DEVELOPMENT OF *IN VITRO* ASSAYS FOR DETECTION OF ANTHELMINTIC RESISTANCE IN CATTLE NEMATODES

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DEVELOPMENT OF *IN VITRO* ASSAYS FOR DETECTION OF ANTHELMINTIC RESISTANCE IN CATTLE NEMATODES

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ABSTRACT

The principle aim of the current research was to modify the larval development assay (LDA) for use with *Cooperia* from cattle. A series of experiments were conducted in order to modify the LDA protocol to determine the most appropriate culture media and incubation temperature.

These initial experiments concluded that, of the protocols examined, a culture medium of $^1/_{8^{th}}$ the concentration of *E. coli* (EC) + $^1/_{4^{th}}$ the concentration of yeast extract (YE) as generally used to culture sheep nematodes, at a culture temperature of 18ºC, resulted in the optimum number of *Cooperia* larvae developed to the third larval stage (L_3). However, the number of eggs that developed to L_3 was still generally low. A comparison was then made using isolates from a farm with a history of resistance in *Cooperia* to ivermectin (IV) and benzimidazoles (BZ) and two farms with a history of no resistance in this parasite. These experiments were undertaken using $^1/_8$EC + $^1/_4$YE media protocol and $^1/_2$EC + $^1/_2$YE concentration of the standard culture media for sheep nematodes.

These three isolates were cultured at temperature of 18ºC and 25 ºC in the commercially available DrenchRite® 96-well microtitre assay plates which contained BZ, levamisole (LV) and IV in doubling dilutions within an agar matrix. The LD_{50} values were determined from a dose response curve. The resulting LD_{50} values were very variable, especially for the IV analogues. There was no obvious difference between the resistant and susceptible farms for the LD_{50} values of BZ or IV. A secondary aim of this research was to investigate the potential usefulness of the larval feeding inhibition assay (LFIA).

This was adopted as published and it was determined it could be used to distinguish between susceptible and resistant *Teladorsagia circumcincta* with a resistance ratio of at least six. This research concluded that further research is required to fully optimise the LDA for *Cooperia* in cattle but adequate dose response curves were determined to indicate it struggles to distinguish BZ and IV resistance.

The LFIA deserves to be further investigated as it offers some scope to detect ivermectin resistance in cattle nematodes as the dose response curves demonstrated a good repeatability for *T. circumcincta* from sheep. Comparing LDA and LFIA, both assays seemed to be useful but the latter was considered to have greater potential.

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LIST OF ACRONYMS AND SYMBOLS

α	Alpha
AR	Anthelmintic resistance
β	Beta
BZ	Benzimidazole
ºC	Temperature in degrees centigrade
CET	Controlled efficacy test
Cm^3	Cubic metres
Co.	Company
DMSO	Dimethyl sulfoxide
DR	DrenchRite
EBSS	Earle's balanced salt solution
EC	*E. Coli* bacteria
ECYE	*E. coli* yeast extract
EHA	Egg hatch assay
FEC	Faecal egg count
FECRT	Faecal egg count reduction test
FITC	Fluorescein isothiocyanate
g	Weight in grams
GABA	Gamma-aminobutyric acid
GIN	Gastrointestinal nematodes
GIT	Gastrointestinal tract
IC_{50}	Dose response curve$_{50}$
ISSO	International Student Support Office
IV	Ivermectin
IVABS	Institute of Veterinary, Animal and Biological Sciences
kg	Weight in kilograms
LDA	Larval development assay
LD_{50}	Lethal dose$_{50}$

LFIA	Larval feeding inhibition assay
LV	Levamisole
ML	Macrocyclic lactones
mg	Weight in milligrams
ml	Volume in millilitres
µg	Weight in micrograms
µl	Volume in microlitres
µm	Diameter in micrometres
ng	Nanograms
No. / No.s	Number / Numbers
NZ	New Zealand
NZAID	New Zealand Aid for International Development
PBS	Phosphate buffer saline
PCR	Polymerised chain reaction
PI	Post infection
SOP	Standard operational procedure
TBZ	Thiabendazole
UK	United Kingdom
USA	United States of America
WAAVP	World Association for Advanced Veterinary Parasitology
Xg	Centrifugation revolution in gravity
γ	Gamma
YE	Yeast extract

CHAPTER ONE

1.0 INTRODUCTION

Gastrointestinal nematode (GIN) parasitism of ruminants is a global problem (Coles *et al.*, 1992; McPherson *et al*., 1989). Nematode infestations can result in poor growth rates or even death, with a consequent reduction in farm income. A variety of strategies are used to limit the impact of nematode parasitism, but most include the use of anthelmintics (Jackson *et al.*, 2006). Anthelmintics are drugs that act against helminths and they can be used prophylactically or therapeutically (Sharma, 2004).

In recent years the impact of nematode parasitism has been more widely recognized with the result that there has been an overall increase in anthelmintic use since the 1980s (Coles *et al*., 1992; Bisset, 1994). This increased use of anthelmintics has unfortunately resulted in the development of an anthelmintic resistance (AR) problem. In New Zealand, the AR problem has been reported and discussed as a serious threat to the livestock sector (Vlassoff and Kettle, 1980; Brunsdon *et al*., 1983; Jackson *et al.*, 1987; Leathwick and Vlassoff, 1996; McKenna, 1996; Leathwick *et al.*, 2001; Pomroy, 2006). AR was first reported in sheep in New Zealand in the late 1970s (Vlassoff and Kettle, 1980) and in cattle in the mid 1980s (Jackson *et al.*, 1987). Since first reported in New Zealand, the overall level of AR has increased dramatically (McKenna, 1996), with most reports coming from sheep (McKenna, 1995a; Pomroy *et al*., 1992). Although certainly not a new phenomenon, it is now recognised as a serious threat to the whole New Zealand livestock sector, including cattle.

Since most reports of resistance are from sheep, more work on diagnostic assays has been conducted with sheep nematodes compared to cattle nematodes. Most studies relating to sheep rely on a limited number of different tests to detect AR and it is generally accepted that these tests are not ideal. Insufficient research has been conducted to assess AR in cattle nematodes. Therefore, there is a need for an increased effort to study anthelmintic resistance in cattle nematodes and in particular the development of sensitive assays to detect this problem.

A number of different methodologies have been employed to test for AR in nematodes and there are two main types of test: those conducted *in vivo* and the *in vitro* assays. The first developed and the most commonly used methods for AR detection are *in-vivo* tests such as the faecal egg count reduction test – FECRT (Taylor *et al*., 2002a) and the controlled efficacy test – CET (McKenna, 1997; Taylor *et al*., 2002a). The latter test is the gold standard test but as it requires animals to be killed, it is a very expensive test in terms of both animals and labour. Consequently, it is rarely used in the field. The FECRT continues to be widely used but there are problems with both sensitivity and specificity. These tests have been replaced, in part, by the *in vitro* tests. This is because *in vitro* tests are often more sensitive, reliable and cost effective.

Amongst the *in vitro* tests, the larval development assay (LDA) has been shown to be most effective and it is validated to detect AR in sheep nematodes (Coles *et al*., 1992; Taylor *et al.*, 2002a; Coles *et al*., 2005; Tandon & Kaplan, 2004). This is the only *in vitro* assay that is commercially available for sheep nematodes and there is no similar commercially validated LDA for cattle nematodes. Alternative *in vitro* assays have also been considered useful and these include the larval feeding inhibition assay (LFIA), which shows some promise to be useful for AR detection in sheep nematodes. Similarly, it could also prove useful for detecting resistance to some types of anthelmintics in cattle.

To date there have been only a few studies attempting to optimise the LDA for use with cattle nematodes (Johnson, 2000). Since *in vitro* assays have been proven useful with sheep nematodes, there is a reasonable expectation that these assays could also be useful with cattle nematodes. This is particularly relevant, since the FECRT is not reliable for use with cattle nematodes.

Although early attempts at using the LDA in cattle nematodes achieved poor results, it is logical that with more work it should be possible to successfully optimise these assays for the detection of AR in cattle nematodes as discussed by Tandon & Kaplan (2004) and Coles *et al.* (2005).

This thesis represents research conducted to optimise two *in vitro* assays in order to detect AR in cattle nematodes and it essentially reports attempts to optimise the standard LDA protocol, used for sheep nematodes to also be effective with cattle nematodes, particularly with *Cooperia oncophora*. This species is the most commonly diagnosed resistant nematode in cattle in New Zealand. This thesis will also report on some initial studies conducted to evaluate the usefulness of the LFIA to detect macrocyclic lactone (ML) resistance in sheep nematodes. This research was considered a preliminary attempt to use it for detecting ML resistance in cattle nematodes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Gastrointestinal nematodes of cattle in New Zealand

2.1.1 Introduction

New Zealand is in the Pacific Ocean in the Southern Hemisphere, lying between 34-46 degrees latitude south and 166-180 degrees longitudes east (Bowling, 2004). New Zealand has a moist, temperate climate and it generally has year-round rainfall that is beneficial for livestock production (Anon, 1992).

The New Zealand economy is an agrarian economy with that sector contributing about 5% of the total GDP. Although the contribution from agriculture is low compared to 27% and 68% from industries and services respectively, it does play an important role in boosting these other two sectors (Anon, 1992). The most important agricultural activities in New Zealand involve production from sheep and cattle.

The estimated cattle population in New Zealand is about 10 million animals with the gross production exceeding NZ$3x$10^9$ per year (Familton, 2001). Parasitism is probably the major animal health problem for these cattle. In 1993, New Zealand farmers spent about NZ$30x$10^6$ on anthelmintics for GIN in cattle alone (Bisset, 1994) whilst in 1999 these costs had risen to approximately NZ$42X$10^6$ (Familton, 2001). Although the latter figure is at least nine years out of date it does give some indication of the scale of the problem that internal parasitism in cattle represents.

Despite the fact that parasitism in cattle in New Zealand is not a new problem, surprisingly little is known about the epidemiology of cattle nematode parasites in this country (Bisset, 1994). The problem of internal parasites of cattle in New Zealand was reported as early as the 1890s. However, the current understanding of the problem still mainly relies on research work conducted in the mid 1960s to late 1980s, with some later efficacy and productivity trials conducted by animal health companies. Therefore, the control recommendations in cattle nematodes are largely based on this limited local information and on the extrapolation of overseas research, combined with the understanding of internal parasites in sheep (Brunsdon, 1964; Bisset, 1994).

The control of GIN parasitism using anthelmintics has inevitably resulted in increased levels of AR (see Section 2.3 for the details). Yet, this increase in prevalence of AR in cattle has highlighted the inadequate and unreliable assays used to detect it. At the moment, diagnosis of resistance to anthelmintics in cattle nematodes is emerging as a major concern in the cattle sector. Since diagnosis of AR using FECRT is unreliable, developing more reliable assays to diagnose this problem presents a new challenge. An argument can also be made for a review of anthelmintic use and for a better understanding to be obtained of the epidemiology of GIN parasitism in New

Zealand. Both facets will be required as the basis to maintain effective control measures of these parasites and lower the chances of further increases in AR.

2.1.2 Epidemiology of GIN of cattle in New Zealand

There are 24 GIN species reported from cattle in New Zealand (Brunsdon, 1960; Bisset, 1994). Among these species, only three: *Ostertagia ostertagi*, *Trichostrongylus axei* and *Cooperia oncophora* are common and these have the greatest and most widespread economic importance. Of these three species, *O. ostertagi* is considered as the most pathogenic while *C. oncophora* is the least (Bisset, 1994).

The prevalence, distribution and economic importance of GIN parasitism in cattle depends largely on the ecological requirements for growth and survival of stages outside the host. This is influenced by the local climate and farm management practices. Vlassoff *et al.* (2001) discussed these factors in detail for sheep nematodes and similar factors will affect the prevalence, distribution and economic importance of GIN parasitism in cattle. Although New Zealand experiences different climates between the North and the South Island, with the North Island being warmer, Bisset (1994) indicated clearly that almost all the GIN species that affect cattle in New Zealand, occur to a greater or lesser extent throughout the whole country.

The yearly trend in levels of pasture contamination in New Zealand is influenced by which animals previously grazed in the paddocks and the seasonal change of the local climate. Bisset and Marshall (1987) clearly described the infection trend in calves in New Zealand. Most calves in New Zealand are born from August to September. Therefore, the seasonal pattern of nematode parasitism in New Zealand is considered from the perspective of a spring–calving dairy herd, but with a minimal drenching. Immediately after the calves start to graze, they are exposed to larvae that have survived the winter: most of which come from the eggs deposited on the pasture by the previous calves that grazed in the paddocks in autumn.

Although calves eat relatively little pasture when they start to graze for the first time, which means they ingest few larvae, their immunity at this stage is low and this leads to a relatively high egg output during spring. Charleston (1994) further described that the infection trend of nematodes in calves is severe when they feed on these infected pastures. This early infection of calves results in the deposition of eggs on the pasture during spring or early summer, giving rise to the next generation of worms. If these worms are not controlled, re-infection in calves will occur and this leads to a further and larger generation of worms which will result in a major autumn/winter pasture larvae peak in May/June.

In mid-spring, temperatures are favourable for a high rate of larval development to occur. This high larval development rate leads to higher concentrations of larvae on the pastures during late spring (Bisset and Marshall, 1987). At this time pasture growth is also rapid and tends to dilute the

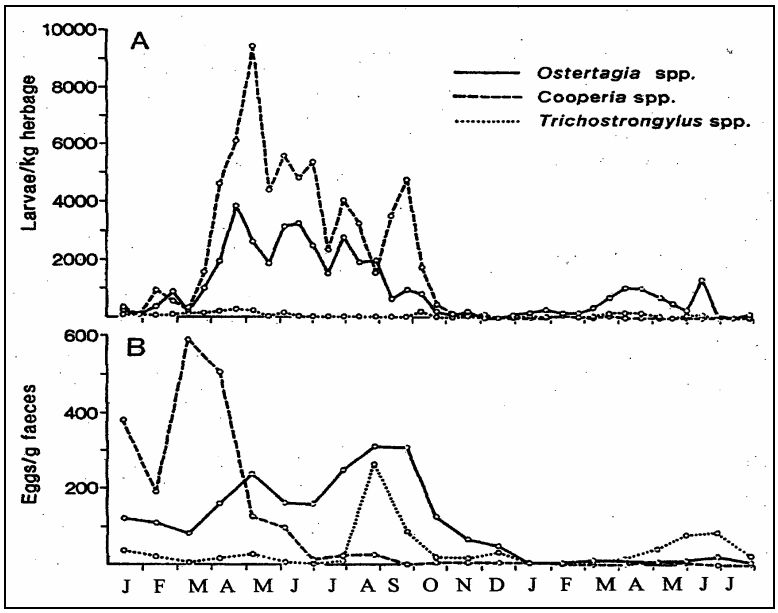
larvae in the pasture. However, calves are also now older and eating more, meaning that more larvae are also ingested and, hence, high infection rates occur despite a low concentration of larvae in the pasture. The increasing infection rate in calves during spring tends to increase egg output, this leads to high egg deposition in the pasture during summer (January/February). At this time, however, larval development and migration is usually impaired by drier environmental conditions and many larvae die due to desiccation. Thus, infection at this time is often minimal compared to the spring time.

As there is usually more rainfall in late summer/early autumn (particularly in March), when egg output is at its peak, there is generally a high larval development rate at this time. However, egg counts start to decline dramatically in late autumn, usually by April, particularly for *Ostertagia* species. The main reason for this being the calves have begun to build up immunity. Even though the worm challenge remains high at this time, host resistance is increasing and at least limiting the fecundity of individual worms (see Figure 2.1).

In the mid-autumn there are several factors operating to affect numbers of larvae on pasture. Temperatures are still warm and the moisture levels are also high. Warmth and

moisture facilitate high development rates (Pandey 1989). However, this coincides with the normal occurrence of reduced egg output in the late autumn and hence a reduction in number of larvae that are developing from this time on (Brunsdon, 1980). Thus pasture larval levels tend to peak in the late autumn or early winter. By the end of autumn/early winter there is poor pasture growth, typical of the winter season. Poor pasture growth results in lower pasture mass which tends to result in concentration of larvae in the pasture, even though egg counts are low and the cooler weather in winter tends to results in fewer larvae developing. This high level of larvae in the pasture indicates that, during winter the infection rate may also be high, as a result of larval concentration in the pasture.

Figure 2.1: A figure representing the typical trend of egg counts and pasture larval levels of the three most important GIN species of cattle in New Zealand, This experiment was conducted in a group of calves with minimum drenching (Adapted from Bisset and Marshall, 1987).



Key: A = Population of larvae in pasture; B = egg output.

As animals head into autumn/winter, there is, however, a common phenomenon in many host-parasite systems whereby an inhibition of larval development may occur in the L_4 stage during the parasitic phase of the nematode in the final host. Brunsdon (1971; 1972) described that under New

Zealand climatic conditions, inhibition in *C. oncophora* results in peak numbers of inhibited larvae during late winter and early spring.

Bisset (1983) described that although *Ostertagia* in cattle are the most affected species, but *Cooperia* species are also affected.

For *Ostertagia*, thousands of these inhibited larvae may accumulate in the abomasal mucosa without causing disease but at a later stage resume their development, usually in small numbers. Occasionally, in circumstances that are not clearly understood, massive numbers may resume their development simultaneously and cause clinical disease (Type II ostertagiosis).

Reduced egg production because of fewer parasites in the GIT reduces pasture contamination and ultimately reduces the number of infective stages attained (if all factors are constant), which leads to lower infection in the subsequent season. For *Cooperia* the fate of inhibited larvae is less well understood.

2.1.3 Factors influencing larval development and survival in the pasture

Eggs from the GIN may hatch and develop to infective third larval stage (L_3) in the faeces if favourable conditions are present (Southcott *et al.*, 1976). The majority of these conditions are

environmental, i.e. conditions the developing stages would naturally encounter in the microhabitat within and around the faecal pellets or dung pat, although some factors are apparently intrinsic. Logically, the microclimate is more important than the macroclimate except in the sense that the latter influences the former. Therefore, it is this microclimate that is considered as a prime factor in egg development and survival (Stromberg, 1997). The macroclimate includes the temperature, moisture (from rain or irrigation), wind, sunlight and oxygen, all within a certain range.

These factors are extremely important as they influence the occurrence, distribution and population of nematode species in a given region or locality. For instance, egg development and larval motility in the pasture is greatly influenced by major factors: temperature, moisture and oxygen supply (Ciordia and Bizzel, 1963; Smith *et al*. 1986; Stromberg, 1997) and these factors vary as the climate changes.

In ruminants, an investigation into the relationship between egg hatching and temperature reveals that members of the commonly occurring GIN differ in the time taken to reach the infective stage at different temperatures (Crofton, 1965; Pandey, 1989; Abdalla and Pomroy, 2007). It is also evident that there are defined lower and upper temperature limits for egg development for each genus (Gronvold, 1989). A similar situation in cattle is evident as earlier described by Pandey (1989). However, any extremely low or high temperature is definitely harmful to the eggs.

For instance, below 9ºC and above 30ºC, development of most species including *Cooperia* in cattle is adversely affected (Ciordia and Bizzel, 1963; Soulsby, 1982). Ciordia and Bizzel (1963) further described that, at 5ºC, eggs of many species including *C. oncophora*, *O. ostertagi*, *Trichostrongylus colubriformis* and *Trichostrongylus axei* are totally inhibited. At 10ºC these authors recognised that it takes about 41 days for more than 50% of the larvae of most of these species to become L_3. At a temperature of 15ºC, most species took about 19 days for the eggs to hatch, whilst at temperatures of 20ºC, 25ºC and 32ºC the numbers of L_3 recovered were about 22%, 30% and 5%, respectively. The generally recommended temperature for optimum egg development is 25ºC. At a higher temperature, most of the eggs hatch rapidly but they are likely to die at the 1st larval stage, whilst at a low temperature larval development ceases.

In one of the early studies of nematode ecology, Dinaburg (1944) described the importance of moisture, as being secondary to temperature for egg development, but a critical factor for larval survival in the field. Later, other authors (Soulsby 1982; Stromberg, 1997) have generally agreed with this theory, confirming that moisture and temperature are the prime factors in egg development.

The moisture content of the cattle dung pats, which is initially the most important, range from 70% to 80% (Rose, 1962). This moisture content may be affected by for example, an alteration in diet and the rate of water loss, which in turn depends on the dung size, ambient temperature and relative humidity. Rose (1962) further observed that, a cattle faecal pat took a month to dry out

completely, and that, from Day 4–5 after the faecal matter is deposited, a dry crust forms on the surface of the dung pat. This crust serves to keep the moisture inside the dung constant and enables most of the eggs to develop within the faecal pat. However, this situation depends on the climate and season of the locality in question. For instance, some areas have long dry periods when the faecal pat may totally dry out and this dry weather can become a primary limiting factor for egg development.

The effect of the dry weather is lack of adequate moisture in the dung which results in desiccation of the eggs. Consequent to desiccation, only a low number of eggs can finally develop to infective stages. For instance, in winter rainfall areas of Australia, dry spells during summer prevent egg development and larval survival with higher numbers of larvae recovered from plots during either autumn or spring (*T. axei*), autumn or winter (*Trichostrongylus vitrinus*) or spring (*H. contortus*) (Callinan, 1978 and 1979; Besier and Dunsmore, 1993). If rainfall occurs during summer, the picture of egg development in the environment is different with *H. contortus* and *T. circumcincta* larvae found more abundantly in the pastures at this time (Southcott *et al.*, 1976).

Moisture also facilitates movement of larvae to the surrounding herbage (Krecek and Murrell, 1990) by providing a surface tension layer in which they can move. However, too much moisture limits larval motility. It has been described that more than 0.12ml of water per cm^3 of soil can hinder larval motility (Soulsby, 1982).

Oxygen supply is another important factor in egg development. Nematodes do not develop beyond the "morula" stage when passed through the host's gastrointestinal tract (GIT). A low oxygen supply in the GIT of ruminants inhibits egg development, since eggs require oxygen for hatching (Silverman and Campbell, 1959). Therefore, oxygen supply immediately after the dung is passed out of the GIT where oxygen is naturally abundant, influences the development of the viable eggs. Rose (1961), Williams and Bilkovich (1971) and Smith *et al*. (1986) all achieved a significantly high percentage of larval development from plots with disseminated faeces, compared to those with faeces in the form of faecal pats, indicating that higher oxygen supply in the disseminated faeces enhanced egg hatch rate.

Similarly, Roberts and O'Sullivan (1949) found the cattle pat is too compact to provide aeration and this limits the oxygen supply. Mixing cattle faeces with vermiculite is the basis for faecal cultures in the laboratory in order to attain higher development rates.

Neither light intensity nor wind contributes greatly to egg development but these two can contribute greatly to larval survival and their distribution in the herbage. Nematode larvae are capable of responding to external stimuli. Larvae may be negatively geotropic and crawl up the leaf blades of the herbage or be positively phototropic to mild light and crawl down the leaf stem. Strong sunlight stimulates them to move down further into the soil (Soulsby 1982). However,

wind probably has a more significant negative impact on larvae. This is because wind dries up the leaf herbage. Any larvae trapped in these upper levels are likely to become desiccated whilst other larvae in the lower parts of the stem and in the soil remain viable. Therefore, the combined effects of sunlight and wind together affect larval distribution and survival.

2.1.4 General overview of *Cooperia* infections in cattle in New Zealand

The aspects of epidemiology and distribution of Cooperia in NZ in relation to the world

The major nematode species causing gastrointestinal disease in ruminants all belong to the order Strongylida. This order is divided into many superfamilies including the Super-family Trichostrongyloidea and within this super-family is the Family Trichostrongylidae. The Trichostrongylidae includes the Genus *Cooperia* in which finally the parasite species *Cooperia oncophora* is classified. Although earlier there was confusion that *Cooperia surnabada* and *C. oncophora* are two different species, Newton *et al.* (1998) later confirmed that these morphologically distinct parasites are genetically the same species.

The genus *Cooperia* is discussed in this section because the species of interest in this current research is found in this genus. Part of the epidemiology of GIN of ruminants including *C. oncophora* can be found in Section 2.1.2 of this thesis. The *Cooperia* species are found in many countries in the temperate, sub-tropical and tropical climate areas. However, in cattle, *C. oncophora* is the dominant and prevalent species found mainly in the temperate regions, including New Zealand (Brunsdon, 1964; Bisset, 1994).

A survey conducted in New Zealand in the early 1960s showed that about 100% of the cattle population were infected with *C. oncophora* (Brunsdon, 1964. However, there have only been a limited number of other studies involving this species and its epidemiology is not yet adequately studied in New Zealand (Bisset, 1994).

Although there is a slight difference in the seasonal pattern in the development of *C. oncophora* in temperate countries between the northern hemisphere and southern hemisphere, there is the general picture that *C. oncophora* develops best at lower temperatures. Lower temperatures prolong the egg development as well as the survival of the larvae in the pastures. Comparing studies in New Zealand, and Germany, for example, there is similarity in the seasonal pattern of larval contamination of pasture for *C. oncophora* but differences in the intensity and exact timing of peak herbage contamination (Brunsdon, 1971; Hertzberg *et al.*, 1992). These slight differences almost certainly result from differences in weather conditions or other epidemiological conditions between these two countries.

Although on its own *C. oncophora* is considered only a mildly pathogenic parasite, when found in a mixed infection with other parasitic nematodes such as *Trichostrongylus* and *Ostertagia* species, it can contribute and make disease more severe (Familton, 2001). The most severe cases of

infections of *C. oncophora* are generally restricted to young animals less than 12 months of age (Parmentier *et al.*, 1994).

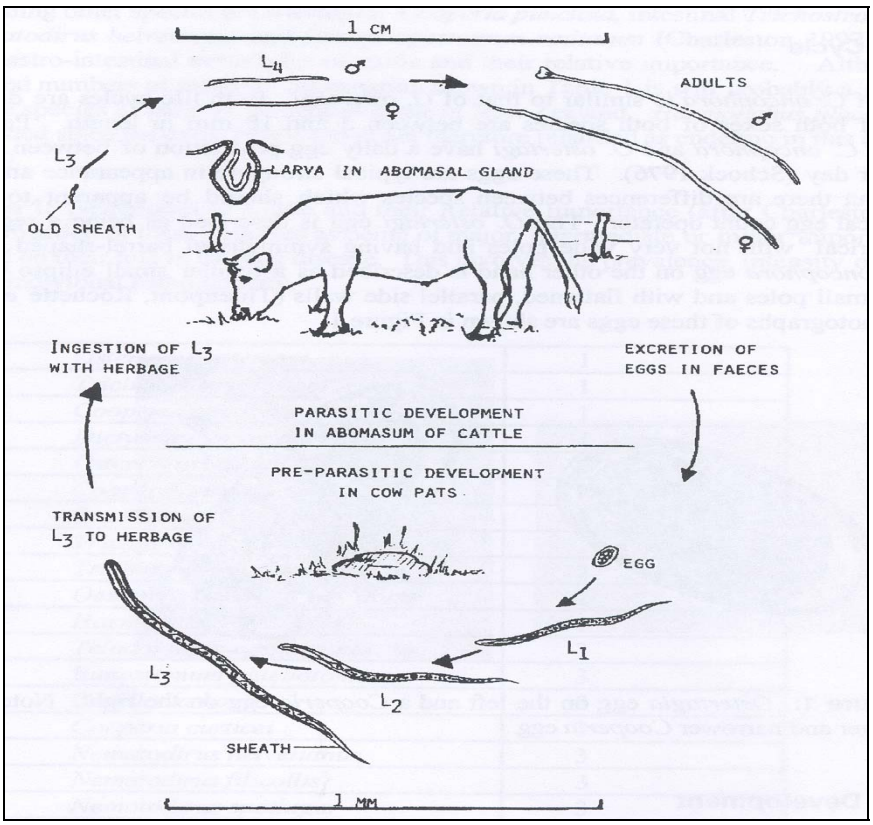
The life cycle

Several authors have described the life cycle of trichostrongylid nematodes. Gronvold, (1989) described *C. oncophora* as a typical trichostrongylid with a direct life cycle. A diagrammatic representation of the trichostrongylids life cycle is presented in Figure 2.2. It can be seen in this figure that two phases occur: the "parasitic" and "pre-parasitic" phases. The egg, the first larval stage (L_1), the second larval stage (L_2) and the third larval stage (L_3) are also known as free living stages and they develop outside the host animal. The fourth larval stage (L_4) develops in the host animal and the adults live in the gastrointestinal tract. For *C. oncophora* the parasitic stages live in the small intestine. Each stage initially grows and is followed by a resting period (lethargus), after which moulting occurs (Dunn, 1978). Usually, there are four moults, two outside the host and two inside the host (Soulsby, 1982). The pre-parasitic life cycle is very important in the development of *in vitro* assays.

One *C. oncophora* female is capable of shedding approximately 100-400 eggs per day (Gronvold, 1989). It is difficult to differentiate the eggs of *C. oncophora* from other trichostrongylids, unless they are cultured to the L_3 stage (Shock, 1976). Since it has been difficult to identify these species through their eggs, there has been a problem experienced during epidemiological study of this species through egg identification.

The *C. oncophora* eggs hatching occur when the L_1 has developed inside the eggs then emerges from the eggs. Eggs will only hatch in the environment under favourable conditions. This hatching event generally occurs within 16-17 hours at 26-30□C. At lower temperatures hatching will take longer. Larvae of *C. oncophora* emerge from the egg tail end first, although larvae of other species, such as *H. contortus*, emerge from the eggs head first (Silverman and Campbell, 1959). After emerging from the eggs, the L_1 stage further develops in the faecal pat or in the soil (Ahluwalia and Charleston, 1974; Rodgers and Brooks, 1977).

Figure 2.2: A typical diagrammatic representation of the direct life cycle of trichostrongylid nematodes including the *C. oncophora* from the egg to the adult worms showing the two phases of the life cycle: the pre-parasitic and parasitic phases (Adapted from Gronvold, 1989).



Gronvold (1989) described the conditions required by the pre-parasitic phase of *C. oncophora* after the L_1 emerged from the egg. The L_1 begins to actively feed on micro-organisms, mainly bacteria, in the faecal pat. At a temperature range of 26-30□C, these L_1 shed their old cuticle to become L_2 after 28-30 hours. However, at lower temperatures this shedding of the old cuticle may take a further 15 hours (Rose, 1963).

After shedding the cuticle, the L_2s of *C. oncophora* then feed and eventually moult to L_3, this moult occurs after approximately 75 hours at 28□C and 96 hours at a temperature range of 20□-26□C. This suggests that under New Zealand weather conditions this may be longer or shorter depending on the season. It is likely to be longer in the winter and shorter in the summer (if other factors remain constant), but generally in New Zealand it is longer because of the cold climate. During the second moult, the L_3 retains the cuticle of L_2 and thus can not feed, but instead it survives on the food granules stored in its intestines and in this form waits in the pasture for the parasitic life cycle to take place following ingestion by a grazing animal (Rose, 1963; Anderson, 1992).

Gronvold (1989) further described the 'parasitic' phase of the lifecycle of this species. The parasitic life cycle starts as a result of ingestion of ensheathed infective larval stage (L_3) on the herbage/pasture. Ingestion of ensheathed infective larval stages is the typical pattern of infection in all trichostrongylids including *C. oncophora*. Normally, after being ingested by cattle the ensheathed L_3 of *C. oncophora* exsheaths in the rumen. Although exsheathment may take place some days after infection, the process of exsheathing itself takes only 10 minutes (Wharton, 1986). The fate of the exsheathed L_3 has not been accurately described for *C. oncophora* but it is associated with the gland crypts in the small intestine where it differentiates and increases in size. Subsequent stages then develop in the gland crypts including the moult to the L_4 and lastly to immature adult stages and emerge from the glands. Finally the immature adults develop into sexually mature adults and commence to lay eggs in the small intestine. However, there may be inhibition at the L_4 stage as discussed in Section 2.1.2.

2.1.5 Immunity against the GIN in calves

A complete understanding of the immune response mechanism to the GIN of cattle has not been established and many questions remain unanswered. Immune responses are initiated soon after infection. Prasad and Singh (1983) described the effects of acquired immunity on parasites in a sequence as follows: retardation of growth and development of larval stages, depression of reproduction, complete refraction to further infection and eventually elimination of infection or self-cure. Immunity in the animals is never total (Charleston, 1994) which signifies that older animals can also develop clinical signs when infected with small burdens of worms.

Although GIN can affect animals of all age groups, 6-12 month old calves are the most affected group. When born, a calf's immune status is usually low with no acquired immunity. When they start to graze, calves are very susceptible because of the absence of an effective immune response. Development of immunity also is usually a gradual process. A reasonable level of immunity to all common GIN species is normally attained when the calf is about 18-20 months old, although the time when immunity is attained to various trichostrongylids differs (Charleston, 1994).

Immunity to *C. oncophora* is normally established first with egg counts declining to lower levels by about 9-12 months of age. Bisset and Marshall (1987) described *C. oncophora* and *Ostertagia* species burdens as being high throughout the first year of their trial. However, in the second year, *C. oncophora* burdens were low and *Ostertagia* species were dominant with *T. axei* being also common at 12-15 months of age. Despite numbers of infective larvae of *Cooperia* species on pasture being high in late autumn, the number of *Cooperia* eggs produced was falling. Bisset (1994) described a similar trend which confirms this general pattern of establishment of immunity in calves.

In Australia, Barger (1999) described that it is the rule rather than the exception, to find an absence of *Cooperia* species once the calves reach the age of 15 months, despite the same animals having 50,000–100,000 worms (including some *Ostertagia*) at the age of 10 months. Similarly, in Scotland and Belgium similar reports show that there is an age-related immune response in calves to *Cooperia* at this age (Armour, 1989; Dorny *et al.*, 1997).

The developing immunity against *C. oncophora* may also be associated with inhibition of the fourth larval stage's development. Brunsdon (1971; 1972) described inhibition of the L_4 stage in detail and part is described briefly in Section 2.1.2, whereby thousands of these inhibited forms may accumulate.

The nutritional status of the calves is a significant factor in the establishment of the immune response. Young calves on a high plane of nutrition tend to have smaller worm burdens or have high worm burdens but are better able to resist the impact of parasitism. This resistance to the impact of parasitism is attributed to good nutrition aiding the development of the immune response in the first instance or supplying sufficient nutrients to compensate for the reduction in utilization associated with parasitism in the latter case (Pomroy, 1994b). Likewise, poorly fed animals may develop more substantial worm burdens. Good nutrition will positively influence the development of the immune response in calves and enable them to resist the impact of the parasites, hence, speed up the initiation of the immune response. Therefore, it is important that calves should be raised under a good plane of nutrition.

2.1.6 Aspects of monitoring and control of GIN within cattle in New Zealand

Effective monitoring and control of GIN parasites in cattle is achieved a number of ways. Various authors have described approaches that have been considered useful for control of GIN in New Zealand cattle. Charleston, (1994) suggests minimum exposure to infective stages, as a key principle objective though practically in New Zealand it is difficult. Pomroy (1994b) pointed out that early diagnosis based on faecal egg counts (FEC) and clinical signs and treatment in New Zealand are useful though unreliable due to certain factors.

Of particular importance is the fact that cattle nematode egg counts are generally low and the relationship between FEC and the worm burden in cattle is relatively poor. Egg production in nematodes is density dependent, particularly for *Ostertagia* and FEC does not accurately reflect the actual worm burden. Initially, it occurs because some worms have poorer fecundity, but it is also important to note that higher infestations of worms in calves lowers the fecundity in some species and consequently confuses the FEC approach. In *O. ostertagi*, for example, heavy infestation result in fewer eggs per female worm (Brunsdon, 1971). This low egg production may be in part due to competition for nutrients among the worms themselves and in part between their hosts.

As a consequence of the failure to minimise exposure of calves to infective stages and also a potential failure to diagnose early clinical signs of parasitism, the monitoring and control of GIN in cattle has been difficult. These difficulties may be further complicated by the sex and reproductive status of the host animal (Barger, 1993). It is also suggested that in diagnosis of clinical signs other important factors, such as age of cattle should be considered. However, Pomroy (1994a; b) suggested other useful approaches to accompany the FEC technique, such as an estimation of the pepsinogen and plasma albumin levels as indicators of worm burden. In practice, estimating pepsinogen and plasma albumin levels is difficult since it also requires time and funds that make the whole approach more complicated, and in addition to this, pepsinogen is only relevant for abomasal genera like *Ostertagia*. In conclusion, neither minimum exposure to infective stages nor diagnosis of clinical cases before treatment has been completely useful.

Several other factors may contribute to the usefulness of using FEC in cattle though it is not reliable. For example, the costs involved in conducting the tests (McKenna, 1990) and the time consuming nature of the assays (McKenna, 2007) are among of the factors. However, farmers continue to use anthelmintics as a common approach against the common nematodes infections in cattle without proper diagnosis.

2.2 The use of anthelmintics in cattle in New Zealand

2.2.1 General overview

Since GIN parasitism is a global problem in the livestock industry, anthelmintics are commonly and widely used in order to limit the impact of nematodes. Anthelmintics are drugs that act against worms and they can be used prophylactically or therapeutically (Coles *et al.*, 1992).

Various anthelmintics are commercially available although these drugs vary considerably in their range of activity, effectiveness and toxicity. Some of these are only effective against certain parasites or genera whilst others are effective against a wide range of genera. Anthelmintics that are effective against many species or genera are termed “broad spectrum”, whilst those effective against only a few species are termed “narrow spectrum” anthelmintics (Familton, 2001).

Familton, (2001) further discussed that, although an anthelmintic may be considered broad spectrum, it does not necessarily imply that it is effective against all the full range of species or all their stages. For example, an anthelmintic might only remove relatively small proportions of the mature worms or, more commonly, they might be less effective against immature stages such as the larvae. The reason for this inefficacy is not well understood.

Since some anthelmintics are potentially toxic or antimetabolic agents, they may also affect the animals’ metabolism. The issues of toxicity, antimetabolic behaviour of drugs and actual efficacy levels eventually lead to the concept of an “ideal” anthelmintic, rather than a completely safe and effective anthelmintic in the true sense. It is often necessary to accept a compromise where the

range of species for which an anthelmintic is effective and/or the safety margin are smaller than desirable. However, poor efficacy against certain species should not be confused with the development of anthelmintic resistance.

The following anthelmintics are now commonly available for use against GIN parasites of cattle throughout New Zealand.

2.2.2 Benzimidazoles and Pro-benzimidazoles

Basically, benzimidazoles (BZ) and pro-Benzimidazoles (Pro-BZ) are in the tubulin-binding group. Previously, these drugs were the most frequently used drugs in New Zealand but recent publications shows that they are now the least used drugs (Jackson *et al.*, 2006).

The BZs and pro-BZs have a similar molecular structure, with the central structure comprising of a 1, 2-diaminobenzene ring. To achieve a prolonged period in the hosts' body in order to increase its efficacy, recent formulations have the 2-thiazolyl side chain replaced by a 2-methyl carbamate. In addition Arundel (1985) described that they have aliphatic and aromatic side chains at the 5-position. Thiabendazole (TBZ) was the first drug commercially released in 1962 (Prichard *et al*., 1981).

The mode of action of these drugs is to disrupt the formation of microtubules in nematodes at concentrations that are harmless to the animals. Microtubules are structural components of the cell. They are comprised of α and β-tubulin polypeptide subunits, of 450 and 455 amino acid sequences respectively, with a total molecular weight of 50,000. The microtubules form an intracellular cytoskeleton and are associated with various physiological functions such as mitotic spindle formation, cell motility, cellular secretion, nutrient absorption and intracellular transport (Lacey & Prichard, 1986; Lacey, 1988).

There is a state of active equilibrium in the cell where soluble tubulin units are lost or gained at opposite terminals of a developing microtubule. The growth of microtubules depends on the rate of addition (polymerisation) or loss of tubulin units. The BZs bind and act on the β-tubulin, thus preventing the formation of microtubules and hence affecting this state of equilibrium. Loss of microtubule equilibrium as a result of the action of BZs leads to loss of cellular homeostasis and this leads to depolymerisation and finally cell death.

2.2.3 Levamisole and Morantel

Levamisole (LV) and Morantel are categorised as being in the ganglion-blocking group. This group of anthelmintics comprises LV and the tetrahydropyrimidine salts, morantel, pyrantel and oxantel. These drugs are generally highly water soluble and are quickly absorbed and excreted from the animals' body. Although drugs in this group have a similar mode of action, they vary in their

relative efficacy. Prichard *et al.*, (1980) stated that LV is considered as more chemically active and it is the most commonly used anthelmintic in this group.

The mode of action of LV is as a cholinergic agonist, which leads to sustained muscle contraction and paralysis. This eventually results in the removal of the nematodes from the gut by normal peristalsis or from the lungs by cilia (Prichard *et al.*, 1980; Booth & McDonald 1988; Arundel, 1985).

2.2.4 Macrocyclic lactones

The macrocyclic lactone (ML) group comprises the avermectins, which include abamectin, ivermectin (IV), doramectin, eprinomectin and selamectin. In addition, it also includes the milbemycins, which include moxidectin and milbemycin oxime. Drugs in this group are effective against a wide range of internal and external parasites in various animals (Shoop and Soll, 2002) including their larvae, but they are not effective against cestodes and trematodes (Conder and Campbell, 1995).

The avermectins are produced by the fermentation of the actinomycete, *Streptomyces avermitilis* and they are then usually subjected to chemical modification to produce the desired chemical structure (Shoop *et al.*, 1995; Conder and Campbell, 1995). The avermectins were discovered in 1975 and ivermectin (IV) was commercially released in 1981. The milbemycins are produced by the fermentation of related actinomycetes. Although milbemycin was first discovered in 1973, moxidectin was first marketed in 1990. It is a semi-synthetic product of *Streptomyces cyanogriseus*. Both avermectin and milbemycin are chemically related, with a C16 substitution, but later doramectin was produced from *Streptomyces avermitilis*, with a C25 substitution.

Since their discovery, the avermectins have become widely available and have revolutionised parasite control. Ivermectin is a white powder which is highly soluble in organic solvents but insoluble in water. A variety of formulations are commercially available including those given orally, by injection, topical pour-on and in slow release devices.

Earlier studies suggested that the action of the MLs was due to their activity as GABA agonists (McKenna, 1985; Shoop, 1993). It was thought that the action potential of γ-aminobutyric acid (GABA) at the synapses of GABA-ergic nerves in worms, was prolonged, and led to paralysis. However, this does not seem to be relevant when explaining their activity in nematodes, and thus, it is now regarded as invalid. The new theory relating to the action of MLs is associated with the opening of glutamate-gated chloride ion channel in cell membranes (Martin *et al.*, 2002). The opening of a glutamate-gated chloride ion channel in a cell increases its permeability to chloride ions and eventually leads to hyperpolarization to the point that a nerve cell cannot transmit an action potential. This eventually makes the worms unable to move, feed or reproduce.

2.2.5 Other anthelmintics

Apart from the BZs, LVs and MLs, there are other broad-spectrum anthelmintics that have been used for nematode control, such as the acetylcholinesterase-inhibitors. These drugs comprise some of the organophosphates but they are now rarely used. This is because they are either too toxic or they are ineffective anthelmintics. These include dichlorvos, trichlorphon and naphthalofos (Arundel, 1985). Though considered toxic and ineffective, organophosphates may however provide a useful alternative treatment against some worms although their use in cattle in New Zealand has not been reported. In Australia, for instance, dichlorvos and trichlorphon are only used in horses - trichlorphon being combined with BZ/Morantel against *Gasterophilus* species, whilst naphthalofos is used for the treatment of *H. contortus* in sheep. Its use in sheep reduces the selection pressure for resistance to other anthelmintics, although it lacks efficacy against the immature stages.

Organophosphates act as anticholinesterases, which involve the inhibition of acetylcholinesterase enzyme secretion. This results in a build-up of acetylcholine within synapses and a continual stimulation of the nerve endings, resulting in spastic paralysis of worms. Eventually this spastic paralysis leads to the removal of the worms by the normal peristaltic action of the bowel. However, little is known about the potential use of organophosphate drugs in cattle worldwide and research to explore their usefulness against cattle nematodes would be required.

2.2.6 Drenching practices in cattle in New Zealand

Different anthelmintics have been used in New Zealand since the early 1960s and subsequently the use of anthelmintics became more common as revealed by a survey conducted in New Zealand on drenching practices particularly in sheep nematodes in the early 1980s (Brunsdon *et al.*, 1983). However, little information about drenching practices against cattle nematodes is known. Anthelmintics used are already discussed in Section 2.2.2 to 2.2.5 of this thesis.

The early drugs used were BZs and LV, which were available in New Zealand from the 1960s while MLs became available since in the early 1980s. The MLs were first commercially released in New Zealand as an injectable formulation in 1982, followed by an oral formulation in 1984 and a topical pour on in 1990 (Bisset *et al.*, 1990). Latter, due to there effectiveness, the uses of MLs against cattle nematodes became more common than BZs and LV. Ivermectin is now commonly used as an effective anthelmintic though other avermectins and milbemycins (avermectin-related compounds) have also been used and these drugs have shown to be effective and are given at small doses (Samson *et al.*, 1992; West *et al*., 1994; Watson *et al.*, 1995a; 1995b: 1995c; McKenna, 1995b).

It was observed in the later decades of the 20th century that regular drenching had become the common approach for controlling GIN of cattle in New Zealand (McKellar, 1988). This was particularly apparent for young beef cattle in the 1980s, which coincided with the time when the bull beef industry initially flourished. At this time it was widely acknowledged that growth rates were being lowered by sub-clinical parasite infestation (McPherson *et al.*, 1989; Bisset, 1994). Since higher growth rates after drenching resulted in higher returns, regular drenching became common. However, frequency of drenching is considered one of the factors that have resulted in the emergence of an anthelmintic resistance (AR) problem.

2.3 Anthelmintic resistance problem

2.3.1 Definition

Various authors have defined the phenomenon of AR and the majority of them include a common meaning of 'genetic' change. Taylor and Hunt (1989) defined resistance to anthelmintics as a heritable change in the ability of an individual parasite to survive the recommended therapeutic dose. Shoop *et al.* (1995) defined it as a change in the gene frequency of a population, produced by drug selection, which renders the minimum effective dose, previously used to kill a defined portion of a worm population, less effective. Likewise, Conder and Campbell (1995) defined it as a heritable reduction in the sensitivity of a parasite population to the action of a drug, which is expressed as a decrease in the frequency of individual parasites that are affected by exposure to the drug. Sangster and Gill (1999) defined AR as a decline in the efficacy of an anthelmintic against a population of parasites which was initially generally susceptible to that drug and that this decline is genetically determined. The only practical definition that has been used in the field is by the World Association for the Advancement of Veterinary Parasitology (WAAVP), which defined anthelmintic resistance as failure to reduce faecal nematode egg counts by at least 95% (Coles *et al*., 1992). A similar but broader definition was adopted by WAAVP in 2006 (Coles *et al*., 2006).

2.3.2 The causes of AR problem

Since regular drenching became the common approach for GIN control in New Zealand, a number of consequential factors are generally considered to have contributed to the development of AR. These same factors have also been described as the major causes of AR in many other countries around the world. These factors include under-dosing (Taylor and Hunt, 1989; Taylor *et al.*, 2002b), frequent drug exposures at short intervals (Taylor and Hunt, 1989), continuous use of drugs with a similar mode of action (McKenna, 1985), treatment when parasites have a small refugia (Miller, 2006), frequent stock relocation onto clean pasture after treatment and the failure to quarantine drench-purchased stock (McKenna, 1989, Pomroy, 2006). Although broad generalisations are made relating to the development of AR, the underlying issue is an inadequate understanding of the problem and how these various factors interrelate. The occurrence of AR also depends on the mode of action of the drug in question, the dose rate, the species in question and how all these factors are also interrelated.

2.3.3 The mode of resistance to the commonly used anthelmintics

Parasites within a population do not respond uniformly to commonly used anthelmintics. This is probably due to the genetic diversity in the population of each parasite species against the drug in question. The result is also likely to depend on the mode of action of the anthelmintic. Therefore, in order to determine the mechanism of resistance to the commonly used drugs, it is necessary to refer to the ways in which these drugs exert their effect and the way the genes are anticipated to change under this selection pressure.

In reference to the action of BZs, Lacey (1988) revealed through the use of binding assays with radiolabelled-mebendazole and tubulin from sensitive and resistant isolates of *Haemonchus contortus*, that tubulin in the mebendazole-sensitive strain has a higher significant binding level than tubulin from the mebendazole-resistant strains. The mode of resistance to BZs and Pro-BZs at the molecular level involves the substitution of the amino acid phenylalanine (Phe) in place of Tyrosine (Try), at position 200 of the sequence of the β-tubulin gene (Roos *et al.*, 1995; Taylor *et al*., 2002b). Whilst Lubega and Prichard (1990) describe the decrease in β-tubulin restriction fragments and the decrease in high affinity sites in resistant species as clear evidence of genetic changes resulting from resistance, Roos *et al.* (1990) did not find any difference in α-tubulin, between BZ-resistant and -susceptible population indicating that changes in β-tubulin are solely responsible for the genetic changes in resistance.

Unfortunately, there is limited information available relating to the genetic mechanism of resistance to LV and other cholinergenic agonists. Some authors believe that the genetics of resistance to LV is multigenic, whilst others believe it is a sex-linked recessive trait that probably involves a single gene. Initially, Sangster and Gill (1988) described the mechanism of LV resistance and argued that it was thought to be associated with a reduction of nicotinic acetylcholine receptors of nematodes or changes in their binding characteristics. Martin and McKenzie (1990) further suggested that the reason for this happening is a sex-linked recessive trait in *Trichostrongylus colubriformis*. Later, Sangster and Björn (1995) and Dobson *et al.* (1996) described the mechanism of resistance to LV in *H. contortus* as an inherited autosomal recessive trait, which involved more than one gene. These studies may be true and this indicates further research is required in order to understand more about the mechanism of resistance in LV.

Although the mechanism of LV resistance is associated with reduction of nicotinic acetylcholine receptors of nematodes that are sensitive to LV or a change in their binding characteristics, there have not been sufficient studies to determine the significant difference between the ligand-binding properties of LV-resistant and –susceptible *H. contortus* receptors (Sangster *et al.*, 1991), nor have there been well established studies associated with ligand-binding to LV resistance in other nematode species, or selection of the specific allele of nicotinic acetylcholine receptors during selection for resistance in various LV-resistant strains (Hoekstra *et al.*, 1997a, 1997b)

In this ganglion-blocking group, resistance may occur to morantel, whilst LV remains effective. However, in most cases, cross-resistance between LV and morantel occurs (Arundel, 1985) and therefore, parasites resistant to LV are usually resistant to morantel.

