ANTHELMINTIC RESISTANCE IN *Haemonchus contortus*

by

FLAVIO AUGUSTO MENEZES ECHEVARRIA
B.V.Sc., M.V.Sc.

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine
University of Glasgow

Department of Veterinary Parasitology
University of Glasgow
October, 1991

ACKNOWLEDGEMENTS

I am most grateful to Professors J. Armour and J.L. Duncan for their friendship and supportive role as supervisors during the period of these studies.

Professor G. Gettinby from University of Strathclyde is greatly acknowledged for his advice and support on the statistical analysis as well as on the adaptation of his original simulation programme on anthelmintic resistance. The help of Steve Hazelwood on programming in the simulation model is also acknowledged.

I wish to thank Professor G.M. Urquhart, head of this department at the start of these investigations, for providing facilities for these studies.

Special thanks are due to Ken Bairden who provided constant support and friendship throughout this period. The skilled assistance of his technical team is also acknowledged.

Professor A. Tait at the Wellcome Unit of Molecular Parasitology, where the isoenzyme study was carried out, provided facilities and fruitful discussions. I am also grateful for the expert assistance of Mrs. N. Buchanan in his laboratory.

Drs. A. Pinheiro and M.F. Borba collected some of the field data in Brazil and their support is greatly appreciated. Special thanks are also due to A.M. Ferreira and L.C.O. Perez at Embrapa's laboratory.

During these studies I was granted a leave of absence from Embrapa, a scholarship from CNPq and an ORS award (Overseas Research Student Award by the Secretary of State for Education and Science, UK) which are much appreciated.

Finally, I would like to thank my wife Tania and my son Daniel for their moral support and understanding over the time spent in this project, mainly at the times that should have been devoted to them.

DECLARATION

The studies reported here are the work of the author with help of those listed on the acknowledgements. This thesis has not been submitted previously for the award of a degree to any university. The following publication was based on the work contained in this thesis:

Echevarria, F.A.M., Armour, J. and Duncan, J.L., 1991. Efficacy of some anthelmintics on an ivermectin-resistant strain of *Haemonchus contortus* in sheep. Veterinary Parasitology 39: 279-284.

SUMMARY

Studies were carried out on possible control measures using different drugs or management procedures which could help in the control of anthelmintic resistance in sheep nematodes, particularly that of ivermectin (IVM) resistance in *Haemonchus contortus*. Other aspects such as selection for IVM resistance, the effect of IVM on susceptible and resistant larval stages of *H. contortus* and the survival of their free-living stages under laboratory or field conditions were also investigated. In addition, a computer model to simulate the development of anthelmintic resistance in breeding ewes was examined incorporating Brazilian field data. Isoenzyme profiles of susceptible and resistant strains were also analysed.

Three strains of *H. contortus* were mainly used in these studies: one susceptible to all of the common anthelmintics; the same strain selected for resistance to IVM under laboratory conditions and a field strain resistant to a wide range of anthelmintics, including IVM, which was isolated from a farm in South Africa. The IVM selected strain was derived experimentally from the susceptible strain serially passaged in lambs and submitted to successive doses of IVM at 0.02mg/kg. Although the drug was highly efficient until the seventh passage and treatment, the progeny of the adult worms which survived this treatment were highly resistant.

Under controlled laboratory conditions, an experiment was set up to look at the development of eggs and larvae of the original susceptible strain in comparison to the selected strain. This showed that at 22^{0}C the resistant strain had a higher percentage development

from eggs to the infective stage while at 27°C the results were reversed, i.e., a significantly higher percentage of larvae developed from eggs of the original susceptible strain. Under field conditions of Southern Brazil, eggs from two strains of *H. contortus*, one resistant and another susceptible to IVM, were seeded on to pasture plots during three summer months. The results of these field studies indicated that resistant larvae survived better after only one of the contamination periods; at other times there appeared to be no significant difference in development rates. In all cases the recovery rate was low, indicating that during the summer months in Southern Brazil there is limited survival and development of eggs and larvae on pasture and that most of the parasite population is present in sheep during this period.

An experiment designed to exploit the residual anthelmintic effects of salicylanilide/substituted phenols, strategically administered during the summer in the conditions of Southern Brazil, was successful in controlling an IVM resistant population of *H. contortus* thus preventing the predictable outbreaks of haemonchosis in autumn. Of these drugs, nitroxynil gave the best results.

Another approach examined as an alternative method in the control of sheep nematodes under Brazilian conditions was the use of "safe pastures", i.e., areas with a low level of infectivity where young susceptible sheep could be grazed with a low risk of infection. In this case an infected pasture, reseeded after a crop of soya bean, was tested for larval infectivity in spring using lamb and calf tracers. After harvesting a soya bean crop from this area, it was found to be virtually negative for *H. contortus* and other important

nematodes.

Ivermectin treatment of IVM resistant or susceptible *H. contortus* at different stages of development indicated that the resistant strain started to show a lowered susceptibility to the drug as early as the fourth larval stage. On susceptible adult worms the drug was almost 100% effective whereas on the early fourth stage its efficacy was about 96%; this may be an indication that selection is more likely to start at the early stages of the parasitic life-cycle.

Following evidence of reduced efficacy of IVM in a field population of *H. contortus* in Brazil, this strain was isolated and submitted to a controlled anthelmintic test using several anthelmintics; this confirmed the presence of IVM resistance. From the major broad spectrum anthelmintics used, levamisole was the compound with the highest efficacy on both resistant and susceptible strains.

Isoenzyme profiles were used in these studies to analyse susceptible, IVM selected and multiple resistant strains of *H. contortus*. No differences in the electrophoretic mobility of most of the enzymes associated with resistance were detected, indicating a low level of enzyme variation in *Haemonchus*. However, the enzyme propionate esterase showed distinct bands for the multiple resistant strain and a single band for the susceptible strain, while the IVM selected strain showed little propionate esterase activity. This suggested that alterations in this enzyme might be correlated with BZ resistance, but not with IVM resistance.

A model developed to study anthelmintic resistance in sheep in UK was modified and fed with Brazilian field data. Although the model looked only at breeding ewes, it showed that the frequency of

resistant genes were likely to increase slowly during a period of 20 years under the control programme most used for this age group.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The development of chemical compounds effective against undesired organisms which could cause disease in man and in animals or threaten crops was a welcome innovation to methods which had been used previously. Some of these had a major impact in the control of certain agents as they were extremely efficient. For example, in the human sector in the early 1930's, sulphonamides and later penicillin and streptomycin were released for the control of bacterial agents; for the control of crop pests, DDT appeared in the mid 40's and for the treatment of gastrointestinal parasitism of animals, thiabendazole appeared in the early 60's as the first broad spectrum anthelmintic.

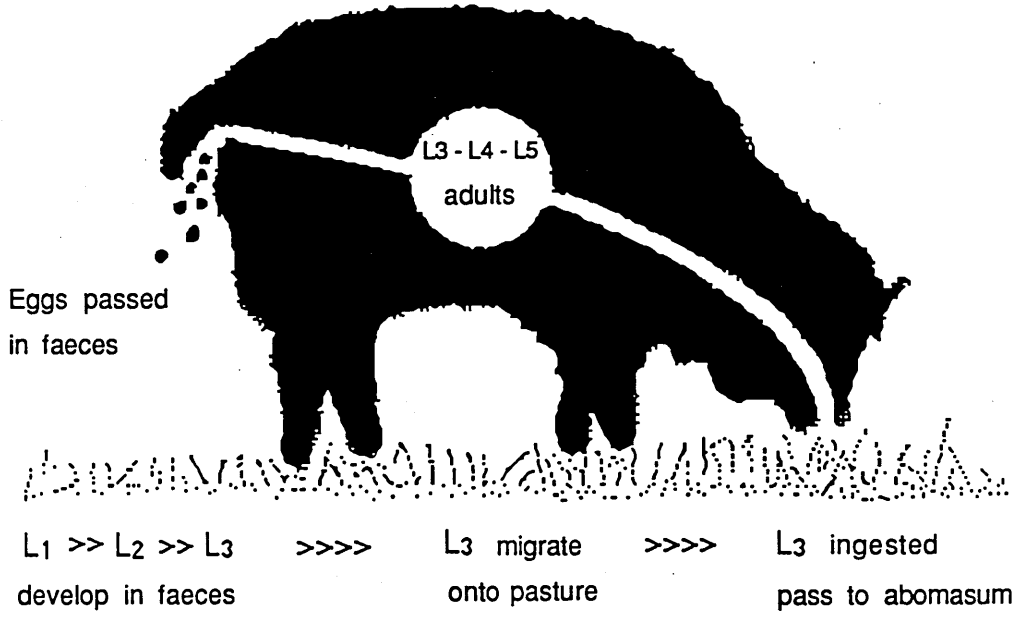
Organisms have what is called biological diversity and this can lead to some individuals, within a given population, having the ability to survive the effects of a given compound. This ability, to survive further exposure to a challenging drug, can be transmitted to their offspring, i.e., resistant individuals develop. Unfortunately this happened with the drugs mentioned above as bacteria became resistant to sulphonamides, penicillin and streptomycin; the housefly to DDT and sheep nematodes to thiabendazole. The appearance of resistance of certain organisms to a given drug may simply be a question of the period of time it has been used as resistance has evolved in a chronological sequence as various drugs have been released. However, more studies are needed to provide a better understanding of the resistance problem so that a better use can be made of existing drugs, and the useful life of new compounds can be

extended in the treatment and prevention of disease in both humans and animals.

The review which follows concentrates on anthelmintic resistance in sheep nematodes which cause some of the most serious health problems facing the sheep industry. As a starting point, it is important to define some expressions which will be used throughout the text and to describe the life-cycle of one of the most important nematodes of sheep, *Haemonchus contortus*.

Nematodes - as the name suggests, belong to the Class Nematoda. Most have a cylindrical form, tapering at either end, and the body is covered by a colourless, somewhat translucent layer, the cuticle. The cuticle is secreted by the underlying hypodermis, which projects into the body cavity forming two lateral cords which carry the excretory canals, and a dorsal and ventral cord carrying the nerves. The muscle cells, which are arranged longitudinally, lie between the hypodermis and the body cavity; the latter contains fluid at high pressure which maintains the turgidity and shape of the body. Most of the internal organs are filamentous and suspended in the fluid-filled body cavity. Locomotion is effected by undulating waves of muscle contraction and relaxation which alternate on the dorsal and ventral aspects of the worm. One of the most important sheep nematodes is *H. contortus* and a diagrammatic representation of its life cycle is given in Fig. 1.1. The females are very prolific egg layers and eggs are passed to the exterior in the faeces. Under suitable conditions of warm temperature and humidity these eggs develop and hatch to first stage larvae (L_1) which moult to the second larval stage (L_2). Both the L_1 and L_2

Fig. 1.1. Life-cycle of *Haemonchus contortus*



are motile and use bacteria as their principal food source. The L_2 then moults to the third larval stage (L_3) which is infective. The L_3 confined within the retained cuticle of the L_2 cannot feed and must survive on stored nutrients acquired by the earlier stages. Development from egg to infective larvae on pasture may be as short as five days but development may be delayed for weeks or months under cool conditions. After ingestion and exsheathment in the rumen, the larvae moult twice (L_4, L_5) in close apposition to the gastric glands. Just before the final moult (to L_5) they develop a piercing lancet which enables them to obtain blood from the mucosal blood vessels. As adults they move freely on the surface of the mucosa. The time from ingestion of L_3 to appearance of eggs in faeces (the prepatent period) is usually 17-21 days.

Anthelmintics - a group of drugs used in the chemotherapy of internal helminth parasites of animals. The ideal anthelmintic should kill both adult and immature stages of helminths without producing toxic effects in the host, have a short withdrawal period, should not cause any environmental hazards and should be inexpensive to use.

Tolerance - defined by Le Jambre (1976) as the natural ability of a helminth species population to withstand its first exposure to an anthelmintic. This ability may vary between populations of the same species and according to the drug used and method of administration, but when these criteria are fixed, it becomes a basic measure of tolerance of such a population.

Resistance - is a significant increase in the tolerance level i.e., an increase in the number of individuals within a population able to withstand doses of a compound which would prove lethal to the majority of individuals in a normal susceptible population of the same species. This increased resistance is a result of changes in gene frequencies caused by breeding from those individuals which have survived exposures to the drug. The most important characteristic of resistance is that it is genetically inherited.

Side-resistance - the term used when individuals in a resistant population are resistant to another compound with a similar mode of action (Kelly and Hall, 1979).

Cross-resistance - resembles side-resistance but involves anthelmintics with different modes of action (Kelly and Hall, 1979).

Multiple-resistance - used to describe parasites that are resistant to two or more different groups of anthelmintics resulting either from independent selection by each group or as a result of cross-resistance (Prichard *et al.*, 1980).

Selection pressure - determined by the dose rate of anthelmintic and by the proportion of the parasite population exposed to the drug. Selection pressure is high when the treatment leaves few survivors and there are few free-living stages which escape exposure (Waller, 1986).

Reversion - used to describe a decrease in the frequency of resistant individuals in a population of helminths following the withdrawal of the selecting anthelmintic (Kelly and Hall, 1979; Prichard *et al.*, 1980).

Counter-selection - describes reversion hastened by the use of an anthelmintic with a different mode of action to that which induced resistance (Waller, 1985).

Resistance factor (resistance ratio) - the ratio of the LD_{50} of the strain suspected of resistance to that of a known susceptible strain. Initially used as a measure of resistance of ectoparasites to insecticides, it is now also used with nematodes to compare levels of anthelmintic resistance between strains.

DETECTION AND MEASUREMENT OF RESISTANCE TO ANTHELMINTICS - METHODS AND LIMITATIONS

Usually, resistance is first suspected when a poor clinical response is obtained after an anthelmintic treatment of a flock. However, before an anthelmintic failure is detected by clinical signs of parasitism and loss of production, selection for resistance has already occurred, as a moderate reduction in anthelmintic efficacy may not be detected by the farmer. If the same anthelmintic, or another with the same mode of action, continues to be used, it will result in further selection of resistant parasites which will ultimately lead to a failure of control and consequently economic

losses (Le Jambre, 1978; Kelly and Hall, 1979; Prichard *et al.*, 1980).

On the other hand, an anthelmintic failure may not necessarily mean resistance as some clinical signs normally associated with gastrointestinal parasitism such as diarrhoea, anaemia and loss of condition are not specific and they could be due to other infectious agents, inadequate nutrition, trace element deficiencies and plant toxicoses (Prichard *et al.*, 1980; Presidente, 1985). Other factors can also contribute to an apparent failure of an anthelmintic treatment without the parasites having become resistant. Some of these include rapid reinfection due to heavily contaminated pastures, presence of developing or inhibited larvae unaffected by the chosen drug, faulty equipment, inaccurate body weight assessment, underdosing, failure to follow the manufacturer's instructions and selecting the wrong drug for the target parasite.

Before reviewing the various tests which can be used to evaluate anthelmintic efficacy, it should be pointed out that, in any investigation of suspected anthelmintic failure, a sound knowledge of the epidemiology of the parasites of the region is required. Also, information should be obtained on the type of worm control practices and drugs used currently and previously, dose rates and frequency of treatment, management history, stock introductions, age of animals and seasonal conditions prior to and at the time of the suspected failure.

Faecal worm egg count reduction test (FECR)

This simple technique is often the first used to evaluate any change in anthelmintic efficacy by comparing pre-treatment worm egg

count levels with those found five to ten days post-treatment. The failure of an anthelmintic to reduce worm egg counts was first reported by Drudge *et al.* (1957a) when sheep infected with *H. contortus* did not show reasonable reductions in worm egg counts after treatment with phenothiazine.

Although this is a cheap and easy way of detecting changes in anthelmintic efficacy, some authors (Kelly and Hall, 1979 and Prichard *et al.*, 1980) have called attention to some drawbacks of the technique such as:

- when animals are harbouring mixed infections, only one genus may be resistant, and therefore post-treatment worm egg counts should be followed by larval culture and differentiation to identify the species of parasites involved;
- immature stages which may survive anthelmintic treatment could develop to adults and then contribute to post-treatment egg counts;
- in some nematode species e.g., *Ostertagia* spp and *Trichostrongylus colubriformis*, the correlation between faecal worm egg count and worm count is not always linear (Sangster *et al.*, 1979a and Martin *et al.*, 1985). This was shown by Hotson *et al.* (1970) in *T. colubriformis* where thiabendazole reduced by 70% the faecal worm egg counts compared with those of controls, but at necropsy it was evident it had had little effect on adult worm burden; in a benzimidazole resistant strain of *O. circumcincta*, treatment with benzimidazoles suppressed egg counts at day seven post-treatment, but at 10 days after treatment worms had resumed egg production and a good correlation with worm burden at necropsy was obtained (Martin *et al.*,

1985);

- in goats, reduced efficacy of levamisole measured by faecal worm egg counts may be due to rapid metabolism in this host and, therefore, inappropiate dose rates rather than resistance (McKenna, 1984).

The efficiency of this test can be improved if, as mentioned above, larval cultures are conducted to determine the genus of the parasites surviving treatment, and also to detect larvae originating from low or negative egg counts due to the lack of sensitivity of the worm egg count technique in detecting very low number of eggs (e.g. less than 50 eggs/gram). The interpretation can also be improved by the addition of an untreated control group; this will provide data on any changes in worm egg counts unrelated to treatment occurring during the monitoring period.

A reduction of less than 90% in worm egg counts following anthelmintic treatment has been used as a marker to indicate the presence of resistance in sheep flocks (Webb *et al.*, 1979; Kettle *et al.*, 1981; 1982; Riffkin *et al.*, 1984) but resistant parasites may also be detected when reductions are greater than 90%, for example, in mixed infections.

Controlled anthelmintic efficacy test

This is the most accurate test to confirm the presence of a suspected nematode resistant population and was described by Moskey and Harwood (1941), Gibson (1964) and Turton and Clark (1974). More recently, The World Association for the Advancement of Veterinary Parasitology set guidelines for such tests including experimental design, animal maintenance, nematode infections, treatment, necropsy

and worm count procedures (Powers *et al.*, 1982). Basically this test involves:

- infection of worm-free animals with the suspected resistant strain;
- at or about 28 days after the infection one group of animals is treated with the anthelmintic under investigation and the other group is left as untreated controls;
- at necropsy total worm counts in the treated groups are compared to those of the untreated groups and the efficacy of the drug is then determined by the formula:

$$\% \text{ efficacy} = (C - T / C) 100$$

where C and T are the mean worm counts of untreated and treated groups respectively.

The inclusion in the test of animals infected with a known susceptible strain has been recommended by Prichard *et al.* (1980) to confirm drug efficacy and thus serve as a positive control group.

When more detailed information about a resistant nematode population is required, a range of dosage rates must be used (usually 0.5, 1.0 and 2.0 times the normal recommended dose) from which the LD_{50} can then be estimated (Le Jambre, 1978).

When anthelmintic efficacy against immature nematodes has to be determined, the controlled anthelmintic efficacy test is used and slaughter is carried out at varying and appropriate times after the infection (Reinecke *et al.*, 1962).

As this is a costly test to perform due to the large number of animals required, laboratory animal models, e.g., guinea pigs have been used to test benzimidazole and levamisole resistance in some

nematodes, e.g., *T. colubriformis* (Kelly *et al.*, 1981). Unfortunately, this is not applicable to other genera because of the host specificity of some parasites.

Although high costs are involved in this kind of test in target animals, it does give very reliable information without the problems of interpretation associated with results from model systems and *in vitro* tests (Boersema, 1983).

In addition to the FECR and the controlled anthelmintic efficacy test, a series of *in vitro* assays have been developed for the detection and measurement of anthelmintic resistance.

In vitro **egg hatch assay**

Based on the fact that benzimidazoles prevent embryonation, hatching and consequently the production of free-living stages of benzimidazole susceptible parasites and that resistant nematodes are refractory to this ovicidal effect, a number of techniques have been developed for the detection of anthelmintic resistance (Le Jambre, 1976; Coles and Simpkin, 1977; Hall *et al.*, 1978; Whitlock *et al.*, 1980a). These tests depend on the separation of eggs from the faeces followed by their incubation in a series of dilutions of a benzimidazole anthelmintic, usually thiabendazole, and consequent determination of the percentage of larvae which embryonate and hatch. From dose response curves on such data an ED_{50} (concentration of drug which prevents development of 50% of the eggs) can be estimated by the use of log dose-probit lines (Le Jambre, 1976) or by arc-sine transformation (Cawthorne and Whitehead, 1983).

It has been demonstrated by Borgsteede and Couwenberg (1987) that

different ED_{50}'s can be obtained not only from different strains but also within a strain. These differences can occur for a variety of reasons including:

- differences in time spent from faecal collection to incubation because, as eggs develop, aerobic metabolism predominates and eggs become refractory to the ovicidal activity of benzimidazole anthelmintics (Le Jambre, 1976; McCallister, 1976);
- different incubation times being used (24-72 hours);
- some authors counting only hatched eggs, others hatched and embryonated eggs;
- some authors using commercial preparations and others the pure compounds;
- different solvents being used to prepare the drug solutions.

To overcome the problem of day to day variability in the test, *in vitro* assays are compared using known resistant and susceptible strains, which act as positive controls, since eggs from resistant strains hatch in higher concentration of benzimidazole than the eggs from susceptible strains (Le Jambre, 1976). Whitlock *et al.* (1980a) have suggested that when the *in vitro* egg hatch assay is used to test a population of parasites, eggs surviving a discriminated concentration should be cultured to the infective larval stage to identify the anthelmintic resistant genus.

As the *in vitro* egg hatch assay is based on the ovicidal effect of drugs, it had been used only with the benzimidazoles. More recently Dobson *et al.* (1986) described an egg hatch assay to be used with levamisole. In this case, freshly collected eggs are incubated in water until one hour before hatching when the drug is then added and

the eggs incubated for a further period.

In vitro larval motility test

Levamisole, pyrantel and morantel act as nerve ganglion stimulants in nematodes leading to rapid tonic paralysis (Van Neuten, 1972). Based on this mode of action Martin and Le Jambre (1979) developed a technique to measure resistance to these anthelmintics in *Ostertagia* spp. by assessing the percentage of paralysed third stage larve after exposure to serial dilutions of such drugs. The motility of *H. contortus* has also been assessed by a micromotility meter (Folz *et al.*, 1987). In this test larvae, after being exposed during 24 hours to different concentrations of a given drug, are placed in a three-channel micromotility meter (developed at Michigan State University). A light located at the base of each channel projects upward through the contents; movement of the larvae causes a disturbance of the light rays and, consequently, a variation in the electrical signal produced by a photodetector. The average deviation of the signal from its mean value is then determined by means of an amplifier, A/D converter and a microcomputer. This average deviation given in arbitrary A/D converter units is the motility index.

Differences in the quantities of cholinesterase enzymes have been found in benzimidazole resistant and susceptible *H. contortus*, *T. colubriformis* and *O. circumcincta*. This has led to the development of a larvae paralysis assay for benzimidazole resistance using incubation of infective *H. contortus* larvae in the presence of physostigmine, which inhibits acetylcholinesterase, resulting in a greater rate of reversible paralysis in those larvae with the least

enzyme content, i.e., those susceptible to benzimidazoles (Sutherland *et al.*, 1988).

The principal problem with this kind of technique is that larvae recover from paralysis after prolonged exposure to these drugs and also atypical dose response curves are obtained, as high concentrations of levamisole are less effective than lower concentrations in immobilising parasites (Coles *et al.*, 1975; Martin and Le Jambre, 1979; Barton, 1983; Coles *et al.*, 1988). Furthermore, there is some subjectivity in assessing whether a larva is paralysed or not, and the assay cannot be preserved for counting at a later time (Dobson *et al.*, 1986).

Tubulin binding assay

Resistance to benzimidazole anthelmintics in nematodes has been associated with a reduction in the ability of these compounds to bind to the structural protein tubulin, in resistant individuals (Lacey, 1985; Lacey and Prichard, 1986; Lacey and Snowdon, 1988). Based on these findings, an assay was developed consisting basically of the measurement of the binding of tritiated benzimidazoles to crude supernatants of third stage larvae (Lacey and Snowdon, 1988). Although, in general, there is a good correlation between the ovicidal activity of the benzimidazoles and their ability to bind to tubulin extracts (Lacey *et al.*, 1987), different correlations occurred between individual benzimidazoles with mebendazole giving the best correlation (Lacey and Snowdon, 1988). According to the authors, this is a sensitive technique to detect minor changes in the susceptibility status of nematode populations. However, the technique is suitable

only for single species infections as in mixed infections only one genus may be responsible for resistance. It also requires large number of larvae.

Larval development test

The *in vitro* egg hatch assay does not allow identification of the resistant parasite because of the similarities in size and appearance of the eggs of related genera. Only infective larvae are sufficiently different and have generic or specific characteristics which allow reliable differentiation. Because of this, tests have been developed (Waller *et al.*, 1986; Coles *et al.*, 1988) in which nematode eggs are exposed to serial dilutions of drugs in special media and allowed to develop to the third larval stage when they are examined and identified. This has been used successfully with the benzimidazole compounds. However, with drugs such as levamisole and ivermectin, a normal dose response curve is not obtained (Coles *et al.*, 1988; Giordano *et al.*, 1988) as at the higher dose concentrations, a reduced percentage kill is observed. Nevertheless, this test is now routinely used by some laboratories (Taylor, personal communication) as their method of choice for screening suspected resistant strains as, although it does not allow determination of ED_{50}'s, it is still possible to detect resistance at a "positive/negative" level.

ANTHELMINTICS

Mode of action

Anthelmintics can be classified according to their mode of action, e.g., drugs which interfere with the neurophysiology of the parasite and those that affect essential energy metabolism. They can also be subdivided according to their spectrum of activity as broad spectrum or narrow spectrum anthelmintics. The efficacy and pharmacology of anthelmintics for use in ruminants have been reviewed by many authors (Prichard, 1978; Prichard *et al.*, 1980; Arundel, 1985; Bogan and Armour, 1986; McKellar and Scott, 1990) and on the basis of this work, drugs are classified in the following groups:

- benzimidazoles and pro-benzimidazoles
- imidothiazoles
- tetrahydropyrimidines
- avermectins
- salicylanilides and substituted phenols
- organophosphates.

Table 1 gives a summary of these groups with some of their respective compounds.

Table 1.1. - Anthelmintics for nematode control in ruminants and their classification according to spectrum and mode of action.

BROAD SPECTRUM
1- Tubulin binding
Benzimidazoles: Thiabendazole, Parbendazole, Mebendazole, Cambendazole, Oxibendazole, Fenbendazole, Albendazole, Oxfendazole, Flubendazole
Probenzimidazoles: Febantel, Thiophanate, Netobimin
2- Ganglion blocking
Imidothiazoles: Tetramisole, Levamisole
Tetrahydropyrimidines: Pyrantel, Morantel
3- Gamma-aminobutyric acid potentiators
Avermectins: ivermectin, oxidectin
NARROW SPECTRUM
4- Uncouple oxidative phosphorylation
Salicylanilides: Clioxanide, Rafoxanide, Oxyclozanide, closantel
Substitute phenols: Nitroxynil, Disophenol,
5- Acetylcholinesterase antagonists
Organophosphates: Haloxon, Dichlorvos, Naphthalophos, Trichlorphon

Adapted from Waller, 1986.

Benzimidazoles and pro-benzimidazoles

The mode of action of this group of drugs was first believed to be based on the inhibition of the fumarate reductase enzyme system which is vital for production of energy in most parasitic helminths. Since this mode of action was proposed by Prichard (1970), there have been many reports of inhibition of this system in nematodes and cestodes by benzimidazoles (Malkin and Comacho, 1972; Romanowski *et al.*, 1975; Barrowman *et al.*, 1984). However, benzimidazoles also cause some other more generalised effects, for example, on the uptake and metabolism of carbohydrates (van den Bossche, 1976), on microtubules (Borgers *et al.*, 1975) and on the secretion of acetylcholinesterase (Watts *et al.*, 1982). The observation by Borgers *et al.* (1975) that mebendazole induced disintegration of the microtubular framework in nematode cells led to the consideration of a possible link between this interaction and the inhibition of glucose absorption and protein secretion. Microtubules are composed of, and exist in equilibrium with, the soluble protein tubulin. During cell division microtubules form the spindle which is responsible for chromosome separation (Lacey, 1985). Using mammalian tubulin, benzimidazole carbamates were observed *in vitro* to be potent inhibitors of the rate of polymerisation of tubulin to form microtubules, demonstrating competitive inhibition to colchicine, a basic microtubule inhibitor (Friedman and Platzer, 1980; Koheler and Bachmann, 1981). The fact that there are differences in the susceptibility of host and parasite to the effects of benzimidazoles might be due to differences between parasite and mammalian tubulin (Dawson *et al.*, 1984). This data provided strong evidence for the involvement of tubulin in the mode of

action of benzimidazoles which was reinforced by the elucidation of the mechanism of benzimidazole resistance in *H. contortus* and *T. colubriformis* (Lacey and Prichard, 1986; Sangster *et al.*, 1985), where a reduction in the binding of [^{3}H] BZ to tubulin protein from resistant strains was observed, as well as effects on microtubules, [^{3}H] colchicine binding and acetylcholinesterase secretion, which was consistent with a reduction in affinity of nematode tubulin for benzimidazoles. Furthermore, Lacey *et al.* (1986) also found a good correlation between inhibition of nematode egg hatch and inhibition of mammalian tubulin polymerization, which is consistent with binding to tubulin being the primary site of action of benzimidazoles.

Thiabendazole (TBZ) was the first broad-spectrum anthelmintic to be developed and it was quickly accepted in the early 60's as the drug of choice to control internal parasites as it was safe to use and highly active on most gastrointestinal nematodes, although it was not effective on inhibited *Ostertagia ostertagi* and *Dictyocaulus* spp. In the following years many other benzimidazole compounds were introduced namely: cambendazole (CBZ), parbendazole (PBZ), oxibendazole (OBZ) and mebendazole (MBZ) and then came a third wave - fenbendazole (FBZ), oxfendazole (OFZ) and albendazole (ABZ) - with an extended spectrum of activity against adult, immature and inhibited larvae of nematodes with some having activity on cestodes and trematodes. It has been suggested that this greater spectrum of activity is a function of their pharmacokinetic behaviour rather than intrinsic differences in activity. As these drugs are less soluble than the earlier benzimidazoles, their rate of dissolution, passage through the gastrointestinal tract and absorption into the systemic circulation is

slower compared with that of for example, thiabendazole, in which maximum plasma concentrations in sheep are reached in four to six hours and excretion is almost complete by 24 hours (Tocco *et al.*, 1964); in contrast, maximum plasma concentrations of FBZ and OFZ occurs between 24 and 36 hours in sheep and cattle (Marriner and Bogan, 1981a,b; Prichard *et al.*, 1978; Ngomuo *et al.*, 1984) and measurable concentrations in plasma and abomasal fluid can be detected for up to five days following a single dose.

Benzimidazole compounds, once administered to an animal, can be metabolised to more active metabolites. For example, febantel, a pro-benzimidazole, is metabolised *in vivo* to FBZ which in turn is reversibly oxidised to OFZ (Marriner and Bogan, 1981b). ABZ is oxidised to the corresponding ABZ sulphoxide and sulphone (Marriner and Bogan, 1980).

Although the benzimidazoles are among the least toxic of all anthelmintics (it has not been possible to determine the LD_{50} of either TBZ or FBZ), four of this group, CBZ, PBZ, OFZ and ABZ, have been found to have teratogenic effects which limits their use in pregnant animals (Bogan and Armour, 1986; Marriner and Armour, 1986).

Recently benzimidazoles have been administered in slow release devices. This has been done with OFZ in a continuous release bolus (Anderson and Laby, 1979) and in a pulse release bolus with five therapeutic doses which are released on five occasions at 21 days intervals, thus providing anthelmintic protection for about 100 days (Bogan *et al.*, 1987; Armour *et al.*, 1988).

The anthelmintic activity of febantel, thiophanate and netobimin is due mainly to the formation of benzimidazoles through *in vivo*

ruminal and hepatic metabolism, and because of this, they are called pro-benzimidazoles (Bogan and Armour, 1986). Thiophanate is metabolised by the rumen microflora to produce a benzimidazole carbamate with similar activity to the early benzimidazoles. Febantel, a phenylguanidine, is absorbed from the gastrointestinal tract and undergoes cyclisation in the liver to form FBZ and OFZ (Delatour *et al.*, 1982). Netobimin, a nitrophenylguanidine, according to McDougall *et al.* (1985), undergoes metabolic reduction from a nitro group to amino group previous to cyclisation to form ABZ in the rumen or in the liver.

Imidothiazoles

The two compounds in this chemical group used as anthelmintics are tetramisole which is a racemic mixture of D and L isomers and levamisole in the pure L form. The efficacy of these drugs is restricted to the L isomer. Levamisole as pure L form is less toxic and it is used at half the dose rate of tetramisole, but both drugs have a relatively low therapeutic index although toxicity is rarely seen. Levamisole is non teratogenic and so it is safe for use in pregnant animals.

The mechanism of action of these drugs is on the parasite's nervous system causing ganglion stimulation which in turn leads to paralysis of the parasites and their expulsion from the host by peristaltic movements (Janssen, 1976). This paralysis may be reversible (van den Bossche, 1976). At very high concentrations levamisole also affects the energy metabolism in nematodes by inhibition of fumarate reductase (van den Bossche, 1976).

Levamisole is quickly absorbed and excreted. Maximum plasma levels are reached within one hour following subcutaneous injection and two to three hours after oral administration. Most excretion of the drug occurs within 24 hours (Bogan *et al.*, 1982).

These drugs have a good activity against adult and developing larval stages of nematodes but not on inhibited larvae and they are not ovicidal like the benzimidazoles. At the moment these drugs are available as tetramisole and levamisole for oral or subcutaneous injection. There are also pour-on, in-feed and bolus formulations of levamisole.

Tetrahydropyrimidines

Pyrantel and morantel are the two members of this group which are similar in structure to the imidothiazole group. The mode of action of this group is believed to be the result of their effect as depolarising muscle relaxants producing paralysis in parasites (Coles *et al.*, 1975). This paralysis has also been shown to be reversible; Martin and Le Jambre (1979) found that *Ostertagia* spp. when exposed to either morantel or levamisole recovered at a faster rate with morantel than with levamisole. This, together with the findings of side-resistance in *Ascaris suum* (Coles *et al.*, 1975) and *Ostertagia* spp. (Martin and Le Jambre, 1979), means that they are probably acting on similar or on adjacent receptors.

Pyrantel and morantel have good efficacy against adult stages of gastrointestinal nematodes but are less active on developing stages and have minimal activity on inhibited larvae (Jones, 1983): they are not ovicidal.

The drugs in this group are sold as oral formulations and from the beginning of this decade morantel has also been available as a sustained release ruminal bolus designed to release drug over 90 days. This bolus has been used strategically at turnout of cattle in Europe and its effectiveness in the prophylaxis of bovine parasitic gastroenteritis has been confirmed (Armour *et al.*, 1981; Jones, 1981; Taylor *et al.*, 1985).

Avermectins

The discovery of this chemically distinct group of anthelmintics by Burg *et al.* (1979) has provided a welcome alternative for nematode control. The avermectins are a group of naturally occurring macrocyclic lactone disaccharides produced by *Streptomyces avermitilis*; ivermectin (IVM) which is currently the most active of those synthesised, being effective at doses measured in micrograms/kilogram, has been marketed since 1981.

This group, including IVM, is currently thought to paralyse nematodes by potentiating the release and binding of the neurotransmitter gamma aminobutyric acid (GABA) allowing accumulation of GABA at the nerve synapse and thus blocking nerve impulse transmission. In insects, GABA is an inhibitor of neurotransmission acting at neuromuscular junctions. Ivermectin potentiates the action of GABA at these sites and causes reduced muscle membrane resistance so that neuromuscular transmission is inhibited and the organism is then unable to move (Fritz *et al.*, 1979). The lack of effect of IVM on the mammalian central nervous system, at concentrations which are therapeutic against nematodes is probably because GABA receptors are

restricted to the central nervous system and the high molecular weight and polarity of IVM prevents the molecule from passing through the blood-brain barrier. Nevertheless, in some breeds of dogs, e.g., collies, there have been reports of some defective transport mechanisms in the blood-brain barrier and IVM has been shown to cause neurotoxicity in these animals (Pulliam *et al.*, 1985).

Ivermectin has no activity against cestodes and trematodes because they do not appear to possess GABA-mediated nerves and IVM has no action on cholinergic nerves.

As ivermectin is mainly excreted through the faeces, regardless of the route of administration (Halley *et al.*, 1989), this may delay degradation of faecal pats by causing mortality of important fauna in the faeces (Wall and Strong, 1987). However, this may depend on the duration of the treatment (e.g., single oral doses compared with bolus formulations) and also on the climatic region as McKeand *et al.* (1988), in the West of Scotland, did not find differences in the breakdown of faeces from cattle which received three routine treatments of IVM at five week intervals compared with faeces from untreated animals.

Ivermectin is highly efficient against all common gastrointestinal nematodes of sheep and cattle and against *D. viviparus*. It is also effective against some external parasites (Nolan *al.*, 1985). Currently, ivermectin is marketed as a subcutaneous injection and pour on for cattle, as an oral drench for sheep and as an oral paste for horses. The drug persists in tissues for some time and it has been shown that it protects against the development of infective larvae of some genera, ingested for about two weeks after

a single administration (Barth, 1983; Bremner *et al.*, 1983; Armour *et al.*, 1985) as well as being highly effective against most of the nematodes present at the time of treatment. The oral formulation for sheep is also highly efficient against nematodes but, because it is more rapidly eliminated when given *per os*, an injectable formulation is recommended for treatment of psoroptic mange (Campbell, 1985). Ivermectin is also safe for use in pregnant animals (Campbell and Benz, 1984).

Salicylanilides and substituted phenols

The drugs in this group act by uncoupling oxidative phosphorylation which in turn causes energy depletion (Cornish *et al.*, 1977; Prichard, 1978b). They attach to plasma protein (Broome and Jones, 1966) and this may explain their restricted activity against blood sucking parasites (Sinclair and Prichard, 1975; Prichard, 1978a,b), the exception being niclosamide which is poorly absorbed from the gastrointestinal tract and is only active against cestodes and immature paramphistomes.

Due to the long half-life of disophenol and closantel, their anthelmintic activity extends for periods of four to six weeks against *H. contortus* (Hall *et al.*, 1981). Because of this long half-life their withdrawal periods are usually long and, in consequence, some have not been registered for use in a number of countries.

Organophosphates

The mode of action of the drugs in this group (naphthalophos, trichlorphon, haloxon and dichlorvos) is by the inhibition of the

activity of the acetylcholinesterase enzyme system which interrupts the mechanism of neurotransmission in nematodes, thus increasing acetylcholine and resulting in continued stimulation of the nerve endings leading to a spasmodic paralysis (Lee and Hodsden, 1963; Hart and Lee, 1966; Knowles and Cassida, 1966).

The drugs in this group have a low therapeutic index (Prichard *et al.*, 1980). Their narrow spectrum of activity in ruminants, mainly against *H. contortus*, means that they should be used strategically when animals are harbouring mainly infections of this parasite.

GENETICS

Development of resistance

The rate at which resistance will develop depends upon factors such as genetics, e.g., type of inheritance, dominance, level of resistance and relative fitness, biological or ecological factors, e.g., life-cycle, reproduction, generation turn-over, stage exposed to the discriminating drug and proportion of population in refugia and operational factors, e.g., type and efficiency of anthelmintic, intensity of application, mode of action, rotation of drugs, dose rates and grazing management.

The appearance of anthelmintic resistant strains of parasites may be explained by the evolutionary theory where the original population of worms contained a few rare individuals carrying the capacity (genes) to survive the treatment. These individuals must be rare and not linked to genes conferring some fitness advantage, e.g., survivability, otherwise they would have spread through the population and a new drug would not have been successful in the first place

(Crow, 1957). As the drug kills all the susceptible individuals, the next generation will consist of the progeny of those few resistant parasites which survived the treatment and many of these will have inherited the ability to survive anthelmintic exposure.

A resistant population will be obtained rapidly if resistance is controlled by a single gene or at a slower rate if it is produced by several genes in which case they would need to work together to express the resistance trait (Le Jambre, 1985). This inference has been drawn from the work with insects but in helminths, where a worm population may have a higher proportion of free-living individuals on pasture than in the parasitised host and in consequence not exposed to drug selection, this may be different. Unfortunately there have been only a few genetic studies reported on helminths; Le Jambre *et al.* (1979a) studied resistance in a population highly resistant to TBZ and concluded that thiabendazole resistance in *H. contortus* was inherited as a polygenic autosomal semi-dominant trait. The second genetic study on *H. contortus* was carried out by Herlich *et al.* (1981) on a CBZ resistant strain which contained about 40% susceptible worms; these authors concluded that resistance was not sex-linked and was probably inherited recessively but was also due to a multigenic trait. Martin *et al.* (1988b) studying the inheritance of TBZ resistance in *T. colubriformis* concluded that it was due to more than one gene and inherited as a co-dominant character with a strong maternal influence. This is in general agreement with the previous theoretical proposal of McKenzie (1985) that selection acting on variation found within the normal distribution of individual drug tolerances, would act preferentially on polygenic variation. In

another study on a levamisole resistant strain of *T. colubriformis* (Waller *et al.*, 1985), from which data was later fed into a computer for modelling by Dobson *et al.* (1987), it was suggested that the high degree of levamisole resistance in *T. colubriformis* was due to a single gene but "vigour tolerance" or low level polygenic resistance was also observed.

Although most of the work conducted so far has provided more support for the polygenic inherited trait theory, Oppenoorth (1983) has questioned whether benzimidazole resistance in the field is really polygenic as most studies have been conducted in the laboratory under intensive pressure.

Depending upon the behavioural and physiological characteristics of the parasites, a proportion of the population may be in refuge, escaping exposure to, and consequently selection by a chemical. Georghiou and Taylor (1977a), in a model simulating evolution of insecticide resistance, found that when 20% of the population were in refugia, 50% of the individuals become resistant after more than 20 generations, but when the number of individuals escaping treatment was zero, 50% of the population were resistant in one generation. In nematodes, free-living stages, and some developing parasitic stages are not affected by some drugs and so are said to be in refugia. In *H. contortus*, Martin *et al.* (1981) found that when only up to 10% of the population were in refugia, resistance developed very rapidly but when 30 or 75% were in refugia, resistance was significantly delayed. The stages in refugia must produce offspring before any anthelmintic selection occurs in order to contribute to the delay of appearance of resistance. This will depend upon the interval between treatments in

relation to the pre-patent period of the parasite and the rate of development and survival of the free-living and parasitic stages (Donald, 1983a). Le Jambre (1978) estimated that during an outbreak of haemonchosis in summer rainfall areas of Australia, about 3% of the total parasite population are harboured by the sheep and so anthelmintic treatment restricted to such times allows the largest proportion of the free-living population to escape exposure to selection pressure.

Reversion

Based on priciples drawn from the study of insecticide resistance (Brown and Pal, 1971), it can be expected that once the drug or the group of drugs to which a parasite population has become resistant is withdrawn from use, this population may return to the initial level of tolerance at a rate that will depend upon the *relative biological fitness* of the resistant and susceptible individuals involved and also on the *frequency of resistant parasites* within the population.

In benzimidazole resistant nematodes reversion appears to be very slow or apparently non existent (Colglazier *et al.*, 1974; Le Jambre *et al.*, 1979a; Whitlock *et al.*, 1980b; Berger, 1980). Kelly and Hall (1979), working with a BZ resistant population of *H. contortus* that had not been exposed to that chemical group of drugs during five years, found that when it was reintroduced under laboratory conditions, it was 95% effective at the first passage but by the third its efficiency had fallen to less than 10%.

Waller *et al.* (1986) reported on a strain of *T. colubriformis* which was resistant to TBZ but had apparently reverted to

susceptibility after six years of levamisole use, in the meantime it had become highly resistant to levamisole. Passages of this strain under laboratory conditions without further anthelmintic treatments resulted in a reduction in the level of LEV resistance, this reversion being faster when counter selected with TBZ. Nevertheless, TBZ "counter" selection resulted in a return to TBZ resistance at a faster rate than occurred in another strain with no prior resistance history.

Donald *et al.* (1980) and Waller *et al.* (1983) described a BZ resistant strain of *Ostertagia* spp. which when exposed to levamisole, under both field and laboratory conditions, showed a significant reduction in BZ resistance. This reduction was also significantly greater than that of the progeny of worms not exposed to any anthelmintic treatment for six months. According to the authors, this was due to levamisole either selecting against BZ resistance or simply removing the resistant worms which, because of the prevailing conditions, were replaced by BZ susceptible worms. Similar results were also reported by Martin *et al.* (1988a), who working in a dry region of South Australia and using only levamisole on a TBZ resistant population of *Ostertagia* spp., found that this decreased the level of TBZ resistance. Nevertheless, an anthelmintic assay on this population showed that its level of resistance was still too high to allow adequate control by TBZ.

An important point that has to be considered is that in laboratory work, resistant strains are continuously passaged from one host to another without exposure to natural environmental conditions. This protects the resistant worms from competition and natural selection with other strains by exposure in the environment.

Nevertheless, there is sufficient information to suggest that the rate of natural reversion does not offer much hope for reintroduction of the anthelmintics to which resistance has developed within a reasonable period of time without the risk of further rapid selection for resistance.

Physiological differences between resistant and susceptible individuals

One of the central problems in the assessment of the development of anthelmintic resistance concerns the general fitness of resistant and susceptible individuals, fitness being defined as the relative reproductive success (e.g., egg production and survival) of a parasite genotype within a population.

The study of anthelmintic resistance in nematodes is in its infancy in comparison to studies in insecticide resistance and, because of this, many concepts are drawn from work on insects. In studies of insecticide resistance it has been shown that resistant individuals are usually at a reproductive disadvantage compared with susceptible ones in the absence of drug selection, otherwise they would be much more common and drugs would not be effective in the first place (Crow, 1957; Abedi and Brown, 1960; Georghiou and Taylor, 1977b).

In nematodes, studies related to differences in fitness between resistant and susceptible stains have shown conflicting results. Kelly *et al.* (1978) studying some of these differences in *H. contortus* resistant or susceptible strains found fecundity, pathogenicity and

survival of free-living stages to be greater in the resistant strain. On the other hand, Prichard *et al.* (1978b) found that a susceptible strain of *H. contortus* and *T. colubriformis* caused 32% mortality and clinical parasitism in surviving sheep within six weeks of infection, whereas in sheep infected with the same number of larvae from a resistant strain (VRSG as used by Kelly *et al.*, 1978) no deaths or clinical signs were found. Also Vlassoff and Kettle (1980) reported that an ABZ resistant strain of *H. contortus* had an establishment rate of only 8% compared with 30% in the susceptible stain. With this resistant strain, although smaller worm burdens developed, faecal egg count levels were similar to that produced by the susceptible strain which suggested a higher egg fecundity. In a study by Berger (1975) a resistant strain of *H. contortus* had similar establishment rates to a susceptible strain (58% vs. 55%), although the egg counts were higher in the susceptible (3330 e.p.g.) than in the resistant strain (1975 e.p.g.). Martin *et al.* (1988a), after four years of use of levamisole on a field population of *Ostertagia* spp resistant to TBZ found that the resistance ratio (based on *in vitro* tests) had fallen from 14 to 4.5, but when thiabendazole at 88 mg/kg was used again on naturally infected animals, the level of resistance was still too high for adequate control by TBZ. In their study no significant reversion occured in this strain when TBZ treatment ceased, or in the laboratory when passaged in the absence of anthelmintic, or when "counter" selected with levamisole. According to these authors levamisole did not select *in vivo* against TBZ resistance but rather the use of levamisole in the field might have altered the population dynamics, suggesting that parasites with the BZ resistant phenotype

were less fit than susceptibles during their free-living stages, especially their ability to survive the hot dry summer conditions characterististic of the Australian winter rainfall region.

These conflicting results may be due to natural differences as some strains had been kept under laboratory conditions and others were only recently isolated from the field. They may also be due to differences in levels of resistance, as the work on insecticide resistance has also shown that intensive drug selection may alter the background genome around the resistant allele leading to a greater fitness of the resistant individuals (McKenzie *et al.*, 1982).

NEMATODE CONTROL PROGRAMMES AND ANTHELMINTIC RESISTANCE

The ways in which anthelmintics are used have implications on the development of nematode resistance.

Therapeutic treatments - In many cases anthelmintic treatments are given when sheep are showing some clinical signs of parasitism. In such conditions the adult parasites have already made a great contribution to the environmental contamination with offspring which were not exposed to selection pressure by any drug. For example, Le Jambre (1978) estimated that during an outbreak of haemonchosis, in some areas of Australia, sheep would be harbouring only 3% of the total parasite population; anthelmintic given at such times would have little impact on the total population.

Suppressive treatments - Suppressive anthelmintic treatment regimes have been shown to increase animal production due to a reduction in the total parasite population below a level necessary to produce losses (Anderson, 1972; 1973; Johnstone *et al.*, 1979). These programmes may be applied in sheep flocks during short periods as a way of avoiding losses during periods known to be favourable for worm transmission. However, if such programmes approach monthly drenching (Johnstone *et al.*, 1976; 1979; Van Wyk *et al.*, 1987), selection pressure is likely to increase markedly because as drenching intervals approach the pre-patent period of nematodes, the resistant worms will be able to continue uninterrupted reproduction in the host, whereas the susceptibles will have little opportunity to infect animals, reach maturity and produce eggs before they are exposed to the next treatment (Donald, 1983b). Examples of such programmes influencing the development of anthelmintic resistance to benzimidazoles and levamisole have been shown by Barton (1980; 1983), Martin *et al.* (1982; 1984) and more recently with ivermectin, Van Wyk and Malan (1980). These latter authors described a population of *H. contortus* highly resistant to ivermectin and closantel which had been used at intervals of less than a month over a period of 2.5 years. During this time ivermectin was administered 11 times and by the end of the 2.5 years its efficacy was only 33%; this strain of *H. contortus* also showed multiple resistance to closantel and benzimidazoles. It is interesting to note that although levamisole was also used 22 times on the same population during the same period, resistance to levamisole did not develop.

Strategic treatments - The basic aim of strategic nematode control programmes is to administer drugs when the parasites are at their lowest level on pasture or at times when the weather is providing the least ideal conditions for the survival of free-living stages. By eliminating environmental contamination, animals grazing these areas would be subsequently exposed to very low numbers of infective larvae and in consequence effects on production and need for treatments would be reduced. This epidemiological concept has been used in some countries and the best and most recent example has been in Australia where two programmes named "Wormkill" and "Drenchplan" have been developed. These programmes require fewer drug treatments than have been used in the past (Dash *et al.*, 1985; Waller, 1986) thus decreasing selection pressure for resistance and at the same time reducing the costs of worm control by at least 25% (Waller, 1986). These programmes rely on strategic use of anthelmintics, such as closantel, to control *Haemonchus* and according to Waller (1987) they are successful mainly because they are effective and offer savings in cost and time to the farmer. Closantel, however, has a prolonged anthelmintic effect associated with a slow decline in blood levels which could select rapidly for resistance in *Haemonchus*. For example, Van Wyk and Malan (1988) have reported a case of resistance to closantel in *H. contortus* from a property in South Africa where this drug was used only four times during 1983 and then once in January 84, January 85 and May 86, when the compound was reported to have an efficacy of only 41.3%. Nevertheless, anthelmintic resistance in farms in Australia which have been using these strategic programmes have not been reported so far.

Sustained or controlled release devices - anthelmintics have been incorporatated into sustained or controlled release devices to prolong the period that the drug is in contact with the parasites. One of the main advantages of these new technologies of worm control is that they may decrease costs by reducing the number of occasions that animals have to be handled for treatment with consequent savings in labour (Anderson, 1985). Current sustained or controlled release devices are administered orally as boluses, remain in the rumen and operate either on a diffusion or on a dissolution principle. An example of a diffusion system is the morantel bolus for cattle. This bolus consisted originally of a stainless steel tube filled with a blend of morantel and a carrier giving continuous release over a period of about 90 days (Jones, 1983). The ends of the tube had a semi-permeable membrane which allowed slow diffusion from the reservoir. Recently the stainless steel tube was replaced by a rolled sheet of laminated ethylene vinyl acetate containing morantel which is wrapped with cellophane tape. Once administered the tape disintegrates and allows the laminate to unroll and diffusion, then, starts to take place. Diffusion down a concentration gradient means the release rate will not be constant. The release of the drug with this system has been shown to decline from a mean of 171 mg/kg during the first 30 days after administration to a mean of 47 mg/kg between days 60 and 90 (Jones, 1983). Also, as the animals are growing throughout the season, the dose, on a bodyweight basis, will obviously be reduced. This decline in drug concentration over a prolonged period of time associated with the fact that the morantel bolus does not completely prevent parasite reproduction (Brusdon and Vlassoff, 1981; Jones,

1981) suggests that the release rate may be in a discriminating range and could select for resistance.

