SOME ASPECTS OF

THE POPULATION DYNAMICS OF

COOPERIA ONCOPHORA

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Abstract

To develop better control strategies for *Cooperia oncophora* a detailed understanding of the population dynamics is desirable. To achieve this, aspects of the life cycle have been studied in a series of experiments, including the free-living and the parasitic phases.

To investigate the development from egg to third stage larvae, faeces containing *C. oncophora* eggs gathered from different donor animals were incubated at different constant and variable temperatures as well as under natural conditions in the field. To examine the survival of *C. oncophora* third stage larvae they were kept under similar conditions as for the development experiments. Based on the results at constant temperatures, parameters were calculated for a model to simulate the effect of temperature during the free-living phase of this nematode. A further experiment was conducted over an 11 month period to investigate the effect of host age and previous exposure on the establishment rate of third stage larvae in 3 groups of young calves. Two groups received a high or low dose of trickle-infection and the third remained as an uninfected control group.

At lower temperatures the development rate and success from egg-third stage larvae were both low but increased with higher temperatures. At 8°C 50% development was reached in 56 days with a success rate of 5.5% compared to 5 days and 26.4% respectively at 32°C. The highest development success rate of 37.4% was observed at 28°C. For larval survival, the median survival was 512.2 days at 8°C and decreased to 6.4 days at 37°C. Both development and survival were significantly ($p<0.05$) influenced by the host animal from which the faeces were sourced. Utilising these parameters in a model provides a useful tool to further understand the effect of temperature on the free-living stages.

The establishment rate of *C. oncophora* in the trickle-infected groups declined rapidly compared to the control group but was not significantly different ($p>0.05$) to the control group if the existing worm burden was removed before challenge. A decline in

establishment rate over the 11 month period of the experiment in the control animals was due to the age of the larvae.

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Our responsibility is to do what we can,
learn what we can,
improve the solutions,
and pass them on.

Richard P. Feynman

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List of Abbreviations

Abbreviation	Description
°C	Degree Celsius
µg	Microgram
µm	Micrometre
AM	Ageing model for *C. oncophora* third stage larvae
AR	Anthelmintic resistance
cm	Centimetre
DM	Development model for *C. oncophora* egg to third larval stage
DT50	Time to reach 50% development
epg	Eggs per gram
FEC	Faecal egg count
FMC	Faecal moisture content
g	Gram
h	Hours
IVM	Ivermectin
L1-L4	Larvae of stage 1-4
L3, [L3]	Ex- and ensheathed third stage larvae
LT50	Median survival time
m	Metre
mg	Milligram
min	Minutes
ml	Millilitre
ML	Macrocyclic lactone
nm	Nano metre
R^2	Coefficient of determination
RES	Ivermectin-resistant *C. oncophora* isolate
SEM	Standard error of the mean
sg	Specific gravity
SUS	Ivermectin-susceptible *C. oncophora* isolate
UV	Ultraviolet light
WAAVP	World Association for the Advancement of Veterinary Parasitology

Preamble

The results of a national survey of beef cattle farms on the North Island of New Zealand in 2004/2005 indicated that *Cooperia* spp. was the most abundant species of parasitic nematode and was already showing a high prevalence of resistance to ivermectin and benzimidazole anthelmintics. At that time there was no resistance to levamisole but this was considered an imminent risk. For the development of future control strategies a more detailed knowledge of the dynamics of this species was necessary. In 2008 the research involved with this thesis was commenced as a cooperation of Massey University and AgResearch Grasslands in Palmerston North, New Zealand. The funding was provided by the Foundation for Research Science and Technology under contract C10X0714.

From the beginning of October 2008 a group of six young calves were kept on the Massey University Farm Tuapaka on a weekly rotation of oral ivermectin treatment and re-infection with larvae to maintain a constant source of ivermectin-resistant *Cooperia oncophora*. The first experiment commenced in October 2008 and investigated the survival of third stage larvae kept under constant temperatures as this was expected to continue for a significant time. The development of egg to third stage larvae at constant temperatures was commenced early the following year. The number of constant temperatures in this experiment required this experiment to be split in two parts as the time needed to analyse the samples was a limiting factor. The survival of third stage larvae in the field was investigated in June 2009 (winter) and larval contaminations continued once a month for all seasons to March 2010. The first animal experiment investigating the efficacy of ivermectin for two *C. oncophora* isolates took place in the first half of 2010 and was repeated with another six animals in the second half of 2011, whereas the establishment of *C. oncophora* in calves was examined from September 2010 to September 2011. The last experiments on development of eggs to third stage larvae and the survival of the larvae at variable temperatures in the laboratory were conducted from May 2011 onwards.

All experiments involving the use of cattle have been approved by the Massey Animal Ethics Committee or the AgResearch Animal Ethics Committee.

Several chapters of this thesis (Chapter 2, 3, 4 and 6) describing different aspects of the life-cycle of *C. oncophora* are, or will be, submitted as publications in peer reviewed journals. All Chapters 2-6 are therefore included in a publication style format. However, the formatting as well as the style of the charts and figures have been partly changed for the purpose of being integrated into one document with section numbers etc. aligned to the particular chapter number in the thesis. These changes include also cross-references between sections and to additional information in the appendices. All references have been integrated into a single reference section.

Chapter 1

Introduction and Literature Review

1.1 Introduction

Gastro-intestinal nematode infections of livestock are found in the farming-industry worldwide and are of major economic significance due to decreased productivity and increased farming associated costs. A national survey of 62 New Zealand beef cattle farms in the North Island of New Zealand from mid-2004 to mid-2005 identified *Cooperia* spp. not only as the most commonly found nematode but also as the parasite with the most severe level of anthelmintic resistance (Waghorn et al., 2006a). Similar findings were found at about the same time for *Cooperia oncophora* in northern Europe (Demeler et al., 2009). These findings indicated the possible need for alternative control strategies, other than just the use of anthelmintics, in the future. However, these issues are in contrast to the limited scope and number of published reports investigating this parasite's population-dynamics.

The general life-cycle can be divided into two phases:

- The non-parasitic free-living phase during which the eggs develop to the infective third stage larvae.
- The parasitic phase starts with the establishment of these larvae in the host where they develop to adult nematodes, mate and produce eggs thus completing the cycle.

The free-living phase can again be divided in two parts, during the first part the eggs develop to the third or infective stage larvae inside the faecal pat and the second when the third stage larvae is waiting on the herbage to be picked up by a new host. During the free-living phase, climatic factors have a substantial influence on population-dynamics. Temperature and moisture content in the faecal pat play an important role in the development rate and success of these larval stages. For the non-feeding third stage larvae in the herbage temperature is the main factor influencing the consumption of stored nutrients and therefore the lifespan.

The establishment of the third stage larvae marks the first step of the parasitic phase of the life-cycle. For *C. oncophora* it is argued that age and the immune response of the host are important factors influencing the establishment rate of incoming larvae, fecundity of established nematodes and other aspects of the dynamics inside the host. However, some of these aspects have not been adequately investigated such as the influence of an existing worm population on the establishment of incoming larvae or the effect of larval age on infectivity.

The combination of abundance and the potential ability to develop resistance to a broad spectrum of anthelmintics make it desirable to investigate the population dynamics of *C. oncophora* in more detail. The aim of this work was to:

1. Provide additional data and information on the effect of temperature on the free-living phase and develop a model of these temperature effects on larvae
2. Investigate the effect of host age and different trickle-infection doses on the establishment rate in the host.

A better general understanding of the population dynamics of *C. oncophora* will allow more effective and sustainable control strategies for this parasite to be developed.

1.2 Parasitic nematodes in cattle

Infections with parasitic gastrointestinal nematodes are commonly encountered in livestock worldwide and are considered to be a major cause for production loss and farming associated cost. Cattle are parasitised by a number of nematodes which differ in their pathogenicity but are relatively site specific (Table 1.1). In New Zealand three of these nematode species are commonly encountered in cattle, these being *Ostertagia ostertagi* and *Trichostrongylus axei* in the abomasum and *C. oncophora* in the small intestine. The host animal develops an immune response to control these parasites, however, this immunity has to be acquired first and young calves especially are susceptible to infections for some time.

