Risk factor epidemiological studies of ivermectin resistant *Ostertagia circumcincta* on Western Australian sheep farms

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Abstract

This study was designed to investigate the farm level epidemiology of ivermectin resistant *Ostertagia circumcincta* on Western Australian sheep farms. The study involved a postal survey and the results of that survey were used to develop statistical models to identify risk factors associated with ivermectin resistance.

The survey was mailed to farmers in July 2001 who had conducted faecal egg count reduction tests on their properties in 1999 and 2000. The questionnaire contained questions about farm management practices, particularly those pertaining to worm control. Some mail and telephone follow-up was conducted.

The response rate to the survey was 54%. The period prevalence for ivermectin resistance in Western Australia 1999 – 2000 as defined in this study was 38% (95% CI 29%, 46%) and for the period 1999 – 2001 was 44% (95% CI 39%, 58%) as some farms were diagnosed with ivermectin resistance in 2001.

Two main effects models of anthelmintic resistance at the farm level were developed: a logistic regression model for risk factors for a farm having been diagnosed with ivermectin resistant *Ostertagia circumcincta* by 2000, and a Weibull parametric survival model studying the effective life defined as time to onset of resistance, for those farms using ivermectin.

The logistic regression model contained three main effects variables: selling 10% more sheep in 2000 than is the usual policy (OR = 4.00), farm purchased since 1975 (OR = 2.34), and number of winter flock anthelmintic treatments in the previous 5 years (OR = 1.04). A secondary logistic-regression model assessed risk factors for farms selling 10% more sheep than usual in 2000; these farmers appeared less committed to their sheep enterprises than other farmers.

The survival analysis model contained four main effects variables: winter drenching frequency, 0-2 vs. 3+ flock treatments in 5 years (RH 0.52); availability of alternative effective anthelmintic classes on the farm (RH 0.30); always using safe pastures (RH 0.23); and veterinarians as the primary source of worm control advice (RH 0.58).

A major outcome of the study has been to identify that the farmer’s management of worm control in the sheep flock has an important influence on whether or not the farm develops anthelmintic (ivermectin) resistance.

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This thesis has seen the coalescence of two interests of mine: my long-standing, active veterinary interest in matters ovine and of anthelmintic resistance, and the other, latent interest in matters statistical and epidemiological. To bring these interests together was the result of the desire of my wife, Elizabeth, to return to Western Australia to raise our family, from Victoria where I had been pursuing the former interest for many years. To have enabled the coalescence of these two interests of mine I am indebted to her drive. To my seniors at Murdoch University in Perth I also owe a great deal, for participation in a program of part time post-graduate study at a foreign university whilst contracted to work solely as a veterinarian in their institution shows patience, tolerance, and understanding. In particular I must mention the (former) Dean of Veterinary Science, Professor John Yovich, the Head of the School of Veterinary Clinical Studies, Dr. John Bolton, the leader of the Production Animal group at Murdoch University, Associate Professor Helen Chapman, and the Chairman of the Murdoch University Veterinary Clinic and Hospital Committee and my direct ‘boss’, Dr. David Fraser, who acquiesced to my embarkation upon this course of study. I trust that their faith in me is repaid into the future.

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This study was granted Human Ethics Approval by the Ethics Committee of Murdoch University, Permit Number 2001/190.

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Chapter 1

INTRODUCTION

The macrocyclic lactone class of anthelmintics would appear to be the most recent of the modern broad-spectrum anthelmintics to be available for use by sheep producers in the world (Geary *et al*, 1999). The prototypical member of this class, ivermectin, was released in Australia in late 1987, about 6 years after the release in other sheep raising regions of the world. During the 1990's two other members of the class have been released in Australia for use in sheep, moxidectin in 1994, and abamectin late in the decade.

In two sheep raising areas of the world, northeast South Africa and Western Australia, ivermectin resistance in the gastrointestinal parasites of sheep has arisen quickly after its release, and the prevalence has reached alarming proportions in a short period of time (Palmer *et al*, 2001; Van Wyk, 2001). In the three provinces in northeast of South Africa the resistant species has been *Haemonchus contortus,* whilst in the intensive sheep production region of southwest Western Australia the species concerned is *Ostertagia (Teladorsagia) circumcincta*.

The first report of a field case of resistance in Western Australia occurred within 4 years of the anthelmintic becoming available (Swan *et al*., 1994). By the turn of the century, 12 years after the release of ivermectin, the farm-level prevalence of resistant *O. circumcincta* was reported as being 38% (Palmer *et al*, 2001). For the two predecessor classes of anthelmintics it took much longer to reach this level of prevalence in Western Australia. In a survey in the early 1980's Edwards *et al* (1986a) reported *O. circumcincta* resistant to the benzimidazoles were diagnosed on 41% of farms, over 20 years since the release of thiabendazole. From the same survey it was estimated that it had taken 18 years for resistance to the imidazothiazole class (e.g. levamisole) to reach a similar prevalence in this species.

Australian scientists, veterinarians and farmers have been very proactive in their approach to dealing with anthelmintic resistance. Throughout Australia control programs were extended to producers based upon scientific hypotheses or knowledge of the disease (Waller *et al*., 1995; Besier, 1997b). Despite the world-leading approaches that have been taken in Australia the rate at which ivermectin resistance appeared has stimulated a reappraisal of scientific knowledge and changes to the control programs used by farmers (Besier, 1997b; Van Wyk, 2001; Besier and Love, 2003).

A major shortcoming in our knowledge of anthelmintic resistance is the lack of epidemiological studies as to why some farms develop anthelmintic resistance sooner than others (Coles, 2001). There appears to have been only a few such studies conducted, that of Edwards *et al*. (1986b) in Western Australia in the early 1980s, and recently of Bartley *et al*. (2003) in Scotland and Ancheta *et al*. (2004) in the Philippines. The knowledge of anthelmintic resistance that we do have has been gained through laboratory and field experiments and computer simulation modelling.

The purpose of this study was to develop epidemiological models of ivermectin resistance in *O. circumcincta* in southwest Western Australian sheep farms at the farm level – in other words, to answer the question:

"Why have some farms developed ivermectin resistance whilst others are yet to do so?"

A questionnaire was developed and mailed to farmers, and the results of the questionnaire used to develop a model of risk factors and conduct a survival analysis.

Chapter 2: Literature Review

ANTHELMINTIC (IVERMECTIN) RESISTANCE IN GASTROINTESTINAL NEMATODES OF SHEEP

Introduction

At the end of the 20th century Barger (1997) made the following observation, based upon what had transpired during the past 100 years:

"The challenge for pest and disease control in the 21st century is essentially similar in the fields of parasitology, entomology, bacteriology and weed control, namely, how to use biocides in such a way that the rate at which they become ineffective through selection for resistance does not exceed the rate at which new active compounds can be developed ..." (page 204)

For weed control, new herbicides continue to be developed and farmers apparently have few concerns about the rapid emergence of herbicide resistance (Heap, 2002). This is not the case for anthelmintics where no new chemical classes have been commercialised since the discovery of ivermectin in 1975 (McKellar and Benchaoui, 1996). Prior to that time new anthelmintics had been released at approximately 5 yearly intervals, but due to the exceptional potency, safety margin and spectrum of activity of the macrocyclic lactones (e.g. ivermectin), and the relatively small size of the worldwide market for small ruminant anthelmintics (11% of the $3.4 billion world animal anthelmintic market), the drive to discover and commercialise new anthelmintics has been limited (McKellar and Benchaoui, 1996; Coles, 2001).

Anthelmintic resistance has been estimated to cost $4 per animal per year (Sangster and Dobson, 2002) and is projected to cost the Australian sheep industry $700 million by the year 2010 (Welsman, 2001). Some authors believe that anthelmintic resistance is inevitable under many parasite control systems (Leathwick *et al*, 2001; Geary *et al*, 1999). This is based partly upon the historical precedent where the release of a new class of anthelmintic has been invariably followed by the future discovery of parasites resistant to its action in some situations. It is also recognised that nematode parasites of sheep are genetically heterogeneous, with a diverse pool of allelotypes from which to select survivors of anthelmintic treatment (Sangster and Dobson, 2002; Silvestre and Humbert, 2002).

One of the earliest definitions of anthelmintic resistance was of there being a “greater frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species, and is heritable.” (Prichard *et al*, 1980). Shoop *et al* (1995) defined anthelmintic resistance as "a change in the gene frequency of a population, produced by drug selection, which renders the minimal effective dosage previously used to kill a defined portion (e.g. 95%) of the population no longer effective". Sangster and Dobson (2002) provide a definition that combines these two and differentiate between what can be measured pharmacologically (as in the previous two definitions) and clinically by field tests such as the faecal egg count reduction test (FECRT) which only detects resistance once 25% of alleles in the worm population are resistant (Martin *et al*., 1989).

By 1980 the potential extent of anthelmintic resistance was widely recognised, benzimidazole resistance was reported throughout the world, and levamisole resistance had also emerged. Several reviews published in that year promoted the use of integrated pest management programs to minimise anthelmintic use, utilize safe pastures and rotate between chemical classes (Brunston, 1980; Morley and Donald, 1980; Prichard *et al*, 1980). Prichard *et al* (1980) also raised the potential importance of “*refugia*”, refugia being a part of the life cycle where parasites are not exposed to the selective pressures of anthelmintic treatment and may hence dilute resistant parasites.

The relevance of the role of refugia in selection for anthelmintic resistance was not generally recognised until revisited by Van Wyk (2001) and others, as an explanation of the appearance of ivermectin resistance at a greater rate and higher prevalence in sheep producing areas of the world with hot, dry summers despite the reduction in treatment frequency since the 1980s. The predominant hypothesis of the cause of anthelmintic resistance until then had been that of treatment frequency, which had been addressed by the adoption of strategic drenching programs such as the double summer drench program in southern Australia.

In Western Australia, in particular, investigations have focused on the selection pressure associated with limited refugia for non-resistant worms (Besier, 1996; Besier, 1997a, Wroth, 1995), since the discovery of clinically ivermectin resistant *Ostertagia circumcincta* on a sheep farm at Esperance, only 4 years after the release of this anthelmintic onto the Australian market (Swan *et al*, 1994).

However, despite the opportunities to study adaptation by parasites presented by the recent development of anthelmintic resistance (Sangster and Dobson, 2002), few field epidemiological studies into causal relationships have been conducted (Edwards *et al*, 1986b; Coles, 2001). Surveys of resistance, commonly unstructured and based upon laboratory submissions, are the exception and continue to be reported from throughout the world (Barger *pers comm*). Computer modeling is one facet of epidemiology utilized in this field of study as a means to test hypotheses. Barger (1997) describes 6 different models used by groups around the world, with the WormWorld model (Barnes and Dobson, 1990) and the AgResearch model (Leathwick *et al*, 1995) being two commonly used models.

The anthelmintics

To review the history of the development of anthelmintic resistance, the time at which particular anthelmintics first became available for use by sheep owners and a brief discussion of their pharmacology, is relevant. Phenothiazine, the first anthelmintic to show reasonable efficacy against gastrointestinal nematodes of sheep was first used in the 1930's, with one report of a 27% reduction in *Trichostrongylus* species after a dose of 300 mg/kg (Forsyth, 1962). In 1955 micronised phenothiazine was introduced to improve the efficacy, with at least 90% of the particles below 10 micron in size (Hebden and Setchel, 1962). Unfortunately this reduced the safety margin of the anthelmintic, and toxicity problems became an issue (Hebden and Setchel, 1962).

Thiabendazole, the prototypical benzimidazole and the first of the modern anthelmintics, was released onto the Australian market in 1961. The exceptional efficacy and safety made it the drug of choice over phenothiazine. Dunsmore (1962) showed that thiabendazole produced a 99% and 97% reduction against immature and mature *Ostertagia* spp. respectively, while ultrafine micronised phenothiazine approached these efficacies only for adult *Ostertagia* spp. Levamisole was released in Australia in 1967, and apart from the brief period when morantel was available, it is the only example of the imidiazothiazole class of anthelmintics used.

Ivermectin, the fermentation by-product of the saprophytic soil fungus *Streptomyces avermitilis* (McKellar and Benchaoui, 1996), is the prototype member of the macrocyclic lactone class of anthelmintics. Discovered in 1977, it was generally released in 1981, although the release was delayed until late 1987 in Australia.

Moxidectin was released in the early 1990's in Australia, although it had been in use as an insecticide in cropping applications in the early 1970's.

Anthelmintics are classified into broad groups both by basic chemical structure and having the same mode of action or biochemical target site in target (and non-target) species. Thus all of the benzimidazoles bind to ß-tubulin, and the imidiazothiazoles bind at the acetylcholine receptor and mimic the action of this nerve transmitter (Sangster and Dobson, 2002). The macrocyclic lactones all share a base of a 16-member lactone ring, but are split into two groups based on chemical differences, the milbemycins (such as moxidectin) and the avermectins (such as ivermectin and abamectin) (McKellar and Benchaoui, 1996). They are believed to act at glutamate gated chloride channels (Sangster and Dobson, 2002), inhibiting nervous impulses. Mammals (as well as cestodes and trematodes) lack the appropriate receptors, explaining the superior margin of safety of the macrocyclic lactones in mammalian hosts, where they exhibit exquisitely potent activity against nematodes and arthropods (McKellar and Benchaoui, 1996). In general, the mechanism of action of anthelmintics was not discovered for many years after commercial release of the first examples; for example, the accepted mode of action of the macrocyclic lactones was only recently modified from the originally perceived action at GABA receptors (McKellar and Benchaoui, 1996). Chemical substitution at various points on the parent molecule confers different pharmacokinetic characteristics, potency and some variation in the spectrum of activity between anthelmintics within the same group. By sharing a common target site, anthelmintics within one group will share cross-resistance once resistance has evolved in a parasite population.

Different members of anthelmintic groups have been released sequentially into the market place, often commercially targeted at a perceived weakness in the activity of predecessors. For example, moxidectin does not appear to adversely affect dung beetles that break down cattle dung, and can claim minimal meat residues compared to ivermectin; both attributes were used for a marketing advantage when released in Australia (McKellar and Benchaoui, 1996). Pharmaceutical companies also release their patented chemicals in different formulations for different target species, or in stable compositions of multiple drug combinations. Both ivermectin and albendazole have been released as the active ingredient in slow release capsules, which reside in the sheep's rumen and release a steady, low dose of active ingredient for 100 days. Ivermectin has now been released as an external parasiticide to control sheep lice (*Bovicola ovis*) and blowflies (principally *Lucilia cuprina*). Moxidectin is available for use in sheep as an oral drench, and as an injection to be given in conjunction with clostridial vaccines.

All modern anthelmintics mentioned above are "broad-spectrum", in that they control most commercially important sheep nematodes as well as many minor species, and often have applications in other fields (for example, the macrocyclic lactones are used to control the microfilarial stages of filarial worms in various host species, including humans and dogs) (McKellar and Benchaoui, 1996). They typically exhibit remarkable efficacy against non-resistant populations, frequently better than 99.9% reduction in the naïve (non-exposed) worm population.

Some anthelmintics have more narrow spectrums of activity: triclabendazole is efficacious only against liver fluke (*Fasciola hepatica*), and closantel (a salicylanilide), is a persistent anthelmintic that offers good control only of blood sucking gastrointestinal parasites such as Barber's Pole worm (*H. contortus*), *Bunostomum*, *Gaigeria* and *Fasciola* spp. Naphthalophos, a narrow spectrum organophosphate anthelmintic with a low margin of safety, has good activity against *H. contortus*, and moderate activity against *Ostertagia* and *Trichostrongylus* species. More effective control of these species can often be accomplished by using combinations of naphthalophos with either or both a benzimidazole and levamisole (Cooper, 1995). Recently it has been demonstrated that the macrocyclic lactones commercially available in Australia can be mixed with napthalophos to make an anthelmintic drug combination. The use of multi-drug combinations often provides effective control of nematode parasites on properties where worms are resistant to the individual component anthelmintics, although eventually the parasites may develop resistance to the combination (Anderson *et al*., 1988, Anderson *et al*., 1991). The effectiveness of the drug combination is usually due to an additive effect, rather than synergism (Anderson *et al*., 1991). The first example of the use of commercially prepared drug combinations was the release of a combination of oxfendazole and levamisole in the mid-1980's, to combat the rising prevalence of resistance to both of these anthelmintics on Australian farms. More recently (2003), ivermectin was released in a multi-drug combination with albendazole and levamisole (Wroth, 2003).

The History of Anthelmintic Resistance

Several authors (Prichard, 1990, Hennessy, 1997, Sangster, 1999) have reviewed the prevalence of anthelmintic resistance in sheep nematodes throughout the world. This review considers the development of resistance in each class of anthelmintic, in particular from the perspective of sheep nematodes in Western Australia.

In the following discussion of the development of anthelmintic resistance the diagnosis of field cases is usually made on the basis of 'clinical resistance' as defined by Sangster and Dobson (2002). In these situations the field test indicates that treatment of sheep with the anthelmintic of interest reduces the resident worm population (usually measured by faecal egg count or total worm burden) by less than 90% or 95% dependant upon the definition of resistance used. This will be discussed in more depth when dealing with tests for resistance.

Phenothiazine

The first report of anthelmintic resistance, to phenothiazine, arose from a natural infection in the USA (Drudge *et al*, 1957a), and was later studied in an artificial infection of *Haemonchus contortus* (Drudge *et al*, 1957b). Pure infections of the resistant *H. contortus* required a four times higher dose to achieve comparable control to the non- resistant parasites.

There have been no reports of phenothiazine resistance in Australia, presumably because this anthelmintic was quickly superseded by the first of the benzimidazoles, thiabendazole (Le Jambre, 1978). Interestingly, Forsyth (1962) reported on the use of a mixture of phenothiazine and phenizole (2'phenylbenzimidazole) to obtain vastly superior efficacy. When either was used alone their efficacy against either *Ostertagia* or *Trichostrongylus* spp. was of the order of 35%, but the mixture removed greater than 99% of worms of both species. This is of historical interest, because the use of anthelmintic mixtures has been used since the 1980's to provide alternatives in the face of rising resistance when new drugs were not forthcoming.

Benzimidazoles

As the anthelmintic group that has been around for the longest, the benzimidazoles have been the most studied. The history of the development of benzimidazole resistance has been mirrored in subsequent chemical groups. Similarly, the issues of cross-resistance amongst members of the group have been debated along the same lines for the later released macrocyclic lactones, along with argument, frequently commercially motivated, regarding differences between members of each group. It has been long recognised that anthelmintic resistance to one member of the benzimidazole group confers cross-resistance to other members of the group.

Typically, the first reports of benzimidazole resistance arose in *H. contortus,* and on farms where this is the dominant worm species, as was the case for phenothiazine. Thus, the first report in Australia was in 1968 from the northern tablelands of NSW (Le Jambre, 1978), and in South Africa in 1975 (Van Wyk *et al*, 1997). Resistance in other species in these countries followed at a later date. Le Jambre (1978) cites the first recorded benzimidazole resistance in *Trichostrongylus* species on research properties in NSW in 1970, but it was not until 1979 that the first reports of resistance in *Ostertagia* species emerged (Prichard *et al*, 1980). In South Africa the first cases of resistance in *Ostertagia* spp. were discovered in 1983 (Van Wyk *et al*, 1997).

The number of recorded cases of benzimidazole resistance increased at a rapid rate after the first discoveries in any country. Edwards *et al* (1986a) surveyed farms in Western Australia in the early 1980's and found that 18%, 41% and 48% of farms had resistance in *H. contortus*, *Ostertagia,* and *Trichostrongylus* spp. respectively. On 17% of these farms, resistance was found to the two available chemical classes, levamisole and the benzimidazoles. Overend *et al* (1994) surveyed 881 of the larger properties throughout Australia in the early 1990's, finding resistance to benzimidazoles on 85%. There was a variation in prevalence between states. Palmer *et al* (1998) reported benzimidazole resistance in submissions from 99.7%, 89% and 23.5% of farms for *Ostertagia*, *Trichostrongylus*, and *H. contortus,* respectively in Western Australia. Van Wyk *et al* (1997) reports that 90% of farms in South Africa had resistance to at least one chemical group by 1990, and Barger (*pers comm*) cites benzimidazole resistance as occurring on 79% farms in that country by 2002. In other sheep rearing nations there was a longer delay before the first reports of anthelmintic resistance. For example, Himanos and Papadopoulous (1994) report the first cases of benzimidazole resistant *Ostertagia* and *Cooperia* spp. in sheep in Greece, in sheep imported from England. Scott *et al* (1991) reports very few cases in Scotland, with most of these traceable to imports from England. They cite no detected resistance in a survey in northern England in 1983/84, but by 1990 eight of 37 farms had benzimidazole resistant *Ostertagia* spp. Coles (1997) reports that the prevalence of benzimidazole resistance varied throughout the United Kingdom from 14% to 61%; by 2001 Bartley *et al* (2001) reported benzimidazole resistance on 60% of farms in Scotland. A survey of South American nations, summarised by Waller *et al* (1996), showed that the farm prevalence of benzimidazole resistance varied from 1.2% in Uraguay to 70% in Paraguay. In major sheep countries, the delay in finding the first case in a country appears not be due to the failure to look for it, as scientists have been keen to publish the first example, but is often due to the lack of structured drench resistance monitoring programs.

Levamisole

The first report of levamisole resistance in Australia was on a property where benzimidazoles, levamisole and morantel were used in a rotation (Le Jambre, 1978). This was in *Ostertagia*, before the realisation that cross-resistance occurred between levamisole and morantel. Prior to that conclusion there had been reports within the scientific literature of apparent control by morantel of levamisole resistant worms. The authors reporting on the prevalence of benzimidazole resistance have also reported on the situation in regard to levamisole. Edwards *et al* (1986a) found levamisole resistant *H. contortus on* 10% of Western Australian properties. For *Ostertagia*, *Trichostrongylus*, and *Nematodirus* spp., the figures were 41%, 24% and 10% respectively. Overend *et al* (1994) found levamisole resistance on 65% of farms surveyed throughout Australia. Palmer *et al* (1998) reported levamisole resistance on Western Australian farms of 94% for *Ostertagia* spp., 96% for *Trichostrongylus* spp. and 11% for *H. contortus*.

Overseas, Van Wyk *et al* (1997) reported that the first case of levamisole resistant *Haemonchus* in South Africa was identified in 1989; by 2000 the farm prevalence had risen to 23% (Barger, *pers comm.*). Southworth *et al* (1996) reported that 2 out of 3 farms in New Zealand had levamisole resistance in a survey conducted between 1990 and 1993.

Ivermectin

The first report of ivermectin resistance in South Africa was in *H. contortus* in 1986, within 5 years of its commercial release in that nation (Gill and Lacey, 1998). The first reported case of resistance in *Ostertagia* spp. was at Esperance in Western Australia, within 4 years of its release (Swan *et al*, 1994). In this case ivermectin had been used 3-4 times per year, but produced a reduction only of 72% in a slaughter trial.

Overend *et al* (1994) found no evidence of ivermectin resistance in an Australia-wide survey conducted at the same time, but by 1996 Besier (1996) reported a further 3 cases in Western Australia, all in *Ostertagia* spp. Palmer *et al* (2000) reported ivermectin resistance in 38% of submissions from farms in Western Australia, diagnosed using a discriminating dose test - a half-dose of ivermectin in a FECRT. Barger (*pers comm*) cites the farm prevalence throughout the rest of Australia as 95% for *Ostertagia* spp. on Kangaroo Island and 20% in western Victoria, and for *H. contortus*, at 10% in south east Queensland and 60% in northern NSW. Hucker and Turner (2001) found a farm period prevalence of 4.6% in south-eastern Australia (the full dose of ivermectin was used in a FECRT) from laboratory submissions over 1998 to 2000. Using the discriminating (half dose) test, the prevalence was 28%. The first case of a moxidectin-resistant strain of sheep parasite in Australia was reported in a field case (Love *et al* 2003), in which resistance to both moxidectin and ivermectin was found in *H.*

contortus. In this case, FECRT reductions were 55% for ivermectin, and 86% and 98%, respectively, for the half and full doses of moxidectin.

There had been considerable discussion regarding the potential for cross-resistance between the first two of the commercial macrocyclic lactone products. Kieran (1994) listed the known cases of ivermectin resistance in Australia, New Zealand, the United Kingdom and USA, and commented that many of these isolates came from properties where goats were raised. He reported that moxidectin at the recommended dose controlled all of these isolates, and postulated that this may be due to its increased potency. One *H. contortus* population in sheep derived from goats in the UK had been reported by Pankavich *et al* (1992), where ivermectin had an efficacy of 57% and moxidectin 100%. Pomroy and Whelan (1993) reported a similar finding for *Ostertagia* spp. derived from goats in New Zealand.