In the case of MLs, the level of understanding of the mode of resistance is still poorly understood (Jackson *et al.,* 2006). This reflects, in part, the more recent development of resistance to this family of anthelmintics. As described in Section 2.2.4 of this thesis, the action of MLs was earlier considered to be due to their activity as a GABA agonist which later recognised that it is not the

case but the opening of a glutamate-gated chloride ion channel in a cell, which increases its permeability to chloride ions and eventually leads to hyperpolarization. Nevertheless, the mechanism of resistance in MLs id believed to occur as a result of selection for the genes that lead to (either or both) the reduced uptake of MLs and reduce access to the various glutamate-gated chloride ion receptors in the cell (Martin *et al.,* 2002; Prichard, 2002). These authors described that there is also an issue of changes in ability of the MLs to bind the glutamate-gated receptors as a potential mode of resistance. However, more research about the mode of resistance in MLs is still required.

2.3.4 The classification of resistance

Three sub-classifications have been described: side resistance, cross-resistance and multiple resistance. Side resistance occurs amongst chemically related compounds with the same mode of action. For example, it can be seen within each of the BZ, salicylanilide and ML groups. Cross-resistance occurs to chemically unrelated compounds, as a result of selection with one chemical group that renders the nematode resistant to another. An example of cross-resistance can be seen between LV and morantel. A multiple resistance occurs when one nematode species becomes resistant to at least two drugs with different modes of action such as between BZ and LV or ML (Taylor & Hunt, 1989; Green *et al.*, 1981; Coles *et al.*, 1992; Pomroy *et al.*, 1992).

More reports of AR are available in sheep nematodes in New Zealand compared to those from cattle. However, some reports on cattle nematodes have been published and they have demonstrated the occurrence of side resistance to BZ (McKenna, 1991; Hosking and Watson, 1991), oxfendazole (Jackson *et al*., 1987); side resistance to MLs (West *et al.*, 1994; Loveridge *et al.*, 2003; Vermunt *et al.*, 1995) and multiple resistance to IV and oxfendazole (Vermunt *et al.*, 1996). These reports indicate that almost all types of resistance have been reported in cattle nematodes in New Zealand.

2.3.5 The background of AR problem in New Zealand

The first case of AR worldwide was reported in USA in 1957 and it involved *Haemonchus* in sheep against the anthelmintic phenothiazine (Drudge *et al.*, 1957; Drudge *et al.*, 1964). This case occurred only 3 years after phenothiazine was commercially introduced. The first case of resistance to a BZ was reported in 1964 against *Haemonchus* species (Kelly and Hall, 1979), which was also only three years after it was commercially released, whilst the first case of LV resistance was reported in the late 1970s in the same species (Le Jambre *et al.*, 1977). Since that time, many other reports have been released worldwide, with the majority of these involving sheep nematodes (Waller, 1997).

Until the late 1970s, there were likely no published reports of resistance in cattle nematodes worldwide. The lack of such reports raised a debate as to whether or not resistance did occur in cattle nematodes (Kelly and Hall, 1979). However, the first case of resistance in cattle nematodes

was reported in the early 1980s in the United States of America (USA). This report involved *O. ostertagi* resistant to LV, initially diagnosed by a FECRT but later confirmed by a slaughter study (Lyons *et al.*, 1981; Lyons *et al.*, 1983).

In New Zealand, the early signs of resistance were suspected in the late 1960s, but confirmed in 1979 in sheep (Vlassoff and Kettle, 1980). In cattle, the first report of AR in New Zealand was in the mid 1980s (Jackson *et al.*, 1987). This report involved *C. oncophora* resistant to oxfendazole following the observation of a post-drench drop in the growth rate of calves and an increased post-drenching rate in egg counts. Resistance was later confirmed by FECRT and the slaughter of one calf. Since then, many more other cases of AR against LV in New Zealand have been identified.

In New Zealand, MLs were first commercially released as an injectable formulation of ivermectin in 1982, followed by an oral formulation in 1984 and a topical pour-on in 1990 (Bisset *et al.*, 1990). The first case of resistance to MLs was reported in New Zealand in the early 1990s against *Teladorsagia* species in goats (McKenna *et al.*, 1990; Pomroy *et al.*, 1992) whilst the first case of resistance to MLs in cattle was reported in *Cooperia* species in the mid 1990s (Vermunt *et al.*, 1995; Familton *et al.*, 2001). Whilst AR in other drugs in nematode species of cattle such as *Ostertagia* has been reported only to benzimidazoles (Hosking *et al.*, 1996) further resistances to MLs in cattle *Cooperia* were reported by Loveridge *et al.* (2003).

Since first reported in cattle in New Zealand, AR is recognized to become a threat to this sector countrywide. Continued regular drenching with little or no laboratory diagnosis before treatment has exposed many farms to the risk of an AR problem (McKenna, 1996). Nevertheless, little knowledge on the problem of AR still leads many farmers to continue drenching their animals, even when it is not necessary, such as when the infectivity of a pasture is low (McKenna, 1996; Taylor and Hunt, 1989; Shoop *et al.*, 1983).

Two species: *O. ostertagi* and *C. oncophora* are the most commonly reported to develop resistance in cattle although there are a few reports of AR in *Trichostrongylus*. Earlier cases of resistance to these species were recognised to be more common to BZ than to LV or the MLs (Conder and Campbell, 1995; Winterrowd *et al.*, 2003). Both *O. ostertagi* and *C. oncophora* have been shown to have high allelic diversity and this probably includes existing genes for BZ resistance that are then amenable to genetic selection.

However, more recent results have shown that resistance to IV is very common, at least in *Cooperia* species (Jackson *et al.*, 2006). The reason for this is not well established, although it is thought to be due to current drenching practices, where the MLs have become the more commonly used anthelmintics. In addition, Jackson *et al.* (2006) further described that anthelmintic treatment applied to animals with higher levels of immunity, or those, which become immune whilst the drug is active, are likely to select for resistance much faster than treatments applied to non-immune stock, since the rate of dilution of surviving nematodes is slower. These two factors

might be the possible reasons for higher selection to IV resistance in cattle nematode in New Zealand in recent years.

Generally, the AR problem in cattle has increased at a slower rate compared to the situation in sheep nematodes (Conder and Campbell, 1995). The factors which could explain this slow development of resistance in cattle nematodes are not well understood but they are probably related to differences in the treatment frequencies in different age groups and differences in other management practices between sheep and cattle.

The current situation in general indicates that there might be no new anthelmintics for nematode control in the near future (McKenna *et al.*, 1996; Leathwick *et al.*, 2001; Mason and McKay, 2006 and Waghorn *et al.*, 2006). Though advice has been given to use non-chemical methods and education in combating these problems of GIN and AR (Pomroy, 2006; Van Wyjk 2006), review of the uses of the currently commercially available anthelmintics is important. Of more important also is the review of the diagnostic methods for detecting the AR problem to monitor the situation and to limit the occurrence of this problem.

2.4 Diagnosis of anthelmintic resistance

Various tests for AR detection have been described. To date, a wide range of parasitological, biochemical and molecular techniques are available for the detection of AR. The methods for AR detection have been reviewed (Taylor *et al.*, 2002a) and these tests are divided into two categories: the *in vivo* and the *in vitro* tests. *In-vivo* tests are those that directly involve animals and in some tests they include the slaughtering of the animal. The two *in vivo* tests commonly used for AR detection are: the faecal egg count reduction test (FECRT), and the controlled efficacy test (CET). The i*n vitro* tests do not involve slaughtering the animals for the test. To date, a number of *in vitro* tests have been developed to test for resistance in ruminants and they have been successfully used in sheep nematodes to diagnose AR problem (Small and Coles, 1993).

The *in vitro* assays include: the egg hatch assay (EHA), larval paralysis and larval motility assays, adult development assay, larval feeding inhibition assay (LFIA), biochemical assays, molecular/genetic tests and the larval development assay (LDA). The *in vitro* assays are generally more precise, reliable and in addition, they are more economical than the *in vivo* assays. Those cited above are not all equally useful for all anthelmintics (Coles *et al.*, 1992). Some of them have only been validated for a limited range of nematodes or indeed not validated at all. Section 2.4.1 to 2.4.2 discusses the *in vivo* assays whilst Section 2.4.3 to 2.4.8 discusses the *in vitro* assays. The larval development assay (LDA) and the larval feeding inhibition assay are discussed separately in Sections 2.5.1 and 2.5.2 respectively, since they are the basis for the research in this thesis.

2.4.1 Faecal egg count reduction test

This is a simple test for diagnosing resistance in nematodes and has been used in many countries, including New Zealand. This assay is useful for a variety of animal parasite species and drugs. The test relies on the relationship between the egg count and the worm burden. The fundamental principle of this test is to compare egg counts in a group of animals before and after treatment with an anthelmintic. It is now considered important to also undertake pooled larval cultures of each group before and after treatment (McKenna, 1994; McKenna, 1997). This allows information to be obtained on efficacy against the different genera. The efficacy can be calculated by a variety of different approaches (Miller *et al*., 2006) but the more commonly used method is to compare the arithmetic mean of egg counts 7-14 days post-treatment to those of Day 0 using the formulae $(T_1\text{-}T_2/T_1)\ X\ 100$: where T_1 is the pre-drenching/treatment egg count (Day 0) and T_2 is the post-drenching egg counts.

One limitation with FECRT is the lack of a common criterion for interpreting the results. The test has been regularly modified and several protocols have been described. Consequently, it is difficult to compare results from different studies (Taylor *et al*., 2002a; McKenna, 1997). The use of an adequate number of animals and the proper dose rate are amongst the factors to consider when conducting a FECRT. Most protocols recommend the use of 10-15 animals per treatment group and each animal should be weighed and dosed according to its body weight (McKenna, 1987; McKenna, 1990). However, under the New Zealand farming system, this is often not practical, since several veterinarians tend to opt to dose at the weight of the heaviest animal in order to reduce labour force, hence, reduce the amount of time required to conduct the test. To reduce laboratory time and costs, a "composite FEC technique" has been proposed (Morgan *et al*., 2005; McKenna, 2007). This composite FEC technique enables a greater number of animals to be included in the treatment group and thus provides "reliable" results. Whilst some protocols include a control group, others do not. Therefore, in this case efficacy is assessed by comparing the post-treatment with the pre-treatment egg counts with or without considering the change in egg counts of a control group.

A variety of nematode genera produce strongylid eggs, which are practically indistinguishable from each other morphologically. These nematodes also vary considerably in their egg laying capacity (McKenna, 1997), and this variation occurs even within the same genus. Variation in egg laying capacity contributes to false interpretation in FECRT (McKenna, 1994), particularly if bulk larval cultures are not conducted.

Post treatment sampling is crucial when it comes to the interpretation of the results. It is advisable to undertake sampling 7-10 days post treatment (McKenna, 1994). Sampling too soon will confuse the interpretation, since there is often a temporary reduction in fecundity of the worms that were not killed. Sampling after 14 days post treatment will also confuse the interpretation, since new

infections may be contracted from the environment and become patent from about two weeks. Therefore, sampling both too early and too late is not advisable because it is difficult to establish the source of the variation during interpretation.

The weakness of the FECRT is that it may fail to detect low levels of resistance (McKenna, 1997). Studies have shown that when gene frequency for resistance is low, resistance may not be detected with a FECRT (McKenna, 1990; Coles *et al.*, 1992; McKenna, 1997). However, despite all of the above-described discrepancies, the FECRT is accepted and recommended by WAAVP as a useful tool for testing for resistance.

2.4.2 Controlled efficacy test

The controlled efficacy test is the gold standard and it is the fundamental test for efficacy of a product (Powers *et al.*, 1982; Coles *et al.*, 2006). However, the test requires a large number of animals to be killed in order to test for resistance (Le Jambre *et al.*, 1976). Animals are usually grouped into a control and a test group, with at least six animals per group. Animals are infected with L_3 about 21-28 days before they are treated with the anthelmintic whilst the control group is not treated. Alternatively, naturally infected animals can be used. Animals should be held in a facility where they cannot become reinfected after treatment. Animals in each group are slaughtered 10-14 days after treatment and the worm burden is estimated. The formula *(C-T)/C X 100* is used, where *C* and *T* are arithmetic mean of the counts from untreated and treated groups respectively.

The cut-off-point to declare resistance is an important element in the test (Coles *et al.*, 1992). It is assumed that the cut-off-point to declare the presence of AR is 95%, similar to the FECRT (Coles *et al.*, 2006). This way assumes that the anthelmintic has previously been shown to have a very high efficacy.

Although CET is a good test, issues such as the cost involved can be a limitation. Reducing the number of animals for the test in order to reduce the cost generally reduces reliability to unacceptable levels (Boersema *et al.*, 1987). In addition, other factors such as pharmacokinetic variation due to drug behaviour or physiological phenomena, differences in parasite burden and presence of arrested larvae may all confuse the results (Lacy and Snowdon, 1988). However, CET remains the most reliable test for testing the efficacy of anthelmintics and is the test to which all others are compared eventually.

2.4.3 Egg hatch assay for BZs

This is one of the first i*n vitro* tests developed to specifically test for AR in livestock and it has been used in past years as a routine test but is only generally suitable for the BZ group of anthelmintics. The test is based on the ovicidal properties of BZs. The principle of the test is to incubate eggs at 26ºC for 24hrs, in serially diluted solutions of thiabendazole, together with a control as a

correcting factor to establish the natural hatch rate of the eggs (Le Jambre, 1976; Johansen, 1989; Coles and Simpkin, 1977). Lugol's iodine solution is added when the hatched eggs are counted to kill all stages and establish the proportion of eggs that fail to hatch. The principle is that the resistant genotypes can embryonate and hatch in higher concentrations of the drug compared to the susceptible genotypes.

The limitation to EHA is that it is only suitable for the BZs and the eggs need to be as fresh as possible, preferably from faeces collected direct from the rectum. An important idea in this case is that eggs should be used not more than one hour after they have been collected (Hall *et al.*, 1978) and that they have not commenced aerobic development. However, various authors have described other situations under which an exception to the use of fresh eggs applies. For instance, Hunt and Taylor (1989) described storing faeces in ice bags or anaerobic containers whilst transporting samples to the laboratory or whilst waiting for processing useful. Anaerobic storage of faeces for up to seven days has been shown to have no significant effect on the test. This means that the test can be useful when the farm is located a distance far from the laboratory.

The eggs needed for this test are recovered from the faeces and should be as clean as possible. A variety of different methods have been described for egg recovery. A common problem for most of these methods is the potential for salt or sugar solutions to kill the eggs, due to their high osmotic pressure. Any extraneous sugar can also act as a nutrient for contaminants (Obendorf *et al.*, 1986). Therefore, care needs to be exercised when using these methods to recover eggs. Provided the eggs are thoroughly separated from faecal debris before they are used, there is little probability of any significant negative effects.

2.4.4 Egg hatch assay for LV

Unlike the EHA for BZ, the EHA for LV relies on the principle that the eggs are first incubated under controlled conditions until just prior to the commencement of hatching (approximately one hour) at which time serially diluted LV is added. The principle of this test is that the resistant strains will hatch at higher concentrations than the susceptible strains (Dobson *et al.*, 1986).

In brief, the procedure for the EHA for LV involves concentrating freshly recovered eggs, incubating them at 26ºC in micro-titre plates for 23 hours until they are about to hatch and then adding serially diluted LV. Incubation is then continued for a further 24 hours followed by snap cooling at -15ºC for five minutes, preferably by using chilled formaldehyde. Afterwards, the number of eggs hatched in the plate wells is established. The crucial issue here is the time at which it is appropriate to add the drug. Since it is difficult to accurately assess when LV should be added makes this test difficult for use in the field. It is also generally necessary to run a control culture with a known susceptible strain simultaneously with the test plates for comparison purposes, which adds to the complexity of the test.

2.4.5 Larval paralysis and motility assays

Larval paralysis and motility assays measure the ability of anthelmintics to paralyse the infective third larval stage. Therefore, the overall principle of these assays relies on the assessment of larvae motility. A variety of methods for larval paralysis and motility assays have been described. The first assays described were for LV and morantel (Martin and Le Jambre, 1979). The procedures for larval paralysis assays involve recovering the L_{3s} from faecal cultures and incubating them in a serial dilution of anthelmintics for 24 hours. The larvae are then examined using a microscope at 100X magnification, to establish whether they are normal (moving) or paralysed (not moving). The percentage of paralysed larvae is then estimated and LD_{50} values determined.

Of particular interest is the method described by Bennett and Pax (1986). These authors developed a microprocessor technology instrument to assess larval motility by measuring the light refraction at the meniscal interface, whereby the angle of light refraction entering the photodiode is altered by the larval movement. Folz *et al.* (1987; 1988) had a similar idea when they developed a different instrument to observe the differences in motility between BZ resistant and susceptible strains but it was effective to differentiate between LV resistant and susceptible strains of *H. contortus*. Sutherland and Lee (1990) described a slightly different approach for the detection of thiabendazole resistance. In their test, they paralysed the larvae in the acetylcholinesterase inhibitor eserine. They found that, larvae of resistant strains were paralysed more slowly than the susceptible strains.

Despite the potential usefulness of these approaches to measure motility to test for resistance, in all the methods, there is the limitation that too few larvae produce insufficient movement for significant effect, whilst too many larvae also move the dead ones and lead to exaggerated results.

Later, Gill *et al.* (1991) described a larval motility inhibition assay that was specific for IV. The procedures involve incubating ensheathed L_3s in a dark place on an agar matrix with serially diluted IV for 24hrs at 25ºC. Then, the larvae are exposed to sufficient light for about 1-2 minutes, which is enough to activate more than 90% of the larvae in the control wells. The larvae are examined under the microscope at 100X magnification and the non-motile larvae are recorded. The proportion of the total larvae recorded is estimated and the LD_{50} values determined.

2.4.6 Adult development assay

Unlike most *in vitro* assays, the adult development assay involves culturing eggs completely through to the adult stage, instead of only to the infective larvae. There are several reports that *H. contortus* has been cultured through to the sexually mature stage (Stringfellow (1984; 1986, 1988; Small and Coles, 1993). Latter, Taylor *et al*. (2002a) successfully cultured the larvae of other nematodes to the adult stage. However, only minor progress has been achieved in trying to develop this method to test for resistance in nematodes. Failure in this can be generally related to

the complexity of the culture technique which suggests a limitation to further development of this method.

2.4.7 Biochemical assays

Biochemical assays include those used to detect resistance through biochemical means. To date, these assays have only been used for the BZs. These assays are based on the principle that BZ-resistant strains are associated with a reduced tubulin-binding affinity for BZs (Taylor *et al.*, 2002b). One example is the measurement of the binding of tritium-labelled BZ to tubulin. This exercise relies on the high affinity of BZ for β-tubulin.

The procedure for this assay includes the incubation of tubulin extract with the tritium-labelled BZ until equilibrium is reached (Lacey and Snowdon, 1988). The free drug is removed with charcoal, leaving the tritium-BZ-tubulin complex, which is then estimated by a liquid scintillation counter. This assay is considered as being rapid, robust, highly reproducible and sensitive to minor changes in the resistance status of the parasite population, although it requires relatively large numbers of larvae. The requirement of expensive laboratory facilities and the need for well-trained personnel, limits the suitability of this test for routine fieldwork.

2.4.8 Molecular/genetic assays

These assays rely on detecting known changes in DNA, which are associated with the occurrence of AR. At present these tests are limited to detecting BZ resistance, since the changes associated with LV and ML resistance remain largely unknown.

Roos *et al.* (1990) reported investigations into the DNA polymorphism of BZ susceptible and resistant populations of the genome of larvae and adults of *H. contortus*. In this experiment, these authors used cloned α and β-tubulin genes of *H. contortus* as a probe to analyse DNA prepared from mixtures of infective larvae and adult worms. A maximum of six different fragments in a susceptible worm population were identified using α tubulin whilst only one or two fragments taken from several resistant populations from several geographical sources could be identified with the β-tubulin, indicating that BZ-resistant genotypes have an altered or reduce complement of β-tubulin compared to the susceptible genotypes. These authors described that BZ resistance is associated with an amino acid substitution of Phenyl to Tyrosine at position 200 in β-tubulin isotype gene in *H. contortus*. This change has also been noted in other nematodes. These findings suggest that molecular/genetic assays can be used successfully to detect AR for the BZs and these molecular/genetic changes can be detected with polymerase chain reaction (PCR) technology.

2.5 Diagnostic assays investigated in this thesis

2.5.1 Larval development assay

The development of the larval development assay (LDA) was a result of a prolonged period of research to develop suitable diagnostic assays. In the mid 1990s, the LDA was commercially

released as an assay to detect AR in sheep nematodes to all available broad-spectrum anthelmintics. Compared to other types of assays the LDA is a potentially very sensitive assay.

The basic principle of the LDA test is to culture eggs through to the L_3 stage in the presence of serially diluted anthelmintics. The actual time of sampling in this test is not essential, although fresh eggs could give better results (Coles *et al.*, 1988; Lacey *et al.*, 1990; Coles *et al.*, 1992). The proportions of larvae, which successfully develop to L_3 stage, are plotted against the $\log_{10}$ of the concentration of anthelmintic and a dose response curve is then developed. The LD_{50} values of anthelmintics can then be estimated and used for interpretation of the results.

The LDA technique has undergone several modifications since it was first described. Various authors have described this test using a variety of different culture media, and using different types of apparatus for the cultures, as well as various ways to recover eggs. All of these changes were attempts to make this assay more reliable.

Ibarra and Jenkins (1984) were the first to describe an LDA using the free-living stages of trichostrongylid nematodes. Since trichostrongylid larvae normally feed on bacteria, these authors used one part of distilled water to one part of a diluted suspension of rat faeces. Rat faeces contain high proportion of bacteria, hence good source of bacteria to feed the larvae. The test was conducted in 1.5ml glass wells with diameters of 8mm. The cultures comprised of 0.2ml of egg suspension containing about 100 eggs plus 0.16ml of rat faecal suspension, and in addition to it, 0.04ml of diluted drug. The glass wells were incubated for four days at 25ºC. It was observed that, with enough oxygen supply, approximately 92% of the eggs developed through to L_3 in the control wells. At about the same time, Hubert and Kerboeuf (1984) described another LDA protocol, which used yeast and Earle's salt solution as a nutritive media but the progress of this particular protocol were less successful.

Following the earlier report of Ibarra and Jenkins (1984), Coles *et al.* (1988) developed a similar assay in order to detect resistance to the BZs. These authors used a uracil-mutant isolate of *E. coli* bacteria, which had been grown in a nutrient broth. They heat-treated these *E. coli* and used them as a nutrient source, instead of rat faeces. An unspecified number of eggs were added in multi-well plates and incubated at 22ºC for 24 hours. After this incubation time, a maximum of 5µl of anthelmintic was added to each well. It was observed that the development to L_3 in the control wells was about 64%. The only problem in this assay was the growth of contaminant micro-organisms in some of the wells. This problem suggested further modifications were required such as sterilising the eggs in some way before using them, or the use of lyophilised *E. coli* rather than a nutrient broth. These suggestions paved a way for other authors to improve the LDA described by Coles *et al.* (1988).

Apart from this assay used to detect resistance to BZs, Giordano *et al.* (1988) attempted to use the same protocol described by Coles *et al.* (1988) to detect resistance to IV but the results were not very successful.

Further publication using LDA did not occur until in the early 1990s when Taylor (1990) described another variation using yeast extract and Earle's balanced salt solution. The difference with this particular protocol to that of Giordano *et al.* (1988) was that the eggs were first hatched in a volume of 1ml in a tube at 27ºC, before being placed in a 5cm diameter petri dish with a depth of 0.5cm agar. Then, 0.99ml nutritive medium and 10µl of anthelmintic solution were added and incubated at 27ºC for a further six days. It was found that the overall egg development was below 50% and the LD_{50} could not be determined for all three classes of anthelmintics. In this case, the authors described the results as a minimum inhibitory concentration (MIC), which is the minimum drug concentration required to completely inhibit larval development. Similarly, in a sequence of trials, Hong *et al.* (1992; 1996) used this protocol, (Giordano *et al.* 1988) and that described by Coles *et al.* (1988) to report MICs and to apparently successfully interpret their results.

At this same time Hubert and Kerboeuf (1992) once again modified their previous assay, by the use of *E. coli* instead of yeast extract and Earle's salt solution. The assay was conducted in 5ml tubes with 11mm diameters. About 20µl of nutrient media and 60-80µl of egg suspension, with approximately 100 eggs, plus 20µl of *E. coli* suspension were added. After incubation at 23ºC for 48hrs, 50µl of anthelmintic was added into the tubes. The overall hatching was about 85% and a linear relationship was observed, which then allowed for an LD_{50} to be determined. These reports were encouraging and as a result, Amarante (1994) used a similar technique and achieved reliable dose response curves for all three classes of anthelmintics whilst Maingi *et al.* (1996) using a similar technique, managed to successfully carry out a survey of anthelmintic resistance in Denmark.

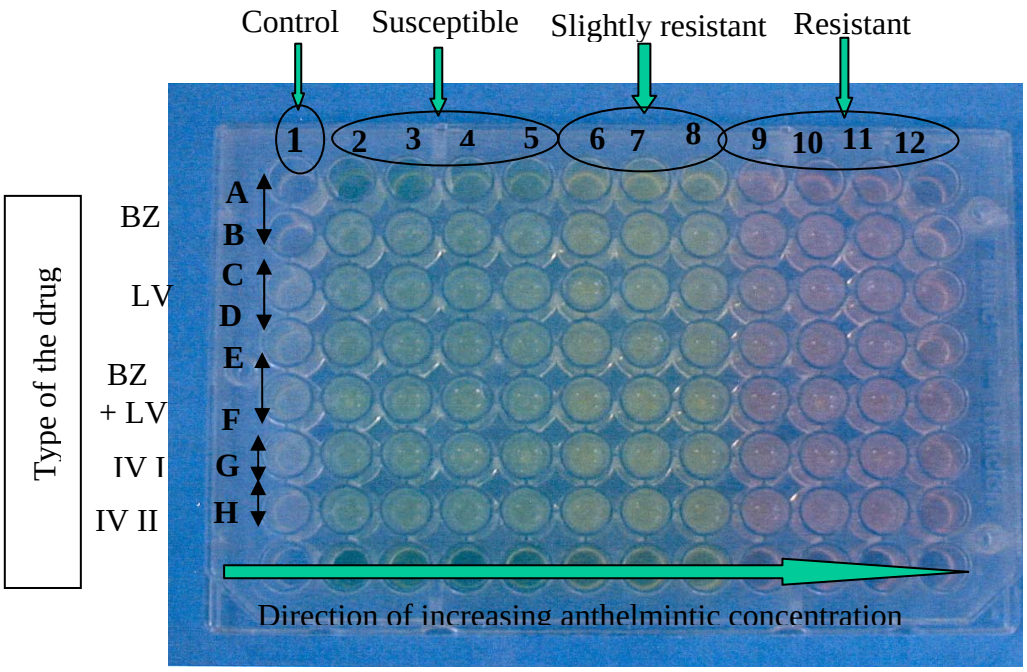
Subsequent to these favourable achievements, several other authors were encouraged to develop a more reliable assay. Gill *et al.* (1995) successfully developed an assay for the three anthelmintic groups. The assay was carried out in 96-well microtitre plates with 200µl of 2% agar matrix. Approximately 20µl of egg suspension with approximately 80 eggs plus serially diluted anthelmintics were added to each well, followed by incubation at 25ºC. On the next day, 20µl of yeast extract nutritive media in a ratio of 9:1. (9 parts yeast extract and 1 part Earle's balanced salt solution) was added into the wells followed by further incubation for six days.

Following the work of Gill *et al.* (1995), a commercial kit for AR detection in sheep and goat nematodes was made available. The kit, called standard 'DrenchRite' LDA plates was produced by the Commonwealth Scientific and Industrial Research Organization (CSIRO) with funding from International Wool Secretariat and marketed by Horizon Technology Pty (1996) Limited, Australia.

Basically, the assay involves culturing the eggs in the wells in 96-well microtitre plates (see Figure 2.3). The plate has coloured zones to differentiate the four zones of interpretation in the LDA plate. The assay is interpreted by counting developed L_3 and determines the wells where there are half as many as in the control wells (effectively the LD_{50}). The first column (colourless) with no anthelmintic is the control, whilst columns 2 to 12 (with anthelmintics) are the test wells. Columns 2-5 are the green zone, indicating susceptibility, whilst columns 6-8 are the yellow zone, indicating moderate resistance and columns 9-12 are the red zone, indicating a high level of resistance. Most drugs are investigated in duplicate (see Figure 2.3), with rows A & B both containing BZ, whilst C & D contain LV and row E & F contain a combination of BZ + LV. The difference is in the last two rows, which are not in duplicate as each row contains different analogues of IV, one in the wells in row G and the other one in the wells in row H.

This commercial kit is considered to be suitable for all classes of drugs. However, the results from the two IV analogues have not been totally validated although indications are that it is able to detect IV-resistant *H. contortus*. At the time this variation of the LDA was developed, IV resistance in *O. circumcincta* was largely unknown. It has subsequently been demonstrated that this LDA cannot reliably detect IV resistance to this species in the field (Besier, 1999). There is also limited information on the ability of this commercial assay to detect AR to any anthelmintic in sheep and goats considered to be minor including the genera *Nematodirus*, *Oesophagostomum*, *Chabertia* and *Cooperia* (Besier, 1999).

Figure 2.3: A diagrammatical representation of a 'standard' 96-well DrenchRite LDA microtitre plate indicating the control wells, the three zones of judgement and the type of anthelmintics that are used in the plates including the control wells (***Note*:** This figure is not adapted)



In New Zealand, particularly at Massey University, authors such as Sharma (2004) and Johnson (2000) developed LDA methods for AR diagnosis in sheep and cattle nematodes respectively using their own prepared concentrations of drugs in 96-well microtitre plates. These types of assays are referred to as 'in-house' LDA assays. Both authors used a nutrient medium that was a combination of yeast extract and *E. coli* (ECYE). A similar nutrient media has also been used for horse nematodes at Massey University (Pomroy, personal communication). These authors recommended that in-house LDA method would be useful in developing an assay for detecting resistance in cattle nematodes in the future. In this thesis, similar types of nutrients were used and this relatively similar method is referred to as "the ECYE protocol".

The 'standard' protocol for the commercially validated LDA method (DrenchRite) has also been successfully modified and used to test for AR in parasites of animals other than sheep and goats. Vârady *et al.* (1996) developed an LDA for pig nematodes, whilst Craven *et al.* (1999) developed an LDA for horse nematodes. The absence of an established LDA for cattle most likely reflects the low prevalence of AR in cattle nematodes recorded around the world. Therefore, the development of an LDA for cattle nematodes remains a challenge.

2.5.2 Larval feeding inhibition assay

This is a relatively new technique which is still under development to detect AR. It has been used to detect resistance to ML and LV in sheep trichostrongylids and it relies on the ability of ML and LV to paralyse the pharyngeal muscles and thus prevent feeding. In the previous work, LFIA has been used successfully to test for resistance in sheep nematodes (Álvarez-Sánchez *et al.*, 2005).

The detail on LFIA is presented in Chapter Six. Briefly, this test is based on the study of the reduction of food ingested by the first larval stage (L_1), hatched by incubation at 22ºC for 24 hours and then incubated at 25ºC for a further 18 hours in serial dilutions of anthelmintics together with fluorescein-labelled *E. coli* (Jackson and Coop, 2000). Larvae are then transferred to a standard microscope slide and examined with a fluorescent microscope to see if fluorescein-labelled *E. coli* is visible in the intestine, and if seen, this indicates the larvae is still feeding. Control wells containing no anthelmintics are used for comparison. The proportion of non-feeding larvae estimated can then used to develop a dose response curve of larval feeding inhibition against the log concentration of the anthelmintic. The concentration inhibiting feeding by 50% (IC_{50}) can then be estimated.

The disadvantages of using LFIA are the difficult in identifying the L_1 stage in the cultures since larvae are not leaved to develop all through to L_3 (which is used for morphological identification) and that this assay requires access to a fully susceptible strain for comparison.

CHAPTER THREE

3.0 CYCLING *C. ONCOPHORA* IN SHEEP

3.1 Introduction

C. oncophora is essentially a cattle parasite, but evidence suggests that it can also be cycled through sheep. There are several advantages of using lambs rather than cattle to cycle *C. oncophora*: lambs are easier to house and feed, and it is easier to collect faecal samples. It also allows *Cooperia* to be cycled during the late winter and spring months when it is difficult to find this parasite in naturally infected cattle. The research documented in this chapter reports on the cycling of *Cooperia* through lambs under experimental conditions.

3.2 Methodology

3.2.1 Sampling group and the source of eggs

The targeted group was male lambs of 3-6 months old. By this age, it is unlikely they will have developed a substantial level of immunity to any parasites and males were used to facilitate faecal collection since bags attached to the rear of the animals collect faeces without any urine contamination. At different times, three groups of weaned lambs were placed in indoor pens (four animals in each group). These animals were fed hay and lucerne pellets. All animals were effectively treated with anthelmintics to remove existing infections (see Appendix 3.5) before being infected with *C. oncophora.* To confirm the success of drenching, faecal samples were collected 10-14 days post-drenching and were examined for the presence of eggs both by the modified McMaster and floatation techniques (Appendix 3.1 and 3.2).

Three sources of larvae were used. Initially, infections were with L_3 from older cultures originally collected from young cattle on Massey University's Tuapaka Farm. The history of this farm suggests these larvae would have a high level of resistance to both BZs and MLs. These cultures had been stored at about 7ºC for several months prior to use. Later, fresh L_3 were obtained from cultures collected from two farms considered to have fully susceptible *Cooperia*: Farm H and Farm BR (Rhodes *et al.*, 2006).

3.2.2 Procedure for infecting the lambs

The lambs were infected with L_3 of *C. oncophora*, 21 days post-drenching. The lambs were infected at various times with different larval doses as shown in Appendix 3.5. Larvae were administered intraruminally via stomach tube.

3.2.3 Data collection

Fresh faecal samples were collected daily starting from Day 12 post-infection (PI) in order to monitor the trend of egg production. The modified McMaster technique (Appendix 3.1) was used to estimate the egg count.

3.3 Results

The individual patterns of egg production are shown in Table 3.1. Data is not presented from several animals that were infected but did not shed eggs. For example, three lambs (No.s 524, 368 and 6245) were infected with a single dose of 30,000 larvae but did not produce any eggs. Similarly, two lambs (6285 and 6305) were infected in the first round with a single dose of 10,000 larvae each but did not produce any eggs from this challenge (see * in Table 3.1) but lamb 6285 produced eggs when infected with high dose in the second round while lamb No. 6305 still did not produce any eggs even when the dose of larvae was increased. Other lambs infected at this time successfully produced eggs. The egg counts ranged from 0-1250eggs/g. None of the lambs produced eggs prior to Day 12 PI. However, by about Day 14 PI, more than 50% of the lambs were producing eggs. Egg production was characterised into three groups (low, medium and high) with their percentages in parentheses: 0-500eggs/g (84%), 501-2000eggs/g (16%) and >2000eggs/g (0%). The maximum egg count was 1250eggs/g and was in lamb No. 6056 in the second round o infection on Day 34.

Table 3.1: The trend of egg production for *C. oncophora*, which was cycled through 3-4 months old lambs demonstrating the egg production of each animal in each single infection.

	A N I M A L I D												
	503*c* (#)	531*c* (#)	6056*a* (#)	6056*b* (#)	6064*c* (#)	6066*a* (#)	6066*b* (#)	6070*a* (#)	6070*b* (#)	6248*a* (- +)	6248*b* (- +)	6285*b* (x)*	6305*b* (xx)*
12	0	0		0	0	0	0	0	0	0	0	0	
13	0					50		0			0		
14	50	0				150		0		0	50	0	
15	0	0	0	0	0	150	0	200	150				
16	0	0				50		100		0		0	
17	0		0	0	50	150		100					
18	0		0		0								
19	50	0					0		50		50		
20		0				400		250		100		100	
21				0		50	50	150	100		50		
22												150	
23			0	500	50	500		200					
24			50		50	700		250					
25										100			
26		0									0		
27		0		700			150		0			0	
28						250		50		0			
29			0	500	0		150		0		0	150	
31										0			
32		0	0		0		350		0				
33			50		50						0		
34				1250						0			
35		50	50		150							0	
36			50	600	50								
37			50		50						0		
38		0	50				50		150				
39		100	0		0								
40		0	0	1000	0		0					50	
42		0			0						50	50	
43		0					50		250				
47												0	
49				250								100	
50							50		250				
52							150						
54												150	
55							100		100				
56				250									
57				0									
58												100	
60				50									

KEY:

a = 1st round data

b = 2nd round data

* = see Section 3.3

DPI = Days post infection

ID = Identity

The number of larvae used to infect the lambs

(+) = Each one of this mark represent 15,000 larvae

(-) = Each one of this mark represent 10,000 larvae

(x) = Represent 37,750 larvae

(xx) =Represent 22,250 larvae

(#) = Represent 30,000 larvae

(*c*) = Infected only once

3.4 Discussion

Although not intended as a specific experiment, these studies give some indication of the success rate of cycling *Cooperia* larvae through sheep. Weaner lambs were used in this experiment, as they were considered unlikely to have any effective anti-parasite immunity, which might otherwise compromise the establishment of infections (Donald and Waller, 1982). The hypothesis was that lambs would readily accept infections of cattle *Cooperia* because of their low immunity; however, this was in fact not the case. The majority of the lambs were infected twice and only produced eggs successfully in the second round. However, the infective dose in the second round was higher and not in divided doses and these larvae was from fresh cultures whilst in the first round they were from older cultures preserved in the refrigerator for some months prior to being used. McKenna (1985), Donald and Waller (1982) and Wagland *et al*. (1984) have also described that at high dose of L_3s the effectiveness of the infection tend to be high and better results are achieved when fresh larvae are used.

As indicated by the egg counts, the established burdens were generally small and tended to persist for about 60 days. Overall, using the criteria for egg count interpretation by McKenna (1987) and McKenna and Simpson (1987), this level of egg production can be considered low at all times during the infection. Although the size of the sampling group was small, using the standards described by these authors, most animals (84%) were in the low egg production category and very few (16%) were in the medium category, whilst none were in the high egg production category (>2000eggs/g).

A possible explanation for this difference in performance between stored and fresh infective larval stages relates to the potential effect of prolonged storage at cold temperatures on the larvae. It is possible that some larvae may have been induced to become inhibited in their development within the lambs as has been described in cattle during autumn and winter (Bisset 1983). Since, no animals were killed for worm counts to examine for the presence of inhibited larvae this cannot be confirmed. Alternatively it is perhaps likelier that the larvae simply failed to establish in good numbers.

Morphologically, the majority of larvae recovered from these animals were identified as *Cooperia,* but in two of the lambs with higher egg counts, *Haemonchus* species was also isolated. **NB**: larvae from these lambs were not used for any subsequent experiments. The finding of *Haemonchus* species does indicate that the sheep may have contracted extraneous infections from an unidentified source. This also suggests that, although larvae were confirmed as being morphologically consistent with the genus *Cooperia*, infection from species other than *C. oncophora* might have occurred. Since no lambs were killed in order to allow the adult worms to be speciated, this cannot be ruled out, but is nevertheless unlikely.

Another explanation for the low egg counts may be that *C. oncophora* are not as fecund when not in their normal host. Shock (1976) described that in cattle *C. oncophora* can produce between 100-400 eggs/worm/day/worm. There is as yet no comparative information on egg production by this species in sheep.

Although not formally planned as an experiment in its own right, this compilation of egg count data provides some insight into the success rate of infection in sheep with *Cooperia* from cattle. In general, sheep do not seem to be very permissive of *Cooperia* of cattle origin, which is generally consistent with observations in the field. Nevertheless, the main purpose of this work, to provide a source of eggs for subsequent experiments, was served satisfactorily.

CHAPTER FOUR

4.0 OPTIMIZATION OF LARVAL DEVELOPMENT ASSAY FOR USE WITH *COOPERIA* FROM CATTLE

4.1 Introduction

The principle of the larval development assay has already been discussed in previous chapters. In summary, the LDA involves culturing nematode eggs in a nutritive medium, layered over an agar gel that contains anthelmintics in 96-well microtitre plates (Gill *et al.*, 1995). The experiments described in this chapter sought to establish a relationship between different nutrient media, the incubation temperature and the number of eggs that successfully developed to L_3 in the wells. A key part of the assay was a consistent distribution of eggs into the wells. Poor egg distribution creates discrepancies and hence false interpretations. In order to develop a technique to ensure consistent egg distribution into the wells eight experiments were conducted but not reported in this thesis.

4.2 Experiment I: THE USE OF AGAR AND MEDIA ON LDA

4.2.1 Introduction

Agar is principally included in the LDA as a suitable matrix for the inclusion of anthelmintics. Comparison has previously been reported on the use of agar for the growth of larvae (Gill *et al.*, 1991) against not using agar (Giordano *et al.*, 1988). Johnson (2000) also compared both these approaches and reported that there was no significant difference between using and not using agar, but nevertheless recommended the use of agar. However, in those reports, only non-sterilised agar was used.

In two different previous studies, Johnson (2000) did not use sterilised media, whilst Sharma (2004) used sterilised media that was then stored at -20ºC prior to use. The impact of using or not using sterilised media was not discussed in either report. It is likely that there are no published reports regarding the use of sterilised versus non-sterilised agar or media when undertaking an LDA. Hence, it was considered important to establish whether any differences occur. Therefore, an initial experiment was undertaken to investigate the use of sterilised versus non-sterilised agar or media for the successful culturing of eggs in an LDA.

4.2.2 Aim

The aim of this experiment was to investigate and establish whether there are differences between the use of either sterilised or non-sterilised agar or media in the LDA.

4.2.3 Methodology

Source of eggs

Eggs for this experiment were obtained from 24, 4-6month old sheep divided into two groups of 12 animals each. They were housed and infected with either *H. contortus* or *Teladorsagia circumcincta* isolates respectively. Twelve fresh faecal samples were collected, six from each group. A modified McMaster egg counting method was used to estimate egg counts and the details are presented in Appendix 3.1. The decision to use sheep nematodes in this experiment was due to the unavailability of cattle nematodes at the time of running this experiment and that the principle being examined was likely to be independent of species.

Egg recovery

Egg recovery was carried out as described by Sharma (2004) and a detailed procedure is described in Appendix 3.3. In brief, a pooled faecal sample from two animals with higher egg counts was suspended in water and slurry sieved through a 250µm, 100µm and finally on a 20µm aperture mesh. The obtained slurry containing the eggs was then centrifuged at 1500Xg for seven minutes in a test tube, with 10% and then a 25% sucrose solution layered on the top. Eggs were recovered from the interface of the two sucrose concentrations, washed thoroughly using deionised water over a 20µm sieve and then transferred to a clean centrifuge tube using a jet of deionised water from a squeeze bottle. The tube was then left to stand on a rack to allow the eggs to settle. After 20-30minutes, the supernatant fluid was discarded by aspirating it with a vacuum system, thus leaving clean eggs in approximately 5–10mls of water at the bottom of the tube. The number of eggs contained in the tube was then estimated by finding the average of 10 counts of 20µl aliquots, using 100X magnification with a compound microscope.

Experimental design

Two protocols involving different culture media were compared in this experiment: the *E. coli* + yeast extract (ECYE) protocol and the commercial DrenchRite (DR) assay/Protocol. For each protocol there were two categories of agar (sterilised or non-sterilised) and two categories of media (sterilised or non-sterilised). Each variation was compared for *Haemonchus* and *Teladorsagia*. At each time point, counts were made from three wells for each preparation of agar, protocol and species. Larval counts were made daily for eight days. All cultures were undertaken at 25ºC, and each variation was accommodated in a separate 96-well microtitre plate.

Preparation of agar and media

Sterilised agar was made by mixing while heating 2g of agar powder with 98mls of distilled water. Whilst still liquid, 100µl of the agar was dispensed into each well (see Appendix 4.1a). Sterilised agar was made by autoclaving the plates after filling. Non-sterilised agar was used without autoclaving the plates.

For the ECYE protocol, the nutritive media was made from yeast extract and *E. coli* at the standard concentration as used for sheep parasites. For the DR protocol, the media was as recommended for the commercial assay with sheep parasites. This process involved preparation of yeast extract nutritive media by mixing yeast extract with Earle's balanced salt solution (EBSS) at a ratio of 9:1 (9mls of yeast extract and 1ml of EBSS). A detailed procedure is described in Appendices 4.1a and b. Sterilised media was made by autoclaving the universal bottles with the media before they were used while the non-sterilised media was used immediately after preparation.

Preparation of the cultures

All cultures were undertaken in 96-well microtitre plates containing 100µl of 2% agar but with no anthelmintics. These kinds of plates are described as in-house LDA plates as is also described in Section 2.5.1. The egg suspension was adjusted to 100egg/100µl of water, which included 25% Amphotericin at a final concentration of 0.15% (w/v) for the ECYE protocol and 80eggs/20µl for the DR protocol. For the ECYE protocol, a total of 100µl comprising 60µl of egg suspension plus 40µl of nutritive media was added to each well on top of the agar. For the DR protocol, 20µl of egg suspension was added to each well with 20µl of yeast extract nutritive media added on Day 2 of incubation when hatching was about 80%.

Data collection and analysis

The plates were read each day for eight days and the numbers of eggs and of each larval stage were recorded. To assess the effect of using either sterilised or non-sterilised agar or media in both the ECYE and DR protocols for growing *Haemonchus* and *Teladorsagia*, the number of eggs that reached L_3 in both species was compared.

For counting, the contents in the wells were transferred to a microscope slide and a few drops of Lugol's iodine were added to kill the larvae and a cover slip applied on top. The slides were then examined at 100X magnification. Killing the larvae facilitates counting as there will be no movement of larvae that will confuse the counting process. The difference between the normally dead larvae and the larvae killed by Iodine was that the larvae killed by iodine were fresher and almost all the expected morphological features were visible while the dead larvae had lost their morphological appearance.

For analysis, the proportion of the L_3s on Day 8 was first ArcSin transformed, as the data were a proportion and not continuous data. The software computer programme SAS was applied to process the data. The model included in the analysis included the main effect of species, agar (sterilised or non-sterilised), media (sterilised or no-sterilised) and the protocols used.

4.2.4 Results

The detailed raw data is presented in Appendices 4.2a and b. The numbers of eggs in the sheep faecal samples were estimated using the modified McMaster technique. For *Haemonchus* species,

egg counts ranged from 7,600-26,950eggs/g with an average of 9,992eggs/g whilst those for *Teladorsagia* species ranged from 400–14900eggs/g with an average of 3,342eggs/g.

The numbers of eggs distributed into the wells were about 51 (46-55) and about 47 (42-55) eggs per well for *Haemonchus* and *Teladorsagia* respectively, for the ECYE protocol. The actual number of eggs hatched in the wells in ECYE protocol for *Haemonchus* was 39–67eggs/well with an average 46eggs/well (90%) and for *Teladorsagia* was 33–59eggs/well with an average 45eggs/well (96%).

For the DR protocol the numbers of eggs actually added to each well were 88 and 55eggs/well for *Haemonchus* and *Teladorsagia* respectively. The actual number of eggs hatched in the wells for *Haemonchus* was 40–96eggs/well with an average 81eggs/well (92%) and *Teladorsagia* was 40–65 with an average 50eggs/well (91%). Therefore, hatching rate was considered good for both protocols.

The summarised results of the analysed data are presented in Table 4.1 below. It can be seen in the table that the Least Square (LS) means of the number of L_3s of *Haemonchus* was significantly higher than that of *Teladorsagia* (0.75–0.87 and 0.69–0.81 respectively). Sterilised and non-sterilized agar did not differ significantly in their LSmeans (0.71-0.84 and 0.72–0.84). Also, sterilised and non-sterilized media did not differ significantly (0.74–0.85 for sterilised media against 0.70–0.83 for non-sterilised media).

Table 4.1: A summary of the back transformed least square means of the proportion of the eggs which developed to the L_3 stage for *H. contortus* and *O. circumcincta* from sheep using the ECYE and the DR protocols with sterilised and non-sterilised agar and nutritive media.

Dependent variable	Independent variable		LSmeans			Probability (P)
			Transformed	Lower limit	Upper limit	
Ensheathed 3rd larval stage (L_3)	Species	*Haemonchus*	0.81	0.75	0.87	0.03
		Ostertagia	0.76	0.70	0.81	
	Agar	Sterilised	0.78	0.72	0.84	0.35
		Non-sterilised	0.79	0.72	0.84	
	Media	Sterilised	0.80	0.74	0.85	0.27
		Non-sterilised	0.77	0.70	0.83	
	Protocol	DR	0.84	0.78	0.89	0.0006
		ECYE	0.72	0.65	0.78	

4.2.5 Discussion

This experiment analysed the effect of using sterilised or non-sterilised agar and media in cultures with two different protocols and two species of parasites. The parameters of interest were the number of eggs that developed through to the L_3 stages. It is important to note that only the least

mean squares and probability was used to interpret the results assuming that other factors were constant. The detailed results presented in Table 4.1 show that species (P=0.03) and protocol (P=0.0006) had significant effects on the growth of eggs in the culture, this suggests that each species grow differently when subjected to different media or protocols.

The difference in larval development between DR and ECYE protocol (P=0.0006) may relate to the concentrations of the media used or to the different volumes of media used in these two protocols (20µl in DR against 100µl in ECYE). The DR protocol uses a smaller volume of media than the ECYE protocol and this may facilitate a better oxygen uptake in the wells which is a very important factor in egg hatching and development. These results are similar to the explanation by Coles *et al.* (1992) and Johnson (2000).

Generally, hatching and development for ECYE and DR protocols varied between the two species with *Haemonchus* being 90 and 92% respectively whilst for *Teladorsagia* it was 96 and 91% respectively. However the 25ºC culture temperature probably didn't suit *Teladorsagia* as well as it did *Haemonchus.* The main reason for this variation may be the differences in the preference in the temperature requirements between these two species. *Teladorsagia* prefers to grow at temperatures lower than 25ºC whereas *Haemonchus* prefers higher temperatures. Abdalla and Pomroy (2006) reported that 17ºC is the optimal temperature for development of *Teladorsagia circumcincta*.