Table 1.1 – Common parasitic nematodes in cattle

Lung	*Dictyocaulus viviparus*
Abomasum	*Ostertagia ostertagi* *Haemonchus* spp. *Trichostrongylus axei*
Small intestine	*Cooperia oncophora* *Trichostrongylus* spp.
Large intestine	*Oesophagostomum radiatum* *Trichuris* spp.

The most abundant species is *C. oncophora*, a nematode species parasitising the small intestines of mainly young calves. In New Zealand it has been identified to be the predominant parasitic nematode in cattle (Brunsdon, 1964; Waghorn et al., 2006a). Interestingly, there is a strong discrepancy between the abundance of *C. oncophora* and the number of published reports about scientific work on this species. The main reason for this limitation is probably due to *C. oncophora* being considered to be of low pathogenicity (Coop et al., 1979; Herlich, 1965) compared to other parasitic nematodes such as *O. ostertagi* and *T. axei* and also that calves normally acquire immunity to *C. oncophora* relatively fast (Smith and Archibald, 1968a).

1.3 The nematode *Cooperia oncophora*

The nematode *Cooperia oncophora* (Railliet, 1898) belongs to the trichostrongylid family of parasitic nematodes which all share a similar life-cycle. The eggs develop free-living to the third or infective larvae (L3), which then infect a host, mature through the fourth larval stage to the adult and close the life-cycle by sexual reproduction. During the parasitic phase the whitish to pinkish adult nematode is located in the first third of the small intestine of mainly cattle. The adult female (9-12mm) can be distinguished from the smaller adult males as the latter have a

recognisable bursa (Isenstein, 1963). A simplified taxonomy for *Cooperia* is displayed below (Table 1.2).

Table 1.2 – *Cooperia* classification (Anderson, 2000)

Animalia, Eumetazoa, Bilateria, Protostomia

Phylum:	Nematoda
Class:	Secernentea
Order:	Strongylida
Superfamily:	Trichostrongyloidea
Family:	Trichostrongylidae
Subfamily:	Cooperiinae
Genus:	*Cooperia*
Species:	*Cooperia oncophora (Cooperia surnabada)*
	Cooperia curticei
	Cooperia pectinata
	Cooperia punctata

1.3.1 Polymorphism in Cooperia oncophora

Although there have been multiple species described in the genus *Cooperia*, there is evidence that some of them are not different species. *Cooperia surnabada* (Antipin, 1931) and *Cooperia mcmasteri* (Gordon, 1932) are two morphological types of *Cooperia* found in cattle that were initially considered to be unique species. The original description of *C. mcmasteri* as a separate species caused confusion and can still be found in publications today (Sevimli, 2013), but was generally rejected as a separate species and has been regarded a synonym for *C. surnabada* (Allen and Becklund, 1958; Burtner and Becklund, 1971; LeRoux, 1936). However, mating experiments between *C. oncophora* and *C. surnabada* indicated that these are polymorphs of the same species (Isenstein, 1971). This was later supported when the ITS-2 (second internal transcribed spacer) sequence of ribosomal DNA was compared between these two species and found to be the same (Newton et al., 1998). Based on these findings *C. surnabada* (syn. *mcmasteri*) is regarded as a minor morph-type of *C. oncophora* in this thesis.

1.3.2 Life cycle overview

The first detailed description of the life-cycle of *C. oncophora* was published 60 years ago (Isenstein, 1963). It is within the trichostrongylid family and has a direct life-cycle without any intermediate host or migration in the definite host. The overall life cycle can be divided into two phases (Figure 1.1). The first phase is non-parasitic and free-living and includes the development from the egg to the L3 in the faeces and on the pasture. The second is the parasitic phase from the ingestion of the infective stage larvae by the host to the adult worm in the host animal (Mehlhorn, 2002; Taylor et al., 2007).

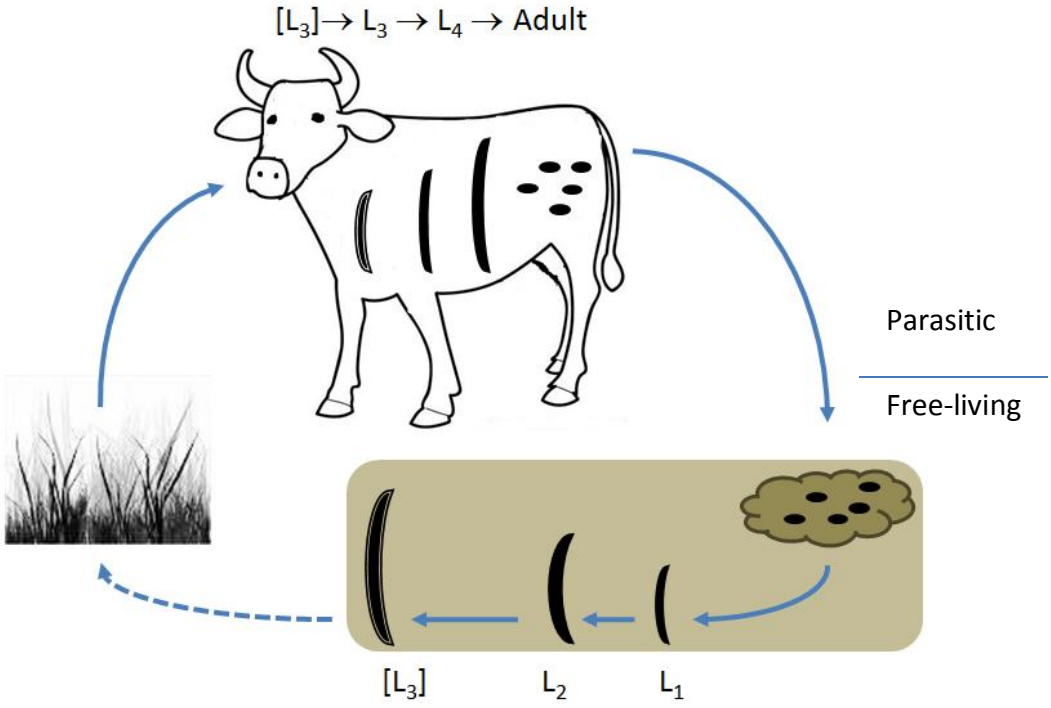

Figure 1.1 - The life cycle of *Cooperia oncophora* can be divided into two phases, a non-parasitic free-living phase outside the host and a parasitic phase.

The eggs of *C. oncophora* are shed with the faeces from the host and hatch in the faecal pat to the first stage larvae (L1). The L1 moults to the second larval stage (L2) which finally moults to the infective third larval stage but retains the old cuticle of the second stage as a sheath ([L3]). At 26 to 30°C the first [L3] have developed by 124 hours or approximately 5 days (Isenstein, 1963). Whereas the first two stages L1 and

L2 are both feeding in the faeces on bacteria, the [L3] does not feed and stays in the cuticle of the second stage larvae, which contributes to its protection and resistance to adverse weather conditions. Under moist conditions the [L3] migrates from the faecal pat into the herbage where it eventually gets ingested by the grazing host. During this period the [L3] has to rely on the nutrition stored during the first two larval stages. The typical size of the [L3] at this stage is approximately 1mm in length (Table 1.3).

Table 1.3 – Average size (µm) for the different stages of *C. oncophora* (Isenstein, 1963)

	Stage						
	egg	L1	L2	[L3]	L3	L4	adult
Size	84.3x42.4	375	808	951	1220	♂ 1930-3700 ♀ 2110-4590	♂ 4730+ ♀ 6510+

Once in the host animal the [L3] ex-sheath and moults to the fourth stage larvae (L4) in the small intestine after approximately 4 days. Around 10 days following infection the final moult to the adult nematode takes place, which in its early stage is also called the fifth stage larvae or L5 by many authors. In this thesis L5 will not be used as a stage in the life cycle as no moults are involved to separate it from the adults. The prepatent period of *C. oncophora*, the time following infection of the host until the adult female will start excreting eggs, is between 17 to 22 days (Isenstein, 1963). Thus the minimum generation interval is under one month under ideal circumstances.

1.4 The free living phase

During the free-living phase of nematodes a wide range of different conditions influences the development and survival of trichostrongylid nematodes. In the main these conditions include the climate in and around the faecal pat during development. However, these factors change slightly with the translocation of the third larval stage from the faecal pat into the herbage. There are also indications that this phase is influenced by the host animal of the parental generation (Jørgensen et al., 1998; Jørgensen, 2000).