Shoop *et al* (1993), working in the USA, reported a case of both ivermectin and moxidectin resistance in *Trichostrongylus* and Ostertagia spp., to support the contention that cross-resistance does occur. Bridi *et al* (1997) reported a strain of *H. contortus* from Brazil for which the three macrocyclic lactone products tested (i.e., abamectin, ivermectin and moxidectin), whether given orally or by injection, failed to give acceptable efficacy. In New Zealand, Leathwick *et al* (2000) reported two cases of ivermectin resistance that had arisen without a history of cross infection between sheep and goats. They raised the issue of a tier of potency for the three macrocyclic lactones tested where ivermectin gave 42% reduction in a slaughter trial (18% in FECRT), abamectin 96% and 92%, and moxidectin >99% and 100% respectively. This same order of potency has been confirmed by Wooster *et al* (2001). It is now accepted that cross-resistance does occur amongst the macrocyclic lactones, but that due to varying potencies of the anthelmintics within the class, more active members (e.g. moxidectin) may remain effective for a period after diagnosis of resistance to a less potent one (e.g. ivermectin).

Elsewhere in the world, Sargison *et al* (2001) reported the first case of ivermectin resistance in the UK, and Gopal *et al* (1999a) reported the first isolation of a strain of *Trichostrongylus* resistant to ivermectin, which had come from a goat property in New Zealand.

Multiple-drug resistant parasites

Of major concern is the emergence of strains of parasites resistant to multiple anthelmintic groups. Van Wyk and Malan (1988) reported one such case in South Africa in *H. contortus* where ivermectin, the benzimidazoles, closantel and rafoxinide were ineffective. In the survey of Overend *et al* (1994), 34% of farms showed resistance to a combination benzimidazole – levamisole combination, and only 9% of the 881 farms surveyed did not show resistance to the 4 anthelmintics tested. As benzimidazole resistant parasites were present on 85% of farms, and levamisole resistant on 65%, parasites resistant to both anthelmintics are likely to have occurred on a large percentage of them (probably more than the 34% reported above). This occurred on the Esperance property on which the first known case of ivermectin resistance in *Ostertagia* spp. developed, leading the farmer to rely solely on ivermectin for the next 4 years (Swan *et al*, 1994). Interestingly, this farmer participated in the survey of this Master's thesis and has continued to achieve good worm control by using either moxidectin or a naphthalophos, benzimidazole and levamisole combination (B. Besier, *pers comm*). Love *et al* (2003) report a strain of *H. contortus* from a property in northern New South Wales that is resistant to all three available macrocyclic lactones, benzimidazoles, closantel and naphthalophos; fortunately levamisole and benzimidazole, levamisole combinations are still effective alternatives. Varady *et al* (1993) report the finding of a multi-resistant *Ostertagia* population in goats in Czheckoslavakia that had been imported from England. This strain was resistant to benzimidazoles, levamisole and ivermectin. Van Wyk *et al* (1997) reported a second severely resistant strain of *H. contortus* in South Africa, in this case resistant to 4 of the 5 anthelmintics available for use at the time (1990). By 1999 a survey of 52 South African properties from 3 provinces showed that 16% of farms showed *H. contortus* resistant to three of the four anthelmintics tested, with half of these resistant to all four (Van Wyk *et al*, 1999). This was described as perhaps the highest prevalence of multi-drug resistant parasites recorded anywhere in the world.

Summary of the history

With no new anthelmintics on the horizon (Geary *et al*, 1999) the emergence of 'super worms' resistant to multiple anthelmintic groups is of grave concern. What is also apparent from the review of the history of the development of anthelmintic resistance is that resistance usually occurs firstly in *H. contortus* and then shortly afterwards in *Ostertagia* spp. There has been a growing appreciation that goat farms are likely to develop anthelmintic resistance more rapidly than sheep farms; the cross grazing of these species can allow resistant worms to emerge in sheep. The first reports of resistance have appeared in Australia or South Africa, well ahead of the rest of the world. For example the first case of ivermectin resistance in the UK was reported when resistance was present on nearly 40% of farms in Western Australia, although farmers in the UK had access to this anthelmintic for 5 years longer than their WA counterparts. This may reflect the variation in selection pressures in the different environments. It is also apparent that for each new anthelmintic class the first case of resistance within a country is reported sooner and prevalence of resistance rises to a certain level more rapidly.

Biocide resistance in other pests of human endeavour

It is worthwhile to briefly review biocide resistance in other pest species of human endeavour. As discussed by Barger (1997) in the opening quotation of this review, the challenge posed by emerging resistance to biocides across the range of human endeavours is that of slowing the rate of the emergence of resistance to less than the rate at which new biocides can be developed.

Anthelmintic resistance of sheep nematodes has been one field in which resistance has emerged faster than in other areas of parasitology. The speed at which this has emerged in recent years has been described as giving scientists a chance to study evolution in progress (Sangster and Dobson, 2002). Hence, parasitologists in other disciplines look to the science of sheep parasitologists when grappling with emerging resistance in their parasite species(Coles, 2001). Human parasitologists review the work of their colleagues studying sheep when devising programs to control emerging resistance, and their programs mirror those recommended by sheep parasitologists (Geerts and Gryseels, 2001). In a similar manner, sheep parasitologists have looked to other fields, principally entomology, to gain insights that may assist their resistance control activities. Prichard *et al*. (1980) referred to the computer modelling work of entomologists Georghiou and Taylor (1977a; 1977b) in making their initial recommendations for the control of anthelmintic resistance in sheep. Coles and Roush (1992), the latter an entomologist, also turned to entomology for insights in the early 1990s, and again a decade later (Coles, 2002a, 2002b).

With that background of looking across disciplines for insights, a brief review, but by no means exhaustive, of biocide resistance in other pests is warranted. More attention will be spent on herbicide resistant annual ryegrass (*Lolium rigidum* Gaud), the world's worst resistant weed. That resistance is focussed in WA, where *L. rigidum* is a severe pest of cereal crop farming, whilst also being the main fodder plant for sheep grazing.

Cattle gastrointestinal parasites

In cattle, the parasite *Cooperia oncophora* appears to be the dose-limiting parasite for the macrocyclic lactone anthelmintics, so cases of cooperiosis have emerged since these anthelmintics have become the standard for bovine worm control (Coles *et al*., 2001; Familton *et al*., 2001; Coles, 2002a). *Fasciola hepatica* resistant to a range of trematicides have been described in a range of host species, and emergence of resistance to the most efficacious product triclabendazole being of particular concern (Mitchell *et al*., 1998; Thomas *et al*., 2000; Coles and Stafford, 2001). Three chemicals have been widely used for the control of bovine trypanosomiasis over the past 40 years in Africa, and resistant biotypes are now emerging (Geerts *et al*., 2001). The recommended program to control the emergence of resistance in African cattle trypanosomes is a mirror of the principles espoused by sheep parasitologists.

Human Parasites

In human medicine some resistant parasites are emerging (Geerts and Gryseels, 2001). Those biotypes recognised are a mebendazole resistant *Nector americanus* from Mali, a pyrantel resistant *Ancylostoma duodenale* from Australia, a praziquantel resistant *Schistosoma mansoni* from the horn of Africa, and ivermectin resistant *Onchocerca volvulus* in West Africa. I will not enter the field of antibiotic resistant bacteria in human medicine.

Sheep ectoparasites

The other major area of sheep husbandry in which resistance has emerged is in the external parasites of sheep; the sheep blow fly *Lucilia cuprina* and the sheep louse *Bovicola* (*Damalinia*) *ovis*. Levot (1997) has reviewed the history of resistance in these pests, with some observations on the evolutionary processes at work. Seventy percent of isolates of *L. cuprina* had developed resistance to organochlorines by 1958. With the withdrawal of organochlorines from the market some reversion has occurred, to the extent that only 3% of populations studied showed resistance in 1997. The organophosphates supplanted the organochlorines for fly control – about 20% of field isolates were resistant in 1965, but resistant biotypes exceeded 98% by 1970. This was despite this biotype having a fitness disadvantage over non-resistant flies when organophosphates weren't used. Cyromazine, an insect growth regulator (IGR), was released in 1979, but as yet no resistant flies have been reported. Diflubenzuron, another IGR, has been released in the 1990s. Resistance to this chemical is easily induced *in vitro*, and is a cross-resistance with organophosphate resistance. Paradoxically, the sheep louse has not developed resistance to organophosphates despite having being used widely for both lice and fly control for over 30 years. Yet the synthetic pyrethroid lousicides, the first of which was released as a 'backliner' product in 1981, selected resistant lice rapidly, the first case being reported in 1985 (Levot, 1997).

Herbicide resistant annual ryegrass

The world herbicide market dwarfs the sheep anthelmintic market by the order of fifty times (Thrill, 2001; Coles, 2001), and new herbicides continue to be released at frequent intervals. It is this frequent discovery and release of new mode-of-action chemicals to control weeds that delays the adoption by farmers of integrated weed management programs designed to counter the emergence of herbicide resistance (Llewellyn, 2002). The website www.weed-science.com contains a continually updated list of the known biotypes of herbicide resistant weeds throughout the world. Since 1980 new reports have been made at a rate of about 9 per year worldwide, and 2 per year in Australia (Heap, 2002). By August 2002 there were 235 reported herbicide resistant biotypes in

47 countries (Heap and Le Baron, 2001). Yet the first of the herbicides released, the auxin inhibitors 2,4-D and MCPA released in 1947, were of a type to which few reports of resistance have emerged (Heap and Le Baron, 2001). Their success and longevity gave plant breeders the opportunity to breed higher yielding crops; that higher yield coming at the expense of plant competitiveness, as the newer breeds didn't grow high, dense swards (Heap and Le Baron, 2001).

Against that background, *Lolium rigidum* Gaud has attained the title of the world's most resistant weed, as the agricultural area affected by resistant biotypes exceeds 1 million hectares, and there are some biotypes resistant to chemicals from 8 different mode-of-action classes (Heap, 2002). The attributes of *L. rigidum* that have enabled it to achieve this ranking as the worst of weeds include its inherently high genetic variability and instability, its plasticity as a plant (the ability to colonise every available space in the pasture or crop), its large seed production, and its good survivability over summer so that 40-80% of the seed produced germinates the following autumn. At the same time, it was deliberately introduced over large areas of Australia in the early 1900s for sheep grazing into a climate with mild winters and hot dry summers that favours the survival of *L. rigidum*. Further, in the relatively infertile Australian soils, a plant that rapidly germinates in autumn into the open canopies of the 'non-existent' pasture at that time has an advantage, especially a species endowed with the plasticity of *L. rigidum*. Farmer's activities also aid its survival – to grow crops successfully in an environment with a short growing season, crops are sown at the season's break, at the same time that *L. rigidum* is germinating. They also rarely use weed free fallows, thus ensuring that there is a large seed bank to germinate in competition with their crops the following autumn (Gill, 1996).

The main factors contributing to the emergence of herbicide resistance are the use by farmers of simple crop rotations that favour a few dominant weeds; the tendency of multiple use of a single mode-of-action herbicide class; and the resistant weeds tend to exist at high densities, to be widely distributed, and to have high genetic variability (Thrill, 2001). Diggle and Neve (2001) used computer modelling to study the evolution of herbicide resistance, and listed six factors as being the most important. Uppermost was the intensity of selection; herbicides being intense selectors having an efficacy of 90-99% when released. At lower efficacies the weed population density remains too high, although the rate of selection for resistance is lower. Other factors of importance include the initial frequency of resistant allelotypes, the mode of inheritance of resistance (in general being of the single gene dominant or semi-dominant type), the relative fitness of resistant biotypes in the presence or absence of the herbicide, the rate of gene flow both within and between the treated populations of weeds, and the intrinsic population dynamics of the weed. In the latter factors, the existence of a persistent seed bank was seen as a buffer against selection for resistance, as some of the plants in later germinations may not have been selected for resistance by prior herbicide use.

The strategies proposed to control herbicide resistance reflect the proposed mechanisms of its development. Foremost is to rotate between mode-of-action chemical classes annually (i.e., with each successive crop). Computer modelling of this strategy has shown that the onset of resistance is delayed, but when it does occur it occurs to both chemical classes at about the same time, and quickly becomes fixed (Diggle and Neve, 2001). The use of mixtures of chemicals, or sequences such as the ‘double-knock’ is also highly recommended (Heap, 2002). The double-knock aims to achieve reduced weed densities, lowered seed production, and a reduction in seed-bank size, to expressly reduce the opportunity of resistant biotypes surviving into the next generation (Thrill, 2001). It does not necessarily imply the use of two chemical applications to achieve the double-knock; one of the non-chemical methods can be used. These non-chemical methods, or integrated weed management (IWM) strategies, include the use of crop rotations, cultivation (rather than the accepted no-till cropping), delaying sowing, stubble burning, using spray-topping/crop-topping/hay making, harvest seed capture methods, and the establishment of a highly competitive crop (Heap, 2002). The relative efficacies of some of these IWM to control *L. rigidum* in WA has been estimated, and are far lower than that achieved by effective herbicides (Stewart *pers comm*):

Table 2.1 The relative efficacy of Integrated Weed Management strategies for the control of *Lolium rigidum* in WA (Stewart *pers comm*)

IWM strategy	Efficacy (range)	IWM strategy	Efficacy (range)
Seed catching	60% (45-75)	Tickle cultivation	35% (15-55)
High seeding rate	40% (25-50)	Delay seeding, then knockdown	50% (35-70)
Grazing	70% (30-95)	Spray-topping	70% (50-90)
Crop-topping	75% (50-95)	Green manuring	98% (90-99)
Swathing	35% (15-50)	Stubble burning	40% (10-90)

Due to the poorer efficacies of these IWM strategies, the profits foregone using them, and the extra expense in terms of time, labour and equipment to implement them, farmers are reluctant to adopt them until herbicide resistance appears on their property, or whilst there are alternate mode-of-action herbicides available (Llewellyn, 2002). At the same time it has been observed that sheep farmers in WA who crop are more familiar with the concepts of biocide resistance and more readily adopt IWM strategies than their crop-only counterparts, probably from their exposure to the problems of anthelmintic resistant sheep parasites (Llewellyn, *pers comm*).

Many of the principles touched upon above will be dealt with in more detail in the forthcoming sections of this review, as anthelmintic resistance in sheep is dealt with specifically.

Hypotheses as to the cause of anthelmintic resistance

"Resistance is probably an inevitable consequence of the use of anthelmintics." (Dash *et al*, 1985). The Trichostrongylid family of nematodes exhibit a large genetic diversity (Geary *et al*, 1999); selection for resistance to anthelmintics, which are severe threats to survival of the species, is therefore a pre-adaptive state (Le Jambre, 1978). As a consequence:

> "The dilemma is (that) as a general rule the more effective an anthelmintic treatment program, the greater the potential for development of resistance" (Dash *et al*, 1985)

Drenching frequency

Prior to the release of thiabendazole in 1961 farmers relied on frequent treatment with inefficacious anthelmintics to control nematodiasis in their flocks. Thereafter, frequent treatment when worm challenge was high was the norm, and economic production gains with treatment intervals as little as two weekly were promoted to farmers (Dash *et al*, 1985; Edwards *et al*, 1986b). Prichard *et al*. (1980) first hypothesised that frequent anthelmintic treatment would cause anthelmintic resistance. This is now believed to be the major cause of resistance in the benzimidazole and imidazothiazole classes of anthelmintics, and against all anthelmintics in *Haemonchus contortus* (Besier and Love, 2003). Subsequent field experiments have demonstrated the selection pressure that is imposed by increasing the frequency of treatment (Martin *et al*, 1982; Barton, 1983; Martin *et al*, 1984; Wroth 1992, 1995), and there is support for this hypothesis in computer simulation modelling (Echevarria *et al*; 1993, Barger, 1997). It has been observed that increasing the frequency of treatment within the same environment increases the selection pressure, but that the frequency of treatment to exert the same pressure varies between environments (Barger, 1997; Leathwick *et al*, 2001).

Under-dosing

Prichard (*et al*, 1980) also proposed that the use of lower doses of anthelmintics was a cause of anthelmintic resistance. That farmers often underestimate the dose to use was demonstrated in WA (Besier and Hopkins, 1988), and the use of low doses of thiabendazole was demonstrated to select for resistance (Martin, 1989), although there is a lack of further field experiments directed at this hypothesis. Computer modelling has shown that under-dosing was equally selective as salvage treatment or preventative programs (Smith *et al*, 1999). The potential for this to be an issue in third world countries was highlighted by Waller (*et al*, 1996) who report that adulteration of proprietary mixtures was commonplace in South America.

Goats

That the first reports of anthelmintic resistance were often from goat derived worm populations led to speculation as to the differences between goats and sheep (Gopal *et al*, 1999b). It is believed that in goats anthelmintics have different pharmacokinetics than in sheep, particularly a shorter half-life, thus it is believed that goats are effectively under-dosed when treated at sheep does rates (Jackson, 1993). Unlike sheep, they develop a less effective immunity to worms so more frequent treatments are required, and this may be another reason that resistance appears in goats before sheep (Coles, 2002a). It has been shown that the prevalence (based upon laboratory submissions) of ivermectin resistance in *Ostertagia circumcincta* is higher in goat than sheep flocks in Victoria (Veale, 2002).

Rotation of anthelmintic classes

A rotation between anthelmintics has been proposed as a method to slow the development of anthelmintic resistance (Le Jambre 1978; Prichard *et al*., 1980), and though there has been some support for this proposal in Australian field trials (Donald *et al*., 1980; Waller *et al*., 1988; Wroth, 1992, 1995), this has never been validated scientifically (Coles, 2002b). Computer modelling has shown that rotation between anthelmintic classes delays the development of resistance for approximately the sum of the time it would take to develop in each individual class (Barger, 1997). The same overall life of two classes could be achieved by using each anthelmintic to exhaustion, as suggested by experimental work in WA (Wroth, 1992, 1995).

Use of anthelmintic combinations

The use of anthelmintic combinations (or mixtures) has been studied in computer models, which demonstrate that if the combination is used prior to any selection pressure having been exerted on the worm population by prior use of either of the anthelmintics in the mixture, this will substantially delay the development of resistance because genes conferring resistance to both drugs in any individual nematode are rare (Barger, 1997). The problem with this proposal in current times is that the drug combinations being used inevitably contain at least one anthelmintic to which resistance has begun to develop. However, the combination provides an alternative ‘anthelmintic’ to others being used at the time, and delays the development of resistance in the ‘protected’ class.

‘Head’ vs. ‘Tail’ selection

The differences in pharmacokinetics between anthelmintics within one chemical class and between different formulations has led to consideration of the relative effects of ‘head’ vs. ‘tail’ selection. Some anthelmintics are short acting (e.g. orally administered ivermectin, which has a half-life of about 25 hours and a plasma depletion curve typical of a single compartment distribution) and do not have an appreciable 'tail' to their plasma depletion curve (Shoop *et al*., 1996). These are examples of an anthelmintic that doesn't persist within the sheep's body; any selection for resistance comes from the worms that survive the short-lasting, high concentration phase immediately after treatment. This is termed 'head-selection'. Other more persistent anthelmintics do have a significant 'tail'. Moxidectin, the example most often studied, has a plasma depletion curve typical of a two-compartment distribution having both a 'head' with a half-life of a similar duration to ivermectin, and a 'tail' with a half-life of 15 days (Shoop *et al*, 1996). The concern with the 'tail' effect is the long exposure of nematodes to low doses of anthelmintic, which allow the accumulation only of parasites resistant to moxidectin (Abbott *et al*, 1995). Ivermectin and albendazole controlled release capsules fall into a similar situation, of long-term release of a low dose of anthelmintic, but without the 'head' effect (Barger, 1993, 1997).

Moxidectin is accepted as being more potent than ivermectin (Kieran, 1994), and therefore has a greater 'head' effect (Sutherland *et al*, 1999). As macrocyclic lactone resistance develops within a worm population the protective effect of the 'tail' of moxidectin to prevent the establishment of ingested L3 is lessened, and the period of protection shortens (Rolfe and Fitzgibbon, 1996; Sutherland *et al*, 1997a). Over the 'tail' period susceptible strains of parasite are eliminated, providing a reproductive advantage to resistant strains (Sutherland *et al*, 1997b; Le Jambre *et al*, 1999; Sutherland *et al* 2002).

Computer models show that the potency of the 'head' period is the most significant factor influencing selection for resistance (more potent anthelmintics will select at a slower rate, which is analogous to the under-dosing hypothesis), followed by the length of the ‘tail’, and then the degree of selection of incoming larvae (Dobson *et al*, 1996; Dobson *et al*, 2002).

Quarantine drenching

Quarantine drenching is the term describing the anthelmintic treatment of purchased sheep to remove any nematodes resident prior to their introduction onto a property. By this strategy any resistant nematodes will be prevented from entering the new property should they have already arisen on the property of origin. Several cases are cited in the literature where the occurrence of anthelmintic resistance, previously unknown or uncommon in one country, can be traced to the importation of sheep or goats from another country with pre-existing anthelmintic resistance (Scott *et al*., 1991; Varady *et al*., 1993; Himanos and Papadopoulos, 1994). A study of goat farms in France showed a positive correlation between the number of herds of origin and the prevalence of anthelmintic resistance (Silvestre *et al*, 2000). Contrasting that finding is the greater prevalence of anthelmintic resistance on the Greek islands when compared to the mainland where it is almost non-existent; this was explained by the relative isolation of the island flocks compared to the mainland flocks which intermingled and shared common grazing, allowing the admission of susceptible nematodes (Papadopoulos *et al*, 2001; Coles, 2002b). Running sheep flocks as a closed population had been described as phenotypic restriction, an important effect on the development of anthelmintic resistance (Jackson, 1993). In an allelotyping study of goat flocks in France that were assumed to be closed (no gene flow), the pre-existing presence of rare resistant allelotypes was found to be the main source of anthelmintic resistance, rather than the occurrence of recent mutations in the face of anthelmintic use (Silvestre and Humbert, 2002). The high prevalence of ivermectin resistance in sheep flocks on the small Kangaroo Island of the coast of South Australia would appear to support the hypothesis of lack of gene flow preventing the dilution of resistant genes (Rendell and Lehmann, 2001). Van Wyk and Van Schalkwyk (1990) introduced non-resistant *Haemonchus contortus* onto a sheep farm in South Africa in an attempt to dilute the resident resistant genotypes with some success, although there has not been widespread acceptance of this practice.

What appears uncertain is whether isolation of the nematodes of a flock by quarantine drenching is good or bad for the occurrence of resistance on that property. There are cases where flock isolation is suggested as the contributor to the high prevalence of resistance, through the failure to introduce a diversity of non-resistant genotypes. At the opposite end of the spectrum is the quarantine drench argument of preventing any genotypes (whether resistant or not) from entering the property, particularly those resistant genotypes if they are becoming prevalent in sheep parasites in the farming community.

'Refugia' hypothesis

In a direct lifecycle parasite such as sheep nematodes, a portion of the life cycle is not exposed to anthelmintic whenever the flock is treated. This portion is composed of the free-living stages, from the egg to the infective larval stage (L3); these stages are considered to be in 'refugia' from the anthelmintic treatment that is administered to remove resident parasites within the host sheep. The proportion of the population in refugia will vary throughout the year; in a Mediterranean environment there will be very few worms in refugia during summer and autumn, and a large proportion there at the end of winter and into spring. Thus the refugia hypothesis suggests that the development of anthelmintic resistance is inversely related to the number of worms in refugia at the time of treatment; if there are few (as with summer drenches) then the next generation of worms will largely come from the anthelmintic resistant survivors of that treatment (Van Wyk, 2001). An alternate way to leave some worms in refugia is not to treat the entire flock, so that unselected worms are able to contribute to the next generation.

This aspect of the selection pressure applied to the worm population was first considered in the 1980s (Le Jambre, 1978; Prichard *et al*, 1980; Martin, 1989), then overlooked as strategic treatment programs were utilised to overcome the concerns about the frequency of treatment. One laboratory experiment with *Haemonchus contortus* addressing this issue showed that the proportion of the worm population that needs to escape treatment has to be quite large to have any influence on the rate at which resistance emerges (Martin *et al*, 1981).

As the prevalence of ivermectin resistance in some areas of the world – notably in those regions with hot, dry summers - began to rise, attention refocussed on the role refugia played in the development of anthelmintic resistance (Besier, 1997a; Van Wyk, 2001; Coles, 2002b). It is now believed to be the main explanation for the rapid emergence of ivermectin resistance in *Ostertagia circumcincta* in WA (Besier and Love, 2003).

Various aspects of the role of refugia in the development of anthelmintic resistance have been studied in computer models. A study of the influence of season of treatment used to control *Haemonchus contortus* showed that treatment of ewes at the start of summer (low levels of refugia) was much more selective than at the end of summer (Echevarria *et al*, 1993). Breed of ewe and the degree of periparturient relaxation of immunity would influence the rate of development of resistance; in Romney ewes where no such relaxation is usual, there would be few worms in refugia on the pasture when compared to Merino ewes at that time (Leathwick *et al*, 1999), thus anthelmintic treatment of Romney ewes around the time of lambing would select for resistance to a greater extent than treatment of Merino ewes then. When studying the ‘tail effect’ of persistent anthelmintics versus non-persistent ones in a further computer model study, the size of the refugia was one of two factors found to be of importance (Leathwick *et al*, 1997). A subsequent model experiment showed that leaving 1-2% of the flock of sheep undrenched would slow the development of resistance by 2-3 times relative to an entire flock treatment in summer (Dobson *et al*., unpublished data, 2002).