The alternative to diffusion is the dissolution system in which the choice of carrier matrix determines the rate of dissolution and thus drug delivery. Examples of this are the bolus described by Laby (1978) used for releasing OFZ in cattle (Anderson and Laby, 1979) and sheep (Anderson *et al.*, 1980) and the commercially available cattle "Autoworm" bolus which also contains OFZ. The Laby bolus, recently marketed in Australasia and in some European countries, is a cylindrical polypropylene capsule containing a core of alternate pellets of carrier matrix and active ingredient held against an orifice by a tension spring. On contact with ruminal fluid the matrix forms a gel at the orifice which dissolves into the digesta and releases the drug. This system has also been developed in the "Autoworm" bolus where a cylindrical device made of PVC contains five or six individual cells with a corroding central alloy core; surrounding the central alloy core each individual cell comprises a white circular, hollow-centered tablet containing either 750 or 1250 mg of OFZ and a silicone rubber sealing washer. In this dissolution system, release rates are linearly related to the area of the dissolution orifice and remain approximately constant during the steady state of release; this is followed by a relatively rapid decline after dissolution is completed so that dose rates below the effective level are much reduced in comparison to the diffusion system thus decreasing the risk of selection for resistance (Anderson, 1985). These newer dissolution devices should be administered at crucial times to prevent pasture contamination or when larval numbers are

declining on pasture so that a high level of control can be expected. By using high release rates, selection for resistance may be delayed as any heterozygotes will be killed thus rendering resistance effectively recessive (Anderson, 1985).

Anthelmintics in feed and water - Since Gordon (1939) reported that daily low intakes of phenothiazine produced a reduction in the egg laying capacity of nematodes, a number of formulations incorporating that drug in licks and blocks have been developed for prophylactic control of nematode infections. Modern anthelmintics have also been used at low doses in feed supplements (Jones *et al.*, 1978), drinking water (Downey *et al.*, 1974; Downey and O'Shea, 1977; Nielsen *et al.*, 1983) and in feed blocks (Thomas, 1978) to provide an alternative method of limiting nematode populations. All these, however, rely on voluntary intake and so depend on each animal or a sufficient number of animals ingesting an adequate amount of drug each day to achieve the desired effect. In one study it took 3 to 35 days for sheep to consume a therapeutic dose of FBZ which had been incorporated into a feed block (McBeath *et al.*, 1979). Also, a wide range in plasma concentration of levamisole was found in heifers having access to drinking water containing this drug, 20% of these animals having plasma levels well below those thought necessary for anthelmintic effect (Nielsen *et al.*, 1983). All these methods of "self treatment" have one problem in common which is the range of drug intake, which means suboptimal dose rates, thereby reduced efficacy and in consequence selection for anthelmintic resistance.

Integrated control systems - Anthelmintic treatment combined with stock relocation to safe pastures with low levels of infective larvae, i.e. those previously grazed by adult stock or aftermath grazing after a silage or hay crop has been removed, has been recommended as beneficial in worm control. However, a small proportion of nematodes may survive treatment given at the time animals are moved and these, including potentially resistant individuals, may be the only source of further infections on the new pasture. Examples of increased resistance derived from such integrated control practices have been reported by some authors (Vlassoff and Kettle, 1980; Cawthorne and Whitehead, 1983; Martin *et al.*, 1985). Martin (1986) reported data from an experiment established to quantify the increase in resistance in *Ostertagia* spp. resulting from a "dose and move" strategy. Dosing with TBZ elevated the level of resistance in eggs of a partially TBZ resistant strain by 2.7 times compared with eggs from similarly infected but undrenched groups. Although this drench was shown in slaughtered animals to reduce *Ostertagia* worm burdens by an estimated 33%, this was not statistically significant nor did it reduce the worm egg output from grazing sheep. According to the author this study showed that once a partial level of resistance is present, a dose and move strategy will have little impact on the size of the parasite population but will increase the level of resistance.

PREVALENCE OF ANTHELMINTIC RESISTANCE IN DIFFERENT GEOGRAPHICAL AREAS

Australia - most reports on anthelmintic resistance come from Australia; whether its prevalence is higher there than in other countries, or whether this is simply a reflection of the fact that more surveys have been conducted there than elsewhere is not certain.

The first survey for resistance conducted by Webb *et al.* (1979) in the Northern Tablelands of New South Wales showed that in 48% of sheep flocks treated with TBZ at 66 mg/kg, the depressions in worm egg counts were less than 90% and that the main parasite involved was *H. contortus*. This apparent lowered efficacy of benzimidazole drugs led sheep farmers to use levamisole and morantel. More recently, Love (1985) conducted a survey on the same farms and reported that oxfendazole produced satisfactory control of *Haemonchus* which suggested some reversion to benzimidazole susceptibility following the dramatic fall in use of benzimidazoles and a switch to levamisole and morantel in the region. At the same time, however, *Trichostrongylus* spp. had become resistant to levamisole/morantel in more than 80% of those flocks (Love, 1985) and *Ostertagia* spp. in about 60% (Waller, 1985). In another survey in the same area (Newman, 1984) it was reported that on average ewes were treated 6.9 times/year and lambs 8 times/year which could be considered as a strong selection pressure for resistance. Also, according to Waller (1985), these frequent treatments were aimed mainly at the control of *Haemonchus* and the increase in resistance in *Ostertagia* spp. and *Trichostrongylus* spp. may have been exacerbated by a manufacturer's recomendation that only

half the normal dose rate of levamisole was necessary for the treatment of *H. contortus* (Waller, 1985).

Widespread reports of anthelmintic resistance to benzimidazoles and levamisole/morantel in *Ostertagia* and *Trichostrongylus* spp. have also been reported in other areas of Australia such as Victoria (Cameron *et al.*, 1984) and the south-west region of Western Australia (Edwards *et al.*, 1986a). In contrast, Riffkin *et al.* (1984) surveyed 104 farms in western Victoria and concluded that resistance in *Ostertagia* and *Trichostrongylus* spp. was not prevalent in that region. However, this survey was conducted during winter and spring at which time faecal egg counts in lambs are usually low and only 10 flocks had sufficiently high egg counts to allow a faecal reduction test to be carried out: this raises doubts about the conclusions of this survey.

New-Zealand - Surveys have shown that the level of BZ resistance in *Haemonchus* and *Trichostrongylus* populations are relatively low, and although resistance was confirmed in appoximately 20% of the flocks it was at a level unlikely to cause treatment failure (Kemp and Smith, 1982; Kettle *et al.*, 1981; 1982). Around the same time, a nationwide survey of anthelmintic usage by 614 sheep farmers (Brunsdon *et al.*, 1983) showed that they had failed to implement recommended strategic prophylatic control programmes for parasitic gastroenteritis continuing, instead, with frequent anthelmintic treatments. This is considered to be responsible for the increase in control failures due to the development, under these conditions, of highly resistant *Ostertagia* spp. (Hughes and Seifert, 1983) and *Nematodirus* spp.

(Middleberg and McKenna, 1983).

South Africa - Anthelmintic resistance of *H. contortus* to benzimidazoles (Berger, 1975; Van Wyk and Gerber, 1980a), rafoxanide (Van Wyk and Gerber, 1980a) and closantel (van Wyk *et al.*, 1982) have been reported. Although there have been no extensive surveys of anthelmintic resistance in South Africa, Van Wyk *et al.* (1987) reported that anthelmintics are used intensively on irrigated pastures and usually are the only form of parasite control employed. This may have led to the development of resistance of *H. contortus* to ivermectin in at least four properties within three years of its initial use.

Britain - Evidence of BZ resistance in *H. contortus* on seven out of 52 farms was detected in a survey conducted by Cawthorne and Cheong (1984) in the south of England and three BZ resistant strains of *O. circumcincta* have also been isolated from sheep in this region (Britt, 1982; Cawthorne and Whitehead, 1983).

More recently, a survey conducted on 40 farms in the north of England by Evans *et al.* (1988) using the *in vitro* egg hatch assay failed to find evidence of BZ resistance. In contrast, a survey in the south of England carried out on 55 farms, selected for having a history of long usage of benzimidazoles, showed that 34 had BZ resistant nematodes, mainly *O. circumcincta* and *H. contortus* (Taylor and Hunt, 1989). In Scotland, resistance to the BZ's has also been shown to be present by Mitchell *et al.* (1991) who screened 37 sheep farms throughout the country; using either the *in vitro* egg hatch

assay or a faecal egg count reduction test they reported that 24.3% of those farms had evidence of BZ resistance, *O. circumcincta* being the predominant resistant nematode.

Europe - Isolated cases of resistance to anthelmintics have been reported in Switzerland (Jordi, 1980) and in The Netherlands (Boersema *et al.*, 1987; Eysker *et al.*, 1982; 1983). In The Netherlands low levels of BZ resistant *H. contortus* were detected in more than half of the flocks examined (Boersema *et al.*, 1987).

USA - Although anthelmintic resistance of *H. contortus* to phenothiazine (Drudge *et al.*, 1954; 1957a; 1957b) and to thiabendazole (Drudge *et al.*, 1964) were first reported in the USA, there have been no reported surveys on the prevalence of resistance in commercial flocks and most of the reports are restricted to research farms. Strains in one of these farms at Ohio University were found to be resistant to benzimidazoles (Herd, 1980) and following a changeover to levamisole, which was used over a period of 10 years, they also became resistant to this drug (Herd and McClure, 1988).

EPIDEMIOLOGY OF NEMATODE PARASITES OF SHEEP IN SOUTHERN BRAZIL AND THE EMERGENT PROBLEM OF ANTHELMINTIC RESISTANCE

Brazil has a sheep population of between 16 and 17 million animals of which 55% are in the south region, mainly in the state of Rio Grande do Sul where 95% of the wool sheep are grazed (Fig. 1.2). The main breeds are Corriedale, Merino and Romney Marsh which are almost entirely reared on natural pastures and on mixed grazing with beef cattle. Climatically in the south there are four seasons: summer (December, January, February), autumn (March, April, May), winter (June, July, August) and spring (September, October, December). Normally the rainfall (1200-1300 mm/year) is evenly distributed throughout the year (Fig. 1.3) but there is high evaporation during the summer and occasional long droughts. The normal mean temperature varies between a maximum of 30.5^{0}C for January and a minimum of 8.1^{0}C for July (Fig. 1.4).

This type of climate suits many of the gastro-intestinal nematodes of sheep though *H. contortus* is the most important, causing outbreaks of disease, usually in autumn.

Epidemiological studies, using either tracer lambs at different times of the year or untreated permanent grazing lambs, have been carried out in four different areas in Rio Grande do Sul (Fig. 1.2) namely Bagé: (Pinheiro *et al.*, 1987), Guaíba (Gonçalves, 1974), Itaqui (Santiago *et al.*, 1976), Uruguaiana (Carvalho, personal communication 1990).

Fig. 1.2. Location of four epidemiological studies carried out in the State of Rio Grande do Sul

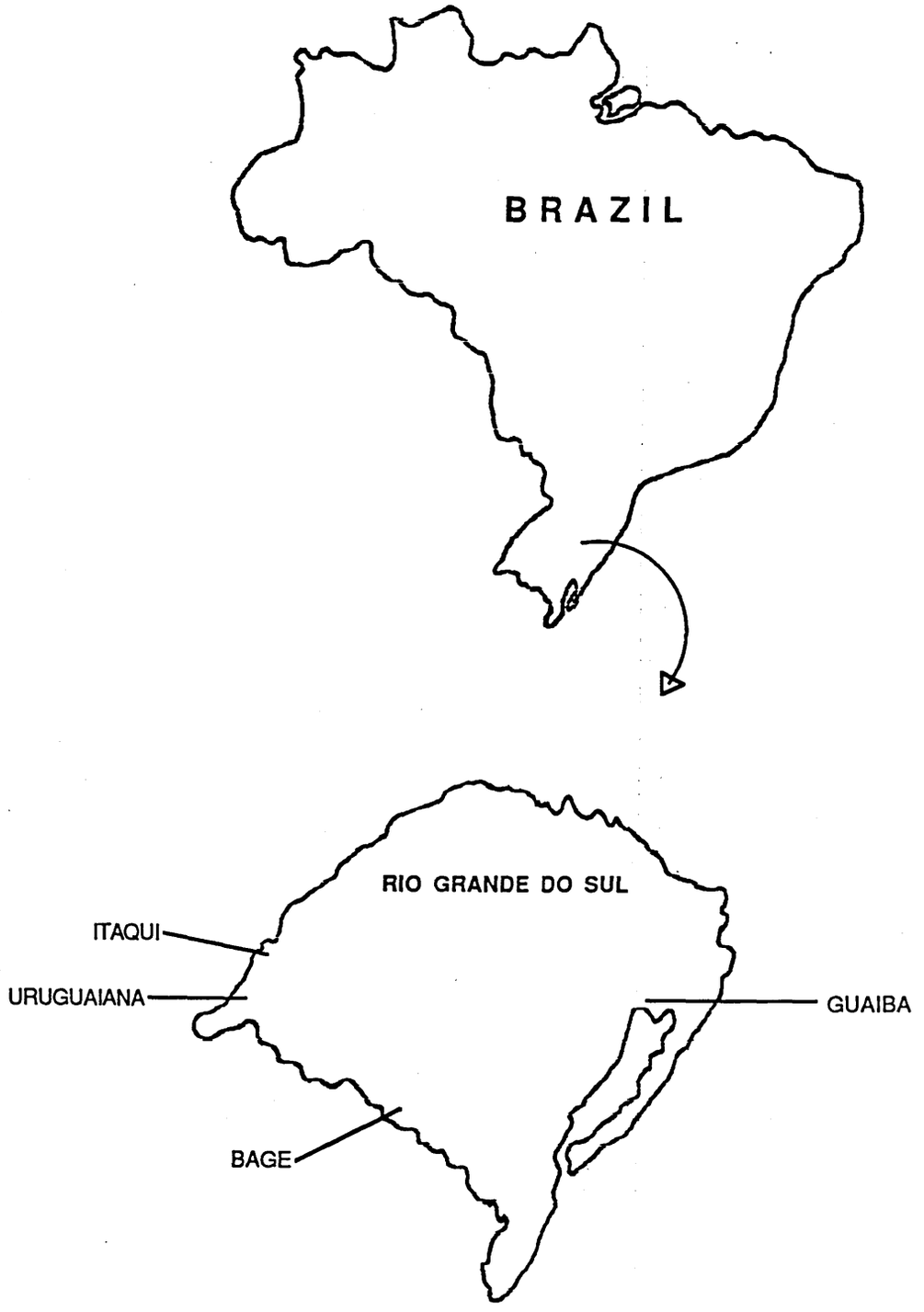


Fig. 1.3. Climatic conditions in Bagé
(Mean 1974-89)

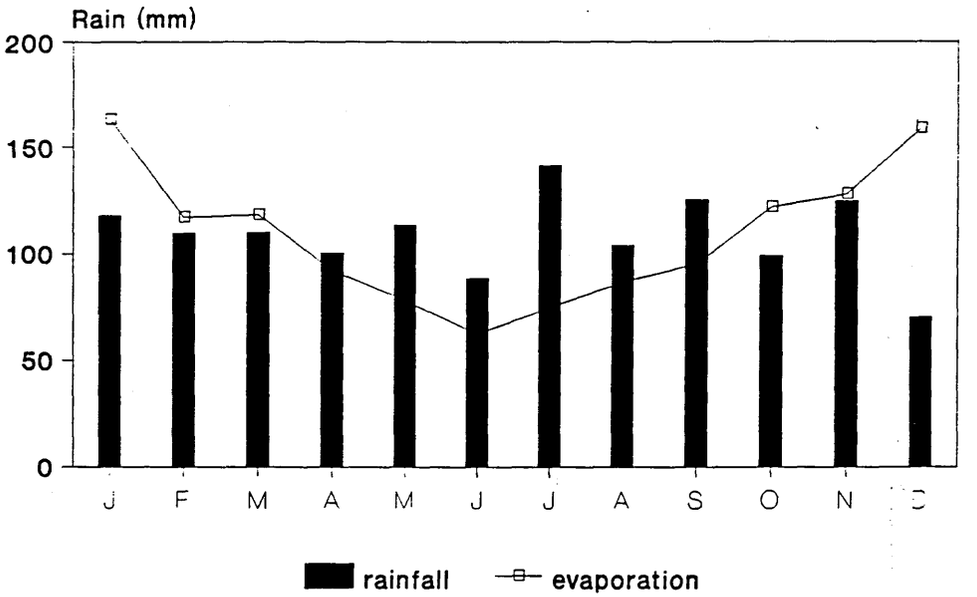
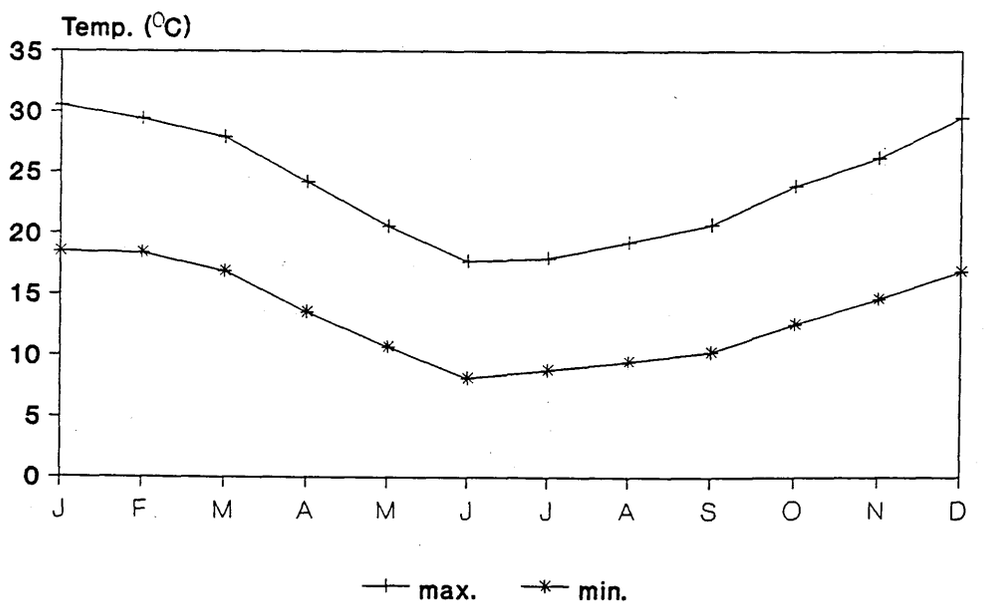


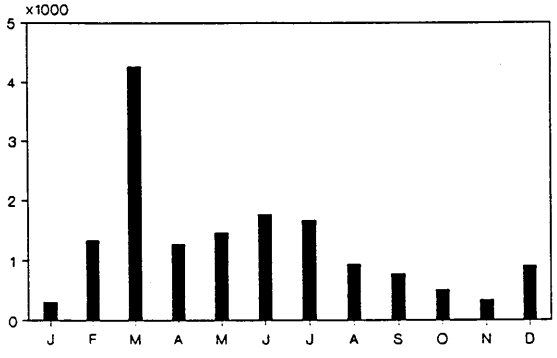
Fig. 1.4. Climatic conditions in Bagé
(Mean 1974-89)



From these studies (Figs. 1.5 to 1.12) it is evident that *H. contortus* is the most pathogenic species of nematode in sheep and outbreaks of haemonchosis occur from mid-summer up to mid-winter (Figs. 1.5 to 1.8). The most favourable weather conditions for the development of *H. contortus* are during the autumn when minimum temperatures are above 10°C and there is a good balance between rainfall and evaporation (Figs. 1.3 and 1.4). As mean minimum temperatures drop to just below 10°C during the winter (Fig. 1.4), sufficient L_3 can be present on pasture to produce clinical infections in June/July. Inhibited larvae have only been confirmed in one area, Uruguaiana, where tracers had arrested fourth stage larvae throughout the year with the highest levels being detected from March to August i.e., autumn/winter (Fig. 1.6). After this time many inhibited larvae were found to resume normal development to adult worms in winter thus contributing to contamination in spring, when weather conditions were favourable for larval development and survival; as a consequence of this build up of infective larvae on pasture, the highest peak occurs in autumn when haemonchosis is often detected (Figs. 1.5 to 1.7).

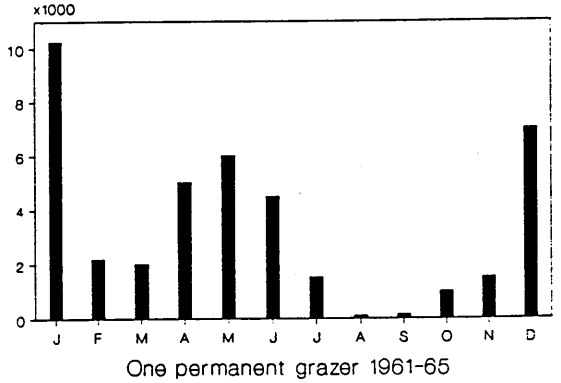
In all areas lambs can become infected while they are with their dams in spring, but it is usually after weaning (Dec/Jan) that they are exposed to a greater challenge and losses occur during their first autumn. Adult sheep do not develop a good immunity to *Haemonchus* (Santiago *et al.*, 1976) and can also show acute clinical disease in autumn. Other important nematodes occuring in Southern Brazil (Figs. 1.9 to 1.12) are *Ostertagia* spp. (mainly *O. circumcincta*) and *T. axei* in the abomasum and *T. colubriformis* and

Fig. 1.5. Epidemiology in Bagé
Haemonchus



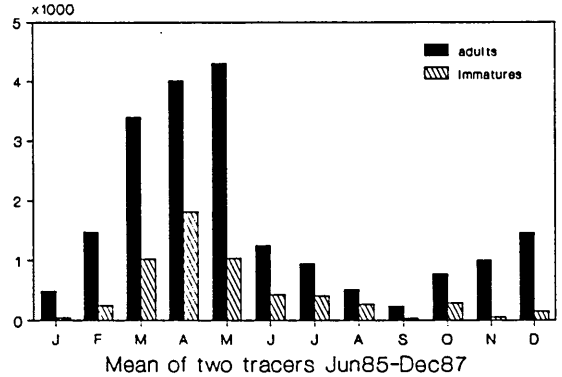
Mean of four tracers 1976-79

Fig. 1.7. Epidemiology in Guaíba
Haemonchus



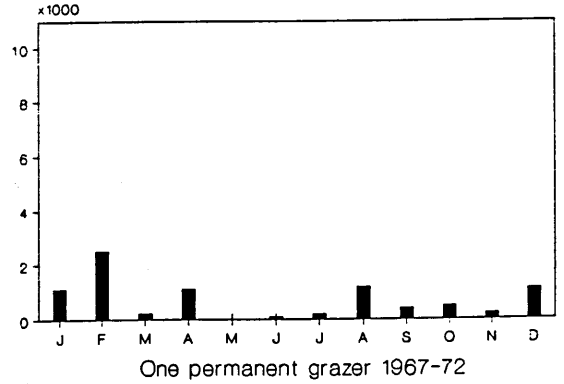
One permanent grazer 1961-65

Fig. 1.6. Epidemiology in Uruguaiana
Haemonchus



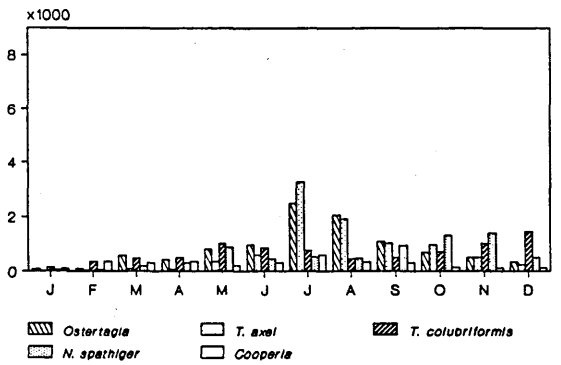
Mean of two tracers Jun85-Dec87

Fig. 1.8. Epidemiology in Itaqui
Haemonchus



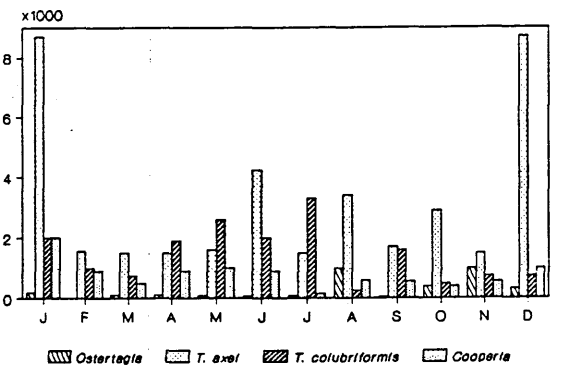
One permanent grazer 1967-72

Fig. 1.9. Epidemiology in Bagé



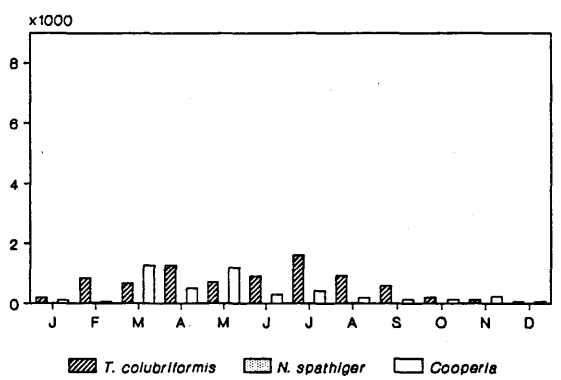
Mean of four tracers 1976-79

Fig. 1.11. Epidemiology in Guaíba



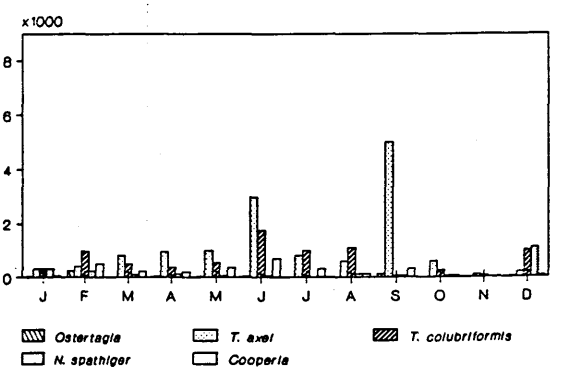
One permanent grazer 1961-65

Fig. 1.10. Epidemiology in Uruguaiana



Mean of two tracers Jun85-Dec87

Fig. 1.12. Epidemiology in Itaqui



One permanent grazer 1967-72

Nematodirus spathiger in the small intestine. In areas where they are commonly found, *Ostertagia* spp. occur in highest numbers in winter, whereas the highest numbers of *T. axei* and *T. colubriformis* are usually detected between autumn and spring. *N. spathiger* is found in Bage at mid autumn and again in spring coinciding with the lambing season; in other areas it was found in very low numbers and its pathogenic importance is not established.

Other gastrointestinal nematodes such as *Strongyloides papillosus*, *Cooperia* spp., *Moniezia expansa*, *Oesophagostomum columbianum*, *O. venulosum* and *Trichuris ovis* occur in low numbers, as do the lungworms *Muellerius capillaris* and *D. filaria* which are not normally regarded as pathogenic. Some of these such as *D. filaria*, *M. capillaris* and *O. columbianum* have disappeared from many farms and although the reason for this decline is not known, the use of effective broad-spectrum anthelmintics may be an important factor.

The earliest epidemiological work in Rio Grande do Sul was conducted by Gonçalves (1974) at Guaiba which is drier than most other areas with a local rainfall of 750mm compared with a state average of 1200-1300 mm/year. Two annual treatments were recommended on the basis of this work: the first, at the end of spring, to control infections of *Haemonchus* and *Trichostrongylus* and the second, at the beginning of autumn, to control the new build up of infections of *Haemonchus* and *Trichostrongylus*, especially *T. axei* (Figs. 1.7 and 1.11).

A second limited study at Itaqui (Fig. 1.8 and 1.12) showed infection present, albeit at low level, throughout the year and six treatments were recommended starting in January and then at

intervals of approximately 60 days (Santiago *et al.*, 1976).

Neither of the above recommendations have been adequately tested in the field and their value may be limited as they were based on data from untreated permanent grazing animals with necropsy worm burdens from only one animal per month. A further two more recent studies at Bagé (Pinheiro *et al.*, 1987) and at Uruguaiana (Carvalho, personal communication, 1990) used 2-4 worm-free tracer lambs throughout the year providing more detailed results. The findings from the two regions showed similar patterns, especially for *Haemonchus* with the highest numbers of parasites being recovered in autumn (Figs. 1.6 and 1.7). Weaning usually takes place in early to mid-summer and based on the above studies, Echevarria *et al.* (1988), recommended the use of drugs with residual protection against *Haemonchus* (e.g., disophenol or closantel) at that time and again 8 weeks later with the aim of reducing the summer contamination of pastures: this effectively eliminated haemonchosis during the autumn. Integrated with these treatments, the use of "safe pastures" i.e., those grazed by cattle during the three months before weaning of the lambs, has also being recommended. After the dose in March it was recommended that farmers should use the parasitology service provided by the wool cooperatives in south Brazil to monitor monthly faecal worm egg counts. In this system 8-10% of animals are sampled monthly for faecal worm egg counts and when these counts reach 500 e.p.g. treatment is recommended. This system of two strategic drenches associated with monthly worm egg count monitoring was tested under field conditions over a period of four years and found to be effective in controlling gastrointestinal parasitism. In some years

sheep needed only the two strategic drenches and in others a further one or two drenches were required. Benefits other than the savings in costs of treatment came from the increased production including improvements in body weight gains, wool quality and reduced mating age for females from 30 to 18 months.

In Southern Brazil, the high level of pasture contamination with trichostrongyle larvae during the autumn has encouraged farmers to administer an excessive number of treatments to lambs during their first year of life. Some may drench at 20 day intervals during autumn and from then on once a month. Although this policy had been successful in controlling parasitism and in doing so provided production benefits, it has precipitated the appearance of anthelmintic resistance.

All reports of anthelmintic resistance in Brazil come from the state of Rio Grande do Sul where 95% of wool sheep farming is based and where *H. contortus* is the parasite of major importance.

The first case of BZ resistance in *Haemonchus* in Brazil was reported over twenty years ago (Dos Santos and Franco, 1967). Later Santiago *et al.* (1979) described a different field population resistant to levamisole which when tested in the laboratory was found to be resistant to a dose of 15 mg/kg (Santiago and Da Costa, 1979). Strains of *T. colubriformis* resistant to levamisole and dl-tetramisole have been reported (Santiago *et al.*, 1977; 1978; Santiago and Costa, 1979). Also, a field strain of *O. circumcincta* resistant to levels of 7.5 mg/kg and 15 mg/kg of levamisole has been reported by Santiago *et al.* (1979): when first tested, 7-8 mg/kg levamisole removed 99% of this field strain of *Ostertagia* (Santiago

et al., 1971).

The same group of authors (Da Costa *et al.*, 1985) tested six benzimidazole compounds against a field strain of *Nematodirus spathiger* and found that their efficacy ranged from 10.2% to 75.8%.

In the only survey on the prevalence of anthelmintic resistance in Brazil, Echevarria and Pinheiro (1989) examined 31 sheep flocks. Based on controlled faecal egg count reduction tests and larval cultures on Day 0 and Day 7 post-treatment with either thiabendazole (TBZ), dl-tetramisole or untreated controls, they found that 38.7% of the flocks showed resistance to benzimidazoles, 25.8% to tetramisole and 19.4% showed multiple-resistance; only 16.1% were susceptible to both drugs. Larvae which survived the treatment with TBZ were mainly *Haemonchus* while those surviving tetramisole treatment were *Trichostrongylus* spp. and *Ostertagia* spp.

Recently, a field population of *H. contortus* has been shown to have developed resistance to ivermectin following 32 drenches given at variable intervals over a period of 4.5 years (Echevarria and Trindade, 1989).

NEMATODE CONTROL IN THE PRESENCE OF ANTHELMINTIC RESISTANCE

After resistance to an anthelmintic is detected on a property, then the drug or group of drugs involved should be avoided (Prichard, 1978; Le Jambre *et al.*, 1979b) and an effective anthelmintic with a different mode of action should be chosen. If *Haemonchus* is the only resistant parasite and other species are not important at that time of the year, then narrow-spectrum anthelmintics can be used. However, the report by Van Wyk and Malan (1988) that *Haemonchus* developed resistance to closantel after only a few drenches may cast doubts on this recommendation although this drug has been apparently used successfully in strategic control programmes in Australia for a number of years.

The question of reversion of BZ resistant strains towards susceptibility by the use of levamisole has already been discussed but, unfortunately, does not offer much hope of practical significance. Reversion can reduce the degree of benzimidazole resistance to a level at which these drugs could be effectively reintroduced but what has been shown is that once the BZ drug is again applied resistance quickly regains its original level.

A population of *Ostertagia* spp. resistant to levamisole was eradicated by the use of a double dose of either OFZ or ABZ at 14 day intervals during three years (Le Jambre, 1981). This is an extreme option and economically it is not likely to be acceptable. Donald (1983b) discussed the use of increased dose rates in relation to resistance and concluded that high dose rates are likely to delay the development of resistance if drugs are used only a few times each year

and if alleles for resistance are in relatively low numbers in the worm population before anthelmintic selection is imposed (Le Jambre, 1978).

The use of integrated control systems which use alternate grazing of sheep and cattle with limited anthelmintic treatments may help to prevent anthelmintic resistance. In this context, Le Jambre (1979) carried out hybridization experiments between *H. contortus* from sheep and H. *placei* from cattle and showed that hybrids, produced by the mating of thiabendazole resistant female *H. contortus* x non-resistant male H. *placei*, were also resistant. However, it was also shown that the F_1 males of the mating between male *H. contortus* and female H. *placei* were sterile and so were the F_2 of the reciprocal cross. The female hybrid from these generations had a low level of fertility when backcrossed to males of either parent strain.

It was shown by Waller and Lacey (1986) that an insect growth regulator, triflumuron, had potent larvicidal effects against free-living stages of *T. colubriformis* and the future development of such compounds may provide an alternative method for the control of resistant parasites.

Although there is an urgent need for the development of new anthelmintics with different modes of action to those currently in use, it is very unlikely that these will appear in the near future since the development process is slow and the costs involved are extremely high. Also, the fact that resistance to ivermectin has emerged so quickly is a clear indication that resistance may evolve quickly to any new drugs. There is, therefore, a great need for continuous research on the effective use of anthelmintic compounds

linked to other measures to reduce dependence on chemical control of nematodes and so extend the useful life of currently available drugs. There is also a need for continued studies of a range of resistant nematode populations in order to provide knowledge to allow the design of appropriate control measures.

The aim of this thesis was to study some aspects of the development and control of anthelmintic resistance in strains of *H. contortus* resistant to ivermectin. Studies were also conducted on the behaviour of free-living and parasitic stages of resistant strains compared with those of susceptibles. Isoenzyme techniques were used to look at differences between susceptible and resistant strains and a computer model was also used to monitor different nematode control programmes on sheep flocks in Brazil and their possible effect on the selection for anthelmintic resistance.

CHAPTER 2

GENERAL MATERIALS AND METHODS

A number of methods were common to more than one experiment and these are described in this general section. Special techniques and experimental designs used in individual studies will be referred to in the appropriate sections.

Animals

The breeds of lambs used in the experiments were Corriedale in Brazil and Suffolk x Scottish Blackface and Finn Dorset x Suffolk crosses in Scotland and all were castrated males. When housed, animals were given 1kg of concentrate/day plus hay and water *ad libtum*. When on grass no supplementary feeding was given.

Housing

At Glasgow, animals were kept in an animal house where they were bedded on clean straw which was replaced every two days; in Brazil the animal house had slatted wooden floors raised 1.5m above ground level. Animals were thus kept in conditions likely to preclude reinfection with parasites.

Faecal analysis

Faeces collected from the rectum were examined for helminth eggs by a modified McMaster flotation technique (Gordon and Whitlock, 1939). In this technique three grams of faeces were homogenised with 42ml water and the resultant suspension passed through a coarse mesh sieve of aperture size 250 microns (Endecotts Ltd., London) which retained the larger particles of debris and allowed the passage of nematode eggs (size range 70 - 165 microns). After the thorough mixing

of the filtrate 15ml samples were transferred to a flat-bottomed centrifuge tube and centrifuged at 2000 rpm for two minutes. The supernatant from the tube was then discarded and the remaining faecal mass boken up by rotary agitation (Whirlmixer, Scientific Industries Ltd.). The tube was then filled to its former level with saturated salt solution and after inverting six times a volume of the suspension sufficient to fill both chambers was quickly transferred by pipette to a McMaster slide (Gelman Hawksley Ltd., Northampton). The numbers of eggs under both etched areas of the slide were counted and the result multiplied by 50 to give the number of eggs per gram of faeces according to the following calculation:

3 grams of faeces in 42ml gives 1 gram in 15ml.

Volume under one square equals 0.15ml.

No. of eggs seen in one square x 100 = no. of eggs/gram

and no. of eggs seen in two squares x 50 = no. of eggs/gram.

Larval cultures

Faecal pellets were placed in disposable cartons and incubated at 27°C. After seven days the cartons were removed from the incubator, filled with lukewarm water and left to settle for 4 hours. The larval suspension was transferred into a beaker and drawn through a coarse filter paper (Whatmans Grade 113, 18.5 cm) using a Buchner funnel and vacuum pump. A single milk filter (Maxa Milk Filters, A. McCaskie Ltd., Stirling) was put on top, and the whole inverted and placed on a Baerman apparatus. After standing for about six hours larvae were withdrawn, counted, differentiated and stored at 4°C. The criteria for

larval identification were those detailed in Technical Bulletin No. 18 (Ministry of Agriculture Fisheries and Food) and used by Keith (1953) i.e. body length, prolongation of the second larval sheath beyond the tail of the third stage larvae and presence of refractile structures.

Cryopreservation of infective larvae

Third stage larvae (L_3) of *H. contortus* were exsheathed using 5% Milton's fluid (Milton 2, Richardson-Vicks Ltd., England) equivalent to a final concentration of 0.1% w/v sodium hypochlorite (NaOHCl). The larvae were suspended in this solution with continuous air bubbling for five minutes then centrifuged three times at 100G for five minutes, the supernatant being replaced with distilled water each time. From the sediment a maximum of 0.5ml was transferred into 1ml polypropylene tubes with screw caps and frozen in the gas phase of liquid nitrogen in a semen storage tank (Union Carbide, UK). When needed, larvae were thawed in a water bath at 50°C until a small piece of ice remained; they were then transferred to water at room temperature.

Packed cell volume estimation (PCV)

Blood was collected from the jugular vein by using disposable needles and vacutainer type tubes containing heparin. Heparinised blood was loaded into plain capillary tubes (Gelman Hawksley Ltd.), one end plugged with cristaseal and the tube spun in a micro-hematocrit centrifuge (Gelman Hawksley) for 5 minutes. The PCV reading was then estimated using a Hawksley micro-hematocrit reader.

Necropsy technique

Animals were slaughtered by stunning and exsanguination and the entire gastro-intestinal tract was removed from the body cavity after ligaturing the pyloric sphincter/duodenal junction to prevent mixing of the abomasal and small intestinal contents.

After opening, the abomasal and small intestinal contents were washed separately into buckets and the volume made up to a standard 2 litres (except when an unusually large amount of material was present when a greater volume of water was required) and samples of 200ml withdrawn, formalised (5%) and retained for subsequent examination. The abomasal mucosa was digested with a 1% hydrochloric acid/pepsin mixture for six hours at 42°C to recover the larval stages present within the mucosal glands. The digested mixture was again made up to 2 litres and 200ml samples withdrawn as before. After staining with iodine and decolourising using sodium thiosulphate, the trichostrongyles present in aliquots of the 200 ml samples were counted and classified as adults, late fourth stage (LL_4) or early fourth stage (EL_4) larvae depending on bursal or vulvar development, the presence of a sheath projection and size. Fifth larval stages were counted as adults.

The iodine solution was prepared by dissolving 907 grams of potassiun iodide in 650 ml boiling water; 510 grams iodine crystals were added and the volume made up to 1 litre. The sodium thiosulphate solution was prepared by dissolving 100 grams of sodium thiosulphate in 5 litres of water.

The large intestine and its contents were poured, a little at a time, on to a 150um sieve which was then washed with a stream of water

from a rubber tube attached to the tap. When all of the material had been screened and washed in this way, the sieve was inverted over a beaker and by means of a stream of water, debris and worms collected on the sieve were washed into it. The contents where then formalised and retained for total worm counting.

Statistical analysis

Worm counts and faecal egg counts were transformed to $\log_{10}$ (count + 1) in order to reduce the error of variance. One-way analysis of variance was used to test for significance between treatments. When a significant difference was found, a Newman Keuls multiple range test was used to test for group differences.

Body weights, PCV's and worm egg counts when measured at different times were submitted to a two-way analysis of variance to provide treatment x time interaction, group differences and time differences.

CHAPTER 3

STUDIES ON FACTORS AFFECTING IVERMECTIN RESISTANT *Haemonchus contortus* IN SHEEP

Experiment 3.1

EFFICACY OF OTHER ANTHELMINTICS ON IVERMECTIN RESISTANT ADULT *Haemonchus* contortus

INTRODUCTION

Over the last 10-20 years anthelmintic resistance of sheep nematodes to the widely used benzimidazole and levamisole groups of drugs has become an increasingly important problem in the major sheep rearing areas of the world (Waller, 1987). The discovery of a chemically distinct group of anthelmintics, the avermectins (Burg *et al.*, 1979), provided a welcome alternative but since their introduction in the early eighties, sheep nematodes resistant to one of these compounds, ivermectin, have been identified. There are two field reports of *H. contortus* resistant to ivermectin - from South Africa (Van Wyk and Malan, 1988) and Brazil (Echevarria and Trindade, 1989). Under laboratory conditions ivermectin resistance has been selected for in *H. contortus* (Egerton *et al.*, 1988), where a high level of resistance was detected after seven generations of exposure to one tenth of the normal recommended dose, and in *T. colubriformis* (Giordano *et al.*, 1988), after four generations subjected to various ivermectin dose rates.

The objective of this experiment was to evaluate the anthelmintic sensitivity of the ivermectin-resistant strain described by Echevarria and Trindade (1989) to benzimidazole and levamisole treatments under controlled test conditions; a known susceptible strain was

included for comparison.

EXPERIMENTAL DESIGN

Eighty 8-9 month old lambs from a commercial farm were housed on slatted wooden floors raised 1.5m above ground level at The National Institute for Sheep Research at Bage, which is part of The Federal Research Organisation of Brazil (EMBRAPA). Lucerne hay and a commercial sheep concentrate ration were available *ad lib.* On arrival the lambs had a mean worm egg count of 330 e.p.g. and were immediately drenched with morantel 10mg/kg and trichlorphon 100 mg/kg; this treatment was repeated the next day.

A second faecal examination 15 days later showed all animals to be negative for nematode eggs. The lambs were then split at random into two groups of 40 - Groups R and S; the Group R animals were infected with 5,000 L_3 of the known resistant strain of *H. contortus* (Echevarria and Trindade, 1989). Following isolation, this field strain had been subjected to a further treatment with ivermectin at 0.2mg/kg and the survivors of this treatment produced the resistant larvae (R-IVM) used in the present experiment. No benzimidazole or levamisole treatments had been applied for at least five years, ivermectin being the only drug used on the farm where the ivermectin-resistant strain was isolated; previously, however, both groups of drugs had been used fairly intensively to control *H. contortus* during the annual autumn peak of infection and resistance to the benzimidazoles had been confirmed. The animals in Group S were infected with 5,000 L_3 of a susceptible strain

(S-IVM) of *H. contortus*.

At day 28 post-infection animals in Groups R and S were allocated, according to their faecal egg counts and at random, to one of four treatment sub groups as follows:

Group	Treatment	Dose rate mg/kg	Animals/treatment
R	R1 - levamisole	7.5	10
infected	R2 - albendazole	3.8	10
with	R3 - ivermectin	0.2	10
R-IVM	R4 - control	-	10
S	S1 - levamisole	7.5	10
infected	S2 - albendazole	3.8	10
with	S3 - ivermectin	0.2	10
S-IVM	S4 - control	-	10

Levamisole - Ripercol Pó, Cyanamid , Brazil
Albendazole - Valbazen Pó, Smith Kline, Brazil
Ivermectin - Ivomec Oral, MSD, Brazil

The anthelmintic doses were calculated individually according to body weights and were administered orally.

The animals were slaughtered on Day 7 after treatment (Day 35 post infection) and total worm burdens estimated as recommended by Powers *et al.* (1982).

Statistics

Egg and worm counts were transformed to $\log_{10}$ (count+1) and analysis of variance performed for testing the main effects and

interactions between strain of parasite and treatment. When there were any significant strain versus treatment interaction, multiple comparisons were conducted using Fisher's Least Significant Difference Test.

RESULTS

Results of mean worm counts and faecal egg counts are shown in Table 3.1.1 and Table 3.1.2 respectively while individual data is found in Appendices 3.1.1 to 3.1.5. These results show clear differences in efficacy of ivermectin and albendazole against the two strains.

From the worm burdens of the controls there were obvious differences in establishment rates of the two strains: 43.7% for the susceptible against 23.4% for the resistant strain. This was reflected in the worm egg counts on the day of treatment which were also significantly lower in animals infected with the resistant strain ($P<0.05$); seven days later there was no statistical difference in faecal egg counts between the two control groups, although the resistant strain still showed a much lower output than the susceptible strain (Table 3.1.2).

The S-IVM strain was equally susceptible to levamisole, ivermectin and albendazole while the R-IVM strain showed a high level of resistance to both ivermectin and albendazole. With the resistant strain there was no significant difference in worm burden reduction between groups treated with ivermectin or albendazole, these drugs showing an efficacy of only 10.4% and 14.7%

respectively. Levamisole on this resistant strain reduced worm counts by 99.8% (Table 3.1.1).

Worm egg output (e.p.g.) was reduced by almost 100% by all three drugs in the animals infected with the susceptible strain: in the resistant strain levamisole was also 100% effective while there was no reduction in egg output in the animals treated with ivermectin. Albendazole significantly reduced egg counts by 92.5% but this reduction was not reflected in the worm counts where this drug was only 14.7% effective.

DISCUSSION

The results obtained in this experiment clearly confirm the high degree of ivermectin resistance of a Brazilian strain of *H. contortus* (Echevarria and Trindade, 1989). They also show that this strain had retained benzimidazole resistance despite the fact that benzimidazoles had not been used for at least five years on the farm where it was isolated.

Albendazole was highly efficient against the susceptible strain (98.9%) but on the resistant strain, although treatment depressed egg output by 92.5%, it only reduced worm burdens by 14.7%. These results confirm previous observations on the effect of benzimidazoles on BZ-resistant strains, i.e, a temporary supression of worm egg counts without a corresponding worm loss (Hotson *et al.*, 1970; Le Jambre *et al.*, 1979; Martin *et al.*, 1985).

With regard to establishment rates of *H. contortus* these have been reported to vary between 12.7% to 44.1% (Reinecke, 1973; Hogarth-Scott

et al., 1976; Van Wyk and Gerber, 1980b; Adams, 1988). Many factors may be responsible for these differences such as time after infection at which it is determined, age and immune status of the host, larval age and larval storage conditions. In the present study there were clear differences in the establishment rates of the two strains used i.e., 43.7% for the S-IVM versus 23.4% for the R-IVM. Although different establishment rates have been reported between resistant and susceptible strains (Kelly *et al.*, 1978; Van Wyk and Malan, 1988; Maingi *et al.*, 1990) Scott and Armour (1991) could not detect significant differences between a drug susceptible and a South African multiple resistant strain of *H. contortus* and it remains questionable whether these differences reflect anthelmintic resistance or susceptibility or whether they are simply due to natural differences between ecotypes since the larvae used are often from different origins.

Reversion towards susceptibility has been reported in some nematode populations by using different anthelmintics e.g. Waller *et al.* (1983) and Martin *et al.* (1988) found that levamisole selected favourably for reversion to benzimidazole susceptibility of a BZ-resistant strain of *Ostertagia*, while thiabendazole treatment decreased the level of levamisole resistance in a levamisole-resistant population of *T. colubriformis* (Waller *et al.*, 1985). The results obtained for the efficacy of albendazole on the resistant strain of *H. contortus* in this study indicated that there had been no reversion to benzimidazole susceptibility by the continued use of ivermectin in the field over a five year period.

Ivermectin was highly effective on the susceptible strain but in the

resistant strain its efficacy was only 14.7%. This efficacy is much lower than the 59.4% reported for the original field population by Echevarria and Trindade (1989). This may be due to the fact that the field population, when brought into the laboratory, had been exposed to a further treatment with ivermectin prior to culture of the larvae used in this study.

Although levamisole has been on the market as long as the benzimidazole drugs and has also been widely used it has been shown that *H. contortus* has not developed resistance against levamisole to the same extent as it has to the benzimidazoles (Waller, 1987). In the present study, of the three chemically distinct broad spectrum anthelmintics used, levamisole was the only drug which proved highly effective on both the susceptible as well as the ivermectin-resistant strain. The reasons for the varying rates at which different genera of gastro-intestinal nematodes acquire resistance to the distinct chemical anthelmintic groupings are not known but clearly elucidation of the mechanism of resistance to these groupings is a pre-requisite if methods to control the advent of resistance are to be developed.

TABLE 3.1.1. Mean worm burdens[1](range) of sheep infected with two strains of *H. contortus* and their percentage reduction after treatment with levamisole, ivermectin and albendazole compared with untreated controls.

Strain	Treatment	Mean worm count	% Reduction
S	Levamisole	0[a] (0 - 0)	100
S	Ivermectin	0[a] (0 - 0)	100
S	Albendazole	23[a] (0 - 90)	98.9
S	Control	2183[c] (850 - 4100)	-
R	Levamisole	2[a] (0 - 20)	99.8
R	Ivermectin	1046[b] (40 - 3020)	10.4
R	Albendazole	996[b] (360 - 1870)	14.7
R	Control	1168[b] (310 - 1770)	-

S = ivermectin-susceptible; R = ivermectin-resistant.

[1] Values shown are arithmetic mean worm counts but comparisons were made on log transformed data.

[a,b,c] Results followed by different letters are significantly different ($P<0.05$).

TABLE 3.1.2. Effect of albendazole, ivermectin and levamisole on worm egg output[1] in sheep infected with two strains of *H. contortus*

Strain	Treatment	Mean egg count		Reduction
		Day 0	Day 7	%
S	Levamisole	27430[a] (11100 - 81200)	0[a] (0 - 0)	100
S	Ivermectin	24760[a] (6300 - 72200)	0[a] (0 - 0)	100
S	Albendazole	25840[a] (2000 - 76400)	44[a] (0 - 400)	99.8
S	Control	22570[a] (8900 - 38100)	34760[b] (20000 -68200)	+54
R	Levamisole	13890[b] (1900 - 39000)	0[a] (0 - 0)	100
R	Ivermectin	13910[b] (3600 - 27700)	14870[c] (0 - 56900)	+6.9
R	Albendazole	14410[b] (3300 - 30700)	1080[d] (100 - 2500)	92.5
R	Control	14140[b] (2100 - 31200)	20178[b] (8500 - 34000)	+42.7

S = ivermectin-susceptible; R = ivermectin-resistant.

[1] Values shown are arithmetic mean worm egg counts but comparisons were made on log transformed data.

[a,b,c,d] Results followed by different letters in the same column are significantly different ($P<0.05$).

Experiment 3.2

THE RESPONSE TO IVERMECTIN TREATMENT OF PARASITIC STAGES OF *Haemonchus contortus* RESISTANT OR SUSCEPTIBLE TO IVERMECTIN

INTRODUCTION

Ivermectin has been shown to have an impressive spectrum of activity against endo- and ectoparasites at very low dose rates (Egerton *et al.*, 1979) and to be particularly effective against the most important gastrointestinal nematodes of cattle (Armour *et al.*, 1980), horses (Klei and Torbert, 1980) and sheep (Armour, *et al.*, 1982). However, as with other classes of broad spectrum compounds (levamisole, morantel and the benzimidazoles) anthelmintic resistance to ivermectin has also developed. Ivermectin resistance was selected for under laboratory conditions both in *H. contortus* (Egerton *et al.*, 1988 and in *T. colubriformis* (Giordano *et al.*, 1988) and has also been detected in *H. contortus* in the field in both South Africa (Van Wyk and Malan, 1988) and Brazil (Echevarria and Trindade, 1989). All these reports were based on loss of effect of the drug on adult worms but it was not known at which larval stage of development these nematodes started to show anthelmintic resistance.

The following experiment was set up to assess the efficacy of ivermectin on different parasitic stages of both resistant and susceptible strains of *H. contortus*.

MATERIALS AND METHODS

Strains. The ivermectin susceptible (S-IVM) and resistant (R-IVM) strains of *H. contortus* used for this experiment were the same as those used and described in Experiment 3.1.

Experimental design. Sixty-six male helminth free Corriedale lambs, aged three months, were divided at random into two groups of 33 animals each (Groups R and S) and submitted to the following experimental protocol:

Day 0 a - 20 lambs - Group Rt (t= treated)
13 lambs - Group Rc (c= untreated control)
all infected with 5,000 L_3 of the R-IVM strain

b - 20 lambs - Group St
13 lambs - Group Sc
all infected with 5,000 L_3 of the S-IVM strain

Day 6 a - at random 5 lambs from group Rt and 5 lambs from group St were treated with 0.2mg/kg of IVM. Groups named: Rt-6 and St-6 respectively.

b - at random 2 lambs from group Rc and 2 lambs from Sc were killed to determine the stage of larval development of both strains.

