathophysiology) is required to elicit stimulatory effects in animals, observed as a reduction in MFB non-eaters.

A negative relationship between WEC and MFB intake was observed for both ewes and wethers. This means that while animals with a GIN infection were more likely to eat the MFB, those animals with a higher WEC (or most affected) were eating less of the MFB. This observation was not surprising. It is well known that GIN infection often causes depression in feed consumption (Parkins and Holmes 1989; van Houtert and Skyes 1996) and because of the fenbendazole-resistant GIN, infected ewes and wethers would not have experienced any positive consequences of consuming the MFB. However, without further investigation if cannot be determined whether infection with anthelmintic-susceptible GIN would have changed this outcome.

Regardless of resistance status of the GIN, ewes and wethers did not consume enough MFB to obtain a therapeutic dose for fenbendazole susceptible contemporary strains of GIN (discussed in Chapter 5). For example, albendazole controlled release devices release 23 mg/day to sheep in the weight range 20–40 kg to control sensitive GIN. MFB consumption provided daily amounts of 13 and 6 mg/day of fenbendazole for ewes and wethers respectively. Inclusion level of fenbendazole (in Chapter 5) was deliberately set to a low level (1 mg/g block) so that positive

benefits would only be achieved with higher rates of MFB intake. This allowed better discrimination in MFB intake between infected and uninfected sheep. As discussed, the lack of curative benefit probably limited MFB intake of infected sheep. However, if these levels of MFB intake were observed with sensitive GIN, then a therapeutic dose would be provided if fenbendazole concentration in the MFB was increased to 3-4 mg/g and further increases in fenbendazole concentration could be used to minimise the extent of sub-dosing.

6.4 Refugia

Using the results collected in the experiments described in this thesis it is possible to calculate the effects of MFB and self-medication on provision of anthelmintic susceptible GIN. It was assumed that all animals that consume the MFB receive a therapeutic dose. This would be achieved through using a higher concentration of anthelmintic in the MFB. For example, if 10% of the mob did not eat the medicated feed block (non-eaters), mean WEC were 600 epg and fenbendazole was 95% efficacious then the percentage of GIN escaping anthelmintic treatment would be 69%. Martin *et al.* (1981) calculated that refugia levels of 30% and above significantly slow the development of anthelmintic resistance compared to levels 10% and below, while refugia levels above 75% are more effective at slowing resistance development than 30%.

The effect of refugia in untreated animals is also dependent on the amount of infective GIN larvae on pasture. In situations where there are low populations of infective, anthelmintic-susceptible larvae on pasture initially, leaving 4% of adult stock untreated was predicted to delay anthelmintic resistance without affecting production (Dobson *et al.* 2011a). If there are higher numbers of anthelmintic-susceptible larvae on pasture the effect of leaving 10% of animals untreated may not delay resistance enough to justify the risk to production (Dobson *et al.* 2011a). Similarly, refugia in the host will have a greater effect on resistance development while anthelmintic efficacy is high. As resistance to an anthelmintic increases the number of GIN in refugia to substantially dilute the resistant genotype becomes too large and compromises control (Dobson *et al.* 2011a). Other factors such as mob size and timing of MFB use will also change

the effect that refugia in the host has on anthelmintic resistance (Gaba *et al.* 2006; Gaba *et al.* 2010). An anthelmintic product like the MFB would be most useful in slowing anthelmintic resistance in situations where there are low numbers of infective larvae on pasture, resistance to the anthelmintic remains low and the MFB is provided at periodic intervals.

6.5 Voluntary targeted selective treatment

Voluntary TST using self-medication relies on two factors. The first factor is that GIN infection increases exploratory activity and as a consequence more infected sheep consume the MFB. The second factor relates to daily MFB intake which relies on a positive post-ingestive consequence. In the presence of infection that is responsive to treatment, sheep are able to display both increased exploratory activity and greater daily intake and as GIN infection is reduced (by anthelmintic) the post-ingestive benefit also lessens and so increases in MFB intake would only be transient. In the presence of anthelmintic resistant GIN, the effects on phobia remain but the effects of positive consequence driving intake are gone.