One field experiment compared the selection pressure imposed by summer drenching to winter drenching in a Mediterranean environment, (Wroth, 1992, 1995). In this study, one or two summer drenches selected more rapidly than the same number administered in winter, although four winter treatments at monthly intervals selected for resistance more rapidly than summer drenches.

Summary of the hypotheses

Two hypotheses appear to have the most support from field and computer modelling experiments to explain the development of anthelmintic resistance: the drenching frequency hypothesis and the refugia hypothesis. The degree of influence of each of these factors varies according to the environment in which sheep are farmed (Barger, 1997). Other hypothetical factors appear to have less influence on the rate of development of anthelmintic resistance.

<u>Testing for anthelmintic resistance</u>

Defining anthelmintic resistance

One of the first definitions of anthelmintic resistance was of there being a:

"Greater frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species and is heritable." (Prichard *et al*., 1980). This definition recognises that a selection process has occurred, and that there is a genetic basis for it. Such changes in frequency of individuals in the population could be measured when dose response curves were produced when testing pure worm populations. When comparing the ED_{50} for unselected and resistant worms, a resistance factor (RF) can be calculated.

This definition was expanded by Shoop (*et al*., 1995) to:

"A change in the gene frequency of a population, produced by drug selection, which renders the minimal effective dosage previously used to kill a defined portion (eg 95%) of the population no longer effective"

Over the interim between these definitions, Presidente (1985) had reviewed the available tests to determine if anthelmintic resistance had occurred in mixed, field populations of worms, and had proposed that a definition of resistance, as measured by the faecal egg count reduction test (FECRT), was if less than 90% reduction in faecal egg count occurred after treatment with the test anthelmintic. Martin (*et al*., 1989) proposed a variation on this definition, subsequently adopted by Lyndall-Murphy (1993) of raising the cut-off to 95% reduction in faecal egg count, but also with the lower 95% confidence interval for the calculated percentage reduction being below 90%.

Such a definition has become standard when describing a worm population as having anthelmintic resistance. Sangster and Dobson (2002) describe this as a clinical definition, whereby anthelmintic treatment has failed to remove worms, even though a change in the worm population's resistance factor can occur without having clinical resistance. It is also recognised that the FECRT is a relatively insensitive test when asked to detect early shifts in the dose-response curve (or low RFs), so that a measured 90% reduction on FECRT means that the anthelmintic fails to remove approximately 50% of the original worm population resident within the host sheep (Martin *et al*., 1989).

Reviews summarising testing protocols

Several authors have given comprehensive reviews of the tests used to detect or measure anthelmintic resistance, and the reader is referred to these for further detail. Presidente (1985) reviews a number of the tests available for measuring benzimidazole and levamisole resistance, particularly the *in vitro* tests. Coles (*et al*., 1992) review all of the tests available at the time, and provide guidelines for their conduct. These guidelines were updated by Wood (*et al*., 1995), particularly the statistical calculations involved, and again, subsequently by Taylor (*et al*., 2002). Lyndall-Murphy (1993) provides the detail of the conduct of FECRTs, and those protocols are still followed today. Reviews such as McKellar and Benchaoui (1996) and Sangster and Dobson (2002) touch briefly on the various tests used and their advantages and disadvantages. Most tests suffer the disadvantage of poor sensitivity when anthelmintic resistance is emerging. Only the modern PCR-based tests overcome this burden (Geerts and Gryseels, 2001).

Faecal egg count reduction test

This test has become the mainstay of field investigations into anthelmintic resistance. Despite it being a cumbersome and laborious test to conduct, it offers the advantages of being able to test field populations against as many different anthelmintics and anthelmintic combinations as the scientist requires, without the sacrifice of sheep or elaborate equipment to conduct the test. To summarise the conduct of the test, sheep with a reasonable worm burden (as measured by faecal egg count) are randomly allocated to treatment groups of about 15 sheep. A control (untreated) group is included. Groups are then dosed with anthelmintic (‘drenched’), and then faecal samples are collected from treated and control animals 10 – 14 days later. Faecal egg counts are performed on these samples, a group arithmetic mean arrived at, and the relative reduction in faecal egg count compared to the control group calculated. Larval culture is recommended to ascertain if the resistant worms derive from one genus only (Lyndall-Murphy, 1993).

The inherent problems with this test are easily apparent (Palmer *et al*., 2000, Hucker and Turner, 2001, Palmer *et al*., 2001). Firstly, it relies on a good correlation between faecal egg output and total worm numbers. This correlation is only appropriately sufficient in young sheep that haven't yet developed immunity to worm infestations; and was estimated to be low (r^2 = 0.39-0.46) even in this class of sheep (Presidente, 1985). Secondly, faecal egg output should be constant throughout the day, and equal amongst worm genera. The anthelmintic treatment used should not suppress egg output without killing the resident worms; there has been a report that this is not the case with ivermectin (Pankavich, *et al*., 1992), or benzimidazoles and imidiazothiazoles (Taylor *et al*., 2002). The resistant worm genera should be present to a sufficient proportion of the mixed field population that the failure to remove them doesn't change faecal egg count sufficiently to be detected by the test. This problem has been largely overcome by the recommendation to perform larval culture on the faecal samples to look for relative reductions in one of the genera present (Lyndall-Murphy, 1993). A further refinement has been the use of discriminating doses of anthelmintic to detect an early shift in the dose-response curve, such as the half-dose of ivermectin or one-third dose of closantel (McKellar and Benchaoui, 1996, Palmer, *et al*., 2001, Love, *et al*., 2003).

Controlled slaughter trial

This is the "gold standard" test for anthelmintic resistance (Sangster and Dobson, 2002), but is limited by the expense, particularly as sheep have to be sacrificed and total worm counts performed. The trial is conducted in a similar manner to the FECRT, by random allocation to treatment groups. Instead of solely collecting faecal samples at the conclusion of the trial, the sheep are slaughtered. The test can be modified to use pure cultures of the suspect strain of worm compared to a reference strain of known sensitivity to the anthelmintic to derive resistance factors, and various doses of the anthelmintic can be used to derive dose response curves. The latter are known as either dose-titration or dose-confirmation trials, depending on the desired outcome (Wood, *et al*., 1995).

Many of the first reports of resistance of a particular worm species to an anthelmintic in a country include both a FECRT and a controlled slaughter trial (Swan *et al*., 1994, Pomroy and Whelan, 1993; Gopal *et al*., 1999a; Bairden *et al*., 2001; Vickers *et al*., 2001). As was recognised by Martin (*et al*., 1989) the FECRT overestimates the reduction in worm population when compared to the controlled slaughter trial in all of these examples.

***In vitro* tests**

By no means an exhaustive listing of the available (or formerly available) tests, these are the commonly used *in vitro* tests for anthelmintic resistance. The most studied anthelmintic class are the benzimidazoles, and the largest number of tests is available to measure resistance in this class. There are fewer tests for imidazothiazole or macrocyclic lactone resistance. PCR tests are becoming available for benzimidazole and macrocyclic lactone resistance. Taylor (*et al*, 2002) gives a review of many of the tests available.

Benzimidazole resistance. The egg hatch assay, in various forms, has been widely used to study hypotheses about anthelmintic resistance (Donald *et al*, 1980; Martin *et al*, 1982; Barton, 1983; Martin *et al*, 1984; Boorgsteede and Duyn, 1989; Martin, 1989,), and to assess field cases of resistance (Sutherland *et al*, 2000). The tubulin binding test has also been used to measure benzimidazole resistance, and compared with the FECRT and the larval development assay (Martin *et al*, 1989). Larval development assays have been developed; a commercialised test (the Drench-Rite ® assay) is available that assesses ivermectin, benzimidazole and levamisole resistance in the one procedure. A PCR for benzimidazole resistance has been developed (Elard *et al*, 1999).

Imidazothiazole resistance. Apart from the larval development assay, the egg hatch assay has been used for testing for resistance to this anthelmintic class (Dobson *et al*, 1996; Boorgsteede and Duyn, 1989; Hoekstra *et al*, 1997). A larval paralysis test has also been described (Taylor *et al*, 2002).

Macrocyclic lactone resistance. Allelotyping of the P-glycoprotein gene has been used to investigate macrocyclic lactone resistance (Blackhall *et al*, 1998; Xu *et al*, 1998).

Larval development assay (Drenchrite ®)

Separate mention of this test is warranted, as it is a commercially available test for resistance to benzimidazoles, levamisole and ivermectin. It offers the simplicity of the farmer just having to collect 200 g of sheep faeces, and the larvae are hatched in wells on the test plate with titrated amounts of the test anthelmintic, and thus the test became widely used after its release in 1995. The shortcomings of the test became apparent quickly. Firstly, the prevalence of benzimidazole and levamisole resistance on Australian farms was so high that it was uncommon for this test to indicate that a farmer could use either of these anthelmintic classes (Overend *et al*, 1992; Palmer *et al*, 1998). When field cases of ivermectin resistance became more common, it was found that the Drenchrite ® test was insensitive at detecting clinical resistance (Palmer *et al*, 1998; Maingi *et al*, 1998; Hucker and Turner, 2001). This test is now infrequently used in Australia, but is still used in other countries where the prevalence of resistance is lower (G. Hood *pers comm*).

Strategies to control anthelmintic resistance

The Australian economy had relied upon the performance of the sheep industry, in particular wool production, until the mid-1980s. It is not surprising, then, that Australian sheep parasitologists have lead the field in researching anthelmintic resistance and developing programs to slow the rate at which resistance develops. The vast production and animal health and welfare improvements bought by the use of the modern anthelmintics when compared to their much less effective predecessors would have prompted the drive to maintain these chemicals as effective weapons in the battle to control the effects of gastrointestinal parasites of sheep.

It was not until the 1990's that a study was conducted to quantify the effects that anthelmintic resistance would have on sheep production (Besier *et al*., 1995). In this study hogget Merino sheep were monitored for 12 months from after weaning through their first summer and into the second spring of their life. Three anthelmintics of different efficacies were compared (100%, 85% and 65%) and used for each treatment group. Five percent more deaths were recorded in the sheep treated with the least efficacious anthelmintic, and fifteen percent of that flock were considered unsaleable by the conclusion of the study. These sheep required a salvage anthelmintic treatment during their second winter to prevent further deaths. They produced 10% less wool than the sheep treated with the highly efficacious anthelmintic. Treatment with the intermediate anthelmintic resulted in an increase in scouring, and an insignificant reduction in sheep value and wool production.

Prichard (*et al*., 1980) reviewed the hypotheses relating to the control of anthelmintic resistance and proposed strategies to control the further development of this problem. These strategies became the basis of the programs extended to farmers throughout Australia.

Reducing treatment frequency

The general practice of farmers until then had been to treat sheep at frequent intervals throughout the winter period when exposure to worms was highest (in the winter rainfall areas of Australia). This had probably been a continuation of the practices necessitated prior to the release of thiabendazole when poorly efficacious anthelmintics were all that farmers had to use.

Based upon his studies of the epidemiology of sheep nematodes in western Victoria, Anderson (1973) is credited with proposing the use of 'summer drenches' to reduce the parasite population so that fewer winter treatments were needed. Gordon (1948, 1958) had made similar observations although they were not implemented due to the lack of efficacious anthelmintics to make the proposal work. Both recognised that a weakness in the epidemiology of sheep nematodes existed at the end of spring; the contributors to the following winter's worm population came from those that survived the summer within sheep. This lead to the proposal to strategically treat sheep in summer at a time when few worm problems occurred lessening the impact of nematodiasis the following winter and reducing treatment numbers. A field study demonstrated that this program would reduce the number of anthelmintic treatments needed without impacting upon sheep health or farm financial performance (Anderson *et al*., 1976). Three treatments given over two summers resulted in 6% deaths in the hoggets studied compared to no deaths in the sheep treated every 2 weeks, but the former was economically superior to the frequent treatment program. No difference in wool production was noted between these programs. Although this proposal had been promoted to Western Australian farmers, one third of those surveyed in the early 1980's did not perform summer drenches (Edwards *et al*., 1986b). Since then it has been widely adopted throughout southern Australia.

A similar strategy is to treat sheep and then move them to a pasture with few worm larvae, so-called ‘safe pastures’. These two uses of anthelmintics are referred to as ‘strategic drenches’ and share the characteristic of minimal larval uptake after the treatment (Barger, 1999). Sheep treated this way are not further contaminating the pasture with worm eggs, so that the interval to next treatment can be extended quite significantly because of no larval uptake. Safe pastures are defined as having not been grazed by worm producing sheep (periparturient ewes, and young sheep under 18 months of age). It has been estimated to take 3 months in summer and 6 months in winter to produce a safe pasture (Southcott and Barger, 1975), but it has been problematic to extend this concept to the farming population.

Rotation between anthelmintics

Le Jambre (1978) proposed that anthelmintics should be alternated or rotated between classes, rather than persisting with the use of one chemical. Prichard (*et al*., 1980) suggested that the interval for rotation between classes should be yearly. This strategy was based upon the idea that reversion from resistance to effectiveness would occur when the worm population was exposed to an alternate chemical, as had been demonstrated at the time with *Ostertagia* (Donald *et al*., 1980).

Several field experiments attempted to determine if rotation delayed resistance. A five-year field trial in southeast Australia studied alternation between classes at each treatment versus an annual rotation as well as comparing strategic treatments (3 times per year) to suppressive treatments (8 treatments annually). No difference in the rate at which anthelmintic resistance arose was found between these programs (Waller *et al*., 1989). Another experiment in Western Australia that studied rotation, along with other strategies, concluded that annual rotation did slow the rate of development of resistance (Wroth, 1992, 1995). This study was equivocal as to whether reversion occurred.

A study of one property over sixteen years reported that resistance to one anthelmintic class occurred after 7 years, and that after changing to an alternate some reversion did occur, only for resistance to rapidly re-emerge once the first anthelmintic was used again (Waller *et al*., 1988).

Computer simulation models suggest that rotation does not ultimately reduce the time to the development of resistance to both chemicals, but it is longer before resistance develops to either one, due to the periods when the anthelmintic is not being used (Smith, 1990; Barger, 1997). These modelling simulations do suggest that using the anthelmintics in combination will slow the rate at which resistance will develop, and that if such mixtures were available and used before any selection pressure had been applied then resistance would arise very slowly indeed (Barger, 1997).

State Agriculture Department extension programs

All sheep producing states in Australia developed worm - and anthelmintic resistance - control programs in the 1980's following the principles noted by Prichard (*et al*., 1980). These programs were given catchy names such as Wormkill (NSW) or Wormplan (Vic) (Dash *et al*., 1985; Waller *et al*., 1995; Besier, 1997b). They all shared common elements, such as:

- Testing to determine which anthelmintics work,
- Reduce treatment frequency by using strategic treatments integrated with grazing management, and faecal egg count monitoring to determine if supplementary treatments are needed,
- Annual rotation of chemicals
- Giving the correct dose by weighing sheep and calibrating the drench gun,
- Use narrow spectrum anthelmintics where possible, and
- Preventing the introduction of resistant worms (quarantine drenching).

It is believed that the simpler portions of these programs, such as using strategic drenches and ensuring the correct dose of anthelmintic, have been widely adopted by sheep producers (Waller *et al*., 1995; Besier, 1997b). The concern is that there has not been uniform adoption of the entire program, e.g. only 20% of producers have conducted anthelmintic resistance tests (Besier, 1997b). Such programs do not exist in other nations, and this deficiency may be putting those sheep industries in peril (Love and Coles, 2002), although they may not be achieving their stated aim in Australia.

Maximising refugia

The possible role of refugia in reducing selection pressure was recognised by Prichard (*et al*., 1980) and other workers at the time (Le Jambre, 1978). Martin (*et al*., 1981) tested this in a laboratory experiment and concluded that a large proportion of the population needed to escape treatment for refugia to have any influence on the rate at which resistance developed.

Several strategies for increasing refugia have been mooted including administering the 'summer' treatment earlier, before the end of spring so that some larval pick-up can occur after treatment, and leaving a proportion of the flock undrenched (Besier, 1997a). What is problematical is the size of the undrenched portion, with computer modelling experiments showing that 10% will result in clinical disease (Besier and Love, unpublished data). The same study compared the selectivity of individual macrocyclic lactones and when only 1-2% of the flock are left undrenched. The latter slowed resistance at a rate comparable to the intermediate potency abamectin (2-3x slower)), with moxidectin at a slower rate still (5x slower). This study showed that maximal reduction would occur if a potent macrocyclic lactone was used in combination with a benzimidazole and levamisole and the small portion of the flock was left undrenched

(25x-32x slower). There is currently a field trial underway in Western Australia to observe the result of introducing such changes (Woodgate *pers comm*).

Using Integrated Pest Management strategies

The Integrated Pest Management (IPM) strategies used in the control of internal parasites has been defined as 'non-chemotherapeutic alternatives and adjuncts' by Waller (1999), although the concept was initially proposed in the review of Prichard (*et al*., 1980). Such strategies are considered important due to the nexus between parasite control with chemotherapeutics and the development of resistance as has been identified by a number of authors (Dash *et al*., 1985; Besier, 1997a; Coles, 2002b):

"The objective, *to ensure effective worm control*, conflicts with the objective, *to minimise selection for .. resistance*, particularly when the former is based around the use of anthelmintics" (Dobson *et al*., 2002).

Initial IPM strategies involved various grazing management programs, particularly those of 'drench-and-move' to use of safe pastures produced by farm use by other than worm producing sheep. Such pastures can have been grazed by adult dry sheep or cattle (but not goats), or have been used for other farming activities such as cropping or laying fallow – the important point is that these alternative uses need to have been done for the required length of time. That such strategies minimise refugia has become a concern recently – do they actually hasten the selection for resistance (Barger, 1999)?

The breeding of sheep with an enhanced ability to cope with nematodiasis has been advanced during the 1990's. It is recognised that this takes two forms: host resistance, which is moderately heritable and is easily measured by faecal egg count, and resilience of sheep in the face of larval challenge, which is more problematical to test for, and has a lower heritability (Bisset *et al*., 2001). CSIRO has developed the NEMESIS program that is being used in Australia by sheep studs and producers and provides Estimated

Breeding Values to rank animals on their ability to reduce faecal egg counts in their progeny. Certain breeds of sheep, such as the Red Massai, are recognised as being more resilient, although they lack the production advantages of the Westernised breeds. Some effort is being made to identify genetic markers for these traits (Bisset *et al.*, 2001).

The other IPM strategy that is in field use is the South African “FAMACHA ©” scheme (Bath and Van Wyk, 2001). In this strategy only those sheep with clinical haemonchosis, as evidenced by anaemia of the mucous membranes, are treated at any one time.

There has been intense investigation into the use of nematophagous fungi, in particular *Duddingtonia flagrans* (Waller and Faedo, 1996). *D. flagrans* is but one of more than 100 such fungi studied for their effects upon the free-living stages of nematodes and field trials with this species have been conducted; it would appear that there are problems with delivery and establishment of this fungus in sheep pastures.

Interest has been focussed upon the use of nutrition as an IPM strategy, reviewed by Sykes and Coop (2001). The availability of amino acids to be partitioned for maintenance, production and immunity is seen as important – high quality protein diets will enhance resilience to worms. There is also some association between trace minerals and immunity, apart from the use of copper as an anthelmintic.

There are a number of other strategies that have been tried including:

- Reintroducing non-resistant worms onto a property (Van Wyk and Van Schalkwyk, 1990, Bird, *et al*., 2001),
- Development of a vaccine to immunise sheep, increasing either resilience or resistance,
- The grazing of plants that contain condensed tannins. These are assumed to work by increasing rumen outflow rates and ingesta flow rates through the

gastrointestinal tract, decreasing the time available for infective larvae to establish (Sykes and Coop, 2001), and

- The use of copper products, and a number of other alternative or naturopathic chemicals, as anthelmintics (Waller, 1999; Trengrove, 2001).

'Smart Grazing'

The 'Smart Grazing' program has been recently developed in Victoria to improve the effectiveness of the double summer strategic drenching program, which in the southern portions of the state is rarely as effective as experienced in the parts of Australia with true Mediterranean environments (Niven *et al*, 2002).

In this program a paddock is designated for grazing by weaner sheep the following winter, and is subject to the 'Smart Grazing' program in the preceding summer. The program consists of drenching wethers onto the designated paddock and stocking them at 5-10 times the usual stocking rate for one month after each of the two, regular summer treatments. During this time they bare the paddock of any pasture (and worm larvae), exposing those remaining larvae to the summer's heat and ultraviolet radiation. With the regular summer rainfall received in the region, the pasture will have regrown sufficiently to enable this stocking rate to be sustained at the second treatment. The paddock is left unstocked until weaners are introduced after the autumn rains have produced sufficient pasture biomass to sustain their winter grazing. Improved production in the weaners was reported when compared to those grazing a pasture with a routine summer drench treatment, and continuous grazing.

Whether such a program will increase the rate of development of anthelmintic resistance or not is uncertain. On the 'smart-grazed' paddocks there will be little refugia; on the paddocks onto which the wethers deposit the worm eggs harvested from the 'smart-grazed' ones there will be increased refugia. Or, perhaps, the farmers that are capable of implementing smart-grazing may also be able to implement other aspects of worm control that moderate any risk posed by this technique. It has been postulated that one of the reasons that ivermectin resistance is relatively uncommon in Victoria when compared to Western Australia is because of the extent of the refugia provided for nematode parasites in the less Mediterranean environment of Victoria (Besier, 1997a).

The genetics of anthelmintic resistance

The nature of the genes coding for anthelmintic resistance are predicted to have an influence on the rate at which resistance develops. Work with benzimidazoles and imidiazothiazoles suggested that anthelmintic resistance was polygenic (Jackson, 1993). Modelling studies show that the number of genes required to produce a resistant genotype has a profound effect on the rate at which resistance develops. If three genes are required to mutate resistance will develop to a particular point (e.g., 50% resistant alleles in the worm population) in 30 years, with 2 genes that point is reached in 12 years, and with one gene it is reached in 6 years (Barger, 1997).

The type of inheritance also influences the rate that resistance develops, with resistance coded by dominant genes developing fastest, by recessive genes the slowest, and by semi- or partially- dominant genes at an intermediate rate (Barger, 1997).

Where or how the resistant genes arise also has an influence. Such genes can be already present in the population at low frequency (pre-adaptive), occur by mutation (whether random or directed), or can enter a population by gene flow or migration (Bacquero and Blaquez, 1997; Silvestre and Humbert, 2002). Although the estimations of mutation rates in sheep nematodes are quite high (once every 20 days in *Haemonchus contortus*, every 200 days in *Ostertagia circumcincta* and every 1000 days in *Trichostrongylus colubriformis*) it is accepted that it is the pre-adaptive presence of resistance genes that is the operative process in the development of anthelmintic resistance (Jackson, 1993;

Sylvestre and Humbert, 2002). There is a large degree of genotypic variation in nematodes, these resistant genotypes occur with surprising frequency in unselected, susceptible populations. Studies of unselected *Haemonchus contortus* showed that the two benzimidazole resistant genes were present in 12% and 46% of the population, and ivermectin resistant genes in 10-20% of the population (Geerts and Gryseels, 2001). Finally, as anthelmintic resistance develops in the nematode population the predominant genotype changes from the naïve, susceptible population with rare heterozygotes, through the intermediate phase of mainly heterozygotes to the final phase where resistance has become fixed in the population due to the fact that the majority of worms are homozygote resistant (Jackson, 1993). If reversion to a susceptible, anthelmintic responsive population were to occur, it could only occur during the heterozygote phase; and there is little field evidence to support the notion of reversion (Waller *et al*, 1988; Boorgsteede and Duyn, 1989; Leathwick *et al*, 2001).

Estimations as to the mode of inheritance can be made with dose-response studies of crosses and subsequent generations using isolates of known resistance status (for an example of the method, refer to Le Jambre *et al*, 2000). Benzimidazole resistance in a variety of parasites has been shown to be a single mutation of tyrosine substitution for phenylalanine at amino acid 200 of isotype 1 ß-tubulin, preventing the binding of the anthelmintic to tubulin (Silvestre and Humbert, 2002). In *Haemonchus contortus* there are multiple genes coding for this change with an intermediate dominance, whilst in *Trichostrongylus* spp. the genes are incompletely recessive (Le Jambre *et al*, 2000; Sangster and Dobson, 2002). Levamisole resistance in *Haemonchus contortus* is a recessive, autosomal trait that probably requires more than one gene, and one isolate of *Trichostrongylus* inherited resistance by a single, sex-linked recessive gene (Le Jambre *et al*, 2000).