Day 10 - procedures for Day 6 were repeated with another seven lambs (5+2) infected with each strain. Groups named: Rt-10 and St-10 respectively.

Day 16 - procedures for Day 6 were repeated with another seven lambs (5+2) infected with each

strain. Groups named: Rt-16 and St-16 respectively.

Day 21 - procedures for Day 6 were repeated with another seven lambs (5+2) infected with each strain. Groups named: Rt-21 and St-21 respectively.

Day 28 - all remaining animals were slaughtered for total worm counts, i.e, 20 animals from Group Rt, 5 lambs from Rc, together with 20 animals from Group St and 5 from Group Sc.

Ivermectin. The drug used was an 0.08% formulation of ivermectin (Ivomec Oral, MSD) and it was administered *per os*, using disposable syringes, according to individual body weights.

All laboratory procedures were carried out as recommended by Powers *et al.* (1982).

Statistics. Worm counts were transformed using $\log_{10}(count+1)$ before being submitted to one-way analysis of variance followed by the Newman Keuls multiple range test for group differences.

RESULTS

Results from the necropsies carried out on the untreated animals on Days 6, 10, 16 and 21 after infection are shown in Table 3.2.1. On Days 6 and 10 post-infection low numbers of immature parasites were detected in comparison with the numbers of adults obtained on Days 16

and 21 but this may be a reflection of the sensitivity of the technique. It can be seen that on Day 6, all larvae independent of the strain used, were either early or late fourth stage, with the majority being at the late fourth stage of development. By Day 10, early fourth stages were no longer detected but late fourth stages were still present and young adults, i.e., early fifth stages, were beginning to appear. By Days 16 and 21 all worms were adults.

All treated groups together with the untreated controls were necropsied on Day 28 post-infection and the results are presented in Table 3.2.2. There were no significant differences between the worm burdens obtained from the untreated controls in both groups ($p>0.05$).

Animals infected with the resistant strain and treated at Day 6 post-infection, had the highest percent reduction in worm burden, 26.3%, in comparison with the untreated control group but this was not significant ($p>0.05$). Those treated on Day 10 had a reduction of around 10% while animals treated on Days 16 and 21 had counts similar to or greater than those untreated controls.

With the susceptible strain IVM was highly efficient, reducing worm burdens significantly at all stages of development in the treated groups in comparison with the untreated controls. The lowest efficacy of 96.2% was registered for treatment given against fourth stage larvae on Day 6 but this was not significantly different from treatment given at the other stages of development where IVM had efficacies over 98.7%.

Five animals were lost at the early stages of the experiment due to pulpy kidney disease and their counts were not included in the analysis of the data (Table 3.2.2).

DISCUSSION

The times chosen for the ivermectin treatments in this experiment were selected to coincide with development to the early fourth, late fourth, fifth larval stages and to the adult stage: these times were based on the work of Reinecke (1973). However it appears that the strains of *H. contortus* used in the present study may have had a more rapid development rate than those studied by Reinecke as the necropsies of the animals on the days selected for treatment showed that the majority of worms had developed further than expected. Thus, in animals killed six days after infection most of the parasites were already at the late fourth stage; by Day 10 post-infection the first young adults started to appear and by Day 16 all the parasites were found to be at the adult stage: this is in agreement with the pre-patent period for *H. contortus* (Soulsby, 1982).

The activity of the oral formulation of IVM on developing fourth stages of susceptible strains of *H. contortus* was reported by Benz *et al.* (1989) to be 98%. Similar efficacies against L_3, L_4 and adult stages of BZ-resistant *H. contortus* and *O. circumcincta* have also been reported with an oral preparation of IVM by Swan *et al.* (1984). However, a lower efficacy (46%) of IVM on immature nematodes of sheep has been obtained against early fourth stages of naturally acquired infections of abomasal nematodes of sheep as well as against experimental infections with nematodes of the small intestine (Westcott and LeaMaster, 1982).

The precise mechanism of anthelmintic resistance to IVM has not yet been determined (Prichard, 1990) although resistance to this compound

has been reported both in the field and in the laboratory. Egerton *et al.* (1988) produced a population of *H. contortus* resistant to IVM after eight generations by treating animals 28 days after infection when parasites were at the adult stage. On the other hand selection pressure with IVM repeatedly applied six days after *T. colubriformis* infection, when larvae were at the fourth stage, produced a resistant population to this drug after four generations (Giordano *et al.*, 1988). In the field where different stages of nematodes were exposed to the drug, IVM resistance has been reported by Van Wyk and Malan (1988) in South Africa after 11 treatments over 27 months and by Echevarria and Trindade (1989) in Brazil after 32 treatments over a 54 month period.

The field population of *H. contortus* which was the origin of the strain used in this trial was derived from a flock of young sheep routinely treated with IVM over a period of 4.5 years (Echevarria and Trindade, 1989). Under such conditions the selection pressure was imposed on all stages of development and this certainly led to resistance evolving in immature and adult *H. contortus*. The results obtained in this experiment confirmed that in this strain of *H. contortus* anthelminintic resistance was shown as early as the fourth larval stage. On the other hand ivermectin applied on the S-strain at the same time produced a reduction of over 96% which is in accordance with the efficacies reported for susceptible strains of *H. contortus* (Benz *et al.*, 1989).

IVM was suspected by Echevarria and Trindade (1989) to have some efficacy on the immature stages of the resistant field strain they originally identified. This was not confirmed in the present study

and may be due to the fact that in their earlier observations low numbers of immature *H. contortus* were present in the untreated controls which made interpretation difficult. In addition, the earlier report by Echevarria and Trindade (1989) was on a field population suspected of IVM resistance while the larvae used in the present studies were from the field isolate which was then subjected to an additional IVM treatment possibly imposing further selection pressure on the original strain. Also in this experimental study sufficient numbers of worms at different stages of development were present at the time of treatment so ensuring a more conclusive result.

It is believed that provided there is enough genetic variability within any given population of nematodes resistance will develop. However the speed of selection for resistance is known to be influenced by many factors such as genetics, e.g. type of inheritance, dominance, level of resistance and relative fitness; biological or ecological factors, e.g. life-cycle, reproduction, generation turnover, stage exposed to the discriminating drug and proportion of population in refugia, and operational factors, e.g. type and efficiency of anthelmintic, intensity of application, mode of action, rotation of drugs, dose rates and grazing management (Prichard, 1990). Under laboratory conditions, where a very small proportion of a given population is under pressure these variables are much reduced and the main factors may be the presence of sufficient genetic variability and intensity of drug selection. If the dose applied is not sufficient to kill heterozygous resistant worms only the susceptibles will be killed and resistance may evolve rapidly.

It has been shown that anthelmintic treatment at sub-optimal dose rates can rapidly select for benzimidazole resistance in sheep nematodes. An example of this is the report by Martin (1989) using thiabendazole at 44mg/kg instead of the optimal recommended dose rate of 66-88mg/kg. Although TBZ proved 90% effective at the first administration, in a summer drench programme of two treatments over two successive years it was found that after the third treatment the drug had become ineffective against *Ostertagia* spp.

In the present experiment IVM had an efficacy of 96.2% against fourth stage larvae of IVM-S *H. contortus*. This lower efficacy on immatures, although well accepted under practical conditions, may be the starting point in the process of selection for anthelmintic resistance to IVM. According to Prichard (1990) unless an anthelmintic is always one hundred per cent effective, the possibility remains that treatment will select for anthelmintic resistance. Individual parasites that survive treatment are more likely to have biological characteristics which render them resistant to the effects of the drug. These characteristics may be transmitted to offspring and so repeated treatments may select for an increasing proportion of resistant individuals.

Selection pressure will also affect the speed of development of resistance and will be highest when the proportion of resistant to susceptible genotypes increases. If the ratio of resistant/susceptible individuals in the original population is low, selection will be most rapid if all of the heterozygous resistant and the homozygous resistant worms survive treatment. For this reason anthelmintic dose rates should be high enough to kill heterozygous resistant nematodes

at all stages, thus rendering resistant genes effectively recessive.

Table 3.2.1. Necropsy worm burdens and stage of development* of ivermectin resistant or susceptible strains of *H. contortus* in lambs on Days 6, 10, 16 and 21 post-infection with 5,000L_3.

Day	Strain	Animal	Worm burden
6	S-IVM	1	70 L4L
	S-IVM	2	30 E4L + 50 L4L
6	R-IVM	1	60 L4L
	R-IVM	2	300 L4L
10	S-IVM	1	0
	S-IVM	2	120 L4L + 20 L5L
10	R-IVM	1	720 L4L
	R-IVM	2	690 L4L + 160 L5L
16	S-IVM	1	490 adults
	S-IVM	2	650 adults
16	R-IVM	1	1050 adults
	R-IVM	2	1170 adults
21	S-IVM	1	530 adults
	S-IVM	2	650 adults
21	R-IVM	1	900 adults
	R-IVM	2	1610 adults

* E4L= early fourth larval stage; L4L= late fourth larval stage; L5L= fifth larval stage.

Table 3.2.2. Worm burdens obtained 28 days after lambs had been infected with ivermectin resistant or susceptible *H. contortus* and either treated with IVM at 0.2mg/kg on Days 6, 10, 16 or 21 days after infection or left as untreated controls.

Strain	Day 6	Day 10	Day 16	Day 21	Untreated
R-IVM	1100	1420	1060	1520	920
	1170	1370	1900	930	1290
	#	720	1400	1820	1700
	570	470	620	1650	830
	890	1440	1130	1520	1210
mean=	877[a]	1084[a]	1222[a]	1488[a]	1190[a]
% reduc.=	26.3	8.9	+2.6	+25	-
S-IVM	60	#	0	0	810
	20	0	0	0	920
	#	0	0	20	730
	30	10	50	10	660
	10	10	0	##	##
mean	30[a]	5[a]	10[a]	8[a]	780[b]
% reduc.=	96.2	99.4	98.7	98.9	-

Mean worm burdens on the same line followed by different superscript are significantly different ($p<0.05$). Worm burdens obtained from the untreated controls were not significantly different ($p>0.05$).

Died 4 days after treatment and ## 2 days after finfection due to pulpy kidney disease.

Experiment 3.3

COULD THE STRATEGIC USE OF SALICYLANILIDES CONTROL AN IVERMECTIN RESISTANT STRAIN OF *Haemonchus contortus* IN GRAZING LAMBS IN BRAZIL?

INTRODUCTION

Ideally sheep should be treated strategically against gastrointestinal nematodes according to epidemiologically based prophylactic control programmes. Treatments recommended by these programmes should be given at specified times aimed to prevent the seasonal rise in worm burdens and thus reduce pasture contamination. The consequence of such strategies is that the frequency of anthelmintic treatments can be reduced, while maintaining effective control (Gordon, 1948). Throughout the world however, most sheep are empirically treated with anthelmintics rather than on the basis of local epidemiological information. Treatments may be given at short intervals during the periods when larvae are at high levels on pasture or when sheep are brought in for husbandry practices such as weaning.

With the frequent use of anthelmintic treatments, resistance has evolved to all broad spectrum compounds (Prichard, 1990); consequently strategic prophylactic control programmes based on the use of salicylanilides, which have a residual effect against *H. contortus*, have been under investigation in various countries. The aim of these programmes is to reduce pasture contamination and to reduce the need for frequent treatments (Dash *et al.*, 1985; Echevarria *et al.*, 1988).

This section reports the results from an experiment set up to assess the potential of strategic treatments using salicylanilide drugs to control a strain of *H. contortus* resistant to both ivermectin and benzimidazoles.

MATERIALS AND METHODS

Site: This experiment was carried out at The National Institute for Sheep Research at Bage, which is part of The Federal Agriculture Research Organisation of Brazil (EMBRAPA).

Experimental design: Two zones, each of 3ha within a 16ha paddock where ivermectin (IVM) resistance of *H. contortus* had been detected previously in set stocked lambs (Echevarria and Trindade, 1989), were fenced off. These two zones were approximately 150m apart and separated by a narrow stream. Zone 1 was on high ground whereas zone 2 was adjacent to the stream on low ground. The zones were each subdivided into 6 plots of 0.5ha to provide replicate fields for the following treatments:

NTXN+IVM - Nitroxynil at 10mg/kg + ivermectin at 0.2mg/kg

DISO+IVM - Disophenol at 10mg/kg + ivermectin at 0.2mg/kg

CLOS+IVM - Closantel at 5mg/kg + ivermectin at 0.2mg/kg

IVM - Ivermectin at 0.2mg/kg

ABZ - Albendazole at 3.8mg/kg

CONTROL - Untreated

Each treatment group consisted of 10 lambs, five per replicate. Lambs were treated according to the above schedule in February, at weaning, and again 56 days later in April. This period was chosen to

span what is considered to be the estimated prophylactic period provided by most salicylanilide drug treatments (Hall *et al.*, 1981). Fifty-six days after the second anthelmintic treatment, these lambs were necropsied to determine their worm burdens under the different control systems. Ivermectin treatments were also carried out in the salicylanilide groups to control other gastrointestinal nematodes, while the IVM and ABZ groups acted as monitors on the level of resistance to these drugs in the *H. contortus* population.

Animals. Sixty Corriedale weaners aged three months were obtained locally from a farm where IVM had never been used. These animals were harbouring mixed natural infections and they were treated orally with IVM at 0.2 mg/kg to eliminate existing infections. The animals were then kept indoors for 14 days, to preclude further reinfection. After this period of housing they were sampled to confirm negative worm egg counts, weighed, ear tagged, ranked by weight and allocated at random to one of the zones. In each zone, two tracers grazed with the 30 principal lambs for 14 days to provide an indication of the level of contamination at the beginning of the trial in mid-January: the tracers were then housed for 14 days before being slaughtered. Initially, all of the lambs grazed together for 14 days on each zone in an attempt to standardise the initial exposure to infection. At the end of this open grazing period, the animals were weighed again, allocated at random to one of the six treatment paddocks within their zone, retagged and treated according to the specified schedule. The first treatment was given in February to simulate the dose usually given at weaning. To assess

the level of pasture contamination, 56 days after the second treatment, five worm-free tracers grazed each of the experimental zones for 14 days. These tracers were again housed for 14 days before being necropsied.

Vegetation. At the experimental site the main species were perennial grasses including *Paspalum notatum*, *Axonopus affinis*, *Erygium horridum*, *Cyperus* spp. and *Carex* spp.

Drug administration. Animals were treated at the manufacturer's recommended dose rates according to individual body weights. Disposable syringes were used to deliver the correct doses of the following drugs:

Nitroxynil - NTXN (Dovenix 34; Rhodia-Merieux S.A.)
34% solution for subcutaneus injection.

Disophenol - DISO (Disophenol; Paraquimica S.A)
20% subcutaneous injection formulation.

Closantel - CLOS (Closantel 10; Fatec)
10% oral solution.

Ivermectin - IVM (Ivomec; Merck Sharp & Dhome)
0.08% oral formulation.

Albendazole - ABZ (Valbazen ; Smith Kline-Enila Ltd.)
31.6g sachet diluted in 1000ml water.

Feeding. At grass no supplementary feeding was given. When indoors the animals were offered 0.3 kg of concentrate/lamb/day with hay and water *ad lib.*

Data collection. Faecal samples were examined using the McMaster technique described by Gordon and Whitlock (1939). Larval cultures were carried out using approximately 10 grams of faeces incubated for seven days at 27°C; at least 50 larvae were counted and identified. Body weights, worm egg counts and packed cell volumes (PCV) were monitored at two week intervals until 56 days after the second anthelmintic treatment.

Packed cell volume was measured on heparinized jugular blood samples by the micro-haematocrit method and recorded as a percentage.

Gastro-intestinal worm burdens were estimated as recommended by Powers *et al.* (1982).

Data Analysis. Worm counts were transformed using logarithms and analysed using two-factor analysis of variance. This provided F-tests for differences between treatment groups, between zones and treatment group x zone interaction. When a significant interaction between treatment and zone was detected, results for each zone were analysed separately using a one-way analysis of variance. Significant treatment differences were further investigated using a Newman Keuls Multiple Range Test.

Body weights, PCVs and worm egg counts, the latter being log transformed, were tested for differences using a nested factorial design suitable for comparing 2 zones, each containing 6 treatment

groups of 5 animals with repeated time measurements on each animal. Significance levels of 5% were used unless otherwise stated.

RESULTS

Necropsy of the two tracer lambs from each zone, which grazed the experimental areas during the open grazing period, revealed levels of infection which were extremely low. The results are shown in Table 3.3.1. Two of the four animals were infected with *N. spathiger*, two with *Trichuris ovis*, and only one with *H. contortus*. A positive worm egg count was only detected in one animal.

Table 3.3.1. Worm counts and worm egg counts (e.p.g.) of tracer lambs which grazed the experimental zones during the open grazing period.

Zone	Animal no.	Nematodes			E.P.G.
		H. contortus	*Nematodirus*	*Trichuris*	
1	41	0	20	0	0
1	229	3*	0	3*	200
2	26	0	40	0	0
2	57	0	0	8*	0

* Total counts.

Worm counts from the permanent grazers, which had grazed for 16 weeks, are summarised in Table 3.3.2. *Ostertagia* spp., *T. axei*, *Cooperia* spp. and *T. ovis* were absent or present at very low numbers in all treatment groups and therefore were not subjected to statistical analysis. *H. contortus* and *N. spathiger* were the two most prevalent nematodes found. No analysis was undertaken on the *N. spathiger* counts as it is known that the salicylanilides have little effect against this non-blood sucking species. From the *H. contortus* counts it was obvious that the treatment groups did not respond similarly in the two zones. Examination of the results from treatment groups in zone 1 revealed that individual worm counts were significantly different ($F_{5,24}=18.4$, $p<0.001$). The IVM group had the highest counts and the NTXN+IVM had the lowest. Only the ABZ and NTXN+IVM groups had counts lower than the control group. Further analysis indicated that all groups had significantly lower counts than the IVM group. The ABZ and NTXN+IVM groups had significantly lower counts than the DISO+IVM and CLOS+IVM groups. Moreover, the NTXN+IVM group had significantly lower counts than the controls.

In zone 2, worm counts were also significantly different between groups ($F_{5,24}=21.2$, $p<0.001$). The IVM group again had the highest counts whereas the ABZ group had the lowest. Only the IVM group had counts higher than the control group. Multiple range tests indicated that as in zone 1, all groups had significantly lower counts than the IVM group. Apart from the IVM group all other groups had significantly lower counts than the control group.

The tracers, which grazed for two weeks after the permanent grazers had been removed, were found to have low or negative *Ostertagia*

spp., *T. axei* and *Cooperia* spp. counts whereas the *H. contortus* and *N. spathiger* counts were high (Table 3.3.3). This was consistent with the patterns seen with the permanent grazers. Once again in zone 1 there was evidence that the counts from the IVM group were significantly higher than all other groups ($F_{5,24}$=6.7, $p<0.05$). In zone 2 the results were different ($F_{5,24}$=11.5, $p<0.01$) in that the IVM and control groups had significantly higher counts than all other groups. In addition the DISO+IVM group had lower counts than the NTXN+IVM group.

Early and late fourth stages of *H. contortus* recovered by digestion of the abomasal mucosa, were found in very low numbers in only a few animals, the maximum count being in one of the permanent grazers in the IVM group in zone 1 which had 280 immature worms. In the tracers only two animals, one from the IVM treated and another from the controls, had late fourth stages. Because of this all immature forms were added to the adult counts for analysis.

Average worm egg counts for different treatment groups in each zone over the 16 week period are shown in Figs. 3.3.1 and 3.3.2. The statistical analysis confirmed that treatment groups responded differently over time in each zone. Worm egg counts remained at generally low levels until Day 84 after the first treatment. Thereafter the counts started to increase. The IVM group showed the highest egg counts in both zones. Analysis of the IVM group versus the other treatment groups in the same zone confirmed that the IVM counts were significantly higher. Furthermore, the animals receiving IVM in zone 1 had higher counts than those receiving IVM in zone 2. The mean counts for IVM treated animals in zones 1 and 2 were 20860

e.p.g. and 4270 e.p.g. respectively.

Due to the higher worm counts in the groups treated only with IVM in comparison with the controls, the egg output per female *Haemonchus* in these two groups were estimated. As *H. contortus* has a higher egg production in comparison to the the other nematodes present, and because, with the exception of *Nematodirus*, these other nematodes were only found in very low numbers, the egg output per female *H. contortus* was calculated using numbers of female worms and faecal egg counts (excluding *Nematodirus* eggs) on day of necropsy. This analysis gave a mean output of 8.76 (±11.9) eggs per female worm for the IVM group and 3.68 (±1.88) for the controls but these differences were not significant due to the high individual variation (Appendix 3.3.13).

Results from the larval cultures revealed that almost 100% of the larvae recovered were *Haemonchus*, the exception being 14% and 19% of *Ostertagia* larvae detected on Day 70 in cultures from the DISO+IVM group and controls respectively.

It can be seen from Figs. 3.3.3 and 3.3.4 that there was a trend of decreasing percentage values for PCV's in all permanent grazing lambs. These decreases ranged from 22 to 30% reduction and coincided with the time when there was an increase in the worm egg counts observed during the grazing period. Although statistically significant differences between treatments were detected in terms of the mean PCV values at the end of the grazing period these only ranged from a mean of 26 in the control group, to 28 in both DISO+IVM and ABZ groups. This was not considered of any clinical significance.

Mean body weights of all groups are shown in Figs. 3.3.5 and 3.3.6 for zones 1 and 2 respectively. Analysis indicated that there were

significant differences in mean body weights of the different treatment groups over time (Appendix 3.3.11). However when analysing only the total accumulated live weight gains over the 112 day grazing period there were no significant differences between treatments or zones. The highest mean weight gain was obtained from animals treated with NTXN+IVM and the lowest from the group medicated with IVM.

All individual data is given in Appendices 3.3.1 to 3.3.8 while a summary of the statistical analysis is presented in Appendices 3.3.9 to 3.3.12.

DISCUSSION

The weather conditions at the begining of this experiment in Southern Brazil were not conducive to the development and survival of *H. contortus* larvae. Despite high evaporation rates in the summer in this region, enough moisture is usually present for the survival of infective larvae which can then, if ingested, develop in the sheep and subsequently produce peak pasture contamination levels of eggs and infective larvae in the autumn. It is at this time that clinical signs of disease become common, particulary in young sheep. In the present study, rainfall was only half of the expected level during the year preceding the start of the experiment and only resumed normal levels one week after the start of the experiment in February. The effect of this drought on pasture larval populations was evident from the worm counts recovered from the tracers which had grazed the experimental sites during the open grazing period at the beginning of the trial; at this time only one tracer was found to harbour *H.*

contortus. This very low level of larvae on pasture was reflected in a delayed process of infection and subsequent pasture contamination so the levels of parasitism in the permanent lambs did not reach those capable of causing severe clinical signs. With the exception of some animals in the IVM group which were most affected, all the others, including the controls, showed only a slow decrease in PCVs and poor live-weight gains by the end of the experimental period. It is probable that if the experiment had continued for a few more weeks clinical signs would have been more in evidence. The low initial infection was also responsible for a delay in the rise of worm egg counts in the untreated animals. Nevertheless, the results show that even a very few surviving larvae can initiate the whole process of animal reinfection and pasture contamination and lead to considerable worm burdens in excess of 2000 *H. contortus*. This highlights the need for strategic anthelmintic treatments when worm larval populations on pasture are most susceptible to adverse weather conditions as this can lead to a reduced number of drug treatments and thus reduce the selection pressure for resistance.

Although the results obtained from the statistical analysis carried out on worm counts did not indicate significant differences between some of the treatments it is clear that the population of *H. contortus* under study was highly resistant to IVM as animals treated with this drug always harboured significantly higher worm counts.

Possible synergistic effects of the combinations of different compounds could not be analysed as the numbers of lambs available for the experiment precluded the inclusion of a group given the salicylanilide/substituted phenol drugs alone. Such interactions may

require further studies under experimental conditions.

The fact that animals treated solely with IVM produced the highest worm counts in tracers and permanents in both replicates (Tables 3.3.2 and 3.3.3) is very interesting. A possible explanation for the higher worm counts in the animals in the IVM groups may be that pasture larval contamination in the replicate areas allocated to these groups were initially higher than in other areas. However this is unlikely as animals in all the groups were managed in the same manner and were exposed to an open grazing period prior to the subdivision of the zones and random allocation to replicated plots.

Another and more likely explanation is that the build up of infection on the IVM plots resulted from the higher, although not significant ($p<0.05$), egg output produced by female worms in the IVM group in comparison with that of the female worms in the controls; this may have led to a heavier contamination of experimental plots grazed by the group treated only with IVM. The reason for the higher egg output by the worms in this group are unknown. Alternatively it may be the result of some immunological interference between the host and the parasite. Adams (1978) showed that active infections by *H. contortus* causes a depletion of peripheral blood lymphocytes which are reactive to worm antigens enabling *H. contortus* to evade the immunological reactions of the sheep and thereby promoting the longevity of infections by this parasite. The same author also showed that this could be reversed by effective anthelmintic treatment. In the present situation, as the *H. contortus* population was resistant to the drug administered, this reaction may not have occurred and the infections therefore continued to build up and cause increased

contamination of these plots. One last although remote possibility is that IVM may have some direct and unknown effect on the resistant worms which may modify their egg production. This is unlikely as the original population in all experimental plots was resistant and it is difficult to conceive that the two extra treatments with IVM were responsible for producing worm populations with greater fecundity than the controls. Nevertheless resistance to IVM is new and as the mechanisms involved are not yet understood, further investigations are required to answer this question.

The use of salicylanilide drugs at strategic times has been previously used in the control of *H. contortus* (Dash *et al.*, 1985; Echevarria *et al.*, 1988). In this trial, closantel, disophenol and nitroxynil were used in association with IVM and both permanent grazers and tracer lambs from groups treated with these mixtures, showed significantly lower burdens of *H. contortus* than the animals treated only with IVM.

Although there were significantly fewer parasites in the permanent grazers treated with disophenol and closantel compared with the group treated with IVM, these two salicylanilide drug treated groups had higher worm counts than the group treated with nitroxynil and these differences were also evident in the worm burdens of the tracers which grazed the different paddocks at the end of the grazing period. Of the three salicylanilide drugs administered only disophenol had been previously used in the area selected for this trial some eight years earlier. The salicylanilides act by uncoupling oxidative phosphorylation which leads to energy depletion. Nitroxynil and disophenol are substituted monophenols and this has been suggested by

Jeannin *et al.* (1990), as a possible explanation for a better efficacy of nitroxynil in comparison with closantel against a South African IVM resistant strain of *H. contortus*. In this study the overall reduced efficacy of disophenol compared with nitroxynil may be due to previous exposure of the *H. contortus* population to disophenol although no sign of resistance had been detected when its use ceased more than eight years earlier.

The efficacy obtained with ABZ in this study was rather surprising since a previous controlled anthelmintic test (see Exp. 3.1) had shown that its efficacy, against the same strain of *H. contortus*, was only 14.7%. The use of benzimidazole drugs had ceased six years earlier when BZ resistance was first detected and IVM was then used as the sole anthelmintic in the area (Echevarria and Trindade, 1989). Although in the earlier experiment it was shown that this strain of *H. contortus* was still highly resistant to ABZ and that no reversion towards susceptibility had taken place despite using only IVM for 6 years, under the field conditions of the present experiment, ABZ appeared as efficient as the combination of nitroxynil and IVM. Reversion towards anthelmintic susceptibility has been reported in some resistant sheep nematode populations (Donald *et al.*, 1980; Waller *et al.*, 1983; Waller *et al.*, 1985; Waller *et al.*, 1989; Martin *et al.*, 1988) and one could suggest that this was the reason for these conflicting results. However reversion is usually a slow process and in the present situation the two experiments were carried out only eight months apart. Furthermore, it has been shown that even if a drug when first reintroduced gives a high efficacy, previous resistance levels may soon be re-established (Kelly and Hall, 1979; Egerton *et*

al., 1988; Waller *et al.*, 1989). Further studies on the behaviour of different nematode strains and interactions of benzimidazole resistant strains with IVM, which is a relatively new drug, might provide an explanation for these findings.

The use of salicylanilide and substituted phenol drugs, with their prolonged anthelmintic activity, has been advocated as a new approach to keeping populations of *H. contortus* at sufficiently low levels to merit reducing the number of treatments. It is even possible that some *Haemonchus* populations could be eradicated as has been achieved with the use of closantel by Barger *et al.* (1990). Nevertheless resistance to closantel has evolved (Van Wyk *et al.*, 1982; Van Wyk and Malan, 1988; Rolf and Boray, 1990) and the reasons for this may be their prolonged plasma life which could in turn predispose to development of resistance.

Although it is known that salicylanilide and substituted phenols bind to plasma proteins and are toxic to parasites only when blood is ingested, in the present study only small numbers of immature stages of *H. contortus* were found. This is most likely due to the overall reduction in pasture larval contamination resulting from the various treatment regimes.

N. spathiger was found in fairly high numbers in all groups and further studies are necessary to assess the significance of this parasite and its susceptibility to the different anthelmintics used in this experiment.

In the study reported here the salicylanilide and substituted phenol drugs were efficient in keeping a field population of IVM resistant *H. contortus* at low levels and nitroxynil was found to be

the most active drug in this respect. Nevertheless, care must be taken in the use of these drugs as resistance has been previously shown to develop to at least one member of this group and given the high biotic potential of *H. contortus*, a build up of infection could occur very quickly under favourable conditions, even with the low worm burdens detected in this study.

TABLE 3.3.2. Mean worm counts (± sd) from five permanent grazers/zone at the end of a 112 day grazing period.

		Treatment					
Nematode	ZONE	IVM	CLOS+IVM	DISO+IVM	ABZ	NTX+IVM	CONTROL
H. contortus	1	5122 (±1497.2)	2198 (±336.9)	2976 (±1115.0)	1276 (±289.9)	950 (±232.6)	1914 (±579.8)
	2	1784 (±677.2)	200 (±66.6)	226 (±81.4)	162 (±105.7)	296 (±92.0)	736 (±258.7)
Ostertagia	1	10 (±15.5)	4 (±4.9)	0 -	2 (±4.0)	2 (±4.0)	4 (±8.0)
	2	0 -	0 -	0 -	12 (±14.7)	0 -	2 (±4.0)
T. axei	1	22 (±25.6)	66 (±65.3)	86 (±78.4)	20 (±17.9)	4 (±8.0)	60 (±47.7)
	2	2 (±4.0)	40 (±25.3)	18 (±18.3)	34 (±35.6)	44 (±24.2)	192 (±109.3)
Cooperia	1	6 (±12.0)	12 (±12.6)	4 (±8.0)	0 -	0 -	0 -
	2	0 -	0 -	0 -	0 -	4 (8.0)	30 (±36.9)
N. spathiger	1	2614 (±2474.8)	2990 (±2800.7)	2296 (±1303.3)	1798 (±1656.6)	502 (±440.2)	1958 (±1077.7)
	2	1548 (±1055.8)	340 (±219.0)	170 (±44.3)	1348 (±336.2)	966 (±504.3)	1182 (±443.3)
T. ovis	1	2.8 (±2.7)	8 (±16.0)	1.8 (±2.2)	0.8 (±1.6)	1.2 (±1.6)	4.6 (±3.4)
	2	1.0 (±1.3)	0.6 (±1.2)	1.0 (±0.9)	1.0 (±2.0)	0 -	2.8 (±2.8)

TABLE 3.3.3. Mean worm counts (± sd) from five tracer lambs/zone grazed for 14 days at the end of a 112 day grazing period.

		Treatment					
Nematode	ZONE	IVM	CLOS+IVM	DISO+IVM	ABZ	NTX+IVM	CONTROL
H. contortus	1	8152 (±8530.2)	1536 (±255.5)	1260 (±602.8)	680 (±347.0)	490 (±140.4)	1948 (±1114.5)
	2	2008 (±1329.2)	152 (±40.2)	76 (±50.0)	158 (±64.3)	240 (±81.2)	1172 (±330.8)
Ostertagia	1	258 (±345.9)	46 (±51.2)	52 (±44.5)	16 (±20.6)	6 (±12.0)	26 (±25.0)
	2	30 (±60.0)	22 (±17.2)	8 (±9.8)	0 -	16 (±16.2)	36 (±30.1)
T. axei	1	52 (±65.2)	32 (±32.5)	10 (±20.0)	0 -	8 (±9.8)	10 (±11.0)
	2	0 -	22 (±16.0)	18 (±22.3)	0 -	4 (±8.0)	402 (292.5)
Cooperia	1	0 -	8 (±11.7)	0 -	0 -	0 -	18 (±36.0)
	2	0 -	0 -	0 -	0 -	4 (±8.0)	16 (±20.6)
N. spathiger	1	480 (±920.1)	3150 (±4047.5)	422 (±749.7)	1180 (±1485.7)	198 (±270.8)	1206 (±1802.3)
	2	536 (±433.3)	278 (±147.4)	224 (±146.5)	322 (±390.0)	282 (±441.6)	2180 (±1831.8)

Fig. 3.3.1. Egg counts from permanent grazers in zone 1

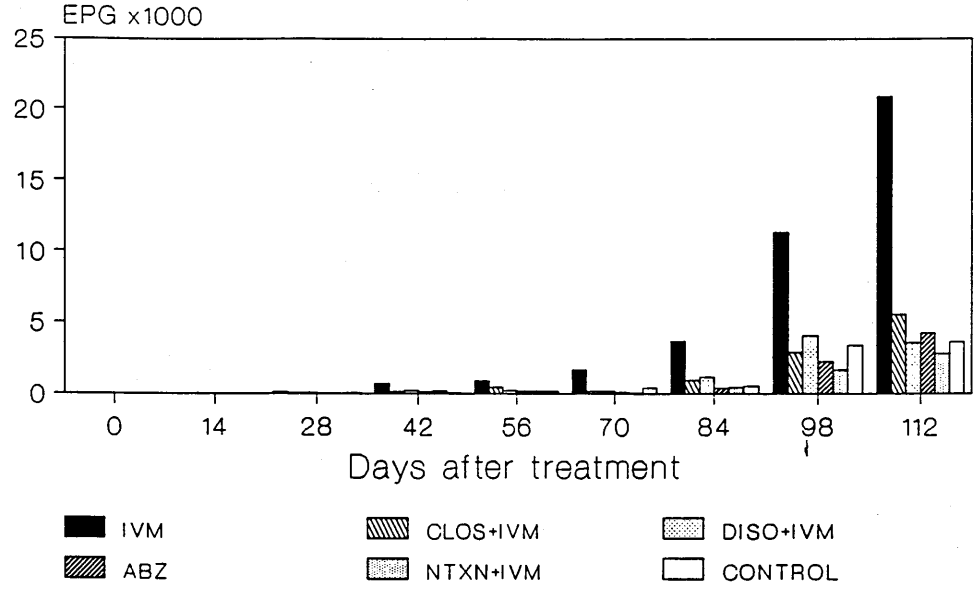


Fig. 3.3.2. Egg counts from permanent grazers in zone 2

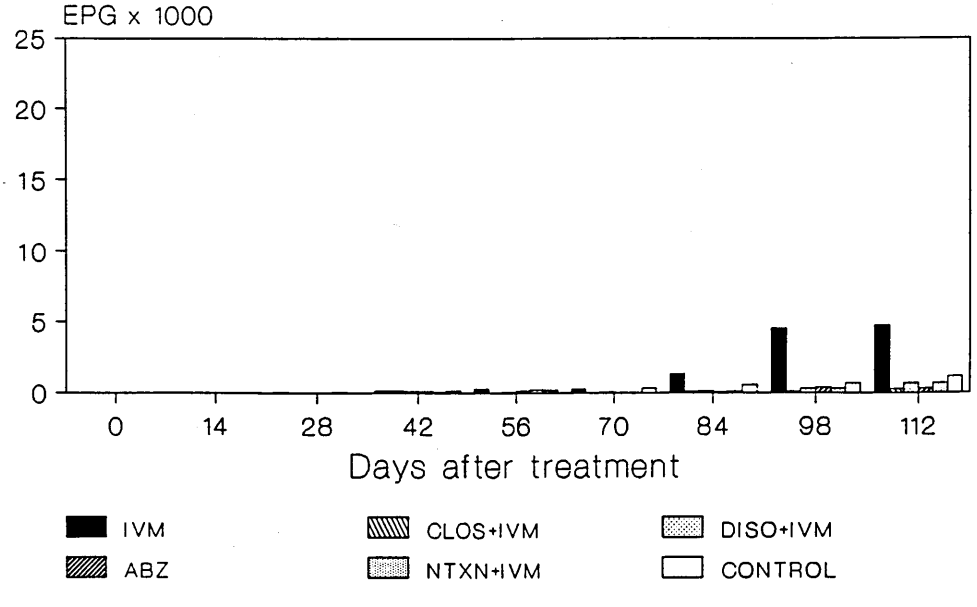


Fig. 3.3.3. Mean PCV from permanent grazers in zone 1

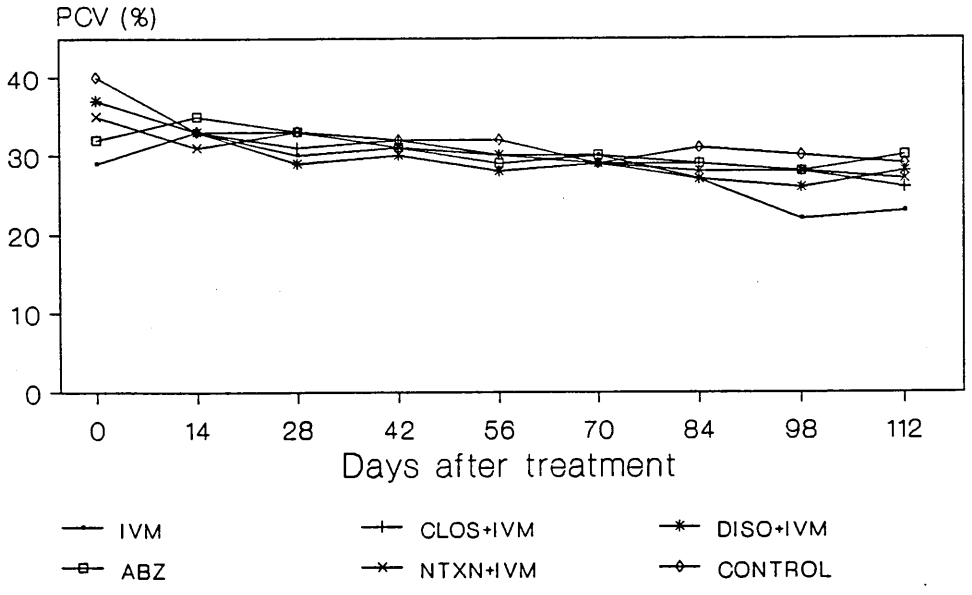


Fig. 3.3.4. Mean PCV from permanent grazers in zone 2

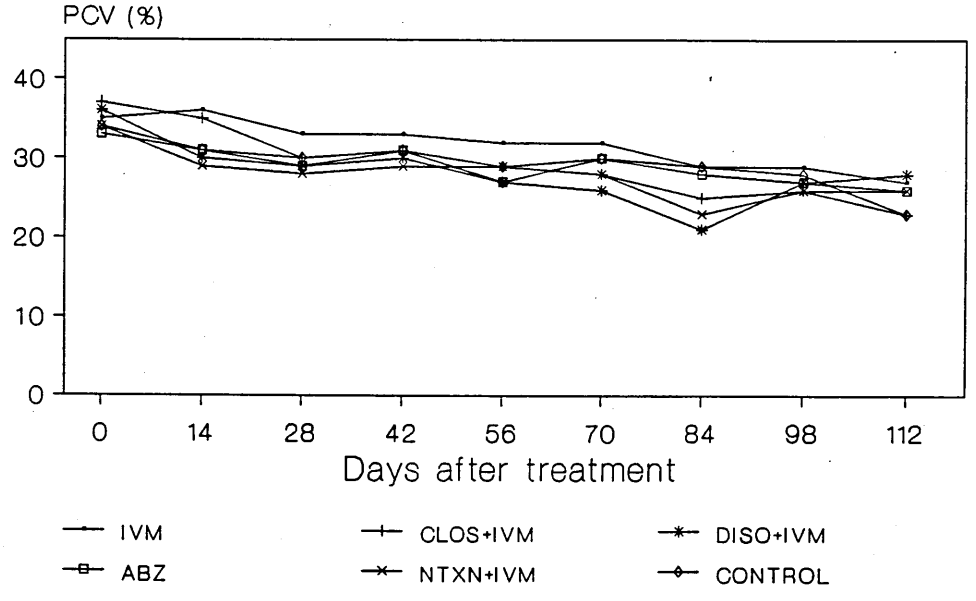


Fig. 3.3.5. Mean body weights from permanent grazers in zone 1

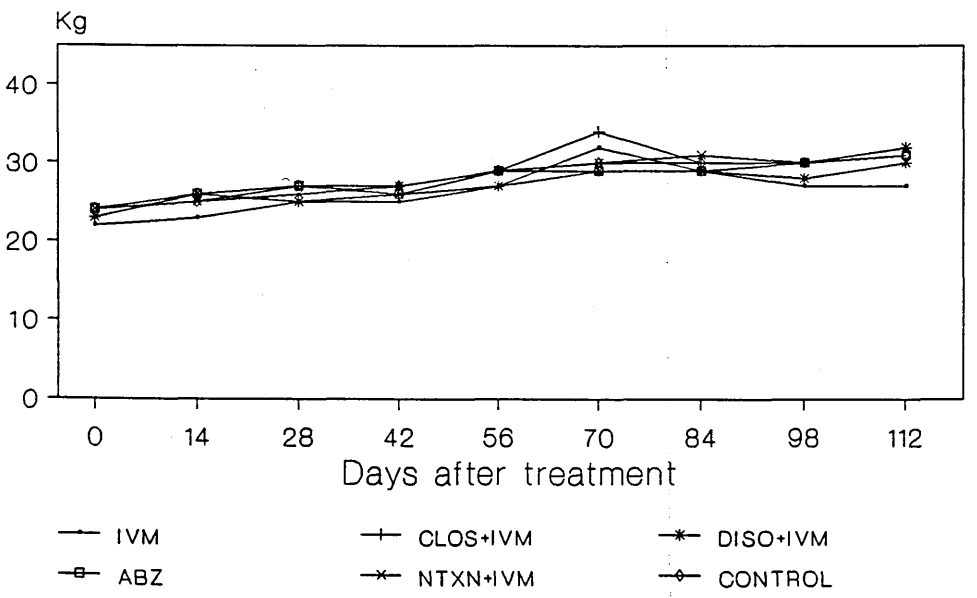
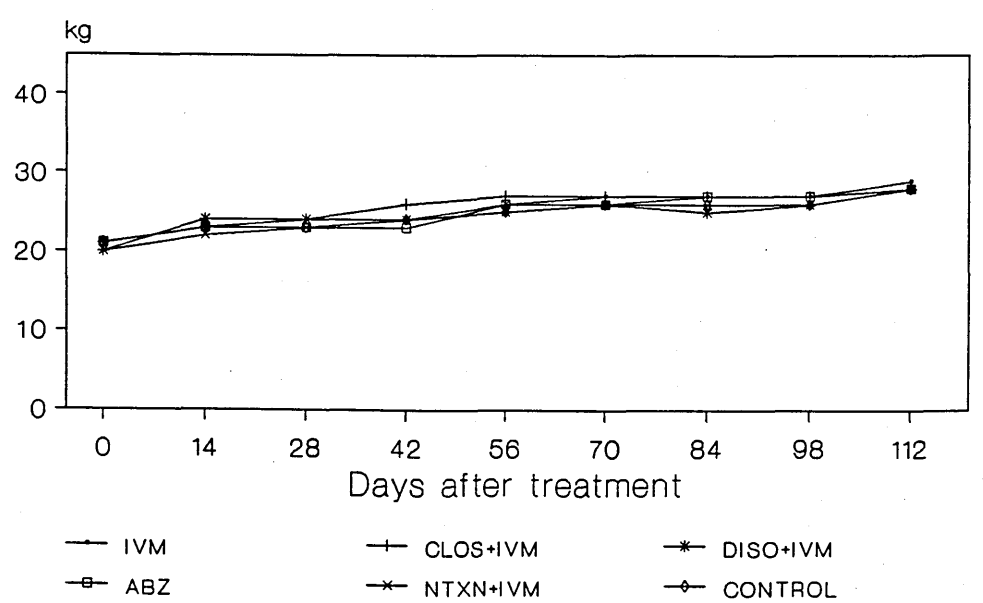


Fig. 3.3.6. Mean body weiths from permanent grazers in zone 2



Experiment 3.4

HOW EFFECTIVE ARE RESEEDED PASTURES AS AN AID IN THE CONTROL OF GASTROINTESTINAL NEMATODES IN SHEEP?

INTRODUCTION

In the mild climate of Southern Brazil *H. contortus* is present almost all year round and leads to outbreaks of haemonchosis during the autumn and early winter months. To avoid some of the economic losses associated with infection farmers treat their young animals at frequent intervals, especially during the autumn, which results in animals being treated approximately nine times/year. As a consequence there are recent reports of resistance of *H. contortus* to the benzimidazoles (BZ) and levamisole (LEV) (Echevarria and Pinheiro, 1989) and to ivermectin (IVM) (Echevarria and Trindade, 1989).

In some parts of Europe good gastrointestinal nematode control in ruminants has been achieved by the division of a given farm into blocks which are used on the basis of an annual rotation between either sheep, cattle and crops, sheep and crops or cattle and crops (Hood and Bailie, 1973; Rutter, 1975; Armour, 1978; Anon 1980, 1981; Mitchell and Fitzsimons, 1983). Before the adoption of such an approach in other parts of the world, it is important that such rotational grazing systems are investigated locally as the species of nematodes present together with prevailing weather conditions may greatly influence the results obtained.

In Southern Brazil, although most farms have large numbers of sheep

and cattle and in many of these some form of crop agriculture is also undertaken, the use of "integrated worm control systems" e.g. dosing with anthelmintics prior to the movement of animals to pastures where the presence of nematodes may be at very low levels, have not yet been exploited.

This study was initiated to investigate whether nematode larvae were present on pastures reseeded after a crop of soya bean had been harvested, and thus assess the possible use of such pastures as an aid for nematode control. This would have the advantage of reducing anthelmintic usage and thus reducing selection pressure for anthelmintic resistance.

MATERIALS AND METHODS

This trial was conducted at Embrapa's Research Centre in Bagé, State of Rio Grande do Sul in Southern Brazil. The climate is temperate with four well-defined seasons of summer (December, January, February), autumn (March, April, May), winter (June, July, August) and spring (September, October, December). Normally the rainfall (1200-1300 mm/year) is evenly distributed throughout the year but there is often high evaporation during the summer and occasionally long droughts. The normal mean temperature varies between a maximum of 30.5°C for January and a minimum of 8.1°C for July.

An area of 26ha which had been grazed by beef cows and their calves during the winter and spring was ploughed and seeded with a soya bean crop in mid November. The crop was harvested at the end of May and the soil then lightly ploughed and seeded with *Avena strigosa*. This

winter/spring grass was ready for grazing by late August when cattle were allowed to graze the area. At this time, four tracer lambs and four tracers calves were introduced to graze the area to assess the level of nematode contamination on this reseeded pasture. The four calves were 10-11 month old Hereford castrated males while the four lambs were 9-10 month old Corriedale wethers. The calves, which were housed and treated twice at an interval of five days with ivermectin at 0.2mg/kg were then released onto pasture 21 days after the second treatment. The tracer lambs had been housed for more than four months and they had also been treated with ivermectin at housing. Samples of faeces from all the tracers were checked and found to be negative for gastrointestinal nematode eggs on the day they were put out to pasture. After a 14 day grazing period all of the tracers were housed for a further 14 days when they were slaughtered for estimation of total worm counts (Powers *et al.*, 1982); worm egg counts were carried out on faecal samples collected at necropsy.

RESULTS

The results are presented in Table 3.4.1. With the exception of one calf, which was found to be harbouring 20 *Cooperia punctata*, all of the remaining tracers had negative worm counts at necropsy. All faecal worm egg counts carried out on the day of necropsy were negative.

TABLE 3.4.1. Necropsy worm burdens and worm egg counts of tracer lambs and calves which grazed reseeded pastures for 14 days were then housed and killed 14 days later.

	Ovine			Bovine		
Tracer	Abomasum	S.Intestine	E.P.G.	Abomasum	S.Intestine	E.P.G.
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	20*	0
4	0	0	0	0	0	0

No immature nematodes were recovered from the eight abomasal digests.
**C. punctata.*

DISCUSSION

The results reported here indicate clearly that the use of reseeded pastures following a crop of soya bean in Southern Brazil produces pastures where there is little risk of nematode infection to sheep and cattle.

In Europe the use of integrated nematode control systems which incorporate minimal anthelmintic treatments with the use of "safe pastures" i.e., pastures with a very low level of nematode infectivity, have been used for some time. The appearance of anthelmintic resistance of sheep nematodes to all broad-spectrum anthelmintics has emphasised the importance of alternative approaches in order to reduce the requirement for frequent anthelmintic treatments. However the technique of moving animals onto clean pastures after anthelmintic treatment has been questioned since any nematodes which survive such treatment would be likely to

include resistant individuals and thus could result in a greater selection pressure for anthelmintic resistance than more traditional empirical treatments on permanent pastures (Le Jambre, 1978; Michel *et al.*, 1983). On the other hand this may not always be the case as these safe pastures could be a "dead-end" for resistant parasites if lambs grazing such areas go to slaughter or if the pastures are subsequently grazed by cattle or cropped the following year (Donald, 1983). That this may be the case is supported by the lack of evidence of widespread anthelmintic resistance in such systems in the United Kingdom where integrated nematode control programmes have been in use for a long time on some farms.

In the Brazilian situation there are also some practical considerations which should be taken into account. First of all, sufficient areas of reseeded pastures or aftermath may not be available to provide safe grazing for all susceptible animals on a farm. In such cases economic factors must be considered together with parasitological aspects of the problem as nematode-safe pastures must also guarantee growth and production at appropriate levels. One important advantage of the use of reseeded pastures in spring is that they could be grazed by ewes and lambs, the latter being sent to slaughter when 3-4 months of age with no need for anthelmintic treatment. Female lambs to be kept for breeding would be a small proportion of the total flock and could be transferred to other areas of the farm where other management systems could be used such as alternate grazing with either adult cattle or with immune adult sheep. The use of other options should also be investigated ie., other types of crop aftermath which could be available at other times of

the year, particulary towards the end of summer, so that infection of susceptible young animals could be prevented during the high risk autumn period.

It has been said that farmers are often unwilling to adopt integrated control programmes (Newman, 1984; Edwards *et al.*, 1986b) despite the considerable benefits which may be obtained (Barger and Southcott, 1978; Donald *et al.*, 1987). However if resistance becomes a major problem in farming and society continues to demand a decrease in the use of prophylactic medicines, the emphasis for a more natural approach may influence farmers to adopt alternative strategies.

CHAPTER 4

STUDIES ON THE BIOLOGY OF IVERMECTIN RESISTANT *Haemonchus* contortus

Experiment 4.1

LABORATORY SELECTION FOR IVERMECTIN RESISTANCE IN *Haemonchus contortus*

INTRODUCTION

Over the last two decades the rapid spread of resistance of nematodes to many anthelmintics has threatened gastrointestinal parasite control programmes in some of the most important sheep grazing areas of the world (Waller, 1987). Approximately 10 years ago the discovery of a chemically distinct group of anthelmintics, the avermectins (Burg *et al.*, 1979) provided a welcome alternative for use in nematode control. Ivermectin, which is currently the only avermectin compound available, is highly efficient not only against all common gastrointestinal and lung nematodes of sheep and cattle but also against some external parasites, at doses measured in micrograms/kilogram (Campbell, 1985). Nevertheless sheep nematodes have been shown to develop resistance to this new compound. For example, resistance to ivermectin has been selected for under laboratory conditions in both *H. contortus* (Egerton, *et al.*, 1988) and *T. colubriformis* (Giordano *et al.*, 1988) and has also been reported in the field with *H. contortus* both in South Africa (Van Wyk and Malan, 1988) and in Brazil (Echevarria and Trindade, 1989).

Since the precise mechanism of anthelmintic resistance to IVM has not yet been determined (Prichard, 1990) it is important to study various aspects of the biology of different strains of nematodes subjected to anthelmintic selection with this drug. Information thus

obtained could be used in further investigations such as the rate of development of resistance, the inheritance of resistance, the identification of resistant strains and its implications on nematode control programmes. For this purpose it would be helpful if a strain of parasite selected for resistance to ivermectin over a known number of generations were available.

This is a report of an experiment in which a susceptible strain of *H. contortus* was subjected to a series of passages in parasite-naive lambs and treated with ivermectin until significant resistance to the drug was obtained.

MATERIALS AND METHODS

Parasites. An anthelmintic susceptible strain of *H. contortus* was used which was originally obtained from The Moredun Institute, Edinburgh and kept at Glasgow for more than 5 years by repeated passage through worm-free lambs.