1.4.1 Development of egg to [L3]

In the first part of the free-living phase the trichostrongylid eggs, which have been excreted with the faeces, hatch and develop from the L1 to the [L3] inside the faecal pat. The development rate or speed of development and the development success or how many eggs successfully develop to [L3] depends on two major factors, this being temperature and moisture (Levine and Todd Jr, 1975; Rossanigo and Gruner, 1995). A third factor is the availability of oxygen which is linked to moisture content (Silverman and Campbell, 1959). To understand the relationship between development and these factors is crucial for the understanding of the population dynamics of the free-living stages. For *C. oncophora* the faecal moisture content (FMC) for successful development to the [L3] has been indicated to have a range between 40% to 85% with the highest development success of 54 L3 developing per 100 eggs occurring at a FMC of 65% (Rossanigo and Gruner, 1995). In addition, for the development of *C. oncophora* the humidity conditions inside a normal cattle faecal pat are relatively stable compared to sheep pellets as the type of faeces can greatly influence the development success (Rossanigo and Gruner, 1994). If too thin the cattle pat can rapidly dry out inhibiting development and if too thick and moist the low oxygen content has been identified to reduce development for multiple species of nematodes (Rose, 1961; Shorb, 1944; Silverman and Campbell, 1959). Although moisture is required for the development of egg to [L3] there are indications that the ambient temperature has a greater impact on the development in temperate regions (Fiel et al., 2012) as opposed to in arid areas such as Kenya where the available rainfall is the limiting factor for successful development (Ng'ang'a et al., 2004). Under New Zealand conditions recent findings have indicated that the temperature is of greater importance for the development of parasitic nematodes under natural conditions (Reynecke et al., 2011).

1.4.1.1 The effect of temperature on eggs

To date there are no published reports on the effect of temperature specifically on the development of *C. oncophora* eggs. In most reports the development of egg to [L3] is studied as one step without investigating the separate developmental stages in

between. Hatching of eggs may be compromised when eggs are exposed to cold but above freezing temperatures (McKenna, 1998). This study indicates for *Cooperia* eggs recovered from sheep that the effect of 4°C on their viability follows a relatively linear decline to minimal levels within 12 days. In comparison to other nematode eggs the genus can "be ranked in the order of *Trichostrongylus* > *Ostertagia* > *Oesophagostomum*/*Chabertia* > *Haemonchus* > *Cooperia*" with *Cooperia* being most susceptible to cold temperatures (McKenna, 1998). This also has practical implications for the proportions of identified species in cultures when the samples have been stored in a refrigerator before processing. For common trichostrongylid parasites of sheep in England the relation of egg hatching and ambient temperature has been investigated (Crofton, 1965). Interestingly the reported minimum hatching temperature of 16°C for *C. curticei* differs from that of 10°C reported by Ahluwalia and Charleston (1974) for a New Zealand isolate. In the latter report the possible adaptation of the nematode to the conditions encountered in New Zealand is discussed. The authors also state that the average temperature in New Zealand was approximately 3°C higher compared to England which may contradict an adaptation resulting in a lower minimal hatching temperature. However, the adaptation of an organism is not only subject to the average climate but to all encountered environmental conditions which may have influenced the tested New Zealand isolate.

For *O. ostertagi* hatching of eggs was investigated at different temperatures which indicated that at 40°C none of the eggs hatched compared to at 35°C where about 35% of the eggs hatched within 12 hours reaching a maximum of 94% hatching after 2 days (Pandey, 1972). When the eggs were kept at a temperature of 4°C hatching was delayed with the first eggs recognised after 30 days with an overall low hatching rate. Similar studies investigated the hatching of *Teladorsagia* (=*Ostertagia*) *circumcincta* where at 35°C all eggs hatched within 12 hours but no observations were made at 40°C (Pandey et al., 1989). Interestingly, at 4°C the first eggs of *T. circumcincta* hatched after 2 days and more than 90% of eggs hatched after 5 days compared to the low and delayed hatching of *O. ostertagi* reported before. However, there is an indication of considerable variation of egg hatching for low temperatures (Ciordia and Bizzell, 1963; Young et al., 1980a). For *Haemonchus contortus* cold temperatures of 7.2°C not only

decreased the embryonation rate of eggs but inhibited hatching whereas at 0°C no embryonation occurred at all whilst at 9°C normal development including hatching occurred (Silverman and Campbell, 1959). For the egg development of *C. oncophora* Rose (1963) noted that during cold temperatures most eggs died and only a small proportion of the eggs embryonated, however, further development was retarded. This is consistent with other findings for different trichostrongylid nematodes, including *C. oncophora*, where development of eggs at 5°C was normally incomplete resulting in their degeneration (Ciordia and Bizzell, 1963)

1.4.1.2 The effect of egg size on hatching

For the eggs of *H. contortus* a significant size difference was found between different morphological types and has been discussed as an adaptation to a wider range of environmental factors, including temperature (LeJambre et al., 1970). Waller and Donald (1970a, b) investigated the effect of desiccation on *Trichostrongylus colubriformis* eggs and found that smaller eggs have a higher hatching probability, despite the fact that they should be more prone to water loss based on their unfavourable surface to volume ratio. However, the smaller eggs also developed faster compared to larger eggs at constant temperatures which is similar to the findings for the eggs of *H. contortus* (Crofton and Whitlock, 1965a, b). This is consistent with the relationship between development to temperature and body mass which follows a general rule for organisms indicating that development time decreases with temperature but increases with size (Gillooly et al., 2002). It is therefore possible that the faster development of the smaller eggs gives them an advantage over a potentially higher water loss compared to larger eggs. This also has implications for the observed differences of the reported minimal temperatures for egg hatching between isolates discussed before as egg sizes may differ between isolates influencing the dynamics during embryonation. However, egg sizes are normally not documented in reports on trichostrongylid nematode development.

1.4.1.3 The effect of temperature on egg to [L3] development

Isenstein (1963) recorded the development for the different free-living stages of *C. oncophora* at 26 to 30°C in laboratory experiments which showed eggs hatched within 16.5 hours, the L1 would undergo a moult to L2 at about 28 hours and the L2 to the [L3] at about 124 hours after hatching, giving a total development time of 140 hours in this temperature range. In most other reports the development from egg to [L3] has not been measured as separate steps for the stages but as one single step from the egg to the [L3]. For trichostrongylid nematodes the development rate in general increases exponentially with an increase of temperature as measured for different parasitic nematodes (Ahluwalia and Charleston, 1974; Fiel et al., 2012; Pandey, 1972; Pandey et al., 1989; Rose, 1961). For *C. oncophora* controlled laboratory experiments at constant temperatures on the development from egg to [L3] follow the reported pattern (Ciordia and Bizzell, 1963; Rose, 1963).

Temperature is not only a factor for the development rate but also for development success with similar effects to that reported for egg hatching. For *O. ostertagi* the development success at different temperatures has been reported starting with a lower limit of 10°C with 27% development success, which increases with rising temperature, reaching an optimum at 25°C with 67% development success followed by a rapid decline with only 37% success at 35°C (Pandey, 1972). In contrast to these results, Rossanigo and Gruner (1995) found that for the same species nearly no development occurred at 10°C or above the maximum limit of 30-35°C. However, the optimal temperature was found to be similar to the one formerly reported. For *C. oncophora* the relationship between temperature and development is comparable with a minimal temperature limit at which development to the [L3] occurs of 6°C and a success rate of 1.3%, whereas the highest success rate of 30% was found at 25°C followed by a rapid decline to 0.03% success rate at 35°C (Ciordia and Bizzell, 1963). This study also investigated several other species and reported the results for the development to be uniform between trichostrongylid species which is questionable based on differences described above.