Although not parameters of interest in this experiment, the effect of the number of days incubation on egg development and of the interaction of agar, media and protocol were also analysed and both were significant (P=0.0001).

With regards use of sterilisation, these results indicated that there was no significant difference between using sterilised or non-sterilised media or agar. This indicates that either sterilised or non-sterilised agar or media can be used. In conclusion, these findings suggest therefore that, any of the two categories of agar or media can be used to achieve the desired growth. However, it is important to note that if the media or agar is to be stored for a long time before being used, there is a need to be sterilised before storage to avoid growth of microbes.

4.3 Experiment II: - COMPARISON OF DIFFERENT TEMPERATURES BETWEEN THE ECYE AND DR PROTOCOLS

4.3.1 Introduction

The larval development assay involves many components. To optimise this assay for *C. oncophora*, a systematic approach is required. In this first experiment, the traditional culture temperature of 25ºC was compared with a lower temperature to determine if this simple change enhanced the

proportion of eggs developing to L_3. Subsequent experiments then examined variations to the media used in both the ECYE and DR protocols.

4.3.2 Aim

The aim of this experiment was to initially determine if the fundamental problem in culturing *C. oncophora* from cattle was the temperature of incubation.

4.3.3 Methodology

Source of eggs

Four fresh bovine faecal samples from young cattle 5-6months of age from Massey University's Tuapaka Farm were received in the laboratory for this experiment. The modified McMaster egg counting technique (as described in Appendix 3.1) was used to estimate the number of egg/g in faeces.

Egg recovery

Egg recovery was carried out as described in Section 4.2.3 and detailed in Appendix 3.3. This was recommended by Sharma (2004). About 30g of the sample was weighed out from the pooled (bulk) faecal sample and processed as described in Section 4.2.3.

Bulk cultures and L_3 collection

The four faecal samples which were taken from cattle from the field were assumed to have mixed infections. A comparative bulk culture was made in order to identify the genera present to establish the proportion of *Cooperia* eggs in the faeces being assayed. Faeces were mixed with vermiculite and cultured at 25ºC as described in Appendix 3.4a and larvae collected by Baermann's technique as described in Appendix 3.4b.

Experimental design

Two LDA protocols were used in this experiment: the ECYE and DR assays. Two temperature environments were prepared at 18ºC and 25ºC. An incubator was used to culture at 18ºC whilst a culture room maintained at 25ºC was used. The latter is the recommended temperature for the commercially available DR assay. Wells were assessed daily for larval development from Day 2 to Day 9. Six replicates per day were examined for each protocol at each temperature. The first six rows of the wells in each plate were used for the ECYE protocol, whilst the following six rows were used for the DR protocol

Preparation of media

In relation to the ECYE protocol, the nutritive media was made from yeast extract and *E. coli* at standard concentrations as used for sheep parasites including Amphotericin B at a final concentration of 0.15% (w/v). For the DR protocol, the media was as used in the commercial assay with sheep parasites. This media includes preparation of yeast extract nutritive media by mixing

yeast extract with Earle's balanced salt solution (EBSS) at a ratio of 9:1 (9mls of yeast extract and 1ml of EBSS). A detailed procedure is described in Appendices 4.1a and b.

Preparation of cultures

Prepared in-house plates were used. These plates comprised 100µl of 2% agar per well in 96-well microtitre plates. No anthelmintics were added. For the ECYE protocol, 60µl of egg suspension (about 60eggs/well) plus 40µl of nutritive media were added to each well. For the DR protocol, 20µl of egg suspension (about 60eggs/well) plus 20µl of nutritive media (yeast extract nutritive media only) were added. The nutritive media for the DR protocol was added into the wells on the second day. A detailed procedure is as described in Section 4.2.3 as well as in Appendices 4.1a & b.

Data collection and analysis

The contents of six wells for each protocol were removed each day and pipetted onto a microscope slide and examined as described in Section 4.2.3. The number of undeveloped eggs and those which successfully hatched, the numbers of each larval stage including those which finally reached the infective stage (L_3), together with the number of dead larvae were recorded. These L_3s were then corrected for the proportion of the *Cooperia* species present in the bulk cultures which was assumed to be constant for each stage.

The number of eggs distributed in the wells was estimated by placing an aliquot of the egg suspension onto a microscope slide at intervals for counting. In addition, 10 wells in each plate were sacrificed and the number of eggs each contained was immediately assessed soon after the plating process was over.

4.3.4 Results

Egg count estimates ranged from 450–900eggs/g with an average 650eggs/gram. The genera isolated in the bulk culture were *Cooperia* (97%) and *Ostertagia* (3%). The average number of eggs in the wells in the ECYE protocol was estimated about 56eggs/well whilst for the DR protocol it was about 50eggs/well. The hatch rate for both protocols was above 50% with ECYE protocol ranging from 74-99% and DR protocol ranging from 74-97%. Development at 18ºC was slower than at 25ºC. Approximately 50% of eggs developed to L_3 in both protocols at 25ºC but the death rates were also high. Development to L_3 at 18ºC was slower and very few larvae had reached L_3 stage by Day 9. The data for the comparison of the percentage of *Cooperia* eggs developed to L_3 for the ECYE and DR protocols in this experiment are shown in Table 4.2.

Table 4.2: The comparison of the percentage of *Cooperia* eggs developing to L_3 for the ECYE and DR protocols at 18ºC and 25ºC (Percentages corrected for the results of the bulk culture).

	ECYE protocol												DR protocol											
	18ºC						25ºC						18ºC						25ºC					
Day	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
2	96	0	0	0	0	96	94	0	0	0	0	94	95	0	0	0	0	95	77	15	0	0	0	92
3	91	0	0	0	0	91	68	26	0	0	0	94	33	61	0	0	0	94	3	91	0	0	0	94
4	11	69	0	0	0	80	6	13	62	0	0	81	5	23	0	0	0	28	1	4	67	0	0	72
5	7	3	74	0	0	84	2	3	76	0	0	81	2	3	79	0	0	85	1	3	80	0	0	84
6	2	0	86	0	0	88	0	0	71	0	0	71	1	1	76	0	0	78	0	0	73	0	12	85
7	1	0	76	4	0	81	37	0	0	37	2	76	0	0	81	0	0	81	0	0	48	2	27	78
8	0	0	68	8	0	76	0	0	1	42	40	83	1	0	71	2	0	74	0	0	0	31	36	68
9	0	0	69	1	1	71							0	0	74	4	9	88						

Key: E = unhatched eggs; L1, L2 = 1st & 2nd larval stages; L3 = infective larval stage; D = dead larvae; T = total.

4.3.5 Discussion

Although there was some development in both protocols at both temperatures, it was generally observed that the overall success percentages of egg development to L_3 for both protocols were poor (0-1% and 0–42%) for ECYE protocol against (0–4% and 0-32%) for the DR protocol at 18 and 25ºC respectively. Although very few larvae had reached L_3 stage by Day 9, this experiment revealed that the number of days required to reach the infective stage was longer at 18ºC than at 25ºC. However, the number of deaths until Day 9 was also still low at 18ºC compared to at 25ºC and this suggested that more incubation time would have allowed for some further development to L_3. Though the percentage of L_3s at Day 8 was higher at 25ºC in both protocols, the death rate was also higher. This suggests that 25ºC is not an appropriate temperature to grow *Cooperia*. Though the lower temperature was probably more appropriate, the media solution used in these cultures may also not be ideal because of the concentration may have been higher to support egg development. Overall, the low number of L_3s recovered suggests that the media concentration should be modified. In this protocol, the number of dead larvae was added to the number of eggs that are no longer present, which had apparently disintegrated during plating.

4.4 VARYING NUTRIENTS BETWEEN AND WITHIN ECYE AND DR PROTOCOLS

4.4.1 Introduction

The standard DR protocol and the ECYE protocol have been designed differently. The ECYE protocol differs from the DR protocol in type, concentration and volume of media solution used. Whilst the ECYE protocol incorporates a mixture of yeast extract and *E. coli* bacteria to develop the nutrient solution, the DR protocol only uses yeast extract. Moreover, the ECYE protocol contain a total volume of 100µl per well, comprised of 60µl of egg suspension plus 40µl of nutrient solution

whilst the DR protocol contain a total volume of 40µl per well comprised of 20µl egg suspension plus 20µl of nutrient media.

A series of experiments to investigate changes in concentration of nutrient media in both protocols were conducted in an attempt to determine the most suitable combination of the nutrient components. The preliminary tests for both protocols were carried out in the in-house 96-well microtitre plates with no anthelmintics included in order to ascertain the most effective media concentrations. As for this experiment the decision was made to continue with a culture temperature of 25ºC. At this temperature the assay would take less time to incubate than at a cooler temperature and the results would become available in a shorter period.

4.4.2 Aim

The aim of these experiments was to compare variations in the concentrations of the different media components for both the ECYE and the DR protocols and discover which one will most successfully culture *C. oncophora* to the infective third larval stage.

4.4.3 Experiment III: VARYING NUTRIENTS IN CULTURES

4.4.3.1 Methodology

Source of eggs

The samples for this experiment were obtained from cattle from Massey University's Tuapaka Farm by walking in the paddocks and carefully collecting fresh faecal samples from the ground. A total of nine samples were collected. Egg counts were then estimated by using the modified McMaster method (Appendix 3.1) and three faecal samples with high egg counts were pooled together for egg recovery. A bulk culture was also made simultaneously, by mixing faeces with vermiculite (Appendix 3.4a) and culturing it at 25ºC in order to determine the composition of genera in the sample. Larval collection was carried out using Baermann's technique for larvae recovery as described in Appendix 3.4b.

Egg recovery

Egg recovery was carried out as previously described in section 4.2.3 and further details are presented in Appendix 3.3.

Preparation of the media solution

A checkerboard approach was used to vary the different components of the ECYE nutritive media solution and seven categories of nutritive media solutions were prepared. This approach is represented in Table 4.3.

Table 4.3: Variation in concentration of various components of the media used in the culture. The baseline 1 refers to the standard concentration used for sheep nematodes.

Media sol.		*E. coli*		Yeast
1	=	1	+	1
2	=	$1^1/_2$	+	$1^1/_2$
3	=	$1^1/_2$	+	1
4	=	1	+	$1^1/_2$
5	=	½	+	½
6	=	½	+	1
7	=	1	+	½

Experimental design

In this experiment, only the ECYE protocol was investigated and all cultures were incubated at 25ºC. Six replicates of each media category were counted at each time point with a total of 42 wells counted per day. The contents in the wells were transferred to a microscope slide and counted as described in Section 4.2.3. Counts were made every two days up to 14 days, and the number of eggs, live and dead larvae found in the wells recorded.

Preparation of the cultures

The 96-well in-house microtitre plates with 100µl of 2% drug-free agar were used. This procedure involved the addition of 60µl of egg suspension on top of the agar in each well, giving about 60eggs/well plus 40µl of the nutrient media solution from the seven categories. Amphotericin B (25%) was added to achieve a final concentration of 0.15% (w/v) in the mixture of eggs and media in each well. The plates were then incubated at 25ºC.

Data collection and analysis

Contents from six wells from each media were withdrawn every two days and the eggs/larvae were counted. Counts were corrected for the results of the bulk cultures to get the estimate of the *Cooperia* species present in the cultures. The whole counting procedure is as described in the data collection and analysis parts of Sections 4.2.3 and 4.3.3 of this thesis. The number of larvae for each well was recorded separately. Then, the averages and the percentages of the larvae that had developed in the wells for each medium were calculated in order to determine the most effective

media concentration in the protocol that had achieved the most successful growth rate. The number of dead larvae was added to the number of eggs that are no longer present, which had apparently disintegrated during plating.

4.4.3.2 Results

The egg count estimates of the nine animals range from 0-400eggs/g with an average 100eggs/g. On average about 63 eggs (ranged from 58-75) were dispensed into each well during plating. The actual results of *C. oncophora* larvae corrected to the percentage of the larvae identified in the bulk culture are as shown in Table 4.4. The bulk culture revealed that the proportion of *Cooperia* eggs in the faeces was 97% with *Ostertagia* being 3%. The raw data of this experiment are represented in Appendix 4.3. Only L_3s of *Cooperia* were identified. Unfortunately it was not possible at this time to identify if earlier larval stages also included *Ostertagia*.

The percentage of eggs that had developed to L_2 in the wells was higher compared to the percentage of eggs that matured to the L_3 stage. For instance, until Day 14, in media concentrations 1, 2, 3, 4, and 7, only 4% of the eggs had developed to L_3 whilst in media concentrations 5 and 6, only 3% had matured to L_3. This was very low compared to the percentages of L_2s, which were 45%, 54%, 42% 49% and 57% against 38% and 46%, respectively. The percentage of larvae dying ranged from 22% to 38% with media concentration 1 associated with the most deaths and media concentration 6 having the least. Death rates rapidly increased as the incubation was continued, until at Day 14 the deaths rates approximated to the percentage of L_2s that were found in the wells.

Table 4.4: The development of *C. oncophora* for each component of the seven media concentrations used in the experiment. Plates were cultured using the ECYE protocol at 25ºC. Each value represent the mean number of eggs (n=6) of L1, L2, L3 and the dead larvae corrected for the proportion of *Cooperia* in the bulk culture expressed as a percentage of the number of eggs initially added to each well.

Well	1						2						3						4					
Day	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
2	105	0	0	0	0	106	104	0	0	0	0	105	95	0	0	0	0	96	97	2	0	0	0	98
4	6	54	34	0	0	94	8	64	23	0	0	95	6	52	32	0	0	90	6	52	33	0	0	92
6	5	23	59	0	0	86	6	11	102	0	0	120	8	3	91	0	0	102	3	2	112	0	0	117
8	6	5	62	0	3	74	6	3	96	0	3	108	3	2	87	0	9	99	5	0	92	0	5	101
10	6	0	75	0	12	94	3	0	76	0	6	85	3	0	73	0	20	96	5	0	101	0	9	113
12	5	0	53	0	30	87	6	0	60	0	30	95	6	0	47	0	21	74	6	0	57	0	24	88
14	5	0	45	4	38	92	5	0	54	4	33	96	5	0	42	4	32	82	8	0	49	4	28	88

5						6						7					
E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
97	2	0	0	0	98	98	2	0	0	0	99	103	1	0	0	0	103
5	34	50	0	0	89	6	35	49	0	0	90	8	17	64	0	0	89
5	3	83	0	0	91	6	18	68	0	0	93	6	17	67	0	0	90
6	2	98	0	3	106	5	0	84	0	1	90	2	6	72	0	3	82
3	0	73	0	12	89	5	0	101	0	8	113	6	0	82	0	10	98
5	0	59	1	20	84	5	0	54	0	25	84	5	0	70	1	17	92
2	0	38	3	28	71	3	0	46	3	21	73	5	0	57	4	22	86

Key:
E = Eggs
L1, 2 = 1st & 2nd larval stages
L3 = Ensheathed larvae
D = Dead larvae
T = Total

4.4.3.3 Discussion

Generally, there was a good hatching percentage in this experiment but the number of larvae that had reached L_3 in the culture was very poor: 3–8% larvae/well on Day 14. The actual egg hatching ranged (as can be seen in Appendix 4.3) from 31–76eggs/well. However, in this experiment the actual number of eggs found in some of the wells varied from that which was intended to be distributed. The reason for the marked variation in the total number of eggs which developed or counted in some of the wells was due to inconsistency in the egg distribution due to a mechanical problem with the dispensing pipette during plating. It was also observed that few eggs were still found in the wells on Day 14. Most larvae easily developed up to the L_2 stage but were not able to mature to L_3. Even with a longer culture period, larvae were dying rather than moulting to L_3. This indicates that because of the failure to develop to L_3s and higher death rates in the wells, neither the media concentration used nor the temperature (25ºC), were appropriate to grow *Cooperia* species. Therefore, further study to determine a favourable media concentration and temperature to grow *Cooperia* was required.

4.4.4 Experiment IV: A FURTHER VARYING OF NUTRIENTS IN BOTH ECYE AND DR PROTOCOLS

4.4.4.1 Introduction

The procedure for the ECYE protocol in this experiment was similar to that in Experiment III except that in this experiment a further two media dilution combinations were included, thus making a total of nine combinations compared to the seven combinations used in the previous experiment. In addition, variations to the DR protocol were also investigated.

4.4.4.2 Aim

The aim of this experiment was to repeat the exercise in experiment III for ECYE using further dilution combinations of the original sheep protocol and to investigate the success of using dilutions in the DR protocol.

4.4.4.3 Methodology

Source of eggs

Samples for egg recovery for this experiment were obtained from the bulls from Massey University's Tuapaka Farm by walking in the paddocks and carefully collecting fresh faecal samples from the ground. A total of twelve faecal samples were collected and submitted to the laboratory for this experiment. The egg counts were estimated by using the modified McMaster method (Appendix 3.1) and faecal samples with high egg counts were pooled together for egg recovery. A bulk culture and larval recovery was carried out as before (Section 4.5.1) to determine the proportion of *Cooperia* present in this mixed culture.

Egg recovery

Approximately 50g of a pooled faecal sample from three animals with high egg counts was weighed into a separate container. The egg recovery procedure was carried as described in Appendix 3.3 and a brief description is also given in Section 4.2.3.

Preparation of media solution

A similar approach to Experiment III was used in order to vary the different components of media in the ECYE protocol and nine categories of media solutions were prepared as shown in Table 4.5.

For the DR protocol, yeast extract at ½, 1 and 1½ times the concentration used for sheep nematodes were compared in this experiment.

Table 4.5: Variation in concentration of various components of the media used in the experiment. Thebaseline 1 refers to the standard concentration used for sheep nematodes.

Media sol.		*E. coli*		Yeast
1	=	0	+	2
2	=	2	+	0
3	=	1	+	1
4	=	$1^1/_2$	+	$1^1/_2$
5	=	1½	+	1
6	=	1	+	$1^1/_2$
7	=	½	+	½
8	=	½	+	1
9	=	1	+	½

Experimental design

The design for this experiment involved the ECYE protocol and was similar to that in Section 4.4.3.1 but this time nine categories of media solutions were used, each with six replicates per time point. These cultures made up a total of 54 wells counted each day. For the DR protocol three media variations at concentrations of ½, 1 and 1½ the standard DR sheep protocol were used each with six replicates counted per time point. This made up a total of 18 wells counted each day in the DR protocol. These cultures were incubated at 25ºC and the duration of culture was 21 days.

Preparation of cultures

The 96-well in-house LDA plates with 100µl of 2% agar but with no anthelmintics were used. The preparation of the cultures for ECYE was similar to that in Section 4.3.3 and also in Section 3.4.3 except that specific media prepared for this experiment were used. Details of the preparation of the various components of the media are presented in Appendices 4.1a and 4.1b. For the DR protocol, 20µl of egg suspension was added to each well on Day 1 to achieve about 60eggs per well. Yeast extract was added on Day 2 after approximately 80% of the eggs had hatched. To achieve a concentration equivalent to ½, 1 and $1^1/_2$ times that used with sheep nematodes, volumes of 10µl, 20µl/ and 30µl/ of yeast extract were added to each well (see the procedure in Appendix 4.1a) and to ensure that the final total volume remained at 40µl the egg suspension for $1^1/_2$ media solution was concentrated into 10µl. Approximately 90µl of 25% Amphoterin B (Fungizone) per ml of egg suspension was added.

Data collection and analysis

Data collection and analysis was performed as described in Section 4.4.3.1 of this thesis. Moreover, the whole counting procedure is also as described in data collection and analysis part of Sections 4.2.3 and 4.3.3 of this thesis.

4.4.4.4 Results

The egg count estimates of the twelve animals ranged from 0-250eggs/g with an average of 21eggs/g. The actual number of eggs dispensed to each well was about 70eggs/well for the ECYE protocol and about 60eggs/well for the DR protocol. Some of the wells in this experiment particularly for the DR protocol (including a few wells in ECYE protocol) became dehydrated at some stage and therefore about 10µl of distilled water was added to those wells over the course of the experiment to prevent them becoming totally dry. The raw results of this experiment are presented in Appendices 4.4a, b, and c. The summarised data of the larval development corrected for the number of *Cooperia* isolated in the bulk culture are represented in Tables 4.6 and 4.7. The bulk culture had 97% *Cooperia* and 3% *Ostertagia* species.

It can be seen from Table 4.6 that for the ECYE protocol, at the end of the incubation period at Day 21, the success of egg development through to L_3 ranged from 20%–44%. Media 1 had the highest success (44%) followed by Media 9 which had (40%). The media with the lowest percentage of larval development was Media 8, which had 20%. In all cases, development was under 50% but still higher than was observed in Experiment III with most of the wells having a higher percentage of L_3s and a lower percentage of L_2s compared to the previous experiment. It can also be seen that the death rates were also increasing rapidly in all media concentrations from Day 9 with all wells having death rates ranging from 16%–32%, a level generally lower than that observed in the previous experiment.

Table 4.6: A summary of the development rate of *C. oncophora* for each of the nine media concentrations of the ECYE cultured at 25ºC for 21 days. Each value represent the mean count in percentage (n=6) of stages L1, L2 L3 and dead larvae corrected for the number of *Cooperia* obtained in the bulk culture.

Media	1						2						3						4					
Da	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
3	62	30	0	0	0	91	96	0	0	0	0	96	95	0	0	0	0	97	101	0	0	0	0	101
6	0	20	78	0	0	98	8	23	71	0	0	102	1	1	106	0	0	108	1	6	97	0	0	103
9	0	0	67	0	16	83	3	0	94	0	8	104	0	0	97	0	11	109	0	0	85	0	21	107
12	0	0	33	11	24	68	0	0	59	7	27	93	0	0	53	21	20	94	0	0	54	16	20	90
15	0	0	36	18	12	65	0	0	46	26	23	96	0	0	48	14	24	86	0	0	41	17	27	85
18	0	0	18	38	10	65	0	0	18	34	35	87	0	0	19	24	31	75	0	0	17	30	34	82
21	0	0	12	44	16	65	0	0	7	31	27	65	0	0	7	34	29	71	0	0	10	25	28	64

5						6						7						8					
E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
107	0	0	0	0	107	94	0	0	0	0	94	94	0	0	0	0	94	95	0	0	0	0	96
1	3	100	0	0	104	0	3	106	0	0	109	0	0	112	0	0	112	0	0	89	0	0	90
0	0	67	0	14	82	0	0	96	0	10	105	0	0	88	0	11	99	0	0	86	0	8	94
0	0	63	14	24	101	0	0	76	6	12	94	0	0	74	8	15	97	0	0	59	12	23	94
0	0	47	16	26	90	0	0	53	12	18	83	0	0	42	19	25	86	0	0	40	16	31	87
0	0	14	29	22	67	0	0	11	17	29	57	0	0	21	17	24	62	0	0	12	16	26	54
0	0	8	33	29	71	0	0	8	26	26	60	0	0	10	29	34	72	0	0	4	20	30	54

9					
E	L1	L2	L3	D	T
95	0	0	0	0	96
0	0	98	0	0	99
1	0	88	0	11	100
0	0	59	8	23	90
0	0	39	15	26	80
0	0	18	22	33	72
0	0	7	40	32	79

Key: (Table 4.6 & 4.7)
E = Eggs
L1, 2 = 1st, 2nd larval stages
L3 = Ensheathed larvae
D = Dead larvae
T = Total
Da = Day

Table 4.7: A summary of the development of *C. oncophora* for each concentration of yeast extract used in DR protocol with the percentages corrected for the bulk culture. Media 1, 2 and 3 represent ½, 1 and 1½, times the standard media concentration used in the commercial assay.

Media		1					2						3					
Day	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
3	88	2	0	0	0	91	107	2	0	0	0	107	110	4	0	0	0	115
6	0	0	121	0	0	121	0	5	107	0	0	112	0	5	126	0	0	131
9	0	0	107	0	0	106	0	0	112	0	2	113	0	0	129	0	15	144
12	0	0	49	0	15	63	0	0	81	11	30	122	0	0	81	8	36	125
15	0	0	26	34	16	75	0	0	26	28	29	84	0	0	39	44	36	118
18	0	0	5	17	16	37	0	0	20	43	34	96	0	0	25	36	34	96
21	0	3	0	36	20	61	0	0	13	49	28	91	0	0	18	27	20	65

Key: (See Table 4.6)

For the DR protocol, the death rates were high and close to the number of larvae that developed to L_3 in the wells. The percentage of larvae maturing to L_3 was increasing but the death rates were also increasing more rapidly than the L_3s could develop. Ensheathed third stage larvae were first observed from Day 12 with the ECYE protocol. Death rates were observed from Day 9 of incubation period. Higher deaths occurred with the ECYE protocol than with the DR protocol. By the end of the experiment, the DR protocol maintained a constant death rate, which was lower than that seen with the ECYE protocol. However, the data was variable and this variation suggested that there was no apparent difference between the ECYE and DR protocol in terms of death rates.

4.4.4.5 Discussion

Overall results of this experiment using the ECYE protocol showed an improvement in the percentage of L_3s developed in the wells compared to the previous experiment though the development was still minimal. The percentage developed through to L_3 for the ECYE protocol, ranged from 20-44%, which was higher than the 3–4% seen in the previous experiment. In addition, for the ECYE protocol there were two media concentrations (1 and 9) which had higher percentages of L_3 development but there was still <50% development indicating that there is scope for further refinement of the technique in order to determine the most appropriate conditions that will grow *Cooperia*. There was no apparent consistency between the two most successful media. Media 1 had no *E coli* but more yeast extract whereas Media 9 had a standard amount of *E. coli* but less yeast extract than the standard sheep media concentrations.

Using the DR protocol, larval development to L_3 ranged from 20–49%, with the highest development observed in Media 2 (49%) and the lowest in Media 3 (20%). Thus, Media 2 in the standard sheep DR protocol was the most successful of the three.

In general, Media 1 ECYE (with no EC + 2mlYE) and Media 2 DR (20µl/well) had the highest percentages of L_3 at the end of the experiment. In comparison to the previous experiment, a longer culture time allowed more L_2s to complete development to the L_3 stage, although even after 21 days there were still some L_2s present. In general the number of L_3s continued to increase up to 21 days supporting the longer incubation period but this was counteracted by the increasing numbers of dead larvae. Another factor which can't be accurately considered is the effect of concentration of media due to evaporation. In at least some of the wells there was notable evaporation requiring addition of additional water.

It was concluded that the overall percentage development to L_3 stage for both these protocols and all the variations compared was still fewer than 50% indicating that none of these media were ideal to culture *C. oncophora* although some, with lower concentrations, were arguably better than others.

4.4.5 Experiment V: VARYING NUTRIENT CONCENTRATIONS AT DIFFERENT TEMPERATURES IN ECYE AND DR PROTOCOLS

4.4.5.1 Introduction

Consequent to the improved growth rate observed in lower concentrations in the cultures in the previous experiment with the ECYE protocol, further experiments were conducted to determine an appropriate media to grow *C. oncophora* successfully. In addition, there was also a need to investigate alternative culture temperatures for both the ECYE and DR protocols instead of the only temperature used in the previous experiment (25ºC). Therefore, two incubation temperatures, 18ºC and 25ºC, were used in this experiment to establish the more favourable temperature to grow *Cooperia.*

4.4.5.2 Aim

The aim of this experiment was therefore to continue investigations to determine an appropriate media to culture *Cooperia* by further lowering the concentrations of nutritive media investigated in the previous experiment in both the ECYE and DR protocols by using two different culture temperatures.

4.4.5.3 Methodology

Source of eggs

Eggs for this experiment were obtained from young cattle of 6-8months of age from Christensen's farm, with naturally-mixed-infection with the expectation that *C. oncophora* species was involved. The samples were picked from the ground by walking around the paddocks and carefully collecting fresh faeces in individual plastic bags. Eight samples were collected and submitted to the laboratory for processing. A modified McMaster method was performed to estimate the number of eggs/g in each sample. A bulk culture was also made to identify the genera present (Appendix 3.4a). The isolated larvae were recovered by Baermann's technique as described in Appendix 3.4b.

Egg recovery

Egg recovery procedure was carried out as described by Sharma (2004). About 50 grams of faeces from a pooled faecal sample from two animals with the highest egg counts were weighed out and processed as described briefly in Section 4.2.3 and details presented also in Appendix 3.3.

Experimental design

Cultures for both protocols (ECYE and DR) were carried out in two temperature environments of either 18ºC or 25ºC. An incubator set at 18ºC was available for low temperature cultures but for the higher temperature cultures, a culture room at 25ºC was used. Temperature in the culture room was monitored using a thermometer. The incubation period was 21 days with plates examined every three days. Three replicates for each media concentration per time point were removed from the wells and counted.

Preparation of media solution

The concentrations of the components of the media that were used for the ECYE protocol are listed in Table 4.8. The original concentration based on the 'standard' protocol

Table 4.8: The concentrations of media used in ECYE protocol developed using a checkerboard approach compared to the 'standard' nutrient medium for DR protocol for sheep nematodes. The baseline value of 1 refers to the concentrations used with the standard DR protocol for sheep assay.

Media soln.		*E.coli*		Yeast	Media soln.		*E coli*		Yeast
1	=	0	+	0	9	=	$^1/_4$	+	0
2	=	0	+	$^1/_8$	10	=	$^1/_4$	+	$^1/_8$
3	=	0	+	$^1/_4$	11	=	$^1/_4$	+	$^1/_4$
4	=	0	+	$^1/_2$	12	=	$^1/_4$	+	$^1/_2$
5	=	$^1/_8$	+	0	13	=	$^1/_2$	+	0
6	=	$^1/_8$	+	$^1/_8$	14	=	$^1/_2$	+	$^1/_8$
7	=	$^1/_8$	+	$^1/_4$	15	=	$^1/_2$	+	$^1/_4$
8	=	$^1/_8$	+	$^1/_2$	16	=	$^1/_2$	+	$^1/_2$

described for sheep trichostrongylids is designated as '1' and the designated media used were variations of these. Based on the 'standard' protocol for sheep nematodes, the volume of media added to each well was 40µl (20µl *E. coli* + 20µl yeast extract, made by mixing yeast and Earle's BSS at a ratio of 9:1). As necessary, dilutions of the nutritive media were prepared by adding distilled water with each component mixed as indicated in Table 4.8. For the DR protocol,

variation of the media was achieved by increasing and decreasing the amount of yeast extract to that normally used in the commercially available DR plates for sheep trichostrongylids. This was achieved by adding 0, 10, 20 and 30µl of yeast extract to achieve concentrations equivalent to respectively 0, ½, 1, and $1^1/_2$ times the 'standard' nutrient medium in the DR protocol. To maintain the total volume at 40µl, the egg solution was concentrated into 10µl when necessary.

Preparation of cultures

The standard in-house 96-well microtitre plates with 100µl of 2% agar but no anthelmintics were used in the experiment. For the ECYE protocol, a total of 100µl was added to each well comprised of 60µl of egg suspension (to give approximately 70eggs/well) plus 40µl of the relevant nutrient solution. Fungizone (25% amphotericin B) was added to the egg suspension to give a final concentration of 0.15% (w/v).

For the DR protocol, a total volume of 40µl was added to each well. This was comprised of 20µl (or 10µl) of egg suspension (with 90µl Fungizone per ml of egg suspension) to give approximately 80eggs/well plus 20µl (30µl) of nutritive media (yeast extract only) similar to that used in the previous experiment. For the variation with no media, only distilled water was incorporated. The nutrient media for the DR protocol was added on the second day of incubation when approximately 80% of the eggs had hatched.

Data collection and analysis

Data collection and analysis was performed as described in Section 4.4.4.3 of this thesis. Counts were then corrected according to the bulk culture results to get the estimate of the *Cooperia* species present. For more details, the whole counting procedure is as described in the data collection and analysis part in Sections 4.2.3 and 4.3.3 of this thesis.

4.4.5.4 Results

The egg counts ranged from 50–350eggs/g, with an average of 444eggs/g. The bulk culture revealed mixed infection of 73% *Cooperia*, 24% *Ostertagia* and 3% *Trichostrongylus*. For the incubator at 18ºC, the temperature was constant but for the culture room at 25ºC the temperature actually varied from 22ºC-25ºC. In this experiment there was a variation in development in the cultures depending on the media concentration within and between the sixteen media concentrations. The raw data of the experiment are presented in Appendices 4.5a-d. The summarised results of larvae from cultures in different variations of media used corrected to the bulk culture results are presented in Tables 4.9a-c.

It can be seen from these summary tables that hatching in the wells were 45-91% and 17-88% for the ECYE protocol at 18ºC and 25ºC respectively and 38-83% and 28-81% for the DR protocol at 18ºC and 25ºC respectively. Although no statistical analyses were undertaken at this stage the mean results indicate hatching appeared to improve with the lower temperature at 18ºC than with

25ºC for both protocols. The percentage of hatching in the DR protocol was lower than it was in the ECYE protocol particularly at 25ºC and the death rates were also high.

The percentage of eggs developed to L_3 stage after 21 days ranged from 0–15% and 0–24% for the ECYE protocol at 18ºC and 25ºC respectively, and 0–6% and 0-9% for the DR protocol at 18ºC and 25ºC respectively. Part of the way through the experiment, it became apparent that some larvae may have been incorrectly identified as being at the L_2 stage when they were in fact at the L_3 stage. No correction was made for any discrepancy occurred since it did not apply to later time points. These cultures had a large amount of debris and undefined round bodies assumed to be spores of certain fungi and they were highly contaminated with earthworm eggs.

Table 4.9a: A summary of the variations of different media used to culture *Cooperia* using the ECYE protocol at 18ºC for 21 days showing the egg development in percentage corrected to the bulk culture.

Media	1						2						3						4					
Da	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
3	0	77	0	0	0	77	1	76	0	0	0	77	6	56	0	0	0	62	58	4	0	0	0	63
6	0	71	2	0	0	73	0	83	0	0	0	83	0	5	76	0	0	81	0	62	0	0	0	63
9	0	7	57	0	0	64	0	5	82	0	0	87	0	0	79	0	0	79	0	0	83	0	0	83
12	0	0	64	0	0	64	0	0	54	1	15	71	0	0	58	0	13	71	0	0	50	1	21	71
15	0	0	61	0	3	64	0	0	40	11	11	62	0	0	38	8	15	61	0	0	42	2	10	54
18	0	0	49	0	6	56	0	0	39	15	7	61	0	1	33	15	6	55	0	0	39	1	6	47
21	0	0	57	0	3	60	0	0	33	7	10	51	0	0	43	9	8	60	0	0	39	6	8	53

5						6						7						8					
E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
2	71	0	0	0	73	0	74	0	0	0	74	36	0	0	0	0	70	40	5	0	0	0	45
0	83	4	0	0	86	1	2	83	0	0	86	0	0	71	0	0	71	0	0	55	0	0	55
0	0	66	0	0	66	0	3	72	0	0	74	0	0	64	0	0	64	0	0	70	0	0	70
0	0	65	0	5	70	0	0	55	1	12	68	0	0	49	0	11	61	0	0	53	0	21	74
0	0	57	0	6	64	0	0	42	5	16	63	0	0	46	3	13	63	0	0	28	4	26	58
0	0	51	2	6	58	0	0	32	13	15	60	0	0	34	14	16	63	0	0	31	8	22	60
0	0	51	0	11	62	0	0	37	11	18	66	0	0	41	10	9	60	0	0	40	13	7	60

9						10						11						12					
E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
1	90	0	0	0	91	0	74	0	0	0	74	23	33	0	0	0	56	52	2	0	0	0	55
0	60	18	0	0	78	0	2	76	0	0	78	0	0	59	0	0	59	0	0	60	0	0	60
0	13	52	0	0	66	0	1	51	0	0	53	0	0	74	0	0	74	0	0	66	0	0	66
0	0	64	4	0	69	0	0	55	0	14	69	0	0	54	1	10	65	0	0	53	0	15	68
0	0	61	1	4	66	0	0	27	13	47	88	0	0	44	8	16	68	0	0	36	5	10	51
0	0	32	0	14	46	0	0	44	4	15	63	0	0	47	7	7	61	0	0	25	10	23	58
0	0	46	0	15	61	0	0	33	4	22	60	0	0	39	9	13	61	0	0	36	15	9	60

13						14						15						16					
E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
0	75	0	0	0	75	0	76	0	0	0	76	18	48	0	0	0	66	57	0	0	0	0	57
0	54	5	4	0	64	1	8	78	0	0	87	1	2	62	0	0	64	2	5	65	0	0	72
0	16	43	0	0	59	0	2	66	0	0	69	0	1	61	0	4	66	0	0	66	0	0	66
0	0	63	0	1	64	0	0	45	0	15	59	0	0	44	0	15	59	0	0	40	0	15	55
0	0	56	0	4	61	0	0	55	4	16	75	0	0	38	6	16	60	0	0	40	2	13	56
0	0	57	0	5	63	0	0	37	7	16	60	0	0	40	6	7	54	0	0	36	4	10	50
0	0	48	0	14	62	0	0	23	7	27	57	0	0	34	13	18	66	0	0	28	4	21	53

Key: E = Eggs; L1, 2 = 1st & 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total.

Table 4.9b: A summary of the development variations in growth rate for *Cooperia* (in %) using different media by the ECYE protocol at 25ºC for 21 days in percentages corrected for the bulk culture

Media	1						2						3						4					
Day	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
3	0	83	0	0	0	84	0	71	0	0	0	72	0	78	0	0	0	78	55	12	0	0	0	67
6	0	63	0	0	0	63	0	6	68	0	0	74	0	3	76	0	0	79	0	6	63	0	0	69
9	0	8	34	0	0	42	0	0	36	0	22	59	0	0	11	9	46	66	0	0	23	0	18	41
12	0	0	18	0	3	20	0	0	12	0	5	17	0	0	18	8	17	42	0	0	19	1	26	46
15	0	1	32	0	18	51	0	0	25	9	24	58	0	0	28	9	17	54	0	0	40	2	12	54
18	0	0	25	0	27	53	0	0	19	7	42	68	0	0	37	9	20	65	0	0	28	6	19	53
21	0	0	32	0	20	52	0	0	18	10	11	39	0	0	22	14	18	53	0	0	37	9	12	57

5						6						7						8					
E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
0	73	0	0	0	73	1	77	0	0	0	78	1	86	0	0	0	88	57	1	0	0	0	58
0	50	3	0	0	53	0	6	65	0	0	72	1	8	67	0	7	83	0	5	56	0	3	64
0	6	24	0	4	34	0	2	38	1	19	61	0	1	38	2	14	54	0	0	39	2	18	59
0	0	19	6	0	26	0	0	23	2	13	38	0	4	6	1	11	22	0	0	20	0	20	41
0	0	30	1	23	54	0	0	29	7	25	61	0	0	22	16	26	64	0	25	13	23	0	61
0	0	34	0	15	49	0	0	35	7	21	63	0	0	24	8	18	50	0	0	37	3	9	49
0	0	28	0	25	53	0	0	25	9	15	50	0	0	35	9	18	61	0	0	28	7	18	53

9						10						11						12					
E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
0	37	0	0	0	37	1	75	0	0	0	76	28	45	0	0	0	73	62	21	0	0	0	83
0	47	16	0	0	63	0	3	61	0	3	67	0	5	74	0	0	79	0	3	64	0	5	71
0	7	29	0	12	48	0	0	56	2	11	70	0	0	41	0	15	56	0	1	41	0	19	61
0	0	15	0	8	23	0	0	38	1	5	44	0	0	33	5	7	44	0	0	32	1	26	59
0	0	42	1	25	68	0	0	35	7	23	64	0	0	23	25	20	68	0	0	34	9	9	51
0	0	24	0	28	52	0	0	23	7	27	58	0	0	25	10	20	55	0	0	23	15	11	49
0	0	14	0	17	31	0	0	26	6	32	63	0	0	25	16	9	50	0	0	20	24	17	61

13						14						15						16					
E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
3	45	0	0	0	48	0	83	0	0	0	83	50	35	0	0	0	85	68	2	0	0	0	70
0	33	10	0	3	45	0	2	69	0	3	75	0	9	70	0	1	80	0	1	63	0	3	67
0	8	20	0	9	37	0	3	47	0	17	66	0	0	42	0	22	64	0	1	60	0	12	74
0	0	29	0	8	37	0	0	25	1	15	41	0	0	20	0	12	32	0	0	28	2	15	44
0	0	28	0	12	41	0	0	34	9	15	58	0	0	44	6	18	67	0	0	26	5	16	47
0	0	14	0	13	27	0	0	35	7	19	61	0	0	37	8	17	62	0	0	23	4	15	42
0	0	5	1	9	15	0	0	30	13	19	62	0	0	32	4	27	63	0	0	35	3	27	66

Key: E = Eggs; L1, 2 = 1st & 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total.

Table 4.9c: A summary of the variation in growth rate of *Cooperia* cultured in different media at 18ºC and 25ºC by the DR protocol for 21 days showing the development in percentage corrected for bulk culture. Media 1, 2, 3 and 4 represent respectively 0, ½, 1 and 1½ times the standard concentrations of yeast extract used for sheep nematodes.

Temp	Media	1						2						3						4					
(ºC)	Da	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
18	3	0	64	0	0	0	64	0	64	0	0	0	64	0	83	0	0	0	83	0	78	0	0	0	78
	6	0	57	4	0	0	62	0	3	63	0	0	65	1	3	77	0	1	81	0	2	76	0	0	78
	9	0	10	52	0	0	62	1	3	54	0	9	68	0	5	71	0	0	76	0	1	68	0	3	72
	12	0	0	57	0	0	57	0	0	43	0	18	61	0	0	51	1	29	81	0	0	61	0	19	79
	15	0	0	45	0	4	49	0	0	47	3	11	61	0	0	44	7	19	70	0	0	35	4	27	66
	18	0	0	42	0	5	47	0	0	26	3	20	49	0	0	26	8	31	65	0	0	28	8	28	64
	21	0	0	21	0	16	38	0	0	29	5	12	46	0	0	23	6	18	47	0	0	30	6	15	51
25	3	0	28	0	0	0	28	2	70	0	0	0	72	1	73	7	0	0	81	1	52	24	0	0	76
	6	0	37	6	0	0	42	0	3	49	2	9	63	0	2	60	1	9	72	0	5	50	0	3	58
	9	0	0	38	0	4	43	0	0	53	0	18	71	0	0	24	0	26	50	0	3	28	1	16	47
	12	0	0	30	0	21	51	0	0	39	2	27	68	0	0	23	2	19	44	0	0	18	6	19	43
	15	0	0	44	0	9	52	0	0	46	5	19	70	0	0	23	6	21	50	0	0	15	4	22	41
	18	0	0	22	0	17	39	0	0	37	2	17	55	0	0	21	10	21	51	0	0	16	4	20	40
	21	0	0	31	0	5	37	0	0	13	5	22	40	0	0	24	9	31	64	0	0	29	4	25	58

Key: E = Eggs; L1, 2 = 1st & 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total.

It can be seen from the tables that the success rate for development to L_3 was lower than the previous experiment. The maximum percentage of L_3 at 25ºC was 24% for media concentration 12 ($^1/_4$ *E. coli* + $^1/_2$YE). At 18ºC development to L_3 was lower but again Media 12 had the highest development rate of 15%. The success rate for the DR protocol was also poor by comparison with the previous experiment and showed a maximum of 9% for Media 3 (standard sheep concentration). Death rates were generally low at 18ºC for the ECYE protocol with Media 12 being about 9% on Day 21. It was evident that with the ECYE protocol at 25ºC more dead larvae were found than developed L_3s.

For both protocols and at both temperatures there were more L_2 stages remaining in the wells at the end of the experiment on Day 21 than there were L_3s. However, these L_2 stages could not be differentiated as to which genera they were.

4.4.5.5 Discussion

This experiment was expected to give a clearer picture of which protocol and which media concentration would be more appropriate to grow *C. oncophora*. Overall, the ability of eggs to hatch was similar to the previous experiment; however, the success rate with the ECYE protocol was generally lower than the previous experiment at 25ºC where the concentrations of nutrients were higher. The reasons for these variations were not clearly established. At times, the temperature in the culture room varied and was lower than 25ºC but the overall effect of this is not likely to be great in itself.

In some of the wells particularly those with no EC or YE (Media 1) and those with very little EC or YE were noted to have no or had few L_3s recovered, suggesting that the reduced supply of nutrients limited larval growth. Media 12 ($^1/_4$ EC + ½ YE) had a better rate of development of L_3s (24%), but also had a relatively high death rate. However, these results were different to those of Johnson (2000) who previously suggested that at 25ºC media concentration $^1/_8$ EC + ¼ YE (equivalent to Media 7 in the present experiment) was superior to the other combinations tested.

In the present experiment, death rates were generally low compared to the previous experiment and presumably some of these larvae were *Ostertagia* and *Trichostrongylus*, since none of these were observed to develop to L_3 within these culture conditions. Overall, it is apparent that 21 days was not a long enough time for any of these protocols.

The high contamination of cultures with earthworm eggs and certain kind of fungal cells suggests this could be one reason for the failure of the eggs/larvae to develop well since contamination can prevent the development of eggs due to inadequate oxygen supply in the cultures. In addition, the release of by-products from contaminating micro-organisms can also potentially kill the larvae. In turn, the presence of contamination tended to increase the time taken for counting each well and even made the identification of larval stages more difficult, particularly for L_1 stage at Day 2. The results in Day 2 had a slightly higher number of larvae than the subsequent days suggesting that some of these may have been earthworm larvae. However, the extent to which this contamination contributed to the lower development rate and even the death rate at this stage has not been well established.

Johnson (2000) had previously recommended the equivalent of Media 7 as superior to the other dilutions compared at that time. In the current experiment, this particular media combination was not obviously better than others at either 18ºC or 25ºC.

In relation to the time of adding the nutritive media, Hubert and Kerboeuf (1984) described that adding eggs and media on the same day resulted in a low hatch rate. In the present experiment the hatch rate was generally high, even though the media and the eggs were added together on Day 0 suggesting that this was not a problem with these variations in the time of adding the media in ECYE protocols.

The results for the DR protocol for this experiment are somewhat at odds with the previous experiment. The results for Media 2, 3 and 4 in the current experiment (Experiment V) should be directly comparable with Media 1, 2, and 3 respectively in the previous experiment (Experiment IV). However, the success rate of development to L_3 by Day 21 is slightly lower. For instance, the standard sheep concentration in the current experiment at 25ºC had only 9% *Cooperia* developed to L_3, compared to 49% in the previous experiment. There was no one obvious reason for these differences. It has already been noted that there was more particulate matter in the egg solution in

the current experiment, which may have allowed more contaminants, and these, then delayed development in some wells.

The typical feature of this experiment was the slow rate of maturation of the L_2 to the L_3 stage in the ECYE protocol, particularly at 18ºC. Nevertheless, the decision was made to use Media 7 and 12 at 18ºC in the ECYE protocol for the subsequent experiments.

CHAPTER FIVE

5.0 COMPARISON OF THE SUSCPTIBLE AND RESISTANT STRAINS OF *C. ONCOPHORA* FROM THREE FARMS IN NEW ZEALAND

5.1 Introduction

Based on the previous work discussed in Section 4.4.5.5, Media 12 (i.e. $^1/_4$EC + $^1/_2$YE) cultured at 18ºC appeared to be more successful in supporting the growth of *C. oncophora* than other media. It was now timely to extend this development of the LDA for cattle by incorporating anthelmintics and explore whether dose response curves could be calculated for different anthelmintic groups. Media 7 (i.e. $^1/_8$EC + $^1/_4$YE) in the previous experiment was not clearly better although it did show the ability to support the growth of *Cooperia*. Yet, Johnson (2000) commented that a media with equivalent components to Media 7 was more successful in supporting the growth of *C. oncophora* than other media compared at that time. Therefore it was proposed to run an experiment using the ECYE protocol with both Media 7 and Media 12 using two temperature environments (18ºC and 25ºC) for this research. A decision to make a comparison with the standard DR protocol as used for sheep trichostrongylids was also proposed.

This chapter reports on a comparison between resistant and susceptible isolates of *Cooperia* from cattle. The first experiment (Experiment VI) was expected to explore the ability of the commercially available DrenchRite LDA plates containing different anthelmintics to develop a dose response curve with *Cooperia* from cattle from a farm with long history of multiple resistance in this species. This experiment was to be the basis for further experiments to compare these dose response curves with those from *Cooperia* from other farms in New Zealand that are considered to have fully anthelmintic-susceptible *Cooperia.*

5.2 Experiment VI: AN LDA TEST ON RESISTANT STRAINS OF *C. ONCOPHORA* FROM TUAPAKA FARM

5.2.1 Introduction

Massey University's Tuapaka Farm is located in the North Island of New Zealand. A survey conducted in previous years showed that this farm has high levels of resistance to BZs and MLs (Pomroy, 1994b). This farm was used as the source of larvae to infect the young lambs that were used to supply eggs for this experiment (refer Chapter 3).

5.2.2 Aim

The aim of this experiment was to assess the usefulness of the commercially available DrenchRite plates with *Cooperia* from Massey University's Tuapaka farm which has a known high level of resistance to both MLs and BZ and to determine if dose response curves could be obtained for the various anthelmintic groups.

5.2.3 Methodology

Source of eggs

The *Cooperia* eggs used in this experiment were from an isolate originally obtained from cattle from Massey University's Tuapaka farm. This isolate had primarily been cycled through lamb Nos. 6056 and No. 6066. Faeces were collected from lambs using faecal bags attached to body harnesses. The bags were then removed and faeces recovered in plastic bags and submitted to the laboratory for egg counts and egg recovery for this experiment. These *Cooperia* eggs from the lambs were then used in this experiment and some larvae were also used to reinfect a second group of about 3-4 months lambs for the subsequent use. The composition of the larvae used to infect these lambs was primarily determined based on larval morphology and confirmed to be 100% *Cooperia.*

Eggs recovery

The required number of eggs for this experiment was determined and a sample of faeces to provide sufficient eggs was weighed and processed. The eggs were recovered from the faeces as previously described by Sharma (2004) and briefly described in Section 4.2.3 as well as detailed in Appendix 3.3. The remaining faeces were mixed with vermiculite and cultured at 25ºC to confirm the species composition in the samples.

Preparation of media solution

Media 7 and 12 were chosen for use with the ECYE protocol as described previously in the introduction. In addition, plates were run using the standard sheep DR protocol. Media for the DR protocol were prepared as previously described in Section 4.3.3 and more details given in Appendix 4.1a. Media used in this experiment were not sterilised but good hygiene was observed.