Animals. For the selection study, worm-free Suffolk x Scottish Blackface lambs aged 4-6 months were kept under conditions such as to preclude reinfection and fed 1kg/day of concentrate plus hay and water *ad lib.*

Selection procedure. For each parasite generation one lamb was used. For the first generation the infection dose was 10,000 L_3 of *H. contortus* but, due to low numbers surviving treatment, for passages two to five 2,000 L_3 were used: for the sixth passage the dose used was 4,000 L_3. On Days 28 and 35 post-infection the worm egg

counts of the lambs were determined using the McMaster technique. Also on Day 28 post-infection animals were treated with 0.02mg/kg of an oral formulation of ivermectin (Oramec, MSD). The dose rate used, i.e. one tenth of the normal recommended dose, was chosen as it had been shown to produce reductions in the order of 95% in the egg output after treatment (Egerton *et al.*, 1988). From Day 7 post-treatment onwards the total daily faecal output was harvested and incubated in a culture room at 22°C for a period of 10 days. At the end of this period the faeces were baermanized to collect infective L_3 for the subsequent passages.

The first adult generation to be subjected to the selection procedure with ivermectin was designated as S-IVM while larvae which survived the first treatment were designated as F1, the second F2 and so on.

Once resistance was suspected, i.e. poor reduction in worm egg counts following treatment at 0.02mg/kg, the same animal was given ivermectin at the normal dose rate of 0.2mg/kg and the L_3 which survived this exposure (R-IVM) were used in an experiment designed to confirm that resistance to normal dose rates of IVM had developed. A group of 12 Finn-Dorset and 8 Scottish Blackface worm-free lambs aged four months were allocated at random to two groups of 10 animals each: the allocation was such that each group comprised a representative proportion of each breed involved. The first group (R) was infected with 10,000 L_3 which survived the full treatment dose (R-IVM) and the other infected with 10,000 L_3 of the original strain which had been repeatedly passaged through sheep (S-IVM). On day 21 post infection all animals within each group were faecal sampled and

according to their individual worm egg counts they were allocated to the folowing sub-groups:

Rt - infected with R-IVM strain and treated with IVM at 0.2mg/kg

Rc - infected with R-IVM strain but not treated

St - infected with S-IVM strain and treated with IVM at 0.2mg/kg

Sc - infected with S-IVM strain but not treated

On day 14 after treatment the individual animals with the highest and lowest egg counts in each sub-group were slaughtered for total worm counting.

Worm egg counts were monitored at 2-3 day intervals from day 21 post-infection until 2 weeks after treatment and the results, after log transformation, submitted to two-way analysis of variance followed by Newman Keuls Multiple Range Test.

RESULTS

The effect of the selection pressure of IVM at 0.02mg/kg on a susceptible strain of *H. contortus* is shown in Table 4.1.1. Ivermectin was highly effective against the population tested during five passages with worm egg counts, measured 7 days after treatment, showing a 100% reduction. With such high efficacy very few eggs were produced at each generation which in turn led to a longer period of collection of faeces in order to achieve the target of 2,000 L_3 to infect a new worm-free lamb for the subsequent generation.

The animal infected with the fourth generation larvae however produced an egg count of 100 e.p.g. and it was decided not to treat this animal, instead, larvae were harvested to provide infective material for the following generation, F5. After the sixth treatment positive egg counts by the MacMaster method were regularly obtained and from this point onwards larvae were more easily obtained to provide a dose of 4,000 L_3 to infect subsequent lambs. By the eighth treatment the infected lamb showed no reduction in worm egg counts after dosing. Four weeks later this animal was treated with the manufacturer's recommended dose of 0.2mg/kg, and this treatment reduced egg counts by 85% when measured at seven days after dosing. Larvae which survived this treatment (R-IVM) were used in the confirmation study: the results of worm burdens and worm egg counts of sheep used in this study are shown in Table 4.1.2 and in Figure 4.1.1.

Statistical analysis of the worm egg counts during the 14 day period following the last treatment showed that worm egg output from animals infected with the selected strain and treated with IVM was not significantly different from that of animals infected with the same strain but which remained untreated. Those infected with the S-IVM had their egg counts significantly reduced by the treatment in comparison to the controls (Fig. 4.1.1). No significant differences were detected betweeen the egg output obtained from the two untreated control groups ($P>0.05$).

Figure 4.1.1 shows clearly the reduction in mean egg count five days after treatment in animals infected with the susceptible strain of *H. contortus* while the resistant-treated group maintained their

egg counts during the entire experimental period. Individual results are given in Appendix 4.1.1.

Results obtained at the necropsy of two lambs from each group 14 days after treatment, revealed that animals infected with the selected resistant strain and treated with IVM had similar worm burdens to those of the untreated controls. Those infected with the original susceptible strain, and treated in the same way, had their worm counts eliminated with the exception of one animal which had 10 *H. contortus* (Table 4.1.2).

DISCUSSION

The results obtained from this study clearly revealed that resistance to IVM had developed in a laboratory maintained strain of *H. contortus* under the selection procedure applied. Although only two animals from each group were killed in the confirmation trial, it was obvious that the drug was highly effective on the original susceptible strain while heavy burdens were present in the animals infected with the selected resistant strain and treated with IVM at the recommended dose rate of 0.2mg/kg.

It is believed that provided there is enough genetic variability within any given population of nematodes resistance will develop. However, the speed of selection for resistance will be influenced by many factors such as genetics, e.g. type of inheritance, dominance, level of resistance and relative fitness; biological or ecological factors, e.g. life-cycle, reproduction, generation turnover, stage exposed to the discriminating drug and proportion of population in

refugia, and operational factors, e.g. type and efficiency of anthelmintic, intensity of application, mode of action, rotation of drugs, dose rates and grazing management (Prichard, 1990). Under laboratory conditions, where a very small proportion of a given population is under pressure these variables are much reduced and the main factors may be the presence of sufficient genetic variability and intensity of drug selection. If the dose applied is not sufficient to kill heterozygous resistant worms only the susceptibles will be killed and resistance may evolve rapidly.

In the present studies the dose of IVM was chosen based on the work of Egerton *et al.* (1988) who obtained 95% reduction in the infecting parent population with 0.02mg/kg. However the strain of *H. contortus* used here seemed to be more susceptible than Egerton's since worm egg counts were reduced by 100% with this low dose until the fourth treatment and only after the fifth dose were *H. contortus* eggs detected by the McMaster technique. Clearly IVM is a very effective anthelmintic against susceptible strains of *H. contortus* even at one tenth of the recommended dose rate but resistance can develop rapidly if the drug is used repeatedly at low dose rates.

The appearance of anthelmintic resistance after eight treatments in this study is very similar to what was obtained in the work by Egerton *et al.* (1988) where resistance was detected after the seventh treatment with IVM. However, these two studies are quite different from the results obtained by Kates *et al.* (1973) where resistance to cambendazole in *H. contortus* was obtained after only four successive parasite generations exposed in turn to 5, 5, 10 and 20mg/kg of that drug respectively. These differences may be more likely due to some

previous exposure of their strain to benzimidazoles (Colglazier *et al.*, 1970) rather than to differences betweeen strains or drugs.

Following the report of anthelmintic resistance to IVM in *H. contortus* under laboratory conditions (Egerton *et al.*, 1988) field populations have also been found to be resistant (Van Wyk and Malan, 1988; Echevarria and Trindade, 1989) and if new strategies which will rely on less intensive use of IVM are not employed further reports of resistance can be expected in the near future.

Differences in the time required for the appearance of anthelmintic resistance, apart from the required genetic variability, may also be the result of some natural differences between strains, selection pressure and species of nematodes involved. In a selection study carried out by Giordano *et al.* (1988) a mixed population of *H. contortus*, *O. circumcincta* and *T. colubriformis* was exposed to increasing doses from 0.1mg up to 0.225mg/kg of ivermectin during four generations; by this time *T. colubriformis* had become resistant to the drug whereas the other nematode species were eliminated by the first treatment.

These results confirm once more that unless anthelmintics are used at dose rates which will kill all nematodes within the final host, drug resistant individuals may survive treatment and transmit this ability to their offspring. Also if the same product continues to be used repeatedly resistance is likely to become a major problem. Because of this, further studies on the more limited use of drugs in prophylactic control schemes should be investigated in order to extend the useful life span of currently available anthelmintics.

Table 4.1.1. Summary of the selection protocol where animals were infected with *H. contortus* and treated with IVM 0.02mg/kg on day 28 post-infection.

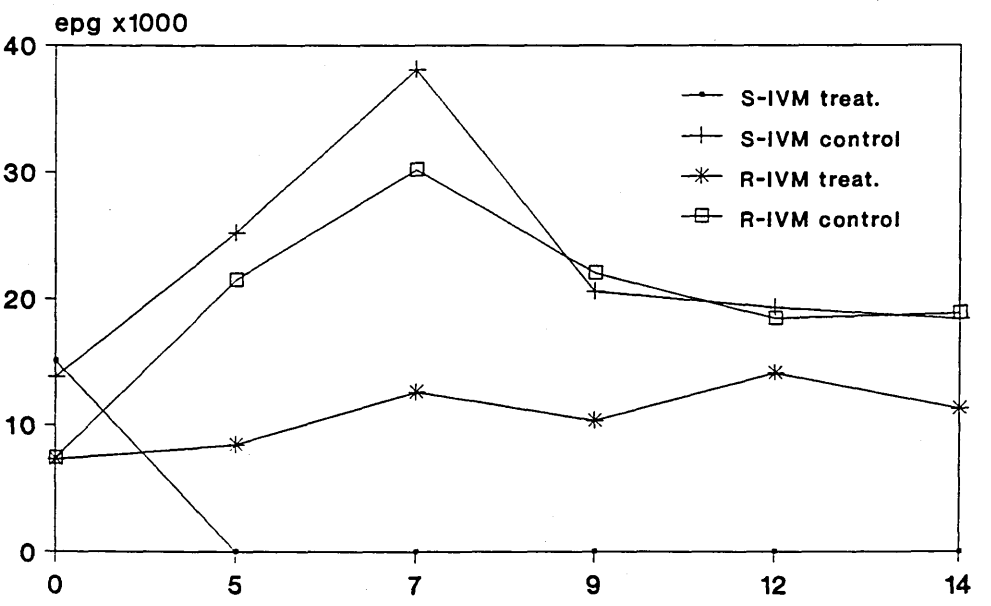
Animal	Passage	Treatment	Generation	E.P.G.	
				day 28	day 35
P81a	1	1	F1	17200	0
P63	2	2	F2	1250	0
W82	3	3	F3	2400	0
W75	4	4	F4	950	0
W91	5	*	F5	100	0
P12	6	5	F6	1350	50
P13	7	6	F7	5400	0
P80	8	7	F8	7000	450
P81	9	8	F9	12800	12400

* Due to the low worm egg count this animal was not treated.

Table 4.1.2. Mean worm burdens and egg counts of animals infected with IVM resistant or susceptible strains of *H. contortus* 14 days after treatment with ivermectin.

Strain	Treatment	Animal	E.P.G.	Worm counts
R-IVM	IVM	1	20400	5200
	0.2mg/kg	2	5800	3610
R-IVM	Untreated	1	21600	3800
	control	2	10200	8920
S-IVM	IVM	1	0	10
	0.2mg/kg	2	0	0
S-IVM	Untreated	1	28300	6670
	control	2	550	620

Fig. 4.1.1. EPG after IVM treatment on resistant or susceptible *H. contortus*



R-IVM treat. x R-IVM cont.= P>0.05; S-IVM treat. x S-IVM cont.= P<0.05

Experiment 4.2

THE PATTERN OF FAECAL EGG OUTPUT IN LAMBS INFECTED WITH A MULTIPLE RESISTANT STRAIN OF *Haemonchus contortus* AFTER TREATMENT WITH ALBENDAZOLE

INTRODUCTION

Changes in nematode worm egg counts following anthelmintic treatment have been commonly used to measure anthelmintic efficacy as faecal examination is simple and does not require sophisticated equipment or facilities. However, there is one important drawback in basing efficacy on worm egg counts as some anthelmintics, particulary the benzimidazole (BZ) compounds, may cause a temporary suppression of egg output in resistant nematodes without a corresponding worm loss. This was first demonstrated in sheep infected with *T. colubriformis* where thiabendazole (TBZ) treatment reduced egg output by 70% five days after treatment but failed to lower worm counts (Hotson *et al.*, 1970). Later Le Jambre *et al.* (1979b) studying strains of *H. contortus* considered BZ-susceptible by Webb *et al.* (1979), on the basis of more than 95% reductions in worm egg counts four days after TBZ treatment, found that these strains were in fact BZ-resistant when screened in an egg-hatch assay. Sangster *et al.* (1979) in a study using various dosing regimes of fenbendazole (FBZ) against a BZ-resistant strain of *H. contortus*, found a good correlation (r= 0.79) between necropsy worm egg counts and worm burdens in lambs when they were killed at day 13 post-treatment.

However different species of parasites may behave differently as the same authors found no such correlation (r= 0.33) in similar studies using FBZ against a BZ-resistant strain of *T. colubriformis*. Martin *et al.* (1985) using two different strains of BZ-resistant *Ostertagia* spp. found that although egg counts returned to pre-treatment levels 10 days after dosing with various BZs, at this time the relationship between worm egg counts and necropsy worm burdens was low (r= 0.46 and 0.39).

In Experiment 3.1 on the efficacy of some anthelmintics on a Brazilian benzimidazole/ivermectin resistant strain of *H. contortus* in sheep, albendazole (ABZ) reduced worm egg counts by 92.5% when measured at Day 7 post-treatment but worm burdens at this time were lowered by only 14.7%. Furthermore where IVM resistance occurs in the field BZ resistance is usually already present. Following these results a small experiment was set up to monitor egg count patterns and necropsy worm burdens of sheep infected with a South African multiple drug resistant (BZ, IVM and closantel) strain of *H. contortus* after treatment with ABZ.

MATERIALS AND METHODS

A group of six, five-month-old Suffolk x Scottish Blackface lambs reared worm-free and fed a ration of hay and lamb finishing pellets *ad lib*, were infected with 5,000 L_3 of a South African multiple resistant strain of *H. contortus* (Van Wyk and Malan, 1988).

Starting 21 days post-infection, worm egg counts were monitored daily using the modified McMaster technique. Based on the worm egg

counts on Day 21 post-infection the animals were allocated to two groups of three animals each. Twenty nine days post-infection (Day 0), animals in one group received ABZ (Valbazen 2.5%; Smith Kline Animal Health Ltd) at 3.8mg/kg, the other group remaining as untreated controls. The dose rate was calculated according to individual body weights and administered *per os*.

All animals were slaughtered 44 days post-infection (Day 15) and total worm counts estimated as recommended by Powers *et al*. (1982).

Larval cultures were carried out on faeces collected on Days 0, 1, 6, 7, 8, 9, 10, 11, and 15 after treatment. Three grams of faeces from individual animals were incubated individually at 27^{0}C for seven days. The faeces were then placed in a Baerman apparatus, the larvae collected and the total number of L_3 estimated by counting 10 aliquots of 40ul: this was then divided by three to give numbers of larvae per gram (lpg) of faeces.

RESULTS

Worm egg counts at the time of treatment and slaughter and necropsy worm burdens are given in Table 4.2.1, while mean egg counts and larval counts post-treatment are shown in Figs. 4.2.1 and 4.2.2 respectively. Individual results are presented in Appendix 4.2.1.

The mean worm egg counts fell by 89% on Day 2 after treatment (from 10250 to 1083 e.p.g.) but from Day 3 onwards they increased until Day 7 when they fell again to a level similar to that recorded on Day 2 post-treatment (Fig. 4.2.1). Over the next eight days there was a general increase in egg output - by Day 10 after treatment the

reduction in egg count was 25.9% and on Day 15 it was 41.1%. However when the animals were killed on Day 15 post-treatment it was found that worm counts were reduced by only 25.2% (Table 4.2.1).

Larval recoveries in the treated group, were reduced by more than 72% when compared to the numbers recovered on the day of treatment; on Day 1 the reduction was 100% (Fig. 4.2.2).

DISCUSSION

Although no statistical analysis was carried out because of the small size of the groups, the results show clearly that ABZ had little effect on total worm burdens at necropsy as they were reduced by only 25.2% (Table 4.2.1). These reductions in worm counts are well within the range of efficacies previously reported for other BZs against this strain of *H. contortus*. For example, Van Wyk and Malan (1988) using FBZ, obtained a reduction in worm burdens of 22.1% and Jeannin *et al.* (1990), also using FBZ, obtained a 36.5% reduction.

Results from the worm egg counts showed that apart from Day 1 when counts were higher, the level of reduction obtained with ABZ was variable being in the range of 23% to 89%. For example reductions on Days 7, 10, 12 and 15 were 89%, 25%, 32% and 41% respectively.

These results indicate that the correlation between worm egg counts and necropsy worm burdens, following treatment of animals harbouring BZ resistant parasites, is low and that, at least in the present study, egg counts were not good indicators of the degree of drug efficacy. This is supported by the contemporaneous findings of Martin *et al.* (1990) who, using composite strains of *T.*

colubriformis and *Ostertagia* spp. which ranged from full susceptibility to 100% BZ-resistance, subjected these to various tests i.e. a worm egg count reduction test, an *in vitro* egg hatch assay and a tubulin binding assay: they found that all of these tests detected resistance where the proportion of resistant strains in the composite was 50% or more, whereas none of the tests unequivocally detected resistance when this proportion was below 25%.

In the present study worm egg counts monitored until Day 15 post-treatment did not return to pre-treatment levels (Fig. 4.2.1) as has been reported for BZ-resistant *Ostertagia* by Martin *et al.* (1985). This may be due to differences between nematode species and/or levels of resistance since Martin's strain was less resistant to ABZ, i.e. an efficacy of 53.8% compared with 25.2% in the present study.

In this experiment larval recoveries did not reflect the changes in worm egg counts (Fig. 4.2.1 versus Fig. 4.2.2) as the former were reduced by over 72%; this was probably due to the presence of non-viable eggs after exposure to ABZ but larval culture of faeces from the untreated controls also showed a marked drop on Day 6 which is probably simply a reflection of the limitations of the larval culture technique. Nevertheless culture would be of vital importance under field conditions where more than one species of nematode may be involved thus requiring proper identification of larvae surviving treatment.

Although the egg counts did not appear to correlate well with worm counts in the present study, a worm egg count reduction test conducted between 7 and 10 days after treatment remains a suitable first-line method to detect drug efficacy under field conditions. Also

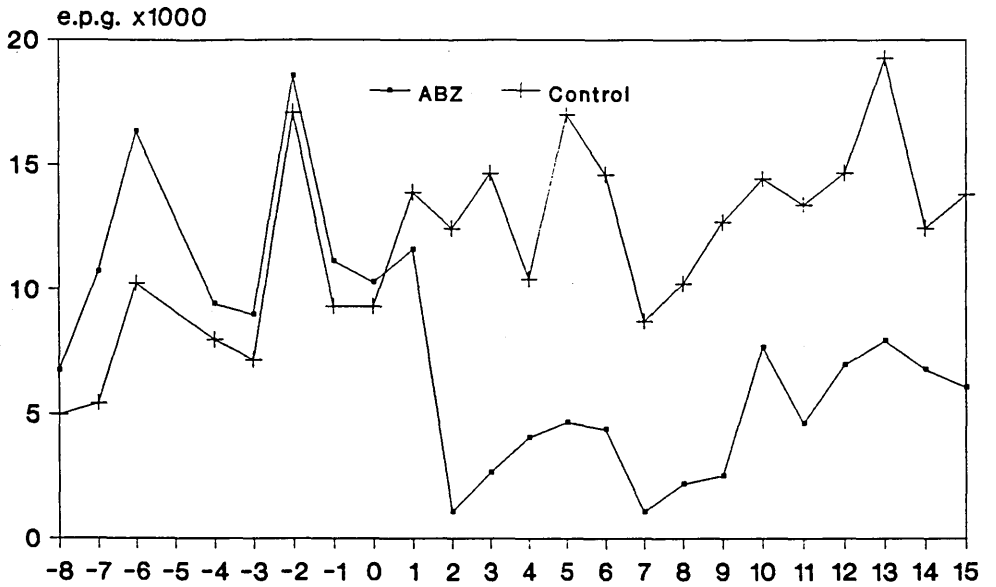
in the case of *H. contortus* which has a high biotic potential, the reduction in worm egg count following treatment must be substantial otherwise surviving eggs may be rapidly translated into infective larvae and thus lead to outbreaks of disease.

TABLE 4.2.1. Worm egg counts and worm burdens of animals infected with a resistant strain of *H. contortus** and treated or untreated with ABZ and slaughtered 15 days later.

Treatment	n	E.P.G.			Worm counts	% Reduction
		Day 0	Day 15	% reduction		
ABZ	1	8050	1100		650	
3.8mg/kg	2	18500	16400		4500	
	3	4200	600		1100	
	mean=	10250	6033	41.1	2083	25.2
Control	1	8800	8200		3100	
	2	13300	23500		3350	
	3	5800	9600		1900	
	mean=	9300	13767	+48.0	2783	-

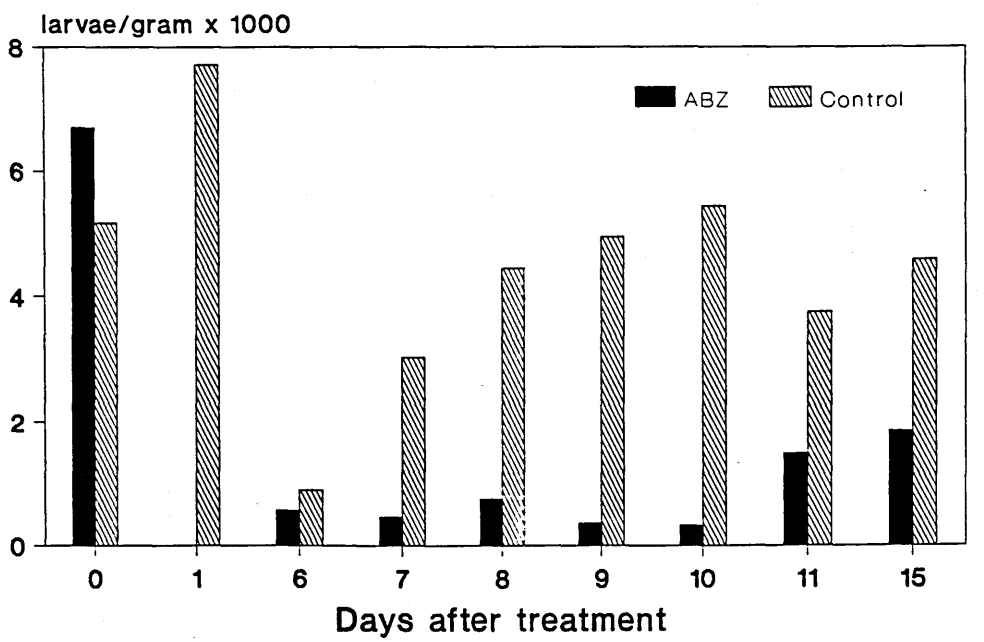
*Resistant to IVM, BZ and closantel (Van Wyk and Malan 1988).

Figure 4.2.1. Worm egg counts after ABZ treatment on a strain* of *H. contortus*



*IVM/BZ-resistant strain (Van Wyk & Malan, 1988)

Figure 4.2.2. *Haemonchus** L3 recoveries from ABZ treated or untreated lambs



*IVM/BZ resistant strain (Van Wyk & Malan, 1988)

Experiment 4.3

THE SURVIVAL AND DEVELOPMENT OF IVERMECTIN RESISTANT OR SUSCEPTIBLE STRAINS OF *Haemonchus contortus* UNDER FIELD AND LABORATORY CONDITIONS

INTRODUCTION

Sheep nematodes have been shown to have sufficient genetic variability to develop anthelmintic resistance to various unrelated compounds. Under field conditions animals are often treated when clinical signs become apparent. This is normally associated with maximum larval availability on pastures. Under such conditions only the parasitic stages within the host will be exposed to the selecting drug while the free-living stages will remain unaffected by the treatment. Le Jambre (1978) estimated that during an outbreak of haemonchosis in the summer rainfall areas of Australia, about 3% of the total parasite population are harboured by the sheep and thus anthelmintic treatment given at such times allows the major proportion of the parasite population present as the free-living stages to escape exposure to selection pressure. The genotype of the free-living population is therefore the key to the progress towards significant anthelmintic resistance. If a high proportion carry R (resistant) genes, progress will be rapid and conversely if the proportion carry S (susceptible) genes is high, then progress towards resistance will be slow.

The relative ability of resistant and susceptible strains to

survive on pasture is also important. Thus, Kelly *et al.* (1978) described a BZ resistant strain of *H. contortus* which survived better on pasture than a BZ susceptible strain. On the other hand Scott and Armour (1991) found that the development of a South African strain of *H. contortus* resistant to ivermectin was slower in comparison to a susceptible strain when subjected to culture at a range of temperatures.

This section describes two experiments carried out to study the behaviour of free-living stages of strains of *H. contortus* either resistant or suceptible to IVM under both laboratory and field conditions.

MATERIALS AND METHODS

Laboratory studies:

A laboratory selected strain of *H. contortus* resistant to IVM, an IVM susceptible and a South African multiple resistant field strain were incubated at different temperatures in order to determine any developmental advantage.

Animals. Three Suffolk x Scottish Blackface lambs aged five months reared worm-free, and fed a ration of hay and lamb finishing pellets *ad lib* were each infected with 10,000 L_3 from one of the different strains of *H. contortus.*

Larval strains. - An ivermectin susceptible strain of *H. contortus* (S-IVM) originally obtained from The Moredun Research Institute, Edinburgh, and kept at Glasgow for more than 5 years by continuous passages through housed worm-naive lambs.

- An IVM selected resistant strain (R-IVM). This was obtained as described in Exp. 4.1 by subjecting the above susceptible strain of *H. contortus* to a selection procedure using IVM at 0.02mg/kg on nine successive generations in lambs. Treatment successfully reduced the worm egg counts by more than 99% up to the seventh treatment. Following the eighth treatment there was no reduction in worm egg counts. The parasites producing these eggs were then exposed to a full therapeutic dose of IVM (0.2mg/kg) and subsequent culture produced the IVM selected resistant larvae which were used in this experiment.
- A multiple resistant strain (R-IVM/SA). This strain, resistant to ivermectin, benzimidazole and closantel (Van Wyk and Malan, 1988) was imported from South Africa and maintained at Glasgow University Veterinary School where four passages of the parasites were carried out in parasite-naive lambs with treatment using ivermectin at 0.2mg/kg being administered during each passage.

Experimental procedures. At day 21 post infection the total faecal output produced overnight by each animal was collected and weighed. Ten aliquots were removed from the faeces of each animal for determination of e.p.g. by the McMaster technique. Two replicated aliquots of 5g of faeces containing each strain were then placed in plastic pots and incubated at 4°C, 10°C, 22°C and 27°C. The numbers of larvae from these pots which hatched and developed to the infective stage at each temperature were measured at weekly intervals from seven days after incubation until Day 72. The percentage hatchability of the three different strains maintained at each temperature was then calculated for each sample time, i.e., the number obtained after incubation was expressed as a percentage of the average number of eggs

present in each pot on the day of incubation.

Statistical analysis. Percentages of larval recoveries were submitted to three-way analysis of variance.

Field studies:

The development and survival of the free-living stages of two strains of *H. contortus* i.e., an IVM resistant and a susceptible strain, were monitored under field conditions in Southern Brazil.

Strains. The IVM resistant and susceptible strains described earlier in Exp. 3.1 were used to infect helminth-free lambs to provide enough material for seeding the pasture plots.

Experimental procedures

Analysis of herbage for the presence of *Haemonchus* L_3. An area of 0.45ha was fenced off two and a half years before the experiment was initiated. After it was fenced off it was never grazed again and herbage assays at the start of these studies showed it to be free of ruminant nematode larvae.

Beginning at the end of November 1990 and continuing for another two months, 90 plots of approximately 0.7m^2 were contaminated with faeces containing the eggs of the ivermectin resistant strain of *H. contortus* and another 90 plots with faeces containing the susceptible strain of *H. contortus*. The faecal material was obtained from two groups of five donor lambs each infected with the different strains. Faeces from 24 hour collections from these lambs were stored at 4^{0}C and throughly mixed, before 30g samples were placed in such a way as to simulate field conditions on the marked plots. Twenty faecal samples were taken at random from each bulk collection to determine

the worm egg count.

Three plots containing each strain were assayed for eggs at three days after contamination, and from then on for eggs and third stage larvae at varying intervals until neither eggs nor L_3 were recovered on three consecutive occasions. Sites to be assayed were selected by use of a table of random numbers.

Since it is believed that the L_3 of all the main gastrointestinal nematodes have a limited migratory capacity (Michel, 1969), a metal ring with a diameter of 38cm was used to encompass a defined area around each lot of faeces. All of the faecal material and grass collected within this area were brought to the laboratory; a soil sample (10cm x 5cm x 5cm) was also collected.

Faeces were weighed and a 3g sample examined by the McMaster technique for the presence of eggs; the remainder was examined for L_3 by the Baerman technique in which 10g of faeces were suspended in a gauze bag in lukewarm water contained in a conical measuring cylinder (Henriksen, 1965). After a minimum of 6 hours the supernatant was drawn off leaving a final volume of 10ml and the gauze with faeces discarded. The numbers of larvae in 1ml were determined and the results expressed as larvae per gram of faeces.

The plastic bag containing the grass collected within the 38cm ring was weighed before adding four litres of lukewarm water and mixing well for 3 minutes. At the end of this period a small incision was made in the bag and the washings allowed to pass through a coarse mesh sieve (apperture size 2mm) into a bucket. The remaining herbage, still in the bag, was then removed and as much fluid recovered from it as possible by squeezing. The grass was then spread on a tray and dried

in an incubator at 70°C. When thoroughly dry the herbage was again weighed and this dry weight used in the final calculation of numbers of larvae per kilogram dried herbage (L_3/kgDM). The washings contained in the bucket were filtered through a 38 micron sieve and the material retained by the sieve transferred to a beaker. The resultant larval suspension was drawn through a coarse filter paper (Whatmans Grade 113, 18.5cm) using a Buchner funnel and a vacuum pump. A single milk filter (Maxa Milk Filters, A. McCaskie Ltd., Stirling) was put on top, and the whole inverted and placed on a Baerman apparatus. After standing for at least 6 hours, 10ml of fluid were withdrawn and the larvae in 1ml differentiated and counted.

RESULTS

Laboratory studies:

Eggs from the three different strains kept at 4°C and 10°C did not develop during the nine week period of observation. Details of the rates of development of the different strains to the infective stage, based on the percentage of L_3 recovered from the initial number of eggs incubated at 22°C and 27°C are given in Appendix 4.3.1 and a summary of the statistical analysis in Appendix 4.3.2.

Analysis indicated that the three strains responded very differently at different temperatures ($p<0.05$). Examination of Figs. 4.3.1 and 4.3.2, which show the percentage development at temperatures of 22°C and 27°C respectively, indicate that there is a large difference in the development of larvae from the different strains. Thus in Fig. 4.3.1 it can be seen that the R-IVM had markedly higher

overall percentage development rates at 22°C compared to the S-IVM and R-IVM/SA over a period of six weeks. In contrast, in Fig. 4.3.2 it can be seen that the overall percentage development rate of the susceptible strain at 27°C was higher although this was confined to the early weeks of incubation. At both temperatures the percentage development of the R-IVM/SA strain was very low and never exceeded 5% whereas the others reached 40% (R-IVM) and 60% (S-IVM).

At 22°C (Fig. 4.3.1) the selected IVM resistant strain showed a higher development rate in comparison to the other two strains used. One week after incubation at 22°C about 30% of the eggs of the R-IVM strain had developed to the infective stage and high recoveries of approximately 15-40% were obtained until the seventh week when a marked drop to around 5% was noticed. In the susceptible strain 20% of the eggs had developed to the infective stage at the first week with numbers being reduced to less than 10% from the second week onwards. The South African strain had by far the lowest development rate from the three strains incubated at 22°C, a maximum of 3% being reached on week one.

The results obtained from the incubation at 27°C (Fig. 4.3.2) showed a different trend. Here, more than 60% of the eggs from the S-IVM strain had developed to the infective stage by the first week and development rates remained high until week four when the recovery rates dropped sharply with few larvae being subsequently recovered. In contrast the other two strains showed very low development rates throughout the nine week period of observation, a maximum of 6.2% being recovered for the R-IVM strain on week three and 4.5% for the R-IVM/SA on week one.

Field studies:

Details of the numbers of larvae which developed to the infective stage and were recovered from pasture and soil after each contamination are given in Appendices 4.3.3 to 4.3.8 while the meteorological data for the period is shown in Figure 4.3.6 and detailed in Appendix 4.3.9; a summary of the statistical analysis is shown in Appendix 4.3.10.

From three days after faecal deposition second stage larvae (L_2) could be detected in both pasture and soil. By the fifth day onwards L_3 were detected but low numbers of larvae were recovered at all times; percentage recoveries were always below 1% independent of the strain used (Figs. 4.3.3 to 4.3.5).

An analysis of the results obtained from the contamination carried out at the end of November (Fig. 4.3.3) revealed that there was no significant difference ($p>0.05$) between the numbers of larvae which developed to the infective stage with the two different strains of *H. contortus*. Recoveries from soil were always higher than those from pasture. Larvae recovered on Day 4 post-contamination were at the second stage while those recovered on Day 6 had reached the infective stage. By Day 47 post-contamination larvae were only detected at very low levels on the plots contaminated with the S-IVM strain and by Day 75 no larvae could be detected.

For the contamination carried out at the end of December (Fig. 4.3.4) there were overall higher ($p<0.05$) numbers of larvae recovered from the samples obtained with the R-IVM strain from both pasture and soil in comparison with those contaminated with the S-IVM strain.

When the contamination was at the beginning of February (Fig.

4.3.5) there were no significant differences ($p>0.05$) between the overall recoveries obtained from the two strains. As in November, recoveries from the soil were generally higher than those from pasture; also the recoveries were usually higher from the plots contaminated with the S-IVM strain compared to those of the R-IVM strain; no R-IVM larvae were recovered from pasture at any of the sampling times.

About 74 days after February contamination there was a period of heavy rain over 4 consecutive days, reaching a total of 308mm; this resulted in the experimental area becoming flooded and as this undoubtedly caused cross-contamination between the plots it was decided to conclude the field studies at this point.

DISCUSSION

The fitness of free-living stages for survival is probably critical in the life-cycle of sheep nematodes as in most climatic zones they form the larger proportion of the total worm population for most of the year. Since the free-living stages are not exposed to anthelmintic treatment they can have an important influence on the susceptibility or otherwise of subsequent generations to anthelmintics.

Kelly *et al.* (1978) reported a BZ susceptible strain of *H. contortus* which yielded less larvae than either a field resistant strain or a laboratory selected resistant strain suggesting that resistance had enhanced its fitness. On the other hand, Waller *et al.* (1989) found that resistance to levamisole in a strain of *T.*

colubriformis reduced the fitness of the parasites and in the absence of drug selection the susceptible strain had a survival advantage suggesting that reversion towards susceptibility would be more likely under field conditions.

The results obtained in the laboratory experiment described here showed a very marked difference ($p<0.05$) in development between the original susceptible strain and the R-IVM selected strain at both temperatures at which development took place. The selected R-IVM strain produced a significantly higher development rate at 22°C while the original susceptible strain was the one which produced most of the larvae at 27°C.

Scott and Armour (1991) studied the pathogenicity and survival of both the multiple resistant South African strain of *H. contortus* and the susceptible strain used in these studies. They found no significant differences in the pathogenicity or parasite establishment and egg production between the two strains. However, they did find a decrease in the number of eggs which survived and developed at different temperatures in the South African multiple resistant strain compared with those of the susceptible strain.

In the present laboratory study *H. contortus* eggs did not hatch at 4°C and 10°C which is in contrast with the findings of Scott and Armour (1991) who reported some development at these temperatures. This may be explained by the different period of faecal collection prior to the incubation at the different temperatures; here, only the overnight faecal ouput was used while in Scott and Armour (1991) study a 24 hour collection period was used during the summer (July) which may have caused differences in the stage of development prior to the

incubation of the eggs used in the two studies.

From the three strains used in the laboratory study the South African multiple resistant strain appears the least fit for development at the temperatures tested. This agrees with the results of Scott and Armour (1991) who also showed that this strain had a lower development rate compared to the same susceptible strain used in this study. The reason for this is not known but it may simply reflect the different origins of the two strains. The resistant strain from the Mediterranean climate of Cape Province in South Africa may be better adapted to temperatures different to those used in this study. On the other hand the temperatures used in this study i.e. 4°C to 27°C, were within the range of those usually found in Cape Province. Another more likely explanation is that the metabolism of this South African strain became significantly altered as it developed resistance to a range of anthelmintics. Studies on the metabolic profile of this and other strains of *H. contortus* from different areas of the world might provide some interesting results. The larval recoveries obtained in the field experiment were low with recoveries of under 1% for both strains. This may have resulted from a low survival of larvae during the summer in Brazil, as eggs and larvae of *H. contortus* are very susceptible to desiccation (Rose, 1964); alternatively it may simply reflect a low sensitivity of the recovery techniques especially when larvae move into the soil where it becomes more difficult to isolate these for counting. However, results similar to those obtained in this experiment have been reported from another study in South America where Maciel (1984), using faeces from cattle infected with *H. contortus*, also recovered less than 1% of the initial

egg deposition. One explanation for the finding of larger numbers in the soil samples than from pasture is that the larvae were washed down into the soil by unusually high rainfall.

Despite low larval recoveries obtained from pasture contamination with faeces from animals infected with either R-IVM or S-IVM strains of *H. contortus* under the summer conditions of Southern Brazil, with the exception of contamination carried out in December where the R-IVM strain produced higher larval recoveries, there were no significant differences in the recoveries obtained from the two strains.

With contamination in February there was a slightly higher recovery with the R-IVM strain although this was not significant. This may be due to small variations in the batches of eggs used, although extra care was taken to ensure similar periods of faecal harvesting for plot contamination, or it may be due to the variability in larval numbers considering the low recoveries in both strains.

Due to the rain which flooded the plots in April it was not possible to continue monitoring the area until the autumn. This was unfortunate as it would have been interesting to ascertain which strain was most likely to make larvae available for the autumn infection. Nevertheless this experiment does highlight the low survival of *H. contortus* larvae during the summer in Southern Brazil and emphasises the importance of treating animals at this time when they may be harbouring a high percentage of the total sheep nematode population.

The question of fitness may be dependent on the particular strain used and its degree of resistance. Thus Maingi *et al.* (1990) found

that a moderately BZ resistant strain of *H. contortus* increased its rate of establishment, egg output and pathogenicity following further BZ selection pressure. The latter involved both *in vitro* and *in vivo* selection over four generations and the degree of resistance, as measured by the egg hatch test, increased by 25%. At the start of the selection process the rate of establishment was 22.8% and by the end of the selection period, i.e., the fourth generation, this had increased to 39.2%. A susceptible strain from a different source which was used for comparison had an establishment rate of 40.8%. Maingi *et al.* (1990) postulated that in the field situation strains which were in the early evolutionary phase of resistance were less fit than susceptible strains but as they became more resistant, fitness and other biological characteristics such as pathogenicity increased and that this might explain observed variations. While this is an attractive theory it does not agree closely with the results of either the present studies or those of Scott and Armour (1991). The latter showed that the pathogenicity and most major biological characteristics of the R-IVM/SA strain were no different to those of the S-IVM strain and this could simply reflect the fact that the R-IVM/SA strain had regained fitness after natural selection for resistance, as in Maingi's experiments. However, the experiments reported here and those of Scott and Armour (1991) on the rates of larval development of several different strains suggest that the R-IVM/SA strain has a decreased ability to develop to the third stage at a range of temperatures, possibly due to poor hatching rates.

Clearly studies on the difference in fitness between anthelmintic resistant and susceptible strains may give conflicting results since

it is difficult to test resistant and susceptible strains which have originated from the same population and that have been submitted to the same laboratory management in order to avoid differences such as the number of passages and generation intervals. It would be interesting to observe if further changes in the biology of the R-IVM laboratory selected strain occurred following additional passages and selection pressure. The selection of multiple resistant strains under laboratory conditions would also provide strains of known history, for comparison with R-IVM laboratory strains.

Fig. 4.3.1. Development *of Haemon*chus L3 at 22^{0}C

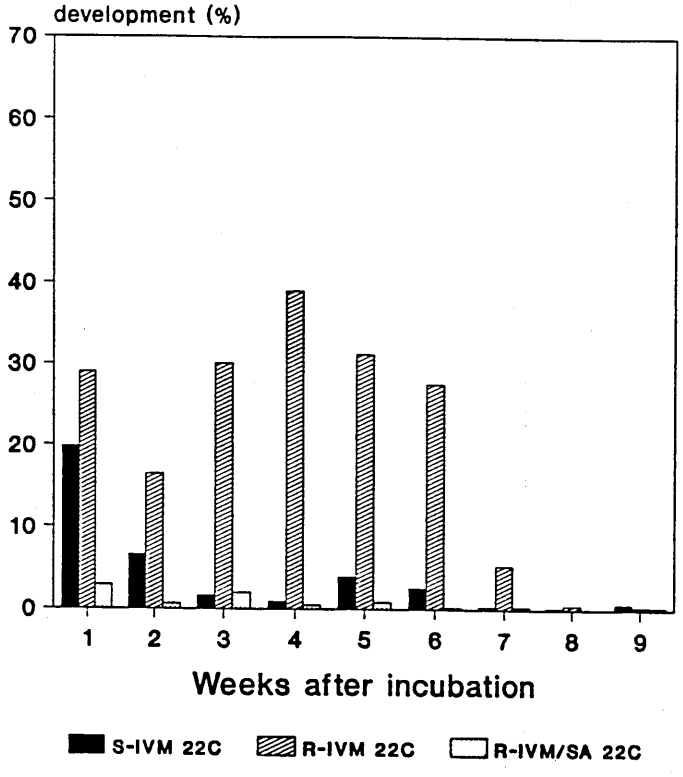


Fig. 4.3.2. Development *of Haemon*chus L3 at 27^{0}C

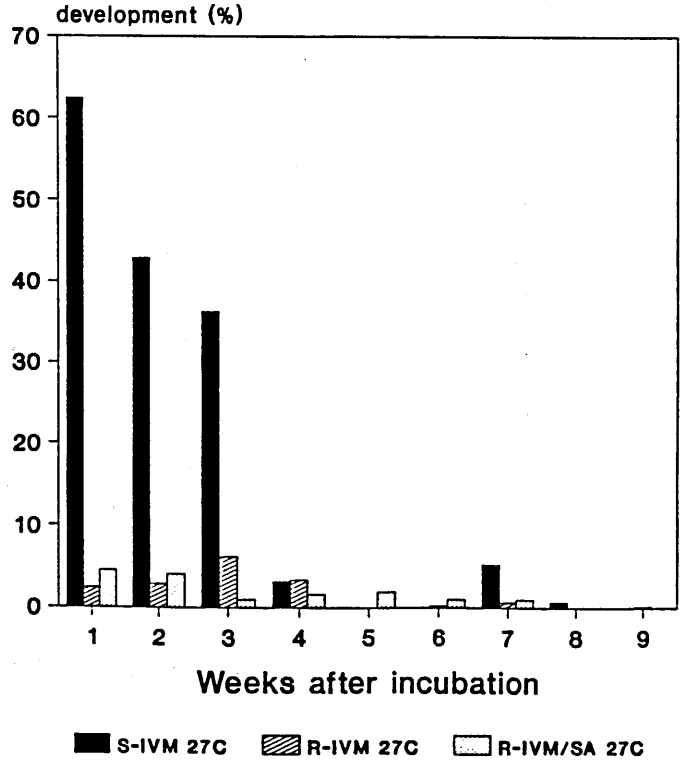


Fig. 4.3.3. L3 of *Haemonchus* recovered from soil and pasture. Contam.: Nov/90

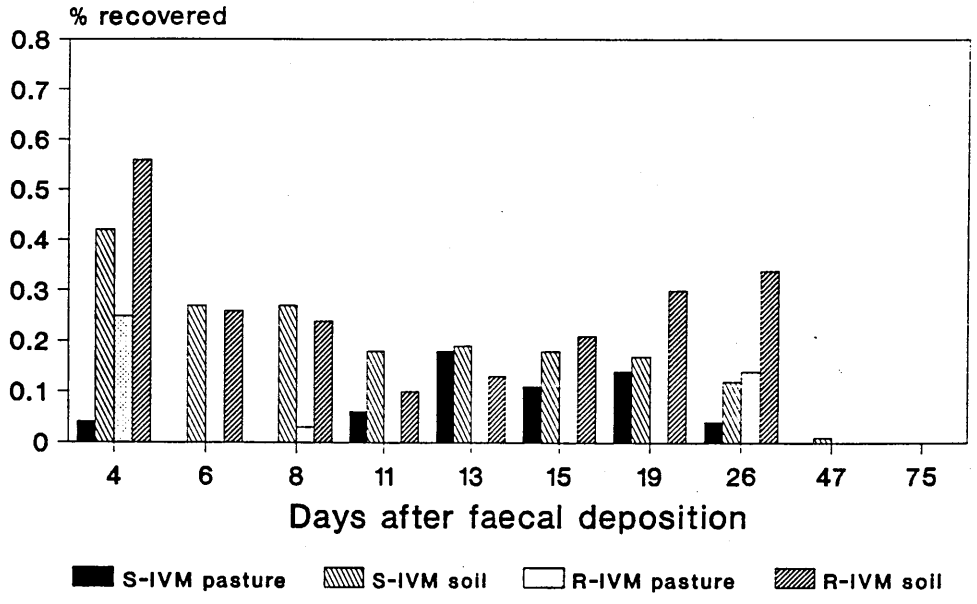


Fig. 4.3.4. L3 of *Haemonchus* recovered from soil and pasture. Contam.: Dec/90

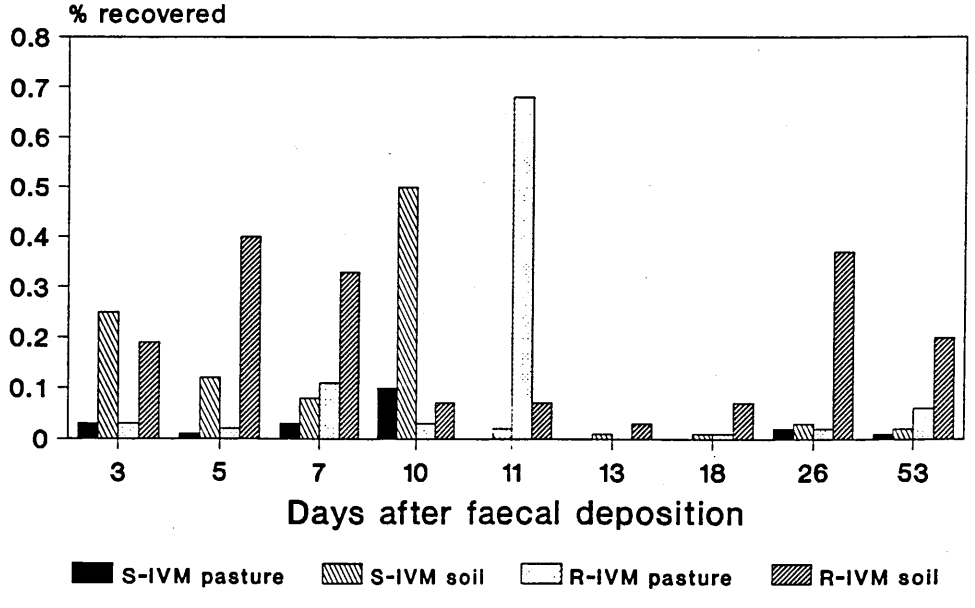


Fig. 4.3.5. L3 of *Haemonchus* recovered from soil and pasture. Contam.: Feb/91

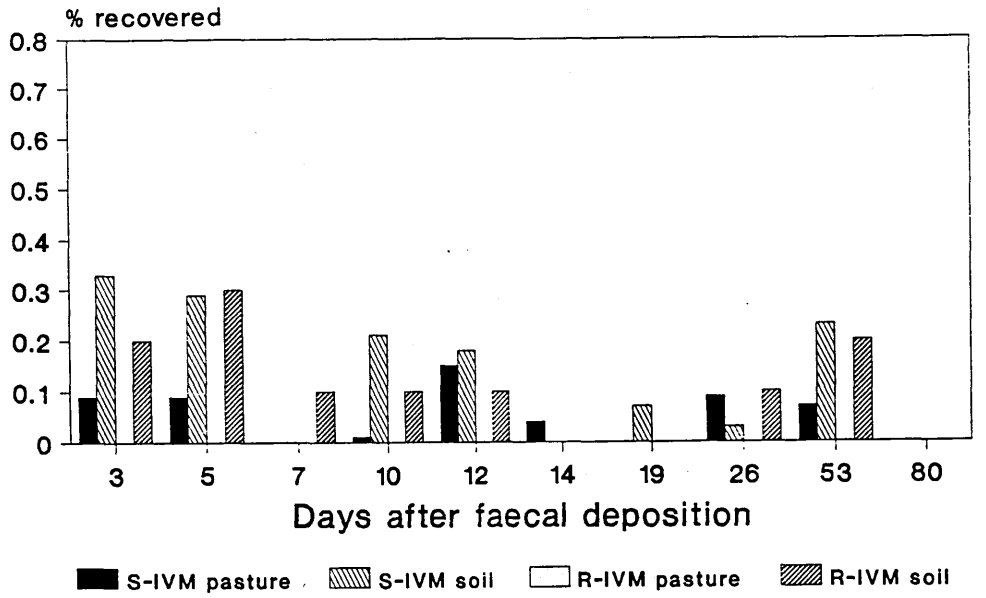
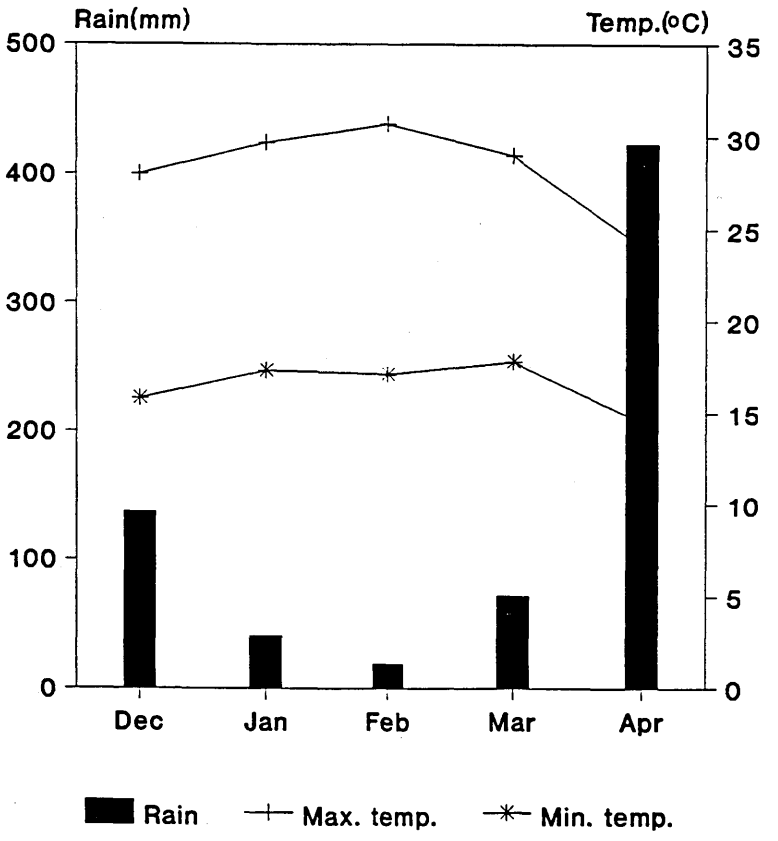


Fig. 4.3.6. Meteorological data during the ecology studies with *Haemonchus*



Experiment 4.4

ISOENZYME ANALYSIS OF *Haemonchus contortus* STRAINS EITHER RESISTANT OR SUSCEPTIBLE TO IVERMECTIN

INTRODUCTION

As anthelmintic resistance in sheep nematodes continues to evolve to all broad spectrum anthelmintics, i.e., benzimidazoles, levamisole, morantel and lately to ivermectin, *in vitro* tests have been developed to differentiate susceptible from resistant strains of parasites to some of the existing drugs (Le Jambre, 1976; Coles and Simpkin, 1977; Hall *et al.*, 1978; Whitlock *et al.*, 1980a; Lacey and Snowdon, 1988; Dobson *et al.*, 1986). Unfortunately these tests only detect resistance when it has reached high levels and even then results may give daily variations (Borgsteede and Couwenberg 1987, Martin *et al.*, 1990). Since IVM is believed to paralyse nematodes, as does levamisole, tests developed to detect levamisole resistance (Martin and Le Jambre, 1979; Dobson *et al.*, 1986) were used for IVM resistance during the present studies. However they did not produce reliable results as the percentage of paralysed larvae or hatched eggs did not produce good dose related responses so LD_{50}'s could not be determined; because of this it was decided to abandon these tests. A technique which would detect resistance at its early stages of development would be very valuable as it would allow other approaches in the control of gastrointestinal parasites to be introduced before widespread anthelmintic failures occurred with resultant economic losses.