1.4.1.4 The effect of the host on development

The effect of the previous host animal on the life-traits of the following generation, especially the free-living stages, is seldom investigated in studies. However, when cattle-sourced *C. oncophora* were cycled either in calves or lambs the [L3] from cultured samples of both groups showed a size difference (Isenstein and Porter, 1964). In another study on trichostrongylid nematodes in sheep, larvae from different age groups of sheep were found to have significantly different development success rates (Jørgensen et al., 1998). This indicates an effect of a developed immune response in the host on the development of the following generation of nematodes. The same author also reported a significant difference in larval development success between different host animals within groups in a following experiment (Jørgensen, 2000). It isn't clear in either of these reports whether these differences were due to an immune response or a difference in faecal composition affecting conditions for larval development. Neither report included any possible effects on other life-traits of the following generation.

1.4.1.5 Other effects on development

The development of the free-living trichostrongylid stages inside the faeces is additionally influenced by a wide range of other factors. Earthworms and dung beetles are known to have a significant impact on the structure of the faeces. Earthworms can cause disintegration of the deposited faeces by feeding on its underside, resulting in a significant impact on trichostrongylid larval development (Grønvold, 1987; Waghorn et al., 2002). The effect of dung beetles is complicated as they normally utilise the faeces as a food source for their larvae in different ways, some species not burying faecal material but tunnelling inside the faecal pat. Historically dung beetle activity has been of no significance for New Zealand (Dymock, 1993). This changed in late 2013 with the first release of dung-beetles into the New Zealand ecosystem which will be continued with the aim to introduce 11 different species (Dung Beetle Strategy Release Group, 2010). The depth the faecal material is transferred into the soil is dependent on the beetle species ranging from less than 10cm up to 40cm into the soil (Dung Beetle Strategy Release Group, 2010). For *O. ostertagi* [L3] were observed to pass across an

obstacle buried 15 cm in the soil indicating they were capable of moving below this obstacle and resurfacing on the other side (Krecek and Murrell, 1988). In addition the stable climate conditions in the soil can positively affect the development success and has been described for New Zealand conditions (Waghorn et al., 2002; Waghorn et al., 2011). However, different studies on the effect of dung beetle activity have identified a substantial decrease of recovered larvae numbers or the infection levels of calves (Bryan, 1973, 1976; Fincher, 1973, 1975; Grønvold et al., 1992). Nevertheless, these stand in contrast to the beneficial effect of larval development found for beetle species tunnelling inside the faecal pat (Chirico et al., 2003). The improved aeration results in an increased hatching rate but can also lead to desiccation of the faecal material under dry conditions inhibiting subsequent larval development (Reinecke, 1960).

Besides the effect of fauna on the development of nematode larvae there is also a known effect of predatory fungi. A wide range of predacious fungi present in New Zealand have been described that will develop in faecal material and affect the viability of nematode larvae (Skipp et al., 2002). The nematode trapping fungus *Duddingtonia flagrans* develops a trapping net when coming into contact with nematodes (Grønvold et al., 1996; Nansen et al., 1988) and can significantly reduce larval numbers recovered from faeces (Fernández et al., 1999; Waghorn et al., 2002; Waghorn et al., 2003). The chlamydospores of *D. flagrans* are partially capable to withstand the passage through the digestive tract of ruminants (Ojeda-Robertos et al., 2009). The subsequent larval development from fungus-treated cattle and sheep has been investigated to notably reduce herbage larval contamination and/or infection rates (Dias et al., 2007; Dimander et al., 2003a; Dimander et al., 2003b; Knox and Faedo, 2001; Larsen et al., 1995; Nansen et al., 1995).

1.4.2 The third stage larvae – [L3]

Once the larvae have developed to the [L3] stage they are no longer able to feed and have to rely on the nutrients stored during their previous development. They actively leave the faeces and move onto the herbage waiting to be ingested by a host animal. The factors that affect this stage are different to those of earlier larval stages.

Studies with *C. oncophora* have shown the developed [L3] migrate to the outer surface of the cattle pat where they aggregate (Rose, 1963). If the crust on the faecal pat is moist and therefore soft the [L3] are able to penetrate it and can actively move onto the herbage. However, if the crust is dry and hard the [L3] seem to aggregate until the crust is softened by sufficient rainfall and subsequently herbage contamination with [L3] increases rapidly (Rose, 1963). Similar findings have been shown in other studies with *O. ostertagi* or mixed infections in faecal pats, where the migration of [L3] from the faecal pat was retarded by a dry and hardened crust but migration was rapid once the crust was softened by rain or irrigation (Barger et al., 1984; Pandey, 1974; Young, 1983; Young and Anderson, 1981). Under arid climatic conditions rainfall has therefore been identified as the most important predictor for the dispersal of larvae (Agyei, 1997). In contrast to the findings of Rose (1963) are the observations by Durie (1961) who found a rapid increase of available larvae after rain for a range of parasitic cattle nematodes but stated that larvae in cattle pats are migrating into the moist middle and bottom parts when the pat starts to dry out. It is possible that this is a desiccation avoiding mechanism of the larvae to the relatively dry conditions encountered in Australia (Durie, 1961) compared to England (Rose, 1963).

1.4.2.1 Dispersal of the [L3]

When discussing the dispersal of such small organisms as a trichostrongylid third stage larva it has to be emphasised that at this scale the importance of the different physical forces encountered change. As gravity acts on the weight and the weight changes with the third power of size the importance of gravity is proportional to size, but the importance of surface forces like adhesion and cohesion compared to gravity increase for small size objects. As an example, for a 1cm cube, gravitation is 50 times higher compared to adhesion. However, this is reversed for a cube with sides of 0.1mm (Went, 1968). For nematodes locomotion is restricted to dorso-ventral sinusoidal movement and dependent on the viscosity of the surrounding medium (Gray and Lissmann, 1964; Wallace, 1968).

The active migration of the 1mm long *C. oncophora* [L3] from the faecal pat into the surrounding herbage is limited to a few centimetres and is linked to a surface water film. This could be problematic for the parasite as ruminants are known to avoid grazing near faecal pats (Cooper et al., 2000). However, once outside the faeces the active movement of trichostrongylid larvae is supported by other factors. *Dictyocaulus viviparus* infective larvae have been found to actively move up the sporangiophores of *Pilobolus* and aggregate on the sporangium and are carried a distance of up to 2 metres away from the faecal pat (Doncaster, 1981) but how important this is overall remains unclear. Nevertheless, for all species rainfall is an important factor for the passive dispersion of the [L3] from the faecal pat over greater distances (Grønvold and Høgh-Schmidt, 1989). With increasing moisture the hardened crust of faecal pats will soften allowing the [L3] to penetrate through it. Rain drops will splash on collision with a surface into multiple smaller droplets. These droplets are capable of carrying larvae from the surface to the surrounding up to a distance of 90cm, with the majority of the larvae translocated between 10-50cm from their origin (Grønvold and Høgh-Schmidt, 1989). Under New Zealand field conditions a substantial contamination of the herbage of up to 45cm in distance around the faecal pat with *Cooperia* and *Ostertagia* has been demonstrated (Boom and Sheath, 2008) indicating larvae can move by whatever means to at least this distance.

Once outside the faecal pat soil can act as a reservoir for infective larvae and lacking the more extreme conditions of the herbage potentially increase the length of their survival (Al Saqur et al., 1982; Krecek et al., 1995; Waghorn et al., 2002). However, the larvae have to be on the herbage to increase the probability of infecting a grazing host animal. The mechanism of gravitaxis in nematodes has not been identified and is apparently missing in some soil dwelling nematodes (Eo et al., 2008) and could not be confirmed for passively ingested larvae such as *H. contortus* (Sciacca et al., 2002) and *Oesophagostomum dentatum* (Ketschek et al., 2004). Nevertheless, vertical migration of trichostrongylid larvae in the herbage can be observed (Pandey, 1974). As gravitaxis has not been confirmed in trichostrongylid nematode larvae other factors have been indicated to affect the migration. This being the microclimate inside the herbage, mainly the temperature and moisture/humidity (Krecek et al., 1995; Silangwa and

Todd, 1964), and the species of plants the herbage consists of (Moss and Vlassoff, 1993; Niezen et al., 1998a).