Experimental design

Two different ECYE protocols were used: Media 12 ($^1/_4$EC + $^1/_2$YE) and Media 7 ($^1/_8$EC + $^1/_4$YE). Two temperature categories were compared for each protocol: an incubator set at 18ºC and a culture room running at 25ºC. In addition, a plate was run using the standard DR protocol with each combination of ECYE media using 18ºC and 25ºC culture temperature. The duration of incubation was determined by differently running in-house control plates containing no anthelmintics. One plate for each media and temperature, containing anthelmintics, were simultaneously run as "test plates". To monitor the control plates, three wells were counted per day starting from Day 2. The decision to remove the "test plates" from the incubator/culture room for counting was based on the time when a maximum number of L_3s was considered to have developed in the control plates with a minimum number of deaths.

Preparation of cultures

The commercially available 96-well "standard" DR plates from Horizon Technology Pty Limited® 1996, Australia, were used with anthelmintics at their normal concentrations. The concentrations

of the anthelmintics in the DrenchRite plates in detail were: TBZ (μM) – 0, 0.010, 0.020, 0.040, 0.078, 0.156, 0.313, 0.625, 1.250, 2.500, 5.000 and 10.000; LV (μM) – 0, 0.195, 0.390, 0.780, 1.560, 3.125, 6.250, 12.560 and four replicates of 25.000; IV I (nM) – 0, 0.500, 0.970, 1.900, 3.900, 7.800, 15.600, 31.250, 62.500, 125.000, 250.000 and 500.000; and IV II (nM) – 0, 0.970, 1.900, 3.900, 7.800, 15.600, 31.250, 125.000, 250.000, 500.000 and 1000.000 (Tandon and Kaplan, 2004). The exact identification of the two ivermectin analogues has not been published. In addition, no published details are available to explain the two rows that contain a combination of BZ and LV. For the purpose of this study, this combination of these drugs was assumed to be a straightforward combination of the BZ and LV concentrations in the same columns.

Each series of anthelmintic concentrations are applied over two rows except for the IV, for which each row contains a different IV analogue i.e. IV I and IV II. Both the ECYE protocol and the standard DR protocol for sheep nematodes were undertaken, largely as previously described. For the ECYE protocol there was a total volume of 100μl in each well comprised of 60μl egg suspension, which included Fungizone (25% Amphotericin B at a final concentration of 0.15%w/v) and 40μl of nutrient solution. The eggs were concentrated so those about 70eggs were distributed to each well. For the DR protocol, there was a total of 40μl comprised of 20μl of egg suspension (aimed at distributing 80eggs/well) plus 20μl of nutrient media (yeast extract only) added on the 2nd day of incubation, equivalent to the standard DR protocol for sheep nematodes. This has previously been referred to as the reference concentration of "1".

To enhance hatching, the cultures were opened for about 5 minutes everyday as from Day 1 to Day 3, and the air gently agitated to encourage oxygen uptake into the media.

Data collection and analysis

For the control plates, three replicates per occasion for each temperature category were removed and counted daily. The contents were transferred to a microscope slide, a few drops of Lugol's iodine being added to kill the larvae and these were then examined at 100X magnification as earlier described in the data collection and analysis parts in Section 4.2.3 and 4.3.3 of this thesis as well as in the experimental design part in this section.

The test plates were removed from the incubator when the maximum number of larvae was considered to have developed to L_3 in the control plates but with a minimum number of deaths. The proportions of L_3s from the test wells with anthelmintics relative to the control wells were plotted against the Log_{10} of the anthelmintic concentration to develop dose response curves. A software computer programme SlideRite (Advanced Graphics Software USA) was used to develop these dose response curves and determine the LD_{50} values. Since the concentrations of anthelmintics in each well of the rows E and F (which was for the BZ and LV combination) were not published, the assumption was made that their concentration was in a doubling manner. However, for the case of LV, the last four wells were not doubled but each well in each replicate had 25.00μM.

5.2.4 Results

The egg count estimate of the faecal sample was 1000eggs/g. The bulk cultures revealed that 100% *Cooperia* was isolated but as there was no sheep slaughtered at this stage for worm counts, hence, it was unfortunately not possible to confirm that these were all *Cooperia oncophora*. It was determined that larval development in the control plates had reached a suitable level of L_3 with minimum deaths by Day 11 at 25ºC and Day 12 at 18ºC. Hence, the decision was made to read the test plates on Day 12 and Day 11 for 18ºC and 25ºC cultures respectively. The raw data for the control plates of this experiment is presented in Appendices 5.1a & b and for the test plates is presented in Appendices 5.2a, b, c & d. The Number of eggs dispensed in the wells was 70 and 80eggs per well for the ECYE and DR protocols respectively.

The summarised results from either Day 11 or Day 12 at 18ºC or 25ºC respectively are presented in Table 5.1 (control plates) and Table 5.2 (control wells in the test plates). It can be seen from the raw data (Appendices 5.1a and b) and (Appendices 5.2a and b) that a moderate number of L_3 stage had developed in the wells compared to the average number of eggs dispensed in the wells (70eggs/well). There was no statistical comparison done, but at this stage based on the percentage hatch and development rate, the results for the two media concentrations are discussed.

Table 5.1: A summary of egg development to the L_3 stage (in %) in the control plates for both the ECYE and DR protocols at 18ºC or 25ºC after 12 and 11 days of incubation, respectively.

Protocol/Type of media	Temp (ºC)	Overall hatching (%)	L2 (%)	L3 (%)	D (%)
ECYE Protocol	18	86	19	34	33
(Media 12)	25	93	17	30	46
ECYE Protocol	18	88	35	46	7
(Media 7)	25	77	31	30	16
DR Protocol (1st Replicate)	18	67	16	33	18
(Yeast extract only)	25	75	15	38	22
DR Protocol (2nd Replicate)	18	78	22	44	12
(Yeast extract only)	25	73	16	51	5

Key: L2 = 2nd stage larvae, L3 = Ensheathed larvae, D = Dead larvae

Table 5.1 shows that both ECYE protocols had an improved hatch rate (>80%) except for Media 7 ($^1/_8$EC + $^1/_4$YE) at 25ºC, for which hatch rate was lower (77%). Although no statistical analyses were undertaken, at this stage the results indicate that for both of the ECYE protocols, Media 7 at 18ºC had the higher percentage development of L_3 (46%) compared to the percentage of dead larvae (7%) while the hatch rate for the DR protocol was somewhat less (<78%) for both temperature variations. However, the most noted feature in this experiment was the higher percentage of L_3s that had developed in the wells than it was for the L_2 stage.

The ECYE protocol with Media 12 had fewer L_2s and a relatively high percentage of L_3s but the number of deaths were increasing, suggesting this media was not as good as Media 7 in this experiment. In general, a higher number of L_3s was reached in both ECYE protocols (though all were <50%) but the death rates were also increasing at a fast rate indicating that to incubate the cultures to a point where more than 50% of the larvae develop to L_3 but with minimum deaths is a challenge.

For the control wells in the test plates (Table 5.2), the hatching ranged from 61–91% for in both protocols being generally higher for the ECYE protocols. The number of L_3s was moderately high (20-55%) with low number of L_2s (0–29%). For the ECYE protocol, Media 12 had the higher hatch rate (91%), with reasonable high numbers of L_3s (34%) but with a high number of deaths (54%); whilst Media 7 at 18ºC had a good hatching percentage (79%), a moderately high percentage of L_2s (39%) with relatively fewer deaths (13%). For the DR protocol the cultures at 25ºC had the highest L_3s (55%) with minimum deaths (9%) but with no L_2s remaining in the wells indicating that this was the most successful protocol in this experiment. Overall the results for the in-house control plates were broadly similar to the control wells of the test plates.

Table 5.2: A summary of egg development to the L_3 stage (in %) for the control wells in the test plates in both the ECYE and DR protocols at 18ºC and 25ºC for 12 and 11 days respectively.

Protocol and type of media	Temp. (ºC)	Overall hatching (%)	L3 (%)	L2 (%)	D (%)
ECYE Protocol	18	70	30	21	19
(Media 12)	25	91	34	3	54
ECYE Protocol	18	79	39	29	13
(Media 7)	25	73	20	19	16
DR Protocol (1st Replicate)	18	69	38	18	14
(Yeast extract only)	25	64	55	0	9
DR Protocol (2nd Replicate)	18	61	36	13	13
(Yeast extract only)	25	61	38	13	11

Key: L2 = 2nd stage larvae, L3 = Ensheathed larvae, D = Dead larvae

The summary of the LD_{50} values determined for each anthelmintic for each variation of protocol and temperature is shown in Table 5.3. Dose response curves for each protocol are shown in Figures 5.1 and 5.2. Of note are the generally high coefficients of determination (r^2) for each curve which overall ranged from 72–99% with many having r^2 value >90% indicating the curves were reasonably good fits for the data. However, the LD_{50} values for each anthelmintic obtained for different combinations of media and temperature were often very variable. For instance, the LD_{50} value for IV I with Media 12 at 18ºC was 15.79nM compared to 3.47nM at 25ºC. This difference became even more exaggerated if compared to the LD_{50} for IV I with Media 7 at 18ºC whereby the value was 35nM.

In contrast, the LD_{50} value for LV was reasonably consistent between protocols at both temperatures, range from 2.15–5.17µM, with the exception of Media 7 at 25ºC and the DR (Media 12 at 25ºC) where the value was >15µM. However, for both of these the r^2 was <90% indicating

variability in the data resulting in a poor curve fit. Similarly, the LD_{50} values for BZ were reasonably consistent between protocol and temperatures.

For the DR protocol, there were two replicates at each temperature allowing limited comparison to be made. The resulting LD_{50} values were very variable for IV I and II and somewhat less so for the other anthelmintics.

In Table 5.3 it can be seen that the LD_{50} values for the ECYE protocol for the BZ/LV combination were also very variable between the different protocols and culture temperatures.

The dose response curves developed with each anthelmintic each media concentration and each temperature category in the ECYE protocol cultures are presented in Figures 5.1a-e while those developed for each anthelmintic in each temperature category in the DR protocol are presented in Figures 5.2a–e. Figures for the ECYE protocol show that the dose response curves were generally typical sigmoid curves. However the dose response curves for BZ/LV and IV at 18ºC with Media 7 ($^1/_8$EC + $^1/_4$YE) in the ECYE protocol declined suddenly with a large slope and did not represent a typical sigmoid curve. The slopes for the dose response curves for other anthelmintics were gentle towards their LD_{50} presented, hence more typical sigmoid curves. Cultures grown at 18ºC by the ECYE protocols developed good response curves indicating they grew well compared to those at 25ºC.

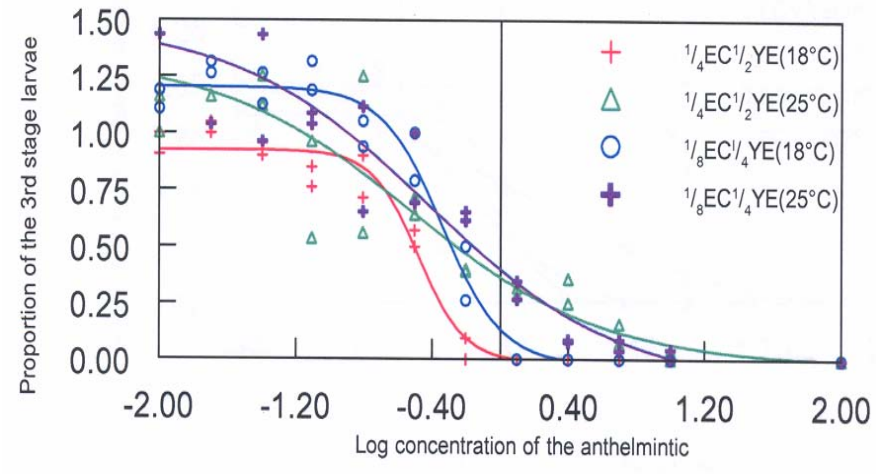
For the DR protocol, all anthelmintics at both temperature developed typical sigmoid curves although LV at 25ºC did not achieve high proportion of L_3s even when lowest concentrations were used. In addition, IV I at 25ºC did not reach the upper limit of the dose response curves. Overall, the DR protocol in this experiment worked well whilst in the previous experiments it was less successful.

Table 5.3: A summary of the LD_{50} values for various anthelmintics for *C. oncophora* cultures from Massey University's Tuapaka Farm are shown. Two ECYE protocols and the standard DR protocol at 18ºC or 25ºC were compared. Values for the BZ and LV are in µM and those for IV are in nM. For the BZ + LV combination the values were in µM.

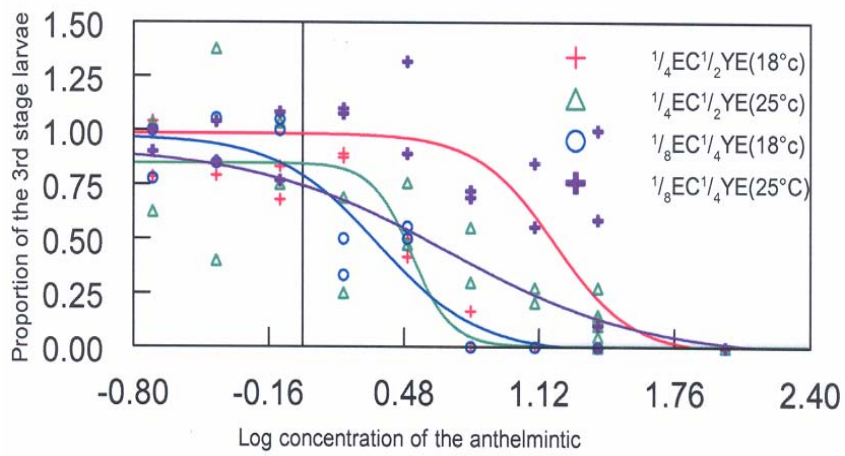
Protocol	Media conc.	Drug	Temp. (ºC)	Incubation time (days)	(r^2)	Log_{10} LD_{50}	LD_{50} values
ECYE	Media 12	BZ	18	12	0.98	-0.51	0.31
		LV	18	12	0.97	0.48	3.04
		BZ/LV	18	12	0.91	1.21	16.08
		IV I	18	12	0.96	1.20	15.79
		IV II	18	12	0.92	1.26	18.06
ECYE	Media 12	BZ	25	11	0.98	-0.29	0.52
		LV	25	11	0.91	0.33	2.15
		BZ/LV	25	11	0.95	0.86	7.22
		IV I	25	11	0.99	0.54	3.47
		IV II	25	11	0.96	0.81	6.52
ECYE	Media 7	BZ	18	12	0.86	0.26	1.81
		LV	18	12	0.69	0.58	3.84
		BZ/LV	18	12	0.83	1.74	55.07
		IV I	18	12	0.92	1.54	34.54
		IV II	18	12	0.89	1.90	78.88
ECYE	Media 7	BZ	25	11	0.90	-0.13	0.73
		LV	25	11	0.76	1.18	15.01
		BZ/LV	25	11	0.79	1.98	94.93
		IV I	25	11	0.93	1.48	30.09
		IV II	25	11	0.85	1.76	57.03
DR	YE only [run with Media 12]	BZ	18	12	0.85	-1.13	0.08
		LV	18	12	0.86	1.23	16.83
		BZ/LV	18	12	0.89	1.42	26.09
		IV I	18	12	0.90	1.34	21.70
		IV II	18	12	0.97	1.49	30.92
DR	YE only [run with Media 12]	BZ	25	11	0.95	-0.52	0.30
		LV	25	11	0.94	0.64	4.32
		BZ/LV	25	11	0.99	1.89	78.24
		IV I	25	11	0.94	0.80	6.36
		IV II	25	11	0.99	0.98	9.60
DR	[run with Media 7]	BZ	18	12	0.98	-0.42	0.38
		LV	18	12	0.92	0.71	5.17
		BZ/LV	18	12	0.96	1.24	17.48
		IV I	18	12	0.97	1.06	11.42
		IV II	18	12	0.96	1.42	26.30
DR	[run with Media 7]	BZ	25	11	0.77	-0.73	0.19
		LV	25	11	0.72	0.68	4.77
		BZ/LV	25	11	0.93	1.44	27.50
		IV I	25	11	0.96	1.09	12.17
		IV II	25	11	0.99	1.39	24.37

Figure 5.1: The LDA dose response curves of different anthelmintics for *C. oncophora* from Massey University's Tuapaka Farm cultured tested using the ECYE protocol in two temperature variations (18ºC and 25ºC). The cultures were read on Day 12 or day 11 for 18ºC and 25ºC respectively. **Note:** $^1/_8$EC + $^1/_4$YE = Media 7 and $^1/_4$EC + $^1/_2$YE = Media 12.

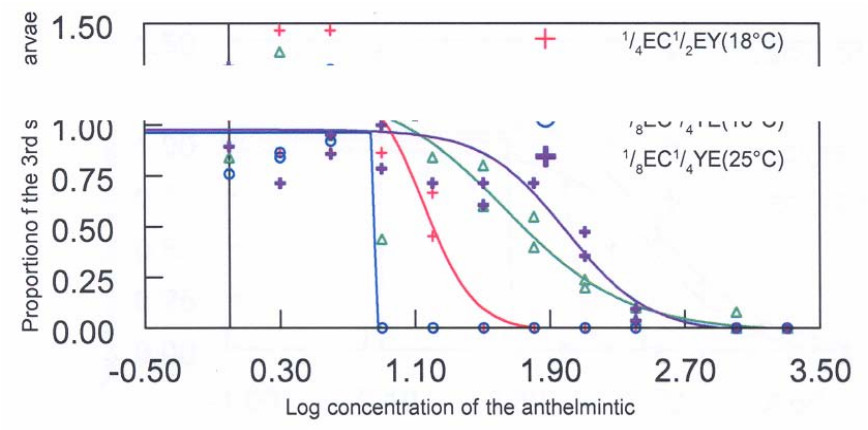
(a) BZ



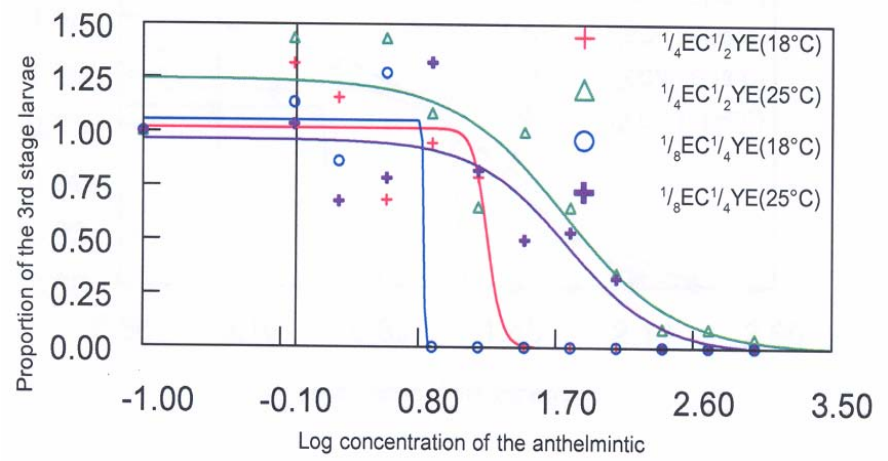
(b) LV



(c) BZ/LV



(d) IV I



(e) IV II

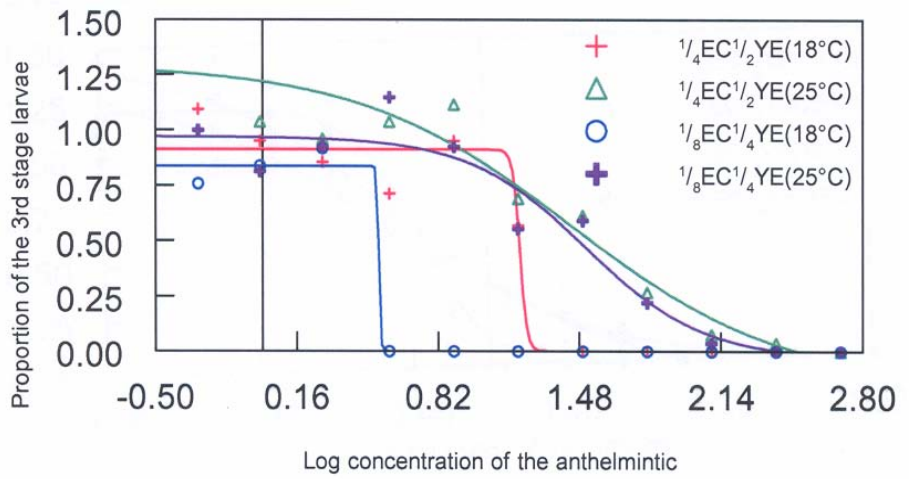
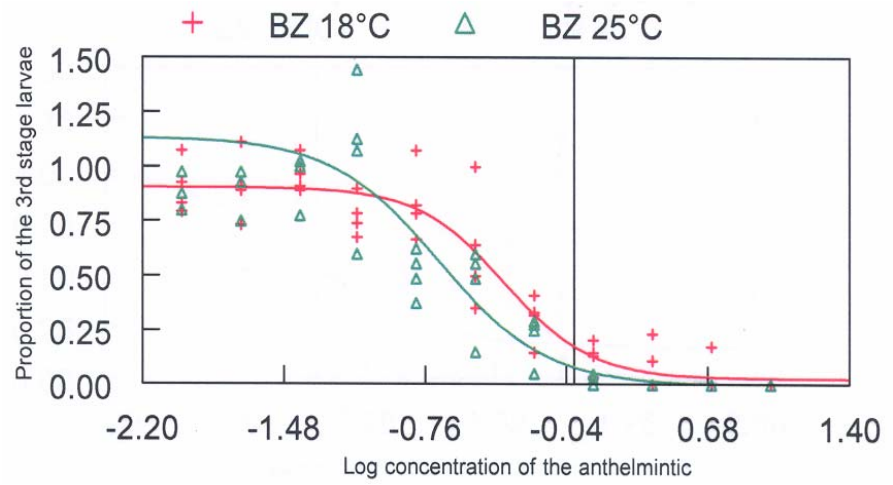
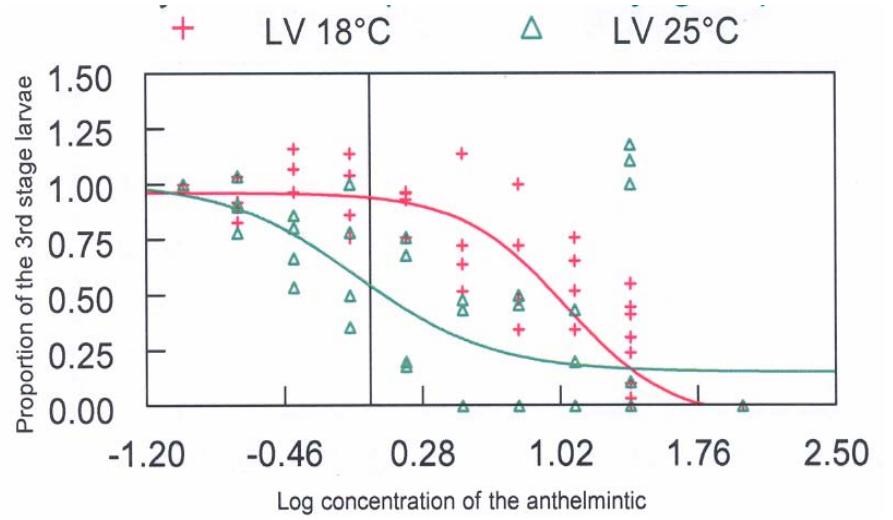


Figure 5.2: The dose response curves for different anthelmintics in the LDA are shown. These were for C. *oncophora* from Massey University's Tuapaka farm tested using the DR protocol at either 18ºC or 25ºC and at varying media and temperatures read at Day 12 or day 11 for 18ºC and 25ºC respectively. (IVM = Ivermectin)

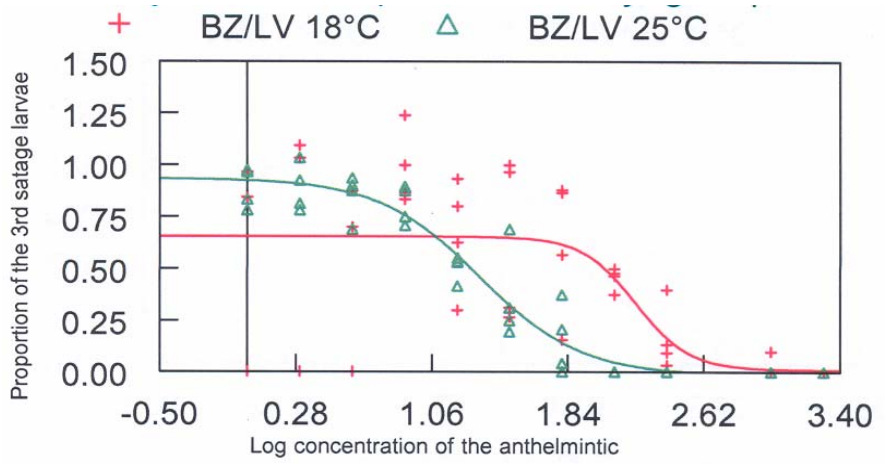
(a)



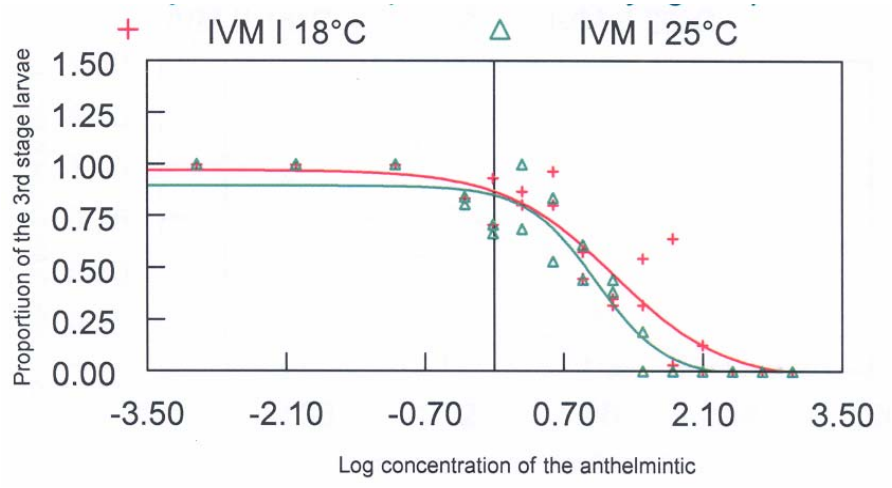
(b)



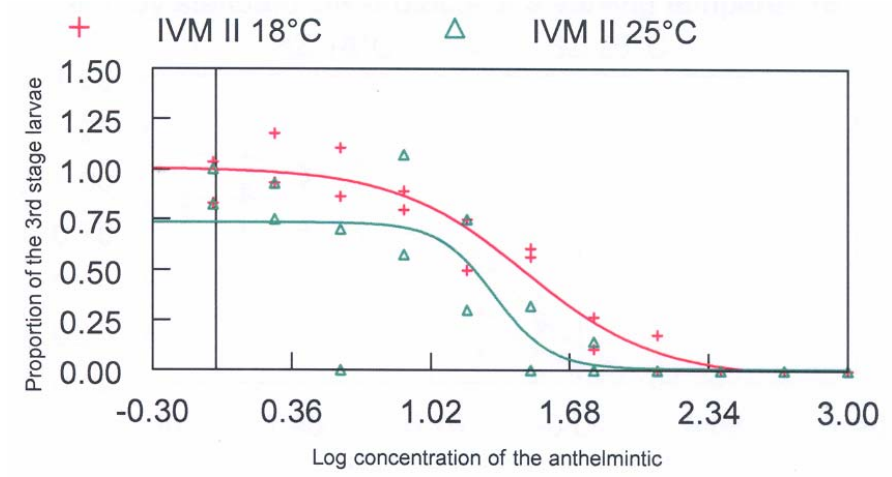
(c)



(d)



(e)



5.2.5 Discussion

The primary aim of this experiment was to assess whether the "standard" commercial DrenchRite LDA plates can be used to culture *Cooperia* to L_3 stage and establish dose response curve for the different anthelmintics included in these plates. It was found that, at the reduced concentration of the media, the ECYE protocol could be useful for culturing *Cooperia* successfully to L_3 using the "standard" commercially available 96-well microtitre plates.

In this experiment, better hatching was observed indicating that there was an overall improvement compared to the previous experiments. However, in the commercial DrenchRite plates, using the ECYE protocol, it was evident that egg development was still low (<50%). The reason for this big difference may be the difference in the volume used with the ECYE protocol compared to the volume used in standard commercial LDA for sheep nematode. The fact that the former involved a higher volume than the latter (100µl against 40µl per well respectively) may be important. Arguably, this higher volume may hinder hatching and even development as a result of

low oxygen tension (Hubert and Kerboeuf, 1984) as oxygen is known to be a critical factor in successful egg development (Smith *et al.* 1986).

It was observed that, by Day 11, the death rate at 25ºC was increasing at a faster rate than L_3 were maturing so a decision was made to read the test plates even though only about 50% of the L_2 had matured to L_3. Similarly, the plates that were cultured at 18ºC were beginning to demonstrate an increasing death rate on Day 12 (Appendix 5.1a & b). Therefore, a decision was made to read these plates on Day 12. In general, cultures incubated at 18ºC showed better development compared to those at 25ºC, which had begun to die at a faster rate, indicating that, this temperature was too high to grow *Cooperia.* However, it is possible that the plates cultured at 18ºC were removed from the incubator too soon as the death rate was still relatively low and more L_2 may have matured to L_3 if further incubation time was allowed. The degree to which the soon removal of the cultures from the incubator affected the dose response curve was not known.

There is no published information on the variability of the LD_{50} for cattle nematodes at different culture temperatures with the standard DrenchRite plates and protocols. The LD_{50} values obtained from the different protocols in this experiment were generally quite variable (Table 5.1a 5.3).

The high coefficients of determination (r^2) of >80% with a range of 72-99% suggests that the dose response curves generally described the distribution of the results reasonably well. It was somewhat surprising to see such high r^2 values given the generally low percentage of larvae that had successfully developed to the L_3 stage. However, the wide range in the r^2 also reflects that some variation with some protocols in this experiment occurred. In some cases the proportion of L_3 stage larvae exceeded one (some up to 140%), which indicates that there was at least some variability in the allocation of eggs in the wells indicating that this might also have contributed to the differences within and between some of the replicates.

In previous research on this farm (Tuapaka) Pomroy (1994b) reported that there were highly resistant *Cooperia* to BZ and ML but susceptible to LV. Likewise, in this experiment, the LD_{50} values for BZ were consistent between the protocols and temperature indicating that there are BZ-resistant *Cooperia* in the farm. Noticeably, in this experiment the results for the BZ/LV combination were quite variable. Though the concentration of BZ and LV used in the DrenchRite assay are not publicly available but one would expect the LV should give similar results to the wells containing just LV only.

For the MLs, IV 1 had a 5-folds difference of LD_{50} values in cultures at 18ºC to those at 25ºC compared to IV II which had zero fold. This difference in LD_{50} values indicate the variability in the data in this experiment but generally the LD_{50} values obtained for IV I and II suggests the repeatability of ML-resistant status in this farm. The analogues of the two MLs used (i.e. IV I and IV II) has not been published so it was difficult to make a comparison with other reports.

It was notable how well the standard DR protocol worked in this experiment contrary to the previous experiments reported by Johnson (2000). The reason for this marked difference is not clear but one possible explanation is that some of the *Cooperia* may have actually been sheep species. As there was no sheep killed to confirm the actual species composition of these parasites this possibility cannot be ruled out. However, based on the general weight of evidence it was considered that the more diluted ECYE protocol Media 7 ($^1/_8$EC + $^1/_4$YE), used at the lower temperature provided a more successful approach to grow *Cooperia* species from cattle.

Overall, the presence of anthelmintics in the DrenchRite plates in this experiment resulted in the proportion of L_3 stage larvae that successful developed to vary from zero to approximately one suggesting that the concentrations used was suitable for this species. The following experiment was undertaken with reputedly susceptible *Cooperia* to enable a comparison with the present results to be made.

5.3 Experiment VII: A LDA WITH SUSCEPTIBLE STRAINS OF *C. ONCOPHORA* FROM TWO FARMS

5.3.1 Introduction

Two farms: namely Farm H and Farm BR, were selected from the list of the beef cattle farms which were tested for AR in the North Island of New Zealand (Waghorn *et al.*, 2006). These farms were used to compare the results from Tuapaka to assess if the LDA could differentiate between resistant and susceptible worms. These farms were used as a source of eggs that were used to infect young sheep. During the survey, Farm H achieved an overall reduction for BZ, LV and IV of 98%, 99% and 98% respectively with the larval culture results indicated the composition was *Cooperia* (77%), *Ostertagia* (17%) and *Trichostrongylus* (8%). Similarly, Farm BR achieved an overall reduction for BZ, LV and IV of 100%, 99% and 98% with larvae culture results being *Cooperia* (87%), *Ostertagia* (13%) and *Trichostrongylus* (0%). These results indicate that the nematodes from these two farms were highly susceptible to all anthelmintics. The difference was the eggs used in the present experiment were collected from the housed young lambs that were infected with *Cooperia* from these two farms.

5.3.2 Aim

The aim of this experiment was to use commercial DrenchRite plates and determine the LD_{50} values for each anthelmintic from two commercial farms: Farm H and Farm BR that were considered to have fully susceptible *Cooperia.*

5.3.3 Methodology

Source of eggs

Faeces were obtained in early 2006 from young cattle 5-6 months old from the two sources: Farm H and Farm BR. These eggs were cultured and the larvae were collected and stored in the refrigerator at 4ºC for about 5months before being cycled through young lambs to provide eggs for

this experiment. Two young lambs, 3-4 months old, from Massey University farm were infected (one with larvae from Farm H and the other from Farm BR).

Before being used to infect the lambs, the generic composition of these larvae was confirmed by morphological identification: for Farm H the larvae were 100% *Cooperia* and for Farm BR they were 93% *Cooperia* and 7% *Ostertagia*. Lamb No. 6285 was infected with larvae from Farm H and lamb No. 6305 was infected with larvae from Farm BR. Faeces from the young lambs were collected direct from the rectum by attaching a faecal collection bag to the lambs in the evening. The bags were then removed the next morning and the samples were submitted to the laboratory for egg counts and egg recovery as well as bulk culture for identification.

Eggs recovery

The procedure for egg recovery was carried out as reported in Sections 4.2.3 and 5.2.3 as well as in Appendix 3.3.

Preparation of media solution

The media concentration chosen was ECYE protocol Media 7, which has a concentration of $^1/_8$EC + $^1/_4$YE. This media was prepared as described in Section 4.4.5.3 (Table 4.8) as well as in Appendix 4.1b. The cultures were carried out in the 96-well standard DrenchRite plates with anthelmintics.

Experimental design

Two assay plates were run, one at a temperature of 18ºC and the other one at 25ºC. The cultures were removed from the incubator on Day 12.

Preparation of cultures

The commercially available "DrenchRite" plates were used in this experiment with their anthelmintic concentrations as described in Section 5.2.3. The procedure for preparation of the cultures was described in Section 5.2.3. Careful observation and efficient aeration of the cultures was carried out throughout the incubation period by inspecting the plates daily and temporarily removing the lid of each plate for about 5-10 minutes to ensure an efficient exchange of air was carried out.

Data collection and analysis

The cultures were removed from the incubator on Day 12. Contents in the wells were transferred to a microscope slide and a few drops of Lugol's iodine were added to kill the larvae. These larvae were then examined using a microscope at 100X magnification and recorded as described in the data collection and analysis part of Sections 4.2.3 and 4.3.3 of this thesis.

A dose response curve was generated by comparing the proportion of L_3s relative to the control wells (column 1 in each row) in the plates to the $\log_{10}$ of the anthelmintic concentration. The software programme SlideRite (Advanced Graphics Software USA) was used to generate dose

response curves. An LD_{50} and r^2 values was also reported to indicate the goodness of fitness of the sigmoid curve.

5.3.4 Results

Egg count results for lamb No. 6285 (Farm H) and lamb No. 6305 (Farm BR) were 200 and 700eggs/g respectively. Both cultures for the samples from either Farm were identified as 100% *Cooperia* based on their morphology but no sheep was killed to confirm they were *C. oncophora*. The egg distribution in the wells was about 60eggs/well (ranging from 45-70) but the total larvae recovered in the wells ranged from 38-70eggs per well.

A summary of the development in both farms: Farm H and BR, in the control wells is presented in Table 5.4 while a summary of LD_{50} values and r^2 values is shown in Table 5.5. For comparison among these farms, the values for Tuapaka calculated from the previous experiment are shown in Table 5.6.

It can be seen in Table 5.4 that the hatching rate was reasonably good for Farm H at both temperatures but somewhat less for Farm BR, but overall development rate was similar to or better than the previous experiments. Generally the percentage of L_3s that developed in the wells was relatively high (ranged from 45-63) with Farm BR having the highest value

Table 5.4: A summary development of eggs to the L_3 stage (in %) in the control wells of the test plates at two different culture temperatures using ECYE protocol. Cultures were read on Day 12 for both 18ºC and 25ºC incubation temperatures.

Protocol/Media solution concentration	Farm ID	Temp. (ºC)	Hatching (%)	L3 (%)	L2 (%)	D (%)
ECYE/ Media 7	Farm H	18	75	47	12	17
	Farm BR	18	60	45	17	12
	Farm H	25	90	55	20	17
	Farm BR	25	75	63	7	10

Key: ID = identity; L2, 3 = 2nd and 3rd larval stages and D = dead larvae

(63%) at 25ºC. The death rates were generally low in both farms in both incubation temperatures.

Table 5.5 show that the r^2 values for the dose response curves ranged from 62-97. There was a notable level of variation in LD_{50} for some anthelmintics between temperatures. For example the LD_{50} for BZ on Farm BR at 25ºC was 0.03µM and 0.2µM at 18ºC, which was a 7-fold difference. Similarly, for Farm BR there was a 2.5-fold variation for IV and a 3-fold variation for IV II.

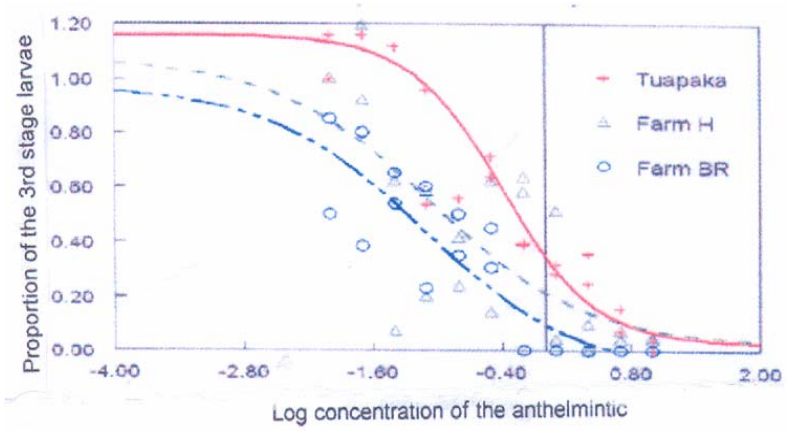
The dose response curves developed are presented in Figures 5.3a–e for cultures grown at 18ºC and Figures 5.4a–e for cultures grown at 25ºC. For Farm H and Farm BR, a common feature in these figures at 25ºC is that the lower concentration of anthelmintics are still generally too high to adequately define the lower portion of the dose response curve. The proportion of larvae for Tuapaka farm reached the L_3 stage in the BZ rows for both culture temperatures (18ºC and 25ºC) was generally lower compared to that of Farms H and BR. For LV there was no obvious difference between the dose response curves for the three farms. Similarly, for BZ/LV there was no obvious difference at 18ºC whilst at 25ºC Tuapaka farm had a curve that was notably to the right of the other two farms. Farm H had a high proportion of larvae not killed by the two IV analogies compared to Tuapaka and Farm BR, suggested that there are early signs of IV resistance in Farm H except for the IV at 25ºC whereby all Farms appeared to respond very well to the dose of IV used.

Table 5.5: The coefficient of determination (r^2) and LD_{50} values for anthelmintics used in *C. oncophora* cultures from Farm H and Farm BR. Two ECYE protocols at 18ºC or 25ºC were compared. Values for the BZ and LV were in µM and for IV were in nM. For the BZ + LV combination the values used were in µM.

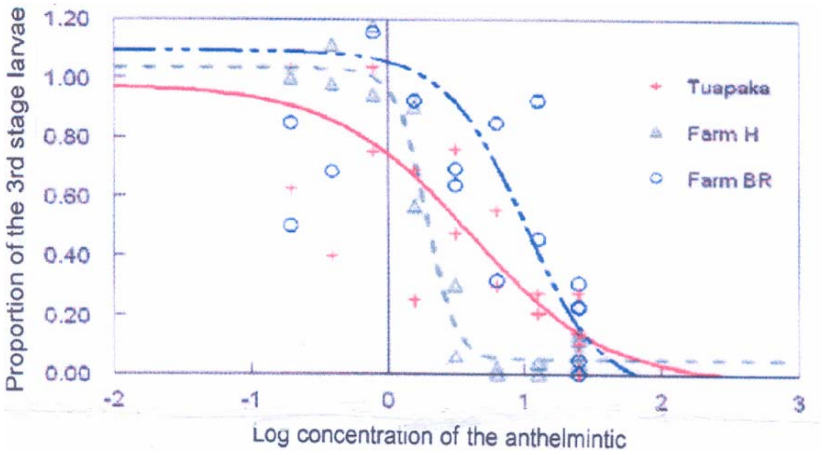
Farm ID	Protocol	Media concentration	Drug	Temp. (ºC)	Incubation time (days)	(r^2)	Log_{10} LD_{50}	LD_{50} values
Farm H	ECYE	Media 7	BZ	18	12	0.68	-1.13	0.07
			LV	18	12	0.96	0.30	2.00
			BZ/LV	18	12	0.91	1.08	12.14
			IV I	18	12	0.85	0.40	2.49
			IV II	18	12	0.95	1.17	14.78
	ECYE	Media 7	BZ	25	12	0.82	-1.02	0.10
			LV	25	12	0.62	1.01	10.16
			BZ/LV	25	12	0.79	1.31	20.23
			IV I	25	12	0.77	1.80	63.39
			IV II	25	12	0.83	0.92	8.37
Farm BR	ECYE	Media 7	BZ	18	12	0.81	-0.69	0.20
			LV	18	12	0.76	0.82	6.62
			BZ/LV	18	12	0.76	1.44	27.86
			IV I	18	12	0.96	1.33	21.43
			IV II	18	12	0.97	1.15	14.01
	ECYE	Media 7	BZ	25	12	0.81	-1.58	0.03
			LV	25	12	0.97	0.45	2.84
			BZ/LV	25	12	0.78	0.73	5.32
			IV I	25	12	0.89	0.93	8.52
			IV II	25	12	0.85	0.55	3.57

Figures 5.3: The comparison of dose response curves between resistant and susceptible *Cooperia* species from three farms in Northern Island of New Zealand on anthelmintics used in cultures tested for resistance using the ECYE protocol at media concentration of $^1/_8$EC + $^1/_4$YE (Media 7) at 18ºC for 12 days.

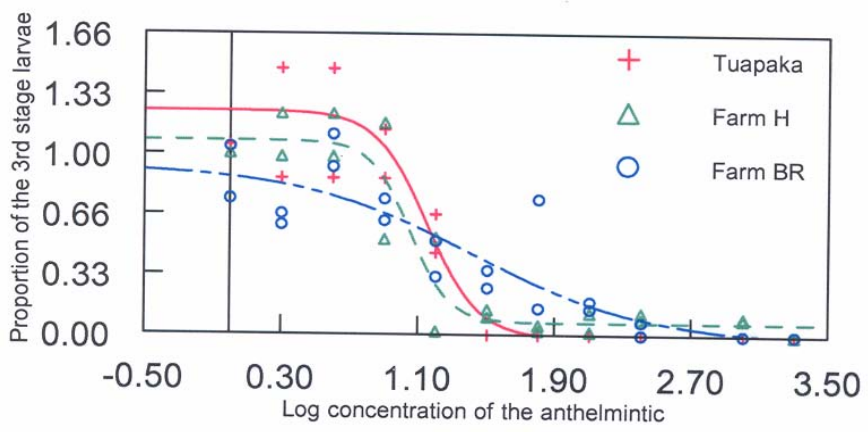
(a): BZ



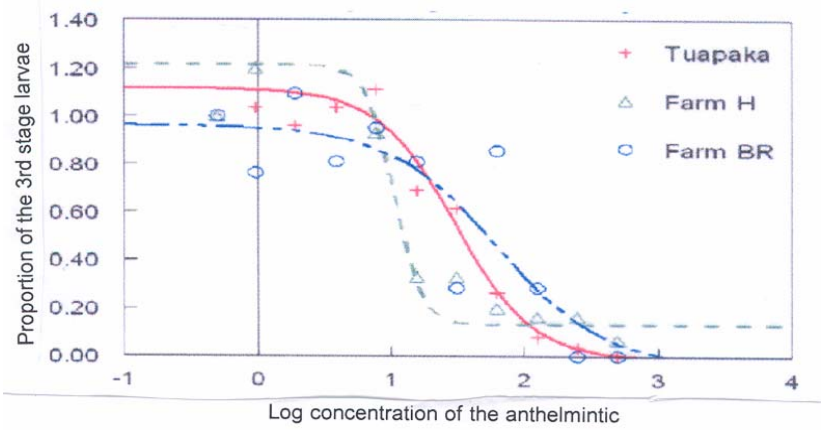
(b) LV



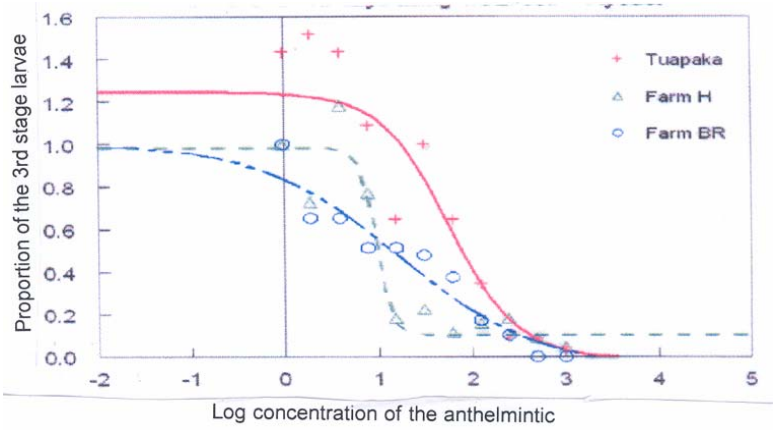
(c) BZ/LV



(d) IV I

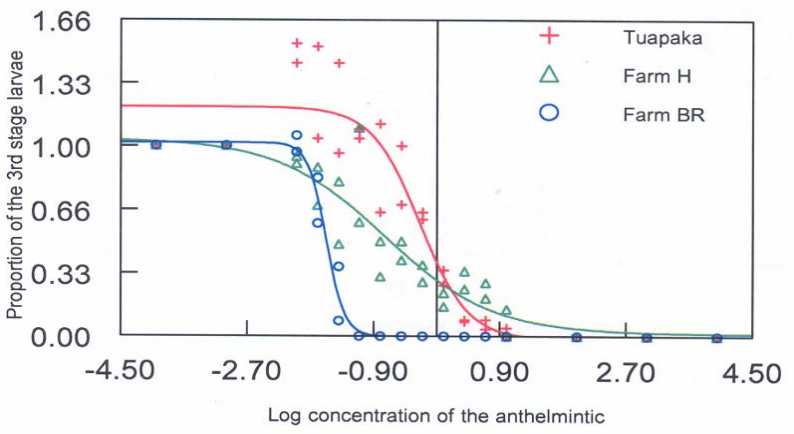


(e) IV II

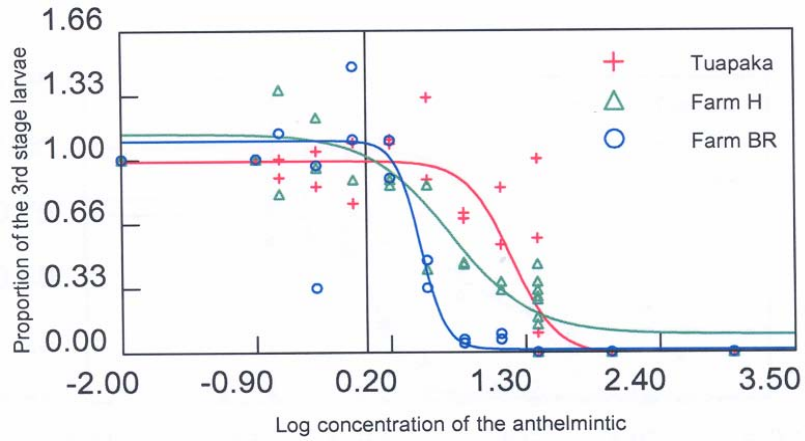


Figures 5.4: The comparison of dose response curves between resistant and susceptible *Cooperia* spp. from three farms in Northern Island of New Zealand on anthelmintics used in cultures tested for resistance using the ECYE protocol at media concentration of $^{1}/_{8}$EC + $^{1}/_{4}$YE (Media 7) at 25ºC for 12 days.