Recently genetic techniques have been used in an attempt to provide an alternative tool to characterise nematode species, strains from different origins or strains of nematodes resistant or susceptible to anthelmintics (Duncan, 1990; Roos *et al.*, 1990). These techniques are intended to assess differences in gene products. Different approaches to the separation of these gene products may be used. For example analysis of differences in size, structure, charge, activity and amino acid sequence can be used. As a preliminary step, nematodes are broken down into more manageable fragments, usually by homogenisation or sonication followed by a separation process such as centrifugation. Isoenzymes, which are different forms of enzymes catalyzing the same chemical reaction, can be assessed by starch gel electrophoresis. Since polypeptides of a given enzyme/isoenzyme are coded for by a single gene sequence, variation in the electrophoretic mobility of the same enzyme in two different individuals implies that the polypeptide structures are different. The analysis of the electrophoretic mobility of an isolate for a range of enzymes may provide a genetic profile which could be used to identify strains within a species (Nomura, 1984). Furthermore, analysis of isoenzymes has given useful information in the identification of zoonotic trypanosome subspecies (Tait *et al.*, 1985) and in the benzimidazole resistance of strains of nematodes (Sutherland, *et al.*, 1988). The latter authors showed differences in the esterase patterns of BZ-susceptible or BZ-resistant strains of *H. contortus*, where the resistant nematodes produced greater acetylcholinesterase activity and an increased number of stained bands for this enzyme.

Benzimidazole resistance in *H. contortus*, is widespread and the

development of ivermectin (IVM), an unrelated compound (Burg *et al.*, 1979) has provided a welcome alternative for use in nematode control. Nevertheless, resistance soon developed against this drug under field conditions in South Africa (Van Wyk and Malan, 1988) and in Brazil (Echevarria and Trindade, 1989).

The following is a report of a study carried out to verify whether isoenzyme analysis could be used as a test to demonstrate differences between a susceptible strain of *H. contortus* and a derivative strain which was selected for resistance to ivermectin.

MATERIALS AND METHODS

The substrate stain systems for isoenzymes analysis are listed in Table 4.4.1 while gel and tank buffers can be found in Table 4.2.2.

Table 4.4.1. Substrate stain systems for isoenzyme analysis.

Glucose-6-phosphate dehydrogenase (G-6-PDH)

10mg Glucose-6-phosphate, 5mg nicotinamide adenine dinucleotide phosphate, 7.5mg MTT (3, (4,5 - dimethylthiazol- 2 - yl) - 2,5 diphenyltetrazolium bromide) and 5mg 0.2M magnesium chloride ($MgCl_2$) were made up to 25ml with 0.2M tris-hydrochloric acid pH 8 and homogenised. 25ml 2% Molten agar and approximately 0.5mg phenazine methosulphate were mixed into the stain before pouring over the cut gel surface. Once the agar had set, the gel was incubated at 37^{0}C in the dark and frequently withdrawn to check for enzyme activity which was shown by a blue-black colour.

Malate dehydrogenase (MDH)

2ml 1M Sodium-1-malate pH 7, 5mg nicotinamide adenine dinucleotide and 5mg MTT were made up to 20ml with 0.1M tris-hydrochloric acid pH 8. The solution was homogenised. Prior to staining 20ml 2% agar and 0.5mg phenazine methosuphate were added and the stains applied to the cut gel surface.

Malic enzyme (ME)

0.5ml Sodium-L-malate, 5mg nicotinamide adenine dinucleotide phosphate, 5mg MTT and 0.5 ml 1M magnesium chloride (4.06g $MgCl_2$ in 20ml distilled water) were made up to 25ml with 0.1M tris-hydrochloric acid pH 7. The solution was homogenised and immediately prior to staining 0.5mg phenazine methosulphate and 25ml 2% agar was mixed in.

Peptidases

Three peptidase substrates were tested namely L-alanyl-L-tyrosine (Ala-Tyr), L-leucyl-L-leucine (Leu-Leu) and L-leucyl-L-alanine (Leu-Ala). 14mg of the respective peptide, 3.5ml o-dianisidine, 4mg snake venom (0.6 units) and 3mg peroxidase (175 units) were made up to 25ml with 0.05M phosphate buffer pH 7.5. In some cases homogenisation was found to be necessary for dissolution. Before application to the cut gel 0.35ml manganese chloride ($MnCl_2$) and 25ml 2% agar was added. High background colouration occurred rapidly if the 0.1M $MnCl_2$ was added too early. Enzyme activity was detected as a red stain.

Phosphoglucocomutase (PGM)

4mg Glucose-1-phosphate, 4mg nicotinamide adenine dinucleotide phosphate, 6mg MTT and 0.5mg 1M magnesium chloride (4.06g $MgCl_2$ in

20ml distilled water) were made up to 20ml with 0.025M tris-hydrochloric acid pH8. Directly before staining, 0.5mg phenazine methosuphate, 10ul glucose-6-phosphate dehydrogenase (140 units/ml) and 20ml 2% agar were added. The solution was swirled to mix and poured onto the cut gel. Blue-black colouration indicated phosphoglucocomutase activity.

Esterase - carboxylic ester - propionate

2mg 4-Methyl-umbelliferyl propionate were dissolved in a few drops of acetone and 10ml of 0.1M phosphate buffer pH 6.5 added. The solution was made up immediately before staining and applied to 3MM paper laid on the gel. The gel was visualised frequently under ultraviolet light (366nm) for the appearance of fluorescent staining.

Table 4.4.2. Gel and tank buffers for starch gel electrophoresis

---

Tris citrate buffer pH 7.0

Tank - 0.9L distilled water was added to 16.35g Trizma and 9.04g citric acid and the pH titrated to 7.0 with concentrated hydrochloric acid. The solution was made up to 1L.

Gel - Distilled water was added to 16.7ml of the tank buffer to bring the volume up to 250ml, maintaining the pH at 7.0.

Tris citrate buffer pH 8.6

Tank - 0.9L distilled water was added to 40g Trizma and 8.71g citric acid and the pH titrated to 8.6 with concentrated hydrochloric

acid. The solution was made up to 1L.

Gel - Distilled water was added to 20ml of the tank buffer to bring the volume up to 250ml, maintaining the pH at 8.6.

Tris phosphate buffer pH 7.0

Tank - 0.9L distilled water was added to 10.89g Trizma and 17.16g sodium dihydrogen phosphate and the pH titrated to 7.0 with concentrated hydrochloric acid. The solution was made up to 1L.

Gel - Distilled water was added to 12.5ml of the tank buffer to bring the volume up to 250ml, maintaining the pH at 7.0.

Tris potassium phosphate buffer pH 9.3

Tank - 0.9L distilled water was added to 21.8g Trizma and 2.72g potassium dihydrogen phosphate and the pH titrated to 9.3 with concentrated hydrochloric acid. The solution was made up to 1L.

Gel - Distilled water was added to 25ml of the tank buffer to bring the volume up to 250ml, maintaining the pH at 9.3.

Strains. - An ivermectin susceptible strain of *H. contortus* (S-IVM) originally obtained from The Moredun Research Institute, Edinburgh and kept at Glasgow for more than 5 years by continuous passages through housed worm-naive lambs was used.

- IVM selected. As described in Exp. 4.1 the above susceptible strain of *H. contortus* was submitted to a selection procedure using ivermectin (IVM) at 0.02mg/kg on nine successive generations in lambs. Treatment successfully reduced the faecal egg counts by more than 99%

up to the seventh treatment. Following the eighth treatment there was no reduction in worm egg counts. The larvae were then exposed to a full therapeutic dose of IVM and the larvae which survived were used in this experiment (R-IVM).

- Multiple resistant (R-IVM/SA). A multiple resistant strain i.e., resistant to ivermectin, benzimidazole and closantel (Van Wyk and Malan, 1988) imported from South Africa and mantained at Glasgow University, Veterinary School (under isolation conditions) with four passages of the strain being carried out in parasite naive-lambs with ivermectin treatment at 0.2mg/kg being given during each passage.
- *D. viviparus*. Third stage larve were obtained from Intervet, UK.

Sample preparation. Third stage larvae (L_3) were exsheathed with 0.1% w/v sodium hypochloride and washed five times with 0.85% sodium chloride. Exsheathed larvae were then homogenized using a glass homogenizer in 0.2mM EDTA pH8 containing the protease inhibitors N-alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK) (50ug/ml), N-1-tosyl-L-phenylalamine chloromethyl ketone (TPCK) (25ug/ml), phenyl methylsulphonylfluoride (PMSF) (1mM), 1,10 phenanthroline (1mM) and antipan (4uM). The homogenate was next centrifuged at 10000g for 10 minutes and the supernatant removed, filtered and stored in Eppendorf vials. Assessment of protein concentration in the samples from the different strains were carried out using the Bradford Protein Assay (Bradford, 1976).

Horizontal starch gel electrophoresis. Reviews on isoenzyme techniques have been carried out by Smith William (1968) and Brewer (1970) and

the following is a brief description of the methodology used in the present study.

Gel preparation. A perspex template 22 x 15 x 0.6cm^3 and a glass plate were sealed together with silicone grease to form a gel mould. Hydrolysed starch (20g) was heated over a Bunsen flame with 250ml of gel buffer (Table 4.2.2) in a 1 litre side-arm flask of toughened glass. The solution was swirled throughout heating to avoid temperature gradients, which might result in glass breakage. Once the starch was fully dissolved the solution thickened and cleared, momentarily clouded and thickened further. Once the starch began to boil, the flask was rapidly removed from the heat. Excess boiling reduces the buffer volume and should be avoided. The solution was degassed by vacuum pump, which causes the starch to boil briefly under reduced pressure, air bubbles are removed and the granule size of the matrix becomes uniform for protein migration. The gel was poured into the mould, so the surface was just proud to the perspex.

The gel was left to set on a level surface for 1 hour at room temperature followed by 1 hour at 4^{0}C, when slots were cut in a horizontal line across the gel $\pm$0.3cm deep. Sample to sample cross contamination was prevented by leaving adequate inter-slot distance and assuring the sample did not contact the glass base plate with too deep a slot. Proteins generally carry a negative charge, so by placing the slots eccentrically the area for separation towards the anode was increased.

The sample carrier filter was a piece of 3MM Chr paper (Whatman) approximately 0.6 x 0.3cm^2 and it was immersed for 10 minutes in the

larval sample which was standardized to have the same amount of protein/piece of carrier filter (which varied between 14 and 20ul of solubilised sample/carrier). The filters impregnated with sample were inserted into the slots using forceps.

Eletrophoresis. The gel in its mould was laid between brass cooling plates. Cooled water (Grant FC25 flow cooler) was pumped (Grant FH15 heater) through a series of copper tubes within the plates. Without cooling the heat produced during the experiment resulted in enzyme denaturation and band distortion, particularly if the temperature exceeded 30 to 35^{0}C. The equipment was set up to pre-cool at least 2 hours in advance of the gel run.

Three sheets of 1 Chr paper (Whatman) were bridged between the buffer tank and the gel surface. The buffer tanks were filled (Table 4.4.2). In a continuous buffer system the salts in the tank are identical to those in the gel, but in a discontinuous system they are different; only continuous buffer systems were used in this study.

A sheet of plastic was applied to the gel surface to minimise buffer evaporation and to provide electrical insulation. A direct current power supply was applied for 3 hours at 250 to 300 volts, i.e., 2 to 3V/cm (0.07 to 0.08A). In an effort to maximise experimental standardisation, conditions were kept as similar as possible between experimental runs, e.g., time for electrophoresis, cooling and sample concentration. Matrix pore size and protein size are similar, consequently, when a potential difference is applied across the gel molecular sieving occurs, although with starch gel electrophoresis the net protein charge has a greater effect on

mobility than mass. The concentration of ions in the gel tank is approximatley ten times greater than in the gel. Lower gel buffer concentrations increase the speed of migration and since the sample carries more current and the buffer proportionately less, buffer dilution leads to increased band width.

After the run, the power was switched off and the gel removed from the cooling plates. The sample carrier filters were withdrawn. The mould was lifted away from the gel and replaced by parallel perspex struts relatively thinner than the gel, which enabled a uniform horizontal section of the gel to be cut to give a staining surface. The original mould was replaced and the substrate stain applied.

Substrate staining. The staining protocols employed were adapted from Harris and Hopkinson (1976) and are entered in Table 4.4.1. Reduction of the tetrazolium dye methyl thiazoyl tetrazolium with the intermediary catalyst phenazine methosulphate results in the formation of formazan, which is blue-black and insoluble. The reagents are light sensitive and the gel should be incubated in the dark. Dehydrogenases are detected with the addition of the coenzymes, nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate, or dehydrogenases may be added as linking enzymes allowing the identification of further enzymes. Alternatively, "positive" fluorescent stains produce fluorescent staining in the presence of certain enzymes and "negative" fluorescent staining produces a non-fluorescent area in a fluorescent field; ultraviolet light (366nm) was required for visualisation. Stains were frequently applied in an agar overlay, a 2% agar solution was prepared by gentle heating. The agar was maintained at 50°C in a water bath until required, when the

rest of the stain was warmed slightly and mixed in. Results were recorded diagrammatically and by photography (Polaroid Cu-5 Hand camera and Hood) on Polaroid 667 film with different filters for tetrazolium dyes (f16 1/16 second, f32 1/125 second).

RESULTS

Figures 4.4.1 to 4.4.9 give a graphical representation of the results obtained with the starch gel elephoresis.

G-6-PDH

This was one of the enzymes which produced high resolution with most of the buffers used. However, there were no differences in band patterns between the three strains of *H. contortus* tested. The highest mobility was detected with tris phosphate buffer, pH 7.0 1/20 when single bands were detected at 6cm from the slot while the lowest mobility was obtained with tris citrate buffer pH 8.6 where bands were detected at 2cm. Tris phosphate 1/20 pH 7.0, tris potassium phosphate pH 9.3, and tris citrate pH 7.0 gave single bands at 2.5, 3.5 and 4.5cm respectively (Figs. 4.4.4 and 4.4.5).

MDH

Here a much lower mobility was detected with the longest distance travelled by the larval samples being 2cm with tris potassium pH 9.3 given single bands or a smear up to 4cm with tris citrate pH 7.0. With tris phosphate pH 7.0 there was a single band just on the slot while with tris citrate and tris phosphate pH 8.6 single bands were found at 1cm and also a clear cathodal band at 0.5cm for the strains used (Fig. 4.4.2).

A sample of *Dictyocaulus viviparus* L_3 was included for comparison in a later stage and it was found that for G-6-PDH with tris citrate pH 8.6 no activity could be detected while when tested for MDH with tris citrate pH 8.6 it produced a clear band of different mobility at 1.5cm (Fig. 4.4.3).

ME

Very faint bands were detected using tris potassium phosphate buffer 9.3 at 2cm and at 1.2cm with tris citrate buffer pH 8.6 while no bands were detected when using tris phosphate 1/20 pH 7.0 or tris citrate pH 7.0.

PGM

Only a single anodal band at 1cm was detected for the selected resistant strain when using tris citrate pH 8.6. Bands were not produced when using other buffers.

Peptidases

When using the peptidases Leu-Leu, Leu-Ala and Ala-Tyr similar single bands were detected for the three strains of *H. contortus*. The three peptidases produced single anodal bands at 7.5cm with the TP 7.0 while Ala-Tyr produced single band at 2.5cm with TC 7.0 and TC 8.6. Ala-Tyr at TC 8.6 showed two faint bands for *D. viviparus* at 1 and 2cm respectively (Figs. 4.4.7 to 4.4.9).

Esterase - propionate

When the three strains of *H. contortus* were assayed with this enzyme stain it was found that with tris citrate pH 7.0 only the R-IVM/SA strain showed a faint band at 1.5cm. At pH 8.6 the R-IVM/SA strain showed a band at the slot point and at 1.5cm while the susceptible only at the slot. The selected resistant strain did not show any

reaction (Fig. 4.4.1).

DISCUSSION

Bullini (1982) has suggested that a large number of enzymes should be examined for reliable evaluation of genetic differences, i.e., multilocus genetic analysis. Based on the work of Duncan (1990), where five strongyle species and eleven isolates of *O. ostertagi* were tested by starch gel electrophoresis, a total of eight enzyme stains and five buffer systems expected to show up possible differences were used to compare different strains of *H. contortus* resistant or susceptible to ivermectin.

Sutherland *et al.* (1988) was able to show differences in the esterase patterns of benzimidazole resistant and susceptible strains of *H. contortus*, where the resistant nematodes exibited greater acetylcholinesterase activity and an increased number of bands for this enzyme by isoelectric focusing. On the other hand, Duncan (1990) when testing a series of enzymes by starch gel eletrophoresis was not able to detect differences in band patterns of *O. ostertagi* resistant or susceptible to morantel, which belongs to a different chemical group of anthelmintics. Ivermectin is from another distinct chemical group and the mechanism of resistance of nematodes to this drug is not yet known.

Apart from the results with the propionyl esterase the results obtained in this study, using enzymes as a method of distinguishing between a strain of *H. contortus* selected for IVM resistance and its original sensitive parental strain, have not been very encouraging.

This is apparent from the similar bands found for the different strains studied.

The results obtained with the propionyl esterase in the present studies are very interesting. The fact that this enzyme was present in the South African strain, which is multiple resistant to ivermectin and to the benzimidazoles, and not present in the selected ivermectin strain may indicate that this alteration is associated with benzimidazole resistance but not with resistance to ivermectin.

Protein electrophoresis as a method of detecting genetic variation has one main limitation, i.e., it needs a series of different buffer systems otherwise it would separate only a limited number of alleles that differ by one amino acid substitution (Kreitman, 1983). In this study, five buffers were employed and even so the technique was not sensitive enough to detect differences between strains of *H. contortus*. Nevertheless, it was sensitive enough to detect differences between *H. contortus* and *D. viviparus*, a lung nematode which belongs to the same superfamily within the Class Nematoda. The fact that differences with most of the enzymes used could not be detected may be due to the fact that the enzymes tested are not involved in the process of IVM resistance. As the appearance of IVM resistance is relatively new and the mechanisms involved are not yet known this question remains to be answered. Another possible explanation may be that refinements of the starch gel technique are necessary in order to show any possible differences.

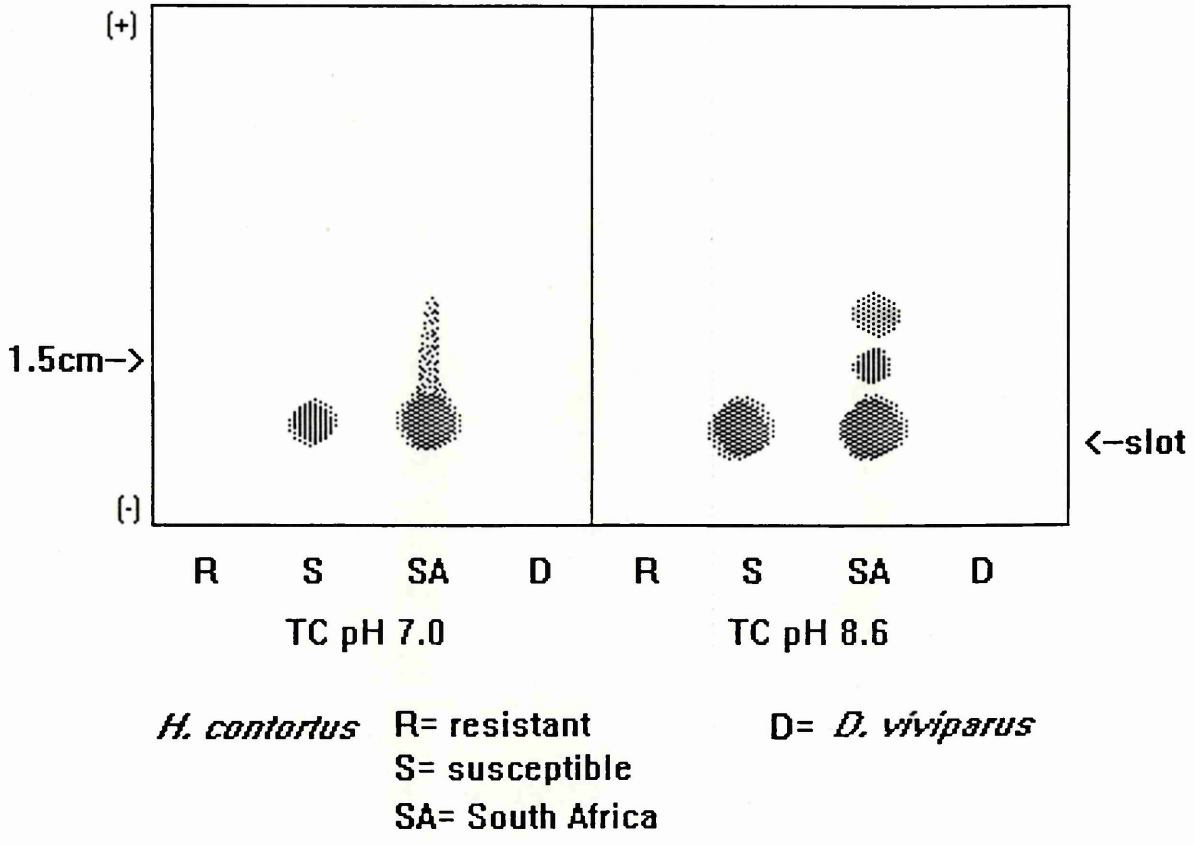
Recent work has suggested that parasites may evolve at a similar rate or at a slightly greater rate than their hosts (Fahrenholz rule) and when parasites are highly host specific this allows them to adapt

to host changes as they would arise (Hafner and Nadler, 1988). Enzyme polymorphism would compensate for alterations in the environment and would confer a genetic advantage on certain groups of parasites. Though differences in the electrophoretic mobility of enzymes could indicate real structural differences, proteins of identical mobility, i.e., electromorphs, may contain amino acid differences that may not be revealed under the electrophoretic conditions (Nadler, 1990). In the case of anthelmintic resistance it is believed that resistant individuals would be at a certain degree of disadvantage when in the absence of a selecting drug, otherwise drugs would not be so effective when first applied as resistant individuals would be more prevalent within a given population. With the continued use of drug pressure the susceptible individuals are eliminated and the fitness of that strain may be altered giving some advantage to the resistant worms. Examples of higher or similar pathogenicity and survival of free-living stages between benzimidazole resistant and susceptible strains have been reported (Kelly *et al.*, 1978; Maingi *et al.*, 1990).

Based on the lack of sufficient polymorphism to most of the enzymes shown in the three populations of *H. contortus* submitted to electrophoresis analysis in the present study, it can be concluded that further investigations on the implications of the esterase group in benzimidazole vs. ivermectin resistance should be undertaken. Also if sensitive markers for ivermectin resistance are to be developed, it may be necessary to identify the target molecule(s) against which ivermectin acts and identify the alterations associated with resistance. The fact that *H. contortus* strains studied seem to exhibit no polymorphism is interesting as these were originally

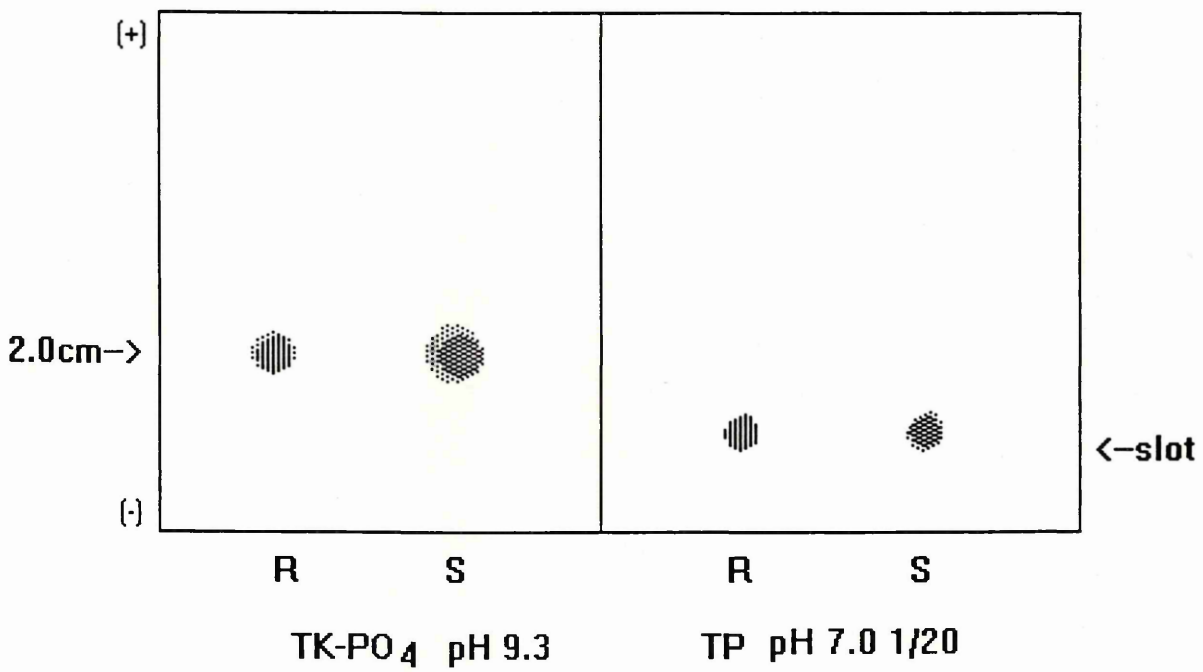
isolated from different geographical regions. This lack of polymorphism in strains from varying geographical origins has also been reported with *O. ostertagi* (Duncan, 1990).

Fig. 4.4.1. Esterase enzyme pattern by starch gel electrophoresis



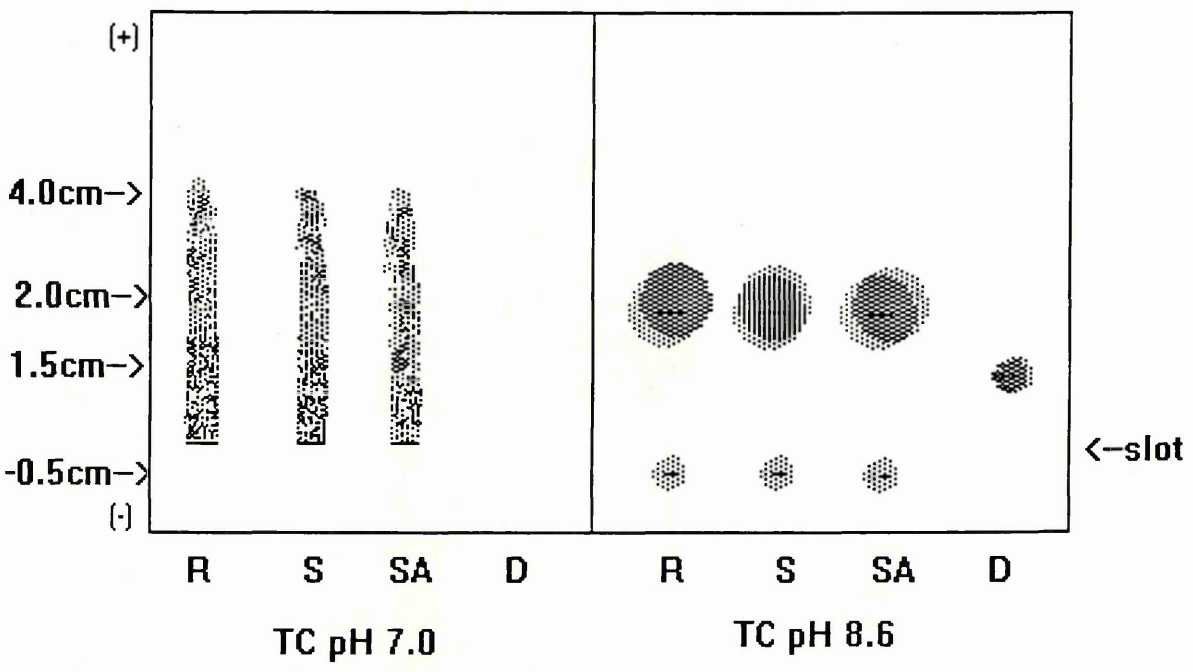
H. contortus R= resistant D= *D. viviparus*
S= susceptible
SA= South Africa

Fig. 4.4.2. MDH enzyme pattern by starch gel electrophoresis



H. contortus R= resistant
S= susceptible

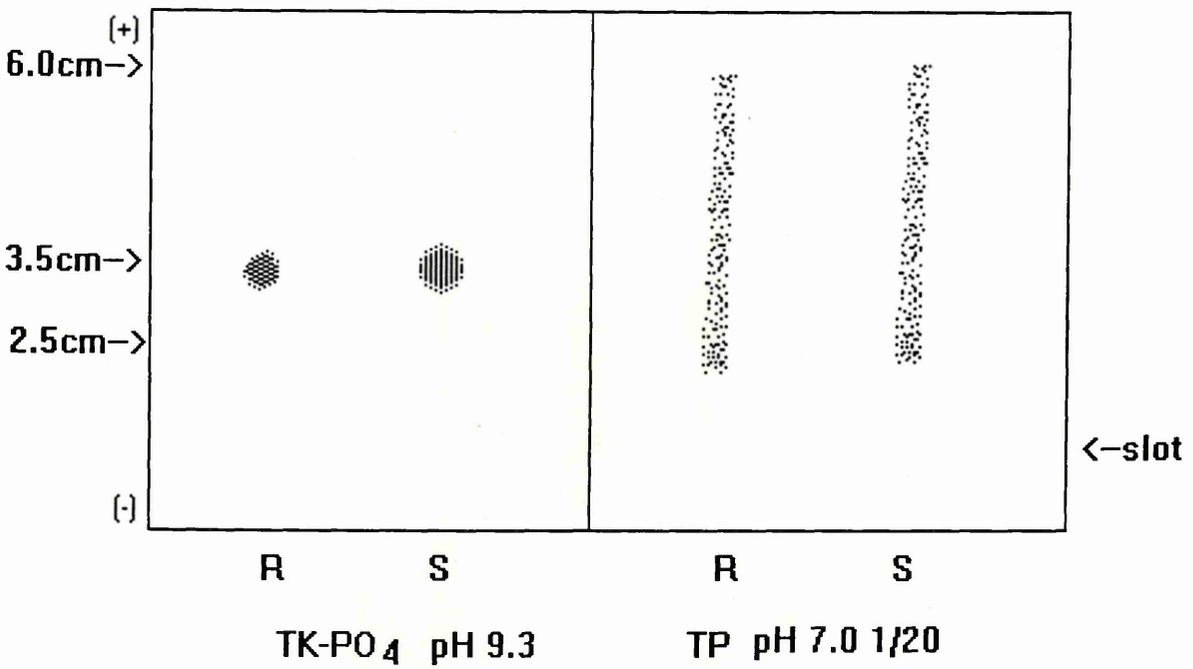
Fig. 4.4.3. MDH enzyme pattern by starch gel electrophoresis



H. contortus R= resistant
S= susceptible
SA= South Africa

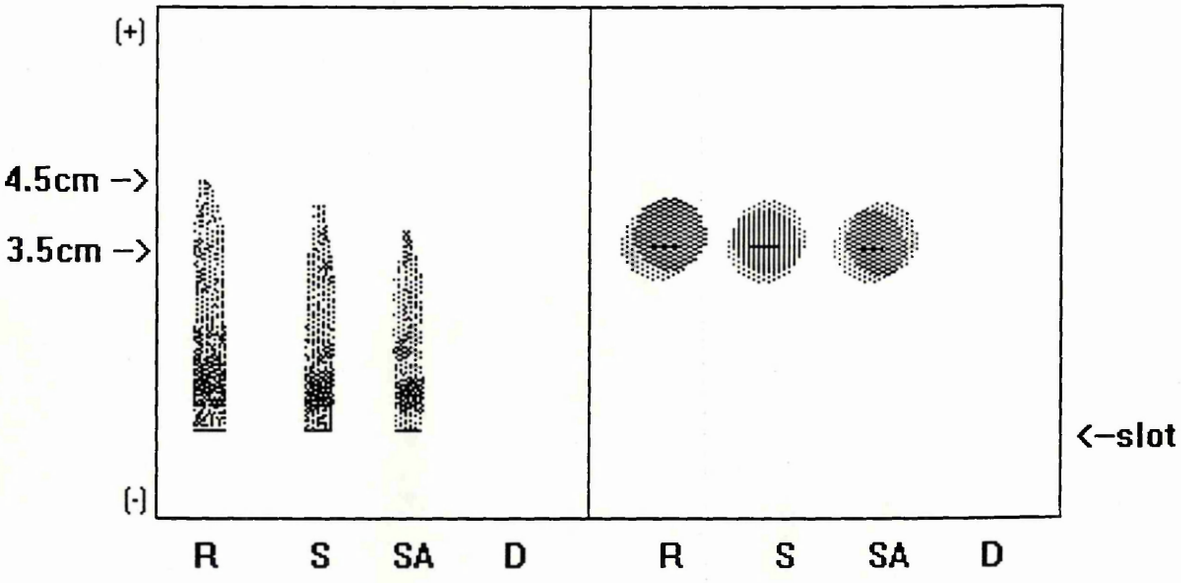
D= *D. Viviparus*

Fig. 4.4.4. G-6-PDH enzyme pattern by starch gel electrophoresis



H. contortus R= resistant
S= susceptible

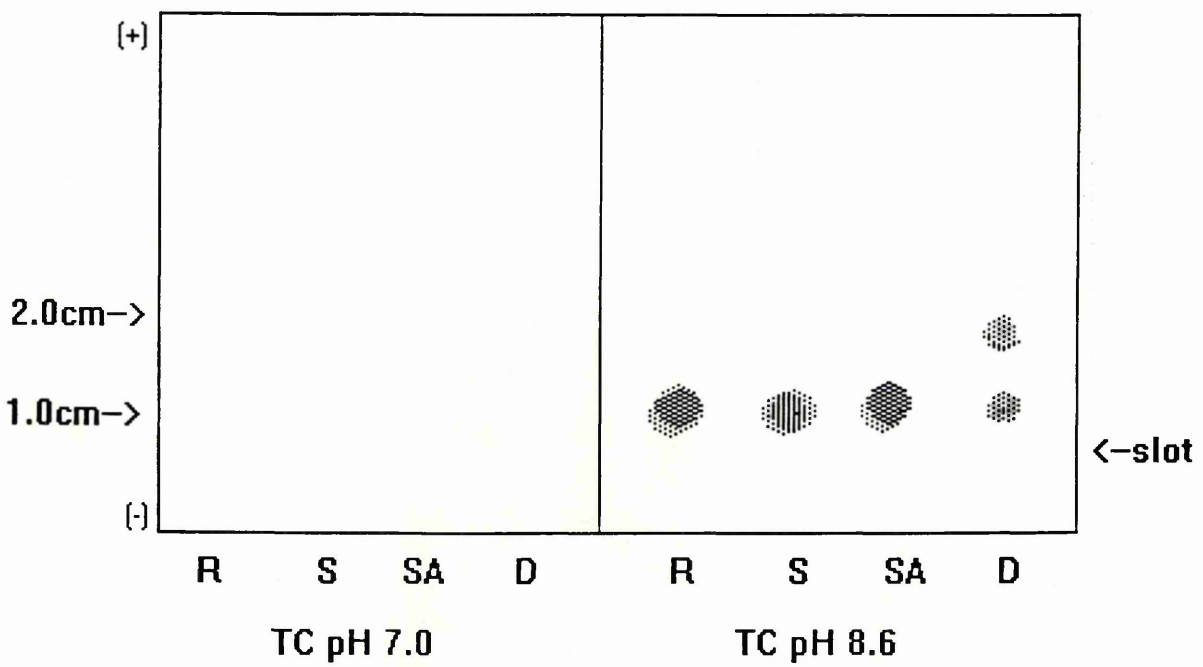
Fig. 4.4.5. G-6-PDH enzyme pattern by starch gel electrophoresis



H. contortus R= resistant
S= susceptible
SA= South Africa

D= *D. Viviparus*

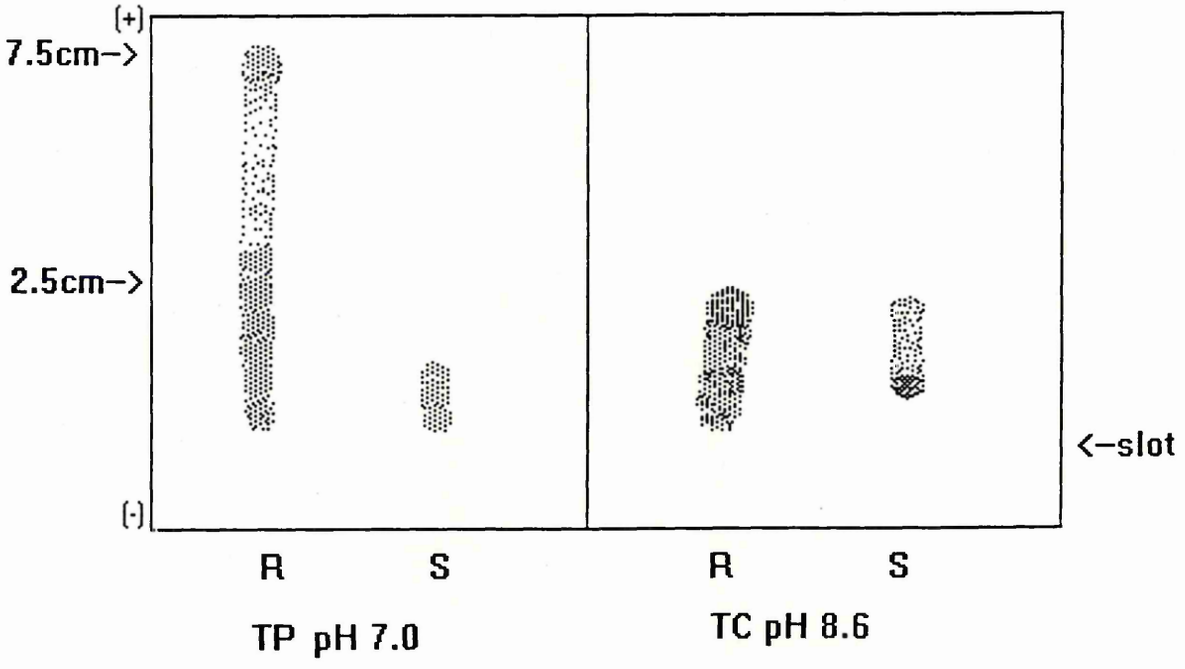
Fig. 4.4.6. Ala-Tyr enzyme pattern by starch gel electrophoresis



H. contortus R= resistant
S= susceptible
SA= South Africa

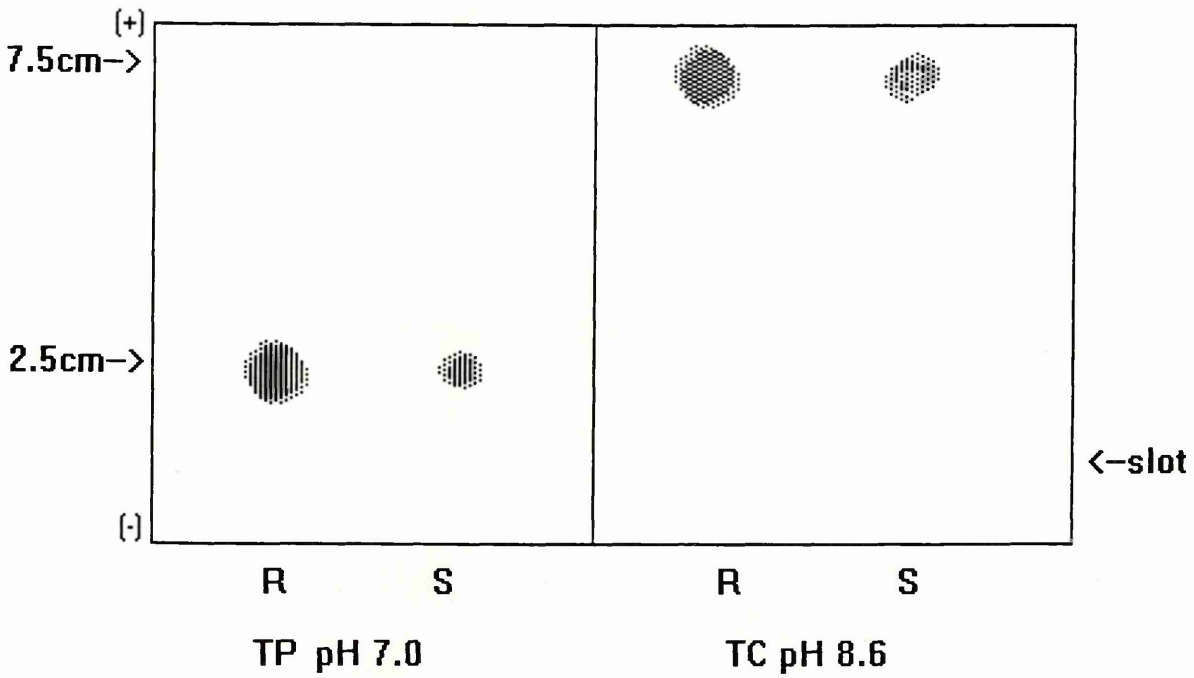
D= *D. Viviparus*

Fig. 4.4.7. Ala-Tyr enzyme pattern by starch gel electrophoresis



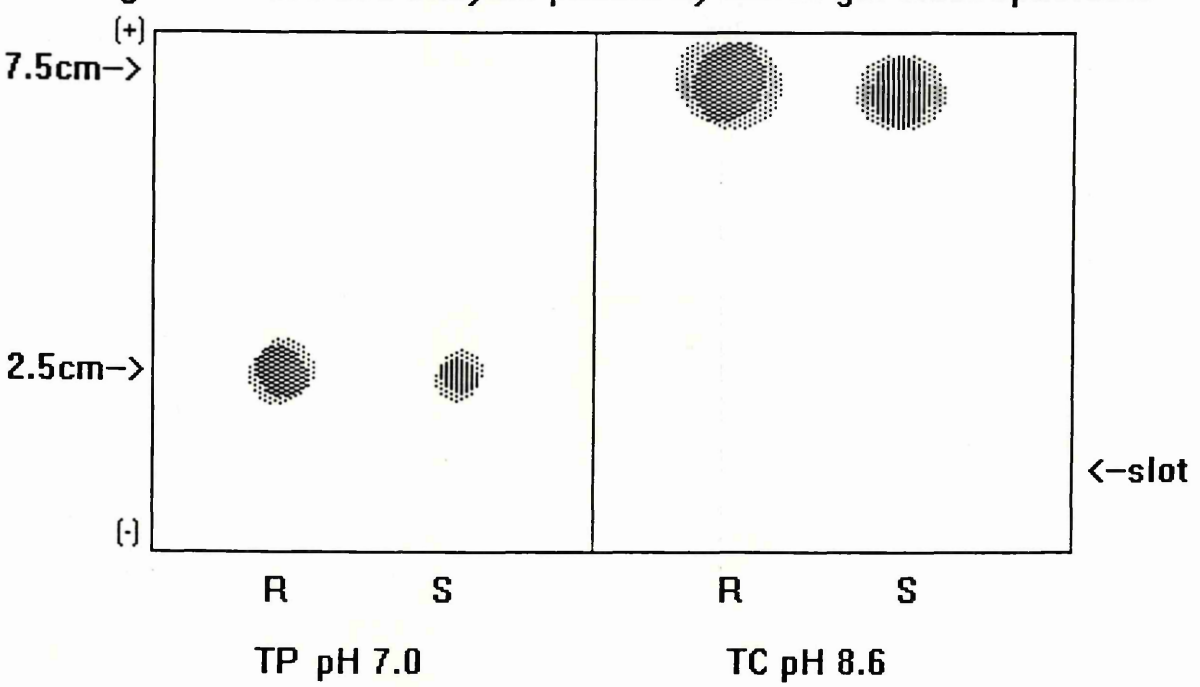
H. contortus R= resistant
S= susceptible

Fig. 4.4.8. Leu-Ala enzyme pattern by starch gel electrophoresis



H. contortus R= resistant
S= susceptible

Fig. 4.4.9. Leu-Leu enzyme pattern by starch gel electrophoresis



H. contortus R= resistant
S= susceptible

CHAPTER 5

COMPUTER MODELLING FOR ANTHELMINTIC RESISTANCE

Experiment 5.1

A DRUG ACTION MODEL IN GENETIC SELECTION FOR ANTHELMINTIC RESISTANCE IN *Haemonchus contortus*

INTRODUCTION

As anthelmintic resistance in sheep nematodes continues to evolve to all broad spectrum anthelmintics there is a need for continuous investigation of the different aspects involved in the process using different approaches. In this respect, mathematical and statistical approaches have been used in the study of the complex process of development of anthelmintic resistance.

Martin *et al.* (1984) and Waller *et al.* (1985) reported on the use of statistical dose-response models to characterise resistant populations. Dobson *et al.* (1987) described a population dynamics model to predict changes in the genetic compositions of *T. colubriformis* populations under drug challenge, using an approach similar to that proposed by Georghiou and Taylor (1977a,b). In addition Gettinby *et al.* (1989) developed a site specific drug action model for genetic selection of anthelmintic resistance in *O. circumcincta* in sheep. This provided a tool for the investigation of the effect of different anthelmintic programmes on nematode resistance under United Kingdom environmental conditions.

This section explores an adaptation of the model of Gettinby *et al.* (1989) as a method of predicting the effect of weather and management factors on the development of anthelmintic resistance in a flock of breeding ewes under conditions typical of Southern Brazil.

MATERIALS AND METHODS

Ovine haemonchosis. Sheep that graze pastures of Southern Brazil are infected with different gastrointestinal nematodes but *H. contortus* is by far the most important as it causes serious losses. This parasite is able to induce 30-40% mortality if lambs are not treated with anthelmintics. Infections reach dangerous levels during autumn when pasture larval availability reaches its highest levels. Under such conditions young susceptible lambs are treated very often. In a survey by Echevarria and Pinheiro (1989) it was shown that lambs in their first year receive on average nine treatments, ranging from 6-12 treatments/year. This in turn has led to the development of anthelmintic resistance to most of the broad spectrum compounds available.

Genetic prediction model. Simple analytical models to describe the nature of genetic change within a parasite population, under field conditons, are difficult to formulate. This is not surprising considering the complex interaction between many factors such as environmental factors, strain differences and management practices that lead to genetic change. Furthermore, every farm is unique and presents a different set of circumstances. Any model of genetic change will therefore have to be site-specific if it is to be useful. The genetic model used in this section was based on the model for anthelmintic resistance in *O. circumcincta* described by Gettinby *et al.* (1989). This model evolved from an earlier population dynamics model proposed and tested by Paton *et al.* (1984).

The following is a brief description of the model with the alterations carried out in order to adapt it to the Brazilian conditions. In many cases, the information required for modelling purposes has not been readily available and consequently some of the following data inputs are estimates obtained from experience of Brazilian field conditions.

Ewes which lamb on the first of September ingest infective larvae from herbage, which is consumed at a rate of approximately 1.4kg of dry matter (DM) per day, whereas herbage consumption of lambs depends on age. Lambs less than 4 weeks old consume about 0.1kg DM per day, and between the age of 4 and 8 weeks about 0.6kg DM per day, thereafter the consumption rate is around 0.7kg DM per day. Adult worms become established within the sheep 16 days after ingestion of infective larvae on pasture according to the density-dependent relationship

$$J = 0.25L$$

where J is the number of infective larvae to become adult worms after 16 days and L is the number of infective larvae consumed 16 days earlier. Egg output from infected ewes during the peri-parturient period is independent of worm burden, but is time dependent. In the first 4 weeks after lambing, the egg output is set at 280 eggs per gram (e.p.g.) of faeces, after week 8 it is set to 1270 e.p.g. after week 12 it is set to 590 e.p.g. In the case of contaminated pasture, ewes deposit E eggs per gram on pasture per day where

$$E = 16A$$

This means that in an average infection each adult female can be expected to produce up to 12,000 eggs per day (i.e., 1 female x 8

eggs/female/gram x 1,500g of faeces/day). This relationship deals specifically with the egg output from *H. contortus* worms and does not take account of interactions which could arise from the presence of other species.

The development of the parasite from the egg and first larval stage to the infective third stage is obtained by adding the daily development fractions. This fraction is the proportion of development that can be expected on a particular day and was determined from daily maximum and minimum temperatures, daily rainfall and herbage conditions using the known development rates under constant temperatures: 10°C requires 28 days; 12°C requires 16 days; 15°C requires 8 days; 18°C requires 6 days; 21°C requires 4 days; 24°C requires 3 days and 33°C requires 3 days.

It is possible to take account of genetic variation within the parasite population over a range of fitness sets associated with a single locus. Each parasite is assumed to have one of three pairs of alleles RR, RS or SS. For a drug administration at a given concentration, parasites with genotypes RR, RS or SS which come into contact with the drug have respective fitness F1, F2 and F3. In this case, fitness is defined as the level of susceptibility of parasites with different genetic composition to the anthelmintic used. For example, if the susceptibility set associated with genotypes RR, RS and SS was [1,1,1] the drug would not be selective and all parasites would survive treatment. In contrast, for a fitness set [1,0.5,0] all parasites with genotype RR would survive treatment, 50% of parasites with genotype RS would be removed and all SS parasites would be removed. After treatment, surviving adult worms within the host are

assumed to mate at random and Mendel's law is used to determine the genotypes of eggs being deposited on pasture. If $p1$, $p2$ and $p3$ are the proportions of adult worms with genotypes RR, RS and SS, respectively remaining after treatment, then the proportions of RR, RS and SS eggs will be P^2, $2PQ$ and Q^2, respectively where

$$P = (p1 + p2/2)$$

and

$$Q = (p3 + p2/2)$$

This is necessary to meet the requirement for Hardy-Weinberg equilibrium.

The genetic prediction model provides an algorithm which enables a day-to-day inventory of the number and genotype of parasites in all stages of development on the pasture and within the sheep to be maintained. The model is controlled by:

a- meteorological conditions which influence the development and survival rates of the parasites on herbage;

b- the stocking density and herbage availability which influence the contact rate between host and parasite;

c- the strain of parasite which influences sheep infectivity and egg output.

Computer implementation: the genetic prediction model. The genetic prediction model has been designed to run on IBM-compatible microcomputers. The program has been written and compiled using PROSPERO Pascal and the programming language C. User entries consist of the days on which the program starts and finishes in each year, the number of ewes and lambs on the plot, the herbage availability, the

days on which sheep are treated with drugs, the daily maximum temperatures, minimum temperatures and rainfall over the grazing period, the proportion of parasites with different genotypes in the first year of grazing, the susceptibility of parasites with different genotypes, and the pasture contamination levels at the start of the grazing period. The genetic prediction model then simulates the day-to-day changes in the parasite population from one year to the next. At the end of the year, it is assumed that the pasture contamination levels on the plot drop to the same starting level as that which exists at the beginning of each grazing year. In order to adapt the model to Brazilian conditions it was necessary to set the beginning of each year to the first of September when lambing takes place. The input allows the user to specify herbage levels and drug action throughout the year and parasite survival. Results for the proportions of different genotypes within the infective larval population on the pasture are obtained at the end of each year. Changes in these proportions from year to year reflect the impact of drug control on selection for resistance.

RESULTS

The genetic prediction model has been used to investigate the effect of anthelmintic control on the development of resistance in a flock of breeding ewes within a paddock in Southern Brazil over a 20 year horizon. Table 5.1.1 lists those control alternatives which have been chosen for examination.

Table 5.1.1. Some management programmes for the control of haemonchosis in breeding ewes under Brazilian conditions, used in the computer simulation

A	Dose on days 60 (marking), 110 (weaning), 194 (mating) and two later treatments on days 244 and 314 after lambing
B	Dose on days 60, 110, 194 and 254 after lambing
C	Dose on days 90, 120, 150, 180 and 210 after lambing
D	Dose at 30 day intervals starting 90 days after lambing and ending 270 days after lambing

All lambs are weaned and removed from the area on day 110 after lambing.

Treatment with an anthelmintic is assumed to be 100% effective in destroying susceptible adult and immature parasite forms. Only those parasites which possess an R allele will survive and the number of survivors will depend on the associated susceptibilities. The genotype susceptibilities used in the computer experiments are given in Table 5.1.2.

Table 5.1.2. Susceptibility sets, in response to treatment, considered for parasites of different genotypes

Parasite genotype			
RR	RS	SS	Expression of R gene
1	0.5	0	Incomplete dominance
1	0	0	Recessive

As the model is site-specific, simulations were undertaken of the changing genetic status of a parasite population on a set paddock of 20ha in Southern Brazil. Meteorological conditions obtained for the year 1987 were used for each year of the simulation. Table 5.1.3 illustrates a set of parameters used as entries to the genetic prediction model. The fitness set [1,0.5,0] corresponds to incomplete dominance of the advantageous allele R and the control programme indicates that ewes are to be treated at marking, weaning, mating and two extra treatments on days 254 and 314 after lambing respectively. It is assumed that lambs are not treated and that they are removed from the experimental area at weaning. Commencing with a worm population with gene frequencies in Hardy-Weinberg equilibrium and containing 0.1% worms with genotype RR, the simulation examines the pattern of resistance which could develop over a period of 20 years.