1.4.2.2 Survival of the [L3]

The survival of trichostrongylid [L3] is strongly related to the ambient temperature as the metabolic rate for living organisms strongly correlates with the body temperature (Gillooly et al., 2001). This is generally supported for trichostrongylid [L3] by measurements of oxygen consumption at different temperatures (Eckert, 1967). However, a significant difference between species was evident; larvae of *H. contortus* consumed significantly more oxygen than larvae of *C. punctata* or *T. colubriformis.* Interestingly, when *C. punctata* larvae were kept at 30°C for 41 days the amount of stored lipids was reduced by 78% whereas the amount of stored protein in the same experiment was reduced by 48% but remained relatively unchanged for the first 32 days, indicating that lipids are the predominantly used source of energy during survival of [L3]. Possible reasons for this preference are that the energy content of lipids is higher compared to proteins or carbohydrates and that the catabolism of lipids generates water which is necessary for larval metabolism and has been confirmed by findings on the [L3] of *Strongylus vulgaris* (Medica and Sukhdeo, 1997). When comparing smaller sized larvae with larger sized larvae the median survival times show no significant differences within species but there was significant difference between species (Rossanigo and Gruner, 1996). The mortality of the third stage larvae of *C. oncophora* and *Chabertia ovina* was lower than for *T. circumcincta* and *T. colubriformis*. This is in contrast to the findings of Boag and Thomas (1985), who investigated the [L3] survival for a wide range of parasitic nematodes and reported a longer median survival for *T. circumcincta* and *T. colubriformis* compared to *C. oncophora* at 20°C. However, when the temperature was 25 or 30°C the median survival for *T. colubriformis* was shorter than for *C. oncophora*.

While in the herbage the [L3] are also exposed to harmful radiation such as ultraviolet light (UV) with significant effects on the survival on a variety of species (van Dijk et al., 2009). This study only exposed the larvae to UV-A (320-400nm) whereas UV-B (280-320nm) and especially UV-C (100-280nm) can cause much more severe damage.

However, in animals melanin plays a crucial role in photoprotection against this damaging radiation (Kollias et al., 1991) and the larvae of *T. circumcincta* have been reported to display melanisation in reaction to sunlight (Baker et al., 2012)

1.4.3 Seasonal patterns

The seasonal patterns of herbage larval contamination are complex as they are affected by the dynamics of the free-living and the parasitic phase. For New Zealand conditions temperature is the most important factor for the development of the free-living phase (Reynecke et al., 2011), however, the dispersal of the [L3] is strongly dependent on rainfall and moisture (Young, 1983). During winter seasons with high rainfall faecal pats are normally subject to a more rapid decomposition compared to summer and could contribute to the low [L3] recovery during the wetter and colder period as there is an inverse relationship between faecal decomposition and [L3] recovery from herbage (Niezen et al., 1998b). In addition, a higher fibre content in the diet of calves during the summer compared to the winter is likely to positively affect the stability of the faecal depositions during summer (Boom and Sheath, 2008). During dry periods the surface of the faecal pat can become hard inhibiting the [L3] from migration until sufficient rainfall softens this crust (Rose, 1963; Young, 1983). In herbage contaminated by sheep the number of recovered larvae also varied considerably throughout the day (Crofton, 1952)

For *O. ostertagi* and *C. oncophora* Rose (1970) investigated the impact of naturally-infected young calves on herbage larval contamination during the summer grazing seasons 1966-68 in England. The results indicate that calves turned out onto the paddock late April became infected by overwintered larvae and started shedding eggs late May. During the following summer months the contamination began to rise, reaching high levels by the end of June. However, by October the climatic conditions had decreased [L3] development inside the faecal pats (Rose, 1970). This is in accordance to similar findings indicating a general pattern with a rise of larval herbage contamination in late summer continuing till winter followed by a decline to the next spring (Bisset and Marshall, 1987; Michel, 1969). Nevertheless, with contamination

levels already starting to decrease in autumn geographical variations of this pattern can be observed (Fiel et al., 2012). Worm burdens of a series of tracer calves grazing pasture contaminated in the previous grazing season demonstrated reasonably high infection rates in spring but these numbers dropped to low levels during the following summer/autumn (Gibbs, 1980; Smith et al., 1969).

The immune status of the livestock grazing the herbage is another important factor influencing the contamination. As the immune response of cattle to *C. oncophora* develops during the first 1-2 years of life, the contamination of the herbage is considerably higher from young animals as demonstrated by faecal egg counts from calves compared to yearlings (Smith, 1970). However, even adult dairy cows can contaminate herbage with *C. oncophora* as a post calving rise in faecal egg counts can be observed (Borgsteede, 1978a, b).

Overall these findings indicate that for New Zealand conditions the pattern of herbage contamination with *C. oncophora* likely resembles that proposed for the trichostrongylid nematodes of sheep (Vlassoff, 1982)

1.5 The parasitic phase

The parasitic phase of the trichostrongylid life-cycle begins with the ingestion of the third stage larvae by the host. The factors influencing the nematode encountered during the free-living phase change drastically once inside the host, such as the temperature that in a healthy cattle is between 38-39°C with limited fluctuations (Wahrmund et al., 2012). However, before the parasite can reproduce the first step is to successfully establish. The ingested [L3] will ex-sheath inside the host to the L3 and undergo two more moults to the L4 and finally to the adult nematode.

1.5.1 Establishment of the nematode in the host

The ingested [L3] ex-sheath in response to the changes in carbon-dioxide and pH in the host (Silverman and Podger, 1964). Isenstein (1963) reported for *C. oncophora* that some ex-sheathed L3 were recovered from the abomasum and small-intestine 13 hours after infection of a calf and after 3 days all larvae were in the small-intestine. By

Day 4 all recovered larvae were in the L4 stage which then underwent their final moult to the adult stage from Day 10 and by 25-31 days after infection 75-88% of the adult nematodes recovered from the small intestine were located in the first 5-6m (Isenstein, 1963).

Table 1.4 – Development time after host infection with *C. oncophora* for different stages and their average size (Isenstein, 1963)

	Stage			
	L3	**L4**	**L4-adult moult**	**adult**
Time after infection (days)	3	4-9	10	11-15
Length ♂	1.22	1.93-3.7	4.05	4.73-6.77 (8.92*)
(mm) ♀	1.22	2.11-4.59	4.9	6.51-9.43 (13.3**)

* length of adult male nematodes 42 days after infection (van Diemen et al., 1997)
** length of adult female nematodes 55 days after infection (Albers et al., 1982)

However, the establishment of nematodes is influenced by a variety of factors such as the host immune system (discussed later), a pre-existing nematode population in the host as well as by the history of the larvae. Although *C. oncophora* is generally regarded to predominantly parasitise young animals there are no indications that this is due to the host age *per se* (Kloosterman et al., 1991), though, there is indication of a rapid development of resistance in older cattle (Smith and Archibald, 1968a). In 7 month old worm-free calves the establishment of *C. oncophora* was reported as >80% when the calves had no previous experience with the parasite compared to establishment rates of approximately 20% in similar calves which had received a priming infection but were effectively treated with an anthelmintic 2.5 months before challenge (Kanobana et al., 2004). Nevertheless, natural trichostrongylid infections normally occur not as discrete events but as continuous intake of the infective stage by the host. In this regard similar experimental results were found in naïve calves accidentally infected with *C. oncophora* where the establishment rate was higher compared to calves which had previously received a trickle-infection with this species for several weeks (Satrija and Nansen, 1993). However, another explanation is that the well-established worm population in the trickle-infected animals affected the establishment of incoming larvae by a density-dependent effect. Such an effect has been indicated for *H. contortus* in young goats (Watson and Hosking, 1993) and in

sheep receiving continuous trickle-infection of four different dose rates and challenge with a dose of radio-labelled larvae (Barger et al., 1985). In the latter experiment the establishment rate in 6 month old lambs was reported to be 40-45% at the beginning of the experiment but rapidly declined after 4-7 weeks to insignificant levels of below 2% after 10 weeks. This decline was independent from the trickle-infection dose rate. Differences in the dynamics of the establishment rate between various trickle-infection dose rates have been recorded for *T. colubriformis* in sheep using a levamisole-susceptible and a levamisole-resistant isolate for trickle and challenge infections respectively (Dobson et al., 1990c). The establishment rate in 21 week old lambs declined rapidly between 6-10 weeks when the sheep received a trickle-infection dose of 200 [L3]/day contrasting to a decline observed between 1-6 weeks in sheep receiving 2000 [L3]/day.