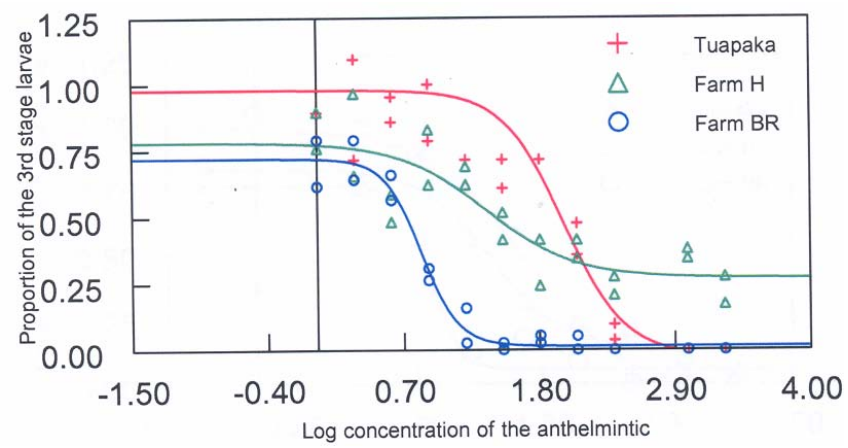
(a) BZ



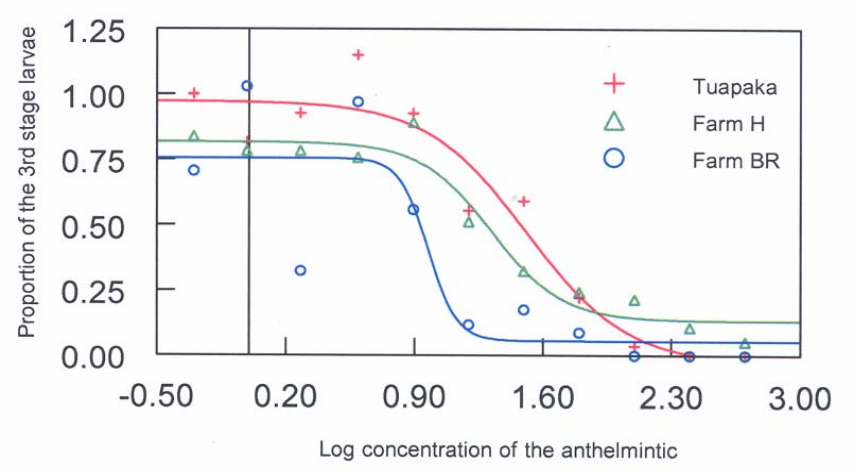
(b) LV



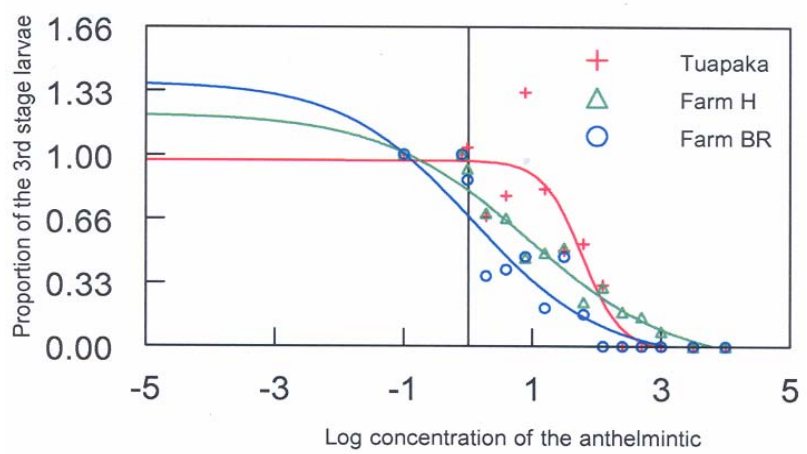
(c) BZ/LV



(d) IV I



(e) IV II



5.3.5 Discussion

The expectation that the media used in the cultures would support the growth of *Cooperia* was fulfilled. The overall development rate was reasonable though some of the control wells demonstrated poor growth rates. The results for these susceptible isolates from Farm H and BR in this experiment therefore provided a basis of comparison with the resistant isolates from Tuapaka farm.

There was little difference between 18ºC and 25ºC in this experiment. Overall, the number of eggs which developed in the wells against the number of dispensed eggs was relatively low. The reasons for this are likely to be similar to those described in the previous experiment in the second paragraph of Section 5.2.5. However, more larvae matured to the L_3 stage in this experiment than in the previous one, particularly at 25ºC. As in the previous experiment, it was possible that the plates cultured at 18ºC were removed too soon from the incubator because L_2 were still present. However, cultures grown at 18ºC generally had high r^2 values indicating a more reliable dose response curve was achieved at 18ºC suggesting this temperature was more appropriate to grow *Cooperia* or at least to run this assay.

The death rate at 18ºC at the time of removing the plates was low and fewer numbers of L_2s were recorded compared to the number recorded in the previous experiment. These larvae could mature to L_3 in the culture if incubation was allowed for some more days. What effect this would have on the resulting dose response curve is not known. However, there appears to be a fine line between achieving the maximum development to L_3 whilst a minimum death rate.

Both Farm H and BR have no history of AR in *Cooperia* as compared to Tuapaka farm, which has a history of resistance to both BZ and IV. The obtained dose response curves are shown in Figures 5.3a–e for cultures incubated at 18ºC and Figures 5.4a–e for cultures incubated at 25ºC. Though the point where the LD_{50} values occur for all three farms were very variable, all the dose response curves appear to be typical sigmoid curves which indicate the reliability of the data in this case.

For comparison, a summary of the LD_{50} values for the three farms are presented in Table 5.6. The LD_{50} values for BZ tend to remain below 1 for all the farms at both temperatures and with different media, although the values for Tuapaka farm were still higher than those of Farm H and BR. This is consistent with BZ resistance being known to occur on Tuapaka but not on Farm H and BR. However, the number of replicates was small and it was not possible to compare the data among the farms statistically.

Table 5.6: A summary comparison of the LD_{50} values between resistant and susceptible *Cooperia* species, Tuapaka and (Farm H and BR) respectively. This comparison among these three farms was made after testing the ECYE protocol at two temperature variations using Media 7.

Farm ID	Protocol	Media concentration	Drug	Temp. (ºC)	Incubation time (days)	LD_{50} values
T u a p a k a	ECYE	Media 7	BZ	18	12	1.81
			LV	18	12	3.84
			BZ/LV	18	12	55.07
			IV I	18	12	34.54
			IV II	18	12	78.88
	ECYE	Media 7	BZ	25	11	0.73
			LV	25	11	15.01
			BZ/LV	25	11	94.93
			IV I	25	11	30.09
			IV II	25	11	57.03
F a r m	ECYE	Media 7	BZ	18	12	0.07
			LV	18	12	2.00
			BZ/LV	18	12	12.14
			IV I	18	12	2.49
			IV II	18	12	14.78
H	ECYE	Media 7	BZ	25	12	0.10
			LV	25	12	10.16
			BZ/LV	25	12	20.23
			IV I	25	12	63.39
			IV II	25	12	8.37
F a r m	ECYE	Media 7	BZ	18	12	0.20
			LV	18	12	6.62
			BZ/LV	18	12	27.86
			IV I	18	12	21.43
			IV II	18	12	14.01
B R	ECYE	Media 7	BZ	25	12	0.03
			LV	25	12	2.84
			BZ/LV	25	12	5.32
			IV I	25	12	8.52
			IV II	25	12	3.57

LD_{50} values for LV were relatively similar in all farms especially where the r^2 value was >90%. Since LV was believed to be effective on all three farms, LD_{50} values were expected to be similar. The combination of BZ and LV was not tested by a FECRT (Waghorn *et al.*, 2006) on either Farm H or BR and similarly a combination has never been formally tested on Tuapaka. Nevertheless, it was expected it would be effective on all three farms due to the LV component.

The results for IV were of special interest as an appropriate assay to detect MLs resistance for any cattle parasite. *Cooperia* from Tuapaka farm generally have high levels of resistance to all ML anthelmintics, which has been documented over several years (Pomroy *et al.*, 2002). Results from the national survey (Jackson *et al.*, 2006) suggested that *Cooperia* from Farm H and Farm BR were fully susceptible as tested by FECRT. Although the identity of the two IV analogues in the DrenchRite plates was unknown there can be a substantial difference between the resistance

ratios obtained using different analogues of IV in the LDA. The potential difference between the two analogues is poorly understood even for sheep nematodes and is thus similarly uncertain for any cattle nematodes.

In the previous experiment the LD_{50} values for Tuapaka generally showed the value for IV I was consistently higher than for IV II. However, for Farm H and BR this pattern was not evident. Farm H showed a 30-fold higher LD_{50} for IV I at 25ºC than at 18ºC while there was only a 3-fold difference for Farm BR. For IV II, Farm H had relatively similar values while Farm BR had a 4-fold difference between the LD_{50} values at 18ºC compared to those at 25ºC.

Nevertheless when comparing the LD_{50} values it was found that those for Tuapaka farm were up to 20 times higher than those of Farms H and BR which was consistent with the presence of ML resistance on Tuapaka. The only obvious anomaly to this was the result for IV I in Farm H at 25ºC which showed a relatively higher LD_{50} value (63.4). Why this higher LD_{50} value occurred was not clear but probably reflects the variation with development rates using this protocol. Further development needs to achieve better development rates before any further conclusions can be drawn.

Comparing the results for BZ between the three farms was even less compelling. There was no obvious difference in LD_{50} between any of the variations tested.

In summary, these results indicates that Media 7 ($^1/_8$EC + $^1/_4$YE) as used in this LDA result in a dose response curve with a reasonably good fit to the data as indicated by the r^2 values in these experiments. The conclusion with regard to the usefulness of this particular protocol in detecting BZ and ML resistance in *Cooperia* from cattle is predictable on the assumption that those from Farms H and BR are indeed susceptible and those from Tuapaka are resistant as previously seen. Therefore, further research is needed to confirm these findings by comparing LDA with FECRT, preferably conducted at the same time.

In addition, conducting regular surveys is important not only for research purposes but also for monitoring purposes. Jackson *et al.* (2006) warned about increased chances of IV resistance in cattle nematodes in New Zealand because ML is more commonly and regularly used in many farms than it was in the past years. Therefore, it is important to regularly conduct surveys in these farms to monitor this situation. However, more reliable assays or in combination rather than the use of only FECRT to assess the resistance status is desirable (see Chapter 6 for further experiments to develop other assays of importance in testing AR in cattle nematodes).

CHAPTER SIX

6.0 LARVAL FEEDING INHIBITION ASSAY

6.1 Introduction

Previous chapters have described a series of experiments to modify the standard LDA used for sheep nematodes in order to be suitable for use with cattle nematodes. Another *in vitro* assay, which shows promise, is the larval feeding inhibition assay (LFIA). These two assays: the LDA and the LFIA are considered to be the most likely methods that could be modified for testing for ML resistance in cattle nematodes. There has been less experimental work with the LFIA and it has not yet been made available as a commercial assay. The experiment in this chapter describes research conducted with ML-resistant and -susceptible species from sheep and goats. The ML-resistant parasites used were *Teladorsagia circumcincta* from goats that had survived recent treatment while the susceptible parasites were *T. circumcincta* from housed sheep infected with a known susceptible isolate. Sheep and goat nematodes were used in these experiments because these were clearly identified as resistant and susceptible isolates available and *Cooperia* from cattle was difficult to obtain at the time of the year these experiments were conducted. It has also been established that the LDA is of limited use in detecting ML-resistance in *Teladorsagia* species in sheep (Besier, 1999). Therefore, it was of interest to determine if the reported success of the LFIA in detecting resistance to MLs in *Teladorsagia* could be repeated with these New Zealand isolates.

The LFIA test is not yet well developed and limited information is available relating to this test. The available information can be viewed in Section 2.5.2. Briefly, the LFIA was initially based on the technique described by Geary *et al.* (1993) for adult worms. In the late 1990s, Jackson and Coop (2000) described an LFIA using sheep nematodes based on this earlier technique by Geary *et al.* (1993). This technique by Jackson and Coop (2000) became the basis for a similar technique for sheep nematodes described by Álvarez-Sánchez *et al.* (2005). Although this later technique might have been tried elsewhere, this report is the most recent publication available. Therefore, the protocol described by Álvarez-Sánchez *et al.* (2005) in their report was used as the basis for the experiments described in this chapter. The intention was to mimic the procedure described by Álvarez-Sánchez *et al.* (2005) as far as possible, but some changes were required to fit the suitability of Massey University's laboratory for running this assay.

6.2 Experiment VIII: AN EXPERIMENT COMPARING RESISTANT AND SUSCEPTIBLE ISOLATES OF *OSTERTAGIA CIRCUMCINCTA*

6.2.1 Aim

The aim of this experiment was to assess the usefulness of the LFIA in testing for resistance to IV in *T. circumcincta* by comparing known resistant and susceptible nematode isolates, in addition to generally assessing the usefulness of this approach to detect AR from the isolates from sheep from Massey University farm.

6.2.2 Methodology

A full standard operational procedure (SOP) for the LFIA procedure is presented in Appendix 6.1. The following section offers an overview of the technique and the experimental design.

Source of the eggs and larvae

Eggs of the ML-resistant isolates were obtained from adult goats kept at the Sheep Research Unit of Massey University which had been treated recently (within two weeks) with IV. These were previously confirmed as *T. circumcincta*. Eggs of the ML-susceptible isolates were obtained from a housed sheep that had been experimentally infected with a known susceptible *T. circumcincta* isolate. This sheep faecal sample was a gift from AgResearch, courtesy of Ms. Tania Waghorn. The samples from the goats were freshly collected from the rectum, whilst those from the sheep were obtained by attaching a faecal collection bag to the sheep overnight and then recovering the faeces in the morning of the next day. The eggs from the sheep were not fresh at the time of running the assay since they had been collected a day before and preserved in a refrigerator at 4ºC until the time of use.

Egg recovery

Eggs were recovered from the faecal samples as described by Sharma (2004), and the detailed procedure is presented in Section 4.2.3 as well as in Appendix 3.3. Egg recovery and larvae collection did not follow the procedure described by Álvarez-Sánchez *et al.* (2005). The procedure by Sharma (2004) was found to be less involved and produced cleaner eggs than the procedure described by Álvarez-Sánchez *et al.* (2005). Recovering eggs from the interface of two sugar solutions meant that only the eggs and not other impurities were recovered, compared to recovering eggs on a 37µm sieve in the procedure by Álvarez-Sánchez *et al.* (2005). In addition, floating the eggs in saturated sodium chloride solution will damage the eggs due to the high osmotic pressure. Consequently, larval collection by Baermannization as described in the technique by Álvarez-Sánchez *et al.* (2005) was not necessary.

Hatching the eggs to larvae stage one (L_1) and estimating the number of larvae

The clean eggs were incubated in a petri dish with a loosened lid at 22ºC for 24 hours in order to allow hatching to the first larval stage (L_1). On the next day, the number of L_1 hatched was estimated by counting ten 20µl aliquots of the egg/larval suspension on a cover slide using a microscope at 100x magnification, with a cover slip applied. A few drops of Lugol's iodine were added to the slides in order to kill the larvae to facilitate counting.

Preparation of cultures

The detailed procedure for this experiment is described in Appendix 6.1. After it was established that the larvae had hatched in the petri dish, they were transferred to 24-well plates to continue the assay. In brief, 1980µl of tap water or distilled water was put in the test wells and control wells, respectively, followed by the addition of approximately 10µl of water containing about 100 of L_1. Ivermectin from a commercially available anthelmintic (Ivomec liquid for sheep and goats

from Merial New Zealand, Batch No. 80016) was serially diluted with 1% DMSO (Sigma Chemicals Co.) into 30 dilutions as shown in Table 6.1.

Following this dilution process, 11 dilutions were selected and 10µl of each of these 11 serially diluted IV concentrations from the list was added to the wells. An exception was made for the control wells, to which only 10µl of the 1% DMSO was added. The final dilutions were as presented in Appendix 6.2. All the dilutions were assayed in duplicate. The plates were incubated at 25ºC for 2 hours, followed by the addition of 10µl of fluorescein-labelled *E. coli*. This was prepared by mixing 100µl of concentrated *E. coli* (2250µg of *E. coli* per ml strain ATCC 9637, Sigma Chemicals Co.) with 1mg of FITC into 1ml of bicarbonate buffer (both from Sigma Chemicals Co.) in a 1.5ml Eppendorf tube and incubated at 20ºC for 2 hours. After adding the fluorescein-labelled *E. coli* to the wells, a further incubation at 25ºC for 18 hours followed.

Table 6.1: An illustration of how the dilutions of the IV used in this experiment was prepared. The dilutions were made in a serial manner and then 11 dilutions were selected from the list. The selected drugs were then applied in the cultures in the experiment and all the concentration are in ng/ml.

Serial Number	Concentration of IV (stock solution)	Diluted concentration of IV	Remarks
1	100000	500	-
2	50000	250	Selected
3	25000	125	Selected
4	12500	62.5	-
5	6250	31.25	-
6	3125	15.625	Selected
7	1562.5	7.8125	-
8	781.25	3.90625	-
9	390.625	1.953125	Selected
10	195.3125	0.9765625	-
11	97.65625	0.48828125	-
12	48.828125	0.244140625	Selected
13	24.4140625	0.122070313	-
14	12.20703125	0.061035156	--
15	6.103515625	0.030517578	Selected
16	3.051757813	0.015258789	-
17	1.525878906	0.007629395	-
18	0.762939453	0.003814697	Selected
19	0.381469727	0.001907349	-
20	0.190734863	0.000953674	-
21	0.095367432	0.000476837	Selected
22	0.047683716	0.000238419	-
23	0.023841858	0.000119209	-
24	0.011920929	5.96046E-05	Selected
25	0.005960464	2.98023E-05	-
26	0.002980232	1.49012E-05	-
27	0.001490116	7.45058E-06	Selected
28	0.000745058	3.72529E-06	Selected
29	0.000372529	1.86265E-06	-
30	0.000186265	9.31323E-07	-

Data collection

After 18 hours of incubation, the contents from each well were transferred to a 50ml test tube, which was then centrifuged at 1000Xg for five minutes. The resultant supernatant liquid was discarded by the use of a vacuum system. The sediment containing the L_1 was finally pipetted onto a grooved slide and examined with an epi-fluorescent microscope and the number of larvae that were observed to have fed on *E. coli* bacteria or not fed on *E. coli* bacteria was recorded. A larva was recorded as feeding if any fluorescent material was observed in its intestine.

Data analysis

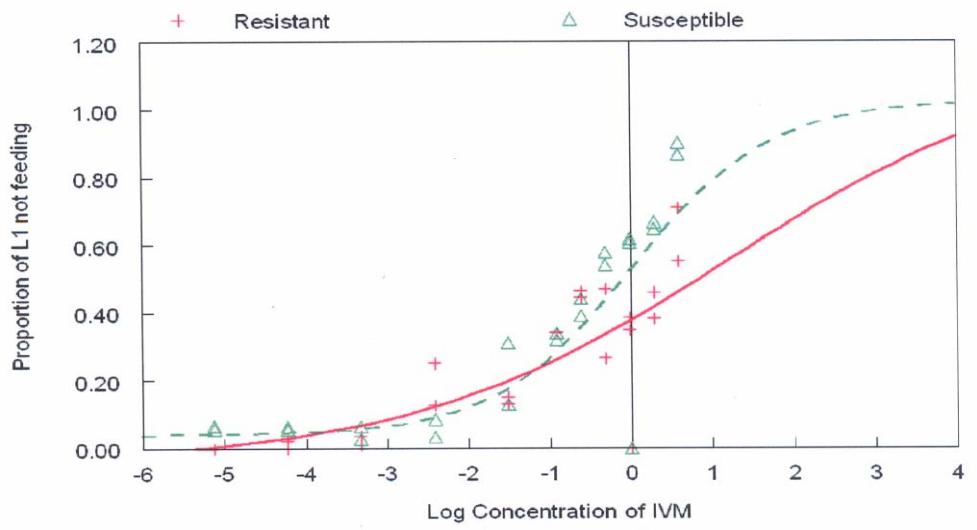
During analysis, the proportion of "non-feeding larvae" for each dilution was determined and plotted against the log of the concentration of IV. A dose response curve was developed for each isolate using the software computer programme SlideRite (Advanced Graphics Software, USA). Each data point was plotted prior to fitting the curve. The concentration of IV that inhibits feeding by L_1 by 50% (IC_{50}) was then determined from the curve in addition to the r^2 value used to assess the fit of the dose response curves.

6.2.3 Results

The egg count estimates from the goat samples were 150–600eggs/g with an average of 350eggs/g whilst that from the sheep was 250eggs/g. During counting, it was found that each well had about 150 and 140 eggs in culture 1 and 2 respectively instead of an intended estimate of 100eggs/well with a range of 127-179 and 102-160 in culture 1 and 2 respectively. The average hatching percentage in both cultures for both isolates was about 98%. The dose response curves for both the IV-resistant and -susceptible isolates are presented in Figure 6.1.

The IC_{50} values for IV were 0.13ng/ml and 6.7ng/ml for susceptible and resistant isolates, respectively, and the r^2 were 0.6 and 0.7, respectively. This resulted in a resistance ratio of 51.5, which is the LD_{50} of the resistant isolate divide by the LD_{50} of the susceptible isolate. It is evident from these two curves that the selected IV concentrations did not fully describe the complete dose response curve for either isolate, particularly the resistant one. The raw data for this experiment is represented in Appendix 6.2.

Figure 6.1: The dose response curve for larval feeding inhibition assay to compare the resistant and susceptible *Teladorsagia circumcincta* from goats and sheep, respectively. The proportion of non-feeding larvae was used when plotting the data.



Key: IVM = ivermectin.

6.2.4 Discussion

This experiment was expected to establish the concentration of IV that would inhibit the larvae of *T. circumcincta* from feeding on *E. coli* bacteria and also enable an IC_{50} value to be determined. Overall, both these objectives were met. However, for the resistant isolate in particular, the dose response curve was not fully described with the available data as the maximum inhibition of feeding was approximately 60%. This probably suggests that in this particular experiment there was a need to use a higher concentration of IV for better representation of the dose response curves (IC_{50}).

Nevertheless, the IC_{50} value for the resistant isolate was similar to that reported by Álvarez-Sánchez *et al.* (2005). However, these authors reported that the IC_{50} value of a susceptible isolate of *T. circumcincta* was about 1.0ng/ml, which was substantially higher than that reported in this experiment. Overall, this initial comparison indicated the potential for this particular assay to detect resistance in MLs in this species, suggesting that this assay may also be possible for cattle nematodes.

The slight variation between the results by Álvarez-Sánchez *et al.* (2005) and the results reported in this experiment might reflect a fundamental difference in the way this assay was conducted. Although the intention was to directly copy the protocol of these researchers as explained earlier in the introduction part of this chapter, some parts of the protocol were changed. The logic that tap water should be used in the test wells whilst distilled water should be used in the control wells as described in their report was not rational. Therefore, in this experiment only distilled water was used in both the control wells and the test wells. The extent to which this affected the dose response curves was not known. However, this assay demonstrated a reasonably high ratio

between the IC_{50} values of the susceptible and resistant isolates, which suggested that it might be capable of detecting resistant isolates in the field. Consequently, there is hope that the LFIA could be used as a basis for detecting IV resistance in other species, particularly trichostrongylids in cattle such as *Cooperia* and *Teladorsagia* species.

6.3 Experiment IX: AN LFIA ON *O. CIRCUMCINCTA* FROM FOUR SHEEP INFECTED WITH A KNOWN SUSCEPTIBLE ISOLATE

6.3.1 Introduction

The previous experiment showed some promise in that it was able to detect IV-resistant isolates of *T. circumcincta*. However, the IC_{50} of the susceptible isolates was only 0.13ng/ml, which was substantially lower value than that reported by Álvarez-Sánchez *et al.* (2005). This experiment was planned in order to explore the repeatability of the IC_{50} for a susceptible isolate of *T. circumcincta.*

6.3.2 Aim

The aim of this experiment was to investigate the repeatability of this assay by determining the IC_{50} from four separate sheep, which had been infected with a susceptible isolate of *T. circumcincta*.

6.3.3 Methodology

Source of eggs

Eggs for this experiment were obtained from four housed sheep infected with a known mono-specific, susceptible isolate of *T. circumcincta* and they were a gift from AgResearch courtesy of Ms. T. Waghorn. These sheep were of similar age, under similar management and all of them had been infected at the same time, with the same dose.

Four samples were collected for this experiment separately (Sheep 1-4). Sheep 2 was the same animal that provided eggs for the previous experiment. The samples were collected on a Saturday evening by attaching a faecal collection bag on the sheep and the bags were removed the next morning. The samples in the bags were emptied into plastic containers and they were preserved in a refrigerator at 4ºC, until the following Monday morning when sequences of assays, one for each sample, were run each day. Therefore, some of the samples remained in the refrigerator for up to six days.

Egg recovery

Egg recovery was carried out as described in Section 6.2.2 with a brief description also in Section 4.2.3 in addition to Appendix 3.3. Each sample from each animal was processed separately. Egg recovery was done on the day of the experiment.

Hatching the eggs to larvae stage one (L_1) and estimating the number of larvae.

Hatching the eggs to larvae stage one and estimating the number of larvae hatched was the same

process as described in Section 6.2.2 and the sample from each animal was handled separately.

Preparation of cultures

The preparation of cultures was the same as described in Section 6.2.2. It is also important to note that in this experiment each sample was cultured in a separate plate and on different days.

Experimental design

In this experiment, the susceptible isolates from the four sheep were used in the cultures and were run separately. All the dilutions were assayed in duplicate as in the previous experiment. The same eleven selected concentrations as described in Section 6.2.2 were used.

Data collection and analysis

Data collection and analysis were similar to those described in Section 6.2.2 in relation to the previous experiment.

6.3.4 Results

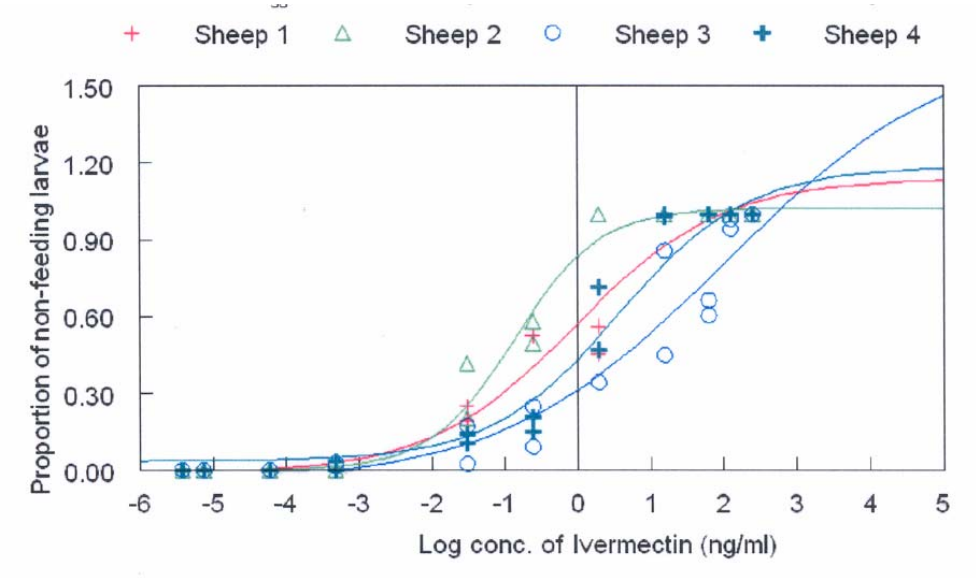
The egg counts from the sheep samples ranged from 250–600eggs/g. with an average of 400eggs/g. The raw data of this experiment are presented in Appendix 6.3. The number of L_1 per well was estimated at 100 larvae/well but there was a slight difference between the number of L_1 dispensed into the wells and the number of L_1 recovered from the wells in this experiment. Sheep 1 and 2 had a range of 58-123larvae/well while Sheep 3 and 4 had a range of 80-132larvae/well, indicating a slight variability of the recovered larvae among these sheep.

The IC_{50} curve of IV to *T. circumcincta* from the four experimental sheep is presented in Figure 6.2 while the raw data of this experiment is presented in Appendix 6.3 though data for feeding larvae was not plotted in the dose response curves.

The larvae were observed to stop feeding as follows: Sheep 1 (well number 8), Sheep 2 (well number 7) Sheep 3 (well number 11) Sheep 4 (well number 8) indicating they stopped feeding at a relatively lower concentration of the drug suggesting that these species are susceptible.

The IC_{50} values (ng/ml) for IV for each sheep are shown as follows: Sheep 1, 0.172; Sheep 2, 0.102; Sheep 3, 0.046 and Sheep 4, 1.34. Only Sheep 4 reached over 1.0ng/ml (1.34) but the remainder of the sheep was lower. The r^2 values for the dose response curves for Sheep 1, 2, 3 and 4 were 0.85, 0.80, 0.91 and 0.92 respectively.

Figure 6.2: The dose response curve (IC_{50}) for four sheep using LFIA to compare the susceptibility of *Teladorsagia circumcincta* against IV drug. The sheep had the same age and same management levels.



6.3.5 Discussion

This experiment demonstrated that there was a reasonable repeatability in the dose response curves between these four sheep. This was clearly reflected in the results, for example in Sheep 2 in the previous experiment the IC_{50} value was 0.13ng/ml with an r^2 of 70% compared to an IC_{50} of 0.102ng/ml and r^2 of 80% in this experiment. The dose response curves showed that there is variation between sheep infected with the same isolate but the IC_{50} for all four sheep was still substantially below that of the resistant isolate shown in the previous experiment indicating that the LFIA is a sensitive test.

When the IC_{50} value of 6.7ng/ml for the resistant isolate from the previous experiment was compared to the highest IC_{50} value from the present experiment the resistance ratio was still at least 5. The r^2 values were generally higher than for the previous experiment although values for Sheep 2 were still <90%. However, the top of the dose response curve was still not described with the concentrations of IV used for Sheep 3. These values would probably be improved if the concentration of IV could be increased to obtain a better representation of the top of the dose response curves in future experiments.

The variation in IC_{50} noted between the four sheep was somewhat larger than the variation noted with time by Álvarez-Sánchez *et al.* (2005). These authors reported some variations with time after infection in individual sheep. In their experiments the values increased by as much as 50% by Day 70 after infection. Although there was no attempt to follow of the changes in IC_{50} with time, this variation in IC_{50} may reflect true individual variation between sheep or normal variation within the assay. However, it was interesting to observe the minimum changes in IC_{50} values for

Sheep 2 in only a week interval between the first and the second experiments.

Although Pandey (1989) described that eggs of *T. circumcincta* could hatch at temperatures as low as 4ºC, the faecal samples were stored in the refrigerator at 4ºC until the 6th day but did not hatch. All eggs were found intact when extracted from the faecal samples indicating that preservation of the samples in the refrigerator did not affect the experiment unduly.

The IC_{50} values for these four sheep (except for Sheep 4) were relatively lower than those reported by Álvarez-Sánchez *et al.* (2005). As a reminder, these authors reported an IC_{50} value of 1.0ng. The reason for this variation was not readily apparent but it may be due to the variations in the technique between laboratories or a completely different isolate was used. Clearly, further validation is required.

Other authors have also reported to use this assay to detect resistance in other species successfully, such as *H. contortus* (O'Grady and Kotze, 2004), *H. contortus*, *Teladorsagia circumcincta*, *Trichostrongylus vitrinus*, *T. colubriformis* and *Cooperia curticei* (Álvarez-Sánchez *et al.*, 2005) and *Necator americanus* and *Ancylostoma ceylanicum* (Richards *et al.*, 1995). These reports represent the usefulness of this assay and suggest that this assay could, if further researched, be also useful in detecting resistance in cattle nematodes.

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CHAPTER SEVEN

7.0 GENERAL DISCUSSION

The preceding chapters present a series of experiments that were carried out to develop *in vitro* assays to detect AR in cattle nematodes. Two assays (LDA and LFIA) were investigated - with two variant protocols for the LDA (ECYE and DR) and various factors within each were modified in an attempt to develop a protocol that could efficiently detect AR in *C. oncophora*. To facilitate a ready access to *Cooperia* larvae the susceptibility of young sheep to this parasite was assessed (Chapter 3) as they were used as donor animals.

To overcome logistical problems including access to young cattle it was determined that young lambs would be used to cycle this parasite. It is acknowledged that sheep may be infected with this parasite but there is minimal published information about the likelihood of these infections. It was generally found that sheep could be successfully infected with *C. oncophora*. However it was clearly demonstrated (see Table 3.1) that sheep infected with these parasites only produce a small number of eggs and also they tended to maintain the egg production for about 60 days. Reinfecting with higher doses was necessary in order to maintain a patent infection. Sheep were used because they are easy to house, feed and collect faecal samples from as compared to cattle and their use would reduce the time and cost of running the assays. In addition, sheep could be infected and produce eggs at the time young cattle would not be available for producing eggs in the field.

The initial factor investigated was whether sterilised or non-sterilised agar and media should be used (Experiment 1). Some previous studies at Massey University (Johnson, 2000 and Sharma, 2004) had used all sterilised media whilst other authors such as Álvarez-Sánchez *et al.* (2005) did not. It was apparent after this work that neither the agar nor the media had to be sterilised, unless the plates were being prepared in advance when sterilisation is then a sensible precaution. If using prepared in-house LDA plates, it seems sensible to prepare them immediately at the time of use. The problem encountered from the use of non-sterilised agar and media is the potential for growth of micro-organisms in some of the wells in the cultures. However, the key point to observe in order to avoid growth of micro-organisms is hygiene at the time of preparing the cultures in addition to a more stringent nematode-egg-cleaning procedure. Moreover, Johnson (2000) has advised the use of antibiotics in the *in vitro* assays. In the current series of experiments, only amphotericin B (Fungizone) was used without any additional antibiotics. Taking particular care to achieve clean egg suspensions was considered more important.

Experiment 1 also showed that the species and the protocol could result in significant effects. By comparing *Haemonchus* and *Teladorsagia* it was apparent that there were differences when culture temperatures and media were altered. Previous reports by Dinaburg (1944) and Crofton (1965) demonstrated that *H. contortus* has an optimum temperature range of 22 - 25ºC whilst recent studies by Abdalla and Pomroy (2006) have shown that *T. circumcincta* has an optimum temperature of approximately 17ºC for development, suggesting that temperature is likely to influence success in an *in vitro* assay.

Although little work has been done on culturing cattle *Cooperia,* comparing two different temperatures in Experiment II showed that *Cooperia* grew better at the lower temperature (18ºC) than at a higher temperature (25ºC). These findings are consistent with the reported work in sheep by Familton and McAnulty (1994), who reported that *Cooperia* in sheep undergo significant development on pasture during the cooler winter months in contrast to some other trichostrongylid nematodes. In terms of the duration of culture, there was a trade-off between the number of larvae that successfully developed all the way to the L_3 stage and the number that died. It was determined that 12 days at 18ºC was a sensible compromise. However, prolonged incubation for more than 12 days as in Experiment II would be needed to enable the maximum proportion of the larvae to mature from the L_2 stage to the L_3 stage.

In the subsequent series of experiments, it was determined that the most appropriate assay protocol and media concentrations to best suit the growth of *Cooperia* (Experiments III, IV and V) were shown to be the ECYE protocol at 18ºC using $^1/_8$th the concentration of *E. coli* and ¼ the concentration of yeast extract recommended for sheep nematodes. These results were similar to those reported by Johnson (2000). The key success to growing the sheep nematodes to L_3 in standard sheep protocol, is the amount of *E. coli* and yeast extract used (Gill *et al.*, 1995). Why the concentration of nutrients generally used for sheep nematodes in the LDA assay is too high to promote the growth of cattle *Cooperia* is not understood. Arguably, the larger volume of liquid (egg suspension and media) used in the ECYE protocol, which is a total of 100µl/well compared to the commercial DrenchRite protocol of 60µl/well could influence the availability of oxygen and affect the egg development. Therefore, reducing the volume may facilitate a better supply of oxygen and this is consistent with the description by Hubert and Kerboeuf (1984). Generally, in nematode ecology Robert and O'Sullivan (1949) and Young *et al.* (1980) described that growth of larvae requires an optimum temperature as well as adequate moisture and oxygen supply to achieve good development (see Section 2.1.3 for a review).

The particular challenge in culturing *Cooperia* in the LDA was the percentage of eggs which complete the moulting from L_2 to L_3. Results achieved in the present experiment suggest that even the protocol recommended here was not ideal and further work is required. It was observed in almost all the experiments that the hatching percentage was reasonable. However, getting L_2 mature to L_3 has been a challenge in all these experiments, and in most experiments death rates increased at this stage of development. The reasons for the difficulties to culture many eggs to L_3 stage need to be clearly established and solutions found.

Nevertheless, these results demonstrate that the LDA shows promise and can be further developed to monitor the occurrence of AR in cattle *Cooperia.* However, little further progress than that described by Johnson (2000) was achieved in modifying the LDA for use with cattle.

The preliminary experiments conducted with the LFIA suggest that this may be a useful assay to

detect ML resistance in sheep and thus possibly in cattle nematodes. The dose response curves and IC_{50} values for ivermectin achieved in this work were similar to the results reported by Álvarez-Sánchez *et al.* (2005). Of particular interest is the apparent ability of this assay to clearly distinguish IV-susceptible *Teladorsagia* in sheep, which was an outcome the LDA cannot achieve. This also suggests that the LFIA may also be useful detecting AR in cattle nematodes. Although sheep nematodes were used, the mechanism of determining the paralysis of the pharyngeal muscles is likely to be similar between sheep and cattle nematodes.

Although the r^2 of the LFIA experiments were high, there is definitely a need to use higher concentrations of the anthelmintic to obtain a more desirable representation of the top of the dose response curves for at least *Teladorsagia,* which is somewhat different from that represented by Álvarez-Sánchez *et al.* (2005). Nevertheless, the report by Álvarez-Sánchez *et al.* (2005) suggests that similar anthelmintic concentrations were useful for several trichostrongylids in sheep indicating that these concentrations may also be useful with cattle nematodes particularly *Cooperia*.

The main problem with the LFIA was the difficulty in identifying the L_1 to genus. One solution to this problem is to utilise PCR technology to determine the genotype of the isolated larvae. However, using PCR technology will make this assay time and money consuming albeit with better results. In addition, it is technically challenging to recover individual non-feeding larvae from a slide while examining the sample with a fluorescent microscope for subsequent identification with PCR. Recovering larvae using a modified Pasteur pipette developed for this experiment required a lot of care.

Overall the studies represented in this report: "The development of *in vitro* assays for anthelmintic resistance in cattle nematodes", illustrate that there is a possibility of successful optimising these two assays, the LDA and LFIA, to detect resistance in cattle nematodes. This work has provided an insight into the initial step towards optimising the LDA (particularly with the use of ECYE protocol) and initial steps to modify the LFIA for detecting AR in cattle nematodes. The important areas highlighted in the discussion in various chapters and here in the general discussion, provide the baseline for further studies. Further studies to further develop these assays are required before they can be successfully used for detecting anthelmintic resistance in cattle in New Zealand and elsewhere.

CHAPTER EIGHT

8.0 CONCLUSION AND RECOMMENDATIONS

Despite access to modern anthelmintics and a better understanding of their epidemiology, GIN infections in cattle are still a problem to the livestock sector in New Zealand. Use of anthelmintics remains the key tool to control these parasites. Regular drenching, sometimes with underdosing, is considered an important risk factor leading to the current AR problem. The impact of parasitism and AR in the cattle sector can be a severe loss in terms of time and money for farmers who use anthelmintics that are no longer effective.

Current indications are that new anthelmintics for use in cattle are not likely in the near future, thus the currently available chemicals need to be used in a more sustainable way. A part of this solution is the development of more sensitive and appropriate assays to monitor the occurrence of anthelmintic resistance.

Both of the assays investigated in this work, the LDA and LFIA, showed the possibility that they could be more reliable assays for cattle nematodes in the future. Generally, it can be concluded from the current experiments and the previous experiments by Johnson (2000) that *C. oncophora* grow better at a lower temperature (18˚C) and using lower concentrations of media components ($^1/_8$ *E. coli* ¼ yeast extract) though the results of the LDA protocols in the current experiments were not better than those of Johnson (2000). The weakness with the LDA protocol was that by Day 12 most of the L_2 had not yet matured to L_3. With the LFIA the principle challenge is likely to be developing a method to easily recover the feeding or non-feeding larvae so they can be identified using PCR technology. Nevertheless, these two assays: LDA and LFIA showed the possibility that they could be extremely useful in the testing of resistance in cattle nematodes in future with LFIA potentially being the best.

In conclusion, more effort is needed to develop better assays to monitor AR in cattle nematodes. The future of the New Zealand cattle farming must consider AR in any parasite control programme.

APPENDICES

Appendix 3.1: Modified McMaster egg counting technique

I: Principle

The modified McMaster counting technique is based on floatation of eggs on the1cm^2 grid chambers of McMaster slide. Strongyle eggs have lower specific gravity than have saturated salt solution (1.2) and therefore float on top of the salt solution in the chambers.

II: Requirements:

Requirements for modified McMaster method involved:

Stainless steel bowls (99 X 3.5cm and 100ml), domestic sieves (1mm aperture, 6cm diameter), plastic spoon, electric weighing balance (Mettler Toledo), saturated salt solution (with specific gravity of 1.2), Pasteur pipettes, McMaster slides (1cm^2, with 0.15ml under each grid), laboratory counter, universal bottles (28ml) and Olympus microscope.

III: Method

1. Bowl, sieve and spoon were place on a balance and press “on” to RE-ZERO it.
2. Then, 2gm of faeces was weighed out on the sieve on the bowl.
3. A universal bottle was filled with 28ml saturated salt solution and this was added into the sieve/bowl, and faeces were broken up with a teaspoon, and then agitated to make an even suspension. The faeces were then pressed through the sieve and the faecal residues remaining on the sieve discarded.
4. While stirring the filtrate to and fro, two samples were removed with a Pasteur pipetteto fill both chambers of McMaster slide.
5. The slides were left for 2minutes to allow eggs to float to the surface before counting.
6. The number of eggs in each grid on both chambers of the McMaster slide was recorded and multiplied by 50 in order to estimate the number of eggs/g.

Appendix 3.2: Faecal egg floatation technique

I: Principle

The faecal egg floatation technique based on floatation of eggs on top of the test tubes with the faecal suspension (liquid) and attach to underneath of the cover slip placed on top of the convex meniscus of the fluid. The principle behind it is that, strongyle eggs have lower specific gravity than have saturated salt solution, which has specific gravity 1.2 that makes the eggs to float on top of the salt solution.

II: Requirements

Requirements for faecal egg floatation method involved:

A stainless steel bowl (99 X 3.5cm and 100ml), domestic sieves (1mm aperture, 6cm diameter), plastic spoon, electric weighing balance, saturated salt solution, glass test tubes (10ml), glass slide, cover slip, laboratory counter, universal bottle (28ml), and Olympus microscope.

III: Method

1. Bowl, sieve and spoon were place on a balance and press "on" to RE-ZERO it.
2. Then, 2gm of faeces was weighed out on the sieve on the bowl.
3. A universal bottle was filled with 28ml saturated salt solution and this was added into the sieve/bowl, and faeces were broken up with a teaspoon, and then agitated to make an even suspension. The faeces were then pressed through the sieve and the faecal residues remaining on the sieve discarded.
4. While stirring to and fro, the faecal suspension was immediately poured in a test tube until the meniscus was convex and then a cover slip was applied.
5. The test tube was left to stand for about 20-30minutes to allow the eggs to float on top of the salt solution under the cover slip.
6. Afterwards, removed by lifting the cover slip upwards quickly while firmly holding the corners and place it on clean-prepared microscope slide.
7. Examination of the slide under the microscope for any egg was performed.

Appendix 3.3: Nematode eggs recovery technique

I: Principle

Strongyle eggs have a lower specific gravity than have 10% sugar solution but not as high as 25% sugar solution. Debris and sand from the faecal slurry have higher specific gravity therefore settled down at the bottom of the test tube leaved the eggs floating on the interface of the two sugar solutions.

A: Sugar solution preparations

I: Requirements:

Requirements for sugar solutions preparation involved:

White sugar crystals (1kg pack of Chelsea sugar brand - NZ Sugar Co. Ltd), yellow and blue food colours, beakers (500mls), measuring cylinder (500mls), universal bottles (28mls), weighing balance, autoclave, tray and distilled water.

II: Method

a) 10% sugar solution

i) 50g of white sugar crystals were weighed into 450mls of distilled water.

ii) Few drops of food colour (yellow) were added in the solution and the mixture was dispensed in 28mls universal bottles.

b) 25% sugar solution

i) 100g of white sugar crystals was weighed into 400mls of distilled water

ii) Few drops of food colour (blue) were added in the solution and the mixture was dispensed into 28mls universal bottles.

NB: Both sugar solutions were sterilized in the autoclave, cooled and stored at room temperature ready for use.

B: Procedure for the actual nematode eggs recovery

I: Principle:

Nematode eggs can penetrate through sieves with a specified dimension and by the help of the force of centrifugation in the centrifuge machine, settle at the bottom of the tubes and finally collected for the intended uses.

II: Requirements:

Requirements for nematode eggs recovery involved

The pooled faecal sample about 200g (depending on the egg counts), weighing balance Mettler Toledo), motor and pestle, flask (500mls, sieves (20µm and 100µm; 250mm), two plastic jugs 3lts), sugar solutions (10 and 25%), Fungizone (25% Amphotericin B), syringe (10 and 20mls), centrifuge (ILEC Cntra-8 International equipment Company), centrifuge tubes (60mls), squeezing bottle with distilled water, microscope slides, graduated pipette, cover slips, laboratory counter (Clay Adams) and microscope (Olympus).

Note: Fresh faecal samples are useful for eggs recovery. After estimating the eggs in all samples (as in Appendix 1), faecal samples with higher egg counts per gram are pooled together for eggs recovery (about 200g pooled sample depends on the number of the egg required for the experiment).

II: Procedure

1. About 200g of pooled faecal sample was put in motor. Carefully and gently the faeces were grounded using a mortar and pestle until a suspension was obtained.
2. The homogenous suspension was then put in a flask containing distilled deionised water (about 200-250ml) for 20minutes to soften it.
3. The faecal suspension was sieved starting with the big sieve (250mm) to remove big particles of the faecal slurry. Repeating this step more than three times will give better results in the next sieving steps.
4. The egg suspension was further sieved through 100µm and finally 20µm sieves into a 3litres plastic jag and the residues were discarded.
5. Further washing while sieving through 20µm sieve was carried out and the eggs were retained on the sieve, washed-off from the screen of the sieve with a jet of water from a squeezed bottle to a clean glass beaker.
6. Sugar gradients were prepared in 60ml centrifuge tubes by first putting 10ml of 10% (yellow) in the centrifuge tube, and then gently add 15ml of 25% sugar solution (blue) at the bottom of the tube using a tip of a syringe while holding the syringe vertically to the bottom of the tube and make sure the sugar gradient does not mix up.
7. By using a 20ml syringe, 10-15ml of faecal slurry was carefully dispensed (depend on the amount of slurry and number of eggs required) on top of the two sugar gradients by holding the tube in slant position and the tip of the syringe against the sides of the tubes to avoid mixing of the slurry with the sugar gradients.
8. The contents in the centrifuge tube were centrifuged at 1500Xg for seven minutes.
9. The eggs were recovered from the interface of yellow and blue sugar gradient phases of the sedimentation by using a pipette, washed through a 20µm sieve and rinsed thoroughly with deionised water from a squeezed bottle and eventually collected in a clean 60ml tube.

NB: Sugar concentration is strong and osmotic pressure can destroy the eggs; so do not leave the eggs in sugar solutions for long period.

10. The tube was then left to stand on a rack for about 20-30minutes to allow for the eggs to settle down. It is good if the tubes are left to stand in the refrigerator to arrest egg development.
11. Then, excess water was aspirated and discarded off from the tube using vacuum system leaving about 5-10ml of egg suspension on the bottom of the centrifuge tube.
12. Afterwards, the eggs were estimated by doing 10 counts of 20µl each on a microscope slide with a cover slip applied, and the average was estimated.

13. The egg suspension was finally adjusted to 100eggs/100µl for the ECYE protocol and about 4000eggs, which is equal to 80eggs/20µl for the DR protocol.
14. Finally, Fungizone was added in the egg suspension at 0.15% and 90µl/ml for the ECYE and DR protocols respectively to prevent bacteria and fungal growth in the cultures.

Appendix 3.4a: Faecal cultures preparation technique

Faecal culture

I: Principle

Nematode eggs can hatch and develop when supplied with optimum temperature, moisture and oxygen. NB: Each species have its favourable requirements of these essential factors and basically under optimum supply of these factors most viable nematode eggs can hatch and develop to infective larvae stage (L_3) successfully. Inadequate supply of one or all of these factors results to the interference in egg development and larvae survival.

II: Requirements

Requirements for faecal culture involved:

Fresh faecal samples (no weight limit), glass jars or plastic containers with lids, mortar and pestle, vermiculite and incubator at 25ºC.

III: Procedures

1. Faeces were grounded in a motor and pestle or in big containers if the amount of sample is large. This was done gently to make sure that it does not damage the eggs.
2. Grounded faeces were then mixed with vermiculate and little amount of water was added just to give it enough moisture. Vermiculite allows air circulation in the cultures to facilitate hatching.
3. Mixed faeces were packed loosely in glass jars or left in the big containers (if large sample is processed) and a lid loosely applied or by leaving an air space at the top to allow for aeration.
4. The jars/containers are transferred to the incubator for 10-14 days. On the second day, the lid was opened and turns the sample to allow more air supply as oxygen to facilitate more hatching of the eggs. Remember to check if the samples are dehydrated every after a day or two. If dehydration noted, the cultures were rehydrate by adding small amount of water using a squeeze bottle according to the level of dehydration. Note that excess moisture affects the texture of the sample, hence affect the egg development.
5. After 10-14 days of incubation, the samples were removed and the Baermann's technique was set in order to recover the eggs.

Appendix 3.4b: Baermann's technique for larvae recovery

I: Principle

Nematode larvae can swim down to the bottom of the funnel to a rubber tube with a forceps/clip at the margin due to the force of attraction caused by a Y-shaped glass funnel. These larvae are then collected in the bottles 18-24hours after setting the Baermann's apparatus.

II: Requirements

Requirements for Baermann's technique involved:

Faecal samples – fresh or from the incubator, tissue papers, glass funnel(s) with rubber tube at the tip, sieves (20µm; 250µm), stand for the funnels, graduated transparent bottles (250ml and 500ml), forceps/clips, plastic jugs or conical flask (1-2lts), light microscope, vacuum system, Pasteur pipettes, graduated-chambered glass slide, microscope glass slide, Lugol's iodine (5% iodine + 10% potassium iodide), cover slip, flame, laboratory counter.