Macrocyclic lactone resistance is believed to be effected at two sites in the cell membrane, the glutamate-gated chloride channels and the P-glycoprotein efflux pumps; which of these sites is the main mechanism and whether the same gene codes for the effect at both sites is yet to be determined (Xu *et al*, 1998; Blackhall *et al*, 1998; Sangster and Dobson, 2002). What is known is that, in general, resistance to this class of anthelmintic is inherited in a dominant manner by a single autosomal gene, which is unprecedented in the field of anthelmintic resistance (Le Jambre *et al*, 2000). There is a body of work that suggests that the mode of inheritance varies not only with parasite species and anthelmintic within the macrocyclic lactone class, but also by the route of administration of the anthelmintic (Leathwick *et al*, 2001). It has been shown that the L3 of *Haemonchus contortus* inherit ivermectin resistance in a completely dominant, autosomal recessive manner; whereas in adults the trait is sex linked, inherited in a partially recessive manner; males having lower resistance possibly due to their smaller body mass (Le Jambre *et al*, 2000). A *Trichostrongylus* isolate from goats was shown to inherit ivermectin resistance in an incompletely dominant manner (Gopal *et al*, 1999b). When comparing the resistance mechanism of L3 of *Ostertagia circumcincta* with persistent macrocyclic lactones moxidectin resistance was inherited in a dominant manner, but the mode of resistance to ivermectin controlled release capsules was of a partially dominant / partially recessive type (Sutherland *et al*, 2003). There is some discrepancy over the mode of inheritance of ivermectin resistance in *Ostertagia circumcincta* with one isolate behaving in dominant manner with no sex linkage, and another in a manner suggestive of a recessive mode of inheritance; although both isolates inherited resistance to moxidectin in a dominant mode (Barnes *et al*, 2001; Leathwick and Sutherland, 2001).

The fact that macrocyclic lactone inheritance appears to be via a single gene locus in a dominant manner is the worst-case scenario. Resistance is predicted to evolve most rapidly to this class of anthelmintic due to these genetic factors (Barger, 1997). The consequences are that the use of increased dose rates to attempt to remove heterozygotes will be an ineffective tactic, and efforts should be undertaken to prevent the introduction of macrocyclic lactone resistant parasites by the use of quarantine drenching (Le Jambre *et al*, 2000).

<u>Epidemiological studies on anthelmintic resistance</u>

The epidemiological studies on anthelmintic resistance can be divided into three areas: studies of prevalence, computer modelling, and studies of associated causes.

Prevalence of anthelmintic resistance

A number of reviews of the prevalence of anthelmintic resistance throughout the sheep producing regions of the world have been published (Prichard, 1990; Coles, 2001; Sangster and Dobson, 2002). The majority of published reports of prevalence are based on the accumulation of results within the local region of authors and are then extrapolated to the nation as a whole. These reports either arise from more structured surveys, or from a summary of laboratory submissions.

One example of the former approach is the survey from the three northeast provinces of South Africa excluding the vast Karoo region where the majority of the country's sheep are raised (Van Wyk *et al*., 1999). Another is the group of papers reporting upon the prevalence in the South American countries of Brazil, Argentina Uruguay and Paraguay (as summarised by Waller *et al*., 1996). The sheep raising regions surveyed were in the area where these four countries meet, and ignoring the vast Patagonian region of southern Argentina, the central region of Brazil, and the entirety of Chile and Peru, areas in which sheep of different types to the study area are raised. The other concern of these reports is that they have been conducted in the warmer, humid regions where *Haemonchus contortus* predominates; extrapolation to other areas within the continent where *H. contortus* is not prominent may lead to erroneous conclusions.

Reports have also been based upon summaries of laboratory submissions in New Zealand (McKenna *et al*., 1995), Australia (Waller *et al*., 1995; Palmer *et al* 2000 and 2001; Hucker and Turner, 2001; Rendell and Lehmann, 2001; Love *et al*, 2003) and Great Britain (Scott *et al*., 1991). Such reports may suffer from one of a number of biases, including being from only the more progressive sheep producers, from farmers believing that anthelmintic resistance is a problem in their flock, from being dominated by the clientele of an enthusiastic advisor, or from a small sample size.

There is a need for random surveys of the prevalence of anthelmintic resistance in sheep producing regions of the world (Sangster and Dobson, 2002). A properly structured survey of the prevalence of anthelmintic resistance was conducted in the early 1980's in Western Australia, where the number of farms surveyed within each shire was stratified on the shire's share of the sheep population within the southwest region of the state (Edwards *et al*., 1986a). Several other reports approach the ideal of being truly random samples without actually meeting that ideal. A nationwide survey of 881 sheep producing properties in Australia was conducted in the early 1990's (Overend *et al*., 1994). The size of the sample makes this the largest survey reported in the world, but the survey was biased through sampling only properties with 5000 or more sheep, twice the national average flock size. No attempt was made to stratify the sample tested within each of the 5 states studied proportional to the state's share of the Australian sheep population. Sample size is also the feature of a study in Greece where 416 flocks were sampled, although no details are given as to the method of selection of study farms (Papadopoulos *et al*., 2001). The report of the prevalence of ivermectin resistance on 19 farms on South Australia's Kangaroo Island could be considered of greater validity than other surveys given the proportion of farms on the island sampled.

Apart from the study of Edwards (*et al*., 1986a) in Western Australia all of the other reports of the prevalence of anthelmintic resistance suffer from sampling errors such as biases in the method of selecting the farms sampled or failing to utilise a random, representative sampling method. Nevertheless, the approaches used to estimate the prevalence do alert the community to the presence of anthelmintic resistance within particular regions, though any attempt to estimate true prevalence from such studies must be done with care.

Computer simulation models

The potential of computer simulation modelling to study the development of anthelmintic resistance was revealed by Prichard (*et al*., 1980) drawing heavily upon the earlier work of entomologists (Georghiou and Taylor, 1977a and 1977b). Since then four computer models have been developed to study anthelmintic resistance in sheep nematodes, each based on the epidemiology of the parasites in the country in which the model was developed (Barger, 1997).

The first published was the model developed by the Universities of Glasgow and Strathclyde in Scotland (Gettinby *et al*., 1989). The models developed at the University of Pennsylvania in the USA, and by CSIRO in Australia were described the following year (Smith, 1990; Barnes and Dobson, 1990). The other model was developed at AgResearch in New Zealand (Leathwick *et al*., 1995). It is the latter two models that have been used most frequently in published work. The major difference between these two is the degree of loss of periparturient immunity estimated for the breed of sheep modelled (Barger, 1997). The AgResearch version is based on the Romney breed, which is modelled as having a smaller loss of periparturient immunity than the Merino as included in the CSIRO model.

These models have been used to estimate the relative rate at which anthelmintic resistant genotypes will develop when subjected to different treatment strategies. They have particularly been used to model the influence of the different pharmacokinetics and potency of the macrocyclic lactones, such as studying whether tail selection is more important than head selection (Dobson *et al*., 1996; Leathwick *et al*., 1997). Various treatment programs and grazing strategies have also been studied (Echevarria *et al*., 1993; Leathwick *et al*., 1995; Smith *et al*., 1999; Leathwick, 2001). Estimations of the influence of refugia on the outcomes have also been included in the modelling simulations (Leathwick *et al*., 1995; Dobson *et al*., 1996; Leathwick *et al*., 1997). The conclusions drawn from these studies have been referred to in previous sections of this review.

Studies of associated causes

There has been a paucity of studies to examine between farm differences into why some farms have developed anthelmintic resistance; this is a major deficiency in the understanding of this disease process (Coles, 2001).

In the process of the stratified random survey of anthelmintic resistance in Western Australia in the early 1980's farmers were surveyed by interview about sheep flock management and anthelmintic use patterns (Edwards *et al*., 1986b). This was the first study to associate farm practices with anthelmintic resistance and identify the significant ($P<0.10$) risk factors. This paper contains a listing of univariable analyses performed with Chi-squared goodness of fit tests for the combinations of benzimidazole or levamisole resistant in farm populations of *Ostertagia* or *Trichostrongylus* nematodes. Unfortunately multivariable techniques to develop main effects models of these risk factors were not used, nor were odds ratios calculated. Nevertheless, the authors summarised the key risk factors to be flock size, percentage of ewes, cattle numbers, type of sheep enterprise (wool only or a mixed wool - prime lamb farm), grazing strategy (set-stocking or rotational grazed), mixed grazing of sheep and cattle, and various factors related to treatment frequency (more than 3 treatments per year, or more than 2 treatments to ewes, or more than 1 summer drench). They concluded that anthelmintic treatment of ewes made the greatest contribution to the resistance status of the farm because they influence the size of the refugia, and any resistant parasites selected by treatment of the ewes are passed on to the lambs.

Bartley *et al*. (2003) sent a mail survey to farmer members of the Moredun Institute in Scotland containing a questionnaire about farm practices and an anaerobic faecal sampling jar. Faeces submitted by the responding farmers were tested by larval development assay and LD_{50} calculated. A generalised linear model was fitted relating LD_{50} to the answers to the questionnaire but no significant relationships were ascribed.

Ancheta *et al*. (2004) produced an analysis of variance model of benzimidazole resistance on sheep and goat farms in the Philippines using the results of LDAs conducted on each of the farms. Variables in the final model were flock size, FEC of the sample tested, recent introduction of stock from a nucleus herd, drenching frequency, years of use of benzimidazole anthelmintics, and access to common grazing. Only the last variable was protective, with farms having access to common grazing likely to have more efficacious benzimidazoles.

Several other surveys of the prevalence of anthelmintic resistance have drawn associations between sheep flock management practices and the occurrence of resistance, although no epidemiological analyses appear to have been performed on the results obtained. In the survey conducted in Argentina associations between drenching frequency and whether the sheep farm also grazes cattle were made (Eddi *et al*., 1996). Subsequent analysis by this author using Chi squared tests of independence suggested that farms that drench four or more times per year to control *H. contortus* in northern Argentina were 10 times more likely to have developed anthelmintic resistance than farms that treat less frequently, but that the association made between mixed sheep/cattle farms relative to sheep only farms and anthelmintic resistance was insignificant (P=0.2). The large survey of Australian farms in 1991/92 reported some associations, but no data were published to enable subsequent epidemiological analysis (Overend *et al*., 1994). This also applies to Greek survey from the late 1990's (Papadopoulos *et al*, 2001).

<u>Conclusions</u>

The rapid emergence of ivermectin resistance in *Ostertagia circumcincta* in Western Australia, and in *Haemonchus contortus* in South Africa has been of great concern to parasitologists. Other southern hemisphere sheep rearing areas are starting to find farms with ivermectin resistance, but as yet there are no reports from the northern hemisphere sheep rearing areas, even in goats. This has led to the resurrection of the refugia hypothesis, in that the environmental differences between these areas could influence the rate at which resistance occurs (Van Wyk, 2001).

In Western Australia the time from release of an anthelmintic until the prevalence of resistance to that class has reached approximately 40% has decreased with each new class of modern, broad-spectrum anthelmintic, from 24 years for the benzimidazoles, through 18 years for the imidiazothiazoles, to only 12 years for the macrocyclic lactones (Edwards *et al*, 1986a, Palmer *et al*, 2001). This rate of emergence is consistent with the predictions of the CSIRO 'WormWorld' model from studies comparing differences in the mode of inheritance of resistance (Barger, 1997). This model is based on the ecology of sheep parasites in the Australian environment. Benzimidazole resistance is known to be polygenic from at least two loci and the model predicts that clinical resistance will arise in approximately 30 years in this scenario (Sangster and Dobson, 2002). Levamisole resistance is recessive, and inherited at two loci, thus resistance was predicted to occur in more than 12 years. With ivermectin resistance having a single autosomal, dominant mode of inheritance this level of resistance is predicted to occur in 6 years (Barger, 1997; Barnes *et al*, 2001). Thus within a given environment the mode of inheritance can be used in computer models to estimate the rate at which anthelmintic resistance will occur. However, it is the ecological and environmental differences between environments (thus, the size of the refugia) that can explain the differences in the rate of emergence of anthelmintic resistance between countries (Besier, 1997a). The current hypotheses fail to explain the reasons why anthelmintic resistance emerges rapidly on some farms and much more slowly on other farms within the same environment. There has been a dearth of epidemiological studies into these between farm differences. The aim of this project was to begin to understand which factors might explain the more important differences between farms in their risk of developing ivermectin resistance and in the rate at which resistance develops in *Ostertagia circumcincta* in Western Australia (Coles, 2002b, Suter *et al*, in press, Suter *et al*, submitted).

Chapter 3

MATERIALS AND METHODS

Thesis Structure

This thesis contains two papers as published or submitted to a peer review journal, and these are presented as such in Chapters 4 and 5. Each chapter is structured as for a stand-alone scientific paper, with its own specific Abstract, Introduction, Materials and Methods, Results, Discussion and Reference List. Chapter 3 of the thesis deals with those aspects of the study's Material and Methods that are not developed fully in those papers, specifically the design and use of the questionnaire which was used to survey participants, and Chapter 6 then discusses the aspects described in Chapter 3. Chapter 7 concludes the thesis with discussion of other aspects of the study findings not addressed in either Chapters 4, 5 or 6, before drawing conclusions and providing suggestions as to future directions of study.

Hypothesis

The hypothesis addressed in this project was:

That there are identifiable differences between Western Australian (WA) sheep farms which explain both the rate at which ivermectin resistance in *Ostertagia circumcincta* develops and the risk of resistance developing on farms by 2000.

Study sample

The Department of Agriculture, WA, had built a database of the results of all of the FECRTs conducted in WA in 1999 and 2000. All farmers on this database were sent a copy of the questionnaire provided that their address could be obtained from secondary sources (see Data, below); in all 235 questionnaires were mailed.

Data

The data used in this study came from four sources, of which a mail questionnaire was the major source utilised. The other sources of data were the lists of faecal egg count reduction test (FECRT) results and farmer addresses supplied by the Department of Agriculture, Western Australia and two private consultants, and a spatial location file from Department of Land Administration (DOLA).

Questionnaire

Design

A mail questionnaire (Appendix 1) was sent to farmers for whom drench resistance test results were available, and was designed to satisfy three data collection requirements:

1. To be able to match replies to the FECRT data obtained from other sources,
2. To be able to locate the farm on the DOLA space file, and
3. To obtain information on farm management practices that might influence the occurrence of ivermectin resistant *Ostertagia circumcincta*.

To meet the first requirement the questionnaire asked respondents to provide their name and their address. From this the questionnaire could be linked to the FECRT files containing the farmer's name, locality and the name of the consultant conducting the test.

The questionnaire also contained four optional questions (see Table 3.1) in which the respondent had the opportunity to list the dates when FECRTs had been performed and the results. This enabled crosschecking should any discrepancy between the records have been encountered.

To be able to locate the farm on the DOLA space file respondents were asked to name the road from which the property driveway originates, and provide details of their land title. Contact with DOLA indicated that this data would be sufficient to locate the property. The land title could be in one of three formats, a Location District and Number, a Plan Number with associated Lot Number, or a Diagram Number with associated Lot Number. All three options were provided to respondents to direct their answer.

The majority of the questionnaire enquired about farm management practices. These questions were based upon the hypotheses surrounding the development of anthelmintic resistance in sheep nematodes and the Department of Agriculture's extension program aimed to control it (the CRACK campaign, Besier and Hopkins, 1988). This entailed 45 questions over 6.5 pages.

The questions in this part included descriptors of the farm's operation in general including enterprise mix, detail about the sheep enterprise including breeds, lambing times, sheep sales and purchases and wool production, and then questions related specifically to worm control practices. These questions addressed quarantine drenching in 2000 and 1995, the current summer drenching program and which anthelmintics have been used historically for summer drenches (both of the older broad spectrum anthelmintics and the macrocyclic lactones specifically), winter drench use, the use of safe pastures, worm egg count monitoring, checking that the correct dose had been administered, previous drench testing, and sources of worm control advice. All of the hypotheses tested were two sided.

The questionnaire comprised 36 open- and 16 closed-type questions, summarised in Table 3.1. In total the questionnaire consisted of 52 questions and 8.5 pages (Appendix 1).

The questionnaire was circulated amongst my two supervisors and two other academics at the School of Veterinary Clinical Studies familiar with either the sheep industry or survey design for suggestions and improvements. Several veterinarians from the

Department of Agriculture provided input with suggestions of phraseology appropriate for WA farmers.

Table 3.1 Summary of questions in questionnaire mailed to WA sheep farmers in 2001 for the purposes of this study.

Number	Question / *hypothesis*	No. closed	No. open
1 & 2	Farmer's name and address		2
3 & 4	Property identifiers		2
5	Annual rainfall - *environment*		1
6	Length of ownership of the farm		1
7 & 8	Farm size		2
9 & 10	Proportion of farm cropped 1995 & 2000		2
Sheep enterprise details			
11	Winter sheep grazed area		1
12	Sheep numbers by age		1
13 & 14	Type of sheep enterprise	1	1
15	Wool micron		1
16 & 17	Sheep sales in 2000	1	1
18 & 19	Sheep purchases in 2000	1	1
20	Wool clip for 2000		1
21	Lambing date and sheep breeds		1
22	Farm enterprise mix		1
Worm control practices			
23 – 25	Quarantine drenching in 2000 – *quarantine*	2	1
26 – 28	Quarantine drenching in 1995 – *quarantine*	2	1
29 - 32	Use of summer drenches – *refugia*	1	
33 – 34	Winter drench use 1996-2000 – *frequency*		2
35	When first used Ivomec® - *frequency*		1
36	Use of Jetamec® - *under-dosing*	1	
37 & 38	Use of safe pastures – *refugia*	1	1
39 – 41	Calculating anthelmintic dose – *under-dosing*	3	
42 – 44	Use of Worm Egg Counting	1	2
45 – 49	Drench resistance testing	1	4
51 – 53	Sources of worm control advice	1	2

Note – Question 50 is missing. Question 50 was inadvertently numbered 51.

Pilot testing of the questionnaire was conducted on 5 farmers who weren't included in the survey group. Suggestions made by these farmers were included in the revised questionnaire as considered appropriate for the study's objectives.

Human Ethics Approval

Murdoch University Human Research Ethics Approval was applied for on 22 May 2001 by submitting the Application Form, a copy of the questionnaire and the proposed

Consent Form to the Human Ethics Committee. The pro forma for the Consent Form was obtained from the web site of the Murdoch University Human Ethics Committee: http://www.research.murdoch.edu.au/ethics/hrec/appendA.asp

Approval to use the questionnaire on human subjects was granted at the Research Ethics Committee's June meeting and Permit Number 2001/190 was granted for the duration of 3 years.

An Annual Permit Renewal was applied for in December 2001. An application to have the Project Closed was made on 28 October 2002.

Distribution

Prior to distributing the questionnaire to farmers, a letter was written to the veterinarians and consultants who had provided the anthelmintic resistance testing results to the Department of Agriculture for their consent for contact with their clients for the purposes of this survey. A sample questionnaire was provided with the letter introducing myself, the project, and its aims and objectives.

The questionnaire in its final form was printed on orange A4 paper and posted to the farmers to be surveyed with an accompanying letter on Murdoch University letterhead paper introducing the survey and describing its aims and objectives. Also included in the envelope was the Consent Form printed on white paper and a Reply Paid envelope for returning the questionnaire. Questionnaires were mailed out in September 2001 to 211 farmers.

Two consultants provided the mailing addresses of their clients who fitted the profile sought for this survey in October 2001; these farmers were duly posted the survey kit. In all, 235 farmers were surveyed for this project.

Follow-up

Letters of follow-up were mailed to non-respondent farmers in December 2001. In February 2002 a further follow-up letter was sent accompanied by another copy of the questionnaire and the Consent Form to the remaining non-respondent farmers which numbered 145 at that point in time.

Further follow-up by letter was conducted in August 2002 to obtain more valid questionnaires that contained the answer to the question "When did you first use Ivomec® on your sheep". Telephone follow-up was conducted in October 2002 to finalise replies to this question. A number of farmers contacted in the spring of 2002 reported the results of drench resistance tests that had been conducted either that spring or the previous one to enable calculation of period prevalence of ivermectin resistance 1999-2002.

Table 3.2 Year of first use of Ivomec® by respondents for which telephone follow-up was attempted (n=35).

Year	n	Year	n
1987	2	1993	
1988	12	1994	
1989	4	1995	
1990		1996	1
1991	2	1997	3
1992	1	Not contacted	10

Data entry

Murdoch University Human Ethics require the identity of the respondents to remain private. To this end data from questions 1 to 17 of the questionnaire (that could be used to identify a farmer) were entered into a spreadsheet and then the printed material relating to these questions was stored separately in a locked safe. A field technician entered the remainder of the responses into a separate spreadsheet. To ensure the entries were correctly linked in the master spreadsheet both portions of each completed questionnaire were given a unique identifier. As a further check the answer to question 20 was included on the first she*et al*ong with the identifier code. Standard data entry procedures were followed. Once data entry was complete the two spreadsheets were merged only on the primary author's computer and the respondent's identifiers deleted and replaced by a number in order of receipt of the response. Any spreadsheets with the identity of the surveyed farmers were stored separately on computer under password protection.

To ensure that transposition errors did not occur during the merging of the two spreadsheets both contained the answer to question 20, the wool production of the farm. Each respondent questionnaire was assigned an ivermectin resistance status based upon the most recent faecal egg count reduction test result where ivermectin was tested. In the majority of cases this was obtained from the electronic files described below. In several cases ivermectin resistance had been diagnosed prior to 1999 so the status was determined from the information provided in answer to questions 46 – 49 of the questionnaire.

Other data sources

The three other sources of data were the lists of faecal egg count reduction test (FECRT) results and farmer addresses supplied by the Department of Agriculture, Western Australia, and two private consultants, and a space file from Department of Land Administration (DOLA).

Department of Agriculture data

The Western Australian Department of Agriculture had provided free faecal egg counting and larval cultures from faecal egg count reduction tests performed in 1999 and 2000 (Palmer *et al*., 2001). In general, these free tests were conducted if the FECRT included the half-dose ivermectin treatment group. The results of this testing were collated by Ms. Jill Lyon at the Regional Veterinary Laboratory, Albany in three File Maker Pro files.

Private consultant data

Two private consultants had also recorded the results of FECRTs conducted on their client's properties during the same period, for a number of clients who had not participated in the free larval culture testing offered by the Department of Agriculture. Both provided their results during spring 2001, and hence their clients did not receive the questionnaire in the original mailing.

DOLA space file

The Department of Land Administration mapping section in Midland, Perth was contacted in autumn, 2002. To minimise file size and associated cost it was determined that the space file could be generated to exclude the Perth metropolitan area and the southwest coastal regions of the state (see Fig. 3.1). A 674 Mb zipped spatial information file was provided on CD-ROM for the fee of $350. No further use was made of this data in this study.

Assigning status to respondent farms

Determination of the ivermectin resistance status of individual farms

It is generally accepted that anthelmintic resistance exists on a farm when there is less than 95% reduction of the mean faecal egg count of the treatment group, relative to the control group, in a faecal egg count reduction test (Coles *et al*., 1992, Lyndall-Murphy, 1993, Taylor *et al*., 2002, Sangster and Dobson, 2002). The use of larval cultures of treatment and control groups enables the differentiation of resistance occurring into worm genera when mixed populations are encountered in field tests (Lyndall-Murphy, 1993). A further refinement is the use of a half-dose of ivermectin treatment group (i.e.

100 ųg/kg ivermectin) to detect resistance at an earlier stage of its development (Palmer *et al*., 2001).

The drench test results available included at least one of the three levels of testing, although many did not include the half dose of ivermectin group (Palmer *et al*., 2001). Farms were classified as "resistant" if they satisfied one of the three criteria in Table 3.3

Table 3.3 Criteria for determination of farm ivermectin resistance status: farms defined as 'resistant' if percentage reduction was less than 95% in a faecal egg count reduction test.

Reduction measured	Stringency of test	Stage of resistance
Strongyle eggs only	Least stringent	Advanced
Ostertagia eggs	Ivermectin resistance confirmed	Well established
Ostertagia eggs after half dose of ivermectin	Most stringent - ML resistance confirmed (Palmer *et al* 2001)	Earliest clinical diagnosis made.

Some FECRTs included abamectin treatment groups; if this treatment was < 95% effective then ivermectin resistance was confirmed because abamectin is a more potent anthelmintic than ivermectin and both are included in the FECRT at the same dose rate (Leathwick and Sutherland, 2001; Wooster *et al*., 2001).

The effective life of ivermectin

The effective life of ivermectin was determined as the time in years from first use of the anthelmintic and either the diagnosis of ivermectin resistance or the year of most recent FECRT if resistance has not been diagnosed on the farm.

A parallel statistic necessary for the Weibull survival analysis was the interval between the last two FECRTs that determined the end of the effective life of ivermectin. In some cases this could be determined in months from the information provided by farmers, but in general it was rounded to years, as for the effective life of ivermectin. The Weibull statistic was then used to estimate at what time during this interval that ivermectin resistance (as defined) occurred.