This thesis provides evidence of both of these self-medication factors in sheep: increased exploratory activity and increased intake of MFB (when MFB was efficacious) in response to positive post-ingestive feedback. Whether or not animals displayed self-medication in these experiments also appeared to be dependent on severity of GIN infection. However, the question of whether MFB consumption between animals is positively related to infection and negatively to host resilience to infection could not be confirmed. For example, the negative relationship between WEC and MFB intake was only observed when sheep were infected with fenbendazole-resistant GIN and the sheep would not have experienced any positive post-ingestive feedback to stimulate consumption. More work is needed to define the above relationships and the suitability of using MFB to achieve voluntary TST.

Another outcome of self-medication is that MFB will only need to be used for short periods (weeks). The increased exploratory activity and MFB intake resulting from GIN infection and positive consequences of eating the MFB means animals with worm burdens are likely to

consume a therapeutic dose within a short period. For example, animals with a susceptible GIN (Chapter 4) consumed the MFB and lowered WEC to negligible levels within one week of access. Shorter periods of access are also preferable to leaving the MFB in the paddock over long periods (months) due to the negative effects of prolonged drug exposure on resistance.

There is potential for MFB to be used to allow animals to selectively treat themselves but key to making this a viable method of GIN control would be the use of an anthelmintic that was efficacious. Resistance to benzimidazole anthelmintics is extensive throughout Australia (90% of properties) (Love 2011). While this thesis was not designed to validate the efficacy of the MFB, but rather the concept of using MFB for voluntary TST, it has highlighted the importance of an effective anthelmintic for use in MFB if they are to be used commercially. This means an alternate anthelmintic to fenbendazole must be used. Fenbendazole was used in these experiments because of its marker properties and suitability for incorporation into a medicated block rather than its efficacy.

6.6 Future research

Fenbendazole has proven to be a useful and accurate marker of MFB intake in grazing sheep over a prolonged period and can be used to examine factors that influence supplement intake in general rather than just MFB, for example, nutritional status (body weight or body condition score) and mineral deficiency (a common justification for use of blocks). The method was recently considered for use with nitrogen blocks to help determine the effect of nitrogen supplementation on methane emission. Other compounds used in animal husbandry may also be useful as markers of intake outside their registered use and may be identified through their pharmacokinetics, metabolism and physical properties.

The experiments presented in the second part of this thesis (Chapter 4 and 5) provide strong evidence of grazing sheep self-medicating for GIN using MFB and pose a range of questions about self-medication in sheep. Villalba *et al.* (2012) suggests that controlled studies of self-medication should encompass four experimental phases. Phase 1, uninfected sheep are

familiarized with supplements and preferences/intake of supplements recorded. Phase 2, animals are parasitised and conditioned to experience the ameliorating effects of the medicinal supplement. Phase 3, preference/intake of supplement is measured in the parasitised animals and phase 4, the animals are treated for parasitism and preference/intake recorded again. The experiments in Chapter 4 did encompass these phases in general through measuring intake of the MFB in animals prior to infection (phase 1), infecting half the animals with GIN and recording MFB intake and WEC (phase 2 and 3) and continuing to record intake and WEC until WEC had declined to negligible levels (phase 4).

This four phase experimental system would be a useful framework for further experiments aimed at defining what the relationship is between MFB intake and severity of GIN infection pathophysiology (which was not confirmed in these experiments). Experimental components similar to those in the experiments in Chapter 5, such as faecal sampling for WEC at 48 h intervals and daily blood sampling, would allow the pattern of intake and changes in WEC to be followed and would also make it easier to identify the relationship between the two. An experiment with treatments of anthelmintic susceptible and anthelmintic resistant GIN infection as well as an uninfected treatment group could be used to examine the differences between intake of medicated and 'un-medicated' (because there would be no positive post-ingestive feedback for sheep infected with anthelmintic resistant GIN) feed blocks. This would help to clarify if the negative relationship between WEC and MFB intake is limited to animals with resistant GIN (i.e. non-medicated).

The effect of MFB use on refugia and anthelmintic resistance development was not directly considered in these experiments. More information on MFB intake dynamics and MFB use over the long term would help to address concerns about anthelmintic resistance. It is also important to determine how the resilience status of the animals affects the use of MFBs in relation to refugia and anthelmintic resistance. The factors that determine MFB intake other than self-medication such as nutritional status, physiological status or mineral deficiency were also not examined. While all animals within each experiment were of a similar sex, age, weight and

grazed on the same paddock it is possible that such factors influenced MFB intake in turn affecting results but this cannot be determined without further investigation.