III: Procedure

1. By using forceps or clips, the rubber tube of the funnels were closed from the bottom.
2. The sieve was layered by fine/tissue paper and the samples were placed on the sieves leaving the fine/tissue papers underneath the samples. During this time the sieves should be put on the top circumference of the funnels and the funnels should be fitted on the stands.
3. The funnels were filled with tap water up to 2cm to the top to make sure it submerges the samples on the sieves.
4. The whole apparatus was left to stand for overnight (18-24hours) to allow for the larvae to settle at the bottom of the funnel.
5. After 24 hours, the forceps/clip was removed and enough amount of the liquid with the larvae was carefully released and tapped off from the funnel into the 2lts plastic jug or conical flask
6. The mixture was sieved and washed through 20µm sieve using tap water and the clean larvae recovered was put into graduated and transparent bottles.
7. The mixture was examined under a stereo microscope to detect any movement. The mixture was left to settle for 30minutes to concentrate the larvae on the bottom and the supernatant removed and discarded using vacuum system. Note the volume of the mixture.
8. **For larvae count**, 1ml of the larvae mixture was pipetted on a graduated-chambered glass slide and few drops of Lugol's iodine was added.
9. The contents on the graduated-chambered glass slide were examined under the microscope and counted using laboratory counter. The number of larvae was record. Larval estimations contained in the liquid in the storage bottles were carried out in relation to the amount of liquid and the larvae present after an average of about 5-10 counts were estimated.
10. **For larval identification**, few drops of the larval suspension were pipetted onto a microscope glass slide.
11. The slide was gently warmed from the bottom and then few drops of Lugol's iodine were added and a cover slip applied.

12. The contents on the microscope slide were gently examined under the microscope and the larvae identification based on the morphological features.

    NB: The bulk of the larvae remained was stored in the refrigerator at 4ºC for future uses.

Appendix 3.5: Detailed procedures and some documentation of the work/logistics that were followed to infect sheep in the process of cycling *C. oncophora* through sheep.

Lamb Group	Date infected	Lamb No.	Sex	Anthelmintic dose rate (Pre-infection dose)	Source and total dose of infective larvae dose (given orally)	General remarks
	5\7\10\13\18\20/04/2006	368[a]	M	All animals in this group drenched with 8ml matrix @ and then 5ml genesis + 2mls albendazole. After 2 days given 6ml genesis +2ml albendazole	30,000 Tuapaka divided into 6	*
1	14/03/2006	503[a]	M		30,000 Tuapaka – divided into two	**: *
	18\20/04/2007	524[a]	M		30,000 Tuapaka – divided into two	*
		531[a]	M		30,000 Tuapaka – divided into two	*
	10\12\14/05/2006	6056[a]	F	6ml genesis+2ml albendazole		
	28/8/2006	6056[b]	F	5mls scanda	30,000 Tuapaka – given at once	*
	10\12\14/05/2006	6064[a]	F	6ml genesis+2ml albendazole	30,000 Tuapaka – divided into three	*
	28/8/2006	6064[b]	F	5mls scanda	30,000 Tuapaka – given at once	
2	12\13/6/2006	6066[a]	M	6ml genesis+2ml albendazole	30,000 Tuapaka - divided into two	
	6/9/2006	6066[b]	M	6ml genesis+2ml albendazole	30,000 Tuapaka – given at once	*
	6/9/2006	6070[b]	F	6ml genesis+2ml albendazole	30,000 Tuapaka – given at once	*
	2/12/2006	6245[a]	M	5ml matrix	30,000 Tuapaka - Cycled through lamb No. 6066 – given at once	* (750 oocysts/g)
	28/10/2006	6245[b]	M	5ml matrix	10,000 Tuapaka - Cycled through lamb No. 6066 – given at once	
3	22/9/2006	6248[a]	M	5ml matrix	15,000 Tuapaka - Cycled through lamb No. 6066 – given at once	*
	22/9/2006	6285[a]	M	5ml matrix	15,000 Tuapaka - Cycled through lamb No. 6066 – given at once	* (1200 oocysts/g)
	28/10/2006	6305[a]	M	5ml matrix	10,000 Tuapaka - Cycled through lamb No. 6066 – given at once	* (500 oocysts/g)
	15/12/2007	6285[b]	M	5ml matrix	37,750 Farm H (Gisborne) – given at once	
	15/12/2007	6305[b]	M	5ml matrix	22,250 Farm BR (Gisborne) – given at once	

Key: * **=** did not produce eggs; ** = positive only by floatation technique; a, b = 1st & 2nd round of infection respectively.

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Appendix 4.1a: Preparation and examination of the cultures – using the DR protocol

I: Requirements:

Requirements for DR protocol involved: 96-wells LDA plates (Commercial/in-house), adjusted egg suspension (with Fungizone), multidose pipette (1ml), pipettes (20µl, 200µl), Incubator (at 25ºC), desiccators, nutrient medium, gentle flame, iodine solution, microscope slide and light microscope.

II: Preparation of the reagents

i) Yeast extract medium

Nutritive media was prepared by weighing 1g of yeast extract into 90ml of 0.85% saline solution. Mixed well and decant 9ml into 10ml bottles. Divide the contents of a whole bottle of Earle's balanced salt solution (EBSS) (E 2888) in aliquots of 1ml each in 1ml Eppendorf tubes. Then store the nutritive media and EBSS aliquots in the freezer until the time of use. At the time of use, a final solution was made by mixing 9ml of yeast Extract and 1ml of EBSS into the bottle to make a total of 10ml. The remaining nutritive media can be stored in the freezer for further use.

ii) Agar, **Amphotericin B and Lugol's Iodine** see the ECYE protocol described in Appendix 4.1b.

II: Procedure

a. The plates were open by cutting the seal and immediately, 20µl of the adjusted egg suspension (Appendix 3.3) was gently added into each well.
b. The plates were covered well by the lid and returned to the original bag and incubated at desired temperature e.g. 18ºC or 25ºC overnight in a water container to avoid desiccation.

2. In the next day, 20µl of nutrient medium prepared as described in II (i) above was added in each well when the control well has about 80% hatching (observe before adding the media).
3. The plates were further incubated and monitored everyday for the change in temperature or any signs of dehydration. If any of the wells seemed to dehydrate, 10µl of distilled deionised water was added and the re-hydrated wells recorded.
4. After the determined incubation, the plates were removed from the incubator and the contents from each well were separately pipetted on a microscope slide starting from the control wells.
5. A drop of Lugol's iodine stain was added on the slide, and the contents were gently heated from the bottom to stretch the larvae for easy identification and a cover slip was applied.
6. The slides were examined under the microscope and the isolated species were identified (based on the measurements and the morphological features) and their numbers recorded.

Appendix 4.1b: Preparation and examination of the cultures – using the ECYE protocol

I: Equipments

Requirements for the ECYE protocol involved:

Measuring cylinders (100ml, 250ml, 500ml), beakers (150ml, 250ml, 500ml), plastic test tubes (50ml), 98-well microtitre plates (300µl), weighing balance, multidose pipette (1ml), pipettes (10µl, 20µl, 250µl), flasks (50ml, 500ml), small bottles (10ml), squeeze bottle (250ml), microwave, sterile camber, distilled water, sieves, desiccators (during incubation), special designated nylon papers (to cover the surface of the plates before storage), Eppendorf tubes (1ml).

Note: For equipments for egg estimation and recovery please refer to Appendix 1 and 2.

II: Chemicals

Agar (Bacto™ – agar Dickinson), *E. coli* bacteria (strain W (ATCC) 9637, Sigma), yeast extract (Y–1000 Sigma), Earle's balanced salt solution (EBSS; E 2888), Amphotericin B solubilised (A-9525, Sigma), phosphate buffer boluses, iodine granules, potassium iodide and distilled water.

III: Preparation of the reagents

1) Agar (2% agar matrix)

Using a weighing balance, 2g of agar powder was weighed and dissolved in 98ml of distilled water by heating the mixture in the microwave until boiled. This was carefully done to avoid overflowing of the media while heating. The media was distributed into the plates by putting 100µl in each well under sterile chamber.

NB:

i. By using a pipette, 100µl of the agar was put in each well in the microtitre plates immediately while the agar is still hot because agar solution solidifies when cool. This needed to prepare and arrange the plates in a manner that will allow for uninterrupted dispensing.
ii. It is important to work under sterile chamber when adding the agar in the plates to maintain sterility in the plates.
iii. After finish pouring the agar in the plates, the plates were left to stand for a while with the lids loosely put in place in order to allow the agar to solidify (about 5 -10 minutes).

2) *E. coli* suspension

15mg of lyophilized E. coli strain was weighed into 100ml of distilled water with the suspension mixed well and stored in a freezer until the time of use.

3) Yeast extract nutritive medium: For preparation of nutritive media refer Appendix 4.1a.

4) Amphotericin B solution

25mg of Amphotericin B powder was weighed into 100ml of distilled water and mixed well. The solution was stored in the freezer until the time of use.

5) Phosphate buffer saline (PBS)

PBS was prepared by dissolving one tablet of phosphate in 500ml of distilled water. The solution was stored at room temperature until the time of use.

6) Lugol's iodine solution

Lugol's iodine was prepared by dissolving 5g of Iodine and 10g of Potassium iodide in 100ml tape water, mixed thoroughly and stored at room temperature until the time of use. NB: Remember to apply a stopper on the bottle, as Iodine is volatile.

IV: Preparation of the plates

The experiments were carried out in 96-well microtitre plates used in the standard DrenchRite plates described by Gill *et al.* (1995). The in-house with no anthelmintics plates were usually first prepared by adding 100µl of hot agar in the wells in the empty sterile plates while working under a sterile chamber (Appendix 4.1b part II). The first step in making in-house plates was the estimation of the number of wells required for the test. This was also useful latter in the estimation of the amount of egg suspension and media required.

Note:

i. Fresh agar was regularly prepared and does not stored agar plates for long periods.
ii. If need to store the plates, the surface of the agar plates was covered on the top with a special designated cover seal after pouring the media in the plates in order to avoid dehydration.
iii. Sterilize by autoclaving the agar that needs to be stored for future uses immediately after plating was performed.

V: Estimating the eggs and adjusting the concentration

1. Eggs were recovered from faces as described in Appendix in 3.3 in steps 1-14.
2. About sion should made available on the bottom of the tubes.
3. The assumption was ECYE protocol requires 60µl of egg suspension each well.
4. (Ensure mixing the egg suspension thoroughly well to maintain the consistent egg dispensing in the wells).
5. The tube containing the egg suspension was shaken well and 100µl of the suspension drawn-off immediately onto a slide to estimate the number of eggs needed - concentration of 100eggs/100µl. **Note:** To achieve the required concentration, if find 75 eggs in each 100µl, decrease the volume to about 7.5ml by first spinning the tube at 1500g for seven minutes. Then, draw off the supernatant fluid by vacuum system and leave about 7.5ml of the egg suspension. If find 175eggs in each 100µl, increase the volume to about 17.5ml simply by adding distilled water.
6. Fungizone (25% Amphotericin B) prepared as described in Appendix 4.1b was
7. added to the egg suspension to a final concentration of 0.15% to prevent the
8. growth of micro-organisms in the wells.

VI: Preparation of the media solution for the ECYE protocol cultures

A: Things to note:

1. The reagents and media solution for the ECYE protocol cultures was first prepared.
2. Each well required 40µl of nutrient medium. The final concentration of the media was prepared and adjusted according to the volume required. So, it was also important to estimate the volume of the media required.
3. The concentration required was at the ratio of *E. coli* and ¼ yeast extract. To achieve this ratio of of *E. coli* and ¼ nutritive medium the following explanation below was helpful.

B: Preparation of the final concentration

1. NB: The components of the media are listed above (Appendix 4.1b part III). Depends on the amount of media required, the proportions were prepared from the initial concentrations of the components of the media for sheep nematodes. The difference was the nutrient medium that was added to each well; working on the basis that 40µl per well was [20µl *E. coli* + 20µl nutrient medium (yeast: Earle's BSS, 9:1)] that is 50% *E. coli* and 50% Yeast extract.
2. Therefore, $^{1}/_{8}$ of 50% *E. coli* and ¼ of 50% yeast extract were estimated according to the volume of media required and mixed together. The remaining $^{5}/_{8}$ of the volume of media required was covered up by adding distilled water to balance the volume of the media required.

Appendix 4.2a: Experiment I.

The raw data for the use of sterilized and non-sterilized agar in cultures in the own prepared LDA agar plates using the ECYE protocol

Day	Agar	Media	Well No.	*Haemonchus*						*Ostertagia*					
				E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
2	Sterilised	Sterilised	1	28	11	0	0	0	39	35	0	0	0	0	35
			2	60	0	0	0	0	60	54	0	0	0	0	54
			3	49	0	0	0	0	49	41	0	0	0	0	41
		Non-sterilized	1	48	0	0	0	0	48	55	0	0	0	0	55
			2	47	0	0	0	0	47	48	0	0	0	0	48
			3	49	0	0	0	0	49	41	0	0	0	0	41
	Non-sterilized	Sterilised	1	65	0	0	0	0	65	40	0	0	0	0	40
			2	60	0	0	0	0	60	47	0	0	0	0	47
			3	46	0	0	0	0	46	55	0	0	0	0	55
		Non-sterilized	1	47	0	0	0	0	47	49	0	0	0	0	49
			2	45	0	0	0	0	45	47	0	0	0	0	47
			3	55	0	0	0	0	55	46	0	0	0	0	46
3	Sterilised	Sterilised	1	44	0	0	0	0	44	45	0	0	0	0	45
			2	50	0	0	0	0	50	57	0	0	0	0	57
			3	49	0	0	0	0	49	40	0	0	0	0	40
		Non-sterilized	1	56	4	0	0	0	60	56	0	0	0	0	56
			2	46	0	0	0	0	46	42	0	0	0	0	42
			3	54	0	0	0	0	54	40	0	0	0	0	40
	Non-sterilized	Sterilised	1	45	0	0	0	0	45	41	0	0	0	0	41
			2	52	0	0	0	0	52	59	0	0	0	0	59
			3	67	0	0	0	0	67	47	0	0	0	0	47
		Non-sterilized	1	42	0	0	0	0	42	48	0	0	0	0	48
			2	33	15	0	0	0	48	35	0	0	0	0	35
			3	17	29	0	0	0	46	28	21	0	0	0	49
4	Sterilised	Sterilised	1	1	50	0	0	2	53	10	34	0	0	0	44
			2	6	38	0	0	0	44	44	11	0	0	0	55
			3	6	45	0	0	2	53	10	36	0	0	0	46
		Non-sterilized	1	6	35	0	0	0	41	44	10	0	0	0	54
			2	3	47	0	0	0	50	7	41	0	0	0	48
			3	6	38	0	0	0	44	11	35	0	0	0	46
	Non-sterilized	Sterilised	1	3	33	0	0	0	36	44	4	0	0	0	48
			2	10	35	0	0	0	45	24	23	0	0	0	47
			3	11	35	0	0	0	47	22	18	0	0	0	40
		Non-sterilized	1	14	35	0	0	0	50	30	18	0	0	0	48
			2	3	38	0	0	0	41	18	24	0	0	0	42
			3	10	43	0	0	0	53	11	25	0	0	0	36
5	Sterilised	Sterilised	1	6	0	34	0	0	40	1	0	52	0	0	53
			2	2	0	36	0	0	38	1	0	48	0	0	49
			3	1	0	51	0	0	52	2	0	29	0	0	31
		Non-sterilized	1	2	0	46	0	0	48	1	0	40	0	0	41
			2	1	0	36	0	1	38	2	0	38	0	0	40
			3	2	0	39	0	0	41	2	0	39	0	0	41
	Non-sterilized	Sterilised	1	3	0	37	0	0	40	1	0	48	0	0	49
			2	1	0	42	0	0	43	1	0	43	0	0	44
			3	3	0	38	0	0	41	2	0	44	0	0	46
		Non-sterilized	1	2	0	39	0	0	41	2	0	39	0	0	41
			2	0	0	40	0	0	40	0	0	45	0	0	45
			3	4	0	42	0	0	46	4	0	43	0	0	47

Appendix 4.2a continued:

6	Sterilised	Sterilised	1	0	0	42	0	0	42	2	0	33	0	0	35
			2	0	0	46	0	0	46	1	0	53	0	0	54
			3	1	0	48	0	0	49	0	0	36	0	0	36
		Non-sterilized	1	0	0	50	0	0	50	2	0	43	0	0	45
			2	0	0	39	0	0	39	1	0	38	0	2	41
			3	3	0	38	0	0	41	2	0	31	0	0	33
	Non-sterilized	Sterilised	1	3	0	37	0	0	40	1	0	48	0	0	49
			2	3	0	50	0	0	53	1	0	38	0	0	39
			3	2	2	45	0	0	49	0	0	46	0	0	46
		Non-sterilized	1	5	0	39	0	0	44	1	0	46	0	0	47
			2	3	0	39	0	6	48	0	0	40	0	0	40
			3	0	0	49	0	0	49	2	0	54	0	0	56
7	Sterilised	Sterilised	1	2	0	53	0	0	55	3	0	38	0	0	41
			2	0	0	50	0	0	50	1	0	48	0	0	49
			3	0	0	44	2	0	46	0	0	44	0	2	46
		Non-sterilized	1	0	0	54	0	0	54	0	0	49	0	0	49
			2	0	0	42	0	0	42	0	0	42	0	0	42
			3	0	0	41	0	0	41	0	0	36	0	3	39
	Non-sterilized	Sterilised	1	0	0	39	5	0	44	0	0	49	0	0	49
			2	0	0	39	0	0	39	0	0	45	0	0	45
			3	1	0	39	2	0	42	3	0	39	0	0	42
		Non-sterilized	1	1	0	40	2	0	43	1	0	42	0	1	44
			2	0	0	51	0	0	51	0	0	45	0	0	45
			3	0	0	39	1	0	40	0	0	38	0	0	38
8	Sterilised	Sterilised	1	0	0	0	51	0	51	0	0	4	35	0	39
			2	0	0	0	50	0	50	0	0	0	51	0	51
			3	0	0	0	57	0	57	0	0	0	45	0	45
		Non-sterilized	1	0	0	0	44	0	44	0	0	0	35	4	39
			2	0	0	0	42	0	42	0	0	0	45	0	45
			3	0	0	0	44	0	44	0	0	0	37	0	37
	Non-sterilized	Sterilised	1	0	0	0	50	0	50	0	0	0	34	7	41
			2	0	0	0	40	0	40	0	0	0	40	6	46
			3	0	0	0	42	0	42	0	0	0	36	3	39
		Non-sterilized	1	0	0	0	39	0	39	0	0	3	43	6	52
			2	0	0	0	41	0	41	0	0	0	50	4	54
			3	0	0	0	38	0	38	0	0	4	34	4	42

Appendix 4.2b: The raw data obtained for testing of the use of sterilized and non-sterilized agar in cultures in the LDA using the DR protocol.

Day	Agar	Media	Well No.	*Haemonchus* E	L1	L2	L3	D	T	*Ostertagia* E	L1	L2	L3	D	T
2	Sterilised	Sterilised	1	26	35	0	0	0	61	13	35	0	0	0	48
			2	15	60	0	0	0	75	4	44	0	0	0	48
			3	30	43	0	0	0	73	5	39	0	0	0	44
		Non-sterilized	1	10	60	0	0	0	70	5	48	0	0	0	53
			2	10	70	0	0	0	80	5	45	0	0	0	50
			3	11	80	0	0	0	91	7	42	0	0	0	49
	Non-sterilized	Sterilised	1	5	56	0	0	0	61	8	32	0	0	0	40
			2	3	74	0	0	0	77	2	59	0	0	0	61
			3	9	83	0	0	0	92	4	33	0	0	0	37
		Non-sterilized	1	8	60	0	0	0	68	3	49	0	0	0	52
			2	16	60	0	0	0	76	3	60	0	0	0	63
			3	5	65	0	0	0	70	4	45	0	0	0	49
3	Sterilised	Sterilised	1	15	0	65	0	0	80	3	0	52	0	0	55
			2	10	0	69	0	0	79	2	0	47	0	0	49
			3	5	0	80	0	0	85	6	0	38	0	0	44
		Non-sterilized	1	6	0	69	0	0	75	0	0	62	0	0	62
			2	15	0	80	0	0	95	3	0	51	0	0	54
			3	28	0	68	0	0	96	0	0	53	0	0	53
	Non-sterilized	Sterilised	1	11	0	80	0	0	91	0	0	60	0	0	60
			2	17	0	72	0	0	89	2	0	47	0	0	49
			3	10	0	80	0	0	90	5	0	44	0	0	49
		Non-sterilized	1	9	0	73	0	0	82	0	0	47	0	0	47
			2	9	0	83	0	0	92	2	0	44	0	0	46
			3	0	0	85	0	0	85	4	0	47	0	0	51
4	Sterilised	Sterilised	1	3	0	84	3	0	90	0	0	49	0	0	49
			2	6	0	67	0	0	73	0	0	46	0	0	46
			3	6	0	68	0	0	74	0	0	65	0	0	65
		Non-sterilized	1	5	0	72	0	0	77	1	0	54	0	0	55
			2	3	0	82	0	0	85	2	0	47	0	0	49
			3	5	0	75	0	0	80	0	0	56	0	0	56
	Non-sterilized	Sterilised	1	0	0	95	0	0	95	3	0	46	0	0	49
			2	0	0	80	0	0	80	0	0	53	0	0	53
			3	0	0	84	0	0	84	0	0	59	0	0	59
		Non-sterilized	1	2	0	86	0	0	88	5	0	43	0	0	48
			2	7	0	87	0	0	94	4	0	46	0	0	50
			3	No growth						2	0	48	0	0	50
5	Sterilised	Sterilised	1	3	0	73	0	0	76	0	0	39	0	1	40
			2	0	0	82	0	0	82	0	0	48	0	0	48
			3	0	0	89	0	0	89	0	0	52	0	0	52
		Non-sterilized	1	4	0	79	0	0	83	0	0	49	0	0	49
			2	0	0	78	0	0	78	0	0	56	0	0	56
			3	3	0	91	0	0	94	0	0	59	0	0	59
	Non-sterilized	Sterilised	1	2	0	89	0	0	91	0	0	63	0	0	63
			2	1	0	84	0	0	85	0	0	45	0	0	45
			3	0	0	89	0	0	89	0	0	57	0	0	57
		Non-sterilized	1	0	0	88	0	0	88	0	0	49	0	0	49
			2	1	0	75	0	0	76	0	0	46	0	0	46
			3	0	0	71	0	0	71	0	0	48	0	0	48

Appendix 4.2b continued:

6	Sterilised	Sterilised	1	4	0	68	0	17	89	0	0	52	0	0	52
			2	0	0	56	0	18	74	0	0	49	0	0	49
			3	0	0	86	0	9	95	0	0	45	0	0	45
		Non-sterilized	1	0	0	69	0	11	80	0	0	49	0	0	49
			2	2	0	50	0	24	76	0	0	60	0	0	60
			3	0	0	78	0	12	90	0	0	56	0	0	56
	Non-sterilized	Sterilised	1	2	0	70	0	6	78	0	0	48	0	0	48
			2	0	0	46	0	31	77	0	0	56	0	0	56
			3	0	0	65	0	25	90	0	0	52	0	0	52
		Non-sterilized	1	0	0	70	0	19	89	0	0	48	0	0	48
			2	2	0	40	0	29	71	0	0	51	0	0	51
			3	0	0	58	0	30	88	0	0	47	0	0	47
7	Sterilised	Sterilised	1	2	0	58	0	19	79	0	0	49	0	0	49
			2	0	0	60	0	20	80	0	0	48	0	0	48
			3	0	1	64	0	10	75	0	0	36	3	0	39
		Non-sterilized	1	0	1	54	0	24	79	0	0	48	0	0	48
			2	0	0	25	0	56	81	0	0	59	0	0	59
			3	0	0	80	0	15	95	0	0	49	0	0	49
	Non-sterilized	Sterilised	1	0	0	30	0	42	72	0	0	55	0	0	55
			2	0	0	64	0	20	84	0	0	47	0	0	47
			3	2	0	30	0	38	70	0	0	44	0	0	44
		Non-sterilized	1	0	0	52	0	28	80	0	0	51	5	0	56
			2	0	0	42	0	46	88	0	0	47	0	0	47
			3	2	0	47	0	30	79	0	0	46	0	0	46
8	Sterilised	Sterilised	1	2	0	17	25	45	89	0	0	0	45	0	45
			2	0	0	0	47	35	82	0	0	0	50	0	50
			3	0	0	0	43	45	88	0	0	0	49	0	49
		Non-sterilized	1	0	0	10	55	20	85	0	0	0	42	0	42
			2	0	0	0	64	18	82	0	0	0	59	0	59
			3	0	0	9	53	28	90	0	0	0	48	0	48
	Non-sterilized	Sterilised	1	0	0	5	55	19	79	0	0	0	45	0	45
			2	0	0	0	70	10	80	0	0	0	60	0	60
			3	1	0	9	60	14	84	0	0	0	50	0	50
		Non-sterilized	1	0	0	0	56	20	76	0	0	0	35	3	38
			2	0	0	0	78	0	78	0	0	0	46	0	46
			3	0	0	0	87	0	87	1	0	0	48	0	49

Key: E = Unhatched eggs

L1, L2 = 1st and 2nd larval stages respectively

L3 = Ensheathed larvae

D = Dead larvae

T = Total

Appendix 4.3: Experiment III: Varying nutrients – raw data for *Cooperia* cultures in seven different components of media concentration using the ECYE protocol at 25ºC for 14 days. This data is corrected to the number of *Cooperia* identified in the cultures.

Note: No. 1-7 = concentration of the media compared to the standard concentration used with sheep at a base line of 1:1: Key for E; L1, L2; L3; D and T is as shown in Appendix 4.2b.

Media		1						2						3					
Day	Well	E	L_1	L_2	L_3	D	T	E	L_1	L_2	L_3	D	T	E	L_1	L_2	L_3	D	T
	1	70	0	0	0	0	70	72	1	0	0	0	73	36	1	0	0	0	37
	2	59	0	0	0	0	59	65	0	0	0	0	65	65	1	0	0	0	66
	3	65	0	0	0	0	65	66	0	0	0	0	66	60	0	0	0	0	60
2	4	75	0	0	0	0	75	67	0	0	0	0	67	66	0	0	0	0	66
	5	76	0	0	0	0	76	68	0	0	0	0	68	75	0	0	0	0	75
	6	66	1	0	0	0	67	69	0	0	0	0	69	70	0	0	0	0	70
	T	411	1	0	0	0	412	407	1	0	0	0	408	372	2	0	0	0	374
	Av	69	0	0	0	0	69	68	0	0	0	0	68	62	0	0	0	0	62
	1	2	42	10	0	0	54	3	20	8	0	0	31	6	36	22	0	0	64
	2	3	22	45	0	0	70	6	25	14	0	0	45	3	54	10	0	0	67
	3	3	34	23	0	0	60	10	35	32	0	0	77	4	28	18	0	0	50
4	4	7	34	20	0	0	61	4	61	10	0	0	75	4	28	20	0	0	52
	5	5	43	14	0	0	62	5	43	17	0	0	65	4	31	17	0	0	52
	6	3	35	22	0	0	60	3	64	9	0	0	76	5	25	37	0	0	67
	T	23	210	134	0	0	367	31	248	90	0	0	369	26	202	124	0	0	352
	Av	4	35	22	0	0	61	5	41	15	0	0	62	4	34	21	0	0	59
	1	4	43	7	0	0	54	1	7	63	0	0	71	8	3	54	0	0	65
	2	4	30	19	0	0	53	5	3	63	0	0	71	5	3	48	0	0	56
	3	2	11	38	0	0	51	3	9	69	0	0	81	7	3	67	0	0	77
6	4	2	4	52	0	0	58	5	14	56	0	0	75	3	5	53	0	0	61
	5	1	1	55	0	0	57	4	8	78	0	0	90	4	0	60	0	0	64
	6	2	1	59	0	0	62	5	3	70	0	0	78	4	0	72	0	0	76
	T	15	90	230	0	0	335	23	44	399	0	0	466	31	14	354	0	0	399
	Av	3	15	38	0	0	56	4	7	67	0	0	78	5	2	59	0	0	67
	1	2	8	36	0	3	49	5	2	71	0	3	81	4	1	60	0	7	72
	2	3	8	37	0	4	52	2	6	60	0	1	69	1	0	54	0	3	58
	3	4	0	34	0	2	40	4	0	65	0	0	69	2	2	59	0	4	67
8	4	5	0	48	0	2	55	3	0	59	0	0	62	1	1	56	0	7	65
	5	3	0	39	0	1	43	4	1	59	0	4	68	2	0	50	0	8	60
	6	4	0	46	0	0	50	5	0	62	0	3	70	2	0	59	0	4	65
	T	21	16	240	0	12	289	23	9	376	0	11	419	12	4	338	0	33	387
	Av	4	3	40	0	2	48	4	2	63	0	2	70	2	1	56	0	6	65
	1	3	0	30	0	12	45	2	0	56	0	3	61	3	0	55	0	14	72
	2	8	0	43	0	14	65	2	0	53	0	5	60	3	0	49	0	14	66
	3	4	0	47	0	8	59	1	0	45	0	6	52	0	0	33	0	19	52
10	4	3	0	60	0	4	67	2	0	42	0	5	49	2	0	56	0	6	64
	5	6	0	59	0	3	68	1	0	54	0	1	56	3	0	38	0	11	52
	6	2	0	53	0	8	63	1	0	47	0	5	53	3	0	53	0	12	68
	T	26	0	292	0	49	367	9	0	297	0	25	331	14	0	284	0	76	374
	Av	4	0	49	0	8	61	2	0	50	0	4	55	2	0	47	0	13	62
	1	0	0	32	0	12	44	3	0	46	0	18	67	8	0	23	0	9	40
	2	5	0	10	0	32	47	5	0	37	0	12	54	3	0	24	0	18	45
	3	3	0	33	0	22	58	3	0	40	0	12	55	2	0	39	0	15	56
12	4	1	0	46	0	23	70	5	0	51	0	19	75	3	0	41	0	12	56
	5	2	0	39	0	15	56	4	0	25	0	33	62	2	0	29	0	15	46
	6	4	0	46	0	14	64	1	0	35	0	21	57	5	0	26	0	13	44
	T	15	0	206	0	118	339	21	0	234	0	115	370	23	0	182	0	82	287
	Av	3	0	34	0	20	57	4	0	39	0	19	62	4	0	30	0	14	48
	1	4	0	12	0	46	62	5	0	36	2	13	56	3	0	5	0	25	33
	2	1	0	20	0	23	44	5	0	37	2	22	66	3	0	12	0	35	50
	3	4	0	30	8	21	63	1	0	39	6	20	66	4	0	34	0	16	54
14	4	4	0	30	4	29	67	2	0	35	4	21	62	4	0	37	3	13	57
	5	2	0	42	2	18	64	3	0	23	1	29	56	2	0	28	8	15	53
	6	2	0	40	2	13	57	3	0	39	1	25	68	2	0	47	6	19	74
	T	17	0	174	16	150	357	19	0	209	16	130	374	18	0	163	17	123	321
	Av	3	0	29	3	25	60	3	0	35	3	22	62	3	0	27	3	21	54

Appendix 4.3 continued: Varying nutrients using the ECYE protocol at 25ºC media components 4-7.

Media	4						5						6						7					
Day	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
2	41	1	0	0	0	42	62	1	0	0	0	63	63	0	0	0	0	63	62	0	0	0	0	62
	65	0	0	0	0	65	71	0	0	0	0	71	73	0	0	0	0	73	63	1	0	0	0	64
	73	0	0	0	0	73	61	2	0	0	0	63	52	0	0	0	0	52	70	0	0	0	0	70
	63	2	0	0	0	65	65	0	0	0	0	65	65	2	0	0	0	67	64	1	0	0	0	65
	65	0	0	0	0	65	61	0	0	0	0	61	61	1	0	0	0	62	75	0	0	0	0	75
	72	0	0	0	0	72	59	0	0	0	0	59	68	0	0	0	0	68	67	0	0	0	0	67
	379	3	0	0	0	382	379	3	0	0	0	382	382	3	0	0	0	385	401	2	0	0	0	403
	63	1	0	0	0	64	63	1	0	0	0	64	64	1	0	0	0	64	67	0	0	0	0	67
4	3	33	20	0	0	56	4	34	19	0	0	57	2	28	20	0	0	50	3	7	50	0	0	60
	3	28	27	0	0	58	4	23	34	0	0	61	4	12	40	0	0	56	8	15	39	0	0	62
	4	29	24	0	0	57	3	19	36	0	0	58	7	14	37	0	0	58	5	6	31	0	0	42
	7	29	14	0	0	50	2	16	33	0	0	51	2	20	31	0	0	53	6	22	32	0	0	60
	3	45	23	0	0	71	2	24	35	0	0	61	2	28	32	0	0	62	5	9	45	0	0	59
	4	40	21	0	0	65	2	18	38	0	0	58	6	35	31	0	0	72	3	9	52	0	0	64
	24	204	129	0	0	357	17	134	195	0	0	346	23	137	191	0	0	351	30	68	249	0	0	347
	4	34	22	0	0	60	3	22	33	0	0	58	4	23	32	0	0	59	5	11	42	0	0	58
6	2	0	72	0	0	74	0	3	67	0	0	70	4	31	27	0	0	62	4	12	44	0	0	60
	5	0	93	0	0	98	2	1	56	0	0	59	4	7	52	0	0	63	3	3	35	0	0	41
	2	0	70	0	0	72	4	2	40	0	0	46	7	9	48	0	0	64	2	8	49	0	0	59
	2	1	71	0	0	74	4	4	40	0	0	48	5	15	32	0	0	52	2	2	54	0	0	58
	2	0	67	0	0	69	4	0	50	0	0	54	2	4	49	0	0	55	3	10	52	0	0	65
	1	4	64	0	0	69	4	2	70	0	0	76	2	6	58	0	0	66	9	30	28	0	0	67
	14	5	437	0	0	456	18	12	323	0	0	353	24	72	266	0	0	362	23	65	262	0	0	350
	2	1	73	0	0	76	3	2	54	0	0	59	4	12	44	0	0	60	4	11	44	0	0	58
8	4	1	52	0	3	60	3	0	64	0	1	68	2	1	50	0	0	53	0	4	59	0	3	66
	4	0	58	0	4	66	2	0	62	0	1	65	2	1	48	0	1	52	1	12	28	0	2	43
	1	0	50	0	3	54	2	3	57	0	1	63	2	0	78	0	1	81	1	2	53	0	1	57
	2	1	66	0	6	75	6	0	69	0	0	75	3	0	42	0	3	48	3	2	46	0	0	51
	3	0	61	0	1	65	6	0	74	0	3	83	4	0	59	0	0	63	0	0	42	0	3	45
	1	0	70	0	3	74	2	0	54	0	3	59	5	0	50	0	0	55	0	4	53	0	1	58
	15	2	357	0	20	394	21	3	380	0	9	413	18	2	327	0	5	352	5	24	281	0	10	320
	3	0	60	0	3	66	4	1	63	0	2	69	3	0	55	0	1	59	1	4	47	0	2	53
10	2	0	62	0	8	72	6	0	44	0	6	56	2	0	62	0	8	72	0	0	30	0	10	40
	3	0	68	0	6	77	2	0	44	0	10	56	3	0	68	0	6	77	5	0	45	0	11	61
	4	0	65	0	5	74	2	0	62	0	8	72	4	0	65	0	5	74	5	0	55	0	2	62
	3	0	72	0	6	81	1	0	56	0	14	71	3	0	72	0	6	81	6	0	69	0	7	82
	3	0	78	0	3	84	1	0	48	0	8	57	3	0	78	0	3	84	3	0	65	0	3	71
	2	0	47	0	5	54	0	0	32	0	4	36	2	0	47	0	5	54	4	0	57	0	6	67
	17	0	392	0	33	442	12	0	286	0	50	348	17	0	392	0	33	442	23	0	321	0	39	383
	3	0	65	0	6	74	2	0	48	0	8	58	3	0	65	0	6	74	4	0	54	0	7	64
12	3	0	29	0	24	56	3	0	31	0	20	54	0	0	17	0	32	49	4	0	50	0	7	61
	5	0	35	0	16	56	4	0	51	0	9	64	3	0	22	0	16	41	1	0	46	0	15	62
	4	0	37	0	14	55	2	0	44	0	10	56	3	0	45	0	18	66	1	0	50	0	12	63
	5	0	34	0	10	49	3	0	39	0	11	53	5	0	54	0	13	72	2	0	56	0	4	62
	4	0	49	0	14	67	4	0	33	0	17	54	4	0	38	0	8	50	4	0	31	2	14	51
	5	0	39	0	15	59	1	0	32	3	9	45	5	0	36	0	9	50	3	0	41	0	16	60
	26	0	223	0	93	342	17	0	230	3	76	326	20	0	212	0	96	328	15	0	274	2	68	359
	4	0	37	0	16	57	3	0	38	1	13	54	3	0	35	0	16	55	3	0	46	0	11	60
14	8	0	31	6	12	57	0	0	29	1	28	58	2	0	20	3	17	42	3	0	30	3	11	47
	6	0	32	2	23	63	2	0	19	2	10	33	3	0	30	0	12	45	1	0	27	4	15	47
	1	0	38	3	21	63	2	0	21	3	12	38	2	0	30	2	14	48	2	0	39	2	12	55
	5	0	30	3	20	58	1	0	28	0	19	48	3	0	32	2	10	47	5	0	43	0	12	60
	3	0	38	1	13	55	0	0	28	3	13	44	2	0	31	1	15	49	1	0	40	0	10	51
	4	0	23	1	19	47	1	0	22	4	28	55	2	0	37	2	13	54	4	0	42	5	24	75
	27	0	192	16	108	343	6	0	147	13	110	276	14	0	180	10	81	285	16	0	221	14	84	335
	5	0	32	3	18	57	1	0	25	2	18	46	2	0	30	2	14	48	3	0	37	2	14	56

Appendix 4.4a: Experiment IV: Further varying of the nutrients - raw data for *Cooperia* cultures in nine different components of media concentration using the ECYE protocol at 25ºC for 21 days. This data is corrected to the number of *Cooperia* identified in the bulk cultures.

	Media	1						2						3					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	53	10	0	0	0	63	65	4	0	0	0	69	63	0	0	0	0	63
	2	39	27	0	0	0	66	54	0	0	0	0	54	65	0	0	0	0	65
	3	52	17	0	0	0	69	80	0	0	0	0	80	71	0	0	0	0	71
3	4	36	33	0	0	0	69	73	0	0	0	0	73	62	0	0	0	0	62
	5	39	21	0	0	0	60	78	0	0	0	0	78	76	0	0	0	0	76
	6	50	21	0	0	0	71	62	0	0	0	0	62	74	0	0	0	0	74
	T	269	129	0	0	0	398	412	4	0	0	0	416	411	0	0	0	0	411
	Av	45	22	0	0	0	66	69	0	0	0	0	69	69	0	0	0	0	70
	1	0	10	54	0	0	64	2	29	54	0	0	85	2	2	91	0	0	95
	2	0	17	57	0	0	74	14	16	35	0	0	65	1	0	76	0	0	77
	3	0	33	35	0	0	68	0	24	50	0	0	74	1	0	88	0	0	89
6	4	0	15	54	0	0	69	7	5	53	0	0	65	0	1	80	0	0	81
	5	1	6	69	0	0	76	7	9	52	0	0	68	1	0	72	0	0	73
	6	1	6	67	0	0	74	4	18	63	0	0	85	0	0	54	0	0	54
	T	2	87	336	0	0	425	34	101	307	0	0	442	5	3	461	0	0	469
	Av	0	15	56	0	0	71	6	17	51	0	0	74	1	1	77	0	0	78
	1	0	0	31	0	9	40	1	0	60	0	3	64	0	0	85	0	11	96
	2	0	0	64	0	12	76	2	0	60	0	15	77	0	0	60	0	11	71
	3	0	0	48	0	11	59	0	0	72	0	3	75	2	0	76	0	5	83
9	4	0	0	50	0	15	65	2	0	83	0	3	88	0	0	78	0	6	84
	5	2	0	55	0	12	69	3	0	59	0	7	69	1	0	59	0	8	68
	6	0	1	38	0	11	50	1	0	72	0	5	78	1	0	62	0	7	70
	T	2	1	286	0	70	359	9	0	406	0	36	451	4	0	420	0	48	472
	Av	0	0	48	0	12	60	2	0	68	0	6	75	0	0	70	0	8	79
	1	0	0	30	10	16	56	0	0	60	4	23	87	0	0	35	20	13	68
	2	0	0	15	19	25	59	0	0	43	5	24	72	0	0	33	13	19	65
	3	0	0	27	12	13	52	1	0	31	3	24	59	0	0	40	14	13	67
12	4	0	0	15	3	15	33	0	0	42	2	16	60	0	0	41	14	13	68
	5	0	0	31	2	27	60	1	0	32	4	20	57	0	0	36	15	10	61
	6	0	0	28	2	6	36	0	0	46	11	11	68	0	0	44	13	20	77
	T	0	0	146	48	102	296	2	0	254	29	118	403	0	0	229	89	88	406
	Av	0	0	24	8	17	49	0	0	42	5	20	67	0	0	38	15	15	68
	1	0	0	21	18	12	51	0	0	38	19	22	79	0	0	35	15	17	67
	2	0	0	16	7	5	28	0	0	45	40	12	97	0	0	47	5	27	79
	3	0	0	41	15	6	62	0	0	33	31	20	84	0	0	24	10	15	49
15	4	0	0	10	5	19	34	0	0	28	5	13	46	0	0	35	10	23	68
	5	0	0	28	26	6	60	0	0	22	6	20	48	0	0	37	12	6	55
	6	0	0	40	7	2	49	0	0	33	12	14	59	0	0	30	8	16	54
	T	0	0	156	78	50	284	0	0	199	113	101	413	0	0	208	60	104	372
	Av	0	0	26	13	8	47	0	0	33	19	17	69	0	0	35	10	17	62
	1	0	0	3	48	5	56	0	0	20	33	25	78	0	0	7	28	31	66
	2	0	0	12	22	5	39	0	0	12	35	24	71	0	0	19	30	21	70
	3	0	0	24	41	14	79	1	0	10	31	19	61	0	0	5	8	14	27
18	4	0	0	8	18	3	29	0	0	18	25	19	62	0	0	23	11	25	59
	5	0	0	12	15	8	35	0	0	6	12	35	53	0	0	24	6	24	54
	6	0	0	16	22	4	42	0	0	10	12	28	50	0	0	6	20	19	45
	T	0	0	75	166	39	280	1	0	76	148	150	375	0	0	84	103	134	321
	Av	0	0	13	28	7	47	0	0	13	25	25	63	0	0	14	17	22	54
	1	0	0	0	28	12	40	0	0	4	21	18	43	0	0	4	25	23	52
	2	0	0	0	25	13	38	0	0	1	28	16	45	0	0	5	16	18	39
	3	0	0	0	25	22	47	0	0	7	20	13	40	0	0	4	23	16	43
21	4	0	0	0	70	10	80	0	0	9	35	20	64	0	0	10	39	20	69
	5	0	0	26	20	5	51	0	0	6	18	24	48	0	0	6	21	23	50
	6	0	0	30	21	6	57	0	0	5	12	27	44	0	0	3	25	26	54
	T	0	0	56	189	68	313	0	0	32	134	118	284	0	0	32	149	126	307
	Av	0	0	9	32	11	47	0	0	5	22	20	47	0	0	5	25	21	51

Appendix 4.4a continued: Further varying of the nutrients using the ECYE protocol at 25ºC for 21 days media components 4 – 6 continued

Day	Media	4						5						6					
	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
3	1	70	0	0	0	0	70	61	0	0	0	0	61	62	0	0	0	0	62
	2	66	0	0	0	0	66	87	0	0	0	0	87	64	0	0	0	0	64
	3	72	0	0	0	0	72	70	1	0	0	0	71	63	0	0	0	0	63
	4	69	0	0	0	0	69	88	0	0	0	0	88	86	0	0	0	0	86
	5	85	0	0	0	0	85	91	1	0	0	0	92	62	0	0	0	0	62
	6	76	0	0	0	0	76	62	0	0	0	0	62	72	0	0	0	0	72
	T	438	0	0	0	0	438	459	2	0	0	0	461	409	0	0	0	0	409
	Av	73	0	0	0	0	73	77	0	0	0	0	77	68	0	0	0	0	68
6	1	0	0	70	0	0	70	2	3	57	0	0	62	0	1	94	0	0	95
	2	1	2	72	0	0	75	2	0	82	0	0	84	0	2	66	0	0	68
	3	0	0	79	0	0	79	2	0	75	0	0	77	1	1	66	0	0	68
	4	1	0	73	0	0	74	1	6	58	0	0	65	0	1	92	0	0	93
	5	1	9	72	0	0	82	0	2	74	0	0	76	0	6	62	0	0	68
	6	0	10	54	0	0	64	1	1	85	0	0	87	0	1	78	0	0	79
	T	3	21	420	0	0	444	8	12	431	0	0	451	1	12	458	0	0	471
	Av	1	4	70	0	0	74	1	2	72	0	0	75	0	2	76	0	0	79
9	1	1	0	62	0	11	74	0	0	61	0	9	70	0	0	67	0	2	69
	2	2	0	43	0	16	61	0	0	36	0	25	61	0	0	71	0	4	75
	3	1	0	60	0	17	78	0	0	52	0	12	64	0	0	74	0	13	87
	4	0	0	51	0	18	69	0	0	56	0	5	61	1	0	71	0	15	87
	5	0	0	74	0	14	88	0	0	35	0	6	41	1	0	69	0	3	73
	6	0	0	78	0	14	92	0	0	52	0	4	56	0	0	62	0	5	67
	T	4	0	368	0	90	462	0	0	292	0	61	353	2	0	414	0	42	458
	Av	0	0	61	0	15	77	0	0	49	0	10	59	0	0	69	0	7	76
12	1	0	0	36	8	19	63	0	0	47	11	20	78	0	0	53	5	4	62
	2	0	0	46	14	14	74	0	0	45	6	13	64	0	0	70	5	9	84
	3	0	0	44	14	9	67	0	0	42	8	21	71	0	0	60	3	10	73
	4	0	0	31	11	14	56	0	0	43	10	16	69	0	0	41	4	16	61
	5	0	0	33	12	20	65	0	0	41	15	12	68	0	0	64	3	9	76
	6	0	0	45	9	10	64	0	0	56	8	22	86	0	0	43	2	6	51
	T	0	0	235	68	86	389	0	0	274	58	104	436	0	0	331	22	54	407
	Av	0	0	39	11	14	65	0	0	46	10	17	73	0	0	55	4	9	68
15	1	0	0	17	16	25	58	0	0	33	10	22	65	0	0	25	6	11	42
	2	0	0	20	0	11	31	0	0	40	12	17	69	0	0	40	8	8	56
	3	1	0	35	13	16	65	0	0	31	11	22	64	0	0	46	11	13	70
	4	1	0	39	16	20	76	0	0	35	10	24	69	0	0	50	6	14	70
	5	0	0	42	14	22	78	0	0	30	17	17	64	0	0	37	11	18	66
	6	0	0	23	15	22	60	0	0	35	10	11	56	0	0	32	10	12	54
	T	2	0	176	74	116	368	0	0	204	70	113	387	0	0	230	52	76	358
	Av	0	0	29	12	19	61	0	0	34	12	19	65	0	0	38	9	13	60
18	1	0	0	8	25	18	51	0	0	6	34	8	48	0	0	1	16	18	35
	2	0	0	6	22	50	78	0	0	11	14	17	42	0	0	3	8	37	48
	3	0	0	10	13	19	42	0	0	8	27	21	56	0	0	14	16	14	44
	4	0	0	4	20	16	40	0	0	19	25	13	57	0	0	10	10	18	38
	5	0	0	24	37	15	76	0	0	6	15	21	42	0	0	9	14	22	45
	6	0	0	21	15	29	65	0	0	12	12	16	40	0	0	10	10	17	37
	T	0	0	73	132	147	352	0	0	62	127	96	285	0	0	47	74	126	247
	Av	0	0	12	22	25	59	0	0	10	21	16	48	0	0	8	12	21	41
21	1	0	0	1	10	28	39	0	0	8	33	18	59	0	0	6	15	20	41
	2	0	0	6	23	16	45	0	0	4	16	20	40	0	0	1	10	22	33
	3	0	0	4	15	25	44	0	0	3	20	23	46	0	0	8	16	18	42
	4	0	0	5	15	16	36	0	0	11	22	16	49	0	0	8	24	20	52
	5	0	0	17	14	18	49	0	0	5	25	32	62	0	0	10	17	28	55
	6	0	0	11	32	19	62	0	0	7	26	15	48	0	0	1	30	5	36
	T	0	0	44	109	122	275	0	0	38	142	124	304	0	0	34	112	113	259
	Av	0	0	7	18	20	46	0	0	6	24	21	51	0	0	6	19	19	43

Appendix 4.4a continued: Further varying of the nutrients using the ECYE protocol at 25ºC for 21 days media components 7 – 9

		7						8							9				
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	75	0	0	0	0	75	54	0	0	0	0	54	65	0	0	0	0	65
	2	69	0	0	0	0	69	69	0	0	0	0	69	55	0	0	0	0	55
3	3	66	0	0	0	0	66	72	1	0	0	0	73	84	0	0	0	0	84
	4	69	0	0	0	0	69	83	0	0	0	0	83	71	0	0	0	0	71
	5	62	2	0	0	0	64	65	0	0	0	0	65	60	0	0	0	0	60
	6	65	0	0	0	0	65	70	0	0	0	0	70	76	0	0	0	0	76
	T	406	2	0	0	0	408	413	1	0	0	0	414	411	0	0	0	0	411
	Av	68	0	0	0	0	68	69	0	0	0	0	69	69	0	0	0	0	69
	1	0	0	79	0	0	79	0	0	80	0	0	80	0	2	70	0	0	72
	2	0	1	72	0	0	73	0	0	61	0	0	61	0	0	75	0	0	75
	3	0	0	76	0	0	76	0	0	64	0	0	64	0	0	68	0	0	68
6	4	0	0	77	0	0	77	2	0	65	0	0	67	0	0	62	0	0	62
	5	0	0	86	0	0	86	0	0	59	0	0	59	0	0	85	0	0	85
	6	0	0	94	0	0	94	0	0	58	0	0	58	0	0	65	0	0	65
	T	0	1	484	0	0	485	2	0	387	0	0	389	0	2	425	0	0	427
	Av	0	0	81	0	0	81	0	0	65	0	0	65	0	0	71	0	0	71
	1	1	0	72	0	4	77	0	0	61	0	0	61	2	0	50	0	13	65
	2	0	0	75	0	8	83	0	0	56	0	3	59	0	0	77	0	5	82
	3	1	0	62	0	17	80	0	0	62	0	8	70	0	0	68	0	12	80
9	4	0	0	57	0	2	59	0	0	64	0	5	69	0	0	60	0	4	64
	5	0	0	55	0	9	64	0	0	65	1	10	76	1	0	67	0	4	72
	6	0	0	60	0	7	67	1	0	65	0	8	74	0	0	58	0	11	69
	T	2	0	381	0	47	430	1	0	373	1	34	409	3	0	380	0	49	432
	Av	0	0	64	0	8	72	0	0	62	0	6	68	1	0	63	0	8	72
	1	0	0	39	3	9	51	0	0	31	9	20	60	0	0	37	8	13	58
	2	0	0	52	4	17	73	0	0	39	16	25	80	0	0	34	13	18	65
	3	0	0	54	4	12	70	0	0	42	8	22	72	0	0	43	3	24	70
12	4	0	0	60	3	10	73	0	0	50	4	3	57	0	0	51	5	8	64
	5	0	0	57	9	9	75	0	0	53	7	15	75	0	0	49	4	21	74
	6	0	0	58	10	8	76	0	0	42	9	14	65	0	0	40	5	16	61
	T	0	0	320	33	65	418	0	0	257	53	99	409	0	0	254	38	100	392
	Av	0	0	53	6	11	70	0	0	43	9	17	68	0	0	42	6	17	65
	1	0	0	28	9	20	57	0	0	19	16	29	64	0	0	18	7	12	37
	2	0	0	31	15	33	79	0	0	33	14	14	61	0	0	31	8	26	65
	3	0	0	30	5	14	49	0	0	42	7	21	70	0	0	33	12	23	68
15	4	0	0	37	12	11	60	0	0	27	7	23	57	0	0	32	12	15	59
	5	0	0	34	21	15	70	0	0	26	7	26	59	0	0	20	9	15	44
	6	1	0	20	21	14	56	0	0	27	17	21	65	0	0	35	17	22	74
	T	1	0	180	83	107	371	0	0	174	68	134	376	0	0	169	65	113	347
	Av	0	0	30	14	18	62	0	0	29	11	22	63	0	0	28	11	19	58
	1	0	0	8	19	19	46	0	0	7	10	19	36	0	0	2	27	31	60
	2	0	0	5	5	20	30	0	0	2	5	26	33	0	0	15	22	28	65
	3	0	0	15	15	19	49	0	0	14	12	16	42	0	0	19	11	31	61
18	4	0	0	29	12	18	59	1	0	13	16	14	44	0	0	8	12	28	48
	5	0	0	13	8	13	34	0	0	8	17	24	49	0	0	15	10	11	36
	6	0	0	22	13	14	49	0	0	7	10	13	30	0	0	17	15	12	44
	T	0	0	92	72	103	267	1	0	51	70	112	234	0	0	76	97	141	314
	Av	0	0	15	12	17	45	0	0	9	12	19	39	0	0	13	16	24	52
	1	0	0	6	10	26	42	0	0	3	25	28	56	0	0	1	37	28	66
	2	0	0	4	42	27	73	0	0	5	10	21	36	0	0	1	20	31	52
	3	0	0	10	27	29	66	0	0	1	14	27	42	0	0	5	36	22	63
21	4	0	0	14	13	28	55	0	0	4	10	16	30	0	0	3	20	21	44
	5	0	0	5	10	20	35	0	0	7	12	15	34	0	0	11	31	19	61
	6	0	0	0	23	17	40	0	0	0	16	21	37	0	0	8	29	17	54
	T	0	0	39	125	147	311	0	0	20	87	128	235	0	0	29	173	138	340
	Av	0	0	7	21	25	52	0	0	3	15	21	39	0	0	5	29	23	57

Key: (No. 1-9 = Media concentration compared to the standard concentration used with sheep nematodes at baseline of 1:1); E = Unhatched eggs; L1, 2 = 1st & 2nd larval Stages respectively; D = Dead larvae; T = Total; Av = Average; NG = No growth.