Geographical location of respondent's farms

Shire: For descriptive purposes prior to progressing to the proposed spatial analysis the period prevalence of ivermectin resistance by shire was displayed graphically (Appendix 2). Farms were located within shires by correlating the response to question 3 (the name of the road upon which the property driveway comes off) with Shire Boundaries as displayed in The West Australian Travellers Atlas (McEvoy *et al*., 1994).

Worm Control Zone: The southwest agricultural area of Western Australia had been divided by Wroth (1996) into 3 climatic zones along approximate rainfall isohyets, although they were also an approximation of the length of the annual pasture growth period. From the Map in Appendix 3 the boundary lines between the zones were plotted on a large wall map of the region. Having found the farm in The West Australian Travellers Atlas it was identified on the wall map and assigned to one of the three Worm Control Zones.

Data quality assessment

The following types of potential data errors were assessed:

Data entry error

The data were saved in a spreadsheet of 134 columns by 387 rows, with the obvious potential for incorrect entries.

Data were checked for correctness against the questionnaire sheets as values were entered, and during early manipulation of the data, including assessment for implausible data (see below). Both data entry people were familiar with farming practices in WA and could ascertain answers that were nonsensical for the question. Problematical responses were recorded separately and the amended data entered into the spreadsheet.

Implausible data

After transposing the data (swapping rows and columns) the entire spreadsheet was sorted in ascending and descending order, each time using a different variable as the sorting variable. After the sort the first few values were then compared to overall summary statistics for that variable and expected results based on knowledge of WA farming systems. This was done to screen the data for obvious errors in either data recording or data entry which might result in an individual cell value being implausible, or being order(s) of magnitude larger or smaller than the remainder of the data values stored in that variable.

The number of errors detected was recorded and calculated as a percentage of the total number of data entries made. It was determined that if there were less than 1% errors found by this method then those identified would be corrected when identified (see below). Checking of the entire dataset against the original paper copies would be required if the error rate was 2% or higher. Three data entry errors were found and corrected, an error rate of 0.005%.

Unusual values were then compared against values in the relevant paper copy and corrected if required. If the data value in the original paper copy was identical to the value in the database, then it was left unchanged.

Outliers

It became apparent in this process that there were three farms whose enterprises would be outliers in many of the analyses, as tabulated below and it was determined to retain these outlier farms within the analysis:

Table 3.4 Summary statistics of enterprise descriptors of respondent farmers compared to those of the 3 identified outlier farm enterprises.

Variable	All responses			Outlier farms		
	First quartile	Mean	Third quartile	Small Suffolk stud	Awassi export farm	Beef feedlot on farm
Farm size (ha)	1200	2640	2700	84	53866	5400
Sheep flock numbers	2590	6274	7204	84	134412	11110
Wool micron	20.0	20.7	21.5	30.0	N/A*	30.2
Wool sold (t)	15300	29459	38240	510	None*	59330
Sheep sales	1059	1690	2662	8	114500	10006
Sheep purchases	5	10	30	41	25000	52
Cattle sales	39	601	2756	none	none	13296

*Awassi are a breed of sheep that shed their fleece, which is a fibre, not wool.

Missing data

Variables in the dataset were screened for blank entries whilst the screening for implausible data was conducted. It was considered that the respondents could have omitted to respond for one of two reasons:

- They did not or could not give an answer to the question. These were considered to be true missing responses, and
- Not providing a number could be considered to be the same as entering a zero.

The number of missing data of each type for the variables is tabulated below:

Table 3.5 Number of missing responses considered likely to be equivalent to '0' by variable in the dataset of responses.

Variable	Number of missing responses
Number of winter drenches	0
Winter drenches with macrocyclic lactones	0
Number of FECs in 2000	12
Sheep numbers	0
Nos. sheep sold in 2000	1
Nos. sheep purchased 2000	3

Table 3.6 Number of missing responses considered to be true missing values by variable in the dataset of 132 responses.

Variable	Number of missing responses	Variable	Number of missing responses
Sources of worm control advice	1	Used quarantine drenches in 1995	10
Years of farm ownership	0	Use summer drenches	1
Farm size	0	Ever used Jetamec®	4
Effective farm size	0	Use of safe paddocks	0
Proportion of farm cropped in 2000	0	Ever used faecal egg counts	3
Proportion of farm cropped in 1995	2	Weigh sheep before drenching	6
Sheep winter grazed area	5	Dose of anthelmintic used	11
Sheep enterprise type	0	Define safe pastures	37
Year first used Ivomec® *	38	First use of FECs	21
Wool micron	7	Performed a FECRT	6
Wool sales in 2000	16	Annual rainfall	0
Calibration of drench gun	2	Main source of advice	5
Quarantine drenches in 2000	8	2nd main source of advice	27
		2000 sheep sales relative to past level of sales	1

*The number of missing responses in the 'Year first used Ivomec ®' variable is prior to the telephone follow-up conducted in Spring 2002. After the telephone follow-up there were 11 responses with missing data.

Duplicate responses

Two responses were received from the one respondent with an interval of about 5 months between responses. When this was discovered they were compared with each other and found to be almost identical, apart from the last digit in 4 or 5 digit responses to questions such as sheep numbers, numbers sold, or kilograms of wool sold. This gave some indication of likely repeatability of responses amongst respondents. One of these responses was deleted from the dataset prior to the epidemiological analyses being performed.

Statistical methods

These are detailed where data are presented in Chapters 4 and 5.

Feedback to participants

An important part of surveys is the provision of feedback to the participants, which will encourage participation in further surveys (Edwards, 1990). To this end, letters briefly explaining the findings have been sent after concluding both studies to both respondent and non-respondent farmers and to the veterinarians or consultants who provided the lists of clients to be surveyed in language appropriate to the readers.

Chapter 4

SHEEP FARM RISK FACTORS FOR IVERMECTIN RESISTANCE IN *Ostertagia circumcincta* IN WESTERN AUSTRALIA.*

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Abstract

On-farm risk factors associated with ivermectin resistance on sheep farms in Western Australia were identified from data derived from a postal survey of 235 farmers who had conducted a faecal egg-count reduction test in 1999, 2000, or both years. A response of 54% was achieved. We developed a logistic-regression model. Contributory main effects in the final model were selling 10% more sheep in 2000 than is the usual policy (OR = 4.00), farm purchased since 1975 (OR = 2.34), and number of winter flock anthelmintic treatments in the previous 5 years (OR = 1.04). A secondary logistic-regression model assessed risk factors for farms selling 10% more sheep than usual in 2000; these farmers appeared less committed to their sheep enterprises than other farmers. These results are discussed in relation to current hypotheses of anthelmintic resistance. This is the first time that the farmer's management of the flock has been implicated in the development of anthelmintic resistance.

Keywords Sheep, Anthelmintic Resistance; Risk Factors; Logistic Regression; Ivermectin; *Ostertagia circumcincta*.

Introduction

The first of the macrocylic lactone family of anthelmintics (ivermectin) was released in Australia in 1987 and resistance in *Ostertagia* in sheep was first reported in Western Australia (WA) in 1992 (Swan *et al*., 1994). By 2000, the farm-level prevalence of resistance in WA had reached 38% (Palmer *et al*., 2001).

Historically in winter rainfall areas, sheep flocks were given anthelmintic treatments frequently during winter at the time of high worm larval challenge. Of factors promoting anthelmintic resistance, the first hypothesis tested was that resistance was caused by frequent anthelmintic use (Prichard *et al*., 1980). This was addressed by adopting strategic drenching, such as the summer drenching proposal of Anderson

(1972). Strategic drenching is used to describe the administration of flock treatments at a time of strategic weakness in the epidemiology of the target species. Summer treatments target a weakness of the life cycle of sheep helminths, when the weather is deleterious to larval survival on pasture. In WA, each summer drench involves anthelmintic treatment of all sheep on the farm and these drenches are administered one or more times during summer. Prichard (*et al*., 1980) also postulated that under dosing could be a cause of anthelmintic resistance. The potential role of under dosing was highlighted by demonstrating that sheep body weights often were underestimated (Besier and Hopkins, 1988). Other hypotheses proposed to slow the development of anthelmintic resistance were to rotate between effective anthelmintic groups, and to reduce the frequency of drenching by using "safe pastures" (Prichard *et al*., 1980). Extension packages such as WORMKILL in New South Wales and CRACK in Western Australia promoted strategic programs to reduce the frequency of treatment, and the message not to under-dose (Besier, 1997).

However, recently it has been postulated that in many situations, strategic drenching has hastened the selection for anthelmintic resistance by allowing the preferential survival of resistant worms in situations where there are few non-resistant worms in "refugia" (Leathwick *et al.*, 1995, Van Wyk, 2001). Refugia is defined as being the fraction of a parasite population not exposed to selection for resistance by anthelmintic treatment; the effectiveness of strategic drenching is because there are few worms in refugia to re-infect treated sheep. The widely used summer-drenching program is considered a major contributor to the rapid development of ivermectin resistance in *O. circumcincta* in WA under the refugia hypothesis (Besier, 1999).

We examined the influence of farm practices (particularly those relating to worm control in sheep) on the development of ivermectin resistance on sheep farms in Western Australia.

Method

A postal survey was sent to all 235 farmers in WA who had conducted an anthelmintic-resistance test (faecal egg-count reduction test; FECRT) in sheep in either 1999 or 2000 (or both). The questionnaire contained 53 questions including descriptors of the farm and its enterprises, and questions addressing the major hypotheses. The refugia hypothesis was tested through questions on summer drenching, the use of safe pastures, and environmental influences (longer, dryer summers). Other hypotheses examined were flock anthelmintic treatment frequency, under-dosing, use of quarantine drenching, sheep trading, and the influence of the farmer's management skill upon the risk of ivermectin resistance. All hypotheses tested were two-sided.

Thirty two of the questions in the questionnaire were open-ended. The questionnaire was pilot tested by 5 farmers who were not surveyed, but repeatability of answers was not tested directly. The questionnaire took 2-4 hours to complete.

The Department of Agriculture Western Australia had collated results of the surveyed farms FECRTs. FECRTs had been conducted according to the protocol of Lyndall-Murphy (1993). Some FECRTs included a half dose ivermectin group as suggested by Palmer *et al*. (2001). Farms were defined as either ivermectin-resistant or not ivermectin resistant. Resistant farms were those that had < 95% reduction in a FECRT by one or more of three criteria:

- Overall reduction of strongyloid eggs,
- Reduction of *Ostertagia* only, or
- Reduction of *Ostertagia* after a half dose of ivermectin was administered.

The farm-level prevalence of ivermectin resistance amongst respondents and non-respondents were calculated, and a chi-squared test for independence between responders and non-responders run.

Associations were evaluated between the binary outcome variable (resistance vs. no resistance) and a variety of farm-level risk factors. Questions that were farm descriptors or respondent identifiers were not analysed. Screening tests were performed using either the chi-squared test for independence or univariable logistic-regression, for categorical and continuous independent variables respectively. Variables with $P < 0.25$ were offered to a multivariable logistic-regression model.

Backwards - stepwise elimination was used for model building, using a threshold $P = 0.10$ for retention of variables. Each of the variables "Sold 10% more sheep in 2000 than is usual", "Month of First Summer Drench", "Worm Control Zone", and those relating to quarantine drenching were aggregated into fewer levels based upon the hypotheses being tested. Linearity in the logit of continuous variables in the model was assessed (Hosmer and Lemeshow, 1989). This necessitated the recoding of the variable "Years of Farm Ownership" as a categorical variable about the point where the plot of the logit changed slope. Contributory main effects variables in the final model were: "Number of winter flock anthelmintic treatments 1996-2000", "Purchase of the farm since 1975", and "Sold 10% more sheep in 2000 than is the usual farm policy". All possible 2-way interaction terms among the explanatory variables were examined after identification of the reduced set of main effects. The goodness-of-fit of the model was assessed with the Hosmer-and-Lemeshow statistic (Hosmer and Lemeshow, 1989). Multi-collinearity between variables in the final model and those identified with $P < 0.05$ in the univariable analyses was tested for using chi-squared test for independence, binary logistic- regression, or linear correlation for categorical, combination binary and continuous, and continuous variables respectively.

It was difficult to explain how one of the main effects - whether or not farmers sold 10% more sheep in 2000 than is usual – would contribute to the development of ivermectin resistance prior to 2000. A secondary logistic-regression model was developed from survey data to define farm-level risk factors for the binary outcome variable “sold 10% more sheep in 2000” vs. “less sheep sold” following the process of univariable and multivariable analysis described above. All questions in the questionnaire were examined in the development of this second model. Variables were retained in this model if $P < 0.15$. Testing for linearity of the logit and for multi-collinearity was conducted as described above.

Statistical analyses were performed using SPSS version 10.1.3.

Results

A response of 54% was achieved (128 from 235). The farm-level period prevalence (1999-2000) of ivermectin resistance amongst respondents was 38% (95% CI 29%, 46%). Respondents were no more likely than non-respondents to have ivermectin resistance on their farm ($P = 0.55$).

The categorical variables tested in the univariable tests to develop the main effects multivariable model are presented in Table 4.1, and the continuous variables in Table 4.2.

Eighteen variables were offered to the multi-variable model, which are asterisked in Tables 4.1 and 4.2. The 18 variables reflected farm enterprise type (n=3), the refugia hypothesis (n=3), the drenching frequency hypothesis (n=4), quarantine drenching use (n=3), sheep trading practices (n=2), if sheep were under-dosed (n=1), the number of effective anthelmintic groups (n=1), and the primary source of worm control advice (n=1).

Table 4.1 Responses of 128 WA sheep farmers by farm ivermectin resistance status to categorical questions in a questionnaire (2000)

Variable	Level of variable	Ivermectin resistance	
		Yes	No
Type of sheep enterprise*	All wool	40	10
	Mixed wool & prime lambs	32	37
Farm has cropping*	Yes	40	72
	No	7	6
Farm runs cattle*	Yes	37	54
	No	10	24
Farm agroforestry	Yes	37	62
	No	10	16
Quarantine drench introduced sheep in 2000	Yes	14	40
	No	20	28
	Sometimes	7	9
Type of macrocyclic lactone quarantine drenches used in 2000*	Moxidectin	11	11
	Abamectin	6	28
Quarantine drench introduced sheep in 1995*	Yes	7	33
	No	29	35
	Sometimes	5	4
Quarantine drench introduced sheep with ivermectin in 1995*	Yes	7	37
	No	40	41
Quarantine drench introduced sheep with macrocyclic lactones 1995	Yes	7	28
	No	5	11
Month first summer drench* administered	Oct-Nov	9	5
	Dec	29	40
	Jan	5	19
	Feb-Apr	2	10
Number of summer drenches administered	One	38	47
	Two	5	25
	Three	2	6
Anthelmintic classes rotated Annually*	Yes	20	24
	No	21	49
Used ivermectin as an external parasiticide?	Yes	6	13
	No	41	65
Frequency of use of safe pastures	Always	4	6
	Usually	23	26
	Half of the time	6	15
	Rarely or never	14	14

Table 4.1 (cont.). Responses of 128 WA sheep farmers by farm ivermectin resistance status to categorical questions in a questionnaire (2000)

Variable	Level of variable	Ivermectin resistance	
		Yes	No
Farmer can correctly define a safe pasture, and used safe pastures?*	Apparently do	17	30
	Obviously don't	52	10
	Indeterminable	30	15
Sheep are weighed to calculate the anthelmintic dose	Yes	35	62
	No	8	14
Calculation of the anthelmintic dose	Guess	37	67
	Dose according to the heaviest in the mob	5	5
Calibration of the drench gun before use (to ensure that the correct dose is administered)*	Always	26	8
	Usually	13	19
	Rarely or never	8	6
Most important source of worm control advice*	Consultant	12	7
	Veterinarian	26	34
	Department of Agriculture	7	8
Worm Control Zone (environment type of farm)*	Zone 1 (coastal with milder, shorter summers)	11	4
	Zone 2	32	50
	Zone 3 (dryer, more inland)	4	20
Number of lambing periods on the farm	One	14	24
	Two	31	53
Sheep sales in 2000 relative to usual sales policy*	10% more	19	9
	The same	18	57
	10% less	9	8
Sheep purchases in 2000 relative to usual purchase policy*	10% more	2	4
	The same	39	62
	10% less	4	1

* Variable offered to multivariable model.

Table 4.2 Responses of 128 WA sheep farmers by farm ivermectin resistance status to questions with continuous answers in a questionnaire (2000)

Variable	Quartile range	Farm ivermectin resistance status	
		Resistant	Susceptible
Percentage of sheep gross income from prime lamb enterprise	0 0	25	37
	1-15	13	23
	17-100	10	18
Number of summer drenches with ivermectin	0-2	16	23
	3-4	12	27
	5-7	8	16
	8-18	8	12
Number of summer drenches with moxidectin	0 0	18	45
	1	13	16
	2-6	18	15
Number of summer drenches with macrocyclic lactones	0-3	11	26
	4-5	11	21
	6-7	14	13
	8-21	11	16
Number of winter flock anthelmintic treatments administered 1996-2000*	0	6	33
	1-2	15	15
	3-5	12	18
	6-66	14	12
Number of winter flock treatments with ivermectin 1996-2000	0 0	26	56
	1	7	10
	2-5	14	12
Number of winter flock treatments with moxidectin 1996-2000*	0 0 0	31	71
	1-4	16	7
Number of winter flock treatments with macrocyclic lactones 1996-2000*	0 0	20	49
	1-2	10	19
	3-8	14	10

Table 4.2 (cont.). Responses of 128 WA sheep farmers by farm ivermectin resistance status to questions with continuous answers in a questionnaire (2000)

Variable	Quartile range	Farm ivermectin resistance status	
		Resistant	Susceptible
Mean number of flock treatments per year*	1-1.1	6	25
	1.2-1.8	11	19
	2-2.6	14	20
	2.8-14.2	17	15
Year ivermectin first used to treat sheep	1987-1988	9	15
	1989-1990	8	16
	1990-1994	10	8
	1995-2000	9	12
Number of faecal egg count flock monitoring tests conducted in 2000	0	11	15
	1-2	16	35
	3	8	16
	4-30	13	13
Date first lambing period commenced in 2000	Feb 1 - May 10	8	22
	May 15 - June 11	15	15
	June 16 - July 1	14	23
	July 5 - Aug 10	8	18
Annual rainfall (mm)	300-400	14	21
	401-470	9	18
	471-550	16	20
	551-770	9	20
Years of ownership of farm	2-15	10	21
	17-25	20	12
	26-38	10	21
	40-131	8	24
Total farm area (ha)	<1200	14	19
	1210-1664	11	19
	1666-2700	14	18
	2900-53846	9	23
Effective farm area (ha) - total less unusable land	<960	14	18
	966-1398	11	20
	1400-2200	15	20
	2246-44736	8	21
Winter grazed area (ha)	<560	15	17
	560-815	10	23
	830-1300	13	17
	1344-35341	10	21

Table 4.2 (cont.). Responses of 128 WA sheep farmers by farm ivermectin resistance status to questions with continuous answers in a questionnaire (2000)

Variable	Quartile range	Farm ivermectin resistance status	
		Resistant	Susceptible
Proportion of farm area cropped in 1995 (%)	<15	11	17
	15-25	14	23
	26-40	12	21
	41-77	9	16
Sheep flock numbers in 2000	<2746	10	19
	2746-4945	11	20
	4950-7274	15	16
	7300-134412	8	22
Sheep winter stocking rate (sheep/ha)	1-2.75	8	23
	2.8-5.75	9	19
	5.8-7.98	16	14
	8-16	12	18
Micron of main fleece line of wool	18.5-20	10	19
	20.1-20.6	11	20
	20.8-21.5	14	13
	21.6-30	6	19
Wool sold in 2000 (t)	<15.8	11	22
	15.8-23.6	15	14
	24-39	13	17
	40-170	8	19
Total sheep purchases in 2000	<5	9	22
	5-9	8	22
	10-21	14	18
	25-1686	16	16
Sheep purchases in 2000 less rams	0		
	0	37	67
	0		
	15-1672	10	11
Total sheep sales in 2000	<1059	14	17
	1060-1680	8	24
	1681-2700	12	20
	2701-114500	14	18
Sheep sales in 2000 less rams	<1000	14	16
	1002-1600	7	24
	1657-2533	13	19
	2540-113000	13	19

Table 4.2 (cont.). Responses of 128 WA sheep farmers by farm ivermectin resistance status to questions with continuous answers in a questionnaire (2000)

Variable	Quartile range	Farm ivermectin resistance status	
		Resistant	Susceptible
Proportion of farm area cropped in 2000 (%)	<16	14	20
	16-31	12	21
	33-44	10	18
	45-82	12	20

* Variable offered to multivariable model.

Table 4.3 Multivariable logistic-regression model of ivermectin resistance on 128 Western Australian sheep farms (2000).

Variable	Values of variable	b	SE(b)	P	OR	95% CI
Number of winter flock anthelmintic treatments in 5 years		0.05	0.02	0.08	1.04	0.99,1.10
Years of farm ownership	> = 25 years				1	
	< 25 years	0.85	0.41	0.04	2.34	1.05,5.22
Sales of sheep in 2000 relative to usual sales policy	Sold the same or less				1	
	Sold >10% more sheep	1.39	0.45	0.002	4.00	1.67,9.58
Constant		-1.6				

Hosmer and Lemeshow test P = 0.14

Fig 4.1 Number of winter flock anthelmintic treatments used on 128 sheep farms in Western Australia between 1996 and 2000.

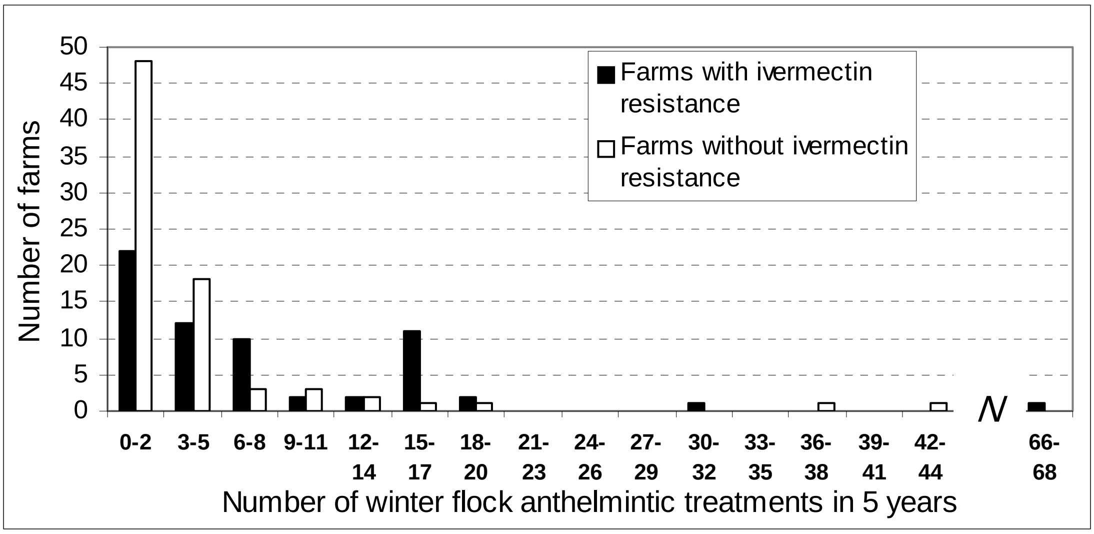

Table 4.4 Multiple collinearities between variables in the final model of sheep farm ivermectin resistance and those variables omitted but significant (P<0.05) in univariable testing (Western Australia, 2000).

	Variables in the final model		
Highly likely variables not included in the final model	Number of Winter Flock Treatments (1996-2000)	Farm purchased since 1975	Sold 10% more sheep in 2000 than the usual policy
Sheep enterprise type (only wool or prime lambs and wool)	P = 0.42	P = 0.79	P = 0.14
Use of macrocyclic lactone quarantine drenches in 2000	P = 0.24	P = 0.92	P = 0.04 OR = 2.32 (1.03,5.21)
First summer drench given before or after January 1st.	P = 0.48	P = 0.16	P = 0.83
Number of winter drenches of moxidectin in 5 years	P < 0.001 r^2 = 12%	P = 0.20	P = 0.06

Table 4.5 Multivariable logistic-regression model of farmers selling more sheep in 2000 than is their usual policy on 128 Western Australian sheep farms (2000).

Variables	**Values of the variable**	**b**	**SE(b)**	**P**	**OR**	**95% CI**
Number of years since farmer first used ivermectin		-0.17	0.08	0.03	0.84	0.72,0.99
Number of Worm Egg Counts performed in 2000		-0.16	0.09	0.08	0.85	0.71,1.02
Farm agroforestry	No				1	
	Yes	0.98	0.63	0.12	2.68	0.77,9.27
Constant		346.47				
Hosmer and Lemeshow test P = 0.98						

The final model of risk factors for ivermectin resistance is displayed in Table 4.3, and the testing for multi-collinearity between the main effects variables and those with P < 0.05 at univariable analysis in Table 4.4. The model to explain the variable “Sold more sheep in 2000 than is usual” is in Table 4.5. The main effects continuous variables in both models were deemed to have suitably linear odds ratios.