6.7 Conclusion

As part of this thesis an accurate method to measure MFB consumption by grazing sheep using fenbendazole was developed. This method was successfully used to examine the effects of GIN infection on MFB intake in sheep. Self-medication using a MFB was demonstrated by sheep infected with anthelmintic susceptible GIN. In the final series of experiments the unexpected establishment of fenbendazole resistant GIN meant that intake of MFB was not curative. Nevertheless, GIN infection appeared to increase exploration for food, depending on severity of GIN infection. While a positive relationship between MFB consumption and severity of GIN infection pathophysiology was not confirmed, these results suggest that MFB may be a prospective tool for achieving voluntary TST.

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Appendix

Validating extraction and HPLC method for analysing fenbendazole and metabolites in plasma.

To determine the precision and accuracy of the extraction and high performance liquid chromatography (HPLC) methods used a number of preliminary experiments were conducted.

Materials and methods

Precision and accuracy of extraction method

1. Four plasma samples taken from sheep after they had received a single oral dose of 5 mg fenbendazole (FBZ)/kg liveweight were used (n=4). Each plasma sample was divided into three 1ml aliquots, each aliquot was then extracted for analysis on the HPLC as described in Chapter 3. The resulting 12 samples (see Table A-1) were analysed on the HPLC in a random order on the same day. The HPLC methods used are described in Chapter 3.

Table A-1: Number of extractions of each plasma sample.

Plasma sample	Extraction		
	1	2	3
1	x	x	x
2	x	x	x
3	x	x	x
4	x	x	x

2. Plasma samples from sheep that had not had a benzimidazole oral drench in the two weeks prior were used. FBZ, oxfendazole (OFZ) and FBZ-sulfone (SUL) standards were prepared by dissolving pure analytical standards in 100% methanol by mixing in a sonicator at 25˚C for 1 h. Three plasma samples were made up to 2, 4, 6 and 8 µg/ml methanol of FBZ, OFZ and SUL by adding the following quantity and concentration of standards to 0.9 ml of plasma:

- 8µg/ml = 0.1 ml of 40µg/ml
- 6µg/ml = 0.1 ml of 30µg/ml
- 4µg/ml = 0.1 ml of 20µg/ml
- 2µg/ml = 0.1 ml of 10µg/ml

Each of the 12 samples (see Table A-2) was extracted and analysed on the HPLC for FBZ, OFZ and SUL concentrations in a random order on the same day.

Table A-2: Concentration of fenbendazole (FBZ), oxfendazole (OFZ) and FBZ-sulfone (SUL) in each sample and the number of times each sample was extracted.

Concentration of FBZ, OFZ and SUL in sample (µg/ml)	Extraction		
	1	2	3
8	x	x	x
6	x	x	x
4	x	x	x
2	x	x	x

Precision and accuracy of HPLC method

Three plasma samples taken from sheep after they had received a single oral dose of 2.5, 5 or 10 mg FBZ/kg liveweight (n=3) were extracted for HPLC analysis. Each sample was then divided between three 4 ml vials with 250 µL glass inserts (n=3) making a total of nine samples. Then the set of nine samples were analysed together on the HPLC three times on three separate days (see Table A-3). The extraction and HPLC methods used are described in Chapter 3.

Table A-3: The number of vials each plasma sample was divided into and the days on which the samples were anaylsed using the high performance liquid chromatography (HPLC) method.

Plasma sample	Vials	Day		
		1	2	3
1	3	xxx	xxx	xxx
2	3	xxx	xxx	xxx
3	3	xxx	xxx	xxx
Total number of samples	9	9	9	9

Analysis

The accuracy and precision of FBZ and OFZ+SUL extraction were analysed separately.

The deviation from the expected percentage of FBZ and OFZ+SUL recovered was calculated for each sample using the following equation:

$$Deviation\ from\ expected\ recovery = 100 - \left(\frac{Concentration\ recovered}{Expected\ concentration} \times 100\right)$$

The mean and standard deviation for each sample was calculated and accuracy and precision were calculated using the following equations:

$$Accuracy = 100 - mean$$

$$Precision = \frac{standard\ deviation}{Accuracy} \times 100$$

Results

Extraction method:

Accuracy = 72.4 %

Precision = 8.8%

HPLC method

Accuracy = 96.2%

Precision = 3.9%

Repeatability for individual samples was 1.4%