Appendix 4.4b: Further varying of the nutrients: Raw data for *Cooperia* cultures using the DR protocol at 25ºC for 21 days corrected to the number of *Cooperia* isolated in the bulk cultures.

Media	⍰	1						2						3					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	40	3	0	0	0	43	62	2	0	0	0	62	30	4	0	0	0	34
	2	26	2	0	0	0	28	67	4	0	0	0	67	67	3	0	0	0	70
3	3	37	0	0	0	0	37	75	0	0	0	0	75	77	2	0	0	0	79
	4	89	0	0	0	0	89	75	0	0	0	0	75	78	0	0	0	0	78
	5	48	3	0	0	0	51	50	0	0	0	0	50	71	2	0	0	0	73
	6	88	0	0	0	0	88	69	0	0	0	0	69	86	3	0	0	0	89
	T	328	8	0	0	0	336	398	6	0	0	0	398	409	14	0	0	0	423
	Av	55	1	0	0	0	56	66	1	0	0	0	66	68	2	0	0	0	71
	1	0	0	92	0	0	92	0	0	42	0	0	42	0	2	69	0	0	71
	2	2	0	107	0	0	109	0	3	80	0	0	83	1	0	69	0	0	70
6	3	0	0	64	0	0	64	0	5	62	0	0	67	0	8	84	0	0	92
	4	0	0	49	0	0	49	0	2	68	0	0	70	0	0	110	0	0	110
	5	0	0	67	0	0	67	1	3	60	0	0	64	1	6	64	0	0	71
	6	0	0	68	0	0	68	0	3	85	0	0	88	0	4	69	0	0	73
	T	2	0	447	0	0	449	1	16	397	0	0	414	2	20	465	0	0	487
	Av	0	0	75	0	0	75	0	3	66	0	0	69	0	3	78	0	0	81
	1	0	0	32	0	0	32	0	0	53	0	0	53	0	0	55	0	0	55
	2	0	0	72	0	0	72	0	0	99	0	0	99	0	0	130	0	9	139
9	3	0	0	60	0	0	60	1	0	54	0	2	57	0	1	64	0	16	81
	4	0	0	69	0	0	69	0	0	64	0	0	64	0	0	76	0	6	82
	5	0	0	77	0	0	77	1	0	73	0	2	76	0	0	76	0	13	89
	6	0	0	85	0	0	85	0	0	71	0	0	71	0	0	78	0	11	89
	T	0	0	395	0	0	395	2	0	414	0	4	420	0	1	479	0	55	535
	Av	0	0	66	0	0	66	0	0	69	0	1	70	0	0	80	0	9	89
	1	0	0	24	1	11	36	0	0	41	9	21	71	0	0	41	7	31	79
	2	0	0	38	0	3	41	0	0	52	9	27	88	0	0	44	8	25	77
12	3	0	0	33	0	9	42	0	0	67	7	3	77	0	0	48	4	18	70
	4	0	0	26	0	8	34	0	0	53	7	20	80	0	0	53	3	26	82
	5	0	0	35	0	20	55	0	0	50	4	27	81	0	0	70	4	15	89
	6	0	0	25	0	0	25	0	0	39	5	13	57	0	0	43	5	19	67
	T	0	0	181	1	51	233	0	0	302	41	111	454	0	0	299	31	134	464
	Av	0	0	30	0	9	39	0	0	50	7	19	76	0	0	50	5	22	77
	1	0	0	2	9	9	20	0	0	7	2	14	23	0	0	18	36	35	89
	2	0	0	9	8	13	30	0	0	10	14	13	37	0	0	26	23	16	65
15	3	0	0	10	18	8	36	0	0	9	12	20	41	0	0	18	23	8	49
	4	0	0	25	25	4	54	0	0	18	19	12	49	0	0	37	24	17	78
	5	0	0	10	18	14	42	0	0	34	36	24	94	0	0	25	27	34	86
	6	0	0	37	48	13	98	0	0	20	20	26	66	0	0	21	30	22	73
	T	0	0	93	126	61	280	0	0	98	103	109	310	0	0	145	163	132	440
	Av	0	0	16	21	10	47	0	0	16	17	18	52	0	0	24	27	22	73
	1	0	0	0	3	6	9	0	0	9	29	20	58	0	0	5	29	22	56
	2	1	0	7	9	6	23	0	0	14	57	18	89	0	0	17	31	18	66
18	3	0	0	2	6	6	14	0	0	0	15	16	31	0	0	11	21	23	55
	4	1	0	0	5	10	16	0	0	10	22	23	55	0	0	13	21	27	61
	5	0	0	3	25	21	49	0	0	20	22	28	70	0	0	23	13	15	51
	6	1	0	4	14	9	28	0	0	20	13	21	54	0	0	25	19	22	66
	T	3	0	16	62	58	139	0	0	73	158	126	357	0	0	94	134	127	355
	Av	0	0	3	10	10	23	0	0	12	26	21	60	0	0	16	22	21	59
	1	1	0	0	20	12	33	1	0	6	37	12	56	0	0	0	18	0	18
	2	0	0	0	30	10	40	0	0	11	36	9	56	0	0	6	19	12	37
21	3	0	0	0	27	17	44	0	0	6	36	25	67	0	0	36	0	15	51
	4	1	5	0	18	16	40	0	0	6	25	24	55	0	0	10	18	13	41
	5	0	1	0	8	10	19	0	0	9	23	17	49	0	0	7	29	13	49
	6	0	8	0	32	10	50	0	0	11	25	17	53	0	0	6	18	21	45
	T	2	14	0	135	75	226	1	0	49	182	104	336	0	0	65	102	74	241
	Av	0	2	0	23	13	38	0	0	8	30	17	56	0	0	11	17	12	40

Key: 1, 2, 3 = Concentration of yeast extract at ½, 1 & $1^1/_2$ compared to the standard concentration used with sheep nematodes; key for E, L1, 2, 3; D, T and Av. is as shown in Appendix 4.4a.

Appendix 4.5a: Experiment V: Varying nutrients – raw data for *Cooperia* cultures in 16 different combinations of media concentration using the ECYE protocol at 18ºC for 21 days corrected to the number of *Cooperia* identified in the cultures.

Media		1						2						3						4				
Days	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D
3	1	0	74	0	0	0	74	0	61	0	0	0	61	5	56	0	0	0	61	45	2	0	0	0
	2	0	81	0	0	0	81	3	73	0	0	0	76	12	44	0	0	0	56	53	3	0	0	0
	3	0	50	0	0	0	50	0	70	0	0	0	70	0	49	0	0	0	49	58	7	0	0	0
	T	0	205	0	0	0	205	3	204	0	0	0	207	17	149	0	0	0	166	156	12	0	0	0
	Av	0	68	0	0	0	68	1	68	0	0	0	69	6	50	0	0	0	55	52	4	0	0	0
6	1	0	75	2	0	0	77	0	89	0	0	0	89	0	13	70	0	0	83	0	52	0	0	0
	2	0	52	4	0	0	56	0	70	0	0	0	70	0	0	65	0	0	65	0	58	0	0	0
	3	0	62	0	0	0	62	0	64	0	0	0	64	0	0	68	0	0	68	1	56	0	0	0
	T	0	189	6	0	0	195	0	223	0	0	0	223	0	133	203	0	0	216	1	166	0	0	0
	Av	0	63	2	0	0	65	0	74	0	0	0	74	0	4	68	0	0	72	0	55	0	0	0
9	1	0	2	46	0	0	48	0	10	66	0	0	76	0	0	72	0	0	72	0	0	65	0	0
	2	0	10	64	0	0	74	0	1	88	0	0	89	0	0	76	0	0	76	0	0	82	0	0
	3	0	7	42	0	0	49	0	3	65	0	0	68	0	0	64	0	0	64	0	0	76	0	0
	T	0	19	152	0	0	171	0	14	219	0	0	233	0	0	212	0	0	212	0	0	223	0	0
	Av	0	6	51	0	0	57	0	5	73	0	0	78	0	0	71	0	0	71	0	0	74	0	0
12	1	0	0	51	0	0	51	0	0	35	4	26	65	0	0	54	0	14	68	0	0	41	0	22
	2	0	0	63	0	0	63	1	1	60	0	0	62	0	0	53	0	15	68	0	0	45	0	19
	3	0	0	56	0	0	56	0	0	49	0	13	62	0	0	47	0	7	54	0	0	47	2	14
	T	0	0	170	0	0	170	1	1	144	4	39	189	0	0	154	0	36	190	0	0	133	2	55
	Av	0	0	57	0	0	57	0	0	48	1	13	63	0	0	51	0	12	63	0	0	44	1	18
15	1	0	0	65	0	0	65	0	0	32	1	8	41	0	0	30	7	14	51	0	0	36	2	9
	2	0	0	45	0	2	47	0	0	38	15	12	65	0	0	31	11	11	53	0	0	41	2	4
	3	0	0	52	0	7	59	0	0	36	14	10	60	0	0	41	4	14	59	0	0	34	1	14
	T	0	0	162	0	9	171	0	0	106	30	30	166	0	0	102	22	39	163	0	0	111	5	27
	Av	0	0	54	0	3	57	0	0	35	10	10	55	0	0	34	7	13	54	0	0	37	2	9
18	1	1	0	56	0	0	57	0	0	28	29	10	67	0	2	5	19	6	32	0	0	28	2	6
	2	0	0	33	0	12	45	0	0	42	0	4	46	0	0	39	12	3	54	0	0	48	0	7
	3	0	0	43	0	4	47	0	0	33	12	6	51	0	0	45	10	7	62	0	0	29	2	4
	T	1	0	132	0	16	149	0	0	103	41	20	164	0	2	89	41	16	148	0	0	105	4	17
	Av	0	0	44	0	5	50	0	0	34	14	7	55	0	1	30	14	5	49	0	0	35	1	6
21	1	0	0	53	0	5	58	0	0	16	11	15	42	0	0	32	13	2	47	0	0	32	2	11
	2	0	0	52	0	3	55	0	0	33	3	4	40	0	0	40	6	11	57	0	0	33	8	7
	3	0	0	46	0	0	46	0	0	39	6	8	53	0	0	43	5	8	56	0	0	39	6	4
	T	0	0	151	0	8	159	0	0	88	20	27	135	0	0	115	24	21	160	0	0	104	16	22
	Av	0	0	50	0	3	53	0	0	29	7	9	45	0	0	38	8	7	53	0	0	35	5	7

Appendix 4.5a continued: (ECYE protocol at 18ºC, media component concentration number 5 - 8)

Media		5						6						7						8					
Days	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	3	68	0	0	0	71	0	69	0	0	0	69	48	12	0	0	0	60	38	2	0	0	0	40
	2	2	45	0	0	0	47	0	68	0	0	0	68	12	59	0	0	0	71	33	9	0	0	0	42
3	3	0	77	0	0	0	77	0	62	0	0	0	62	35	21	0	0	0	56	36	3	0	0	0	39
	T	5	190	0	0	0	195	0	199	0	0	0	199	95	92	0	0	0	187	107	14	0	0	0	121
	Av	2	63	0	0	0	65	0	66	0	0	0	66	32	31	0	0	0	62	36	5	0	0	0	40
	1	0	68	8	0	0	76	1	2	84	0	0	87	0	0	74	0	0	74	0	0	58	0	0	58
	2	0	63	2	0	0	65	2	2	78	0	0	82	0	0	64	0	0	64	0	0	37	0	0	37
6	3	0	90	0	0	0	90	1	1	60	0	0	62	0	0	52	0	0	52	0	0	51	0	0	51
	T	0	221	10	0	0	231	4	5	222	0	0	231	0	0	190	0	0	190	0	0	146	0	0	146
	Av	0	74	3	0	0	77	1	2	74	0	0	77	0	0	63	0	0	63	0	0	49	0	0	49
	1	0	0	63	0	0	63	0	3	64	0	0	67	0	0	58	0	0	58	0	0	53	0	0	53
	2	0	0	47	0	0	47	0	5	63	0	0	68	0	0	68	0	0	68	0	0	71	0	0	71
9	3	0	0	67	0	0	67	0	0	64	0	0	64	0	0	45	0	0	45	0	0	63	0	0	63
	T	0	0	177	0	0	177	0	8	191	0	0	199	0	0	171	0	0	171	0	0	187	0	0	187
	Av	0	0	59	0	0	59	0	3	64	0	0	66	0	0	57	0	0	57	0	0	62	0	0	62
	1	0	0	44	0	7	51	0	0	47	2	13	62	0	0	61	0	6	67	0	0	29	0	9	38
	2	0	0	50	0	2	52	0	0	40	0	12	52	0	0	49	0	11	60	0	0	62	0	21	83
12	3	0	0	79	0	5	84	0	0	59	0	8	67	0	0	22	0	13	35	0	0	51	0	25	76
	T	0	0	173	0	14	187	0	0	146	2	33	181	0	0	132	0	30	162	0	0	142	0	55	197
	Av	0	0	58	0	5	62	0	0	49	1	11	60	0	0	44	0	10	54	0	0	47	0	18	66
	1	0	0	45	0	12	57	0	0	42	6	12	60	0	0	41	1	10	52	0	0	9	1	35	45
	2	0	0	47	0	2	49	0	0	36	8	14	58	0	0	43	6	15	64	0	0	25	1	12	38
15	3	0	0	61	0	3	64	0	0	33	0	16	49	0	0	40	0	11	51	0	0	42	8	22	72
	T	0	0	153	0	17	170	0	0	111	14	42	167	0	0	124	7	36	167	0	0	76	10	69	155
	Av	0	0	51	0	6	57	0	0	37	5	14	56	0	0	41	2	12	56	0	0	25	3	23	52
	1	0	0	48	2	6	56	0	0	19	9	16	44	0	0	28	15	16	59	0	0	38	15	16	69
	2	0	0	37	1	4	42	0	0	41	11	10	62			NG			0	0	0	15	6	32	53
18	3	0	0	51	2	5	58	0	0	25	16	14	55	0	0	32	10	12	54	0	0	29	0	10	39
	T	0	0	136	5	15	156	0	0	85	36	40	161	0	0	60	25	28	113	0	0	82	21	58	161
	Av	0	0	45	2	5	52	0	0	28	12	13	54	0	0	30	13	14	57	0	0	27	7	19	54
	1	0	0	39	0	9	48	0	0	31	11	17	59	0	0	23	13	8	44	0	0	33	11	9	53
	2	0	0	46	0	7	53	0	0	22	6	19	47	0	0	38	4	6	48	0	0	41	8	3	52
21	3	0	0	51	0	13	64	0	0	45	12	12	69	0	0	48	9	11	68	0	0	33	15	7	55
	T	0	0	136	0	29	165	0	0	98	29	48	175	0	0	109	26	25	160	0	0	107	34	19	160
	Av	0	0	45	0	10	55	0	0	33	10	16	58	0	0	36	9	8	53	0	0	36	11	6	53

Appendix 4.5a continued: (ECYE protocol at 18ºC, media component concentration number 9 - 12)

Media		9						10						11						12					
Days	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	0	82	0	0	0	82	0	81	0	0	0	81	35	2	0	0	0	37	55	0	0	0	0	55
	2	2	77	0	0	0	79	1	54	0	0	0	55	31	49	0	0	0	62	40	2	0	0	0	42
3	3	0	81	0	0	0	81	0	62	0	0	0	62	13	37	0	0	0	50	45	4	0	0	0	49
	T	2	240	0	0	0	242	1	197	0	0	0	198	61	88	0	0	0	149	140	6	0	0	0	146
	Av	1	80	0	0	0	81	0	66	0	0	0	66	20	29	0	0	0	50	47	2	0	0	0	49
	1	0	60	1	0	0	61	0	2	69	0	0	71	1	0	65	0	0	66	0	0	45	0	0	45
	2	0	66	11	0	0	77	0	4	75	0	0	79	0	0	59	0	0	59	1	0	57	0	0	58
6	3	0	33	37	0	0	70	1	0	58	0	0	59	0	0	33	0	0	33	0	0	57	0	0	57
	T	0	159	49	0	0	208	1	6	202	0	0	209	1	0	157	0	0	158	1	0	159	0	0	160
	Av	0	53	16	0	0	69	0	2	67	0	0	70	0	0	52	0	0	53	0	0	53	0	0	53
	1	0	13	45	0	0	58	0	0	34	0	1	35	0	0	74	0	0	74	0	0	63	0	0	63
	2	0	15	42	0	0	57	0	0	50	0	0	50	0	0	51	0	0	51	0	0	57	0	0	57
9	3	0	8	52	0	0	60	0	3	53	0	0	56	0	0	72	0	0	72	0	0	55	0	0	55
	T	0	36	139	0	0	175	0	3	137	0	1	141	0	0	197	0	0	197	0	0	175	0	0	175
	Av	0	12	46	0	0	58	0	1	46	0	0	47	0	0	66	0	0	66	0	0	58	0	0	58
	1	0	0	53	6	0	59	0	0	39	0	14	53	0	0	62	3	11	76	0	0	51	0	16	67
	2	0	0	49	4	0	53	0	0	53	0	17	70	0	0	48	0	4	52	0	0	41	0	14	55
12	3	0	0	70	1	0	71	0	0	55	0	6	61	0	0	33	0	13	46	0	0	50	0	10	60
	T	0	0	172	11	0	183	0	0	147	0	37	184	0	0	143	3	28	174	0	0	142	0	40	182
	Av	0	0	57	4	0	61	0	0	49	0	12	61	0	0	48	1	9	58	0	0	47	0	13	61
	1	0	0	40	2	8	50	0	0	25	8	15	48	0	0	42	7	14	63	0	0	23	3	20	46
	2	0	0	58	0	2	60	0	0	28	13	60	101	0	0	36	8	15	59	0	0	28	7	5	40
15	3	0	0	64	0	2	66	0	0	20	15	50	85	0	0	40	7	13	60	0	0	45	3	2	50
	T	0	0	162	2	12	176	0	0	73	36	125	234	0	0	118	22	42	182	0	0	96	13	27	136
	Av	0	0	54	1	4	59	0	0	24	12	42	78	0	0	39	7	14	61	0	0	32	4	9	45
	1	0	0	36	0	14	50	0	0	44	4	9	57	0	0	40	4	7	51	0	0	15	0	36	51
	2	0	0	36	0	6	42	0	0	42	3	11	56	0	0	48	2	5	55	0	0	27	0	32	59
18	3	0	0	13	0	17	30	0	0	32	5	19	56	0	0	38	14	6	58	0	0	25	0	19	44
	T	0	0	85	0	37	122	0	0	118	12	39	169	0	0	126	20	18	164	0	0	67	0	87	154
	Av	0	0	28	0	12	41	0	0	39	4	13	56	0	0	42	7	6	55	0	0	22	0	29	51
	1	0	0	47	0	7	54	0	0	41	3	10	54	0	0	36	12	6	54	0	0	37	16	4	57
	2	0	0	40	0	13	53	0	0	28	6	20	54	0	0	23	10	11	44	0	0	36	13	7	56
21	3	0	0	36	0	19	55	0	0	19	2	30	51	0	0	44	1	19	64	0	0	23	10	13	46
	T	0	0	123	0	39	162	0	0	88	11	60	159	0	0	103	23	36	162	0	0	96	39	24	159
	Av	0	0	41	0	13	54	0	0	29	4	20	53	0	0	34	8	12	54	0	0	32	13	8	53

Appendix 4.5a continued: (ECYE protocol at 18ºC, media component concentration number 13 - 16)

Media		13						14						15						16					
Days	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	0	59	0	0	0	59	0	62	0	0	0	62	44	24	0	0	0	68	58	0	0	0	0	58
	2	0	68	0	0	0	68	0	71	0	0	0	71	2	53	0	0	0	55	42	0	0	0	0	42
3	3	0	74	0	0	0	74	0	70	0	0	0	70	3	50	0	0	0	53	52	0	0	0	0	52
	T	0	201	0	0	0	201	0	203	0	0	0	203	49	127	0	0	0	176	152	0	0	0	0	152
	Av	0	67	0	0	0	67	0	68	0	0	0	68	16	42	0	0	0	59	51	0	0	0	0	51
	1	0	55	8	0	0	63	0	11	73	0	0	84	2	2	56	0	0	60	0	8	46	0	0	54
	2	0	32	0	12	0	44	1	10	70	0	0	81	0	2	50	0	0	52	3	1	70	0	0	74
6	3	0	58	6	0	0	64	2	0	66	0	0	68	0	1	59	0	0	60	No growth					0
	T	0	145	14	12	0	171	3	21	209	0	0	233	2	5	165	0	0	172	3	9	116	0	0	128
	Av	0	48	5	4	0	57	1	7	70	0	0	78	1	2	55	0	0	57	2	5	58	0	0	64
	1	0	14	45	0	0	59	0	4	62	0	0	66	0	0	42	0	10	52	0	0	28	0	0	28
	2	0	17	36	0	0	53	1	0	58	0	0	59	0	1	58	0	0	59	0	0	72	0	0	72
9	3	0	12	33	0	0	45	0	1	57	0	0	58	0	3	62	0	0	65	0	0	77	0	0	77
	T	0	43	114	0	0	157	1	5	177	0	0	183	0	4	162	0	10	176	0	0	177	0	0	177
	Av	0	14	38	0	0	52	0	2	59	0	0	61	0	1	54	0	3	59	0	0	59	0	0	59
	1	0	0	58	0	1	59	0	0	46	0	15	61	0	0	49	0	23	72	0	0	41	0	17	58
	2	0	0	47	0	2	49	0	0	53	0	1	54	0	0	18	0	16	34	0	0	20	0	8	28
12	3	0	0	64	0	0	64	0	0	20	0	23	43	0	0	50	0	2	52	0	0	45	0	15	60
	T	0	0	169	0	3	172	0	0	119	0	39	158	0	0	117	0	41	158	0	0	106	0	40	146
	Av	0	0	56	0	1	57	0	0	40	0	13	53	0	0	39	0	14	53	0	0	35	0	13	49
	1	1	0	43	0	6	50	0	0	47	9	19	75	0	0	29	5	22	56	0	0	40	1	23	64
	2	0	0	49	1	3	53	0	0	43	2	11	56	0	0	40	9	15	64	0	0	35	4	6	45
15	3	0	0	57	0	2	59	0	0	56	1	13	70	0	0	33	1	6	40	0	0	33	0	7	40
	T	1	0	149	1	11	162	0	0	146	2	43	201	0	0	102	15	43	160	0	0	108	5	36	149
	Av	0	0	50	0	4	54	0	0	49	4	14	67	0	0	34	5	14	53	0	0	36	2	12	50
	1	1	0	51	0	3	55	0	0	28	8	9	45	0	0	40	11	9	60	0	0	39	0	9	48
	2	0	0	59	0	4	63	0	0	42	6	7	55	1	0	27	2	3	33	0	0	32	6	7	45
18	3	0	0	43	0	7	50	0	0	29	5	26	60	0	0	40	2	8	50	0	0	25	5	10	40
	T	1	0	153	0	14	168	0	0	99	19	42	160	1	0	107	15	20	143	0	0	96	11	26	133
	Av	0	0	51	0	5	56	0	0	33	6	14	53	0	0	36	5	7	48	0	0	32	4	9	44
	1	0	0	50	0	19	69	0	0	20	9	20	49	0	0	26	22	27	75	0	0	25	3	13	41
	2	0	0	35	0	8	43	0	0	23	3	28	54	0	0	31	6	15	52	0	0	29	7	15	51
21	3	0	0	43	0	11	54	0	0	18	6	25	49	0	0	35	7	7	49	0	0	20	2	27	49
	T	0	0	128	0	38	166	0	0	61	18	73	152	0	0	92	35	49	176	0	0	74	12	55	141
	Av	0	0	43	0	13	55	0	0	20	6	24	51	0	0	31	12	16	59	0	0	25	4	18	47

Key: Number 1- 16 (ECYE protocol = Media concentration (compared to the standard media for sheep nematodes which is baseline of 1:1); E = Unhatched eggs; L1, 2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total; NG = No growth.

Appendix 4.5b: Varying nutrients – raw data for *Cooperia* cultures in 16 components of media concentration using the ECYE protocol at 25ºC for 21 days.

Media		1						2						3						4					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	1	65	0	0	0	66	1	60	0	0	0	61	0	57	0	0	0	57	53	3	0	0	0	56
	2	0	60	0	0	0	60	0	55	0	0	0	55	0	53	0	0	0	53	23	18	0	0	0	41
3	3	0	70	0	0	0	70	0	52	0	0	0	52	0	72	0	0	0	72	54	6	0	0	0	60
	T	1	195	0	0	0	196	1	167	0	0	0	168	0	182	0	0	0	182	130	27	0	0	0	157
	Av	0	65	0	0	0	65	0	56	0	0	0	56	0	61	0	0	0	61	43	9	0	0	0	52
	1	0	49	0	0	0	49	0	8	44	0	0	52	0	3	64	0	0	67	0	4	66	0	0	70
	2	0	43	0	0	0	43	0	0	61	0	0	61	0	5	65	0	0	70	0	9	42	0	0	51
6	3	0	55	0	0	0	55	0	7	54	0	0	61	0	0	49	0	0	49	0	2	39	0	0	41
	T	0	147	0	0	0	147	0	15	159	0	0	174	0	8	178	0	0	186	0	15	147	0	0	162
	Av	0	49	0	0	0	49	0	5	53	0	0	58	0	3	59	0	0	62	0	5	49	0	0	54
	1	0	8	22	0	0	30	0	1	33	1	12	47	0	1	1	10	36	48	0	0	19	0	12	31
	2	0	5	22	0	0	27	0	0	29	0	18	47	0	0	0	9	49	58	0	0	16	0	15	31
9	3	0	6	36	0	0	42	0	0	23	0	22	45	0	0	25	2	22	49	0	0	18	0	16	34
	T	0	19	80	0	0	99	0	1	85	1	52	139	0	1	26	21	107	155	0	0	53	0	43	96
	Av	0	6	27	0	0	33	0	0	28	0	17	46	0	0	9	7	36	52	0	0	18	0	14	32
	1	0	0	7	0	5	12	0	0	9	0	0	9	0	0	31	18	22	71	0	0	15	0	14	29
	2	0	0	18	0	2	20	0	0	10	0	7	17	0	0	0	0	11	11	0	0	19	2	10	31
12	3	0	0	16	0	0	16	0	0	9	0	4	13	0	0	10	0	7	17	0	1	10	0	36	47
	T	0	0	41	0	7	48	0	0	28	0	11	39	0	0	41	18	40	99	0	1	44	2	60	107
	Av	0	0	14	0	2	16	0	0	9	0	4	13	0	0	14	6	13	33	0	0	15	1	20	36
	1	0	0	36	0	15	51	0	0	19	3	17	39	0	0	12	9	19	40	0	0	26	3	6	35
	2	0	2	13	0	10	25	0	0	24	3	16	43	0	0	30	7	14	51	0	0	38	2	11	51
15	3	0	0	26	0	18	44	0	0	15	16	24	55	0	0	23	6	7	36	0	0	30	0	11	41
	T	0	2	75	0	43	120	0	0	58	22	57	137	0	0	65	22	40	127	0	0	94	5	28	127
	Av	0	1	25	0	14	40	0	0	19	7	19	46	0	0	22	7	13	42	0	0	31	2	9	42
	1	0	0	7	0	14	21	0	0	12	0	45	57	0	0	32	10	18	60	0	0	22	4	29	55
	2	0	0	29	0	27	56	0	0	23	14	27	64	0	0	30	6	13	49	0	0	20	2	14	36
18	3	0	0	23	0	23	46	0	0	9	2	27	38	0	0	24	5	15	44	0	0	23	8	2	33
	T	0	0	59	0	64	123	0	0	44	16	99	159	0	0	86	21	46	153	0	0	65	14	45	124
	Av	0	0	20	0	21	41	0	0	15	5	33	53	0	0	29	7	15	51	0	0	22	5	15	41
	1	0	0	32	0	15	47	0	0	8	2	7	17	0	0	20	17	4	41	0	0	32	2	12	46
	2	0	0	11	0	23	34	0	0	12	18	10	40	0	0	10	8	16	34	0	0	23	14	3	40
21	3	0	0	32	0	8	40	0	0	23	3	8	34	0	0	22	7	21	50	0	0	31	5	12	48
	T	0	0	75	0	46	121	0	0	43	23	25	91	0	0	52	32	41	125	0	0	86	21	27	134
	Av	0	0	25	0	15	40	0	0	14	8	8	30	0	0	17	11	14	42	0	0	29	7	9	45

Appendix 4.5b continued: (ECYE protocol at 25ºC, media component concentration number 5 - 8)

Media		5						6						7						8					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	0	63	0	0	0	63	0	62	0	0	0	62	0	57	0	0	0	57	45	0	0	0	0	45
	2	0	49	0	0	0	49	2	58	0	0	0	60	3	66	0	0	0	69	36	2	0	0	0	38
3	3	1	58	0	0	0	59	0	61	0	0	0	61	0	79	0	0	0	79	52	0	0	0	0	52
	T	1	170	0	0	0	171	2	181	0	0	0	183	3	202	0	0	0	205	133	2	0	0	0	135
	Av	0	57	0	0	0	57	1	60	0	0	0	61	1	67	0	0	0	68	44	1	0	0	0	45
	1	0	48	0	0	0	48	0	6	46	0	0	52	0	0	53	0	10	63	0	2	43	0	0	45
	2	0	26	7	0	0	33	0	4	61	0	0	65	1	5	55	0	5	66	0	5	47	0	3	55
6	3	0	43	0	0	0	43	0	5	46	0	0	51	1	13	50	0	2	66	0	4	42	0	3	49
	T	0	117	7	0	0	124	0	15	153	0	0	168	2	18	158	0	17	195	0	11	132	0	6	149
	Av	0	39	2	0	0	41	0	5	51	0	0	56	1	6	53	0	6	65	0	4	44	0	2	50
	1	0	8	14	0	4	26	0	5	25	0	22	52	0	2	42	1	7	52	0	0	21	0	21	42
	2	0	4	20	0	2	26	0	0	33	1	12	46	0	0	22	2	11	35	0	0	27	2	12	41
9	3	0	2	22	0	4	28	0	0	32	2	10	44	0	0	25	1	14	40	0	0	44	2	9	55
	T	0	14	56	0	10	80	0	5	90	3	44	142	0	2	89	4	32	127	0	0	92	4	42	138
	Av	0	5	19	0	3	27	0	2	30	1	15	47	0	1	30	1	11	42	0	0	31	1	14	46
	1	0	0	16	6	0	22	0	0	8	0	4	12	0	0	8	0	2	10	0	0	14	0	14	28
	2		No growth				0	0	0	21	5	19	45	0	0	6	2	7	15	0	0	18	0	16	34
12	3	0	0	14	4	0	18	0	0	24	0	7	31	0	10	0	0	17	27	0	0	15	0	18	33
	T	0	0	30	10	0	40	0	0	53	5	30	88	0	10	14	2	26	52	0	0	47	0	48	95
	Av	0	0	15	5	0	20	0	0	18	2	10	29	0	3	5	1	9	17	0	0	16	0	16	32
	1	0	0	18	0	23	41	0	0	34	9	19	62	0	0	30	10	10	50	0	15	14	23	0	52
	2	0	0	31	2	19	52	0	0	16	4	16	36	0	0	12	8	29	49	0	20	15	15	0	50
15	3	0	0	22	0	11	33	0	0	19	3	24	46	0	0	9	20	22	51	0	24	2	15	0	41
	T	0	0	71	2	53	126	0	0	69	16	59	144	0	0	51	38	61	150	0	59	31	53	0	143
	Av	0	0	24	1	18	42	0	0	23	5	20	48	0	0	17	13	20	50	0	20	10	18	0	48
	1	0	0	51	0	10	61	0	0	29	11	18	58	0	0	22	5	6	33	0	0	36	3	11	50
	2	0	0	2	0	14	16	0	0	31	3	13	47	0	0	16	8	20	44	0	0	21	2	3	26
18	3		No growth				0	0	0	22	2	19	43	0	0	19	5	15	39		No growth				0
	T	0	0	53	0	24	77	0	0	82	16	50	148	0	0	57	18	41	116	0	0	57	5	14	76
	Av	0	0	27	0	12	39	0	0	27	5	17	49	0	0	19	6	14	39	0	0	29	3	7	38
	1	0	0	33	0	15	48	0	0	18	12	8	38	0	0	31	8	21	60	0	0	25	1	16	42
	2	0	0	22	0	26	48	0	0	28	8	17	53	0	0	31	4	12	47	0	0	16	12	13	41
21	3	0	0	11	1	17	29	0	0	13	2	10	25	0	0	19	8	8	35	0	0	24	4	14	42
	T	0	0	66	1	58	125	0	0	59	22	35	116	0	0	81	20	41	142	0	0	65	17	43	125
	Av	0	0	22	0	19	42	0	0	20	7	12	39	0	0	27	7	14	47	0	0	22	6	14	42

Appendix 4.5b continued: (ECYE protocol at 25ºC, media component concentration number 9 - 12)

Media		9						10						11						12					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	0	0	0	0	0	0	2	45	0	0	0	47	12	46	0	0	0	58	0	50	0	0	0	50
	2	0	42	0	0	0	42	0	72	0	0	0	72	52	2	0	0	0	54	92	0	0	0	0	92
3	3	0	44	0	0	0	44	0	58	0	0	0	58	1	57	0	0	0	58	53	0	0	0	0	53
	T	0	86	0	0	0	86	2	175	0	0	0	177	65	105	0	0	0	170	145	50	0	0	0	195
	Av	0	29	0	0	0	29	1	58	0	0	0	59	22	35	0	0	0	57	48	17	0	0	0	65
	1	0	42	12	0	0	54	0	2	52	0	0	54	0	3	59	0	0	62	0	2	60	0	6	68
	2	0	27	13	0	0	40	0	1	49	0	1	51	0	4	65	0	0	69	0	3	51	0	2	56
6	3	0	41	12	0	0	53	0	5	42	0	5	52	0	5	50	0	0	55	0	2	38	0	3	43
	T	0	110	37	0	0	147	0	8	143	0	6	157	0	12	174	0	0	186	0	7	149	0	11	167
	Av	0	37	12	0	0	49	0	3	48	0	2	52	0	4	58	0	0	62	0	2	50	0	4	56
		No growth																							
	1	0						0	0	36	0	6	42	0	0	33	0	12	45	0	1	48	0	16	65
	2	0	10	10	0	5	25	0	0	33	2	10	45	0	0	43	0	16	59	0	0	27	0	20	47
9	3	0	1	35	0	14	50	0	1	63	3	9	76	0	0	19	0	8	27	0	1	22	0	8	31
	T	0	11	45	0	19	75	0	1	132	5	25	163	0	0	95	0	36	131	0	2	97	0	44	143
	Av	0	6	23	0	10	38	0	0	44	2	8	54	0	0	32	0	12	44	0	1	32	0	15	48
	1	0	0	8	0	5	13	0	0	20	0	10	30	0	0	42	1	1	44	0	0	8	1	15	24
	2	0	0	9	0	2	11	0	0	45	1	0	46	0	0	15	10	5	30	0	0	35	1	28	64
12	3	0	0	18	0	11	29	0	0	25	1	2	28	0	0	20	0	10	30	0	0	31	1	19	51
	T	0	0	35	0	18	53	0	0	90	2	12	104	0	0	77	11	16	104	0	0	74	3	62	139
	Av	0	0	12	0	6	18	0	0	30	1	4	35	0	0	26	4	5	35	0	0	25	1	21	46
	1	0	0	37	0	15	52	0	0	20	5	22	47	0	0	22	14	22	58	0	0	36	10	7	53
	2	0	0	40	1	21	62	0	0	35	4	17	56	0	0	15	29	12	56	0	0	18	4	2	24
15	3	0	0	22	1	22	45	0	0	26	7	15	48	0	0	17	16	12	45	0	0	26	6	11	43
	T	0	0	99	2	58	159	0	0	81	16	54	151	0	0	54	59	46	159	0	0	80	20	20	120
	Av	0	0	33	1	19	53	0	0	27	5	18	50	0	0	18	20	15	53	0	0	27	7	7	40
	1	0	0	21	0	27	48	0	0	11	7	21	39	0	0	27	8	9	44	0	0	16	8	9	33
	2	0	0	16	0	17	33	0	0	31	4	15	50	0	0	19	6	13	38	0	0	9	16	8	33
18	3	0	0	19	0	22	41	0	0	13	6	27	46	0	0	12	10	26	48	0	0	28	12	9	49
	T	0	0	56	0	66	122	0	0	55	17	63	135	0	0	58	24	48	130	0	0	53	36	26	115
	Av	0	0	19	0	22	41	0	0	18	6	21	45	0	0	19	8	16	43	0	0	18	12	9	38
	1	0	0	26	1	10	37	0	0	17	5	13	35	0	0	5	2	1	8	0	0	13	23	10	46
	2	0	0	3	0	11	14	0	0	23	5	29	57	0	0	24	17	14	55	0	0	28	20	15	63
21	3	0	0	3	0	19	22	0	0	20	4	32	56	0	0	30	18	5	53	0	0	6	14	15	35
	T	0	0	32	1	40	73	0	0	60	14	74	148	0	0	59	37	20	116	0	0	47	57	40	144
	Av	0	0	11	0	13	24	0	0	20	5	25	49	0	0	20	12	7	39	0	0	16	19	13	48

Appendix 4.5b continued: (ECYE protocol at 25ºC, media component concentration number 13 - 16)

Media		13						14						15						16					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
3	1	4	36	0	0	0	40	0	52	0	0	0	52	58	14	0	0	0	72	63	2	0	0	0	65
	2	2	45	0	0	0	47	0	64	0	0	0	64	1	66	0	0	0	67	46	0	0	0	0	46
	3	1	25	0	0	0	26	0	79	0	0	0	79	59	2	0	0	0	61	51	2	0	0	0	53
	T	7	106	0	0	0	113	0	195	0	0	0	195	118	82	0	0	0	200	160	4	0	0	0	164
	Av	2	35	0	0	0	38	0	65	0	0	0	65	39	27	0	0	0	67	53	1	0	0	0	55
6	1	0	18	10	0	4	32	0	2	56	0	3	61	0	7	64	0	1	72	0	0	66	0	4	70
	2	0	33	6	0	0	39	0	1	53	0	4	58	0	8	53	0	0	61	0	1	43	0	0	44
	3		No growth				0	0	2	53	0	1	56	0	5	48	0	2	55	0	2	39	0	2	43
	T	0	51	16	0	4	71	0	5	162	0	8	175	0	20	165	0	3	188	0	3	148	0	6	157
	Av	0	26	8	0	2	36	0	2	54	0	3	58	0	7	55	0	1	63	0	1	49	0	2	52
9	1	0	6	4	0	14	24	0	0	43	0	0	43	0	0	25	0	18	43	0	1	54	0	10	65
	2	0	3	24	0	3	30	0	6	26	0	19	51	0	0	34	0	20	54	0	2	41	0	9	52
	3	0	9	20	0	4	33	0	0	40	0	21	61	0	0	39	0	13	52	0	0	46	0	10	56
	T	0	18	48	0	21	87	0	6	109	0	40	155	0	0	98	0	51	149	0	3	141	0	29	173
	Av	0	6	16	0	7	29	0	2	36	0	13	52	0	0	33	0	17	50	0	1	47	0	10	58
12	1	0	0	16	0	6	22	0	0	27	0	18	45	0	0	15	0	6	21	0	0	18	4	4	26
	2		No growth				0	0	0	24	2	10	36	0	0	19	0	12	31	0	0	25	0	16	41
	3	0	0	29	0	6	35	0	0	8	0	6	14	0	0	14	0	10	24	0	0	22	0	14	36
	T	0	0	45	0	12	57	0	0	59	2	34	95	0	0	48	0	28	76	0	0	65	4	34	103
	Av	0	0	23	0	6	29	0	0	20	1	11	32	0	0	16	0	9	25	0	0	22	1	11	34
15	1	0	0	20	0	9	29	0	0	35	11	14	60	0	0	39	5	19	63	0	0	15	4	13	32
	2	0	0	15	0	10	25	0	0	19	2	11	32	0	0	32	5	14	51	0	0	30	1	21	52
	3	0	0	31	0	10	41	0	0	25	8	10	43	0	0	31	3	8	42	0	0	16	7	4	27
	T	0	0	66	0	29	95	0	0	79	21	35	135	0	0	102	13	41	156	0	0	61	12	38	111
	Av	0	0	22	0	10	32	0	0	26	7	12	45	0	0	34	4	14	52	0	0	20	4	13	37
18	1	0	0	11	0	9	20	0	0	29	3	12	44	0	0	26	10	5	41	0	0	26	6	14	46
	2	0	0	8	0	12	20	0	0	25	4	19	48	0	0	15	2	27	44	0	0	17	1	13	31
	3	0	0	13	0	10	23	0	0	28	10	13	51	0	0	45	7	8	60	0	0	11	3	7	21
	T	0	0	32	0	31	63	0	0	82	17	44	143	0	0	86	19	40	145	0	0	54	10	34	98
	Av	0	0	11	0	10	21	0	0	27	6	15	48	0	0	29	6	13	48	0	0	18	3	11	33
21	1	0	0	0	0	3	3	0	0	30	4	8	42	0	0	21	5	30	56	0	0	20	3	31	54
	2	0	0	6	2	3	11	0	0	16	6	24	46	0	0	20	3	14	37	0	0	36	3	21	60
	3	0	0	6	0	16	22	0	0	24	21	12	57	0	0	33	2	19	54	0	0	27	2	11	40
	T	0	0	12	2	22	36	0	0	70	31	44	145	0	0	74	10	63	147	0	0	83	8	63	154
	Av	0	0	4	1	7	12	0	0	23	10	15	48	0	0	25	3	21	49	0	0	28	3	21	51

Key: Number 1- 16 (ECYE protocol = concentration of the media used (compared to the standard media for sheep nematodes which is baseline of 1:1); E = Unhatched eggs; L1, 2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total.

Appendix 4.5c: Varying nutrients – raw data for Cooperia cultures in four components of media concentration by DR protocol at 18ºC for 21 days.

Media		1						2						3						4					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	0	65	0	0	0	65	0	70	0	0	0	70	0	83	0	0	0	83	0	75	0	0	0	75
	2	0	69	0	0	0	69	0	57	0	0	0	57	0	90	0	0	0	90	1	77	0	0	0	78
3	3	0	69	0	0	0	69	0	74	0	0	0	74	1	90	0	0	0	91	0	95	0	0	0	95
							20						20						26						24
	T	0	203	0	0	0	3	0	201	0	0	0	1	1	263	0	0	0	4	1	247	0	0	0	8
	Av	0	68	0	0	0	68	0	67	0	0	0	67	0	88	0	0	0	88	0	82	0	0	0	83
	1	0	49	2	0	0	51	0	0	57	0	0	57	0	0	76	0	0	76	0	1	73	0	0	74
	2	0	76	9	0	0	85	0	7	54	0	0	61	2	0	90	0	3	95	0	4	79	0	0	83
6	3	1	55	3	0	0	59	0	1	88	0	0	89	0	8	77	0	1	86	0	2	88	0	0	90
							19						20						25						24
	T	1	180	14	0	0	5	0	8	199	0	0	7	2	8	243	0	4	7	0	7	240	0	0	7
	Av	0	60	5	0	0	65	0	3	66	0	0	69	1	3	81	0	1	86	0	2	80	0	0	82
	1	0	8	36	0	0	44	0	0	48	0	14	62	0	7	72	0	0	79	0	2	42	0	8	52
	2	0	9	66	0	0	75	0	2	44	0	14	60	0	7	73	0	0	80	0	1	89	0	2	92
9	3	0	15	61	0	0	76	3	7	80	0	2	92	0	2	81	0	0	83	0	0	84	0	0	84
							19						21						24						22
	T	0	32	163	0	0	5	3	9	172	0	30	4	0	16	226	0	0	2	0	3	215	0	10	8
	Av	0	11	54	0	0	65	1	3	57	0	10	71	0	5	75	0	0	81	0	1	72	0	3	76
	1	0	0	58	0	0	58	0	0	52	0	0	52	0	0	41	0	43	84	0	0	59	0	26	85
	2	0	0	57	0	0	57	0	0	25	0	29	54	0	0	66	0	18	84	0	0	80	0	7	87
12	3	0	0	66	0	0	66	0	0	59	0	29	88	0	0	55	2	31	88	0	0	53	0	26	79
							18						19						25						25
	T	0	0	181	0	0	1	0	0	136	0	58	4	0	0	162	2	92	6	0	0	192	0	59	1
	Av	0	0	60	0	0	60	0	0	45	0	19	65	0	0	54	1	31	85	0	0	64	0	20	84
	1	0	0	54	0	4	58	0	0	32	5	26	63	0	0	46	8	28	82	0	0	29	3	31	63
	2	0	0	41	0	5	46	0	0	56	2	3	61	0	0	56	6	6	68	0	0	48	7	31	86
15	3	0	0	47	0	3	50	0	0	61	2	6	69	0	0	37	8	26	71	0	0	33	2	25	60
							15						19						22						20
	T	0	0	142	0	12	4	0	0	149	9	35	3	0	0	139	22	60	1	0	0	110	12	87	9
	Av	0	0	47	0	4	51	0	0	50	3	12	64	0	0	46	7	20	74	0	0	37	4	29	70
	1	0	0	66	0	3	69	0	0	10	4	33	47	0	0	31	12	35	78	0	0	23	15	33	71
	2	0	0	51	0	5	56	0	0	24	4	20	48	0	0	23	7	36	66	0	0	25	4	40	69
18	3	0	0	15	0	9	24	0	0	48	3	9	60	0	0	29	7	27	63	0	0	40	6	17	63
							14						15						20						20
	T	0	0	132	0	17	9	0	0	82	11	62	5	0	0	83	26	98	7	0	0	88	25	90	3
	Av	0	0	44	0	6	50	0	0	27	4	21	52	0	0	28	9	33	69	0	0	29	8	30	68
	1	0	0	21	0	5	26	0	0	49	10	11	70	0	0	28	5	19	52	0	0	47	8	21	76
	2	0	0	17	1	16	34	0	0	32	7	18	57	0	0	6	2	10	18	0	0	25	7	15	47
21	3	0	0	29	0	31	60	0	0	10	0	9	19	0	0	38	13	28	79	0	0	23	5	10	38
							12						14						14						16
	T	0	0	67	1	52	0	0	0	91	17	38	6	0	0	72	20	57	9	0	0	95	20	46	1
	Av	0	0	22	0	17	40	0	0	30	6	13	49	0	0	24	7	19	50	0	0	32	7	15	54

Key: Number 1- 4 (DR protocol) = Media concentration (0, ½, 1 and $1^1/_2$ compared to the standard media for sheep nematodes); E = Unhatched eggs; L1, 2 = 1st & 2^{nd} larval Stages respectively; L3 = Ensheathed larvae; D = Dead larvae; Av = Average and T = Total.