Figure 4.1 shows the winter flock anthelmintic use on farms of respondents by resistance status. Number of winter drenches is the sum of the number of drenches administered to different ages and sex groupings (classes) of sheep over the 5-year period 1996-2000.

Discussion

This was a biased sample as the questionnaire was mailed to the 235 farmers in WA who had performed a FECRT during 1999 and 2000. These farmers could be considered to be amongst the more progressive farmers in the State, because the uptake of FECRTs in the WA farming community has been low (between 5 and 10% of farms) (D. Palmer, *pers comm*). In common with most recent surveys, these figures for the prevalence of anthelmintic resistance are based on laboratory submissions, rather than on random surveys. A response of 54% is less than ideal, but acceptable given the length of the questionnaire (53 questions). Coles (1997) conducted a 30-question survey of sheep farmers in Great Britain and achieved a 23% response after offering a monetary raffle prize for respondents.

The FECRT is widely used for the field diagnosis of anthelmintic resistance to all types of anthelmintics. It suffers from relatively poor sensitivity, particularly when resistance is emerging (Geerts and Gryseels, 2001). Anthelmintic resistance is confirmed as being present at the cut-off of 95% reduction. This cut-off is used to make a clinical diagnosis of resistance (Sangster & Dobson, 2002). To improve the sensitivity of the FECRT in the mixed worm populations encountered in the field, larval cultures of control and treatment groups are used to ascribe resistance to strongyle genera (Lyndall-Murphy, 1993). A further refinement is to use a discriminating dose of anthelmintic, such as a half-dose of ivermectin to enhance the identification of emerging anthelmintic resistance (Palmer *et al*., 2001).

Palmer *et al*. (2001) quoted a prevalence of ivermectin resistance in *Ostertagia circumcincta* of 38% from 132 of the tests included in this study (i.e. those using a half dose of ivermectin). This prevalence is much higher than that reported elsewhere in the world (Sangster, 1999; Vickers *et al*., 2001; Coles, 2002). It is believed that the situation in WA is explained largely by the “refugia” hypothesis. The major source of refugia for direct life-cycle parasites such as sheep strongyles is the free-living population from eggs to infective third stage larvae. For a winter-active parasite such as *O. circumcincta*, the proportion of the population in refugia will be at a maximum at the end of winter, and will be at a minimum during summer in a Mediterranean environment. Thus this hypothesis proposes that anthelmintic treatment of the entire flock during summer will exert the greatest selection pressure, because the entire nematode population will be exposed to the treatment. This is supported by experimental work in Western Australia (Besier *et al*., 2001), which suggests that summer drenching in WA carries a severe risk of promoting anthelmintic resistance. All but two respondents had adopted the recommendation of summer drenching, so we could not test the hypothesis that summer drenching contributes to resistance. Even so, summer drenching practices were considered in the development of the model.

By contrast, the role of drenches given in winter as a contributor to the development of ivermectin resistance in WA has not been clear. Under the refugia hypothesis, winter is considered the time when least selection pressure on the worm population is exerted in a Mediterranean environment. The epidemiological studies of Wroth (1992) in WA showed that winter drenching also contributed to the development of anthelmintic resistance although not as strongly as did summer drenching. In our survey, the median number of winter flock anthelmintic treatments given over the last 5 years was 2 (Table 2), which we consider only minimal use of anthelmintics relative to treatment frequencies elsewhere in the world. However, winter drenching was a key for the development of anthelmintic resistance. This model included number of winter drenches in total, rather than number of winter uses of macrocyclic lactone anthelmintics although there was a correlation (Table 4) between the number of winter doses of moxidectin and the total number of winter drenches.

This model includes winter drench usage as a risk factor, which supports the original Prichard *et al*. (1980) hypothesis of increased frequency of anthelmintic use leading to resistance. Winter drenching was probably the main factor that produced anthelmintic resistance to the benzimidazole and imidazothiazole anthelmintics (Besier and Love, 2003), which developed prior to the widespread adoption of summer drenching. The refugia hypothesis is supported by this finding when frequent winter drenching is used in conjunction with summer drenching, as was the practice of the respondent farmers. Generally, the only time large numbers of sheep are purchased is when a farm is commenced and typically the new flock is accumulated from a number of sources. It would be expected that the anthelmintic resistance status of the flock on a new farm would reflect that of a number of worm populations at the time of purchase. This would be particularly so if farmers did not practice quarantine drenching of the introduced sheep (i.e., anthelmintic treatment to remove all worms as sheep are introduced). The length of time the farm has been owned could thus be a risk factor as anthelmintic resistance developed after the release of the modern, broad spectrum anthelmintics. It is also expected that as resistance to one anthelmintic class occurs and farmers are forced to use alternate classes, resistance to the alternatives will arise in a shorter time frame. Presumably the first cases of benzimidazole resistant *O. circumcincta* in WA occurred in the 1970s but resistance was not widespread until the early 1980s, when the prevalence of resistance to this anthelmintic class was 52% (Edwards *et al*., 1986). A survey in the early 1990s showed widespread anthelmintic resistance in WA to three of the four available anthelmintic options of the time. On farms surveyed the prevalence of benzimidazole resistance was 88%, for levamisole 70%, and for a benzimidazole - levamisole combination 65% (Overend *et al.*, 1994). Thus, farms purchased after 1975 would be more likely to accumulate their sheep flocks from sources with pre-existing anthelmintic resistance, making ivermectin resistance more likely to occur than in flocks that were established prior to 1975 and have been closed since then.

We introduced a variable “Farm purchased since 1975” from the question “How long have you owned your farm?” Lack of linearity in the logit for this variable led to recoding as a categorical variable with 1975 being the demarcation point. Without quarantine drenching of the newly purchased sheep in flocks established since 1975, any resistant parasites purchased inadvertently with the sheep would become established in the composite population, so that the farm would commence with pre-existing anthelmintic resistance. Quarantine drenching was a variable considered in developing the model, and was practiced by 32% of respondents in 1995 and 42% in 2000 (see Table 1).

When asked about sheep trading practices, 20% of respondent farmers indicated that they had sold at least 10% more sheep in 2000 than is usual. There could have been peculiarities of the year 2000 which resulted in more sheep sales than is usual, such as drought or exceptional prices. A review of meteorological records for 2000 suggests it was a normal year, and sheep prices were depressed, rather than exceptional.

We introduced a variable reflecting the commitment of the farmer to the sheep enterprise as indicated by the management decisions taken, the variable being “Sold more sheep in 2000 than is usual”. From the secondary model developed to examine risk factors for commitment to the enterprise it is suggested that these farmers had higher odds of using ivermectin early after its release in Australia (at the height of wool prices), to have switched some of their farm area to agroforestry in the late 1990s when wool prices were low, and to have conducted little faecal egg count monitoring of worm burdens in 2000 (see Table 5). These risk factors suggest that decisions about the management of the sheep flock are being made by farmers who have not been as fully committed to their sheep enterprises as other farmers in the survey.

The model developed supports the drenching frequency hypothesis (Prichard *et al*., 1980). It also raises factors previously not considered in the development of anthelmintic resistance: the management decisions made by individual farmers that reflect their commitment to the farm's sheep enterprise, and the length of farm ownership. The latter could be related to the failure to adopt quarantine drenching of newly purchased sheep. Our model does not incorporate issues related to summer drenching, because most respondents had adopted this strategy.

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Chapter 5

THE EFFECTIVE LIFE OF IVERMECTIN ON WESTERN AUSTRALIAN SHEEP FARMS - A SURVIVAL ANALYSIS*

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Abstract

A mail survey of Western Australian sheep farmers who had conducted faecal egg count reduction tests for anthelmintic resistance in 1999 or 2000 was conducted, with some telephone follow-up. A response of 56% was achieved (132 of 235). The period prevalence of ivermectin resistance was 44%. To ascertain contributing factors, we developed a survival model of time to occurrence of resistance. Variables significant in the final model were winter drenching frequency, 0-2 vs. 3+ flock treatments in 5 years (RH 0.52); availability of alternative effective anthelmintic classes on the farm (RH 0.30); always using safe pastures (RH 0.23); and veterinarians as the primary source of worm control advice (RH 0.58). The relationship of these variables to the understanding of anthelmintic resistance is discussed.

Keywords: Ivermectin, anthelmintic resistance, sheep, survival analysis, *Ostertagia circumcincta*.

Introduction

Gastrointestinal parasitism is widely regarded as the major disease problem of sheep in Australia (Love and Coles, 2002). Nematodes from three genera are recognised as being the most pathogenic, causing the majority of production losses: *Haemonchus contortus, Ostertagia* (*Teladorsagia*) *circumcincta* and *Trichostrongylus* spp. The latter two species predominate in Western Australia (WA), which has a Mediterranean environment with winter rainfall. Ivermectin resistance has arisen rapidly in *O. circumcincta* in Western Australia whereas it is uncommon in other regions of Australia (Besier & Love, 2003). Palmer (*et al*., 2001) estimated the farm level prevalence of macrocyclic lactone resistance in WA to be 44%, based on faecal egg count reduction test results where less than a 95% reduction was achieved after treatment with a half dose of ivermectin. On the basis of this test ivermectin resistance was confirmed on half of the properties tested. As yet ivermectin resistance in *Trichostrongylus* species is unreported in Australia, but it is becoming prevalent in *Haemonchus contortus* in those areas where this species is endemic (Love and Coles, 2002).

Ivermectin is a member of the macrocyclic lactone (ML) class of anthelmintics that includes moxidectin and abamectin. Although cross-resistance between members of this class has been demonstrated to occur, resistance occurred first to ivermectin because it has been available for the longest and is the least potent of the MLs against *O. circumcincta* (Leathwick *et al*., 2000). The ML class is the most recent of the modern anthelmintics available to sheep producers (Geary *et al*., 1999), and it is anticipated that resistance to this class will pose a serious problem for farmers as the prevalence of resistance to the older anthelmintic classes, the benzimidazoles and imidazothiazoles, was already over 80% in Australia in the early 1990’s (Overend *et al*., 1994).

State Departments of Agriculture have been active in promoting worm and anthelmintic resistance programs since the 1980’s (Waller, 1995). These programs were based upon the hypotheses summarised by Prichard (*et al*., 1980) and have become best practice parasite management programs followed worldwide in all animal species (Geerts and Gryseels, 2002). These programs recommend testing for anthelmintic resistance, using strategic treatments (anthelmintic treatment at critical times in the parasite life cycle), minimising anthelmintic use, routine laboratory monitoring for parasitism, annual rotation between effective chemicals, and quarantine drenching of introduced stock to prevent the incursion of resistant parasites.

The rapid rate at which ivermectin resistance has arisen has led to a reappraisal of these programs, particularly in respect to the strategic “summer drenching” program that effectively controls these parasites in winter rainfall regions (Besier, 1997). Under the “*refugia*” hypothesis (Prichard *et al*., 1980; van Wyk, 2001) it is proposed that it is the effectiveness of “summer drenching” that promotes the development of anthelmintic resistance. *Refugia* is defined as a situation where part of the parasite population is not exposed to anthelmintic treatment; in WA there is no *refugia* on a farm when all sheep are treated with anthelmintic in summer, because virtually no worm larvae survive on pasture. Hence, the source of the future worm population will be derived chiefly from resistant worms that have survived the summer treatment. In a similar manner, anthelmintic resistance can increase where sheep are drenched and moved to a “safe” pasture, which has low levels of infective larvae (van Wyk, 2001).

There have been few epidemiological studies of anthelmintic resistance at the farm level (Coles, 2002). In a companion study to this, Suter (*et al*., 2004) produced a main effects logistic regression risk factor model for ivermectin resistance on Western Australian sheep farms, from the results of a postal questionnaire sent to farmers who had conducted anthelmintic resistance testing in 1999 - 2000. The main effects were found to be winter anthelmintic treatment frequency, excess sheep sales and duration of farm ownership. In this paper we present a survival analysis model derived from the same data set, enhanced by follow-up telephone interviews with the respondent farmers.

Methods

A postal survey was sent to all 235 farmers in WA known to have conducted an anthelmintic-resistance test (faecal egg-count reduction test; FECRT) in sheep in either 1999 or 2000 (or both). The questionnaire contained 53 questions including descriptors of the farm and its enterprises, and questions addressing the major hypotheses. The *refugia* hypothesis was tested through questions on summer drenching, the use of safe pastures, and environmental influences (longer, dryer summers). Other hypotheses examined were the effects of flock anthelmintic treatment frequency, under-dosing, use of quarantine drenching, sheep trading, and the influence of the farmer's management skill upon the risk of ivermectin resistance. All hypotheses tested were two-sided. Thirty-two of the questions in the questionnaire were open-ended. The questionnaire was pilot tested by 5 farmers who were not surveyed, although repeatability of answers was not tested directly. The questionnaire took 2-4 hours to complete.

Telephone follow-up was conducted with 38 of the respondent farmers in 2002 to determine the year in which they had first used ivermectin on their sheep. When applicable these farmers also provided the results of any FECRT conducted since 2000. Results of the FECRTs had been collated by the Department of Agriculture, Western Australia, which had been conducted according to the protocol of Lyndall-Murphy (1993). Some tests included a half dose ivermectin group as suggested by Palmer *et al*. (2001), to provide better discrimination between farms for ML resistant *Ostertagia*.

Farms were defined as either ivermectin-resistant or not ivermectin resistant. Resistant farms were those with a < 95% reduction in a FECRT by one or more of three criteria:

- Overall reduction of strongyloid eggs,
- Reduction of *Ostertagia* only, or
- Reduction of *Ostertagia* after a half dose of ivermectin.

The farm-level prevalence of ivermectin resistance amongst respondents and non-responders was calculated, and independence assessed with a chi-square test.

The effective life of ivermectin on a farm was defined as the time (in years) from first use of ivermectin until the occurrence of ivermectin resistance. Times to resistance of those farms not yet diagnosed as such when surveyed were right censored at the time of their last FECRT. For those farms found to be resistant the times at which this occurred were interval censored in that they were known to fall in the interval between the last negative and the first positive tests but were not known exactly. As an approximation to the true times, the mid points of these intervals were used for construction of Kaplan-Meier survival plots, obtaining summary statistics and exploratory Cox regression modelling. However, the interval censoring was taken into account for inference purposes. In particular, the effects of various farm level risk factors were modelled under an assumption of proportional hazards (relative risk constant over time) by appropriate parameterisation of the Weibull distribution. Standard Wald tests were used for assessment of the effects. In general, the relative hazard estimates obtained from the parametric regression approach were in close agreement with those obtained from the semi-parametric Cox modelling. Variables with $P < 0.15$ in the univariable analysis were offered to the multivariable survival model. A backwards-stepwise elimination approach was used for development of the model, with a threshold P-value of 0.10 for retention of variables. The suitability of the proportional hazards assumption was checked by application of the test of Grambsch and Therneau (1994) prior to removal of any variable. Where appropriate, aggregation of categorical variables was undertaken based upon *a priori* assumptions after examination of the effect of the covariates on the model. Some continuous variables, such as the number of winter drenches in 5 years, were recoded into categorical variables with the demarcation being the median value. The multivariable model was checked for interactions amongst the main effects variables within the model.

Analyses were performed using S-PLUS (2002, version 6).

Results

A response of 56% was achieved (132 from 235). The farm-level period prevalence (1999-2002) of ivermectin resistance (as defined) amongst respondents was 44%. Respondents were no more likely than non-respondents to have ivermectin resistance on

Table 5.1 Cumulative survival times for the effective life of ivermectin from a survey of Western Australian sheep farmers (data to 2001). Estimates were obtained from unadjusted Kaplan-Meier plots; continuous variables were stratified into intervals determined by their quartile values.

Variable	Level	n	Quartiles of cumulative survival (years)		
			75%	50%	25%
Type of sheep enterprise*	All wool sheep	43	4.8	9.5	-
	Some prime lambs	66	6.0	-	-
Wool micron	18.5-20.0	35	5.5	10.5	-
	20.1-20.5	21	5.0	10.5	-
	20.6-21.4	26	6.0	10.5	10.5
	21.5-30.0	28	9.6	-	-
Does the farm have crop*	No	11	6.0	7.0	10.5
	Yes	104	5.5	11.5	-
Does the farm graze cattle*	No	32	6.0	10.5	-
	Yes	83	5.5	-	-
Worm Control Zone (farm environment)	Coastal, high rainfall	95	6.0	11	-
	Inland, drier	20	4.9	6.5	-
Timing of first summer Drench	Before Dec.	17	5.5	10.5	-
	December	63	6.0	10.5	-
	After Dec.	30	5.5	-	-
Number of summer drenches	0-1	80	5.5	10.5	-
	2	27	6.0	10.5	-
	3-4	8	6.0	10.5	11.5
Always uses safe pastures*	No	89	5.5	9.5	-
	Yes	23	-	-	-
Used macrocyclic lactone quarantine drenches in 2000	No	60	5.5	10.5	-
	Yes	55	6.0	10.5	-
Used quarantine drenches in 1995	No	76	5.5	10.5	-
	Yes	49	6.5	-	-
Used ivermectin in 1995 for quarantine drenching	No	70	5.5	11.0	-
	Yes	44	6.0	10.5	-
Length of farm ownership*	25 years +	58	5.0	9.0	-
	< 25	54	6.5	11.0	-
Main source of worm control advice used on the farm*	Government veterinarian	39	6.0	11.0	-
	Private veterinarian	8	7.5	-	-
	Consultant	54	5.0	10.5	-
	Other	13	3.5	6.5	-

(Cont. on next page)

Table 5.1 (cont.) Cumulative survival times for the effective life of ivermectin from a survey of Western Australian sheep farmers (data to 2001).

Variable	Level	n	Quartiles of cumulative survival (years)		
			75%	50%	25%
Annual rotation of drench classes*	No	70	6.5	-	-
	Yes	45	5.0	9.5	11.5
Number of alternative anthelmintic classes*	0	85	5.5	9.5	-
	1	18	-	-	-
	2	8	10.5	11.5	11.5
Calibrate the drench gun	No	6	4.8	4.9	-
	Yes	107	6.0	10.5	-
Sheep sales in 2000*	The same or less	83	6	11.5	-
	More than usual	30	5	9.6	11.5
Number of winter flock treatments 1996-2000*	0	38	7.0	-	-
	1-2	27	6.0	11.0	-
	3-5	25	3.5	6.0	-
	6-66	25	6.0	9.6	11.5
Aggregated no. winter flock treatments 1996-2000*	0-2	65	6.5	-	-
	3-66	50	4.9	9.5	-
No. winter flock treatments with moxidectin 1996-2000*	0	94	6.0	-	-
	1-4	21	2.5	6.5	10.5
No. winter flock treatments with macrocyclic lactones 1996-2000*	0	64	6.0	-	-
	1-2	28	5.5	8.0	-
	3-8	23	5.0	9.5	11.5
Annual anthelmintic use (average winter treatments plus summer drenches)*	0-1	30	7.0	-	-
	1.1-1.8	28	5.5	10.5	-
	1.9-2.7	28	5.5	-	-
	2.8-14.2	29	5.5	9.5	11.5

- Covariate did not attain the quartile in Kaplan-Meier.
* Variable offered to the multivariable model

their farm (P = 0.55). On average respondents conducted drench resistance tests every 3.2 years, the range being 1-11 years.

Replies were excluded from all analyses if time of first use could not be ascertained or ivermectin had never been used as a sheep anthelmintic on the farm. Further exclusion occurred when relevant covariate values were missing. In total, 108 farms were included in the final model, and those farms had a period prevalence of ivermectin resistance of

48% (95% CI 39%, 58%). From the Kaplan-Meier survival curve of Figure 5.1, the estimated median time to diagnosis of resistance was 10.5 years.

The variables tested in the univariable tests to develop the main effects multivariable model are presented in Table 5.1. Fourteen variables were offered to the multivariable model. These variables reflected farm enterprise type (n=3), the *refugia* hypothesis (n=1), the drenching frequency hypothesis (n=5), sheep trading practices (n=2), the number of effective anthelmintic groups (n=2), and the primary source of worm control advice (n=1). The stepwise selection process that jointly considered these variables yielded a model with 4 main effects for the effective life of ivermectin on WA sheep farms.

The estimated, adjusted hazard rates of the 4 variables remaining as covariates in the final model are provided in Table 5.2. The relative hazards give the relative probabilities

Table 5.2 Multivariable parametric (Weibull) survival model for the effective life of ivermectin on 108 Western Australian sheep farms (data to 2001). The hazard function for the model is of the form $h(t) = \lambda k t^{\gamma}$ where t is years since first use of ivermectin, shape is determined by γ and scale is determined by the baseline parameter k together with $\lambda = exp(\Sigma_i \lambda_i x_i)$. The covariate x_i is a 0/1 indicator for category membership of the *i*th co-variable and the relative hazard (RH) is obtained from $exp(\lambda_i x_i)$.

Variable	**Covariate**	***P***	**RH**	**95% CI**
Number of winter flock treatments in 5 years	0-2	0.04	0.52	0.28,0.97
	3-66		1	
Use of safe pastures	Always	0.02	0.23	0.06,0.83
	Less frequently		1	
Effective alternate anthelmintics available	Yes	0.02	0.30	0.11,0.83
	No		1	
Main source of worm control advice	Veterinarians	0.08	0.58	0.31,1.07
	Other		1	
Log likelihood Chi square	$P < 0.001$			
Shape parameter	1.49			

of developing resistance at any particular time, given survival up to that time and with the other covariate values taking fixed values. The model does not contain any interaction terms or stratification variables.

Figure 5.2 displays the Kaplan-Meier survival plots for the number of the identified anthelmintic resistance control strategies adopted by the respondent farmers (ivermectin resistance management score). The higher this score, the longer before the occurrence of ivermectin resistance (P < 0.001).

Figure 5.1 Cumulative survival plots for the effective life of ivermectin on Western Australian sheep farms (data to 2001). Event times were defined as the occurrence of resistance and were subject to interval censoring. The semi-parametric Kaplan-Meier plot (heavy line) was derived using the midpoint of the inter-test interval preceding the diagnosis of ivermectin resistance. The parametric survival curve (dotted line) belongs to the Weibull family of distributions and was derived using the interval endpoints.

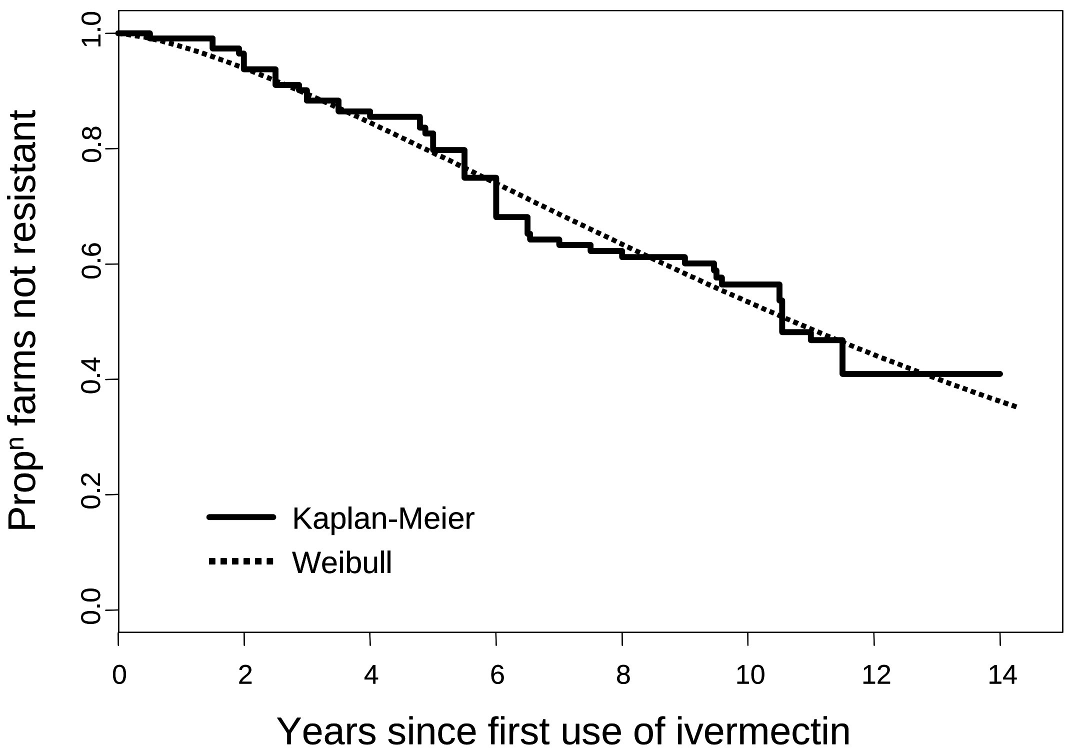


Table 5.3 shows tests of association between the variable “Always uses Safe Pastures” and other survey data that explain the biological significance of this variable.