Each simulation produces a list of the day-to-day worm burdens in

the sheep population for each grazing period and also the number of worms in each of the free-living stages. The number of parasites of each genotype is also recorded. At the end of each year the proportion of worms with genotypes RR, RS and SS found on herbage is used to calculate the distribution of the genotypes. Examination of the proportion of R genes amongst the larvae on pasture at the end of each year provides an indication of whether or not selection for a resistant strain is taking place and the rate at which it is occurring. Figure 5.1.1 illustrates this for a flock of ewes in which programme A, the most used in the region, is implemented yearly. The ewes are dosed at marking, weaning, mating, 60 days after mating and again 60 days later. The results are shown for a population in which all worms with genotype RR and 50% with genotype RS survive drug treatment. The results show that there was a very slow but steady increase in the frequency of R genes in the larval population, starting at 3% and reaching a maximum of 14.6% at the end of the 20 year period.

Results for the comparison of evolution of resistance under the various control programmes and using different degrees of fitness to treatment, i.e., 0,0.5,1 and 0,0,1 for the genetic composition SS, RS and RR respectively are given in Fig. 5.1.2. For treatment A, C and D there was a clear difference in the rate of increase in the proportion of R genes, when the RS is set to 0.5.

The reduced interval between treatments for group C in comparison with A produced a much higher increase in the proportion of R genes at the end of 20 years. On the other hand, the additional two treatments given on programme D did not produce a greater increase in the

frequency of R genes in comparison with C with the same 30 day interval between treatments (Fig. 5.1.2).

Worm burdens under the different simulations are given in Table 5.1.4. With the exception of treatment A, B and Late season treatment all the others had very high mean worm burdens.

The effect of early versus late season treatment was compared by simulating four treatments at 30 day intervals starting 30 days after lambing with the same treatment starting at weaning. Early treatment selected for R genes while the same regime starting at weaning did not produce an increase in R genes (Fig. 5.1.3). However the mean worm burden for the latter treatment was low (Table 5.1.4) and may not represent the field conditions.

As the model produces an extensive detailed output only the results for the programme A is attached as Appendix 5.1.1 to this section.

DISCUSSION

The different anthelmintic programmes used in the simulation model employed in the present studies have shown that they do not select for high levels of resistance very quickly. The model has shown the nematode control programme (option A) most used by the Brazilian farmers would only increase the frequency of R genes from 3% to 20% over a 20 year period. On the other hand, if treatments are given at 30 day intervals between days 90 and 210 after lambing, the frequency of the R gene increases from 3% to 40%. Anthelmintic resistance in weaned lambs in Brazil has been well documented in a survey where it was shown to be highly prevalent and also that lambs harbour heavy

infections and are treated very often (Echevarria and Pinheiro, 1989). However, the level of resistance in breeding ewes is not known and the output produced by the model may be correct as ewes are regarded as more refractory to nematode infections and are treated with a lesser intensity.

The importance of parasite fitness in response to drug exposure is important in the determination of the absolute value of resistance. The comparisons carried out in this experiment have showed that the nematode control programmes select for higher frequencies of R genes when this fitness is set to [0,0.5,1] instead of the [0,0,1] for SS, RS and RR respectively. In this model it is assumed that the drug used is 100% effective in removing all stages of *H. contortus*. In practice, this is rarely obtained so that susceptible parasites can survive and consequently the selection for resistance can be expected to be slower.

The simulation model used in these studies has produced some interesting results. Nevertheless, they have to be interpreted with reservation since although the model has been partially reconstructed for the Brazilian conditons it still needs extensive revisions to provide a better approximation to the field situation. The model, in the version which was used, did not allow treatment of lambs. As young lambs can produce high worm egg counts before they are weaned and moved away, they are an important part of the epidemiological process. Furthermore, under the conditions of Southern Brazil, many farmers treat their lambs at marking, about 60 days after lambing. This is certainly an important factor and should be stressed in further studies on the improvement of the simulating facilities of the model.

Other points to be considered in the future are the inclusion of facilities for treatment and movement of animals. Despite these reservations the model is a potentially powerful tool in the study of the interactions of control programme management and selection for anthelmintic resistance. Furthermore, it has drawn attention to the fact that further studies are also necessary on the biological aspects of the process of anthelmintic resistance in particular, resistant and susceptible populations need to be better characterised with respect to infectivity, pathogenicity, development and survival. Also work is required to discover the genetic mechanisms that can best represent the parasite under field conditions.

Table 5.1.3. Illustration of user entries to the genetic prediction model for *H. contortus*

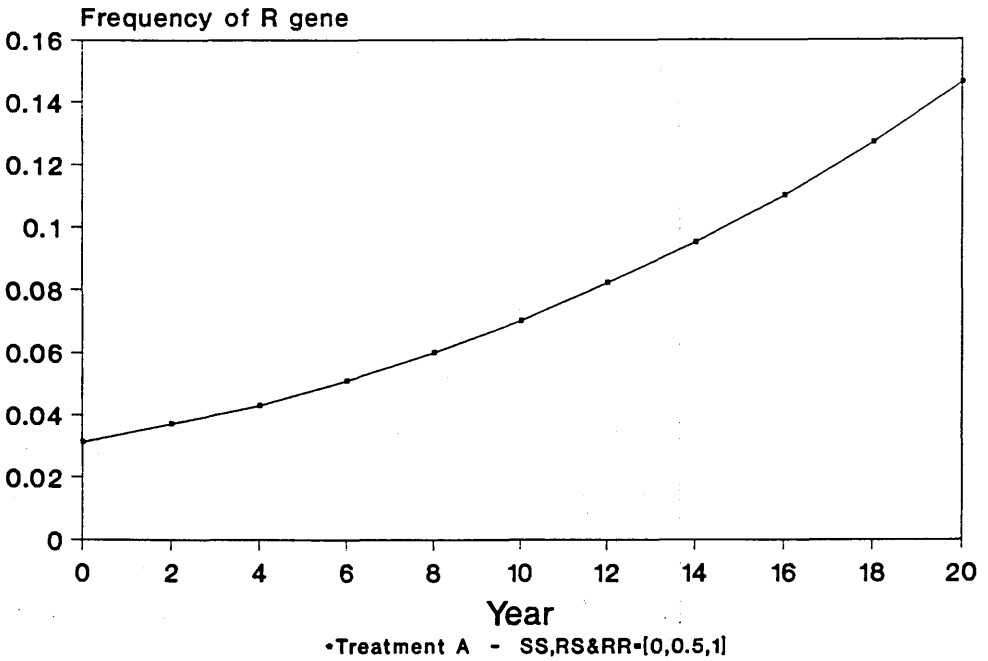
Parameter	Value
Number of:	
ewes	20
lambs	16
ewe replacements per year	4
Ares of land (in hectares)	20
Day of year on which:	
year starts (lambing)	1
Daily survival rate of:	
egg to L_1	0.92
L_1 to L_3	0.92
available infective L_3	0.60
adult worm	0.99
No. of years over which resistance is to be studied	20
Initial pasture contamination levels (L_3/kg DM):	
day 1	150
Control programme implemented	A
Susceptibiliy set of parasitic genotypes (RR,RS,SS) in response to drug treatment	[1,0.5,0]
Proportion of (RR,RS,SS) genotype in parasite population in first year	(0.001,0,061,0,938)

Table 5.1.4. Relationship between resistance after 20 years, anthelmintic control programme, drug usage and worm burden assuming SS, RS, and RR parasites are [0,0.5,1]

Control programme	Frequency of R gene after 20 years	No. of doses/ ewe/year	Maximum worm burden	Mean worm burden
A	0.146	5	5967	278
B	0.058	4	5731	30
C	0.409	5	12292	1276
D	0.415	7	12292	1290
Early	0.315	4	5489	1101
Late	0.040	4	13712	39

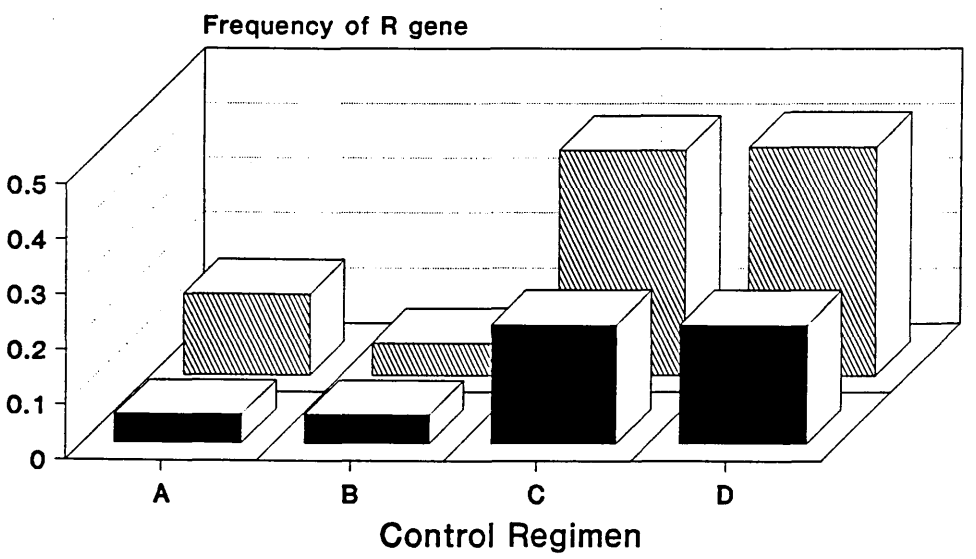
Early= treatments on days 30, 60, 90 and 120 after lambing;
Late= treatments on days 110, 140, 170 and 210 after lambing.

Fig. 5.1.1. Frequency of R gene within a *Haemonchus* population in breeding ewes*



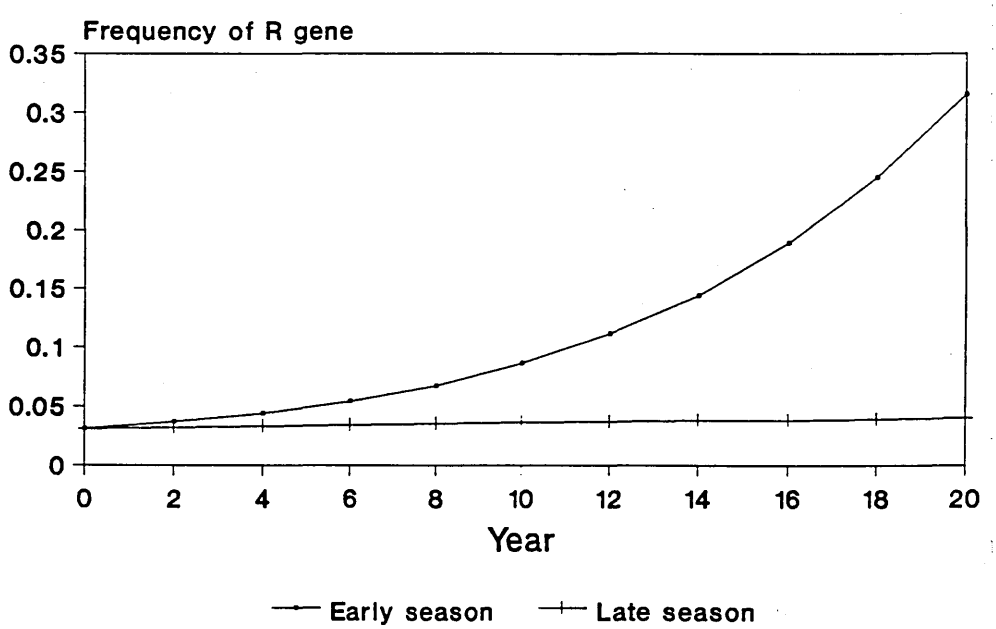
*Treatment A - SS,RS&RR=[0,0.5,1]

Fig. 5.1.2. Effect of different control programme regimes on R gene frequency*



*Selecting regime during 20 years

Fig. 5.1.3. Early x late season treat.:
its effect on the frequency of R gene



CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

The objective of this thesis was to look at a variety of aspects of anthelmintic resistance in *H. contortus*, particularly that of ivermectin resistance, and to explore possible ways in which its control, prevention and diagnosis might be improved.

The practical problem of anthelmintic resistance is that it may take many years to evolve and during this period farmers do not realize the problem exists. If they continue to use the same drug or related compounds, eventually a general failure in parasite control occurs. Before that, however, subclinical losses in terms of reduced weight gains and quantity and quality of wool would certainly have occurred.

Anthelmintic resistance has been most commonly reported from major sheep rearing areas of the world, i.e., Australia, South Africa and Brazil and mostly involves *H. contortus*. In all these areas, climatic conditions favour the development of free-living stages of important parasitic sheep nematodes. Under such conditions farmers usually treat their animals, particularly the young susceptible age groups, many times per year. Although locally appropriate strategic control programmes have been developed for some of these areas, farmers often tend to treat their animals empirically, often at short intervals during the times when larvae are at high levels on pasture. Under such conditions resistance has developed to most of the available drugs.

The use of benzimidazoles was widespread throughout the world when the discovery of an unrelated group, the avermectins, was made in the late 1970's; one avermectin, namely ivermectin, was first marketed in

the early 1980's and proved to be highly efficient against both internal and external parasites. Nevertheless, within a decade, sheep nematodes had become resistant to this drug under both laboratory and field conditions.

Under extensive grazing conditions, it may take a long time for the development of resistance to ivermectin; for example, in Brazil resistance appeared only after 4.5 years of exclusive use (Echevarria and Trindade, 1989). Under more intensive conditions the time required was shorter, for example, in South Africa on intensive grazing on irrigated pastures, where the artificial environment provided excellent conditions for the survival of larvae, resistance was obtained after 2.5 years of alternated use of IVM with other drugs (Van Wyk and Malan, 1988).

This thesis reports on possible control measures using different drugs or management procedures which could help in the control of anthelmintic resistance in sheep. Other aspects such as selection for IVM resistance, the effect of IVM on susceptible and resistant stages and the survival of free-living stages of *H. contortus* under laboratory or field conditions were also investigated as well as a computer model to simulate the development of anthelmintic resistance for breeding ewes in a specific site.

For the purposes of these studies three strains of *H. contortus* were mainly used: one susceptible to all of the common anthelmintics, the same strain selected for resistance to IVM under laboratory conditions and a strain resistant to a wide range of anthelmintics, including IVM, which was isolated from a farm in South Africa.

As described in Exp. 4.1, a susceptible strain of *H. contortus* was

submitted to a selection programme using 0.02mg/kg of IVM, i.e., one tenth of the commercially recommended dose. Even at such a low dose, the drug was highly efficient until the seventh generation when the progeny of the adult worms which survived treatment were highly resistant. A similar selection procedure by Egerton *et al.* (1988) produced resistance after six treatments, but signs of resistance had already appeared by the fourth and fifth generation.

The rate at which resistance develops depends on the selection pressure imposed on a given population in the parasitic phase but it will also depend on the general fitness of resistant and susceptible individuals both in the parasitic phase, as well as in the free-living stage. An experiment set up to look at the development of eggs and larvae of the original susceptible strain, in comparison to the selected strain under laboratory controlled conditions, showed significant differences in the behaviour of these strains at two different temperatures. At 22^{0}C the resistant strain showed a much higher percentage development from eggs to the infective stage while at 27^{0}C the results were reversed, i.e., a significantly higher percentage of larvae developed from eggs of the original susceptible strain.

It is not easy to assess differences between strains of a given population of parasites. Most of the reports published in this area are of strains from different locations and/or origins and so submitted to varying selection pressures, under both field and laboratory conditions. Given the biological variation which seems to occur between strains, definitive data is difficult to obtain. In the present studies, an IVM resistant selected strain was compared to the

original susceptible strain. This undoubtedly is the best way to proceed and there were clear indications that at higher temperatures larvae of the susceptible strain develop and survive better. To select for resistance under field conditions takes much longer so most experiments are done comparing resistant strains with a susceptible strain of different origin. Thus in the current studies eggs from two strains of *H. contortus*, one resistant and another susceptible to IVM, were seeded onto pasture plots during three summer months in Brazil. The results indicated that resistant larvae were obtained in higher numbers only after one of the contamination periods; at others there appeared to be no significant differences in development rates. However, in all cases the recovery rate was low indicating that during the summer months in Brazil there is limited survival and development of eggs and larvae. Under summer conditions, therefore, animals would be carrying most of the parasite population and it is these worms which will make a significant contribution to the autumn rise in pasture larval numbers and consequently to outbreaks of haemonchosis.

An experiment designed to exploit the residual effects of salicylanilide/substituted phenols, strategically administered during the summer in the conditions of Southern Brazil was successful in controlling an IVM resistant population of *H. contortus* thus preventing the predictable outbreaks of haemonchosis in autumn. Of these drugs, nitroxynil gave the best results. Albendazole was also effective but as this population of *H. contortus* had a previous history of resistance to BZs, it would be expected that the use of albendazole would result in the rapid re-appearance of resistance, as

it has been shown that once the original selecting agent is reintroduced, resistance may re-appear quickly (Kelly and Hall, 1979; Waller *et al.*, 1986). This may, however, depend on the fitness of the resistant parasites since resistance to a certain drug and fitness may be selected on different paths e.g., in *Lucilia cuprina*, genetic analysis has localised the fitness modifier(s) to chromosome 3 i.e., on a different chromosome from the diazinon resistance locus (McKenzie and Purvis, 1984).

The fact that the salicylanilide/substituted phenols gave good control of the IVM resistant population should be viewed with caution since some cases of resistance to these drugs have already been reported and if this drug group were to be used without a rational approach, resistance would be likely to develop. However, if its use is restrained to the period when the free-living larval population on the pasture is low, then, although selection for resistance would still be occurring, the numbers of larvae surviving the summer conditions in Southern Brazil would be low and the need of further treatment during the autumn considerably reduced.

Another approach examined in this thesis, as an alternative method in the control of sheep nematodes under Brazilian conditions, was the use of "safe pastures", i.e., areas with low levels of infectivity where young susceptible sheep could be grazed under low risk of infection. In this case a pasture, reseeded after a crop of soya bean, was tested for pasture infectivity in spring using lamb and calf tracers; it was found to be virtually negative for *H. contortus* and other important nematodes. These findings, together with those obtained with the use of salicylanilide drugs, are of great importance

for the Brazilian situation; lambing ewes could be kept on reseeded pastures from spring until lambs were weaned in mid-summer with no need of anthelmintic treatment; after weaning lambs, if necessary, could be treated with salicylanilide drugs so that by autumn, the risk of disease would be very low.

The technique of moving animals onto safe pastures in the spring after treatment may be questioned as any residual population of parasites carried by the animals would probably contain a majority of resistant worms and so selection for resistance could be high. However, in the Brazilian situation the safe pastures could be a dead end for the resistant parasites since the male lambs would be sent to slaughter at weaning and the females lambs after weaning could be treated with salicylanilides during summer. These pastures could then be grazed by adult cattle or cropped in the following year. This proposal is supported by the European experience where alternate grazing has been used for a number of years without any known report of the occurrence of anthelmintic resistance under such management.

The parasitic stage of development at which IVM resistance appears was also investigated. Treatment of developing stages of IVM resistant or susceptible *H. contortus* at different times indicated that the resistant strain started to show a lowered susceptibility to this drug as early as the fourth larval stage. The fact that on the susceptible adults the drug is almost 100% effective whereas on the early fourth stage its efficacy is about 96% may be an indication that selection is more likely to start at the early stages of the life-cycle.

With BZ drugs, treatment has been shown to only temporarily

suppress worm egg output of resistant worms without a corresponding loss of worm burdens (Hotson *et al.*, 1970; Martin *et al.*, 1985). In the present studies, a small experiment was carried out to monitor the egg output of a multiple resistant strain of *H. contortus*, (i.e., resistant to IVM, BZ and closantel) following treatment with a BZ, albendazole, in comparison to an untreated group. Interestingly and in contrast to the results of Martin *et al.* (1985), although egg counts increased after an initial drop, they did not return to previous levels despite considerable worm burdens remaining in the host. This has considerable practical significance and the interpretation of egg counts following treatment with BZs has to be viewed with caution. On the basis of the results of the experiment carried out in this thesis (Exp. 4.2) if there is any rise in worm egg counts following the initial post-treatment drop, consideration must be given to the possibility of BZ resistance being present. The inclusion of larval cultures for the identification of species surviving treatments would also be important in identifying problem species such as *H. contortus* with its high biotic potential.

Following the evidence of reduced efficacy of IVM in a field population of *H. contortus* in Brazil, this strain was isolated and submitted to a controlled anthelmintic test where resistance to IVM was confirmed. The use of levamisole, albendazole, and IVM on this strain as well as a strain of known susceptibility to the major broad spectrum anthelmintics revealed that levamisole was the compound with the best efficacy on both resistant and susceptible strains. However, the results with albendazole, although giving a high efficacy on the susceptible strain, showed that the resistant strain had not reverted

to BZ susceptibility after many years without exposure to this group of drugs indicating, as discussed earlier, that reversion may not be of practical importance once resistance has evolved in a given population.

Levamisole has been on the market for as long as the BZ drugs and although it has been widely used, *H. contortus* has not developed resistance to it to the same extent as to benzimidazoles. In contrast, other species of gastrointestinal nematodes have shown resistance to this drug, but the reasons for the varying rates at which different genera of nematodes of sheep acquire resistance to distinct chemical groups remain unknown. Further investigations are required to elucidate the mechanisms involved in the development of resistance.

With the continued development of anthelmintic resistance to all broad spectrum compounds, there is an increasing need for the development of new techniques to distinguish resistant and susceptible parasites both for diagnosis as well as for a better understanding of the mechanisms involved in the process. Investigations at the molecular level might provide some answers and in studies reported here, examination of isoenzyme profiles were used in an attempt to differentiate susceptible, IVM selected and multiple resistant strains of *H. contortus*. Using a series of stains for eight enzymes separated in different buffer systems, no differences in the electrophoretic mobility of any enzymes associated with resistance could be detected. This indicated a low level of enzyme variation in *Haemonchus* and, although no alterations in most of the enzyme electrophoretic profiles were observed between IVM susceptible and resistant parasites, the propionate esterase showed distinct bands

for the multiple resistant strain and a single band for the susceptible strain, while the IVM selected strain showed little propionate esterase activity. These results suggest that alterations in this enzyme might be correlated with BZ resistance, but not IVM resistance.

Another possible approach in the understanding of the development of anthelmintic resistance and its possible prevention is the use of mathematical models to simulate the alterations in the genetic profiles of worm populations. In this thesis, a model developed to study anthelmintic resistance in sheep under UK conditions (Gettinby *et al.*, 1989) was modified to use some Brazilian field data. Although this model allowed us to look only at breeding ewes, it showed that in the control programme most used by Brazilian farmers the frequency of R genes were likely to increase slowly during a period of 20 years. This, however, is a theoretical approach and until the model is refined and fully adapted to study the problem in weaned lambs where anthelmintic resistance is more prevalent, further work should be directed to obtaining more information on the aquisition of resistance and the environmental profile of resistant worms.

In conclusion the results of the studies described in this thesis have highlighted several important points involved in anthelmintic resistance which could be investigated in the future using the following approaches:

- the epidemiology approach to provide basic data on survival and development of parasitic and free-living stages of resistant and susceptible strains, the efficacy of different anthelmintic treatments, and the use of different control systems under different

climatic conditions;

- the biochemical approach to elucidate the mechanisms of anthelmintic resistance, particularly in the case of ivermectin;

- the genetic approach to determine the genetic profile involved in resistant nematode populations as genetic variation may be population specific;

- the molecular approach to produce sensitive techniques capable of detecting resistance at its early stages of development in order to allow alternative control measures;

- modelling - data generated by some of the above approaches could be used to model realistically the effects of control strategies on nematode populations, and could also lead to a much improved knowledge of the role of operational factors that accelarate or delay resistance. Specific computer models supplied with this information should substantially improve the management of resistance.

REFERENCES

REFERENCES

Abedi, Z.H. and Brown, A.W.A., 1960. Development and reversion of DDT-resistance in *Aedes aegypti*. Canadian Journal of Genetics and Cytology **2**: 252-261.

Adams, D.B., 1978. The induction of selective immunological unresponsiveness in cells of blood and lymphoid tissue during primary infection of sheep with the abomasal nematode, *Haemonchus contortus*. Australian Journal of Experimental Biology and Medical Science **56**: 107-118.

Adams, D.B., 1988. The effect of dexamethasone on a single and superimposed infection with *Haemonchus contortus* in sheep. International Journal for Parasitology **18**: 575-579.

Anderson, N., 1972. Trichostrongylid infections of sheep in a winter rainfall region - I. Epizootiological studies in the western district of Victoria, 1966-67. Australian Journal of Agricultural Research **23**: 1113-1129.

Anderson, N., 1973. Trichostrongylid infections of sheep in a winter rainfall region - II. Epizootiological studies in the western district of Victoria, 1967-68. Australian Journal of Agricultural Research **24**: 599-611.

Anderson, N., 1985. The controlled release of anthelmintics for helminth control in ruminants. In: Resistance in nematodes to anthelmintic drugs (N. Anderson and P.J. Waller, Eds), pp. 127-136.

Anderson, N. and Laby, R.H., 1979. Activity against *Ostertagia circumcincta* of low doses of oxfendazole continuously released from intraruminal capsules in cattle. Australian Veterinary Journal **55**: 244-246.

Anderson, N., Laby, R.H., Prichard, R.K. and Hennessy, D., 1980. Controlled release of anthelmintic drugs: a new concept for prevention of helminthosis in sheep. Research in Veterinary Science **29**: 333-341.

Anonymous, 1980. Grazing plans for the control of stomach and intestinal worm in sheep and cattle. Booklet 2154. Ministry of Agriculture, Fisheries and Food (Publications), Pinner, Midlessex.

Anonymous, 1981. Clean grazing systems for sheep. Sheep husbundry no. 4. Booklet 2324. Ministry of Agriculture, Fisheries and Food (Publications), Alnwick, Northumberland.

Armour, J., 1978. New approaches to the control of parasites. In: Intensive grassland use and livestock health, pages 81-88. British Grassland Association Conference, Berks, 16-17 February, 1978.

Armour, J., Bairden, K., Batty, A.F., Davison, C.C. and Ross, D.B., 1985. Persistent anthelmintic activity of ivermectin in cattle. Veterinary Record **116**: 151-153.

Armour, J., Bairden, K., Duncan, J.L., Jones, R.M. and Bliss, T., 1981. Studies on the control of bovine ostertagiasis using a morantel sustained release bolus. Veterinary Record **108**: 532-535.

Armour, J., Bairden, K., Oakley, G.A. and Rowlands, D. ap. T., 1988. Control of naturally acquired bovine parasitic bronchitis and gastroenteritis with an oxfendazole pulse release device. Veterinary Record **123**: 460-464.

Armour, J., Bairden, K. and Preston, J., 1980. Anthelmintic efficiency of ivermectin against naturally acquired bovine gastro-intestinal nematodes. Veterinary Record **107**: 226

Armour, J., Bairden, K. and Preston, J., 1982. Anthelmintic efficiency of ivermectin against naturally occurring gastrointestinal nematodes of sheep. Veterinary Record **111**: 80-81.

Arundel, J.H., 1985. Veterinary Anthelmintics. Veterinary Review. Post-Grad. Foundation. Veterinary Science, University of Sydney, no. 25.

Barger, I.A., Hall, E. and Dash, K.M., 1990. Local eradication of *Haemonchus contortus* using closantel In: 7th ICOPA, Paris, 20-24 August. Abstracts pg. 746.

Barger, I.A. and Southcott, W.H., 1978. Parasitism and production in weaner sheep grazing alternately with cattle. Australian Journal of Experimental Agriculture and Animal Husbandry **18**: 340-346.

Barrowman, M.M., Marriner, S.E. and Bogan, J.A., 1984. The binding and subsequent inhibition of tubulin polymerization in *Ascaris suum* (*in vitro*) by benzimidazole anthelmintics. Biochemical Pharmacology **33**: 3037-3040.

Barth, D., 1983. Persistent anthelmintic effect of ivermectin in cattle. Veterinary Record **113**: 300.

Barton, N.J., 1980. Emergence of *Haemonchus contortus* resistant to thiabendazole. Australian Veterinary Journal **56**: 46-47.

Barton, N.J., 1983. Development of anthelmintic resistance in nematodes from sheep in Australia subjected to different treatment frequencies. International Journal for Parasitology **13**: 125-132.

Benz, G.W., Roncalli, R.A. and Gross, S.J., 1989. Use of ivermectin in cattle, sheep, goats and swine. In: Ivermectin and abamectin (W.C. Campbell Ed) pp. 215-219.

Berger, J., 1975. The resistance of a field strain of *Haemonchus contortus* to five benzimidazole anthelmintics in current use. Journal of the South African Veterinary Association **46**: 369-372.

Berger, J., 1980. Oxfendazole: anthelmintic activity in sheep artificially infected with nematodes. Results of trials against nine species including benzimidazole-resistant *Haemonchus contortus*. Journal of South African Veterinary Medical Association **51**: 51-58.

Boergers, M., De Nollin, S., De Brabander, M. and Thienpont, D., 1975. Influence of the anthelmintic mebendazole on microtubules and intracellular organelle movement in nematode intestinal cells. American Journal of Veterinary Research **36**: 1153-1166.

Boersema, J.H., 1983. Possibilities and limitations in the detection of anthelmintic resistance. In: Facts and Reflections IV. Resistance of Parasites to Anthelmintics (F.H.M. Borgsteede, Sv. Aa. Henriksen and H.J. Over, Eds). pp. 207-216. Central Veterinary Institute, Lelystad, The Netherlands.

Boersema, J.H., Borgsteede, F.H.M., Eysker, M., Hendrikx, W.M.L., Jansen, J. and Smith-Buys, C.M.C., 1987. Prevalence of benzimidazole resistance of nematodes in sheep in The Netherlands. Research in Veterinary Science **43**: 18-21.

Bogan, J. and Armour, J., 1986. Anthelmintics for ruminants. In Parasitology *Quo Vadit?* 6th ICOPA (M.J. Howell Ed). pp. 483-491. Australian Academy of Science, Camberra, Australia.

Bogan, J.A., Armour, J., Bairden, K. and Galbraith, E.A., 1987. Time of release of oxfendazole from an oxfendazole pulse-release bolus. Veterinary Record **121**: 280.

Bogan, J.A. Marriner, S.E. and Galbraith, E.A., 1982. Pharmacokinetics of levamisole in sheep. Research in Veterinary Science **32**: 124-126.

Borgsteede, F.H.M. and Couwenberg, T., 1987. Changes in LD_{50} in an *in vitro* egg development assay during the patent period of *Haemonchus contortus* in sheep. Research in Veterinary Science. **42**: 413-414.

Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein. Analytical Biochemistry **72**: 248-254.

Bremner, K.C., Berrie, D.A. and Hotson, I.K., 1983. Persistence of the anthelmintic activity of ivermectin in calves. Veterinary Record **113**: 569.

Brewer, G.J., 1970. An introduction to isoenzyme techniques. Academic Press.

Britt, D.P., 1982. Benzimidazole-resistant nematodes in Britain. Veterinary Record **110**: 343-344.

Broome, A.W. and Jones, W.G.M., 1966. A new drug for the treatment of fascioliasis in sheep and cattle. Nature **210**: 744-745.

Brown, A.W.A. and Pal, R., 1971. Insecticide Resistance in Arthropods, Second Edition World Health Organization, Geneva.

Brusdon, R.V. Kissling, R. and Hosking, B.C., 1983. A survey of anthelmintic usage for sheep: a time for change? New Zealand Veterinary Journal 31: 24-29.

Brusdon, R.V. and Vlassoff, A., 1981. A morantel sustained-release bolus for the control of gastrointestinal nematodes in grazing calves. New Zealand Veterinary Journal 29: 139-141.

Bullini, L., 1982. Enzyme variants in the identification of parasite vectors: methodological aspects of the electrophoretic approach. New approaches to the identification of parasites and their vectors. Schwate & Co., A.G. Basel. Edited by Newton, B.N. and Michel, F. pp. 53-69.

Burg, R.W., Miller, B.M., Baker, E.E., Birbaum, J., Currie, S.A., Hartman, R., Kong, Y.-L., Monaghan, R.L., Olson, G., Putter, I., Tunac, J.b., Wallick, H., Stapley, E.O., Oiwa, R. and Omura, S., 1979. Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. Antimicrobial Agents and Chemotherapy 15: 361-367.

Cameron, S., Grant, I.M., Morley, F.H.W., Sackett, D. and Vizard, A., 1984. Anthelmintic resistance in sheep. Australian Veterinary Journal 61: 369.

Campbell, W.C., 1985. Ivermectin: An update. Parasitology Today 1: 10-16.

Campbell, W.C. and Benz, G.W., 1984. Ivermectin: A review of efficacy and safety. Journal of Veterinary Pharmacology and Therapeutics 7: 1-16.

Cawthorne, R.J.G. and Cheong, F.H., 1984. Prevalence of anthelmintic resistant nematodes in sheep in south-east England. Veterinary Record **114**: 562-564.

Cawthorne, R.J.G. and Whitehead, J.D., 1983. Isolation of benzimidazole resistant strains of *Ostertagia circumcincta* from British sheep. Veterinary Record **112**: 274-277.

Coles, G.C., East, J.M. and Jenkins, N.S., 1975. The mechanism of action of the anthelmintic levamisole. General Pharmacology **6**: 309-313.

Coles, G.C. and Simpkin, K.G., 1977. Resistance of nematodes eggs to the ovicidal activity of benzimidazoles. Research in Veterinary Science **22**: 386-387.

Coles, G.C., Tritschler II, J.P., Giordano, D.J., Laste, N.J. and Schmidt, A.L., 1988. Larval development test for detection of anthelmintic resistant nematodes. Research in Veterinary Science **45**: 50-53.

Colglazier, M.L., Kates, K.C. and Enzie, F.D., 1974. Cambendazole-resistant *Haemonchus contortus* strain in sheep: Further experimental development. Journal of Parasitology **60**: 289-292.

Colglazier, M.L., Kates, K.C. and Enzie, F.D. 1970. Comparative response of two ovine isolates of *Haemonchus contortus* to thiabendazole. Journal of Parasitology **56**: 768-772.

Cornish, R.A., Behm, C.A., Butler, R.W. and Bryant, C., 1977. The *in vivo* effects of rafoxanide on the energy metabolism of *Fasciola hepatica*. International Journal for Parasitology **7**: 217-220.

Crow, J.F., 1957. Genetics of insecticide resistance to chemicals. Annual Review of Entomology **2**: 227-246.

Da Costa, U.C., Benevenga, S.F. and Santiago, M.A.M., 1985. *Nematodirus spathiger* resistance to benzimidazoles in sheep in Rio Grande do Sul, Brazil. In: Abstracts of World Association for the Advancement of Veterinary Parasitology, 11th Conference, 1985.

Dash, K.M., Newman, R.L. and Hall, E., 1985. Recommendations to minimise selection for anthelmintic resistance in nematode control programmes. In: Resistance in Nematodes to Anthelmintic Drugs (N. Anderson and P.J. Waller, Eds), pp. 161-169, CSIRO, Glebe, Australia.

Dawson, P.J., Gutteridge, W.E. and Gull, K., 1984. A comparison of the interaction of anthelmintic benzimidazoles with tubulin isolated from mammalian tissue and the parasitic nematode *Ascaridia galli*. Biochemical Pharmacology 33: 1069-1074.

Delatour, P., Daudon, M., Garnier, F. and Benoit, E., 1982. Relationship of metabolism and embryotoxicity of febantel in the rat and sheep. Annales de Recherches Veterinaires 13: 163-170.

Dobson, R.J., Donald, A.D., Waller, P.J. and Snowdon, K.L., 1986. An egg-hatch assay for resistance to levamisole in trichostrongyloid nematode parasites. Veterinary Parasitology 19: 77-84.

Dobson, R.J., Griffiths, D.A., Donald, S.D. and Waller, P.J., 1987. A genetic model describing the evolution of levamisole resistance in *Trichostrongylus colubriformis* a nematode parasite of sheep. IMA Journal Mathematics Applied in Medicine & Biology 4: 279-293.

Donald, A.D., 1983a. The development of anthelmintic resistance in nematodes of grazing animals. In: Facts and Reflections IV. Resistance of Parasites to Anthelmintics (F.H.M. Borgsteede, Sv. Aa. Henriksen and H.J. Over, Eds). pp.15-28.

Donald, A.D., 1983b. Anthelmintic resistance in relation to helminth control and grazing systems. In: Facts and Reflections IV. Resistance of Parasites to Anthelmintics (F.H.M. Borgsteede, Sv. Aa. Heriksen and H.J. Over, Eds). pp. 187-198.

Donald, A.D., Morley, F.H.W., Axelsen, A., Donnelly, J.R. and Waller, P.J. 1987. Integration of grazing management and anthelmintic treatment for the control of nematode infections in young sheep. In: Temperate Pastures: Their production and management. (Wheeler, J.L.; Pearson, C.J. and Robards, G.E., Eds.) pp. 567-569.

Donald, A.D., Waller, P.J., Dobson, R.J. and Axelsen, A., 1980. The effect of selection with levamisole on benzimidazole resistance in *Ostertagia* spp. of sheep. International Journal for Parasitology **10**: 381-389.

Dos Santos, V.T. and Franco, E.B., 1967. O aparecimento de *Haemonchus* resistente ao radical benzimidazole em Uruguaiana. Congresso Latino-Americano de Parasitologia Ist, Santiago, Chile, 1967.

Downey, N.E. and O'Shea, J., 1977. Calf parasite control by means of low-level anthelmintic administered via drinking water. Veterinary Record **100**: 265-266.

Downey, N.E., O'Shea, J. and Spillane, T.A., 1974. Preliminary observations on the use of low-level medication in calves drinking water as a means of endoparasite control. Irish Veterinary Journal **28**: 221-222.

Drudge, J.H., Leland, S.E. and Wyant, Z.N., 1957a. Strain variation in response of sheep nematodes to the action of phenothiazine. I. Studies of mixed infections in experimental animals. American Journal of Veterinary Research **18**: 133-141.

Drudge, J.H., Leland, S.E. and Wyant, Z.N., 1957b. Strain variation in the response of sheep nematodes to the action of phenothiazine. II. Studies on pure infections of *Haemonchus contortus*. American Journal of Veterinary Research **18**: 317-325.

Drudge, J.H., Leland, Jr. S.E., Wyant, Z.N. and Ealm, G.W., 1954. Observations on the effectiveness of phenothiazine in the control of gastro-intestinal nematodes of sheep. Annual Report Director Kentucky Agricultural Experiment Station p. 56.

Drudge, J.H., Szanto, J., Wyant, Z.N. and Elam, G., 1964. Field studies on parasite control in sheep: comparison of thiabendazole, ruelene and phenothiazine. American Journal of Veterinary Research **25**: 1512-1518.

Duncan, M.C. 1990. The molecular diversity of strains of *Ostertagia ostertagi*. PhD thesis. University of Glasgow. 371p.

Echevarria, F.A.M., Pinheiro, A.C. and Correa, M.B.C., 1988. Alternativas para o controle da verminose ovina no Rio Grande do Sul. Embrapa, Comunicado Técnico no. 8 pp. 1-6.

Echevarria, F.A.M. and Pinheiro A.C., 1989. Avaliação de resistência anti-helmíntica em rebanhos ovinos no município de Bagé, RS. Pesquisa Veterinária Brasileira **9**: 69-71.

Echevarria, F.A.M. and Trindade, G.N.P., 1989. Anthelmintic resistance by *Haemonchus contortus* to ivermectin in Brazil: A preliminary report. Veterinary Record **124**: 147-148.

Edwards, J.R., Wroth, R., De Chaneet, G.C., Besier, R.B., Karlsson, J., Morcombe, P.W., Dalton-Morgan, G. and Roberts, D., 1986a. Survey of anthelmintic resistance in Western Australian Sheep flocks. 1. Prevalence. Australian Veterinary Journal **63**: 135-138.

Edwards, J.R., Wroth, R., De Chaneet, G.C., Besier, R.B., Karlsson, J., Morecombe, P.W., Dalton-Morgan, G. and Roberts, D. 1986b. Survey of anthelmintic resistance in Western Australian sheep flocks. 2. Relationship with sheep management and parasite control practices. Australian Veterinary Journal **63**: 139-144.

Egerton, J.R., Ostlind, D.A., Blair, L.S., Eary, C.H., Suhayda, D., Cifelly, S., Riek, R.F. and Campbell, W.C., 1979. Avermectins, new family of potent anthelmintic agents: efficacy of the b_{1a} component. Antimicrobial Agents and Chemotherapy **15**: 372-378.

Egerton, J.R., Suhayda, D. and Eary, C.H., 1988. Laboratory selection of *Haemonchus contortus* for resistance to ivermectin. Journal of Parasitology **74**: 614-617.

Evans, P.G., Soutar, A.J. and Gettinby, G.C., 1988. Anthelmintic resistance: prevalence and current research in The United Kingdom. In: Abstracts of European Federation of Parasitologists, Vth European Multicolloquium of Parasitology, Budapest, 1988. p. 86.

Eysker, M., Borgsteede, F.H.M., Jansen, J. and Kloosterman, A., 1982. Animal husbandry management systems and the use of anthelmintics in the Netherlands. In: Facts and Reflections IV - Resistance of Parasites to Anthelmintics (F.H.M. Borgsteede, Sv. Aa. Henriksen and H.J. Over, Eds), pp. 155-1654, Central Veterinary Institute, Lelystad, The Netherlands.

Eysker, M., Jansen, J., Boersema, J.H. and Lewing-Van der Wiel, P.J., 1983. Development of benzimidazole resistance in a *Haemonchus contortus* strain in the Netherlands following fenbendazole treatment of ewes at parturition. Research in Veterinary Science **34**: 46-49.

Ferguson, A., 1980. Biochemical Systematics and Evolution. Blackie, Glasgow and London. ISBN 0-216-90779-9.

Folz, S.D., Pax, R.A., Thomas, E.M., Bennett, J.L., Lee, B.L. and Conder G. A., 1987. Detecting *in vitro* anthelmintic effects with a micromotility meter. Veterinary Parasitology **24**: 241-250.

Friedman, P.A. and Platzer, E.G., 1980. Interaction of anthelmintic benzimidazoles with *Ascaris suum* embryonic tubulin. Biochimica et Biophysica Acta **630**: 271-278.

Fritz, L.C., Wang, C.C. and Gorio, A., 1979. Avermectin $B_{1}a$ irreversibly blocks postsynaptic potentials at the lobster neuromuscular junction by reducing muscle membrane resistance. Proceedings of *the* National Academy of Sciences of the United States of America **76**: 2062-2066.

Georghiou, G.P. and Taylor, C.E., 1977a. Operational influences in the evolution of insecticide resistance. Journal of Economic Entomology **70**: 653-658.

Georghiou, G.P. and Taylor, C.E., 1977b. Genetic and biological influences in the evolution of insecticide resistance. Journal of Economic Entomology **70**: 319-323.

Gettinby, G. Soutar, A., Armour, J. and Evans, P., 1989. Anthelmintic resistance and the control of ovine ostertagiasis: a drug action model for genetic selection. International Journal for Parasitology **19**: 369-376.

Gibson, T.E., 1964. The evaluation of anthelmintics for the removal of gastro-intestinal nematodes of sheep - an improved form of the controlled test. Parasitology **54**: 545-550.

Giordano, D.J., Tritschler II and Coles, G.C., 1988. Selection of ivermectin-resistant *Trichostrongylus colubriformis* in lambs. Veterinary Parasitology **30**: 139-148.

Gonçalves, P.C., 1974. Epidemiologia da helmintose ovina em Guaíba (Rio Grande do Sul - Brasil) Porto Alegre, UFRGS, Tese a Titular no Departamento de Medicina Veterinária Preventiva. 41p.

Gordon, H.McL., 1939. The anthelmintic efficiency of phenothiazine. Australian Veterinary Journal **15**: 245-251.

Gordon, H.McL., 1948. The epidemiology of parasitic diseases, with special reference to studies with nematode parasites of sheep. Australian Veterinary Journal **24**: 17-45.

Gordon, H.McL and Whitlock, H.V., 1939. A new technique for counting nematode eggs in sheep faeces. Journal of Council for Scientific and Industrial Research, Australia, **12**: 50-52.

Hafner, M.S. and Nadler, S.A., 1988. Phylogenetic trees support the coevolution of parasites and their hosts. Nature **332**: 258-259.

Hall, C.A., Campbell, N.J. and Richardson, N.J., 1978. Levels of benzimidazole resistance in *Haemonchus contortus* and *Trichostrongylus colubriformis* recorded from an egg hatch test procedure. Research in Veterinary Science **25**: 360-363.

Hall, C.A., Kelly, J.D., Whitlock, H.V. and Ritchie, L., 1981. Prolonged anthelmintic effect of closantel and disophenol against thiabendazole selected resistant strain of *Haemonchus contortus* in sheep. Research in Veterinary Science **31**: 104-106.

Halley, B.A., Nessel, R.J. and Lu, A.Y.H., 1989. Environmental aspects of ivermectin usage in livestock: general considerations. In: Ivermectin and abamectin (W.C. Campbell, Ed.), pp. 162-172.

Haris, H. and Hopkinson, D.A., 1976. Handbook of enzyme electrophoresis in human genetics. North-Holland Publishing Co., ISBN 0-444-11203-0.

Hart, R.J. and Lee, R.M., 1966. Cholinesterase activities of various nematode parasites and their inhibition by the organosphosphate anthelmintic haloxon. Experimental Parasitology **18**: 332-337.

Herd, R.P., 1980. Animal health and public health aspects of bovine parasitism. Journal of the American Veterinary Medical Association **176**: 737-743.

Herd, R.P. and McClure K.E., 1988. Sheep anthelmintics: resistance and reversion. In: Abstract of European Federation of Parasitologists, Vth European Multicoloquium of Parasitology, Budapest, 1988. p. 114.

Henriksen, S.A., 1965. En forbedret teknik ved undersogelse for lungeormelarver i faeces. Nordisk Veterinaer Medicin **17**: 446-454.

Herlich, H., Rew, R.S. and Colglazier, M.L., 1981. Inheritance of cambendazole resistance in *Haemonchus contortus*. American Journal of Veterinary Research **42**: 1342-1344.

Hogart-Scott, R.S., Kelly, J.D., Whitlock, H.V., NG, B.K.Y., Thompson, H.G., James, R.E. and Mears, F.A. 1976. Research in Veterinary Science **21**: 232-237.

Hood, A.E.M. and Bailie, J.H., 1973. A new grazing system for beef cattle - The two field system. Journal of the British Grassland Society **28**: 101-108.

Hotson, I.K., Campbell, N.J. and Smeal, M.G., 1970. Anthelmintic resistance in *Trichostrongylus colubriformis*. Australian Veterinary Journal **46**: 356-360.

Hughes, P.L. and Seifert, D.A., 1983. Field comment on anthelmintic resistance of sheep nematodes. New Zealand Veterinary Journal **31**: 183-184.

Jeannin, P.C., Bairden, K., Gettinby, G., Murray, M. and Urquhart, G.M., 1990. Efficacy of nitroxynil against ivermectin, benzimidazole and salicylanilide resistant *H. contortus*. Veterinary Record **126**: 624-625.

Jansen, P.A.J., 1976. The levamisole story. Progress in Drug Research **20**: 348-383.

Johnstone, I.L., Bowen, F.L. and Smart, K.E., 1976. Production in Merino weaners following anthelmintic therapy. New South Wales Veterinary Proceedings **12**: 31-34.

Johnstone, I.L, Darvill, F.M., Bowen, F.L, Butler, R.W., Smart, K.E. and Pearson, I.G., 1979. The effect of four schemes of parasite control on production in Merino wether weaners in two environments. Australian Journal of Experimental Agriculture and Animal Husbandry **19**: 303-311.

Jones, R.M., 1981. A field study of the morantel sustained release bolus in the seasonal control of parasitic gastroenteritis in grazing calves. Veterinary Parasitology **8**: 237-251.

Jones, R.M., 1983. Therapeutic and prophylatic efficacy of morantel when administered directly into the rumen of cattle on a continuous basis. Veterinary Parasitology **12**: 223-232.

Jones, R.M., Pott, J.M. and Cornwell, R.L., 1978. Low level feed administration of morantel tartrate in the prophylaxis of experimental nematode infections in lambs and calves. British Veterinary Journal **134**: 166-170.

Jordi, R., 1980. Untersuchungen zur anthelminthika-resistenz von Trichostrongyliden des schafes. Schweizer Archiv Fur Tierheilkunde **122**: 679-694.

Kates, K.C. Colglazier, M.L. and Enzie, F.D., 1973. Experimental development of a cambendazole-resistant strain of *Haemonchus contortus*. Journal of Parasitology **59**: 777-778.

Keith, R.C., 1953. The differentiation of the infective larvae of some common nematode parasites of cattle. Australian Journal of Zoology **I**, 223-235.

Kelly, K.C. and Hall, C.A., 1979. Resistance of animal helminths to anthelmintics. Advances in Pharmacology and Chemotherapy **16**: 89-128.

Kelly, J.D., Sangster, N.C., Porter, C.J., Martin, I.C.A. and Gunawan, M., 1981. Use of guinea pigs to assay anthelmintic resistance in ovine isolates of *Trichostrongylus colubriformis*. Research in Veterinary Science **30**: 131-137.

Kelly, J.D., Whitlock, H.V., Thompson, H.G., Hall, C.A., Martin, I.C.A. and Le Jambre, L.F., 1978. Physiological characteristics of free-living and parasitic stages of strains of *Haemonchus contortus*, susceptible or resistant to benzimidazole anthelmintics. Research in Veterinary Science **25**: 376-385.

Kemp, G.K. and Smith, C.F., 1982. Anthelmintic resistance survey in New Zealand. New Zealand Veterinary Journal **30**: 141-144.

Kettle, P.R., Vlassoff, A., Lukies, J.M., Ayling, J.M. and McMurtry, L.W., 1981. A survey of nematode control measures used by sheep farmers and of anthelmintic resistance on their farms. Part I. North Island and the Nelson region of South Island. New Zealand

Veterinary Journal **29**: 81-83.

Kettle, P.R., Vlassoff, A., Ayling, J.M., McMurtry, L.W., Smith, S.J., Watson,A.J., 1982. A survey of nematode control measures used by sheep farmers and of anthelmintic resistance on their farms. Part 2. South Island excluding the Nelson Region. New Zealand Veterinary Journal **30**: 79-81.

Klei, T.R. and Torbert, B.J., 1980. Efficacy of ivermectin (22,23-dihydroavermectin B1) against gastrointestinal parasites in ponies. American Journal of Veterinary Research **41**: 1747-1750.

Knowles, C.O. and Casida, J.E., 1966. Mode of action of organophosphate anthelmintics. Cholinesterase inhibition in *Ascaris lumbricoides*. Journal of Agricultural and Food Chemistry **14**: 566-572.

Kohler, P. and Bachmann, R.B., 1981. Intestinal tubulin as possible target for the chemotherapeutic action of mebendazole in parasitic nematodes. Molecular and Biochemical Parasitology **4**: 325-336.

Kreitman, M., 1983. Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster*. Nature (London) **304**: 412-417.

Laby, R.H., 1978. Australian Patent Application No. 35908/78.

Lacey, E., 1985. Synthesis of specifically-labelled tritiated benzimidazole carbamates. Journal of Labelled Compounds and Radiopharmaceuticals **22**: 893-902.

Lacey, E., Brady, R.L., Prichard, R.K. and Watson, T.R., 1987. Comparison of inhibition of polymerisation of mammalian tubulin and helminth ovicidal activity by benzimidazole carbamates. Veterinary Parasitology **23**: 105-119.

Lacey, E. And Prichard, R.K., 1986. Interactions of benzimidazoles (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of *Haemonchus contortus*. Molecular and Biochemical Parasitology **19**: 171-181.

Lacey, E. and Snowdon, K.L., 1988. A routine diagnostic assay for the detection of benzimidazole resistance in parasitic nematodes using tritiated benzimidazole carbamates. Veterinary Parasitology. **27**: 309-324.

Lee, R.M. and Hodsden, M.R., 1963. Cholinesterase activity in *Haemonchus contortus* and its inhibition by organophosphorus anthelmintics. Biochemical Pharmacology **12**: 1241-1252.

Le Jambre, L.F., 1976. Egg hatch as an *in vitro* assay of thiabendazole resistance in nematodes. Veterinary Parasitology **2**: 385-391.

Le Jambre, L.F., 1978. Anthelmintic resistance in gastrointestinal nematodes of sheep. In: The Epidemiology and Control of Gastrointestinal Parasites of Sheep in Australia pp. 109-120. (A.D. Donald, W.H. Southcott and J.K. Dineen, Eds), CSIRO, Melbourne.

Le Jambre, L.F., 1979. Hibridization studies of *Haemonchus contortus* (Rudolphi, 1803) and *H. placei* (Place, 1893) (Nematoda: Trichostrongylidae). International Journal for Parasitology **9**: 455-463.

Le Jambre, L.F., 1981. Eradication of levamisole-resistant *Ostertagia*. Australian Veterinary Journal **57**: 99-100.