Appendix 4.5d: Varying nutrients – raw data for *Cooperia* cultures in four components of media concentration using the DR protocol at 25ºC for 21 days.

Media		1						2						3						4					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
	1	0	25	0	0	0	25	4	52	0	0	0	56	0	84	0	0	0	84	2	25	70	0	0	97
	2	0	35	0	0	0	35	0	80	0	0	0	80	1	74	20	0	0	95	0	70	4	0	0	74
3	3		No growth				0	3	90	0	0	0	93	1	74	3	0	0	78	0	69	1	0	0	70
													22						25		16				24
	T	0	60	0	0	0	60	7	222	0	0	0	9	2	232	23	0	0	7	2	4	75	0	0	1
	Av	0	30	0	0	0	30	2	74	0	0	0	76	1	77	8	0	0	86	1	55	25	0	0	80
	1	0	4	10	0	0	14	0	7	60	2	7	76	0	3	79	0	5	87	0	8	50	0	4	62
	2	0	59	3	0	0	62	0	2	62	0	3	67	0	4	64	0	8	76	0	4	54	0	0	58
6	3	0	53	5	0	0	58	0	0	32	4	19	55	0	0	47	3	14	64	0	5	53	0	7	65
			11				13						19						22						18
	T	0	6	18	0	0	4	0	9	154	6	29	8	0	7	190	3	27	7	0	17	157	0	11	5
	Av	0	39	6	0	0	45	0	3	51	2	10	66	0	2	63	1	9	76	0	6	52	0	4	62
	1	0	0	28	0	0	28	0	0	53	0	15	68	0	0	18	0	52	70	0	5	33	0	18	56
	2	0	0	55	0	4	59	0	0	48	0	20	68	0	0	32	0	19	51	0	0	35	0	29	64
9	3	0	0	38	0	10	48	0	0	66	1	23	90	0	0	25	0	11	36	0	4	20	3	3	30
							13						22						15						15
	T	0	0	121	0	14	5	0	0	167	1	58	6	0	0	75	0	82	7	0	9	88	3	50	0
	Av	0	0	40	0	5	45	0	0	56	0	19	75	0	0	25	0	27	52	0	3	29	1	17	50
	1	0	0	29	0	5	34	0	0	62	3	29	94	0	0	25	0	30	55	0	0	24	8	15	47
	2	0	0	36	0	20	56	0	0	35	2	33	70	0	0	26	3	13	42	0	0	12	9	27	48
12	3	0	0	30	0	40	70	0	0	27	0	23	50	0	0	22	4	17	43	0	0	20	2	18	40
							16						21						14						13
	T	0	0	95	0	65	0	0	0	124	5	85	4	0	0	73	7	60	0	0	0	56	19	60	5
	Av	0	0	32	0	22	53	0	0	41	2	28	71	0	0	24	2	20	47	0	0	19	6	20	45
	1	0	0	30	0	11	41	0	0	72	13	6	91	0	0	17	6	31	54	0	0	20	2	14	36
	2	0	0	54	0	7	61	0	0	48	0	22	70	0	0	15	7	22	44	0	0	8	4	26	38
15	3	0	0	54	0	9	63	0	0	25	2	32	59	0	0	42	5	12	59	0	0	18	7	30	55
							16						22						15						12
	T	0	0	138	0	27	5	0	0	145	15	60	0	0	0	74	18	65	7	0	0	46	13	70	9
	Av	0	0	46	0	9	55	0	0	48	5	20	73	0	0	25	6	22	52	0	0	15	4	23	43
	1	0	0	16	0	14	30	0	0	38	2	13	53	0	0	29	7	14	50	0	0	8	2	33	43
	2	0	0	30	0	20	50	0	0	48	3	9	60	0	0	17	20	21	58	0	0	21	0	14	35
18	3	0	0	23	0	20	43	0	0	30	1	31	62	0	0	20	4	30	54	0	0	23	11	15	49
							12						17						16						12
	T	0	0	69	0	54	3	0	0	116	6	53	5	0	0	66	31	65	2	0	0	52	13	62	7
	Av	0	0	23	0	18	41	0	0	39	2	18	58	0	0	22	10	22	54	0	0	17	4	21	42
	1	0	0	63	0	8	71	0	0	14	5	23	42	0	0	27	11	35	73	0	0	26	3	34	63
	2	0	0	17	0	5	22		No growth				0	0	0	18	10	34	62	0	0	30	2	33	65
21	3	0	0	19	0	4	23		No growth				0	0	0	32	7	28	67	0	0	37	8	11	56
							11												20						18
	T	0	0	99	0	17	6	0	0	14	5	23	42	0	0	77	28	97	2	0	0	93	13	78	4
	Av	0	0	33	0	6	39	0	0	14	5	23	42	0	0	26	9	32	67	0	0	31	4	26	61

Key: Numbers 1- 4 (DR protocol) = concentration of the media used (0, ½, 1 and $1^1/_2$ compared to the standard media for sheep nematodes), E = Unhatched eggs; L1, L2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; Av = Average and T = Total.

Appendix 5.1a: Experiment VI: The raw data for in-house cultures tested by the ECYE protocol with variations of media and temperature as indicated. These were control plates monitored daily from Day 2 with three replicates read per day.

		Media 12												Media 7									
		18ºC						25ºC						18ºC						25ºC					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
2	1	75	0	0	0	0	75	58	0	0	0	0	58	67	0	0	0	0	67	11	47	0	0	0	58
	2	80	0	0	0	0	80	62	0	0	0	0	62	65	0	0	0	0	65	21	41	0	0	0	62
	3	70	0	0	0	0	70	30	0	0	0	0	30	70	0	0	0	0	70	18	58	0	0	0	76
3	1	14	62	0	0	0	62	8	63	0	0	0	71	7	60	0	0	0	67	4	61	17	0	0	82
	2	7	60	0	0	0	67	5	53	0	0	0	58	23	37	0	0	0	60	2	40	26	0	0	68
	3	14	50	0	0	0	64	50	22	0	0	0	72	17	41	0	0	0	58	1	48	20	0	0	69
4	1	2	60	0	0	0	62	2	60	0	0	0	62	0	6	20	41	0	67	0	21	50	0	0	71
	2	10	50	0	0	0	60	17	46	0	0	0	63	2	8	9	41	0	60	2	19	41	0	0	62
	3	5	65	0	0	0	70	7	52	0	0	0	59	2	23	20	10	0	55	2	7	50	0	0	59
5	1	3	70	0	0	0	73	4	3	39	0	0	46	0	2	63	0	0	65	0	0	59	0	0	59
	2	3	66	0	0	0	69	1	20	41	0	0	62	0	4	54	0	0	58	0	0	65	0	0	65
	3	2	63	0	0	0	65	0	25	39	0	0	64	1	3	65	0	0	69	0	0	70	0	0	70
6	1	0	0	76	0	0	76	0	10	50	0	0	60	0	0	59	0	0	59	0	0	54	0	0	54
	2	0	0	60	0	0	60	0	12	51	0	0	63	0	0	68	0	0	68	0	0	69	0	0	69
	3	0	0	59	0	0	59	0	9	60	0	0	69	0	3	67	0	0	70	0	0	69	0	0	69
7	1	0	0	62	0	0	62	0	0	54	0	12	66	0	0	72	0	0	72	0	0	61	0	0	61
	2	0	0	70	0	0	70	0	0	59	0	8	67	0	0	69	0	0	69	0	0	67	0	0	67
	3	0	0	72	0	0	72	0	0	45	0	9	54	0	0	60	0	0	60	0	0	62	0	0	62
8	1	0	0	65	0	0	65	0	0	44	0	28	72	0	0	58	0	6	64	0	0	56	0	6	62
	2	0	0	70	0	0	70	0	0	42	0	19	61	0	0	50	0	8	58	0	0	55	0	4	59
	3	0	0	59	0	0	59	0	0	53	0	13	66	0	0	60	4	6	70	0	0	63	0	5	68
9	1	0	0	63	0	0	63	0	0	41	0	23	64	0	0	57	1	5	63	0	0	49	10	4	63
	2	0	0	65	0	0	65	0	0	54	2	13	69	0	0	44	3	7	54	0	0	40	13	4	57
	3	0	0	59	0	0	59	0	0	46	4	12	62	0	0	55	2	3	60	0	0	34	12	6	52
10	1	0	0	50	0	19	69	0	0	44	8	19	71	0	0	62	1	6	69	0	0	41	18	5	64
	2	0	0	50	0	22	72	0	0	26	10	19	55	0	0	45	6	4	55	0	0	35	22	2	59
	3	0	0	50	0	15	65	0	0	36	9	19	64	0	0	57	3	3	63	0	0	26	20	6	52
11	1	0	0	30	10	28	68	0	0	9	22	30	61	0	0	43	6	8	57	0	0	23	21	8	52
	2	0	0	20	19	32	71	0	0	10	19	35	64	0	0	41	4	3	48	0	0	20	21	10	51
	3	0	0	34	16	21	71	0	0	16	23	32	71	0	0	40	5	5	50	0	0	22	22	15	59
12	1	0	0	18	26	20	64							0	0	26	31	11	68						
	2	0	0	9	27	22	58							0	0	26	30	2	58						
	3	0	0	12	20	28	60							0	0	20	36	2	58						

Key: E = Unhatched eggs; L1 and L2 = 1st and 2nd larval stage respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total.

Appendix 5.1b: The raw data for in-house cultures using the DR protocol with only yeast extract media as well as two temperatures variations as indicated. These were control plates monitored daily from Day 2 with three replicates per day.

		DR (run together with media 12)												DR (run together with media 7)									
		18ºC						25ºC						18ºC						25ºC					
Day	Well	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T	E	L1	L2	L3	D	T
2	1	60	0	0	0	0	60	10	50	0	0	0	60	69	0	0	0	0	69	20	50	0	0	0	70
	2	70	0	0	0	0	70	8	54	0	0	0	62	65	0	0	0	0	65	10	62	0	0	0	72
	3	65	0	0	0	0	65	15	45	0	0	0	60	55	0	0	0	0	55	20	45	0	0	0	65
3	1	18	40	0	0	0	58	2	66	0	0	0	68	3	60	0	0	0	63	3	24	42	0	0	69
	2	24	42	0	0	0	66	5	64	0	0	0	69	4	44	0	0	0	48	3	16	44	0	0	63
	3	17	45	0	0	0	62	3	56	0	0	0	59	2	56	0	0	0	58	4	22	47	0	0	73
4	1	0	10	50	0	0	60	0	61	0	0	0	61	0	20	39	0	0	59	0	12	58	0	0	70
	2	0	11	45	0	0	56	0	56	0	0	0	56	3	21	38	0	0	62	1	10	56	0	0	67
	3	0	12	49	0	0	61	0	59	0	0	0	59	1	30	33	0	0	64	0	7	62	0	0	69
5	1	0	2	55	0	0	57	0	15	41	0	0	56	0	3	62	0	0	65	0	1	58	0	0	59
	2	0	0	60	0	0	60	0	10	51	0	0	61	0	4	57	0	0	61	0	4	66	0	0	70
	3	0	0	54	0	0	54	0	2	49	0	0	51	0	2	55	0	0	57	0	0	68	0	0	68
6	1	0	3	64	0	0	67	0	10	49	0	0	59	1	1	57	0	0	59	0	0	58	0	0	58
	2	2	3	54	0	0	59	0	9	52	0	0	61	0	0	62	0	0	62	0	0	60	0	0	60
	3	0	2	52	0	0	54	0	8	60	0	0	68	0	1	63	0	0	64	0	0	5	0	0	5
7	1	0	3	52	0	0	55	0	0	50	0	4	54	0	0	52	0	0	52	0	0	47	8	6	61
	2	0	0	65	0	0	65	0	0	51	0	10	61	0	0	61	0	0	61	0	0	41	10	7	58
	3	0	0	59	0	0	59	0	0	40	0	9	49	0	0	60	0	0	60	0	0	46	13	0	59
8	1	0	0	68	0	0	68	0	0	42	0	6	48	0	0	63	0	0	63	0	0	10	38	4	52
	2	0	0	58	0	0	58	0	0	57	0	8	65	0	0	65	0	4	69	0	0	14	40	6	60
	3	0	0	52	0	0	52	0	0	50	0	9	59	0	0	58	0	4	62	0	0	14	45	3	62
9	1	0	0	63	0	0	63	0	0	48	2	11	61	0	0	63	1	1	65						
	2	0	0	50	0	0	50	0	0	50	5	8	63	0	0	48	2	0	50						
	3	0	0	70	0	0	70	0	0	47	2	18	67	0	0	59	0	0	59						
10	1	0	0	45	9	5	59	0	0	34	6	14	54	0	0	52	0	2	54						
	2	0	0	38	10	3	51	0	0	32	10	15	57	0	0	55	2	6	63						
	3	0	0	31	28	6	65	0	0	32	7	18	57	0	0	50	4	4	58						
11	1	0	0	31	10	16	57	0	0	10	29	15	54	0	0	41	7	11	59						
	2	0	0	30	13	8	51	0	0	16	30	18	64	0	0	35	12	11	58						
	3	0	0	30	21	10	61	0	0	9	32	20	61	0	0	31	2	16	49						
12	1	0	0	8	25	19	52							0	0	13	30	14	57						
	2	0	0	18	20	10	48							0	0	18	41	10	69						
	3	0	0	12	34	15	61							0	0	21	35	4	60						

Key: E = Unhatched eggs; L1, 2 =1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total.

Appendix 5.2a: Experiment VI: Raw data for *Cooperia* cultures using the ECYE protocol at a concentration of $^1/_4$EC + $^1/_2$YE in the commercial standard DrenchRite plates on Day 12 at 18ºC or 25ºC

Temp (ºC)	Drug	Stage	Cntr		2		3		4		5		6		7		8		9		10		11		12	
18	BZ	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	11	14	10	12	9	12	8	10	12	10	10	8	9	8	2	0	0	0	0	0	0	0	0	0
		L3	21	20	19	20	22	20	20	18	16	17	15	18	12	10	2	0	0	0	0	0	0	0	0	0
		D	16	11	11	9	18	9	21	12	13	12	12	9	9	11	6	0	0	0	0	0	0	0	0	0
		T	48	45	40	41	49	41	49	40	41	39	37	35	30	29	10	0	0	0	0	0	0	0	0	0
	LV	E	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	10	20	10	5	9	10	11	8	10	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L3	24	28	25	22	19	24	20	19	21	25	10	14	4	0	0	0	0	0	0	0	0	0	0	0
		D	15	16	19	20	15	9	10	13	10	7	12	9	11	2	0	0	0	0	0	0	0	0	0	0
		T	49	64	56	47	43	43	41	40	41	39	22	23	15	2	0	0	0	0	0	0	0	0	0	0
	BZ + LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	19	18	15	10	14	11	9	7	8	9	3	0	0	0	0	0	0	0	0	0	0	0	0	0
		L3	15	22	25	23	22	19	22	19	17	19	10	10	0	0	0	0	0	0	0	0	0	0	0	0
		D	17	3	13	14	11	13	8	14	14	10	9	8	0	0	0	0	0	0	0	0	0	0	0	0
		T	51	43	53	47	47	43	39	40	39	38	22	18	0	0	0	0	0	0	0	0	0	0	0	0
	IV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	10	18	10	19	10	10	12	8	9	10	8	8	0	0	0	0	0	0	0	0	0	0	0	0
		L3	21	19	23	25	20	22	18	13	15	18	20	15	12	0	0	0	0	0	0	0	0	0	0	0
		D	10	14	15	15	11	12	10	21	20	12	10	8	5	0	0	0	0	0	0	0	0	0	0	0
		T	41	51	48	59	41	44	40	42	44	40	38	31	17	0	0	0	0	0	0	0	0	0	0	0
25	BZ	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	4	3	3	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L3	19	16	21	19	24	21	24	18	25	19	20	15	15	16	5	8	0	0	0	0	0	0	0	0
		D	40	41	34	44	41	42	43	35	36	9	30	30	25	30	10	9	1	0	0	0	0	0	0	0
		T	63	60	58	65	65	63	67	54	61	28	50	45	40	46	15	17	1	0	0	0	0	0	0	0
	LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	5	2	4	2	3	1	6	5	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
		L3	20	18	20	14	17	19	21	18	10	6	10	10	0	0	0	0	0	0	0	0	0	0	0	0
		D	34	44	37	39	40	44	33	34	25	25	10	19	11	2	0	0	0	0	0	0	0	0	0	0
		T	59	64	61	55	60	64	60	57	35	31	22	29	11	2	0	0	0	0	0	0	0	0	0	0
	BZ + LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	2	3	5	2	4	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L3	25	22	19	25	21	19	23	28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		D	33	35	41	33	20	45	22	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		T	60	60	65	60	45	67	46	68	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	IV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L3	26	31	20	21	22	19	20	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		D	38	38	41	27	38	35	9	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		T	64	70	61	48	60	54	29	27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: E = Unhatched eggs; L1, 2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total; Cntr = Control wells column.

Appendix 5.2b: Experiment VI: Raw data for *Cooperia* cultures using the ECYE protocol at a concentration of $^1/_8$EC + $^1/_4$YE in the commercial standard DrenchRite plates on Day 12 at 18ºC or 25ºC.

Temp		Larval	**W**								**E**				**L**				**L**											
(ºC)	Drug	stage	1		2		3		4		5		6		7		8		9		10		11		12					
18	BZ	E	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L2	20	19	15	15	10	7	14	11	16	32	22	21	16	14	19	12	12	8	4	0	0	0	0	0				
		L3	25	28	29	28	29	45	28	35	24	15	14	35	16	20	10	11	8	8	9	7	4	2	1	0				
		D	5	18	12	6	8	4	6	2	0	9	10	4	5	4	4	6	2	4	4	1	3	2	0	0				
		T	50	65	56	53	47	56	48	48	40	56	46	60	37	38	33	29	22	20	17	8	7	4	1	0				
	LV	E	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L2	12	13	23	18	25	14	17	22	34	30	23	16	20	19	10	8	10	7	0	0	0	0	0	0				
		L3	40	29	25	30	16	40	30	30	10	20	19	22	12	16	11	6	6	8	4	8	2	4	0	0				
		D	5	14	1	6	5	5	17	4	4	6	10	6	7	3	3	8	5	5	5	3	3	2	0	0				
		T	57	56	49	54	46	59	64	56	48	56	54	44	39	38	24	22	21	20	9	11	5	6	0	0				
	BZ + LV	E	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L2	21	19	35	24	6	11	8	32	36	21	17	20	20	17	12	8	0	0	2	1	1	0	0	0				
		L3	25	20	21	22	34	33	31	19	11	25	21	23	20	12	10	11	6	4	1	2	2	0	0	0				
		D	9	11	2	6	7	8	8	2	6	4	9	5	3	7	4	2	3	2	0	2	0	6	1	0				
		T	55	50	60	52	47	52	47	53	54	50	47	48	43	36	26	21	9	6	3	5	3	6	1	0				
	IV	E	0	2	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L2	20	25	15	12	10	14	20	25	30	23	10	29	21	18	19	20	10	6	4	2	0	0	0	0				
		L3	26	23	40	33	27	35	25	33	27	25	29	15	18	23	16	15	7	8	2	2	1	2	0	1				
		D	4	8	9	6	3	2	6	4	2	8	4	5	3	4	3	4	2	5	3	1	2	0	0	0				
		T	50	58	64	51	40	51	51	65	59	56	43	49	42	45	38	39	19	19	9	5	3	2	0	1				
25	BZ	E	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L2	16	13	11	14	12	10	16	10	9	12	20	12	4	6	7	0	5	4	3	1	0	0	0	0				
		L3	22	30	27	25	29	25	23	24	24	29	21	20	16	20	15	10	10	9	1	1	0	0	0	0				
		D	11	9	10	9	10	9	12	10	15	10	9	9	10	9	6	8	4	6	1	2	0	0	0	0				
		T	49	52	48	48	51	44	51	44	49	51	50	41	30	35	28	18	19	19	5	4	0	0	0	0				
	LV	E	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0				
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L2	15	8	13	16	10	11	9	12	10	16	9	10	9	8	5	2	4	0	4	0	0	0	0	0				
		L3	25	31	25	28	26	24	20	26	22	28	29	25	20	18	17	10	10	10	1	0	0	0	0	0				
		D	10	13	7	11	8	15	18	12	10	10	8	12	5	8	8	19	10	12	0	2	0	0	0	0				
		T	51	52	45	55	44	50	47	50	42	54	46	47	36	34	30	31	24	22	5	2	0	0	0	0				
	BZ + LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L2	13	14	12	15	14	20	12	10	15	20	5	13	9	8	7	5	4	3	2	0	0	0	0	0				
		L3	21	28	27	25	23	20	20	24	21	22	35	20	15	17	15	20	10	10	2	1	0	0	0	0				
		D	21	7	10	11	9	12	18	10	12	9	9	11	9	9	7	8	2	8	0	1	0	0	0	0				
		T	55	49	49	51	46	52	50	44	48	51	49	44	33	34	29	33	16	21	4	2	0	0	0	0				
	IV	E	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		L2	15	8	16	11	17	28	14	14	10	10	9	6	5	8	6	7	0	3	2	1	0	0	0	0				
		L3	27	28	27	29	22	19	25	22	31	37	25	23	15	14	16	15	6	9	1	0	0	0	0	0				
		D	9	12	4	13	10	6	8	10	6	7	10	9	9	8	4	6	3	4	0	4	0	0	0	0				
		T	51	48	47	53	49	54	47	46	47	54	44	38	29	30	26	28	9	16	3	5	0	0	0	0				

Key: E = Unhatched egg; L1, 2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead Larvae; T = Total.

Appendix 5.2c: Experiment VI: Raw data for *Cooperia* cultures using the DR protocol at a concentration of 1 (20µl yeast extract only) in the commercial standard DrenchRite plates on Day 12 at 18ºC or 25ºC.

Tem		Larval							W			E			L			L								
(ºC)	Drug	stage	1		2		3		4		5		6		7		8		9		10		11		12	
		E	0	0	0	0	0	0	0	0	0	0	0	0	3	0	2	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	BZ	L2	15	18	20-	16	10	12	9	12	4	11	13	15	18	9	8	5	0	1	3	0	1	0	0	0
		L3	34	27	27	29	31	30	31	29	23	20	28	29	12	27	14	4	7	4	8	3	6	0	0	0
		D	10	8	14	11	8	9	9	13	15	14	8	13	8	9	21	10	13	5	11	6	5	0	0	0
		T	59	53	41	56	49	51	49	54	42	45	49	57	41	45	45	20	20	10	22	9	12	0	0	0
		E	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	LV	L2	19	20	21	15	12	16	10	16	14	11	10	9	11	9	3	7	0	0	0	0	0	0	0	0
18		L3	29	29	30	26	28	31	33	25	28	27	33	21	29	21	22	19	16	13	9	12	7	3	0	0
		D	10	9	8	12	9	15	7	12	9	10	7	14	14	9	18	14	10	22	10	8	8	3	0	0
		T	58	58	59	53	49	62	50	53	51	48	50	44	56	40	43	40	26	36	19	20	15	6	0	0
		E	0	0	0	0	0	0	0	2	0	0	1	0	0	0	3	0	0	1	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	BZ	L2	10	15	18	11	8	13	10	19	11	9	6	10	7	6	8	5	3	3	0	2	0	0	0	0
	+	L3	32	30	27	29	35	31	28	21	32	26	20	9	10	8	5	17	12	14	3	1	0	0	0	0
	LV	D	14	14	10	14	7	8	11	12	13	13	3	10	11	4	7	8	5	4	3	5	0	0	0	0
		T	56	59	55	54	50	52	49	54	56	48	30	29	28	18	23	30	20	22	6	8	0	0	0	0
		E	0	0	0	0	0	0	1	0	0	0	2	0	0	1	0	4	0	0	1	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	IV	L2	10	12	18	10	11	9	6	10	8	12	10	8	5	6	9	6	4	1	0	2	0	0	0	0
		L3	31	28	26	29	29	33	27	31	30	25	14	21	11	17	17	3	20	5	4	0	0	0	0	0
		D	12	10	11	17	9	9	11	13	10	14	5	9	6	6	4	8	5	4	6	2	0	0	0	0
		T	53	50	55	56	49	51	45	54	48	51	31	38	22	30	30	21	29	10	11	4	0	0	0	0
		E	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	BZ	L2	0	0	8	0	5	3	6	4	0	2	1	0	5	2	0	0	0	0	0	0	0	0	0	0
		L3	40	41	35	40	39	38	40	42	45	44	25	20	24	20	10	12	0	2	0	0	0	0	0	0
		D	11	8	6	10	4	7		6	8	8	6	8	4	0	6	4	2	4	1	0	0	0	0	0
		T	51	49	49	50	48	48	46	53	53	54	32	28	33	22	16	16	2	6	1	0	0	0	0	0
		E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	LV	L2	0	0	0	0	2	1	4	3	1	4	1	2	3	1	0	0	0	0	0	0	0	0	0	0
		L3	46	50	42	39	37	43	36	50	35	34	20	24	21	25	20	10	5	0	0	0	0	0	0	0
		D	6	4	13	6	11	7	6	4	12	8	5	5	4	10	9	10	10	10	4	0	0	0	0	0
		T	52	54	55	45	50	51	46	57	48	46	26	31	28	36	29	20	15	10	4	0	0	0	0	0
		E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	BZ	L2	0	0	6	6	3	1	0	2	5	0	0	4	8	4	3	4	5	2	0	0	0	0	0	0
	+	L3	48	41	40	40	39	38	45	37	36	29	20	22	12	8	2	0	0	0	0	0	0	0	0	0
	LV	D	4	7	5	3	5	9	8	6	7	9	10	6	9	14	9	5	2	0	0	0	0	0	0	0
		T	52	48	51	49	47	48	53	45	48	38	30	32	29	26	14	9	7	2	0	0	0	0	0	0
		E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
	IV	L2	0	0	2	10	5	4	7	6	4	1	8	4	6	7	0	0	0	0	0	0	0	0	0	0
		L3	45	40	38	30	30	33	31	30	24	28	20	23	20	12	0	0	0	0	0	0	0	0	0	0
		D	6	10	9	7	2	6	5	4	7	3	10	8	3	11	15	12	0	0	0	0	0	0	0	0
		T	51	50	49	47	37	43	43	40	35	32	38	35	29	30	17	13	0	0	0	0	0	0	0	0

Key: E = Unhatched eggs; L1, 2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead Larvae; T = Total.

Appendix 5.2d: Experiment VI: A duplicate of the raw data for *Cooperia* cultures using the DR protocol at a concentration of 1 (20µl yeast extract only) in the commercial standard DrenchRite plates on Day 12 at 18ºC or 25ºC.

Tem			**W**						**E**				**L**				**L**											
(ºC)	Drug	stage	1		2		3		4		5		6		7		8		9		10		11		12	
		E	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
		L1	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	BZ	L2	9	13	8	10	15	10	12	9	8	6	7	9	6	5	4	2	0	1	0	0	0	0	0	0
		L3	30	28	25	26	22	25	29	25	27	22	20	22	15	18	10	9	4	1	0	0	0	0	0	0
		D	6	8	9	7	12	9	8	16	10	12	13	11	6	9	8	12	2	5	0	0	0	0	0	0
		T	53	50	42	43	49	44	49	50	45	40	40	43	27	32	22	23	6	7	0	0	0	0	0	0
		E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LV	L2	10	9	7	17	15	11	10	12	9	8	6	5	10	3	2	0	0	0	0	0	0	0	0	0
		L3	29	25	24	23	28	29	22	26	22	24	15	16	10	12	10	13	1	0	0	0	0	0	0	0
		D	11	9	12	5	8	9	18	10	9	11	7	8	4	7	8	6	2	0	0	0	0	0	0	0
18		T	50	43	43	45	51	49	50	48	40	43	28	29	24	22	20	19	3	0	0	0	0	0	0	0
		E	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	BZ	L2	10	12	8	6	18	9	17	10	8	5	4	0	2	4	6	2	0	0	0	0	0	0	0	0
	+	L3	25	30	31	25	20	28	25	29	22	26	12	15	10	4	0	3	0	0	0	0	0	0	0	0
	LV	D	15	6	5	10	1	10	12	9	13	11	7	10	4	9	2	4	0	0	0	0	0	0	0	0
		T	50	48	44	41	39	47	55	48	43	42	23	25	16	17	8	9	0	0	0	0	0	0	0	0
		E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	IV	L2	9	8	10	15	11	9	7	9	8	10	7	5	8	2	4	8	4	5	0	3	0	0	0	0
		L3	31	30	26	25	22	28	25	26	25	24	18	15	10	17	10	8	1	0	0	0	0	0	0	0
		D	11	9	9	9	6	13	11	5	9	16	9	12	10	9	5	9	0	3	2	2	0	0	0	0
		T	51	47	45	49	39	50	43	40	42	50	34	32	28	28	19	25	5	8	2	5	0	0	0	0
		E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	BZ	L2	10	16	7	6	8	10	5	9	13	10	4	5	2	4	2	0	0	0	0	0	0	0	0	0
		L3	40	18	32	30	30	29	31	30	24	26	15	10	6	10	2	5	1	0	0	0	0	0	0	0
		D	5	13	9	11	8	12	13	12	8	12	8	11	8	9	2	1	0	2	0	0	0	0	0	0
		T	55	47	48	47	46	51	49	51	45	48	27	26	16	23	6	6	1	2	0	0	0	0	0	0
		E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LV	L2	10	9	4	11	6	8	7	8	12	9	2	6	4	0	1	2	0	0	0	0	0	0	0	0
		L3	30	28	35	31	27	28	27	33	27	29	20	15	15	10	6	5	0	0	0	0	0	0	0	0
25		D	6	5	12	10	10	7	8	9	10	6	9	7	3	9	8	4	0	0	0	0	0	0	0	0
		T	46	42	51	52	43	43	42	50	49	44	31	28	22	19	15	11	0	0	0	0	0	0	0	0
	BZ	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+	L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	LV	L2	9	10	8	12	6	9	4	8	6	9	8	6	3	6	1	1	0	0	0	0	0	0	0	0
		L3	32	29	25	28	25	30	28	20	28	26	17	16	10	20	12	6	0	0	0	0	0	0	0	0
		D	8	12	15	10	9	6	14	12	8	7	4	8	5	3	6	4	2	3	0	0	0	0	0	0
		T	49	51	48	50	40	45	46	40	42	42	29	30	18	29	19	11	2	3	0	0	0	0	0	0
		E	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	IV	L2	7	9	10	9	11	8	10	6	5	8	4	5	0	4	2	1	1	0	0	0	0	0	0	0
		L3	31	28	25	28	22	26	31	29	26	30	19	21	12	9	6	4	0	0	0	0	0	0	0	0
		D	10	11	15	11	7	9	8	13	7	8	11	7	10	9	6	4	1	0	0	0	0	0	0	0
		T	48	49	50	48	40	43	49	48	38	46	34	33	22	22	14	9	2	0	0	0	0	0	0	0

Key: E = Unhatched eggs; L1, 2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead Larvae; T = Total.

Appendix 5.3a: Experiment VII: Raw data for the *Cooperia* cultures from Farms H and BR using the ECYE protocol with media concentration $^{1}/_{8}$EC + $^{1}/_{4}$YE at 18ºC for 12 days.

			W E L L S																							
Farm	Drug	Stg	1		2		3		4		5		6		7		8		9		10		11		12	
ID	Dup		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Farm H	BZ	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	4	4	3	17	11	4	6	52	0	10	5	5	23	46	2	13	14	9	0	9	6	8	0	0
		L3	34	35	50	41	46	49	31	3	10	24	12	17	31	6	29	26	2	21	0	4	2	3	2	2
		D	14	10	9	4	3	3	8	2	5	21	5	5	8	6	6	10	9	5	0	10	1	2	0	0
		T	52	47	62	67	60	56	45	58	15	55	24	27	62	59	37	49	35	35	0	23	9	13	2	2
	LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	5	0	6	4	8	3	3	5	5	5	40	52	51	10	21	20	15	19	31	19	12	10	4	0
		L3	15	22	53	50	59	49	50	59	30	45	16	3	1	0	0	2	2	7	4	6	2	7	1	1
		D	3	10	4	4	7	6	6	2	15	10	5	4	0	0	2	3	0	7	0	3	6	4	0	1
		T	23	32	63	59	74	58	59	66	52	60	61	59	52	10	23	25	17	33	35	28	20	21	5	2
	BZ + LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	7	18	2	6	1	2	4	1	2	4	47	28	20	25	6	8	20	20	15	19	15	10	8	2
		L3	20	22	57	41	56	50	56	50	30	48	1	22	8	4	3	1	1	5	5	5	5	4	0	0
		D	7	13	9	5	5	6	11	11	3	7	5	4	1	2	8	0	6	1	6	4	1	2	2	0
		T	34	53	68	52	62	58	61	62	35	59	53	54	29	31	17	9	27	26	26	28	21	16	10	2
	IV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	9	0	2	12	13	6	3	3	17	20	18	41	38	40	25	20	24	20	18	16	17	15	11	9
		L3	38	0	30	44	36	32	44	52	50	34	28	8	10	10	10	5	6	7	5	8	5	4	2	2
		D	9	0	10	8	4	8	14	2	6	8	9	3	4	1	6	1	0	0	2	1	0	3	0	1
		T	56	0	42	64	53	46	61	57	73	62	55	52	52	51	36	26	30	27	25	25	22	22	23	12
Farm BR	BZ	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	10	5	8	13	15	15	4	10	14	12	1	2	4	1	2	0	0	0	0	0	0	0	0	0
		L3	20	26	17	13	16	10	13	14	12	6	7	13	9	8	0	0	0	0	0	0	0	0	0	0
		D	5	3	9	5	0	19	2	2	0	2	2	1	1	1	0	0	0	0	0	0	0	0	0	0
		T	35	33	34	31	38	44	19	26	26	20	10	16	14	10	2	0	0	0	0	0	0	0	0	0
	LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	6	14	7	5	16	14	13	8	9	13	7	11	2	10	10	7	16	3	20	21	17	16	10	5
		L3	13	22	11	11	27	15	15	27	12	40	9	14	11	7	12	10	3	5	4	1	3	0	0	0
		D	5	7	0	24	11	11	3	3	2	2	2	3	2	0	2	0	2	1	0	4	0	2	0	0
		T	24	33	18	50	54	40	31	38	23	55	18	28	15	17	24	17	21	9	24	26	20	18	10	5
	BZ + LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	9	7	7	8	6	5	1	6	4	1	5	15	3	4	12	6	5	8	21	6	10	15	8	9
		L3	28	27	21	28	17	18	31	25	21	17	9	14	10	7	21	4	4	5	2	0	0	0	0	0
		D	11	8	6	5	8	6	9	7	8	15	2	2	0	0	3	2	2	2	0	0	3	2	1	0
		T	48	39	34	41	31	29	41	38	33	34	16	31	13	12	36	12	11	15	23	6	13	17	9	9
	IV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	13	0	16	15	9	16	4	15	6	10	2	8	7	9	7	8	0	11	9	8	27	15	11	13
		L3	15	0	21	29	16	19	23	19	17	15	20	15	17	14	6	11	18	5	6	3	0	0	0	0
		D	9	0	8	0	15	5	12	2	9	12	16	12	8	2	0	10	5	8	0	3	1	4	2	3
		T	38	0	45	44	40	40	39	36	32	37	38	35	32	25	13	29	23	24	15	14	28	19	13	16

Key: E = Unhatched eggs; L1, 2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total.

Appendix 5.3b: Experiment VII: Raw data for the *Cooperia* cultures from Farms H and BR using the ECYE protocol with media concentration $^{1}/_{8}$EC + $^{1}/_{4}$YE at 25ºC for 12 days.

			W		E		L		L		S													
	Drug	Stg	1		2		3		4		5		6		7		8		9		10		11		12	
Farm ID	Dupl.		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Farm H	BZ	E	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	10	11	7	15	8	12	3	1	0	2	1	0	1	3	3	4	0	0	2	0	0	0	0	0
		L3	32	35	29	33	22	31	26	17	35	21	16	11	16	14	12	10	5	8	8	12	9	7	0	5
		D	9	13	10	4	10	4	7	4	17	10	4	4	4	6	6	7	10	9	10	6	8	11	10	8
		T	51	59	46	52	40	52	36	22	52	33	22	15	21	23	21	21	15	17	20	18	17	18	10	13
	LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	24	8	12	8	15	5	2	2	1	2	1	2	1	2	2	1	1	0	0	0	0	0	0	0
		L3	28	22	38	18	34	21	25	43	25	19	12	19	13	10	9	8	9	10	8	8	0	6	4	4
		D	18	7	12	9	11	6	2	14	12	12	11	5	12	9	8	8	7	9	10	9	9	3	5	3
		T	70	37	62	35	60	32	29	59	38	33	24	26	26	21	19	17	17	19	18	17	9	9	9	7
	BZ + LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	11	3	10	8	8	4	0	0	1	3	2	3	3	2	2	0	1	0	0	0	0	0	0	0
		L3	29	29	22	26	19	28	17	14	18	24	18	20	15	12	12	7	10	12	8	6	10	11	5	8
		D	8	12	7	8	10	7	2	13	2	13	10	10	8	10	10	8	6	8	10	12	3	3	8	5
		T	48	44	39	42	37	39	19	27	21	40	30	33	26	24	24	15	17	20	18	18	13	14	13	13
	IV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	11	13	13	9	11	9	3	4	4	1	2	7	1	3	0	1	0	0	0	1	0	0	0	0
		L3	37	39	31	36	29	27	29	26	28	18	33	19	19	20	12	9	9	12	8	7	4	6	2	3
		D	7	10	11	13	13	8	11	7	9	8	9	11	12	8	11	7	10	8	5	7	8	7	6	9
		T	53	62	55	58	53	44	43	37	41	27	44	37	32	31	23	17	19	20	13	15	12	13	8	12
Farm BR	BZ	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	2	3	4	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L3	30	37	29	39	25	22	11	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		D	16	9	16	13	14	15	12	12	0	5	4	2	0	0	0	0	0	0	0	0	0	0	0	0
		T	48	49	49	52	39	38	24	15	0	5	4	2	0	0	0	0	0	0	0	0	0	0	0	0
	LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	0	10	0	2	0	0	1	0	1	1	5	3	6	5	16	5	0	2	3	0	0	0	0	0
		L3	30	21	34	39	29	7	33	31	27	23	10	10	2	1	2	2	0	0	0	0	0	0	0	0
		D	8	0	19	17	14	14	13	21	9	0	4	4	6	6	5	4	0	12	4	2	0	0	0	0
		T	38	31	53	59	49	21	47	52	37	24	19	17	14	12	23	11	0	14	7	2	0	0	0	0
	BZ + LV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	4	6	0	0	1	0	2	1	0	2	15	5	4	1	2	4	0	0	0	0	0	0	0	0
		L3	38	39	30	24	30	25	25	22	10	12	6	1	1	0	2	1	0	2	0	0	0	0	0	0
		D	2	4	20	18	15	14	15	20	10	5	4	12	4	4	1	7	0	2	0	0	0	0	0	0
		T	46	53	50	42	46	39	42	43	20	19	25	18	9	5	5	12	0	4	0	0	0	0	0	0
	IV	E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		L2	4	1	0	0	0	0	1	0	3	0	0	3	2	3	8	2	0	0	0	0	0	0	0	0
		L3	34	30	24	26	35	11	11	12	33	14	19	6	4	14	6	5	3	0	0	0	0	0	0	0
		D	4	15	25	21	8	5	19	14	14	22	13	9	8	21	13	12	1	0	0	0	0	0	0	0
		To	42	47	49	47	43	16	31	26	50	36	32	18	14	19	27	19	0	0	0	0	0	0	0	0

Key: E = Unhatched eggs; L1, 2 = 1st and 2nd larval stages respectively; L3 = Ensheathed larvae; D = Dead larvae; T = Total.

Appendix 6.1: Procedures for the larval feeding inhibition assay (LFIA).

I: Principle:

The principle of LFIA base on the fact that, ivermectin cause paralysis to the larvae and hence makes them unable to feed. These larvae can be observed in fluorescent microscope and determined whether or not the larvae has stop feeding on fluorescein-labelled *E. coli*. Fluorescence labelling of the bacteria make them easy to be seen under fluorescent microscope.

II: Requirements:

Requirements for LFIA protocol involved:

Monospecific infested faecal samples, Sieves (100µm and 20µm), petri dish (50mls), lyophilised *Escherichia coli* (Sigma Chemicals Co.), FITC (Sigma Chemicals Co), eppendorf tubes (2mls), centrifuge tubes (10mls), microcentaur, bicarbonate buffer [1.5g/lt Nacl, 1.96g/lt Na_2CO_3, 2.66g/lt NaHCO3 made up to one litre)], ivermectin (Sigma Chemical Co.) DMSO, phosphate buffer solution – PBS, distilled water, bench top centrifuge, automatic pipettes (1ml, 250µl, 20µl, 10µl).

III: Method

- The ivermectin concentrations were prepared as shown in Table 6.1.
- Eggs were extracted from the faeces (for sheep, the monospecific-infected animals were used)

  and the whole procedure can be seen in Appendix 3.3.
- After extracted, the eggs were incubated at 22ºC for 24 hours in a covered Petri dish to allow for hatching to the first larval stage (L_1).
- On the next day, concentrated *E. coli* was prepared by mixing 100µl of (2250µg/ml) with 1mg of FITC into 1ml of bicarbonate buffer in a 1.5ml eppendorf tube.
- The eppendorf tube with *E. coli* was incubated at 20ºC for 2 hours.
- The tube was removed from the incubator and centrifuged at 1500Xg for 2minutes.
- Afterwards, the supernatant fluid was removed using vacuum system and 1ml of PBS was added.
- The tube was centrifuged again at 1500Xg for 2mintutes to wash-off the excess fluorescein from the bacteria and this step was repeated 3 times for thorough cleaning.
- Then, the clean *E. coli* bacteria were re-suspended with 1ml PBS.
- The number of larvae present in the egg suspension was estimated by finding the average of 10 counts of 20µl egg suspension each in a ruled petri dish.
- After estimating the larvae, about 100 larvae were added in 1880µl of tap water and 10µl of selected ivermectin concentration in a 24-well cluster plates. For control wells, 1980µl of distilled water and 10µl of 0.1%DMSO was used instead of ivermectin concentration followed by incubation at 25ºC for 2hours.
- Then 10µl of FITC labelled *E. coli* was added in the suspension in the wells and followed by further incubation of the plates at 25ºC for about 18hours.
- On about 18hours of incubation, the plates were removed and the contents pipetted into 10ml centrifuge tubes.

- Contents in the tubes were centrifuged at 1000Xg for 5minutes in a bench top centrifuge and the supernatant fluid removed by vacuum system leaving about 100µl on the bottom.
- Finally, the larvae were put on a microscope slide and examined under an inverted-fluorescent microscope and the number of larvae observed feeding or non-feeding on the fluorescent-labelled *E. coli* recorded.

Appendix 6.2: Experiment VIII:

Larval counts for the resistant and susceptible species of *O. circumcincta* cultured in eleven dilutions of IV in a 24-well plate in duplicate. The larvae was examined using fluorescent microscope, counted and recorded as shown in this table in two categories as feeding and non-feeding larvae on the bacteria cells.

Type of the isolate	Wells	Selected drug (ng/ml)	Final conc. (ng/ml)	Log conc. of the drug	RECOVERED LARVAE				Total larvae in culture		Ratio (NF larvae) Culture	
					Culture 1		Culture 2					
					FE	NF	FE	NF	1	2	1	2
Resistant species (Goats)	Control	0	0	0	138	0	121	0	138	121	0	0
	2	0.0007	4E-06	-5.4288	150	0	140	0	150	140	0	0
	3	0.0015	7E-06	-5.1278	135	0	160	0	135	160	0	0
	4	0.0119	6E-05	-4.2247	145	2	151	1	147	152	0.01	0.01
	5	0.0954	0.0005	-3.3216	129	18	132	22	147	154	0.12	0.143
	6	0.7629	0.0038	-2.4185	111	28	105	28	139	133	0.2	0.211
	7	6.1035	0.0305	-1.5154	132	20	140	19	152	159	0.13	0.119
	8	48.828	0.2441	-0.6124	123	30	113	32	153	145	0.2	0.221
	9	390.63	1.9531	0.2907	109	52	79	80	161	159	0.32	0.503
	10	3125	15.625	1.1938	48	103	28	121	151	149	0.68	0.812
	11	25000	125	2.0969	6	142	2	130	148	132	0.96	0.985
	12	50000	250	2.3979	0	129	0	141	129	141	1	1
Susceptible species (Sheep)	Control	0	0	0	120	0	102	0	120	102	0	0
	2	0.0007	4E-06	-5.4288	118	0	128	0	118	128	0	0
	3	0.0015	7E-06	-5.1278	118	9	114	17	127	131	0.07	0.13
	4	0.0119	6E-05	-4.2247	166	13	119	17	179	136	0.07	0.125
	5	0.0954	0.0005	-3.3216	112	26	107	34	138	141	0.19	0.241
	6	0.7629	0.0038	-2.4185	120	52	91	43	172	134	0.3	0.321
	7	6.1035	0.0305	-1.5154	104	45	79	62	149	141	0.3	0.44
	8	48.828	0.2441	-0.6124	69	73	48	98	142	146	0.51	0.671
	9	390.63	1.9531	0.2907	19	132	9	140	151	149	0.87	0.94
	10	3125	15.625	1.1938	0	150	0	130	150	130	1	1
	11	25000	125	2.0969	0	148	0	122	148	122	1	1
	12	50000	250	2.3979	0	130	0	136	130	136	1	1

Key: FE and NF = Feeding and Non-feeding larvae respectively.

Appendix 6.3: Experiment IX:

Larval counts for susceptible species of *O. circumcincta* from four sheep with the same level of management cultured using 11 dilutions of IV in LFIA in a 24-well plate. The larvae examined using fluorescent microscope, counted and recorded as shown in this table in two categories as feeding and non-feeding larvae on the bacteria cells.

		RECOVERED LARVAE										
Sheep		Selected Drug conc	Final conc.	Log conc. of the drug	Culture 1		Culture 2		Total larvae in culture		Ratio)NF larvae) Culture	
ID	Wells	(ng/ml)	(ng/ml)	drug	FE	NF	FE	NF	1	2	1	2
	Control	0	0	0	76	0	63	0	76	63	0	0
	2	0.0007	4E-06	-5.4288	89	0	91	0	89	91	0	0
	3	0.0015	7E-06	-5.1278	90	0	95	0	90	95	0	0
	4	0.0119	6E-05	-4.2247	58	0	85	0	58	85	0	0
1	5	0.0954	0.0005	-3.3216	102	0	90	0	102	90	0	0
	6	6.1035	0.0305	-1.5154	56	19	80	19	75	99	0.2533	0.1919
	7	48.828	0.2441	-0.6124	44	49	-	-	93	-	0.5269	-
	8	390.63	1.9531	0.29073	49	63	50	42	112	92	0.5625	0.4565
	9	3125	15.625	1.19382	0	75	0	82	75	82	1	1
	10	12500	62.5	1.79588	0	86	0	115	86	115	1	1
	11	25000	125	2.09691	0	96	0	70	96	70	1	1
	12	50000	250	2.39794	0	85	0	101	85	101	1	1
	Control	0	0	0	65	0	95	0	65	95	0	0
	2	0.0007	4E-06	-5.4288	90	0	123	0	90	123	0	0
	3	0.0015	7E-06	-5.1278	99	0	81	0	99	81	0	0
	4	0.0119	6E-05	-4.2247	86	0	75	0	86	75	0	0
	5	0.0954	0.0005	-3.3216	110	0	90	0	110	90	0	0
2	6	6.1035	0.0305	-1.5154	34	24	74	19	58	93	0.4138	0.2043
	7	48.828	0.2441	-0.6124	38	52	53	52	90	105	0.5778	0.4952
	8	390.63	1.9531	0.29073	0	80	0	68	80	68	1	1
	9	3125	15.625	1.19382	0	108	0	89	108	89	1	1
	10	12500	62.5	1.79588	0	99	0	68	99	68	1	1
	11	25000	125	2.09691	0	86	0	107	86	107	1	1
	12	50000	250	2.39794	0	99	0	95	99	95	1	1

	Control	0	0	0	98	0	94	0	98	94	0	0
	2	0.0007	4E-06	-5.4288	117	0	78	0	117	78	0	0
	3	0.0015	7E-06	-5.1278	104	0	113	0	104	113	0	0
	4	0.0119	6E-05	-4.2247	99	0	100	0	99	100	0	0
3	5	0.0954	0.0005	-3.3216	104	2	102	3	106	105	0.0189	0.0286
	6	6.1035	0.0305	-1.5154	90	19	112	3	109	115	0.1743	0.0261
	7	48.828	0.2441	-0.6124	89	9	81	27	98	108	0.0918	0.25
	8	390.63	1.9531	0.29073	73	38	-	-	111	-	0.3423	-
	9	3125	15.625	1.19382	59	48	13	78	107	91	0.4486	0.8571
	10	12500	62.5	1.79588	42	64	36	71	106	107	0.6038	0.6636
	11	25000	125	2.09691	2	99	6	97	101	103	0.9802	0.9417
	12	50000	250	2.39794	0	100	0	102	100	102	1	1
	Control	0	0	0	90	0	80	0	90	80	0	0
	2	0.0007	4E-06	-5.4288	132	0	116	0	132	116	0	0
	3	0.0015	7E-06	-5.1278	98	0	105	0	98	105	0	0
	4	0.0119	6E-05	-4.2247	105	0	103	0	105	103	0	0
4	5	0.0954	0.0005	-3.3216	128	0	84	3	128	87	0	0.0345
	6	6.1035	0.0305	-1.5154	91	11	108	18	102	126	0.1078	0.1429
	7	48.828	0.2441	-0.6124	90	16	99	26	106	125	0.1509	0.208
	8	390.63	1.9531	0.29073	27	68	58	51	95	109	0.7158	0.4679
	9	3125	15.625	1.19382	1	116	0	102	117	102	0.9915	1
	20	12500	62.5	1.79588	0	108	0	97	108	97	1	1
	11	25000	125	2.09691	0	113	0	114	113	114	1	1
	12	50000	250	2.39794	0	94	0	123	94	123	1	1

Key: FE and NF = Feeding and Non-feeding larvae respectively.

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