Reports on the effect of the age of [L3] on their ability to successfully establish in a host animal are sparse. However, there are indications that the establishment rate decreases with larval age (Ciordia et al., 1966; Rose, 1963) and such observations have been reported for different stages of other parasitic nematodes (Skorping, 1982). A correlation between larval size and ability to establish could be detected for total worm burden and number of recovered male worms in one study with *T. circumcincta* (Rossanigo and Gruner, 1996).

1.5.2 The adult nematode in the host

As an adult nematode *C. oncophora* predominantly reside in the lumen of the first quarter of the small intestine without migrating into the mucosa (Coop et al., 1979). The reported pathological changes in calves were limited to compression and distortion of villi in direct contact with the nematodes which is in accordance to previous findings indicating the low pathogenicity of *C. oncophora* (Herlich, 1965). However, these findings stand in contrast to observations of the same species resulting in destruction of the surface epithelium (Armour et al., 1987) and that of other species in the same genus such as *C. pectinata* or *C. punctata* (Herlich, 1965; Rodrigues et al., 2004). Nevertheless, infections by *C. oncophora* can potentially be lethal as indicated by experimental inoculation of calves with 800,000-1,800,000 [L3] given in high daily

dose rates which resulted in a 50% death rate in these calves (Henriksen, 1981). Such infection rates are however, unlikely under natural conditions and common observations following controlled infections with *C. oncophora* include little effect on growth rates but with softening of faeces passed by calves 3-6 weeks post infection (Armour et al., 1987; Coop et al., 1979). Under more severe infections it has also been observed to cause diarrhoea (Herlich, 1965).

The number of adult female to male worms in the host animal is often skewed towards a female dominated population (Poulin, 1997). Such an increase in the proportion of female nematodes can also be found in calves following trickle-infection with *C. punctata* (Yatsuda et al., 2002) or a single infection with *C. oncophora* (Kanobana et al., 2004). *C. oncophora* adult females start to shed eggs 17 days after intake by the host with the majority of females laying eggs by 22 days (Isenstein, 1963). There are other reports of the minimum prepatent period being as short as 14 days (Herlich, 1965; Kanobana et al., 2004). Subsequent to a single infection with 30,000 or 100,000 *C. oncophora* [L3] the number of eggs/g faeces (epg) increased rapidly reaching a peak 21 days after infection at approximately 1700 or 3800 eggs/g, respectively, followed by an exponential decline (Kanobana et al., 2004). In contrast, the faecal egg count (FEC) in calves receiving a weekly dose of 20,000 [L3] reached a peak of only 400 eggs/g and this occurred slightly later at about 50 days followed by a comparable decline (Dorny et al., 1997). This is similar to the general trend found with other trichostrongylids. Sheep 21 weeks of age and trickle-infected with *T. colubriformis* at different dose rates of 200, 632 or 2000 [L3] per day resulted in peak egg counts of approximately 1000 epg after 8 weeks for the lowest daily dose compared to approximately 1500 epg for the highest daily dose (Dobson et al., 1990c). Interestingly, by dividing the observed egg count by the predicted worm count (Dobson et al., 1990a) the fecundity of the female worm was calculated. These calculations indicated that the maximum fecundity of approximately 600 eggs per female per day was reached within 3-4 weeks followed by a decrease in fecundity (Dobson et al., 1990c). However, the total egg output by the nematode population was maintained by more females establishing until the total egg output declined when a threshold population triggering an immune response of 3000 nematodes was reached.

The mortality of trichostrongylids in the host has mainly been investigated in nematodes in sheep. Following a single infection with *H. contortus* of sheep the worm burdens declined exponentially indicating a death rate of 1% per day (Barger and Le Jambre, 1988). A similar result was found for the death rate of *O. ostertagi* in calves, however, there were differences between infection dose rates indicating a density-dependent effect (Anderson and Michel, 1977). When sheep with an already existing *H. contortus* burden were trickle-infected with two different rates of radio-labelled [L3] the decline of the unlabelled worm burden indicated that the death rate was proportional to the intake of new larvae (Barger and Le Jambre, 1988). Similarly, the death rate of benzimidazole-resistant *T. circumcincta* and *T. colubriformis* worm burdens was investigated under a constant trickle-infection with benzimidazole-susceptible isolates (Leathwick et al., 1997). This experiment indicated a death rate of 10.6% per day for *T. circumcincta* whereas *T. colubriformis* numbers did not change significantly in the study time of 6 weeks. In a different experiment (Dobson et al., 1990b) with *T. colubriformis* in sheep there was an effect of dose rate with the death-rate of adult nematodes being similar for higher infection rates of 632-2000 [L3]/day. However, for the lowest infection rate of 200 [L3] the decline in adult worm burden was delayed by 5 weeks but in other respects was similar. In this latter study it was suggested that, as for the decrease of fecundity, a threshold level of the worm population needed to be reached before there was rejection of the nematodes.

1.5.3 Development of the host immune response

The immune response of cattle as the host of a *C. oncophora* infection is known to become effective within the first year of life, thus protecting the animal during its second grazing season (Armour, 1989; Armour et al., 1988; Smith, 1970; Smith and Archibald, 1968a; Smith and Archibald, 1968b). The proportion of *C. oncophora* in the total gastrointestinal nematode population becomes negligible in comparison to other species such as *O. ostertagi* in adult dairy cows (Agneessens et al., 2000; Borgsteede et al., 2000). However, some exceptions have been observed including a temporary rise in faecal egg output of *C. oncophora* 2-4 weeks after calving (Borgsteede, 1978b).

The development of the host immune response seems to be triggered by a threshold, a minimum accumulated number of larval infection for a sufficient period of time. Such a threshold has been indicated for *H. contortus*, *Nematodirus spathiger* and *T. colubriformis* (Dineen et al., 1965a; Dineen et al., 1965b; Dobson et al., 1990a; Windon et al., 1984).The reported threshold for these species were relatively low and if a similar effect functions as a trigger for the development of a host immune response against *C. oncophora* the natural infection by grazing calves during the first season would likely be sufficient. Emery et al. (1992a) estimated that the threshold for *T. colubriformis* equated to approximately 1.8 $x10^5$ 'worm days' (3000 worms x 60d). These authors then suggested it was related to the amount of protein, some of which was acting as an antigen, secreted over these worm days

The host immune system likely affects the L3 and L4 larval stages in the host in a diverse manner as specific antigens of these stages have been identified in *T. colubriformis* in sheep (Emery et al., 1992a, b). Arrested development in the L4 stage within the host has been described for *C. oncophora* (Bisset and Marshall, 1987; Brunsdon, 1971; Kanobana et al., 2004). The reasons for larvae to become inhibited are still poorly defined. In one experiment with adult dairy cows 75% of all recovered *C. oncophora* were arrested in comparison to other gastrointestinal nematodes indicating an influence of the host immune system (Borgsteede et al., 2000). However, the results of Michel et al. (1974, 1975, 1978) indicate a temperature effect on the free-living [L3] influencing the interruption of development in the host.

The host may also influence the development and survival of the free-living stages. Jørgensen et al. (1998) described an effect of the host where the age of the sheep affected the development success of the free-living stages for a variety of trichostrongylid nematodes. A follow-up experiment confirmed these results and further indicated an effect of individual hosts within an age group on the development success of the free-living stages (Jørgensen, 2000).

Following the infection of cattle with *C. oncophora* an altered mucin biosynthesis is induced within 28 days post infection (Li et al., 2009). Mucus of previously infected

sheep has been reported to cause clumping of *T. colubriformis* larvae *in vitro* potentially interfering with larval establishment in the host (Harrison et al., 1999).

The direct immune response of the host to the gastrointestinal nematodes is complex and not fully understood. The role and interaction of IgE, IgA, eosinophils and mast cells will not be discussed here but have been reviewed elsewhere (Claerebout and Vercruysse, 2000; Gasbarre et al., 2001).

1.6 Anthelmintics

Experiments on parasitic nematodes in livestock often require the use of anthelmintics and a short overview of the most relevant anthelmintics for the present work is given in the following.

1.6.1 The different classes and active ingredients

Nowadays a number of different anthelmintics are available to counteract helminth infections. Table 1.5 gives a brief overview of some anthelmintic classes, active ingredients and some commercial products, including those used in these studies.