Le Jambre, L.F., 1985. Genetic aspects of anthelmintic resistance in nematodes. In: Resistance in Nematodes to Anthelmintic Drugs (N. Anderson and P.J. Waller, Eds), pp. 97-106. CSIRO, Melbourne.

Le Jambre, L.F., Royal, W.M. and Martin, P.J., 1979a. The inheritance of thiabendazole resistance in *Haemonchus contortus*. Parasitology **78**: 107-119.

Le Jambre, L.F., Martin, P.J. and Webb, R.F., 1979b. Thiabendazole resistance in field populations of *Haemonchus contortus*. Australian Veterinary Journal **55**: 163-166.

Le Jambre, L.F., Soutchott, W.H. and Dash, K.M., 1978. Effectiveness of broad spectrum anthelmintics against selected strains of *Trichostrongylus colubriformis*. Australian Veterinary Journal **54**: 570-574.

Love, S.C., 1985. Wormkill-strategic drenching programme for Northern Tablelands, NSW. In: Proceedings of 68th Annual Conference of the Association of Veterinary Inspectors of N.S.W. (G.W. Eggleston, Ed), pp. 136-143.

Maciel, S.J., 1984. Studies on the abomasal parasites of cattle in subtropical and temperate climates. PhD Thesis. University of Glasgow. 181p.

Maingi, N., Scott, M.E. and Prichard, R.K., 1990. Effect of selection pressure for thiabendazole resistance on fitness of *Haemonchus contortus* in sheep. Parasitology **100**: 327-335.

Malkin, M.F. and Comacho, R.M., 1972. The effect of thiabendazole on fumarate reductase from thiabendazole-sensitive and resistant *Haemonchus contortus*. Journal of Parasitology **58**: 845-846.

Marriner, S.E. and Armour, J., 1986. Nematode infections of domestic animals - gastrointestinal infections. In: Chemotherapy of Parasitic Diseases (C. William, C. Campbell and R.S. Rew, Eds), pp. 287-305.

Marriner, S.E. and Bogan, J.A., 1980. Pharmacokinetics of albendazole in sheep. American Journal of Veterinary Research **41**: 1126-1129.

Marriner, S.E. and Bogan, J.A., 1981a. Pharmacokinetics of fenbendazole in sheep. American Journal of Veterinary Research **42**: 1146-1148.

Marriner, S.E. and Bogan, J.A., 1981b. Pharmacokinetics of oxfendazole in sheep. American Journal or Veterinary Research **42**: 1143-1145.

McBeath D.G., Preston, N.K. and Thompson, F., 1979. Studies in sheep on the efficacy of fenbendazole administered via a feed-block carrier. British Veterinary Journal **185**: 271-278.

McCallister, G.L., 1976. Effect of haloxon and thiabendazole on the free-living stages of *Haemonchus contortus*. Proceedings of the Helminthological Society of Washington **43**: 89-90.

McDougall, J., Cameron, B.D. and Lichtenwainer, D., 1985. The metabolism and pharmacokinetics of a novel anthelmintic (Sch 32481). Communication to the 3rd Conference of the European Association for Veterinary Pharmacology and Toxicology, Ghent.

McKeand, J., Bairden, K. and Ibarra-Silva, A.-M., 1988. The degradation of bovine faecal pats containing ivermectin. Veterinary Record **122**: 587-588.

McKellar, Q.A. and Scott, E.W., 1990. The benzimidazole anthelmintic agents - a review. Journal of Veterinary Pharmacology and Therapeutics **13**: 223-247.

McKenna, P.B., 1984. Gastro-intestinal parasitism and anthelmintic resistance in goats. Surveillance **11**: 2-4.

McKenzie, J.A., 1985. The genetics of resistance to chemotherapeutic agents. In: Resistance in Nematodes to Anthelmintic drugs (N. Anderson and P.J. Waller, Eds), pp. 89-95. CSIRO, Melbourne.

McKenzie, J.A. and Purvis, A., 1984. Chromosomal localisation of fitness modifiers of diazinon resistance genotypes of *Lucilia cuprina*. Heredity **53**: 625-634.

McKenzie, J.A., Whitten, M.J. and Adena, M.A., 1982. The effect of genetic background on the fitness of diazinon resistance genotypes of the Australian sheep blowfly *Lucilia cuprina*. Heredity **49**: 1-9.

Martin, P.J., 1986. Development and control of resistance to anthelmintics. In: Parasitology *Quo vadit?* 6th ICOPA (M.J. Howell, Ed). pp. 493-501. Australian Academy of Science, Camberra, Australia.

Martin, P.J., 1989. Selection for thiabendazole resistance in *Ostertagia* spp. by low efficiency anthelmintic treatment. International Journal of Parasitology **19**: 317-325.

Martin, P.J., Anderson, N. and Jarret, R.G., 1985. Resistance to benzimidazole anthelmintics in field strains of *Ostertagia* and *Nematodirus* in sheep. Australian Veterinary Journal **62**: 38-43.

Martin, P.J., Anderson, N. Jarret, R.G., Brown, T.H. and Ford, G.E., 1982. Effects of a preventive and supressive control scheme on the development of thiabendazole-resistance in *Ostertagia* spp. Australian Veterinary Journal **58**: 185-190.

Martin, P.J. and Le Jambre, L.F., 1979. Larval paralysis as an *in vitro* assay of levamisole and morantel tartrate resistance in *Ostertagia*. Veterinary Science Communications **3**: 159-164.

Martin, P.J., Le Jambre, L.F. and Claxton, J.H., 1981. The impact of *refugia* on the development of thiabendazole resistance in *Haemonchus contortus*. International Journal for Parasitology **11**: 35-41.

Martin, P.J., Anderson, N., Brown, T.H. and Miller, D.W., 1988a. Changes in resistance of *Ostertagia* spp. to thiabendazole following natural selection or treatment with levamisole. International Journal for Parasitology **18**: 333-340.

Martin, P.J., Anderson, N. and Jarrett, R.G., 1990. Detecting benzimidazole resistance with faecal egg count reduction tests and *in vitro* assays. Australian Veterinary Journal **66**: 236-240.

Martin, P.J., Anderson, N., Lwin, T., Nelson, G. and Morgan, T.E., 1984. The association between frequency of TBZ treatment and the development of resistance in field isolates of *Ostertagia* spp. of sheep. International Journal for Parasitology **14**: 177-181.

Martin, P.J., McKenzie, J.A. and Stone, R.A., 1988b. The inheritance of thiabendazole resistance in *Trichostrongylus colubriformis*. International Journal for Parasitology **18**: 703-709.

Michel, J.F., 1969. The epidemiology and control of some nematode infections of grazing animals. Advances in Parasitology **7**: 211-283.

Michel, J.F., Cawthorne, R.J.C., Anderson, R.M., Armour, J., Clarkson, M.J. and Thomas, R.J., 1983. Resistance to anthelmintics in Britain: husbandry practices and selective pressure. In: Facts and Reflections IV. Resistance of Parasites to Anthelmintics (F.H.M. Borgsteede, Sv. Aa. Henriksen and H.J. Over, Eds.), pp 41-59. Central Institute, Lelystad, The Netherlands.

Middleberg, A. and McKenna, P.B., 1983. Oxfendazole resistance in *Nematodirus spathiger*. New Zealand Veterinary Journal **31**: 65-66.

Ministry of Agriculture, Fisheries and Food Technical Bulletin No. 18., 1971. Manual of Veterinary Parasitological Techniques.

Mitchell, G.B.B., and Fitzsimons, J., 1983. Control of ovine gastrointestinal helminthiasis by the use of clean grazing and strategic dosing in the field. Research in Veterinary Science, **35**: 100-105.

Mitchell, G.B.B., Jackson, F. and Coop, R.L., 1991. Anthelmintic resistance in Scotland. Veterinary Record **129**: 58.

Moskey, H.E. and Harwood, P.D., 1941. Methods of evaluating the efficacy of anthelmintics. American Journal of Veterinary Research **2**: 55-59.

Nadler, S.A., 1990. Molecular approaches to studying helminth population genetics and phylogeny. International Journal for Parasitology **20**: 11-29.

Newman, R.L., 1984. Worm control in sheep on the Northern Tablelands of New South Wales: a survey of farmers opinions. Department of Agricultural Economics and Business Management, University of New England, Armidale, NSW, Australia.

Ngomuo, A.J., Marriner, S.E. and Bogan, J.A., 1984. The pharmacokinetics of fenbendazole and oxfendazole in cattle. Veterinary Research Communications **8**: 187-193.

Nielsen, P., Hansen, J.W. and Nansen, P., 1983. Comparison of three routes of administration of a water soluble anthelmintic: levamisole. Research in Veterinary Science **35**: 122-123.

Nolan, J., Schnitzerling, H.J. and Bird, P., 1981. Evaluation of the potential of systemic slow release chemical treatments for control of the cattle tick (*Boophilus microplus*) using ivermectin. Australian Veterinary Journal **57**: 493-497.

Nomura, T. 1984. Choice of character. KLAS Manual for genetic monitoring of inbred mice. University Press.

Oppenoorth, R.J., 1983. Insect resistance to insecticides as compared to that of parasites to anthelmintics. In: Facts and Reflections IV. Resistance of Parasites to Anthelmintics (F.H.M. Borgsteede, Sv. Aa. Henriksen and H.J. Over, Eds). pp. 31-38.Central Veterinary Institute, Lelystad, The Netherlands.

Paton, G., Thomas, R.J. and Waller, P.J. 1984. A prediction model for parasitic gastro-enteritis in lambs. International Journal for Parasitology **14**: 439-445.

Pinheiro, A.C., Echevarria, F.A.M. and Alves-Branco, F.P., 1987. Epidemiologia da helmintose ovina em Bagé (Rio Grande do Sul - Brasil). In: Coletânea das Pesquisas: Medicina Veterinária/ Parasitologia (EMBRABA/CNPO) **5**: 263-267.

Powers, K.G., Wood, I.B. Eckert, J., Gibson, T. and Smith, H.J., 1982. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) - Guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine and ovine). Veterinary Parasitology **10**: 265-284.

President, P.J.A., 1985. Methods for detection of resistance to anthelmintics. In: Resistance in Nematodes to Anthelmintic Drugs (N. Anderson, and P.J. Waller, Eds), CSIRO, Melbourne.

Prichard, R.K., 1970. Mode of action of the anthelmintic thiabendazole in *Haemonchus contortus*. Nature **228**: 684-685.

Prichard, R.K., 1978a. Sheep anthelmintics. In: The Epidemiology and Control of Gastrointestinal Parasites of Sheep in Australia (A.D. Donald, W.H. Southcott and J.K. Dineen, Eds) pp. 75-107, CSIRO, Melbourne.

Prichard, R.K., 1978b. The metabolic profile of adult *Fasciola hepatica* obtained from rafoxanide-treated sheep. Parasitology **76**: 277-288.

Prichard, R.K., 1990. Anthelmintic resistance in nematodes: extend, recent understanding and future directions for control and research. International Journal for Parasitology **20**: 515-523.

Prichard, R.K., Hall, C.A., Kelly, J.D., Martin, I.C.A. and Donald, A.D., 1980. The problem of anthelmintic resistance in nematodes. Australian Veterinary Journal **56**: 239-251.

Prichard, R.K., Hennessy, D.R. and Steel, J.W., 1978. Prolonged administration: A new concept for increasing the spectrum and effectiveness of anthelmintics. Veterinary Parasitology **4**: 309-315.

Pulliam, J.D., Seward, R.L., Henry, R.T. and Steiberg, S.A., 1985. Investigating ivermectin toxicity in collies. Veterinary Medicine **80**: 33-40.

Reinecke, R.K., 1973. The larval anthelmintic test in ruminants. In: Technical Communication 106, Department of Agricultural Technical Services, Pretoria, 20 pp.

Reinecke, R.K., Snijders, a.J. and Horak, I.G., 1962. A modification of standard procedures for evaluating the relative efficacy of anthelmintics. Onderstepoort Journal of Veterinary Research **29**: 241-257.

Riffkin, G.G., Callinan, A.P.L, Freemantle, A.M., Westcott, J.M., Napthine, D.V. and O'Connor, A.J., 1984. Anthelmintic resistance and sheep management practices in south western Victoria. Australian Veterinary Journal **61**: 248-251.

Rolfe, P.F. and Boray, J.C., 1990. Resistance of *Haemonchus contortus* in sheep to closantel. In: 7th ICOPA, Paris, 20-24 August, pg. 1119.

Romanowski, R.D., Rhoads, J.L., Colglazier, M.L. and Sangster, N.C., Prichard, R.K. and Lacey, E., 1975. Tubulin and benzimidazole-resistance in *Trichostrongylus colubriformis*. Journal of Parasitology **71**: 645-651.

Rose, J.H., 1964. Relationship between environment and the development and migration of the free living-stages of *Haemonchus contortus*. Journal of Comparative Pathology **74**: 163-172.

Roos, M.H. Boersema, J.H., Borgsteede, F.H.M., Cornelissen, J., Taylor, M. and Ruitenberg, E.J., 1990. Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. Molecular and Biochemical Parasitology **43**: 77-78.

Rutter, W., 1975. Sheep from grass. Bulletin no. 13. The East of Scotland College of Agriculture, June 1975. 41 pgs.

Sangster, N.C., Prichard, R.K. and Lacey, E., 1985. Tubulin and benzimidazole-resistance in *Trichostrongylus colubriformis* (Nematoda). Journal of Parasitology 71: 645-651.

Sangster, N.C., Whitlock, H.V., Kelly, J.D., Gunawan, M. and Hall, C.A., 1979. The effect of single and divided dose administration on the efficacy of fenbendazole against adult stages of benzimidazole resistant sheep trichostrongylids. Research in Veterinary Science 26: 85-89.

Santiago, M.A.M., Benevenga, S.F., Costa, U.C., 1976. Epidemiologia e controle da helmintose ovina no municipio de Itaqui, Rio Grande do Sul. Pesquisa Agropecuária Brasileira 11: 1-7.

Santiago, M.A.M. and Costa, U.C., 1979. Resistência de *Haemonchus contortus*, *Trichostrongylus colubriformis* e *Ostertagia* spp. ao levamisole. Revista do Centro de Ciências Rurais 9: 315-318.

Santiago, M.A.M, Benevenga, S.F., Costa, U.C., Santiago, C., Pignataro, I., Santos, M.N. and Tavares, A., 1971. Ação antihelmíntica do levo-tetramisole. I. Ovinos. Revista de Medicina Veterinária 7: 117-130.

Santiago, M.A.M., Costa, U.C. and Benevenga, S.F., 1977. *Trichostrongylus colubriformis* resistente ao levamisole. Revista do Centro de Ciências Rurais 7: 421-422.

Santiago, M.A.M., Costa, U.C, and Benevenga, S.F., 1978. Atividade anti-helmíntica do dl-tetramisole e do thiabendazole em uma estirpe de *Trichostrongylus colubriformis* resistente ao levamisole. Revista do Centro de Ciências Rurais 8: 257-261.

Santiago, M.A.M, Costa, U.C. and Benevenga, S.F., 1979. *Haemonchus contortus* e *Ostertagia circumcincta* resistente ao levamisole. Revista do Centro de Ciências Rurais **9**: 101-102.

Scott, E.W. and Armour, J., 1991. The effect of development of resistance to benzimidazoles, salicylanilides and ivermectin on the pathogenicity and survival of *Haemonchus contortus*. Veterinary Record **128**: 346-349.

Sinclair, K.B. and Prichard, R.K., 1975. The use of disophenol in studies of the pathogenicity of the arrested fourth-stage larvae of *Haemonchus contortus* in the sheep. Research in Veterinary Science **19**: 232-234.

Smith William., I., 1968. Chromatographic and electrophoretic techniques. Heinemann.

Soulsby, E.J.L., 1982. Helminths, Arthropods and Protozoa of Domestic Animals. 7th Edition, Bailliere Tindall, London, 809 pp.

Sutherland, I.A., Lee, D.L. and Lewis, D., 1988. Detection of benzimidazole resistance in Trichostrongylid nematodes. Parasitology Today **4**: 22-24.

Swan, G.E., Schroder, J., Carmichael, I.H., Louw, J.P., Harvey, R.G. and Penderis, I., 1984. Efficacy of ivermectin against internal parasites of sheep. Journal of the South African Veterinary Association **55**: 165-169.

Tait, A., Barry, J.D., Wink, R., Sanderson, A. and Crowe, J.S., 1985. Enzyme variation in *Trypanosoma brucei* subspecies. II. Evidence for *T. brucei rhodesiense* being a set of variants of *T.b. brucei*. Parasitology **90**: 89-100.

Taylor, S.M., Mallon, T.R. and Kenny, J., 1985. Comparison of early season suppressive anthelmintic prophylatic methods for parasitic gastroenteritis and bronchitis in calves. Veterinary Record 117: 521-524.

Taylor, M.A. and Hunt, K.R., 1989. Anthelmintic drug resistance in the UK. Veterinary Record 125: 143-147.

Thomas, R.J., 1978. The efficacy of in-feed medication with fenbendazole against gastrointestinal nematodes of sheep, with particular reference to inhibited larvae. Veterinary Record 102: 394-524.

Tocco, D.J., Buhs, R.P., Brown, H.D., Matzuk, A.R. Mertel, H.E., Harman, R.E. and Trenner, N.R., 1964. The metabolic rate of thiabendazole in sheep. Journal of Medicinal Chemistry 7: 399-405.

Turton, J.A. and Clark, C.J., 1974. The effect of natural worm loss on the estimate of anthelmintic activity in an anthelmintic test with *Haemonchus contortus*. Parasitology 69: 191-196.

Van de Bossche, H., 1976. The molecular basis of anthelmintic action. In: Biochemistry of Parasites and Host-parasite Relationships (H. Van den Bossche, Ed), p. 553-572, North Holland, Amsterdan.

Van Neuten, J.M., 1972. Pharmacological aspects of tetramisole. In: Comparative Biochemistry of Parasites (Van Den Bossche, Ed) pp. 101-115. Academic Press, New York and London.

Van Wyk, J.A. and Gerber, H.M., 1980a. A field strain of *Haemonchus contortus* showing slight resistance to rafoxanide. Onderstepoort Journal of Veterinary Journal 47:137-142.

Van Wyk, J.A. and Gerber, H.M., 1980b. Survival and development of larvae of the common nematodes of ruminants after long-term cryopreservation and investigation of different routes of infestation. Onderstepoort Journal of Veterinary Research **47**: 129-136.

Van Wyk, J.A., Gerber, H.M. and Alves, M.R.R., 1982. Slight resistance to the residual effect of closantel in a field strain of *Haemonchus contortus* which showed an increased resistance after one selection in the laboratory. Onderstepoort Journal of Veterinary Research **49**: 257-262.

Van Wyk, J.A., Malan, F.S., Gerber, H.M. and Alves, M.R., 1987. Two field strains of *Haemonchus contortus* resistant to rafoxanide. Onderstepoort Journal of Veterinary Research **54**: 143-146.

Van Wyk, J.A. and Malan, F.S., 1988. Resistance of field strains of *Haemonchus contortus* to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. Veterinary Record **123**: 226-228.

Vlassoff, A. and Kettle, P.R., 1980. Benzimidazole resistance in *Haemonchus contortus*. New Zealand Veterinary Journal **28**: 23-24.

Wall, R. and Strong, L., 1987. Environmental consequences of treating cattle with the antiparasitic drug ivermectin. Nature (London) **327**: 418-421.

Waller, P.J., 1985. Resistance to anthelmintics and the implications for animal production. In: Resistance in Nematodes to Anthelmintics Drugs (N. Anderson and P.J. Waller, Eds), pp. 1-12. CSIRO, Melbourne.

Waller, P.J., 1986. Anthelmintic resistance in nematode parasites of sheep. Agricultural Zoology Reviews 1: 333-373.

Waller, P.J., 1987. Anthelmintic resistance and the future for roundworm control. Veterinary Parasitology **25**: 177-191.

Waller, P.J., Dobson, R.J. and Donald, A.D., 1983. Further studies on the effect of selection with levamisole on a benzimidazole resistant population of *Ostertagia* spp. of sheep. International Journal for Parasitology **13**: 463-468.

Waller, P.J., Dobson, R.J., Donald, A.D., Griffiths, D.A. and Smith, E.F., 1985. Selection studies on anthelmintic resistant and susceptible populations of *Trichostrongylus colubriformis*. International Journal for Parasitology **15**: 669-676.

Waller, P.J., Dobson, R.J., Obendorf, D.L. and GilLam, R.J., 1986. Resistance of *Trichostrongylus colubriformis* to levamisole and morantel: differences in relation to selection history. Veterinary Parasitology **21**: 255-263.

Waller, P.J., Donald, A.D., Dobson, R.J., Lacey, E., Hennessy, D.R., Allerton, G.R. and Prichard, R.K., 1989. Changes in anthelmintic resistance status of *Haemonchus contortus* exposed to different anthelmintic selection pressures in grazing sheep. International Journal for Parasitology **19**: 99-110.

Waller, P.J. and Lacey, E., 1986. The effect of Triflumuron (SIR8514) on the free-living stages of sheep nematodes. Veterinary Parasitology **21**: 119-126.

Watts, S.D.M., Rapson, E.B., Atkins, A.M. and Lee, D.L , 1982. Inhibition of acethylcholinesterase secretion from *Nippostrongylus brasiliensis* by benzimidazole anthelmintics. Biochemical Pharmacology **31**: 3035-3040.

Webb, R.F., McCully, C.H., Clarke, F.L, Greentree, P. and Honey, P., 1979. The incidence of thiabendazole resistance in field population of *Haemonchus contortus* on the Northern Tablelands of New South Wales. Australian Veterinary Journal **55**: 422-426.

Westcott, R.B. and LeaMaster, B.R., 1982. Efficacy of ivermectin against naturally acquired and experimentally induced nematode infections in sheep. American Journal of Veterinary Research **43**: 531-533.

Whitlock, H.V., Kelly, J.D., Porter, C.J., Griffin, D.L. and Martin, I.C.A., 1980a. *In vitro* field screening for anthelmintic resistance in strongyles of sheep and horses. Veterinary Parasitology **7**: 215-232.

Whitlock, H.V., Sangster, N.C., Gunawan, M., Porter, C.J. and Kelly, J.D., 1980b. *Trichostrongylus colubriformis* and *Ostertagia* spp. resistant to levamisole, morantel tartrate and thiabendazole: Isolation into pure strain and anthelmintic titration. Research in Veterinary Science **29**: 31-35.

APPENDICES

APPENDIX 3.1.1. Number of helminths found at necropsy seven days after treatment - susceptible strain.

Treatment	Ear tag	No. of helminths
Levamisole 7.5 mg/kg	G11	0
	G12	0
	G13	0
	G14	0
	G15	0
	G16	0
	G17	0
	G18	0
	G19	0
	G20	0
Ivermectin 0.2 mg/kg	R51	0
	R52	0
	R53	0
	R54	0
	R55	0
	R56	0
	R57	0
	R58	0
	R59	0
	R60	0
Albendazole 3.8 mg/kg	B51	60
	B52	90
	B53	20
	B54	0
	B55	0
	B56	20
	B57	0
	B58	0
	B59	20
	B60	20
Control	Y91	4100
	Y92	3200
	Y93	1210
	Y94	850
	Y95	2460
	Y96	2850
	Y97	2930
	Y98	1680
	Y99	960
	Y100	1590

APPENDIX 3.1.2. Number of helminths found at necropsy seven days after treatment - resistant strain.

Treatment	Ear tag	No. of helminths
Levamisole 7.5 mg/kg	G01	0
	G02	0
	G03	0
	G04	20
	G05	0
	G06	0
	G07	0
	G08	0
	G09	0
	G10	0
Ivermentin 0.2 mg/kg	R31	2480
	R32	720
	R33	3020
	R34	1440
	R35	970
	R36	1280
	R37	700
	R38	490
	R39	560
	R40	40
Albendazole 3.8 mg/kg	B41	1020
	B42	1870
	B43	1740
	B44	800
	B45	1310
	B46	360
	B47	1040
	B48	940
	B49	390
	B50	490
Control	Y81	1420
	Y82	1350
	Y83	1260
	Y84	1770
	Y85	1290
	Y86	310
	Y87	1510
	Y88	820
	Y89	310
	Y90	1640

APPENDIX 3.1.3. Results of the faecal worm egg counts conducted on days 0, 1, 3, 6 & 7 post-treatment.

			Days post-treatment				
TRAT	STRAIN	EAR TAG	0	1	3	6	7
L	S	G11	81200	8200	0	0	0
E	S	G12	33400	13800	100	0	0
V	S	G13	27900	17600	0	0	0
A	S	G14	23700	10200	100	0	0
M	S	G15	31100	9000	0	0	0
I	S	G16	20900	15500	0	0	0
S	S	G17	16000	5000	0	0	0
O	S	G18	15400	12700	0	0	0
L	S	G19	13600	6400	0	0	0
E	S	G20	11100	3400	0	0	0
I	S	R51	72200	13600	200	0	0
V	S	R52	34200	3000	0	0	0
E	S	R53	22100	8800	0	0	0
R	S	R54	25200	25300	300	0	0
M	S	R55	21700	10300	0	0	0
E	S	R56	19700	11000	700	0	0
C	S	R57	17400	13200	0	0	0
T	S	R58	16000	6100	0	0	0
I	S	R59	12800	5100	0	0	0
N	S	R60	6300	800	0	0	0
A	S	B51	76400	87200	900	*	*
L	S	B52	34000	9900	0	0	400
B	S	B53	33400	16500	100	0	0
E	S	B54	23600	8000	0	0	0
N	S	B55	22000	11600	0	0	0
D	S	B56	19900	9900	0	0	0
A	S	B57	19200	28900	0	0	0
Z	S	B58	14800	11800	0	0	0
O	S	B59	13100	14500	0	0	0
L	S	B60	2000	1400	0	0	0
C	S	Y91	38100	46300	23200	6900	24400
O	S	Y92	36200	50700	36500	9400	36900
N	S	Y93	29200	46400	12200	28500	48200
T	S	Y94	23900	19800	10500	8400	24700
R	S	Y95	23500	20900	10900	28700	44600
O	S	Y96	19400	39000	3900	12100	29300
L	S	Y97	16700	14300	15100	6000	20000
	S	Y98	15600	27200	15200	11400	26000
	S	Y99	14200	10900	2700	400	25300
	S	Y100	8900	14400	16500	6900	68200

cont... APPENDIX 3.1.3. Results of the faecal worm egg counts conducted on days 0, 1, 3, 6 & 7 post-treatment.

			Days post-treatment				
TRAT	STRAIN	EAR TAG	0	1	3	6	7
L	R	G01	39000	14600	100	0	0
E	R	G02	16600	6900	100	0	0
V	R	G03	17700	2300	200	0	0
A	R	G04	16600	5900	100	0	0
M	R	G05	13800	1000	0	0	0
I	R	G06	12700	7200	0	0	0
S	R	G07	9600	2200	100	0	0
O	R	G08	6200	2300	300	100	0
L	R	G09	4800	6400	400	0	0
E	R	G10	1900	900	0	0	0
I	R	R31	27700	8200	11400	8100	36400
V	R	R32	23900	27000	19300	20600	56900
E	R	R33	18600	7100	4700	5300	9600
R	R	R34	16900	11800	5300	5300	10800
M	R	R35	15700	29800	14400	5100	6600
E	R	R36	10300	11700	10500	1900	13400
C	R	R37	9700	9200	2900	3500	7300
T	R	R38	6900	2000	100	400	0
I	R	R39	5800	4800	5100	5100	7700
N	R	R40	3600	1800	300	0	0
A	R	B41	30700	11300	5100	900	1000
L	R	B42	23600	23000	700	5600	2500
B	R	B43	18900	4700	5400	900	800
E	R	B44	17300	3200	2500	200	600
D	R	B45	14600	6700	2600	1400	1800
A	R	B46	11600	1900	500	500	600
Z	R	B47	10000	5400	2400	2800	1700
O	R	B48	7900	1400	1800	500	1500
L	R	B49	6200	2700	2600	200	200
E	R	B50	3300	1300	600	0	100
C	R	Y81	31200	19600	19700	20700	*
O	R	Y82	22500	11000	8800	6500	11900
N	R	Y83	19400	20100	18700	5400	32500
T	R	Y84	16500	10500	12700	9100	34000
R	R	Y85	14100	6700	5800	6300	27400
O	R	Y86	12400	7000	3900	6500	8500
L	R	Y87	10000	7400	16600	9400	15600
	R	Y88	8400	16100	8800	17600	23500
	R	Y89	4800	5400	4600	2000	11000
	R	Y90	2100	500	1600	2900	17200

* No sample for e.p.g.

APPENDIX 3.1.4. Faecal egg count of sheep infected with an ivermectin resistant strain of *H. contortus*

	Days after infection												
An.	17	18	19	20	21	22	24	27	28	29	31	34	35
1	0	0	2900	6500	6200	9300	10500	9800	17300	19600	19700	20700	.
2	0	0	0	200	0	300	900	1600	1900	11000	8800	6500	11900
3	400	2100	3200	27800	16700	28100	26200	26200	39000	20100	18700	5400	32500
4	100	600	1300	11500	3400	9000	14000	10500	8400	10500	12700	9100	34000
5	0	400	4000	25700	13100	12500	25900	8500	23600	6700	5800	6300	27400
6	0	200	1800	4000	3400	21400	4600	7000	12400	7000	3900	6500	8900
7	0	400	2100	7800	2700	6200	7400	5500	6900	7400	16600	9400	15600
8	0	300	4600	7300	9700	9500	12000	16900	12700	16100	8800	17600	23500
9	0	200	4900	18000	12800	15200	14400	19600	19600	5400	4600	2000	11000
10	0	700	3600	15000	10600	11000	10100	12200	10000	500	1600	2900	17200
11	200	400	2300	5900	9000	2500	13300	12500	17700				
12	100	100	100	1200	700	7900	5200	10900	10000	M=10430	10120	8640	20178
13	100	500	1000	5800	6500	7200	14800	18800	14100				
14	0	500	800	3800	2900	10400	7400	11700	9600				
15	200	1200	3000	12000	8600	10000	14200	18200	30700				
16	0	300	4100	12200	4500	8100	20300	20700	31200				
17	200	0	1000	3000	3500	15600	6000	8900	6200				
18	0	300	5000	11400	13900	16800	19700	18300	22500				
19	200	200	4600	9800	15100	1000	18500	27700	14600				
20	0	0	700	2400	600	4400	400	1700	2100				
81	0	200	100	5200	3900	8900	13600	12700	16900				
82	300	1200	2200	3300	5200	22000	6300	7800	5800				
83	200	1000	13000	2500	12900	16500	14900	37200	13800				
84	0	200	6000	8700	13200	19700	16900	27200	18900				
85	0	200	8500	13100	9900	14500	27300	25400	16600				
86	0	200	1800	3300	10500	18500	17800	21600	18600				
87	200	100	5500	10500	6900	10600	17600	13200	16500				
88	200	400	2800	7000	7000	17500	11900	11800	6200				
89	100	200	2300	5300	5500	12200	27700	22200	19400				
90	0	0	3300	8900	3300	3200	10700	18900	4800				
91	0	200	400	2400	2100	3300	3800	5500	3600				
92	0	200	300	1800	900	9500	6200	6000	4800				
93	0	800	100	1900	1200	15000	7700	12500	10300				
94	100	600	1000	7300	2800	26600	13000	28600	15700				
95	400	400	5300	8600	9600	7900	20900	42500	27700				
96	0	400	3200	600	5600	4300	1000	45400	23900				
97	0	200	900	8700	3800	14400	14100	7300	11600				
98	0	0	300	6500	1200	12400	22700	2500	3300				
99	0	.	2500	12700	6200	5000	7500	7300	9700				
100	0	.	1300	5500	6400	1800	8900	4400	7900				
MEAN=	75	392	2795	7878	6550	11255	12808	15680	14163				

On day 16th after infection all animals had negative faecal egg counts.

APPENDIX 3.1.5. Faecal egg counts of sheep infected with an ivermectin susceptible strain of *H. contortus*

	Days after infection												
An.	17	18	19	20	21	22	24	27	28	29	31	34	35
21	0	0	200	300	300	4900	6900	14200	19700	46300	23200	6900	24400
22	0	100	1200	5400	6200	8300	12100	24000	25200	50700	36500	9400	36900
23	200	0	2300	5000	4800	2100	7000	15100	15400	46400	12200	28500	48200
24	0	100	200	100	1400	1800	2800	9900	23700	19800	10500	8400	24700
25	0	100	300	1100	1000	1000	2700	6400	11100	20900	10900	28700	44600
26	0	300	1300	2200	3000	10400	3600	2100	2000	39000	3900	12100	29300
27	0	0	2400	5300	1700	3000	2000	9700	16000	14300	15100	6000	20000
28	0	300	2400	2300	14400	10600	13600	9600	8900	27200	15200	11400	26000
29	0	300	1500	6000	2800	10000	10400	27700	13100	10900	2700	400	25300
30	100	400	3000	8300	4200	7800	20200	10300	23900	14400	16500	6900	68200
31	0	600	1800	4000	7800	10500	11300	20100	22100				
32	200	500	4900	5100	3200	12100	9600	21300	33400	M=28990	14670	11870	34760
33	200	0	3400	5200	2900	21700	5200	.	15600				
34	0	600	2000	3600	3200	4400	4200	15100	23500				
35	200	700	6600	5900	7600	4400	3600	13400	14200				
36	0	.	3700	4700	700	16200	33200	6500	27900				
37	100	0	3000	1100	200	2900	2200	26400	16000				
38	0	800	0	0	12100	1700	400	7300	6300				
39	0	300	5900	14800	11700	24000	64500	12100	72100				
40	0	1100	3200	8600	9600	16200	38900	50700	76400				
81	200	300	8000	4700	3100	43300	15900	56700	36200				
82	100	0	1600	3300	3900	10900	13600	36100	13600				
83	0	0	1000	3200	2300	6000	11900	.	12800				
84	0	600	1900	1800	1600	6400	7600	13700	21700				
85	0	0	5200	2000	5100	1800	4900	11300	16700				
86	200	.	5100	7400	3600	8000	20500	.	17400				
87	200	400	200	2200	4900	9800	10700	11100	19200				
88	0	300	6400	4700	6000	23400	31100	.	29200				
89	0	200	7200	7300	8600	28800	25800	.	81200				
90	0	400	8000	2200	5700	11500	21300	13100	33400				
91	0	0	500	4200	2100	11700	31600	18800	31100				
92	300	100	600	900	1300	7200	9000	27000	20900				
93	0	200	1800	1600	1600	4900	5200	28900	22000				
94	.	200	500	2600	13300	5000	50300	42900	14800				
95	0	0	600	3700	6900	20600	7700	8400	23600				
96	0	100	1200	2600	4100	8000	8400	.	19400				
97	0	0	2800	900	2000	18300	14600	13700	34200				
98	0	100	2700	1500	1700	13100	15900	11900	19900				
99	0	0	1100	4500	11800	19200	14200	10600	34000				
100	0	100	4600	9600	5300	37900	24700	16200	38100				
MEAN=	51	242	2758	3998	4843	11735	14983	18303	25150				

On day 16th after infection all animals had negative egg counts.

APPENDIX 3.3.1. Body weight of lambs entering the experimental areas for "homogenizing grazing" during 14 days before allocation to treatments.

Zone 1		Zone 2	
Ear tag	Body weight	Ear tag	Body weight
61	28.5	33	22.5
63	28.0	58	22.5
38	27.5	65	22.5
58	26.5	69	22.5
68	26.5	95	22.5
98	25.5	87	22.5
66	25.5	46	22.0
84	25.5	20	22.0
94	25.5	47	22.0
88	25.5	64	22.0
97	25.5	62	22.0
90	25.0	89	22.0
23	24.5	54	21.5
56	24.5	42	21.5
32	24.5	39	21.5
67	24.0	85	21.5
60	24.0	91	21.5
57	23.5	96	21.0
82	23.5	27	21.0
22	23.5	29	21.0
49	23.0	34	21.0
31	23.0	100	21.0
45	23.0	53	20.5
59	23.0	21	20.5
99	23.0	25	20.0
92	23.0	43	20.0
86	23.0	35	20.0
28	22.5	36	20.0
48	22.5	81	20.0
37	22.5	57	19.5

APPENDIX 3.3.2. Worm egg counts (e.p.g.) of lambs treated at weaning and again 56 days later.

TREAT	REPLICATE	EAR TAG	DAYS 0	14	28	42	56	70	84	98	112
I	H1	B1	0	0	#	900	1800+200N	1600	2600	18100+400N	6800+100N
V	H1	B2	0	0	200	1800	1100	2800	3800	9000+300T	16200
E	H1	B3	0	0	200	700+300N	1000	800	6200	13300	9700
R	H1	B4	0	0	0	0	400	2000	3700	12400	13400+200N
M	H1	B5	0	0	100	300+200N	200	1100	2000	3800	58200+700N+100T
E											
C	L1	B6	0	0	200	100	700	200	1300	3400+200N	1800
T	L1	B7	0	0	100	100	200	100	2300	5900	6200+300N
I	L1	B8	0	0	0	100	200	100	1200	4800	4100+100N
N	L1	B9	0	0	0	200	300	700	900	5600	6800+200N
	L1	B10	0	0	0	100	0	300	800	2900+100N	4700+100N
C	H2	R231	0	0	0	0	300	600	1300	1900	4900
L	H2	R232	0	0	0+100N	100	300	0	700	1900+100N	4800
O	H2	R233	0	0	0	500	600+200N	100	600	1800+100N	7400
S	H2	R234	0	0	200	0	400	0	900	1400	7500
	H2	R235	0	0	0	100	600+200N	100	1200	7400+400N	3200
+											
	L2	R273	0	0	0	200	0	0	200	100	400
I	L2	R274	0	0	0	400	0	0	0	100	0
V	L2	R275	0	0	0	0	0	0	200	0	600
M	L2	R276	0	0	0	0	0	0	0	200	200
	L2	R277	0	0	0	0	0	0	100	100	200
D	H3	B37	0	0	0	100	400	100	400	700	1500
I	H3	B38	0	0	0	100+100N	100	0	200	2800	2500
S	H3	B39	0	0	100+100N	100	400	600	2400	11900	9000
O	H3	B40	0	0	0	400	200+200N	300	1300	3400	1800
	H3	B41	0	0	100	500+200N	100	0	1600	1500	3100
+											
	L3	B30	0	0	0	100	0	100	300	0	500
I	L3	B31	0	0	0	0	0	0	100	200	300
V	L3	B32	0	0	0	0	200	0	0	0	1000
M	L3	B33	0	0	0	200	0	100	100	800	400
	L3	B34	0	0	0	100+100N	0	0	100	600	1300
A	H4	G41	0	0	0	200+200N	0	0	600	1800+300N	5500+400N
L	H4	G42	0	0	0	100	0	0+100N	500	3900	5200
B	H4	G43	0	0	0	0+100N	100	0	400	1600	4500
E	H4	G44	0	0	0	300	600	200	300	2500	2800
N	H4	G45	0	0	0	0+200N	200	200	300	1400	3200
D											
A	L4	R241	0	0	0	0	100+100N	0	0	300	200
Z	L4	R242	0	0	0	400	200	0	200	700	600+200N
O	L4	R243	0	0	0	0	300	0	0	600	200
L	L4	R244	0	0	0	0	0	0	100	200	200
E	L4	R245	0	0	0	0	0	0	0	300	300
N	H5	R50	0	0	0	0	0	0	400	1500	2300
T	H5	R51	0	0	0	0	200	100	600	1300+100N	3300
X	H5	R52	0	0	0	600+200N	200	100	200	1600	2900
N	H5	R54	0	0	0	400	400	0	400	2600	1700
	H5	R56	0	0	0	0	200	0	600	1300	3800
+											
	L5	G54	0	0	0	100	400	0	0	200	300
I	L5	G55	0	0	100	0	200	0	400	400	700
V	L5	G57	0	0	200	0	0	0	0	400	300
M	L5	G58	0	0	0	0	100	0	0	300	1100
	L5	G59	0	0	0	200+100N	400	0	0	400	1000
	H6	G61	0	0	0	0	0	700	400	3500+600N	6000
	H6	G62	0	0	100N	0	0	200	200	2800	3000
C	H6	G63	0	100N	0	100	200	100	600	3300	1700+300N
O	H6	G64	0	0	200	200+100N	700	600	700	3700	4700+100N
N	H6	G66	0	0	200N	0	0	500+100N	800	3700	2600
T											
R	L6	Y61	0	0	0	0	300	500	900	300+200N	1100
O	L6	Y62	0	0	0	0	200	300	200	1000	600
L	L6	Y63	0	100	0	0	200	600+100N	400	300	1200
	L6	Y64	0	0	0	400	0	300	800	1200	2100
	L6	Y65	0	0	0	200+100N	100	0	300	500	600

No sample for e.p.g.; N Nematodirus spp.; T Trichuris spp.

APPENDIX 3.3.3. Packed cell volume (%) of lambs treated at weaning and again 56 days later.

TREAT	REPLICATE	EAR TAG	DAYS 0	14	28	42	56	70	84	98	112
I	H1	B1	35	37	28	32	32	34	29	26	23
V	H1	B2	30	32	29	30	29	29	27	21	22
E	H1	B3	20	34	32	34	31	31	25	15	24
R	H1	B4	31	32	33	30	28	29	28	25	24
M	H1	B5	31	31	28	28	28	26	26	23	21
E											
C	L1	B6	36	38	33	36	34	34	34	29	32
T	L1	B7	32	34	36	32	31	31	30	27	16
I	L1	B8	35	35	30	32	27	30	28	28	27
N	L1	B9	38	37	35	30	34	35	32	32	33
	L1	B10	33	35	29	35	32	28	23	29	29
C	H2	R231	38	32	32	33	30	30	30	30	31
L	H2	R232	34	35	31	32	31	30	30	28	25
O	H2	R233	37	35	28	31	28	29	28	28	21
S	H2	R234	34	27	30	29	28	28	29	26	27
	H2	R235	40	37	33	34	32	30	30	27	28
+											
	L2	R273	39	35	30	32	29	24	30	29	24
I	L2	R274	42	37	31	31	28	30	27	17	20
V	L2	R275	34	30	30	29	29	28	26	25	24
M	L2	R276	38	36	30	30	31	30	16	28	25
	L2	R277	31	37	29	32	26	28	28	30	23
D	H3	B37	36	32	28	27	26	29	25	26	27
I	H3	B38	40	35	29	32	29	29	28	28	28
S	H3	B39	39	36	28	32	29	29	27	25	28
O	H3	B40	34	30	27	28	28	28	27	25	29
	H3	B41	34	34	32	30	28	29	28	27	29
+											
	L3	B30	34	28	28	29	28	24	20	29	25
I	L3	B31	35	28	28	29	28	28	20	26	29
V	L3	B32	33	27	28	30	26	27	16	25	27
M	L3	B33	39	30	30	31	26	26	24	27	29
	L3	B34	37	35	29	30	27	26	25	28	28
A	H4	G41	34	42	34	34	32	33	33	29	33
L	H4	G42	30	36	38	33	35	33	32	32	32
B	H4	G43	35	32	33	32	28	30	29	28	29
E	H4	G44	30	31	31	29	27	27	27	25	24
N	H4	G45	33	32	27	27	24	25	24	28	33
D											
A	L4	R241	30	27	27	31	23	25	23	27	27
Z	L4	R242	37	31	29	30	27	31	29	25	28
O	L4	R243	32	28	28	31	28	29	26	26	28
L	L4	R244	37	32	32	29	28	33	32	25	28
E	L4	R245	31	35	30	33	27	34	30	30	20
N	H5	R50	37	32	38	35	32	33	32	32	24
T	H5	R51	38	32	39	31	34	31	29	32	28
X	H5	R52	32	29	32	30	30	29	27	24	26
N	H5	R54	34	33	29	27	26	25	25	29	27
	H5	R56	34	30	27	30	29	28	29	23	30
+											
	L5	G54	33	25	25	27	33	27	25	24	25
I	L5	G55	30	28	27	28	32	27	28	26	25
V	L5	G57	35	35	27	29	24	26	14	26	27
M	L5	G58	40	30	30	32	25	31	29	32	24
	L5	G59	32	26	29	30	29	30	18	24	27
	H6	G61	42	30	33	32	31	29	31	32	30
	H6	G62	38	27	29	29	30	18	28	30	31
C	H6	G63	40	35	37	37	36	35	33	35	33
O	H6	G64	40	39	32	29	28	29	28	28	23
N	H6	G66	39	33	33	34	35	35	34	24	27
T											
R	L6	Y61	31	27	29	29	28	30	23	28	17
O	L6	Y62	39	37	35	35	29	33	33	30	32
L	L6	Y63	35	30	30	31	29	30	30	29	23
	L6	Y64	30	27	28	28	30	30	28	26	27
	L6	Y65	34	34	30	31	29	29	30	29	17

APPENDIX 3.3.4. Live weight of lambs treated at weaning and again 56 days later.

TREAT	REPLICATE	EAR TAG	DAYS 0	14	28	42	56	70A	84	98	112A
I	H1	B1	22.0	24.0	24.0	26.0	28.5	34.5	31.5	28.5	30.0
V	H1	B2	23.0	24.0	27.0	27.0	30.0	34.5	31.5	31.5	31.0
E	H1	B3	22.5	22.0	25.0	24.0	26.0	30.0	27.0	27.0	28.5
R	H1	B4	22.0	23.0	25.0	25.5	26.0	31.5	28.0	24.0	25.0
M	H1	B5	20.5	22.0	22.5	23.0	25.5	30.0	26.0	25.0	22.5B
E											
C	L1	B6	22.0	24.0	25.5	25.5	28.5	28.5	29.0	28.5	30.0
T	L1	B7	20.5	24.0	24.0	24.0	26.0	27.0	27.0	27.0	28.5
I	L1	B8	20.0	23.0	24.0	24.0	26.0	27.0	25.5	26.0	29.0
N	L1	B9	20.0	23.0	23.0	25.0	27.0	28.0	28.0	28.5	30.0
	L1	B10	20.5	20.0	21.5	22.5	24.0	24.0	24.0	25.5	26.0
C	H2	R231	25.5	27.0	28.5	28.5	30.0	34.5	30.0	31.5	33.0
L	H2	R232	23.5	25.0	26.0	26.0	28.5	34.5	28.5	31.5	33.0
O	H2	R233	23.0	27.5	27.0	27.0	29.0	34.5	31.5	31.5	33.0
S	H2	R234	22.5	24.0	25.5	25.5	27.0	31.5	28.5B	28.5	29.0
	H2	R235	21.5	24.0	25.5	25.5	28.5	33.0	30.0	28.5	30.0
+											
	L2	R273	25.5	29.0	29.0	33.0	32.0	32.0	31.5	31.5	33.0
I	L2	R274	20.5	23.0	23.0	24.0	24.0	24.0	22.0	21.0	23.0
V	L2	R275	20.0	23.0	23.5	24.0	25.5	25.5	26.0	25.5	27.0
M	L2	R276	19.0	23.0	24.0	25.5	27.0	28.5	28.0	29.0	31.0
	L2	R277	17.5	19.0	21.0	22.5	24.0	25.5	26.0	25.5	27.0
D	H3	B37	26.0	28.0	28.5	28.0	30.0	31.5	31.5	32.0	33.0
I	H3	B38	22.5	27.0	24.0	25.0	27.0	28.5	28.5	28.5	30.0
S	H3	B39	24.5	27.0	25.5	25.5	27.0	28.5	28.0	26.0	28.5
O	H3	B40	22.0	24.0	24.0	24.0	26.0	28.5	28.5	26.0	29.0
	H3	B41	21.5	23.0	24.0	25.0	27.0	28.5	28.5	29.0	30.0
+											
	L3	B30	20.5	23.0	23.0	24.0	25.5	25.0	25.0	26.0	28.5
I	L3	B31	20.5	24.0	25.0	25.5	26.0	27.0	27.0	27.0	29.0
V	L3	B32	21.5	24.0	24.5	25.0	25.5	27.0	26.0	27.0	30.0
M	L3	B33	20.0	24.0	23.0	24.0	24.0	25.5	24.0	24.0	26.0
	L3	B34	19.5	23.0	22.0	22.5	24.0	24.0	25.0	25.0	27.0
A	H4	G41	27.5	32.0	34.0	30.0	32.0	33.0	30.0	33.0	34.5
L	H4	G42	24.5	27.0	28.0	27.0	30.0	30.0	31.5	32.0	33.0
B	H4	G43	24.0	26.0	26.0	27.0	30.0	31.0	31.5	32.0	34.0
E	H4	G44	21.5	21.0	22.0	22.5	25.5	27.0	27.0	27.0	28.5
N	H4	G45	21.5	23.0	23.0	23.0	25.5	25.5	27.0	27.0	27.0
D											
A	L4	R241	22.5	25.0	26.0	25.5	28.0	27.0	28.5	28.5	30.0
Z	L4	R242	22.0	23.0	23.0	24.0	26.0	27.0	28.0	28.5	30.0
O	L4	R243	19.5	21.0	22.0	21.0	22.5	24.0	22.5	22.5	24.0
L	L4	R244	20.5	23.0	23.0	22.5	25.0	25.5	25.0	25.0	27.0
E	L4	R245	19.5	24.0	22.5	24.0	27.0	28.0	28.5	28.5	31.0
N	H5	R50	25.5	29.0	29.0	29.0	33.0	33.0	34.5	34.5	36.0
T	H5	R51	24.0	27.0	27.0	27.0	30.0	31.0	31.0	30.0	31.0
X	H5	R52	23.0	27.0	26.0	27.0	28.5	29.0	30.0	30.0	31.5
N	H5	R54	22.0	23.0	24.0	25.5	27.0	28.5	28.5	29.0	30.0
	H5	R56	23.0	21.0	26.0	25.5	28.5	30.0	29.0	28.5	30.0
+											
	L5	G54	22.5	23.0	24.0	25.5	27.0	27.0	27.0	28.5	30.0
I	L5	G55	20.0	20.0	23.0	24.0	26.0	27.0	27.0	27.0	29.0
V	L5	G57	19.5	21.0	22.5	22.5	24.0	24.0	23.0	24.0	25.5
M	L5	G58	18.0	21.0	23.0	24.0	26.0	24.5	26.0	26.5	28.0
	L5	G59	20.5	22.5	23.0	24.0	26.0	25.5	25.5	25.5	27.0
	H6	G61	27.0	30.0	31.0	30.0	33.0	33.0	32.0	33.0	34.5
C	H6	G62	24.5	26.0	27.0	28.5	30.0	30.0	30.0	31.0	31.5
O	H6	G63	21.5	24.0	25.0	25.5	28.5	28.5	28.0	28.5	30.0
N	H6	G64	23.0	23.0	25.0	25.0	27.0	28.0	28.5	28.0	29.5
T	H6	G66	21.5	24.0	25.5	25.5	28.5	30.0	30.0	29.0	30.0
R											
O	L6	Y61	21.5	24.0	24.0	25.5	27.0	28.0	28.5	28.5	30.0
L	L6	Y62	22.5	24.0	22.5	25.0	26.0	27.0	28.0	28.5	30.0
	L6	Y63	21.0	23.0	22.5	24.0	25.0	25.5	25.5	25.5	28.0
	L6	Y64	21.0	23.0	22.5	24.0	26.0	26.0	26.0	26.5	27.0
	L6	Y65	18.5	20.0	21.0	21.0	22.5	23.0	23.0	23.0	25.0

A - wet live weight.
B - foot-miisis.

APPENDIX 3.3.5. Results of larval cultures obtained from animals drenched at weaning and 56 days later.

TREAT	REPLICATE	NO. OF LARVAE CLASSIFIED DAY								% of *Haemonchus* DAY							
		14	28	42	56	70	84	98	112	14	28	42	56	70	84	98	112
IVM	H	0	52	50	56	56	72	50	50	0	100	98a	100	100	100	100	100
	L	0	55	52	50	52	82	50	50	0	100	100	100	100	100	100	100
CLOS+ IVM	H	0	62	56	50	51	68	50	50	0	100	100	100	100	100	100	100
	L	0	32	51	57	13	64	50	50	0	100	100	100	100	100	100	100
DIS+ IVM	H	0	54	60	58	60	80	50	50	0	100	100	100	100	100	100	100
	L	0	32	57	53	16	32	50	50	0	100	100	100	81b	100	100	100
ABZ	H	0	58	62	58	54	62	50	50	0	100	100	100	100	100	100	100
	L	0	40	50	59	17	54	50	50	0	100	100	100	100	100	100	100
NTX+ IVM	H	0	56	50	54	58	76	50	50	0	100	100	100	100	100	100	100
	L	0	40	54	54	12	64	50	50	0	100	100	100	100	100	100	100
CON- TROL	H	13	52	57	52	70	78	50	50	100	100	100	100	100	100	100	100
	L	20	53	52	51	56	68	50	50	100	100	100	100	86c	100	96d	100

a - 2% *Ostertagia* spp. b - 19% *Ostertagia* spp. c - 14% *Strongyloides*. d - 4% *Strongyloides*

APPENDIX 3.3.6. Worm counts of permanent grazers treated at weaning and again 56 days later.