Table 5.3. Associations between use of safe pastures by respondent farmers and other worm control practices in WA (data from survey, 2001).
Table 5.3a. The ability of respondent farmers to correctly define the time taken to produce a safe pasture in summer and winter by their use of safe pastures.

	Definition of time to produce a safe pasture		*P**	OR	95% CI
Use of safe pastures	Correct	Incorrect			
In summer (correct answer 1-3 months)					
Always	18	10	0.04	2.44	1.04,5.75
Less than always	42	57			
In winter (correct answer 6 months)					
Always	5	23	0.53	1.44	0.47,4.43
Less than always	13	86			

Table 5.3b. Frequency of winter flock treatments by respondent farmers against their use of safe pastures.

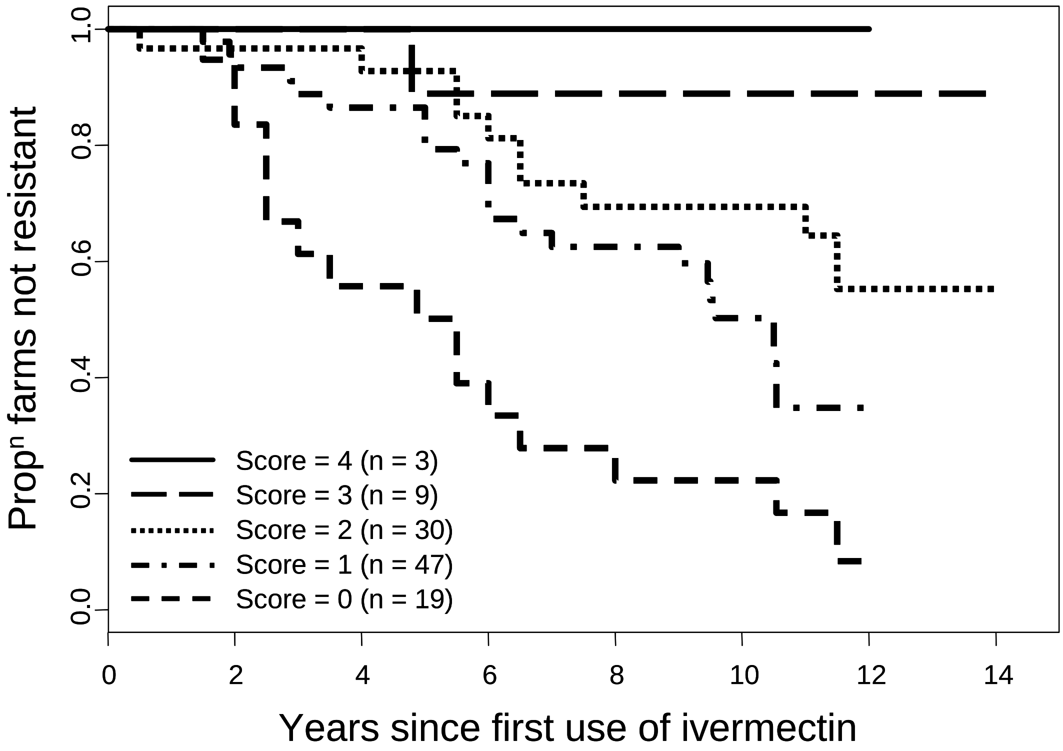
	Winter flock treatments given in 5 years (1996-2000)		*P**	OR	95% CI
Use of safe pastures	0-2	3-66			
Always	19	5	0.01	0.27	0.10,0.75
Less than always	46	45			

*Chi square test of independence.

Discussion

The majority of epidemiological studies on anthelmintic resistance have been surveys to determine prevalence at a particular time point (reviewed by Prichard, 1990, Coles, 2001, Barger, 2002, Sangster and Dobson, 2002). Studies that have examined risk factors for resistance, again for resistance at a particular point in time, include those by

Figure 5.2 Kaplan-Meier survival plots for ivermectin resistance management score on Western Australian sheep farms (data to 2001). The score represents farm management intensity and for each farm comprises the number of protective covariates from the final model (Table 2) adopted: infrequent winter drenching (0-2 drenches in 5 years), availability of alternative effective anthelmintic classes, consistent use of safe pastures, and veterinarians being the primary source of worm control advice ($P < 0.001$).



Edwards *et al*. (1986), Bartley *et al*. (2003), Ancheta *et al*., 2004, and Suter *et al*., 2004. This is the first study to examine the rate at which anthelmintic resistance develops by producing a survival model for the effective life of ivermectin on WA sheep farms.

The final model contained four variables: whether 0-2, or 3+ flock anthelmintic treatments were given in winter from 1996-2000, whether there were any other broad spectrum anthelmintic classes still effective on the farm, consistent use of safe pastures, and the type of worm control advice used by the farmer. The biological significance of these variables can be explained by various hypotheses and through an understanding of anthelmintic resistance.

The significance of inclusion of a variable relating to winter drench use supports the drenching frequency hypothesis proposed by Prichard *et al*. (1980). The logistic regression risk factor model developed by the present authors from this same cohort also contained a variable related to frequency of winter drench usage (Suter *et al*., 2004). Some authors believe that drenching frequency was the main factor in the development of benzimidazole and imidazothiazole (i.e., levamisole) resistance in all genera, and in ML resistance in *Haemonchus contortus* (Besier and Love, 2003). It is considered that the *refugia* hypothesis is more likely than the drenching frequency hypothesis to explain the development of ivermectin resistance in *Ostertagia circumcincta* in WA, as a consequence of the routine practice of summer drenching (Besier, 1999). In this study, all respondent farms practised summer drenching; further winter drenching would provide additional selection pressure to that imposed by the summer drenches. Farms on which less than 3 winter drenches were given every 5 years developed ivermectin resistance at a rate 0.52 times that of farms where more frequent winter drenching was practised.

The lack of alternative effective anthelmintic classes to the MLs in sheep has probably contributed to the appearance of resistance to this anthelmintic class. Prichard *et al*. (1980) proposed that anthelmintics should be rotated between classes to prolong the effective life of each of the anthelmintics. The rotation of anthelmintics was believed to allow reversion to effectiveness of an anthelmintic to which resistance had been developing (Prichard *et al*., 1980). However, field experiments to confirm the rotation hypothesis have been equivocal (Leathwick, *et al*., 2001), and reversion has not been shown to occur in practice (van Wyk, 2002). A computer simulation model has indicated that, under a given worm management program, resistance to one class of anthelmintic will arise after a certain number of uses of the anthelmintic (Smith, 1990). Under that model, if rotation between two classes is practiced without any changes to the management plan then the time to resistance is the sum of the times for each individual class. The study of Suter *et al*. (2004) included a variable, "Farm owned since 1975", which was related to the previous property history and the likelihood of the farm having purchased anthelmintic resistant nematodes at its inception. The variable in this model "Alternative anthelmintics available" is also related to the prior history of the property. In a survey of anthelmintic resistance in the early 1990's it was estimated that 80% of WA farms had resistance to the imidazothiazole class of anthelmintics and 88% to the benzimidazole class (Overend *et al*., 1994). The survival model presented here found that availability of an alternative anthelmintic class on the farm was significantly associated with a reduced rate of development of ivermectin resistance (RH = 0.3).

A "safe pasture" is a pasture carrying very low levels of infective nematode larvae, so that sheep moved to safe pastures do not rapidly become re-infected. These pastures can be prepared in a number of ways including prior grazing by adult dry sheep that are not producing significant numbers of nematode eggs, grazing by cattle (Barger and Southcott, 1978), or by other agricultural use such as cropping and haymaking. The time required to produce a safe pasture by these methods depends upon seasonally variable effects on the survival of worm larvae, and in WA is estimated to be 1-3 months during summer and 6 months during winter. Thus farmers need to plan sheep grazing movements to ensure that safe pastures will be available when flocks need treating. However, the use of safe pastures for grazing sheep after anthelmintic treatment is another instance of a strategic treatment, which like summer drenching, could promote anthelmintic resistance (van Wyk, 2001). The protective hazard rate associated with inclusion of the variable "Always uses safe pastures" would appear to be counter to the *refugia* hypothesis, although if sufficient *refugia* was provided elsewhere on the farm using safe pastures may not select for resistance at a great rate.

An alternative explanation of the biological significance of this variable could be that farmers who always use safe pastures are more capable of managing worm control in their flock than other farmers. By implementing safe pastures all of the time these farmers would reduce the drenching frequency on their farms. Subsequently, infrequent drenching makes it easier for the farm manager to ensure safe pastures are always available for use after drenching.

The variable describing use of safe pastures was derived from the closed question “Do you put your sheep onto a safe pasture after drenching?” for which the answer could be selected from 5 choices: Always, Usually, Half the time, Rarely, or Never. The latter 4 choices were aggregated into the alternate co-variable “Does not always use safe pastures”. A subsequent question asked respondents to define the time it would take to produce a safe pasture in either winter or summer. The respondents who indicated that they always used safe pastures were 2.44 times more likely to correctly define the time required in summer than the other respondents (Table 5.3a), whilst neither group of respondents could accurately state the time required to produce a safe pasture in winter. The respondent farmers who indicated that they always used safe pastures were 0.27 times as likely to give 3 or more flock treatments during winter than farmers who were less rigorous about their use of safe pastures for sheep grazing (Table 5.3b). Respondents who always used safe pastures were thus considered indicative of farmers that were more capable of managing nematodiasis in their sheep flock.

The last variable in the model for the effective life of ivermectin was the source of worm control advice utilised by the farmer. Farmers who used veterinarians as their primary source of worm control advice were 0.58 times as likely to develop ivermectin resistance each year than farmers who relied upon other sources of advice on managing worm control. The covariate “Veterinarians as primary source of worm control advice” included both private veterinarians and government veterinary officers; the other covariate's sources of advice included farm consultants, neighbouring farmers, farm magazines and radio broadcasts. Respondents using veterinarians for advice were considered indicative of farmers that were committed to sourcing the most up-to-date scientific advice for managing worm control for their sheep enterprise. The risk factor model of Suter *et al*. (2004) also included a variable reflecting the farmer's commitment to the management of their sheep flock.

The survival plots of Figure 2 illustrate the significant additive impact obtained by combining the practices that remained as variables in the final survival model. The effective life of ivermectin was clearly prolonged on those farms that scored more highly. In particular, farms where none of these practices were employed had a median survival time about half that of the median of the whole cohort studied, whilst those employing 3-4 of these strategies had only one case of ivermectin resistance during the study period. This indicates the importance of implementing a suite of strategies to control a multi-factorial disease such as anthelmintic resistance, and the difficulty that many farmers have in doing this.

These findings are based on results of FECRT, which despite limitations, is recognised as the most appropriate field test to determine the effectiveness of a range of anthelmintics against field populations of nematodes (Johansen, 1989). These limitations include the fact that it is a cumbersome and time-consuming test to conduct on the farm and in the laboratory, which may largely explain why this test has not been more widely adopted by farmers. A further reason is the failure of farmers to recognise the importance of testing the resistance status of the nematode parasites of their sheep flocks (Besier, 1996). The FECRT is insensitive for the detection of early stages of anthelmintic resistance (Martin *et al.* 1989), but of good specificity if conducted properly (Geerts and Gryseels, 2003). The FECRT protocol indicates a clinical diagnosis of resistance at a particular percent reduction after treatment; in this study a reduction of less than 95% was considered diagnostic for resistance. In mixed nematode populations typically encountered in the field the sensitivity of the test can be improved by using faecal larval cultures to identify resistant genera (Lyndall-Murphy, 1993) and by using discriminating dosages of anthelmintic such as the half dose of ivermectin used in this study (Palmer *et al*., 2001).

The surveyed farmers constitute approximately 2% of WA's sheep farmers, and could be considered amongst the most progressive due to their adoption of anthelmintic resistance testing. This possible bias, along with the small sample size, would suggest that caution against over interpretation of the findings of the present study is warranted. To minimise the cost of the study only those farmers having conducted a FECRT in the years 1999 and/or 2000 were included in the questionnaire mailing list. However, we note that the estimated period prevalence of ivermectin resistance between 1999-2002 is consistent with other recent estimations of the prevalence of this disease in WA (Palmer, *et al*., 2001). The potential bias that responders may be more likely than non-responders to have ivermectin resistance was not observed in this study. The response of 56% was good given the length and complexity of the questionnaire. In a similar recent survey of progressive Scottish farmers a response rate of 43% was achieved (Bartley *et al*., 2003).

Conclusion

This survival analysis has identified possible factors that may contribute to the rapid development of ivermectin resistance on WA sheep farms where routine summer drenching is practised. The present study has provided support for the role of a high frequency of drenching in the development of anthelmintic resistance, and for the proposal that the farmer's management of the sheep flock also plays a role.

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Chapter 6

QUESTIONNAIRE

In this chapter potential biases, data handling issues and problems with the questionnaire design are discussed. The questionnaire used for this study is presented in Appendix 1, the process of data collection described in Chapter 3, and the results summarised in Chapters 4 and 5. The response rate was 56% (132 from 235).

A questionnaire is used to collect data for further analysis; the analysis and any conclusions drawn are dependant upon the quality of the data derived from the questionnaire. The quality of the data can be compromised through biases, invalid responses, poor repeatability, missing data and errors of data entry (Scholl *et al*., 1994, Schukken *et al*., 1989).

Potential biases include sampling errors, biases of the interviewer, and of response rates between affected and non-affected participants (Scholl *et al*., 1994). The first and the last biases are addressed in the papers in Chapters 4 and 5; bias of the interviewer would be related to the design of the questionnaire because this study did not involve interviews *per se*. Such biases or problems are discussed in the section below titled "Design".

Another potential bias is that of recall – those farmers with the disease of interest having additional motivation to participate in the survey and provide answers (Last, 1988). In part this is addressed by the response rates between affected and non-affected participants, which were not significantly different. The existence of this potential bias should lead to some caution when interpreting the results and drawing conclusions.

Assessment of the validity of responses requires comparison with a gold-standard measurement of the same variable (Slater *et al*., 1992). Potentially the only way to achieve that with the data collected in this survey would be for the interviewer to monitor every sheep related activity on the farm over a number of years. Practically this is impossible to do, so the validity of the responses in this survey would always be subject to question.

The repeatability of the questionnaire answers was not assessed in this study, and should have been incorporated into the study design (Scholl *et al*., 1994, Maingi and Gichigi, 2001). Repeatability is commonly assessed using a test-retest protocol where the same questions are put to the same respondents by the same interviewer within a reasonable period of time (Slater *et al*., 1992). The kappa statistic is used to determine the repeatability above chance, where the results are scaled as excellent (kappa>0.75), average to good (kappa 0.4-0.75) and poor (kappa<0.4). Repeatability of a variable of average or better suggests that the data is of reasonable quality and that the conclusions drawn are reliable. One respondent did submit two questionnaires at an interval of several months, with almost complete duplication of results (i.e. excellent repeatability). Scholl *et al*. (1994) considered mail surveys of farmers would result in higher repeatability than face-to-face or telephone interviews, because the farmer would be able to consider the answers in their own time and be able to refer to written records if unsure. It did occur that many of the farmers contacted during the telephone follow-up wished to refer to their records before giving their response several days later.

Problems with the questionnaire

The problems with the questionnaire are detailed below. Edwards (1990) discusses these problems prefaced with:

“It (a questionnaire) should be treated as a delicate scientific instrument and should be calibrated, monitored and used accordingly.”

Despite the attention to detail recommended by Edwards (1990) in the design process, the review by other veterinarians, and the pilot testing on typical farmers the remaining problems were not identified until a wider range of respondents was surveyed.

It was apparent during data entry that respondent farmers would strive to give an accurate answer. Examples of their attempts to give an accurate response included:

- Adding comments when the choices available did not suit their farm,
- The reluctance to answer Q35, particularly if the time of first use had been more than 5 years ago,
- Some respondents telephoned indicating that they would return the questionnaire when they had been able to verify some of their answers.

In designing the questionnaire the inability to recall details of farm events of more than 5 years ago was recognized as being important. Questions of a historical nature were thus designed to ask about 1995 relative to 2000; the only question that dealt with an earlier time was Q35 which resulted in the highest non-response rate. Two surveys of worm control practices and the repeatability of responses have shown good repeatability for description of type of anthelmintic used in the previous 3 years, dropping to poor repeatability for recall 5-8 years ago (Maingi *et al*., 1996, Maingi and Gichigi, 2001).

Missing data

Tables 3.5 and 3.6 classify the variables with missing responses as either ‘true missing values’ or where the lack of a response could be taken as zero respectively.

The variable with the most missing responses (n=38) was ‘Year first used Ivomec’ which was subject to telephone follow-up to improve the dataset for the survival analysis. The issue with this variable and that related to the first use of faecal egg counts (missing n=21) would be recall, as depicted in Table 3.2. 37 farmers did not provide a definition for the time taken to produce a safe pasture, which made the inclusion of this variable in the subsequent analyses unlikely. These farmers may have refrained from answering because they didn't know the answer or understand the question and preferred to leave the response blank.

There were 27 missing responses to the final question asking their second most important source of worm control advice. This may have been due to the respondents only relying upon one source of advice to the exclusion of other extraneous sources. This variable was omitted from subsequent analysis because of the missing values.

Three variables had less missing data, but the amount missing did not exclude them from use in the analysis. These missing responses may have been due the respondent's inability to understand the question or the concept that was being determined. These were 'Dose rate of anthelmintic used' (n=11), and the two questions relating to quarantine drenching (1995 n=10 and 2000 n=8). Inability to recall may have contributed to the higher number of missing responses in the question dealing with quarantine drenching in 1995 relative to 2000.

Questions that could indicate a respondent farmer's potential income can be problematical in surveys (Chapman *et al*, 1991). The two questions that would provide a guide as to income were also amongst those with a moderate number of missing responses. 16 respondents did not provide the quantity of wool sold in 2000, although 1 farmer did not produce any wool because the property ran Awassi sheep that shed their coat. Some farmers may not have sold any wool in that year, but that would be uncommon as sales are usually made shortly after shearing. There were 7 respondents that did not provide the wool micron of their main fleece line. Wool micron is the main determinant of wool price and can be readily accessed through wool brokerage firms and market reports; when micron (price) combined with wool production gross farm income can be estimated reasonably accurately.

Data handling errors

The data entry error rate was determined to be 0.005%, which is considered to be acceptably low. The inclusion of outliers in the analyses reflects the variation in farms and farming practices making the analyses more valid although reducing the power of the study. The way that missing responses have been dealt with is considered acceptable and unlikely to be associated with any bias in the analyses.

Design

The questionnaire contained some questions that presented problems to respondent farmers when answering the questionnaire; these problems were related to the design of the questionnaire. Some problems of this type became apparent during data entry. In some cases the respondents had written their appropriate answer against a closed question when there wasn't an alternative available that suited their farm enterprise. In other cases farmers were answering inappropriately, eg circling two alternatives when one was the expected response. These examples served to indicate that there could have been misinterpretation and confusion amongst respondents, even in those questions or questionnaires that appeared to have been answered appropriately.

The questions that caused such concerns are detailed below:

Q18. How many sheep did you purchase in 2000? This question did not allow for the practice of agistment, nor was agistment allowed for elsewhere in the questionnaire. Agistment is where the host farmer rents grazing land on his farm to another farmer to graze his livestock on. Agistment is generally charged at the rate of cents per head per week or month. Similarly the questionnaire failed to ask whether the respondent farmer had agisted their sheep at another farmer's property.

Agistment usually occurs when one property has an abundance of feed and the other has a shortage. Common causes of feed shortage are drought and fire, occasionally farms carrying a high stocking rate agist in years that are tight but sustainable for more conservative farmers.

In the context of anthelmintic resistance both agistment and sheep trading would have the same effect - allowing the introduction of nematode genotypes foreign to the farm. When agisted sheep are likely to have anthelmintic resistant worms then quarantine drenching upon arrival would be appropriate; if the agistment farm has pre-existing resistance and would hope to dilute the resistant genotypes already present then they may elect to not quarantine drench on arrival but to recommend that the agisted sheep be so drenched upon return to their home farm.

Q22. The total farming enterprise mix in 2000? This question then provided boxes to fill in with three columns. The first column listed alternate enterprises to sheep grazing (but excluding sheep), the second asked the respondent to indicate the winter area used, and the third asked for the number of livestock in winter. There had been previous questions (Q8-12) that had elucidated responses to farm size, effective farm size (less unusable land such as laneways, house and gardens, nature reserves and salt affected land), sheep winter grazed area and sheep numbers in winter.

The first identifiable error was asking for the total enterprise mix when no option was given to sheep producers to answer about the sheep enterprise. The question would have been better if the question had included *(excluding sheep)*. The second error was restricting the responses to winter only, when some farmers will agist or trade in sheep to utilise their excess summer crop stubble. It is traditional agronomic practice in Australia to compare farms in winter, which is commonly the period of shortest feed supplies. The possibility of sheep trading to utilise excess stubbles had been addressed in Q13-19. Even so, it may have been appropriate to ask for details of summer farm enterprise mix. The third problem with this question related to the practice of some farmers to graze sheep and cattle on the same paddocks, making the task of attributing an area to each species difficult.

Q23. Do you give a drench to newly purchased or introduced sheep within 2 days of arriving on your property (a quarantine drench)? This closed question gave 3 choices in the answer – Yes/No/Sometimes – but failed to recognise the farmer that did not introduce any sheep.

Eight farmers did not answer this question; 3 farmers did not answer the question about sheep purchases in 2000. It would be unlikely that a farm was entirely closed and did not purchase any sheep, especially rams. That only 3 farms answered that they did not purchase sheep in 2000 supports that assumption; had respondents been asked to detail sheep purchases over a longer time period this assumption could have been confirmed. The same query was raised in respect to Q26 relating to quarantine drench use in 1995.

Q27. Did you use ML products as part (or all) of your quarantine drenching at that time? (meaning 1995), and

Q28. Which ML products did you use? Some respondents had answered 'No' to Q27 yet had indicated the use of one or more of the specified product brands of ML drench in Q28.

The problem would be the failure of the farmer to recognise what 'ML' meant in Q27, while subsequently recognising the product brand names in Q28, eg Ivomec® or Cydectin®. The data entry person would change the answer to Q27 from 'No' to 'Yes' if use of one an ML product brand was indicated in Q28.

Q30. When do you give summer drenches? Some respondents were circling more than one answer. All responses were accepted, and the number of circled months in the response taken to indicate the number of summer drenches used on the property. Two farmers did not circle any months, and this was taken to indicate that the farmer did not summer drench.

Q31. Please record the number of summer drenches used for each of the years and *Q32. Please record the number of summer drenches with MLs used for each of the years*. These tables listed anthelmintic products across the top of the table, and each of the rows indicated a different year in reverse chronological order. A number of issues arose in completing these tables. Some of the farmers that used drench combinations wrote the combination in (e.g. Ram + white), whereas others just recorded numbers (e.g. placing '1' in the Rametin® column and '1' in the white drench column in the same example). Answering in the latter way was the way respondents were directed to answer in the instructions provided; this method of answering appeared to cause some initial confusion with the data entry person.

Dividing this section of the questionnaire into two questions caused apparent confusion amongst some respondents when they had used, for example, a macrocyclic lactone summer drench in 1998/99. It would appear that some respondents recorded a number in the default column "Can't recall product, but drench used" for Q31, and then filled appropriate cells in Q32. In entering responses in the spreadsheet such double answers were recorded against the products that were indicated as having been used.

Q33. Please indicate approximately how many times during the last 5 years would you have drenched the listed classes of sheep during winter (May-Sept. inclusively)? The data entry person was concerned that the blank cells in the response table may have been missing answers. The frequency of winter drenching amongst respondents was low, with the median being 2 flock treatments in the 5 years. Thus answers with blank cells were a common occurrence.

Q35. If you have used Ivomec®, in which year did you first use this product? This was the question with the most missing answers (n=38) prior to telephone follow-up. Prior questions (Q24, 25, 28, 32 and 34) provided respondents with the opportunity to indicate use of Ivomec®, yet a portion of such respondents had not answered Q35. Such respondents were recorded as having given a blank answer in the spreadsheet. There were 25 farmers who answered this question on telephone follow-up; the remainder of respondents were not be contacted because their phone number could not be ascertained.

It became apparent during telephone follow-up that respondents appeared to want to provide an accurate answer to this question but could not recall with certainty if the first use was 5 or more years ago. The majority of these (n=14) had used Ivomec® around the time of its release (Table 3.2); the year of first use could be ascertained from their verbal response, which was along the lines of:

"I can't remember which year it was, but it was the summer after it was first released."

Knowing that Ivomec® was released in November, 1987 enabled a determination that the first use was either in 1988 or 1999; further questioning could determine which of those years was the appropriate answer. Use of similar non-leading questions enabled the other respondents contacted by telephone to narrow down the time of first use of this product.

Q37. After drenching your sheep are they put onto 'safe' or 'clean' pasture? Several respondents indicated that they put as many mobs as possible onto safe pastures. Such responses were allocated to the 'Half the time' response.