Table 1.5 – Overview of active ingredients of available anthelmintics, their classes and some examples of commercially available products.

Active Ingredient	Class (Acronym)	Active ingredient in
ivermectin	macrocyclic lactone (ML)	Ivomec®
abamectin	avermectin	Startect®, Matrix®
moxidectin	milbemycin	
levamisole	imidazole	Scanda®, Matrix®
albendazole	benzimidazole (BZ)	
oxfendazole		Scanda®, Matrix®
monepantel[1]	amino-acetonitrile derivative (AAD)	Zolvix®
derquantel[1]	spiroindole (SI)	Startect®

1 = not licensed for use in cattle

The class of the macrocyclic lactones (ML) contains the chemical groups of the milbemycins and avermectins. Both are fermentation products from the actinomycete genus *Streptomyces*, which were found to have antiparasitic properties in the mid 1970's by scientists at Merck (Burg et al., 1979; Chabala et al., 1980; Egerton et al.,

1979, 1980). The main structural difference between the two groups is a disaccharide substituent on the C-13 in the avermectins compared to the milbemycins (Shoop et al., 1995). The first commercially available ML for animal use was ivermectin (IVM; 22,23-dihydroavermectin B_{1a} and B_{1b}) which showed a high efficacy against a broad spectrum of invertebrate ecto- and endoparasites as displayed in Table 1.6 (Campbell, 1981; Campbell et al., 1983). In humans IVM is commonly used to control onchocerciasis, lymphatic filariasis and scabies (Hodges et al., 2010; Hotez et al., 2007; Meinking et al., 1995; Mohammed et al., 2006; Molyneux et al., 2003)

Early studies indicated that the mode of action of IVM was by interacting with GABA receptors (Holden-Dye and Walker, 1990). However, subsequent reports revealed a glutamate-gated chloride channel (GluCL) could be sensitive to IVM (Arena et al., 1991) and this was later accepted as the more likely mode of action in nematodes and other invertebrates for chemotherapeutically relevant concentrations of IVM (Cully et al., 1994).

For the treatment of nematode infections levamisole is an important derivate in the class of the imidazole/imidazothiazoles. Historically tetramisole, a racemic mixture of dexamisole and levamisole was used, however, both isomers share the same side effects but only the L-isomer levamisole possesses efficacy against nematodes. On nematodes it acts as an acetylcholine mimetic agent resulting in a prolonged activation of the muscle cells in the body wall of nematode (Atchison et al., 1992; Coles et al., 1975)

The first benzimidalzole available was thiabendazole in 1961 followed by various other derivates such as oxfendazole. The likely mode of action for all benzimidazoles (BZs) is an interaction with the ß-tubulin compromising the cytoskeleton which is subsequently lethal by disrupting the metabolic processes in the nematode cells (Lacey, 1990).

Table 1.6 – Internal and external parasites of cattle against which subcutaneous ivermectin was shown to be effective at a dose of 0.2mg/kg (Shoop et al., 1995).

Nematoda	Arthropoda
Gastrointestinal worms *Bunostomum phlebotomum* *Cooperia oncophora* *Cooperia pectinata* *Cooperia punctata* *Cooperia* spp. *Haemonchus placei* *Mecistocirrus digitatus* *Nematodirus helvetianus* *Nematodirus spathiger* *Oesophagostomum radiatum* *Ostertagia ostertagi* *Strongyloides papillosus* *Toxocara vitulorum* *Trichostrongylus axei* *Trichostrongylus colubriformis* *Trichuris* spp. **Lungworms** *Dictyocaulus viviparous* **Skin worms** *Parafilaria bovicola* **Eye worms** *Thelazia* spp.	**Cattle grubs** *Dermatobia hominis* *Hypoderma bovis* *Hypoderma lineatum* **Screw worm fly larvae** *Chrysomya bezziana* *Cochliomyia hominivorax* **Sucking lice** *Haematopinus eurysternus* *Linognathus vituli* *Solenopotes capillatus* **Biting lice** *Bovicola bovis* **Mange mites** *Psoroptes ovis* *Sarcoptes scabiei* vat. *bovis* *Chorioptes bovis* **Ticks** *Boophilus microplus* *Boophilus decoloratus* *Ornithodoros savignyi*

1.6.2 Pharmacokinetics

Anthelmintics can be administered as a topical application, as a pour-on, by subcutaneous injection or oral application although not all routes are applicable for all types of anthelmintics. The pharmacokinetic profile of an anthelmintic is usually measured as the concentration of the drug in the plasma which is acting as a proxy measurement for the concentration at the site of the nematode. In contrast the time to reach maximal plasma concentration of topically applied IVM is extended by the time needed to penetrate the skin although different formulations have been found to increase the rate of transdermal delivery (Yazdanian and Chen, 1995). Additionally, the

fatty layer of subcutaneous tissue interacts with IVM delivered by topical or subcutaneous application delaying the passage into the plasma (Herd et al., 1996). However, when applied as an oral dose MLs interact strongly with digestive material (Hennessy and Alvinerie, 2002) resulting in a lower systemic availability (Lifschitz et al., 2005), but is otherwise unaffected by the rumen fluid (Andrew and Halley, 1996; Lifschitz et al., 2005). Nevertheless, this has important implications on the efficacy on different gastrointestinal nematodes with subcutaneous and pour-on application showing a decreased efficacy against *Cooperia* compared to oral application (Leathwick and Miller, 2013). When IVM is given to sheep via the subcutaneous or oral route, the plasma concentration was highest after 36 or 24 hours, respectively, and was not detectable 14 days after oral but still detectable 28 days after subcutaneous application (Marriner et al., 1987). This is in accordance with later findings in sheep which also showed that no residues were found in abomasal fluid 96 hours after the oral but still found 21 days after the subcutaneous application (Bogan and McKellar, 1988).

In contrast to IVM, levamisole is fast-acting, reaching the maximal plasma concentration in 1-3 hours followed by a rapid decrease to undetectable levels within 6-10 hours (Forsyth et al., 1983; Manger, 1991; Oakley, 1980; Sangster et al., 1988). For the anthelmintic efficacy of levamisole it is not the duration of exposure but the maximal concentration (Marriner, 1986) whereas the pharmacokinetics of oxfendazole indicate that the maximum plasma concentration is reached later when compared to IVM with a shorter half-life (Marriner and Bogan, 1980, 1981).

1.6.3 Resistance to anthelmintics

Modern farming practice and livestock husbandry relies heavily on the use of anthelmintics to control parasitic infections. However, the intense use of these drugs has resulted in the development of resistance in the helminth parasites, counteracting the efficacy of anthelmintics. Anthelmintic resistance (AR) of a nematode species to anthelmintics is poorly defined. Prichard et al. (1980) provides a widely accepted definition for resistance as follows: “resistance is present when there is a greater

frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species and is heritable". The World Association for the Advancement of Veterinary Parasitology (WAAVP) has developed guidelines for evaluating the efficacy of an anthelmintic but did not define how to determine when resistance had occurred (Wood et al., 1995). They indicate that a highly effective anthelmintic should achieve greater than 98% efficacy using geometric means. However, they also have other categories for efficacy being effective (90-98%) and moderate (80-89%). More recently the International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) produced guidelines for determining the efficacy of anthelmintics and have indicated that an anthelmintic should achieve >90% efficacy also based on evaluation of geometric means (Vercruysse et al., 2001). As for WAAVP, these guidelines make no comment on defining when anthelmintic resistance has occurred. The only internationally accepted guidelines for defining anthelmintic resistance are those from WAAVP and are based only on measuring faecal egg counts and not actual worm burdens (Coles et al., 1992). In these guidelines the definition of anthelmintic resistance for sheep and goats is where the reduction in arithmetic mean faecal egg counts is <95% and the lower confidence limit for this reduction is less than 90%. However, these same guidelines indicate that for cattle a reduction in egg count <90% is indicative of anthelmintic resistance. In a later publication Coles et al. (2006) stated the definition of AR modified such that an efficacy of <95% would indicate AR assuming that the efficacy of the anthelmintic was expected to be ≥ 99% and this applied generally for all ruminants, horses and pigs. Further discussions have occurred as to how to interpret results from a faecal egg count reduction test (FECRT). Dobson et al. (2009) indicated that geometric means may provide a biased efficacy result compared with the use of arithmetic means. More recently Levecke et al. (2012) determined that for a reduced efficacy threshold of 95% the variation ranged from 92.5-97.5% but was affected by the level of excretion and aggregation of egg counts implying the ability to detect small differences with FECRT was limited. Thus comparing these definitions for resistance with those above for evaluating efficacy clearly indicates there are inconsistencies.