			NEMATODES								
TREAT	REPLICATE	E TAG	H. cont	E4L	L4L	Ost	T. axei	Coop	N. spathiger	T. ovis	E.P.G.
I	H1	B1	6620	130	0	0	70	30	5650+L4L 1140+E5L 440	6	6800+100N
V	H1	B2	6610	180	100	40	20	0	930+L4L 120	6	12200
E	H1	B3	5080	170	0	0	20	0	20	0	9700
R	H1	B4	4230	0	0	0	0	0	2520	2	13400+200N
M	H1	B5	2770	0	0	10	0	0	2250	0	58200+700N
E											
T	L1	B6	1410	0	0	0	0	0	840	0	1800
I	L1	B7	1930	0	0	0	0	0	1930	0	6200+300N
N	L1	B8	1670	0	0	0	0	0	620	2	4100+100
	L1	B9	2970	0	0	0	0	0	2740	0	6800+200N
	L1	B10	940	0	0	0	10	0	610	3	4700+100N
C	H2	R231	1940	30	0	10	60	30A	1630+L4L 340	0	4900
L	H2	R232	1930	0	0	0	0	0	5750+L4L 430	4	4800
O	H2	R233	2330	50	0	10	190	0	5660+L4L 800	0	7400
S	H2	R234	2780	0	0	0	30	20	0	0	7500
A	H2	R235	1930	0	0	0	50	0	340	0	3200
+											
I	L2	R273	290	0	0	0	40	0	640	0	400
V	L2	R274	100	0	0	0	40	0	170	0	0
M	L2	R275	250	0	0	0	40	0	420	3	600
	L2	R276	200	0	0	0	0	0	20	0	200
	L2	R277	160	0	0	0	80	0	450	0	200
D	H3	B37	2490	50	60	0	60	0	0	0	1500
I	H3	B38	1900	0	0	0	50	0	2290	4	2500
S	H3	B39	3380	0	0	0	20	0	2120	5	9000
O	H3	B40	2050	0	0	0	60	0	3620+L4L 160	0	1800
	H3	B41	4950	0	0	0	240	20	3160+L4L 130	0	3100
+											
	L3	B30	230	0	0	0	20	0	170	0	500
I	L3	B31	160	0	0	0	50	0	220	2	300
V	L3	B32	200	0	0	0	0	0	200	2	1000
M	L3	B33	160	0	0	0	0	0	90	1	400
	L3	B34	380	0	0	0	20	0	170	0	1300
A	H4	G41	1440	40	0	0	40	0	4800+L4L 210	0	5500+400N
L	H4	G42	1360	20	0	10	40	0	260	0	5200
B	H4	G43	1500	0	80	0	0	0	1500	4	4500
E	H4	G44	760	0	0	0	20	0	1150	0	2800
N	H4	G45	1180	0	0	0	0	0	1070	0	3200
D											
A	L4	R241	130	0	0	30	10	0	940	0	200
Z	L4	R242	370	0	0	30	100	0	1760	0	600+200N
O	L4	R243	100	0	0	0	20	0	1060	0	200
L	L4	R244	80	0	0	0	40	0	1720	0	200
E	L4	R245	130	0	0	0	0	0	1260	5	300
N	H5	R50	1120	0	0	0	0	0	420	0	2300
T	H5	R51	900	0	0	0	20	0	1070	0	3000
N	H5	R52	770	0	0	10	0	0	0	4	2900
X	H5	R54	1300	0	0	0	0	0	70	0	1700
	H5	R56	660	0	0	0	0	0	950	0	3800
+											
	L5	G54	350	0	0	0	0	0	1400	0	300
I	L5	G55	250	0	0	0	50	0	1280	0	700
V	L5	G57	270	0	0	0	40	20A	770	0	300
M	L5	G58	440	0	0	0	60	0	1320	0	1100
	L5	G59	170	0	0	0	70	0	60	0	1000
	H6	G61	1600	0	0	0	0	0	2930	6	6000
	H6	G62	1310	0	0	0	140	0	1840	8	3000
C	H6	G63	1810	0	0	0	50	0	2960	1	1740+300T
O	H6	G64	1840	0	0	20	80	0	2060	0	4700+100N
N	H6	G66	3010	0	0	0	30	0	0	8	2600
T											
R	L6	Y61	950	0	0	0	35	30A	1120	3	1100
O	L6	Y62	710	0	0	0	27	100B	1460	1	600
L	L6	Y63	780	0	0	10	50	0	1630	0	1200
	L6	Y64	980	0	0	0	190	0	360	8	2100
	L6	Y65	260	0	0	0	100	20	1340	2	600

H. cont = H. contortus E4L= early 4th stage; L4L= late 4th stage; Ost= Ostertagia spp
Coop= Cooperia spp; N. spat= N. spathiger ;
All males were classified and found to be: A= 100% C. punctata and B= 84% C. punctata + 16% C. onchophora.

APPENDIX 3.3.7. Worm counts from the abomasum of tracers

TREAT.	REPLICATE	EAR TAG	H. contortus adults	H. contortus L4L	Ostertagia adults	Ostertagia L4L	T. axei adults	T. axei L4L
I	H1	B96	6490	0	100	0	0	0
V	H1	G33	3530	0	50	20	80	100
E	H1	G29	2730	0	70	110	40	0
R	H1	G28	3010	0	0	0	0	20
M	H1	G23	25000	0	940	0	20	0
E								
C	L1	B88	700	0	0	0	0	0
T	L1	G05	1180	0	0	0	0	0
I	L1	G16	2120	0	130	20	0	0
N	L1	G22	4500	0	0	0	0	0
	L1	G12	1520	20	0	0	0	0
C	H2	B100	1520	0	40	0	0	0
L	H2	G32	2030	0	0	0	40	20
O	H2	B86	1400	0	140	0	0	0
S	H2	G19	1330	0	20	30	80	0
A	H2	G20	1390	0	0	0	20	0
+								
I	L2	B84	150	0	10	0	50	0
V	L2	B87	190	0	30	0	30	0
M	L2	G24	130	0	0	0	10	0
	L2	G13	90	0	20	0	10	0
	L2	B81	200	0	50	0	10	0
D	H3	G02	410	0	0	0	0	0
I	H3	B83	2170	0	30	0	10	40
S	H3	B91	1660	0	110	0	0	0
O	H3	G09	1040	0	20	0	0	0
	H3	G14	1020	0	100	0	0	0
+								
	L3	B95	50	0	20	0	0	0
I	L3	G17	0	0	0	0	0	0
V	L3	G07	100	0	20	0	20	30
M	L3	B92	150	0	0	0	0	0
	L3	G08	80	0	0	0	20	20
A	H4	G38	720	0	50	0	0	0
L	H4	G06	80	0	0	0	0	0
B	H4	G41	930	0	0	0	0	0
E	H4	G40	580	0	30	0	0	0
N	H4	B90	1090	0	0	0	0	0
D								
A	L4	B85	190	0	0	0	0	0
Z	L4	G21	70	0	0	0	0	0
O	L4	G27	120	0	0	0	0	0
L	L4	G25	260	0	0	0	0	0
E	L4	G11	150	0	0	0	0	0
N	H5	B94	610	0	0	0	20	0
T	H5	B89	250	0	30	0	20	0
N	H5	G39	480	0	0	0	0	0
X	H5	G10	460	0	0	0	0	0
	H5	G34	650	0	0	0	0	0
+								
	L5	G36	230	0	0	0	0	0
I	L5	G03	200	0	0	0	0	0
V	L5	B99	120	0	10	0	0	0
M	L5	G35	290	0	0	30	0	0
	L5	B97	360	0	0	40	20	0
	H6	G18	2870	590	0	0	30	0
	H6	G04	1250	0	20	0	10	0
C	H6	G37	260	0	0	0	0	0
O	H6	B98	3370	0	60	0	10	0
N	H6	G31	1990	0	50	0	0	0
T								
R	L6	G30	1190	0	40	20	0	0
O	L6	G26	1050	0	0	0	280	50
L	L6	G15	660	0	50	0	150	110
	L6	B82	1680	0	0	0	640	230
	L6	G01	1280	0	70	0	550	0

L4L= late 4th stage;

APPENDIX 3.3.8. Small intestinal worm counts and e.p.g. of tracers

TREAT.	REPLICATE	EAR TAG	T.coubriformis	Cooperia	N. spathiger	E.P.G.
I	H1	B96	0	0	40	2100+100N
V	H1	G33	0	0	0	13500
E	H1	G29	0	0	2320	14000+200N
R	H1	G28	0	0	20	11400
M	H1	G23	200	0	20	38100
E						
C	L1	B88	0	0	410	4500+100N
T	L1	G05	0	0	1370	10300+100N
I	L1	G16	0	0	490	8300
N	L1	G22	0	0	150	36200
	L1	G12	0	0	260	24900
C	H2	B100	0	10	3150	3500
L	H2	G32	0	30	10930	6400+400N
O	H2	B86	0	0	1460	6600
S	H2	G19	0	0	110	7000
A	H2	G20	0	0	100	4700
+						
I	L2	B84	0	0	190	6700
V	L2	B87	0	0	390	1000+100N
M	L2	G24	0	0	270	500
	L2	G13	0	0	60	100
	L2	B81	0	0	480	900
D	H3	G02	0	0	60	3300
I	H3	B83	110	0	100	12300
S	H3	B91	10	0	1920	21500+700N
O	H3	G09	0	0	10	7400
	H3	G14	0	0	20	6300
+						
	L3	B95	10	0	250	8700
I	L3	G17	0	0	0	*
V	L3	G07	0	0	210	300
M	L3	B92	0	0	200	300
	L3	G08	0	0	460	2300+600N
A	H4	G38	0	0	0	8700
L	H4	G06	0	0	0	2900
B	H4	G41	0	0	50	7000
E	H4	G40	0	0	3590	1500+200N
N	H4	B90	0	0	2260	4800+400N
D						
A	L4	B85	0	0	0	1900
Z	L4	G21	0	0	0	1100
O	L4	G27	0	0	710	800+200N
L	L4	G25	0	0	20	2300
E	L4	G11	0	0	880	300+200N
N	H5	B94	0	0	240	3400
T	H5	B89	0	0	0	1400
N	H5	G39	0	0	10	4000
X	H5	G10	0	0	30	2100
	H5	G34	0	0	710	7300+100N
+						
	L5	G36	0	0	0	600
I	L5	G03	0	20	1140	400+100N
V	L5	B99	0	0	0	300
M	L5	G35	0	0	270	1100
	L5	B97	350	0	0	3500+100N
	H6	G18	0	0	30	5500
	H6	G04	0	0	1230	9300+300N
C	H6	G37	0	0	20	700
O	H6	B98	0	0	60	10800
N	H6	G31	0	90	4690	1300+200N
T						
R	L6	G30	0	50	5670	5000+700N
O	L6	G26	0	0	1580	3400+200N
L	L6	G15	0	0	2180	5100+200N
	L6	B82	0	0	560	9800
	L6	G01	0	30	910	19800

* Died on 11-06-90, 14 days after start of grazing; found to be negative to gastrointestinal nematodes.

APPENDIX 3.3.9. One-way analysis of variance for *H. contortus* recovered from permanent grazers in zones 1 and 2.

		DF	MS	F	SIG
Zone 1	treatment	5	0.33	18.4	*
	error	24	0.02	-	
	Singnificant Multiple Comparisons: IVM X CONTROL*; IVM X NTXN+IVM*; IVM X ABZ*; IVM X DISO+IVM*; IVM X CLOS+IVM*; CLOS+IVM X NTXN+IVM*; CLOS+IVM X ABZ*; NTXN+IVM X CONTROL*; DISO+IVM X NTXN+IVM*; DISO+IVM X ABZ*;				
Zone 2	treatment	5	0.84	21.2	*
	error	24	0.04	-	
	Significant Multiple Comparisons: IVM X CONTROL*; IVM X NTXN+IVM*; IVM X ABZ*; IVM X DISO+IVM*; IVM X CLOS+IVM*; ABZ X CONTROL*; NTXN+IVM X CONTROL*; CLOS+IVM X CONTROL*; DISO+IVM X CONTROL*;				

SIG, * = P≤0.05

APPENDIX 3.3.10. One-way analysis of variance for *H. contortus* recovered from tracer lambs in zones 1 and 2.

		DF	MS	F	SIG
Zone 1	treatment	5	0.75	6.7	*
	error	24	0.11	-	
	Significant Multiple Comparisons: IVM X CONTROL*; IVM X NTXN+IVM*; IVM X ABZ*; IVM X DISO+IVM*; IVM X CLOS+IVM*;				
Zone 2	treatment	5	1.92	11.5	*
	error	24	0.17	-	
	Significant Multiple Comparisons: IVM X NTXN+IVM*; IVM X ABZ*; IVM X DISO+IVM*; IVM X CLOS+IVM*; ABZ X CONTROL*; CLOS+IVM X CONTROL*; CLOS+IVM X ABZ*; DISO+IVM X CONTROL*; DISO+IVM X NTXN+IVM*;				

SIG, * = P<0.05

APPENDIX 3.3.11. Statistical analysis for live weight from permanent grazers

	DF	MS	F	SIG
Zone	1	1058.4	106.9	SIG
Treat	5	14.9	1.5	NS
Zone x Treat	5	19.1	1.9	NS
Error	528	9.9		
Time	8	364.2	75.9	SIG
Time x Zone	8	8.4	1.8	NS
Time x Treat	40	14.7	0.6	SIG
Time x Treat x Zone	40	1.9	0.4	NS
Error	432	4.4		

SIG= P<0.05; NS= P>0.05

APPENDIX 3.3.12. Statistical analysis for PCV from permanent grazers

	DF	MS	F	SIG
Zone	1	154.7	81.4	SIG
Treat	5	37.3	19.6	SIG
Zone x Treat	5	127.7	67.2	SIG
Error	528	1.9		
Time	8	458.6	46.1	SIG
Time x Zone	8	16.5	1.7	NS
Time x Treat	40	19.6	1.9	SIG
Time x Treat x Zone	40	10.5	1.1	NS
Error	432	9.9		

SIG= P<0.05; NS= P>0.05

APPENDIX 3.3.13. Number of female worms, e.p.g. and egg ouptut per female* measured at necropsy of permanent grazers from groups treated only with IVM and for untreated controls.

Group	Ear tag	EPG	No. of females	Eggs/female*
I	B1	6800	3310	2.05
V	B2	16200	3305	4.90
E	B3	9700	2540	3.82
R	B4	13400	2115	6.34
M	B5	58200	1385	42.02
E	B6	1800	705	2.55
C	B7	6200	965	6.43
T	B8	4100	835	4.91
I	B9	6800	1485	4.58
N	B10	4700	470	10.00
	Mean=	12790.0	1771.5	8.76
	St. Dev.=	16534.6	1053.1	11.90
C	G61	6000	800	7.50
O	G62	300	655	4.58
N	G63	1700	905	1.88
T	G64	4700	920	5.11
R	G66	2600	1505	1.73
O	Y61	1100	475	2.32
L	Y62	600	355	1.69
	Y63	1200	390	3.08
	Y64	2100	490	4.29
	Y65	600	130	4.62
	Mean=	2360.0	662.0	3.68
	St. Dev.=	1789.6	390.3	1.88

*Differences in the egg output/female between the two groups were not significant (p>0.05).

APPENDIX 4.1.1. Live weights, dose of ivermectin and worm egg counts from animals infected with susceptible or resistant strain of *H. contortus*

Group	Animal	Live-weight kg	IVM ml/lamb	EPG after treatment - days 0	5	7	9	12	14
Susceptible treated	P51	22	5.5	37000	0	0	0	0	0
	P60	28	7.0	17100	0	0	0	50	50
	P55	42	10.5	12000	0	0	50	0	0
	P54	49	12.3	9100	0	0	0	0	0
	P59	52	13.0	150	50	0	0	0	0
	mean=	39	9.7	15070	10	0	10	10	10
Susceptible control	P57	33	-	26000	48000	67100	32900	27000	28300
	P56	23	-	17800	26000	32300	28100	28600	25900
	P58	46	-	13700	26200	52200	20500	21200	19000
	P52	21	-	11500	+	+	+	+	+
	P53	48	-	50	600	700	900	450	550
	mean=	34		13810	25200	38075	20600	19313	18438
Resistant treated	B79	44	11.0	17600	6800	17300	8900	13900	12000
	B73	38	9.5	7200	15800	16300	22300	20600	20400
	B78	42	10.5	5750	3700	5000	7200	11400	6400
	B75	42	10.5	2700	13100	17600	9800	16600	11900
	B80	19	4.8	3100	2700	6900	3500	8200	5800
	mean=	37	9.3	7270	8420	12620	10340	14140	11300
Resistant control	B71	23	-	13500	22700	59000	20900	17000	33700
	B74	43	-	13200	42600	50400	32500	34600	21600
	B76	43	-	4800	22500	23500	24400	13800	10600
	B72	35	-	2300	5500	7400	7000	12600	10200
	B77	34	-	3000	14200	10700	25400	14400	18200
	mean=	36	-	7360	21500	30200	22040	18480	18860

+ Animal P52 died on Day 2 and was found to have 9650 *H. contortus*.

APPENDIX 4.2.1. Individual worm egg counts from animals treated or untreated with albendazole.

Day	ABZ treated			Untreated controls		
	animal			animal		
	1	2	3	1	2	3
-8	3900	13900	2350	5300	3900	5700
-7	5800	22000	4300	6150	7500	2650
-6	13300	22600	13200	10200	15600	4900
-4	10500	13200	4450	5900	10000	NS
-3	5300	15900	5650	7150	8150	6100
-2	20200	26500	9000	13100	29400	8800
-1	11700	15200	6400	9900	13000	5000
0	8050	18500	4200	8800	13300	5800
1	8200	22900	3600	8800	15000	17800
2	150	3000	100	13900	14800	8500
3	650	6900	400	13600	19100	11200
4	1450	9400	1300	3300	18300	9600
5	350	4600	9000	17500	20100	13500
6	1350	10700	1050	16000	13400	14400
7	600	2100	550	10200	8000	7900
8	700	5500	400	10300	12300	8000
9	800	5300	1500	16600	9900	11500
10	1750	18800	2400	10900	13750	18600
11	1500	10100	2200	14600	17600	7900
12	1400	16500	2900	10300	18500	15100
13	1950	20300	1450	19200	24600	14000
14	7500	11900	900	9000	22400	11200
15	1100	16400	600	8200	23500	9600

NS= no sample.

APPENDIX 4.2.2. Mean larval recoveries (lpg) at days 0, 1, 6, 7, 8, 9, 10, 11 and 15 post treatment with albendazole.

Day	ABZ treated	% reduction	Untreated	% reduction
0	6695	-	5167	-
1	0	100	7705	+49.1
6	575	91.4	900	82.6
7	464	93.1	3030	41.4
8	750	88.8	4453	13.8
9	353	94.7	4955	4.1
10	322	95.2	5444	+5.4
11	1497	77.6	3755	27.3
15	1856	72.3	4600	11.0

APPENDIX 4.3.1. Number of eggs from three different strains of *H. contortus*: susceptible to ivermectin, IVM selected and the multiple resistant strain from South Africa and incubated at 22^0C and 27^0C with their respective recoveries rates during 9 weeks.

Week	Strain	Temp.	Eggs/pot	Larvae Recovered	% recovered	Mean
1	S-IVM	22	141250	24400	17.3	
	S-IVM	22	141250	31400	22.2	19.8
	S-IVM	27	78700	37000	47.0	
	S-IVM	27	72404	56400	77.8	62.4
	R-IVM/SA	22	69350	3000	4.3	
	R-IVM/SA	22	69350	1000	1.4	2.9
	R-IVM/SA	27	65189	2200	3.4	
	R-IVM/SA	27	65189	3600	5.5	4.5
	R-IVM	22	196050	76200	38.9	
	R-IVM	22	196050	37200	19.0	29.0
	R-IVM	27	203892	8000	3.9	
	R-IVM	27	192129	1800	0.9	2.4
2	S-IVM	22	135600	14200	10.5	
	S-IVM	22	135600	3600	2.7	6.6
	S-IVM	27	77126	22000	28.5	
	S-IVM	27	73978	42400	57.3	42.9
	R-IVM/SA	22	67963	400	0.3	
	R-IVM/SA	22	69350	400	0.6	0.6
	R-IVM/SA	27	54519	1200	2.2	
	R-IVM/SA	27	50243	3000	6.0	4.1
	R-IVM	22	196050	25400	13.0	
	R-IVM	22	196050	39400	20.0	16.5
	R-IVM	27	53350	2200	4.1	
	R-IVM	27	46560	800	1.7	2.9
3	S-IVM	22	132775	2400	1.8	
	S-IVM	22	141250	2000	1.4	1.6
	S-IVM	27	72404	600	8.3	
	S-IVM	27	77126	49600	64.3	36.3
	R-IVM/SA	22	66576	400	0.6	
	R-IVM/SA	22	69350	1000	1.4	2.0
	R-IVM/SA	27	53450	600	1.1	
	R-IVM/SA	27	50243	400	0.8	1.0
	R-IVM	22	188208	110800	58.9	
	R-IVM	22	203892	2400	1.2	30.1
	R-IVM	27	49470	3000	6.1	
	R-IVM	27	64990	4000	6.2	6.2
4	S-IVM	22	113000	1600	1.4	
	S-IVM	22	144075	400	0.3	0.9
	S-IVM	27	73978	400	5.4	
	S-IVM	27	83422	800	1.0	3.2
	R-IVM/SA	22	69973	0	0.0	
	R-IVM/SA	22	66576	600	0.9	0.5
	R-IVM/SA	27	51312	1600	3.1	
	R-IVM/SA	27	53450	0	1.6	1.6
	R-IVM	22	188208	68600	36.5	
	R-IVM	22	188208	78000	41.4	39.0
	R-IVM	27	51410	1400	2.7	
	R-IVM	27	48500	200	4.1	3.4

cont... APPENDIX 4.3.1.

Week	Strain	Temp.	Eggs/pot	Larvae Recovered	% recovered	Mean
5	S-IVM	22	135600	5600	4.1	
	S-IVM	22	138425	5000	3.6	3.9
	S-IVM	27	75552	0	0.0	
	S-IVM	27	72404	0	0.0	0.0
	R-IVM/SA	22	69963	600	0.9	
	R-IVM/SA	22	58254	400	0.7	0.8
	R-IVM/SA	27	54519	1200	2.2	
	R-IVM/SA	27	50243	800	1.6	1.9
	R-IVM	22	184287	46600	25.3	
	R-IVM	22	188208	69600	37.0	31.2
	R-IVM	27	49470	0	0.0	
	R-IVM	27	45590	0	0.0	0.0
6	S-IVM	22	141250	2200	1.6	
	S-IVM	22	144075	4800	3.3	2.5
	S-IVM	27	81848	0	0.0	
	S-IVM	27	78700	0	0.0	0.0
	R-IVM/SA	22	69350	0	0.0	
	R-IVM/SA	22	72124	200	0.3	0.2
	R-IVM/SA	27	53450	800	1.5	
	R-IVM/SA	27	51312	200	0.4	1.0
	R-IVM	22	192129	58200	30.2	
	R-IVM	22	188208	47000	25.0	27.6
	R-IVM	27	46560	200	4.3	
	R-IVM	27	50440	0	0.0	0.2
7	S-IVM	22	144075	400	0.3	
	S-IVM	22	138425	400	0.3	0.3
	S-IVM	27	83422	3600	4.3	
	S-IVM	27	78700	4800	6.1	5.2
	R-IVM/SA	22	72124	400	0.6	
	R-IVM/SA	22	72124	0	0.0	0.3
	R-IVM/SA	27	53450	600	1.1	
	R-IVM/SA	27	50243	400	0.8	1.0
	R-IVM	22	192129	1400	0.7	
	R-IVM	22	199971	19800	9.9	5.3
	R-IVM	27	66930	800	1.2	
	R-IVM	27	47530	0	0.0	0.6
8	S-IVM	22	138425	200	0.1	
	S-IVM	22	141250	200	0.1	0.1
	S-IVM	27	75552	1000	1.3	
	S-IVM	27	73978	0	0.0	0.7
	R-IVM/SA	22	69350	0	0.0	
	R-IVM/SA	22	69963	0	0.0	0.0
	R-IVM/SA	27	53450	0	0.0	
	R-IVM/SA	27	51312	0	0.0	0.0
	R-IVM	22	184287	1200	0.7	
	R-IVM	22	192129	600	0.3	0.5
	R-IVM	27	57230	0	0.0	
	R-IVM	27	48500	0	0.0	0.0
9	S-IVM	22	144075	200	0.2	
	S-IVM	22	141250	1400	1.0	0.6
	S-IVM	27	75552	0	0.0	
	S-IVM	27	77126	0	0.0	0.0
	R-IVM/SA	22	66576	200	0.3	
	R-IVM/SA	22	67963	0	0.0	0.2
	R-IVM/SA	27	51312	1400	2.7	
	R-IVM/SA	27	53450	0	0.0	0.1
	R-IVM	22	188208	800	0.4	
	R-IVM	22	203892	400	0.2	0.3
	R-IVM	27	49470	0	0.0	
	R-IVM	27	48500	0	0.0	0.0

S-IVM= ivermectin susceptible strain; R-IVM= ivermectin selected strain;
R-IVM/SA= multiple resistant strain from South Africa.

APPENDIX 4.3.2. Summary of the three-way analysis of variance for the experiment of the effect of different temperatures on the development of three different strains of *H. contortus*.

Source of variation	DF	MS	F
Strain	2	1064.8	12.6*
Temperature	1	51.4	0.6
Time	8	521.0	6.1*
Strain x temp.	2	2161.3	25.5*
Strain x time	16	296.6	3.5*
Temp. x time	8	161.5	1.9
Strain x temp. x time	16	194.4	2.3
Error	54	84.8	

* $F_{8,54}=2.85$

APPENDIX 4.3.3. Data from the study on the development and survival of an IVM susceptible strain of *H. contortus* in Southern Brazil starting on 30-11-90.

Day	Plot	E.P.G. on day zero	Larvae in faeces	Grass			soil		
				D.M. (g)	larvae recov.	L3/kg	% recov	larvae	%recov.
+4	65	6,600	0.0	20.1	0.0	0.0	0.00	37.0	0.56
	73	6,350	0.0	14.3	5.0	350.0	0.08	24.0	0.38
	79	14,200	0.0	19.2	7.0	365.0	0.05	46.0	0.32
	Mean=	9,050	0.0	17.9	4.0	238.3	0.04	35.7	0.42
+6	51	3,250	0.0	22.4	0.0	0.0	0.00	18.0	0.55
	63	7,600	0.0	26.0	0.0	0.0	0.00	10.0	0.13
	75	9,900	0.0	20.7	0.0	0.0	0.00	13.0	0.13
	Mean=	6,917	0.0	23.0	0.0	0.0	0.00	13.7	0.27
+8	57	5,150	0.0	22.7	0.0	0.0	0.00	28.0	0.54
	71	9,300	13.0	26.7	0.0	0.0	0.00	11.0	0.12
	77	5,050	0.0	19.6	0.0	0.0	0.00	7.0	0.14
	Mean=	6,500	4.3	23.0	0.0	0.0	0.00	15.3	0.27
+11	45	4,700	0.0	21.3	2.0	94.0	0.04	8.0	0.17
	47	3,500	0.0	18.5	4.0	216.0	0.11	6.0	0.17
	58	6,950	0.0	20.6	3.0	146.0	0.04	13.0	0.19
	Mean=	5,050	0.0	20.1	3.0	152.0	0.06	9.0	0.18
+13	54	2,150	0.0	26.5	5.0	189.0	0.23	8.0	0.37
	67	6,300	0.0	24.3	7.0	288.0	0.11	4.0	0.06
	78	4,150	0.0	31.8	8.0	252.0	0.19	6.0	0.15
	Mean=	4,200	0.0	27.5	6.7	243.0	0.18	6.0	0.19
+15	56	3,700	0.0	23.2	9.0	388.0	0.24	8.0	0.22
	70	7,250	0.0	24.7	4.0	162.0	0.06	12.0	0.17
	80	5,650	0.0	21.1	2.0	95.0	0.04	9.0	0.16
	Mean=	5,533	0.0	23.0	5.0	215.0	0.11	9.7	0.18
+19	49	3,750	0.0	19.2	5.0	260.0	0.13	9.0	0.24
	72	12,600	0.0	17.6	7.0	398.0	0.06	11.0	0.09
	85	4,450	0.0	16.1	10.0	621.0	0.22	8.0	0.18
	Mean=	6,933	0.0	17.6	7.3	426.3	0.14	9.3	0.17
+26	44	5,950	0.0	19.7	0.0	0.0	0.00	9.0	0.15
	74	9,400	0.0	22.0	5.0	225.0	0.05	9.0	0.10
	81	7,950	0.0	21.8	5.0	229.0	0.06	9.0	0.11
	Mean=	7,767	0.0	21.2	3.3	151.3	0.04	9.0	0.12
+47	62	5,750	0.0	38.0	0.0	0.0	0.00	1.0	0.02
	69	4,150	0.0	29.1	0.0	0.0	0.00	0.0	0.00
	86	5,150	0.0	22.1	0.0	0.0	0.00	0.0	0.00
	Mean=	5,017	0.0	29.7	0.0	0.0	0.00	0.3	0.01
+75	66	5,650	0.0	18.5	0.0	0.0	0.00	0.0	0.00
	68	8,800	0.0	20.2	0.0	0.0	0.00	0.0	0.00
	84	7,800	0.0	14.3	0.0	0.0	0.00	0.0	0.00
	Mean=	7,417	0.0	17.7	0.0	0.0	0.00	0.0	0.00

Larvae found on Day +4 were second stage larvae.

APPENDIX 4.3.4. Data from the study on the development and survival of an IVM resistant strain of *H. contortus* in Southern Brazil starting on 30-11-90.

Day	Plot	E.P.G. on day zero	Larvae in faeces	Grass				Soil	
				D.M. (g)	larvae recov.	L3/kg	% recov	larvae	% recov.
+4	59	7,350	0.0	10.2	8.0	784.0	0.11	26.0	0.35
	64	4,850	0.0	16.8	16.0	952.0	0.33	24.0	0.49
	67	3,750	0.0	15.5	12.0	724.0	0.32	31.0	0.83
	Mean=	5,317	0.0	14.2	12.0	820.0	0.25	27.0	0.56
+6	43	6,400	0.0	12.2	0.0	0.0	0.00	11.0	0.17
	79	3,200	0.0	14.3	0.0	0.0	0.00	16.0	0.50
	85	6,800	0.0	15.0	0.0	0.0	0.00	8.0	0.12
	Mean=	5,467	0.0	13.8	0.0	0.0	0.00	11.7	0.26
+8	51	4,900	0.0	17.5	0.0	0.0	0.00	6.0	0.12
	61	6,300	0.0	20.8	0.0	0.0	0.00	17.0	0.27
	91	3,250	0.0	14.1	3.0	213.0	0.09	11.0	0.34
	Mean=	4,817	0.0	17.5	1.0	71.0	0.03	11.3	0.24
+11	44	4,950	0.0	14.0	0.0	0.0	0.00	5.0	0.10
	66	7,550	0.0	22.6	0.0	0.0	0.00	3.0	0.04
	68	4,000	0.0	20.4	0.0	0.0	0.00	6.0	0.15
	Mean=	5,500	0.0	19.0	0.0	0.0	0.00	4.7	0.10
+13	46	3,000	0.0	25.2	0.0	0.0	0.00	5.0	0.17
	49	5,700	0.0	21.2	0.0	0.0	0.00	9.0	0.16
	53	7,200	0.0	19.6	0.0	0.0	0.00	5.0	0.07
	Mean=	5,300	0.0	22.0	0.0	0.0	0.00	6.3	0.13
+15	69	5,100	0.0	26.1	0.0	0.0	0.00	9.0	0.18
	72	5,350	0.0	20.5	0.0	0.0	0.00	7.0	0.13
	83	3,300	0.0	19.1	0.0	0.0	0.00	11.0	0.33
	Mean=	4,583	0.0	21.9	0.0	0.0	0.00	9.0	0.21
+19	57	9,200	0.0	13.9	0.0	0.0	0.00	7.0	0.08
	71	2,300	0.0	14.8	0.0	0.0	0.00	13.0	0.57
	82	3,250	0.0	13.3	0.0	0.0	0.00	8.0	0.25
	Mean=	4,917	0.0	14.0	0.0	0.0	0.00	9.3	0.30
+26	62	4,600	0.0	19.4	6.0	309.0	0.13	10.0	0.22
	76	2,950	0.0	19.0	3.0	158.0	0.10	16.0	0.54
	87	3,450	0.0	22.3	7.0	314.0	0.20	9.0	0.26
	Mean=	3,667	0.0	20.2	5.3	260.3	0.14	11.7	0.34
+47	65	5,650	0.0	24.1	0.0	0.0	0.00	0.0	0.00
	70	2,300	0.0	29.3	0.0	0.0	0.00	0.0	0.00
	77	4,400	0.0	19.8	0.0	0.0	0.00	0.0	0.00
	Mean=	4,117	0.0	24.4	0.0	0.0	0.00	0.0	0.00
+75	47	4,400	0.0	11.0	0.0	0.0	0.00	0.0	0.00
	86	4,450	0.0	8.2	0.0	0.0	0.00	0.0	0.00
	88	3,650	0.0	11.2	0.0	0.0	0.00	0.0	0.00
	Mean=	4,167	0.0	10.1	0.0	0.0	0.00	0.0	0.00

Larvae found on Day +4 were second stage larvae.

APPENDIX 4.3.5. Data from the study on the development and survival of an IVM susceptible strain of *H. contortus* in Southern Brazil starting on 28-12-90.

Day	Plot	E.P.G. on day zero	Larvae in faeces	Grass				Soil	
				D.M. (g)	larvae recov.	L3/kg	% recov	larvae	% recov.
+3	93	9650	2.0	20.2	2.0	99.0	0.02	13.0	0.14
	101	14,200	0.0	22.3	5.0	224.0	0.04	22.0	0.16
	122	6,800	0.0	21.2	3.0	142.0	0.04	31.0	0.46
	Mean=	10,500	0.7	21.2	3.3	155.0	0.03	22.0	0.25
+5	114	21,000	0.0	25.2	4.0	159.0	0.02	17.0	0.08
	115	9,800	1.0	22.9	2.0	87.0	0.01	19.0	0.19
	119	26,650	0.0	21.1	1.0	47.0	0.01	25.0	0.09
	Mean=	19,150	0.3	23.1	2.3	97.7	0.01	20.3	0.12
+7	92	41,950	0.0	32.2	2.0	62.0	0.01	11.0	0.03
	100	10,900	0.0	25.5	5.0	196.0	0.05	14.0	0.13
	105	19,750	0.0	26.8	7.0	261.0	0.04	15.0	0.08
	Mean=	24,200	0.0	28.2	4.7	173.0	0.03	17.7	0.08
+10	88	23,000	0.0	15.9	2.0	126.0	0.01	7.0	0.03
	89	40,700	0.0	16.5	4.0	242.0	0.01	17.0	0.04
	108	14,050	0.0	23.3	2.0	86.0	0.01	8.0	0.07
	Mean=	25,917	0.0	18.6	2.7	151.3	0.01	10.7	0.05
+12	90	24,600.0	0.0	16.2	0.0	0.0	0.00	2.0	0.01
	109	25,650.0	0.0	33.2	1.0	30.0	0.00	11.0	0.04
	111	10,850.0	0.0	24.8	0.0	0.0	0.00	1.0	0.01
	Mean=	20,366.7	0.0	24.7	0.3	10.0	0.00	4.7	0.02
+14	103	20,000.0	0.0	14.6	0.0	0.0	0.00	2.0	0.01
	125	22,850.0	0.0	26.1	1.0	38.0	0.00	5.0	0.02
	129	10,500.0	0.0	18.5	0.0	0.0	0.00	0.0	0.00
	Mean=	17,783.3	0.0	19.7	0.3	12.7	0.00	2.3	0.01
+19	91	34,200.0	0.0	38.1	1.0	26.0	0.00	1.0	0.00
	110	20,800.0	0.0	24.6	1.0	41.0	0.01	1.0	0.01
	121	16,750.0	0.0	35.9	0.0	0.0	0.00	2.0	0.01
	Mean=	23,916.7	0.0	32.9	0.7	22.3	0.00	1.3	0.01
+26	120	23,150.0	0.0	19.8	3.0	152.0	0.01	9.0	0.04
	126	22,250.0	0.0	27.4	5.0	183.0	0.02	10.0	0.05
	128	20,050.0	0.0	28.5	3.0	105.0	0.02	2.0	0.01
	Mean=	21,816.7	0.0	25.2	3.7	146.7	0.02	7.0	0.03
+53	87	43,400.0	0.0	21.4	3.0	140.0	0.01	13.0	0.03
	94	31,200.0	0.0	26.8	2.0	75.0	0.01	6.0	0.02
	104	25,530.0	0.0	34.3	7.0	204.0	0.03	4.0	0.02
	Mean=	33,376.7	0.0	27.5	4.0	139.7	0.01	7.7	0.02

Larvae found on Day +3 were second stage larvae.

APPENDIX 4.3.6. Data from the study on the development and survival of an IVM resistant strain of *H. contortus* in Southern Brazil starting on 28-12-90.

Day	Plot	E.P.G. on day zero	Larvae in faeces	Grass				Soil	
				D.M. (g)	larvae recov.	L3/kg	% recov	larvae	% recov.
+3	100	4,250	0.0	15.2	3.0	197.0	0.07	12.0	0.28
	103	4,350	0.0	15.7	1.0	64.0	0.02	5.0	0.12
	117	4,150	0.0	13.2	0.0	0.0	0.00	7.0	0.17
	Mean=	4,250	0.0	14.7	1.3	87.0	0.03	8.0	0.19
+5	96	3,900	0.0	25.2	0.0	0.0	0.00	5.0	0.13
	119	3,150	0.0	18.3	1.0	55.0	0.03	14.0	0.44
	128	2,850	0.0	24.5	1.0	41.0	0.04	18.0	0.63
	Mean=	3,300	0.0	22.7	0.7	32.0	0.02	12.3	0.40
+7	105	3,000	0.0	20.6	5.0	243.0	0.17	9.0	0.30
	112	1,850	0.0	30.2	1.0	33.0	0.05	6.0	0.32
	127	2,450	0.0	22.3	3.0	135.0	0.12	9.0	0.37
	Mean=	2,433	0.0	24.4	3.0	137.0	0.11	8.0	0.33
+10	95	3,500	0.0	20.1	3.0	149.0	0.09	2.0	0.06
	98	3,150	0.0	23.4	0.0	0.0	0.00	0.0	0.00
	114	2,800	0.0	17.1	0.0	0.0	0.00	4.0	0.14
	Mean=	3,150	0.0	20.2	1.0	49.7	0.03	2.0	0.07
+12	99	3,600.0	0.0	23.2	0.0	0.0	0.00	3.0	0.08
	115	2,750.0	0.0	17.8	1.0	56.0	2.04	1.0	0.04
	130	3,250.0	0.0	17.4	0.0	0.0	0.00	3.0	0.09
	Mean=	3,200.0	0.0	19.5	0.3	18.7	0.68	2.3	0.07
+14	107	1,350.0	0.0	16.1	0.0	0.0	0.00	0.0	0.00
	122	3,450.0	0.0	21.7	0.0	0.0	0.00	2.0	0.06
	116	4,600.0	0.0	10.4	0.0	0.0	0.00	1.0	0.02
	Mean=	3,133.3	0.0	16.1	0.0	0.0	0.00	1.0	0.03
+19	111	1,450.0	0.0	22.1	0.0	0.0	0.00	3.0	0.21
	125	2,950.0	0.0	22.7	1.0	44.0	0.03	0.0	0.00
	133	4,950.0	0.0	24.5	0.0	0.0	0.00	0.0	0.00
	Mean=	3,116.7	0.0	23.1	0.3	14.7	0.01	1.0	0.07
+26	93	4,250.0	0.0	21.4	0.0	0.0	0.00	15.0	0.35
	120	2,150.0	0.0	27.1	1.0	37.0	0.05	7.0	0.33
	129	2,650.0	0.0	18.3	0.0	0.0	0.00	11.0	0.42
	Mean=	3,016.7	0.0	22.3	0.3	12.3	0.02	11.0	0.37
+53	104	3,000.0	0.0	25.6	2.0	78.0	0.07	8.0	0.27
	121	3,850.0	0.0	20.0	3.0	150.0	0.08	7.0	0.18
	134	3,250.0	0.0	18.1	1.0	55.0	0.03	5.0	0.15
	Mean=	3,366.7	0.0	21.2	2.0	94.3	0.06	6.7	0.20

Larvae found on Day +3 were second stage larvae.

APPENDIX 4.3.7. Data from the study on the development and IVM susceptible strain of H. contortus in Southern Brazil starting on 1-2-91.

Day	Plot	E.P.G. on day zero	Larvae in faeces	Grass				Soil	
				D.M. (g)	larvae recov.	L3/kg	% recov	larvae	% recov.
+3	130	1,800.0	4.0	19.4	2.0	103.0	0.11	8.0	0.44
	145	2,550.0	10.0	11.9	2.0	168.0	0.08	6.0	0.24
	168	1,250.0	7.0	17.0	1.0	59.0	0.08	4.0	0.32
	Mean=	1,866.7	7.0	16.1	1.7	110.0	0.09	6.0	0.33
+5	159	1,200.0	0.0	25.1	0.0	0.0	0.00	5.0	0.42
	162	1,850.0	0.0	25.3	2.0	79.0	0.11	2.0	0.11
	167	1,150.0	0.0	23.7	2.0	84.0	0.17	4.0	0.35
	Mean=	1,400.0	0.0	24.7	1.3	54.3	0.09	3.7	0.29
+7	131	2,600.0	0.0	13.5	0.0	0.0	0.00	0.0	0.00
	166	1,500.0	0.0	17.5	0.0	0.0	0.00	0.0	0.00
	170	1,150.0	0.0	19.9	0.0	0.0	0.00	0.0	0.00
	Mean=	1,750.0	0.0	17.0	0.0	0.0	0.00	0.0	0.00
+10	133	2,250.0	0.0	12.1	0.0	0.0	0.00	5.0	0.22
	144	2,500.0	0.0	10.0	1.0	100.0	0.04	3.0	0.12
	169	1,750.0	0.0	11.5	0.0	0.0	0.00	5.0	0.29
	Mean=	2,166.7	0.0	11.2	0.3	33.3	0.01	4.3	0.21
+12	149	3,400.0	0.0	6.1	3.0	492.0	0.09	3.0	0.09
	152	2,050.0	0.0	15.3	0.0	0.0	0.00	4.0	0.20
	164	850.0	0.0	15.6	3.0	192.0	0.35	2.0	0.24
	Mean=	2,100.0	0.0	12.3	2.0	228.0	0.15	3.0	0.18
+14	135	1,900.0	0.0	16.3	2.0	123.0	0.11	0.0	0.00
	138	2,450.0	0.0	21.0	1.0	48.0	0.01	0.0	0.00
	158	900.0	0.0	14.3	0.0	0.0	0.00	0.0	0.00
	Mean=	1,750.0	0.0	17.2	1.0	57.0	0.04	0.0	0.00
+19	142	2,500.0	0.0	18.1	0.0	0.0	0.00	2.0	0.08
	151	2,450.0	0.0	15.2	0.0	0.0	0.00	1.0	0.04
	155	2,100.0	0.0	14.7	0.0	0.0	0.00	2.0	0.10
	Mean=	2,350.0	0.0	16.0	0.0	0.0	0.00	1.7	0.07
+26	140	3,400.0	0.0	20.1	4.0	199.0	0.12	2.0	0.06
	153	2,950.0	0.0	22.4	2.0	89.0	0.08	1.0	0.03
	161	1,500.0	0.0	25.2	1.0	40.0	0.07	0.0	0.00
	Mean=	2,616.7	0.0	22.6	2.3	109.3	0.09	1.0	0.03
+53	132	1,900.0	0.0	22.6	0.0	0.0	0.00	4.0	0.21
	139	950.0	0.0	23.3	1.0	43.0	0.11	3.0	0.32
	141	1,950.0	0.0	18.2	2.0	110.0	0.10	3.0	0.15
	Mean=	1,600.0	0.0	21.4	1.0	51.0	0.07	3.3	0.23
+80	136	3,450.0	0.0	17.0	0.0	0.0	0.00	0.0	0.00
	147	2,550.0	0.0	16.8	0.0	0.0	0.00	0.0	0.00
	150	1,900.0	0.0	16.5	0.0	0.0	0.00	0.0	0.00
	Mean=	2,633.3	0.0	16.8	0.0	0.0	0.00	0.0	0.00

Larvae found on Day +3 were second stage larvae.

APPENDIX 4.3.8. Data from the study on the development and survival of an IVM resistant strain of H.contortus in Southern Brazil starting on 1-2-91.

Day	Plot	E.P.G. on day zero	Larvae in faeces	Grass				Soil	
				D.M. (g)	larvae recov.	L3/kg	% recov	larvae	% recov.
+3	138	5,950.0	6.0	12.9	2.0	155.0	0.07	21.0	0.35
	156	4,300.0	2.0	13.2	2.0	145.0	0.05	8.0	0.18
	171	3,350.0	0.0	14.1	0.0	0.0	0.00	4.0	0.12
	Mean=	4,533.3	2.7	13.4	1.3	100.0	0.00	11.0	0.20
+5	135	5,550.0	0.0	23.7	4.0	169.0	0.07	6.0	0.11
	157	3,950.0	0.0	22.1	1.0	45.0	0.03	16.0	0.41
	166	3,750.0	0.0	25.3	0.0	0.0	0.00	12.0	0.32
	Mean=	4,416.7	0.0	23.7	1.7	71.3	0.00	11.3	0.30
+7	145	3,700.0	0.0	16.8	2.0	119.0	0.05	3.0	0.08
	150	2,850.0	0.0	8.2	2.0	244.0	0.07	2.0	0.07
	173	4,750.0	0.0	14.0	1.0	71.0	0.02	2.0	0.04
	Mean=	3,766.7	0.0	13.0	1.7	144.7	0.00	2.3	0.10
+10	155	5,250.0	0.0	11.2	0.0	0.0	0.00	6.0	0.11
	162	6,000.0	0.0	11.1	0.0	0.0	0.00	4.0	0.07
	170	6,950.0	0.0	11.4	0.0	0.0	0.00	2.0	0.03
	Mean=	6,066.7	0.0	11.2	0.0	0.0	0.00	4.0	0.10
+12	143	4,250.0	0.0	10.0	2.0	200.0	0.05	1.0	0.02
	163	7,350.0	0.0	14.1	1.0	71.0	0.01	2.0	0.03
	137	3,750.0	0.0	14.1	1.0	71.0	0.03	5.0	0.13
	Mean=	5,116.7	0.0	12.7	1.3	114.0	0.00	2.7	0.10
+14	144	4,350.0	0.0	13.9	0.0	0.0	0.00	0.0	0.00
	146	5,900.0	0.0	13.7	0.0	0.0	0.00	4.0	0.07
	165	3,650.0	0.0	19.0	0.0	0.0	0.00	2.0	0.06
	Mean=	4,633.3	0.0	15.5	0.0	0.0	0.00	2.0	0.00
+19	136	4,850.0	0.0	16.8	1.0	60.0	0.02	0.0	0.00
	154	2,250.0	0.0	26.2	0.0	0.0	0.00	0.0	0.00
	176	5,000.0	0.0	23.8	1.0	42.0	0.02	0.0	0.00
	Mean=	4,033.3	0.0	22.3	0.7	34.0	0.00	0.0	0.00
+26	140	5,450.0	0.0	17.5	2.0	114.0	0.04	4.0	0.07
	153	4,300.0	0.0	12.5	0.0	0.0	0.00	3.0	0.07
	160	4,850.0	0.0	17.5	2.0	114.0	0.04	3.0	0.06
	Mean=	4,866.7	0.0	15.8	1.3	76.0	0.00	3.3	0.10
+53	137	4,900.0	0.0	18.8	0.0	0.0	0.00	16.0	0.33
	164	7,150.0	0.0	23.5	0.0	0.0	0.00	4.0	0.06
	174	5,700.0	0.0	23.1	1.0	43.0	0.02	6.0	0.11
	Mean=	5,916.7	0.0	21.8	0.3	14.3	0.00	8.7	0.20
+80	141	6,550.0	0.0	17.6	2.0	114.0	0.03	0.0	0.00
	161	6,300.0	0.0	17.7	2.0	114.0	0.03	0.0	0.00
	168	4,150.0	0.0	21.3	1.0	47.0	0.02	0.0	0.00
	Mean=	5,666.7	0.0	18.9	1.7	91.7	0.00	0.0	0.00

Larvae found on Day +3 were second stage larvae.

APPENDIX 4.3.9. Rainfall, maximum and minimum temperatures during the study on the development and survival of R-IVM and S-IVM larvae of H. contortus under field conditions prevailing in Southern Brazil.

Month	Rain mm	Temperature °C	
		Maximum	Minimum
Dec	137.0	28.0	15.8
Jan	40.8	29.7	17.3
Feb	18.8	30.7	17.1
Mar	72.0	29.0	17.8
Apr	483.4	24.1	14.5

APPENDIX 4.3.10. Summary of the statistical analysis for for the study on the development and survival of larvae of H. contortus under the summer conditions prevailing in Southern Brazil.

November contamination

Source of variation	D.F.	M.S.	F
Factor A	1	0.24	0.18
Factor B	1	20.72	15.80
Interaction AB	1	0.68	0.52
Error	36	1.31	

December contamination

Source of variation	D.F.	M.S.	F
Factor A	1	9.44	10.70
Factor B	1	5.88	6.67
Interaction AB	1	0.01	0.01
Error	32	0.88	

February contamination

Source of variation	D.F.	M.S.	F
Factor A	1	3.48	3.61
Factor B	1	11.66	12.10
Interaction AB	1	2.22	2.31
Error	36	0.96	

Factor A= R-IVM and S-IVMS trains;
Factor B= Pasture/soil
F values bigger than 4.71 are significant ($p<0.05$).

APPENDIX 5.1.1. Output produced by the simulation model for a flock of ewes treated with programme A under Brazilian conditions.

```
    Epidemiological Data :
    Daily survival prob. of              egg to l1 stage 0.92
    Daily survival prob. of               l1 to l3 stage 0.92
    Daily survival prob. of available infective l3 stage 0.60
    Daily survival prob. of                   adult worms 0.99
    Proportion of ss, rs and rr output by ewes 0.9380 0.0610 0.0010
    Fitness value of ss, rs and rr 0.00 0.00 1.00

    Meteorological Data :
    File containing meteorological data 'met87brj.dat'
    Metdata begins 1 and ends 364 with predicted counts until 365
    Area of land is  20.0 hectares
    Long term average daily temperature is  18.0, rainfall is   4.0

    Grass Sample Data :
    No. of grass samples 12
    Day   Weight / Kg
      1      268.0
     30      292.0
     60      332.0
     90      450.0
    120      555.0
    150      499.0
    180      602.0
    210      726.0
    240      762.0
    270      574.0
    300      477.0
    364      328.0

    Site Data :
    No. of lambs and ewes on plot: 16   20
    Net weight of faecal output in gms/day of lambs and ewes: 1000.0
    2300.0
    Day on which ewes are separated from lambs: 110
    Day on which lambs are moved: 110
    No. of ewes replaced each year:       4
    No. of years for which system is run: 20

    Treatment Data :
    Extra Treatments for ewes : none
    5 Treatments per year   carried out on following days :
    60, 110, 194, 254, 314
```

```
    L3 Sample Data :
    Proportion of ss, rs and rr in l3 stage on pasture 0.9380 0.0610 0.0010
    No. of l3 samples 1
    Day    l3 / Kg dry matter
    1          150.0
```

Year	Frequency of R gene	Proportion of ss	rs	rr	Average worm burden	Day	Maximum worm burden
0	0.031	0.938	0.061	0.001			
1	0.032	0.938	0.061	0.001	7.3	59	5731.3
2	0.033	0.936	0.063	0.001	17.0	59	5731.3
3	0.034	0.934	0.065	0.001	17.3	59	5731.3
4	0.035	0.932	0.067	0.001	17.6	59	5731.3
5	0.036	0.930	0.068	0.001	17.9	59	5731.3
6	0.037	0.928	0.070	0.001	18.3	59	5731.3
7	0.038	0.926	0.072	0.001	18.6	59	5731.3
8	0.039	0.924	0.074	0.002	18.9	59	5731.3
9	0.040	0.922	0.076	0.002	19.3	59	5731.3
10	0.041	0.920	0.078	0.002	19.7	59	5731.3
11	0.042	0.918	0.080	0.002	20.0	59	5731.3
12	0.043	0.916	0.082	0.002	20.4	59	5731.3
13	0.044	0.914	0.084	0.002	20.8	59	5731.3
14	0.045	0.912	0.086	0.002	21.3	59	5731.3
15	0.046	0.910	0.088	0.002	21.7	59	5731.3
16	0.047	0.908	0.090	0.002	22.1	59	5731.3
17	0.048	0.906	0.092	0.003	22.6	59	5731.3
18	0.050	0.904	0.094	0.003	23.1	59	5731.3
19	0.051	0.901	0.096	0.003	23.6	59	5731.3
20	0.052	0.899	0.098	0.003	24.1	59	5731.3