Q38. Approximately, how many weeks or months do you believe it takes to produce a 'safe' or 'clean' pasture on your property? Several respondents gave a range in their responses. The data entry person would enter the lower limit of the range in the spreadsheet.

Q39. Do you estimate the weight of sheep to determine the dose of drench to be given?

There appeared to be some confusion over the use of the word 'estimate' in this question. The intention had been to determine whether the farmer used weighing scales to determine the weight of the sheep in the mob in order to calculate the dose of drench to administer. During pilot testing one person indicated that 'estimate' included 'measure' in correct usage of the English language, so the wording of this question was changed. It became apparent that some respondents could have taken the vernacular interpretation of the word 'estimate' as being 'guess'. Responses were recorded in the spreadsheet as given.

Q40. Which method do you use to calculate the dose of drench to give to your sheep?

One of the alternative answers in this closed question was 'guess the weight'. Grammatically this was incorrect, and this alternative should have been 'calculate the dose from a guessed weight of the sheep'. This became apparent when a few responses circled both this answer and another alternative, suggesting that they firstly guessed the weight of the sheep, and then determined the dose to give based upon this guessed weight of the 'heaviest in the mob'. The response indicating calculation of the dose was entered in the spreadsheet.

Q50. There was no question 50 in the questionnaire.

Q51. From which sources do you obtain your information on worm control in sheep?

This question was followed by two questions asking the respondent to indicate the '*most important*' and the '*2nd. most important*' source of worm control information. Several farmers had only given one response to Q51, yet had answered both following questions. In this case, the second answer was entered in the spreadsheet under Q51.

Missing question. Missing from the questionnaire was a question asking for the respondent's telephone number. When the questionnaire was designed it had been the intention not to conduct any telephone follow-up, but this became necessary to obtain sufficient answers to Q35 to provide sufficient power for the survival analysis. It had not been anticipated that this question would have been so difficult to answer. In all 10 respondents could not be contacted by telephone because their telephone number could not be determined from the address details provided on their response.

Conclusion

The multivariable models presented in this thesis were based upon data derived from a questionnaire designed by the author. The discussion above and in Chapters 4 and 5 highlighted problems with the questionnaire that could compromise data quality. Nevertheless, through the attention to design and data entry, and through the achievement of an adequate response rate without any bias between responders and non-responders, the data used to construct the models could be considered of acceptable quality.

Chapter 7

DISCUSSION, CONCLUSION and FUTURE DIRECTIONS

The two papers that form Chapters 4 and 5 contain detailed discussions of the results of the study, although discussion of some other aspects is warranted herein:

Further discussion

In this study the ‘disease’ state was defined on the basis of a clinical diagnosis of ivermectin resistance at the 95% cut-off through the aggregation of results of three levels of testing (see Table 3.3). These tests were performed by different people at different times on different age groups of sheep which could result in observer or measurement bias or bias due to instrument error (Last, 1988). The presence of these biases should be considered in the interpretation of results of the study.

The use of the 95% cut-off could also result in measurement bias, although such a technique for continuous variables with a Gaussian distribution (as in haematological or biochemical clinical chemistry) is a well established method to differentiate ‘normal’ from ‘diseased’ patients (Kaneko, 1988). Admittedly there will be misclassification of some farms with FECRT results near the cut-off which is reflected in the poor sensitivity in the detection of early stages of anthelmintic resistance (Martin *et al*., 1989), but good specificity (Geerts and Gryseels, 2001). Further bias should be acknowledged through the use of data from a retrospective postal questionnaire, in particular through the accuracy of the farmer’s responses or recall bias.

There were particular problems in determining the biological significance of the variables in the logistic regression model (Table 4.3) that may have required the use of a further survey or another survey method (eg personal interview) to better elucidate the conclusions that were drawn. In particular the “winter drenching” variable may be subject to simultaneous-equations bias (Vågsholm, *et al*., 1991) in that it hasn’t been ascertained whether the increased drench use on farms with resistance is due to the presence of resistance and thus reduced worm control, or the cause of the resistance. This bias could also be considered in the inclusion of a variable related to drenching frequency in the study of Anchetta *et al*. (2004) in the Philippines, and in the models presented in the survival analysis (Chapter 5). There could be many other biologically plausible explanations for the variables “Years of farm ownership” and “Sold more sheep in 2000 than is usual” which cannot be deduced from the data in this survey, such as age of the farmer or education level, or a tactical response to seasonal conditions. The explanation for both of these variables could be subject to the bias of interpretation (Last, 1988)

Conclusion

This thesis represents an important contribution to the knowledge of the development of anthelmintic (in particular, ivermectin) resistance through the development of two multivariable models from the analysis of the data derived from a mail questionnaire of sheep farmers in southwest WA. Although the data set was small and failed to test the importance of summer drenching, this does not in any way detract from the validity and usefulness of the conclusions.

These two models, which described the risk of a farm having ivermectin resistance in 2000, and the rate at which ivermectin resistance developed on the study farms, appear to be the among the first multivariable models using observational data to study anthelmintic resistance. As such they have provided an opportunity to test the various hypotheses surrounding anthelmintic resistance on working sheep farms and provide indications as to which of these hypotheses have the more important roles to play in this disease. Scientific papers have been submitted for publication for each of these models, and the logistic regression risk factor model paper accepted.

This thesis has made some important findings:

- Winter drenching frequency appeared important in both the risk of and rate of development of resistance in *O. circumcincta*. This factor seemed to have been overlooked in scientific understanding due to the attention given to summer drenching (Besier and Love, 2003).
- The past history of the property was a factor in the development of resistance to anthelmintics. Past history was reflected in the logistic regression model by the variable "Farm owned since 1975" and in the survival model by the variable "Alternate anthelmintic groups available". The biological significance of the former was related the accumulation of sheep carrying worms with pre-existing anthelmintic resistance at the time of farm purchase; as the latter variable demonstrates, the less alternative anthelmintics available the quicker resistance to newer anthelmintics occurred.
- This thesis has demonstrated for the first time that the farmer influences the occurrence of anthelmintic resistance through the management decisions and commitment that they have to their sheep enterprise. The biological significance of the variable "Sold more sheep in 2000 than is usual" in the logistic regression model was explained this way, as were the two remaining variables in the survival model "Source of worm control advice" and "Always using safe pastures".
- This thesis would appear to have failed to test the refugia hypothesis adequately due to the almost complete reliance on summer drenching of the study farms. Further study is required to investigate the risk of factors associated with the refugia hypothesis.

- This thesis would appear to be the first to have studied the rate at which anthelmintic resistance has developed as described in the survival analysis (Chapter 5). Previous studies have been ‘point-in-time’ studies, such as the prevalence surveys that are commonly performed.

It is important that these findings are communicated to the scientific, veterinary and farming communities around the world so that the rate at which anthelmintic resistance develops is slowed. It is also important that these results are validated by further similar studies.

Further directions

This study has produced two multivariable models of ivermectin resistance on sheep farms in southwest Western Australia, being a logistic regression model for the risk of having resistance confirmed in 2000, and a survival model for the effective life of ivermectin on the study farms. To the author’s knowledge these are among the first two multivariable models studying farm level risk factors for anthelmintic resistance produced anywhere in the world. The paucity of such studies is likely a reflection of the separation of the disciplines of parasitology and epidemiology. Having said that, parasitologists do study the epidemiology of nematodiasis in great detail for a particular nematode life cycle in a variety of environments. What they have not looked at is the effect of differences between farms in worm or other farm management strategies on the development of anthelmintic resistance.

There are three other published studies looking at risk factors; the first two failed to develop multivariable models. Edwards *et al*. (1986b) studied benzimidazole and imidazothiazole resistance on farms in the same area of WA in the early 1980’s and produced tables of univariable associations using Chi-square tests of independence. The study of Edwards *et al*. (1986b) was published before logistic regression became widely available for use by field researchers (Hosmer and Lemeshow, 1989), perhaps explaining why the authors chose not to include a multivariable model to summarise the main risk factors. This author attempted to obtain the data of Edwards *et al*. (1986b) to complement the risk factor model for ivermectin resistance but was unsuccessful. Bartley *et al*. (2003) attempted to produce a generalised linear model for risk factors for benzimidazole resistance on sheep properties in Scotland in 2001 but failed to find any variables to satisfy the model requirements.

Ancheta *et al*. (2004) developed an analysis of variance multivariable model to provide risk factors for benzimidazole resistance on sheep and goat farms in the Philippines where *H. contortus* is the predominant cause of nematodiasis. In their study resistance was measured by LDA which gave a percentage efficacy for each flock as had Bartley *et al*. (2003). In the studies in Western Australia resistance was determined by a cut-off clinical diagnosis as used in this study (Edwards *et al*., 1986b).

The model developed from the Philippines study included a treatment frequency variable, and another being the number of years since first use of a benzimidazole anthelmintic, which are similar to variables in the two multivariable models developed in this study (Ancheta *et al*., 2004). Other variables included farm size, where larger farms were found to have had less efficacious anthelmintics, and the FEC of the sample tested for the study– farms with higher FEC were more likely to have more severe resistance. Farms that had recently imported animals from a nucleus or multiplier farm would have worse resistance presumably because the source farm used anthelmintics more frequently to ensure their animals were in good health for sale. Of interest was the protective effect of flocks having access to common grazing, previously identified by Papadopoulos *et al*. (2001) in Greece.

The refugia hypothesis is believed to explain the rapid emergence of ivermectin resistance in *Ostertagia circumcincta* in WA because of the reliance of farmers upon the practice of summer drenching for worm control. Only two respondent farmers indicated that they did not summer drench so the association between summer drenching and ivermectin resistance could not be studied adequately. Whether these farmers had only recently stopped summer drenching due to the change in the worm control extension message in WA to minimize this practice is unknown.

One purpose in attempting to follow-up the data of Edwards *et al*. (1986b) was to investigate the relationship between summer drenching and the development of anthelmintic resistance. One third of farmers in that 1980's study did not practice summer drenching, one third gave one summer drench and the remaining third gave two. At the univariable level that study showed that giving two summer drenches significantly increased the risk of having either benzimidazole or imidazothiazole resistance. It is unknown if this variable would have been retained in a multivariable model of risk factors for resistance to these anthelmintics.

Having produced these initial models it is necessary to validate these findings by repeating similar studies elsewhere in sheep raising regions of the world as anthelmintic resistance is emerging. For example main-effects risk factor models could be developed for ivermectin resistance in *O. circumcincta* in Victoria or New Zealand and for *H. contortus* in northern NSW or South Africa. Similarly models for benzimidazole resistance could be developed in various parts of the UK or in other sheep rearing nations where the prevalence of resistance to that class of anthelmintic is of a similar level to that of ivermectin resistance in WA. Main effects survival models could also be developed, although ideally subject farms would need to conduct frequent regular anthelmintic resistance testing throughout the study period, something that would have to be built into the protocol of any prospective study.

It may be unlikely that 'point-in-time' models will be able to answer questions about the refugia hypothesis. Although the models presented here do not adequately test the summer drenching link with ivermectin resistance, the survival model does, at the *prima facie* level, refute this hypothesis by including a variable dealing with safe pasture usage. What is likely to occur in the future in WA is a change from one or more blanket summer treatments to all sheep on the farm to a less intensive strategy. Survival analysis of farms not yet resistant may indicate changes in the relative hazard rates occurring at the time farmers change their summer drenching practices; such changes may give clues as to the validity of the refugia hypothesis as indicated by the direction of the change. In the other sheep raising areas of the world different factors may have to be used to assess the refugia hypothesis; for example in Victoria farmers using 'Smart Grazing' could be compared to farmers that give a regular summer drench program of two drenches without intensive grazing management.

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APPENDICES

APPENDIX 1 Questionnaire

Worm control practices on WA sheep farms

Your farm:

These identifying details will be kept confidential and stored separately from the rest of the questionnaire.

1. *Please provide your name* ....

2. *Your postal address* ....

....

Town .... Postcode ☐☐☐☐

Considering your MAIN or CENTRAL PROPERTY (i.e. where your homestead is, or where the main farm buildings are).

3. *What is the name of the road that the property driveway comes off?*

....

4. *This property will be identified by a land title. Please provide the appropriate answer, either*

Location District and Number .... , or

Plan Number .... and Lot Number .... , or

Diagram Number .... and Lot Number .... ?

5. *Give the property's average annual rainfall* .... mm or .... inches

6. *For how long have you operated this property?* .... Years

Now considering your TOTAL FARMING OPERATION,

7. *What is the total size of your farming operation?* .... ha or .... acres

8. *What is the effective area of your farming operation (i.e. land available for grazing or cropping)?*
.... ha or .... acres

9. *What proportion of your total farming operation's effective area was cropped in 2000?* ....%

10. *What proportion of your total farming operation's effective area was cropped in 1995?* .... %

Your sheep enterprise:

11. *What was the sheep winter grazing area of your farm in 2000?* .... % or .... Hectares

12. *How many sheep did you have as of 31/12/00?*

Please fill in the boxes:

Black tag (under 1)		Purple tag (1996)	
Blue tag (1999)		Green tag (1995)	
Red tag (1998)		Orange tag (1994)	
Yellow tag (1997)		White tag and older	

13. *Which of the following best describes your sheep enterprise.*

Please circle one:

All wool sheep   All prime lambs   A mixture   A sheep trading enterprise   A Sheep stud

14. *If your sheep enterprise is a mixture, what percentage of income was from prime lambs in 2000?*
.... %

15. *What was the average micron of your main adult fleece line shorn in 2000?* .... micron.

16. *How many sheep did you sell in 2000?*

Please fill in the boxes:

Shipping wethers		Prime lambs (black tag - 2000)	
Old wethers		Hoggets (blue tag - 1999)	
Cull for age ewes		Store lambs (black tag - 2000)	
Other ewes		Cull rams	

17. *Did you sell more or fewer sheep in 2000 than you usually? Answer THE SAME if it was within 10% of your usual sales policy.*

Please circle one option

MORE THE SAME FEWER

18. *How many sheep did you purchase in 2000?*

Please fill in the boxes:

Wethers		Store lambs (black tag - 2000)	
Ewes		Hoggets (blue tag - 1999)	
Rams		Others, please specify	

19. *Did you purchase more or fewer sheep in 2000 than you usually buy? Answer THE SAME if it was within 10% of your usual sales policy.*

Please circle one option.

MORE THE SAME FEWER

20. *What was the total wool clip for 2000?* .... BALES or .... kg or .... TONNES

21. *When did lambing commence in 2000?*

Please answer one or more, as appropriate:

Lambing date 1 Ewe Breed Ram Breed

Lambing date 2 Ewe Breed Ram Breed

Lambing date 3 Ewe Breed Ram Breed

22. **The total farming enterprise mix in 2000:**

Please fill in the boxes:

	Winter area used (hectares)	Number of livestock in winter (if applicable)
Cropping		
Cattle		
Pigs		
Other livestock - please specify:		
Agroforestry		
Other land uses - please specify		

Your sheep worm control practices:

23. *Do you give a drench to newly purchased or introduced sheep within 2 days of arriving on the property (a quarantine drench)?* YES / NO / SOMETIMES If NO, go to question **26**

24. *What drenches do you currently or usually use as a quarantine drench?*
Please circle <u>one or more</u> of the options below:

WHITE Drench e.g. Valbazen®	CLEAR Drench e.g. Nilverm ®	ML drench e.g. Ivomec ®	Rametin ®	Combination e.g. Scanda®
Systamex ®	Levamisole ®	Cydectin ®		Combi®
Panacur ®	LeviGold ®			
Mebendazole®				

25. *If you use one or more of the ML products as part (or all) of a quarantine drench, which do you use?*
Please circle:

Ivomec ®	Cydectin ®	Virbamec ®	Abamectin ®
Paramectin ®	Rycomectin ®	Other (please specify) ……………	

26. Did you give quarantine drenches to sheep introduced approximately 5 years ago?

YES / NO / SOMETIMES If NO, go to question **29**

27. *Did you use ML products as part (or all) of your quarantine drenching at that time?*

YES / NO / SOMETIMES If NO, go to question **29**

28. Which ML products did you use?
Please circle.

Ivomec ®	Cydectin ®	Virbamec ®	Abamectin ®
Paramectin ®	Rycomectin ®	Other (please specify) ……………	

29. *Do you use a summer drench program for your sheep?* **(Drenches** given during the period when there is usually no green pasture, i.e. between about November and April. Some farmers may describe this as 'giving a drench onto stubble').

YES / NO If NO, please go to the question on 33

30. *When do you give summer drenches?*

Circle one

Oct Nov Dec Jan Feb Mar Apr

Please fill out the two tables below:

An example may be:

If all of the sheep were drenched in November with Scanda ® (Combination), and with Rametin ® and Scanda® in March, then your answer would look like:

	WHITE drench	CLEAR Drench	Combination	Rametin ®
1998/99	0	0	2	1

31. *Please record the number of summer drenches used for each of the years*

Summer Period	WHITE Drench e.g. Valbazen® Systamex ®	CLEAR Drench e.g. Nilverm ® Levamisole ®	Combination e.g. Scanda ®	Rametin ®	Can't recall product, but drench used
2000/01					
1999/2000					
1998/99					
1997/98					
1996/97					
1995/96					

32. *Please record the number of summer drenches with MLs used for each year*

Summer Period	Ivomec ®	Cydectin ®	Virbamec ®	Paramec ®	Rycomectin ®	Abamectin ®	Can't recall product, but drench used
2000/01							
1999/2000							
1998/99							
1997/98							
1996/97							
1995/96							
1994/95							
1993/94							
1992/93							
1991/92							
1990/91							
1989/90							
1998/99							

Winter drench use:

33. *Please indicate approximately how many times during the last 5 years would you have drenched the listed classes of sheep during winter (May - Sept. inclusive)?:*

Class of sheep	Total No. of winter drenches since 1995	Class of sheep	Total No. of winter drenches since 1995
Shipping wethers		Prime lambs	
Other wethers		Hoggets	
Lambing ewes		Store lambs	
Other ewes		Rams	

34. *Over the past 5 years how many times (the total number of times) would you have used any of the following products during the winter period?:*

Ivomec ®		Cydectin*®		Virbamec ®		Abamectin ®	
Paramec ®		Rycomectin ®		Ivomec ® Maximizer capsules		Other	

* include Cydectin ® Weanerguard and Eweguard

35. *If you have used Ivomec®, in which year did you first use this product?* ....

36. *Have you ever used Jetamec® as a drench for your sheep?* YES / NO

37. *After drenching your sheep are they put onto 'safe' or 'clean' pasture?*
Please select one option from those below

ALWAYS USUALLY HALF THE TIME NOT OFTEN NEVER

38. *Approximately, how many weeks or months do you believe it takes to produce a 'safe' or 'clean' pasture on your property?*

In summer? .... Weeks or .... Months Not sure ....
In winter? .... Weeks or .... Months Not sure ....

39. Do you estimate the weight of the sheep to determine the dose of drench to be given?
YES / NO If NO, go to question 41

40. *Which method do you use to calculate the dose of drench to give to your sheep?:*
Please circle one option

Give them plenty more than they need?	The weight of the heaviest sheep	The average of the mob	Guess the weight	Other (please specify) ....

41. *Do you calibrate your drench gun before using it?*
Please circle one option

ALWAYS USUALLY HALF THE TIME NOT OFTEN NEVER

42. *Have you used worm egg counts to monitor the number of worms present in your sheep?*
YES / NO If NO, go to question 45

43. *In what year did you first use worm egg count monitoring?* ....

44. *In 2000, approximately how many flock monitorings were carried out?*
Please circle one option:
None 1 2 3 4 5 > 5 (Please specify...............)

45. *In which years was a drench resistance test done on your farm (Faecal Egg Count Reduction Test or a Drench-Rite ® test)?*
Please circle.

1986	1987	1988	1989	1990
1991	1992	1993	1994	1995
1996	1997	1998	1999	2000

Please record below the results of the most recent drench resistance test done on your farm (record drench group and result)?
46. Month and Year of Test ....

Drench Group	Result	Drench Group	Result

If you have access to previous results, please report these also.

46. Month and Year of Test ....

Drench Group	Result	Drench Group	Result

47. Month and Year of Test ....

Drench Group	Result	Drench Group	Result

48. Month and Year of Test ....

Drench Group	Result	Drench Group	Result

49. Month and Year of Test ....

Drench Group	Result	Drench Group	Result

The sheep worm control advice you receive

50. *From which sources do you obtain your information on worm control in sheep?*
Please circle all appropriate answers

Consultant Veterinarian Agriculture WA Stock Agent

Neighbour Farm Weekly AgNotes Regional Updates

Internet Radio Seminars Television

Other (please specify)? ....

51. Of these sources, which is THE MOST important source of information for you?

....

52. *Of these sources, which is THE 2nd. MOST important source of information on worm control for you?*

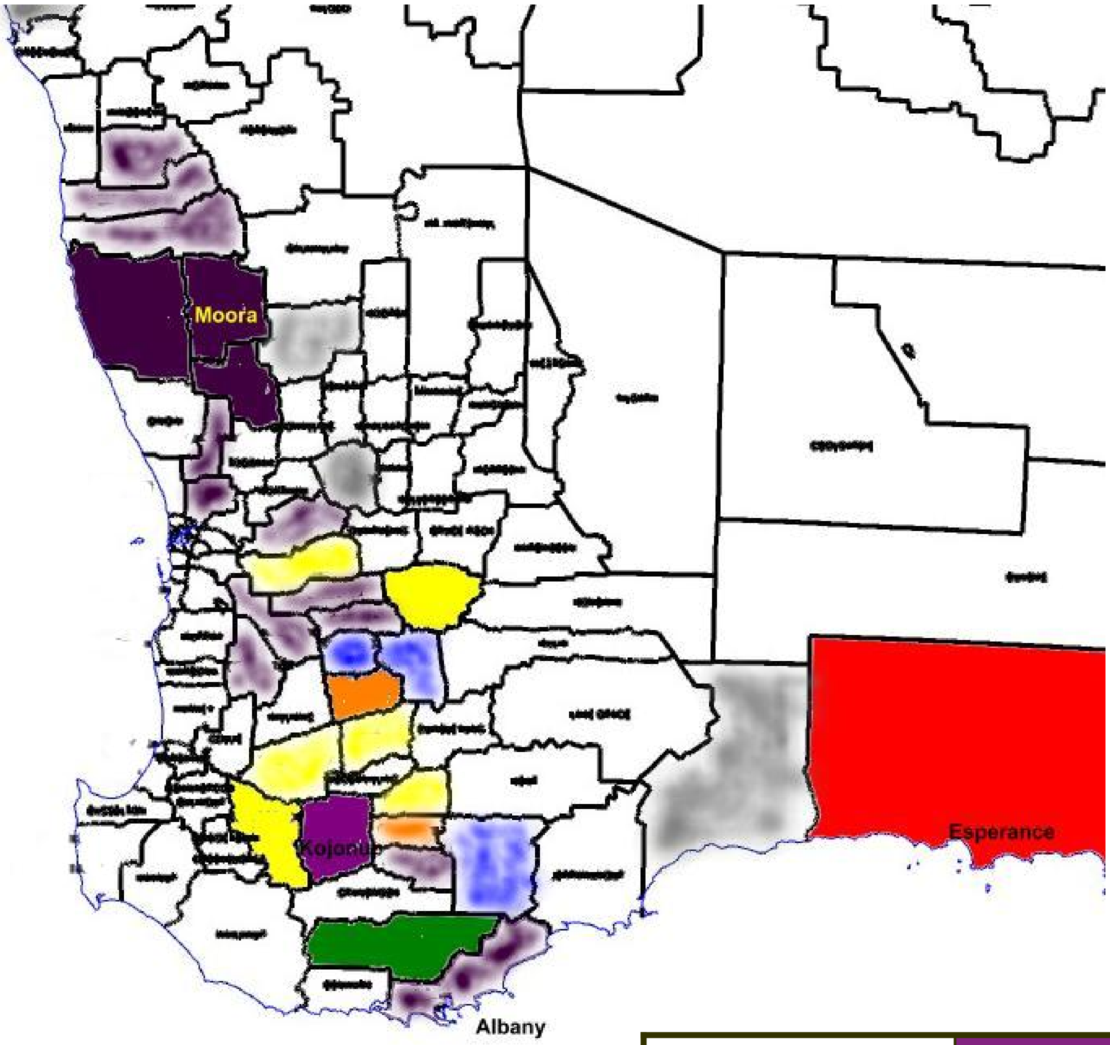
....

Thankyou for helping with this study. Please place the questionnaire (and signed consent form) in the stamped, addressed envelope enclosed and mail it back to:

Dr. Robert Suter
Murdoch University Veterinary Hospital
Murdoch WA 6150.

The information you have provided will help improve worm control practices. You will receive a summary of the results of this survey when data analysis is completed.

APPENDIX 2 **Shire prevalence of ivermectin resistance**



Zero	
0.2	
0.3	
0.4	
0.5	
0.6	
0.7	
0.9	
Not sampled	

Solid colours reasonable sample size;
Shaded areas n < 4.

APPENDIX 3 **Worm control zones in WA** (Wroth, 1996)