New Zealand has a proven history of reported AR for *Cooperia* in cattle with MLs and BZs (Bisset et al., 1990; Hosking et al., 1996; Vermunt et al., 1995, 1996a, 1996b; West et al., 1994). Similar reports can also be found from other parts of the world such as South America (Fiel et al., 2001) and North America (Edmonds et al., 2010) as well as Europe (Demeler et al., 2009; Stafford and Coles, 1999). For IVM, *C. oncophora* was identified as a dose limiting species even before the drug was commercially available (Egerton et al., 1979).

1.6.4 Reason for resistance and strategies to delay it

A number of different factors have been proposed to contribute to the development of anthelmintic resistance. Excessive use of anthelmintics and under dosing are likely to play important roles in the development of AR (Stafford and Coles, 1999). Another aspect, regarding the previous section on anthelmintics, is that for anthelmintics with a long half-life the concentration of the active ingredient decreases slowly giving resistant nematodes an advantage to establish in the host animal enabling them to produce eggs and contaminate the pasture before susceptible nematodes are able to establish, as reviewed by Sutherland and Leathwick (2011). In general this indicates that anthelmintic formulations such as pour-ons should be avoided as a long half-life or persistency increases the probability for AR development (McKenna, 1995). Further, it has been a common farming practice to shift livestock onto less contaminated pasture after they have been yarded for anthelmintic treatment. This can rapidly increase the proportion of resistance genes in the helminth population on that new pasture as shown for sheep in New Zealand (Waghorn et al., 2009).

The development of AR can be slowed by various measures, the first being to avoid the factors leading to AR development described above. One of the most discussed factors to counteract the development of AR is to leave some parasites *in refugia*. Refugium (lat. = hideaway) describes a part of the parasite population in a group of hosts that is not exposed to an anthelmintic treatment thereby avoiding selection for resistance and delaying the development of AR (Martin et al., 1981). However, the use of a refugium to slow AR development has to be incorporated into farming practices before

substantial AR is present (Leathwick et al., 2012). Another factor which has been recently discussed is the use of combination anthelmintics instead of anthelmintics with a single active ingredient (Dobson et al., 2011; Smith, 1990). These modelling exercises calculated gene frequencies in nematode populations with different anthelmintic treatments and concluded that in theory the use of combination anthelmintics can substantially delay AR development. However, when taking into account that AR resistance may also involve a loss of biological fitness of the nematode a reversion towards susceptibility might be possible (Leathwick, 2013b).

1.7 Modelling

The term model in science is encountered with increasing frequency with a large variety of different designs, applications and aims. There are fundamentally two ways to develop models to describe the population dynamics of parasitic nematodes. The first would be to use general observations such as field data to model the general dynamics in the field and subsequently differentiate between model factors ("top-down"). The second is the opposite and uses components of the population dynamics which can then be combined to develop more complex models ("bottom-up"). Well known examples of the second approach to develop simple models are the differential equations describing the relationship between predator and prey by Lotka (1925) and Volterra (1926). More recent approaches to simulate predator-prey systems have considerably grown in complexity and rely on the use of increased computational power (Kelpin et al., 2000). These models provide information to investigate the dynamics on a more detailed level as they can be structured by physiological characteristics such as age and size. They are generally referred to as structured population models (de Roos et al., 1992). Most models of gastrointestinal nematodes in livestock follow the first approach and have proven to be of great interest to predict the population dynamics of a parasite and as a tool to further examine parts of these dynamics. An example is the investigation of different aspects of the life cycle of *T. colubriformis* in sheep with a series of experiments and the construction of models (Barnes and Dobson, 1990a, b; Barnes et al., 1988; Dobson et al., 1990a). Other approaches have examined the dynamics of the [L3] such as mortality and migration

(Grenfell et al., 1986), their development (Chaparro and Canziani, 2010; Hsu and Levine, 1977) or pasture contamination (Chaparro et al., 2011). The use of models in parasitic nematode research is, however, not limited to general population dynamics but also includes models on the development of AR. These latter models provide important tools for the development of new or modified control strategies (Laurenson et al., 2012; Leathwick, 2013b; Leathwick et al., 2012).

The hatching of *H. contortus* eggs could be adequately described by using a non-linear function with temperature as a factor (Young et al., 1980a). However, for modelling the development of *O. ostertagi* from the eggs to the [L3] Young et al. (1980b) developed a multi-stage model based on results gathered in laboratory and field experiments. This model satisfactorily described the development in faecal pats in relation to temperature with the exception of a delay in development resulting from decreased aeration in the faecal pat. It used a single death rate for all stages which proved adequate for simulating the overall development to the [L3]. Onyiah (1985) developed a model for the development of *H. contortus* [L3] based on previously published data. They concluded that it provided a useful tool to facilitate a better understanding which was useful for evaluating control strategies rather than a predictive model *per se*. In a similar way a more recent model investigated the development and survival in relation to temperature of the pre-infective stages of *T. circumcincta, T. colubriformis* and *H. contortus* (Leathwick, 2013a). This model, in contrast to the previous, calculated development as one stage from the egg to the [L3] using a development rate and a survival rate on an hourly time interval. The method used to construct the latter model is based on a more recent technique for structured population models called the Escalator Boxcar Train (de Roos, 1988; de Roos et al., 1992) and has also been used to examine the population dynamics of equine nematodes in relation to temperature (Leathwick et al., unpublished).

1.8 Conclusion

Although the general biology of all trichostrongylid nematodes is similar a detailed knowledge of individual factors is necessary to fully understand the variations for an individual species. One of the most abundant species in cattle in temperate regions is *C. oncophora* which also displays the highest prevalence of resistance to various anthelmintics in these regions. However, not much attention has been paid to examine the basic dynamics of this species and most work has been undertaken a long time ago with the experimental raw-data no longer available. Therefore a detailed investigation and documentation of the population-dynamics is required.

The factors influencing the population-dynamics of *C. oncophora* are extremely diverse as the life-cycle includes two phases with totally different habitats. The free-living non-parasitic phase is influenced by factors related to the faeces and the herbage such as the climate, predation and migration, whilst the parasitic phase inside the host is influenced by factors mainly related to density-dependent effects and the host's immune response. The indications for *C. oncophora* are that compared to other trichostrongylids, this species is capable of developing and surviving in a wide range of temperatures.

The parasitic phase of *C. oncophora* mainly parasitises young animals in which intensive egg production takes place but the establishment rate, fecundity and death rate of the nematode is compromised by the developing host immune response in ageing hosts. However, a threshold population size seems to be necessary for some parasitic nematodes to trigger the development of an immune response. There is also an indication that an existing nematode population in the host can adversely affect the establishment of new incoming larvae.

This Chapter will be submitted to Veterinary Parasitology with the following headings and authors

The effect of temperature on development of the free-living stages of *Cooperia oncophora*

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Chapter 2

The effect of temperature on development of the free-living stages of *Cooperia oncophora*

2.1 Abstract

The effect of temperature on development of *Cooperia oncophora* from egg to third stage larvae was investigated in a series of experiments. Bovine faecal samples containing *C. oncophora* eggs from 3 different host animals were separately cultured under constant temperatures (8, 16, 20, 24, 28 and 32°C) in the laboratory. The number of third stage larvae was regularly counted using a flotation technique (Flotac). Additional faecal samples mixed from multiple host animals were cultured at variable temperatures in the laboratory and the third stage larvae regularly counted as for the constant temperatures. Artificial faecal pats were deposited in field plots to examine development under natural conditions and were examined for third stage larvae at 2-4 weekly intervals. For the constant temperatures used an increase of temperature also increased the development rate. At 8°C the time to reach 50% development was 56 days and the development success was 5.5% compared to 5 days and 26.4% at 32°C. The highest development success was observed at 28°C with 37.4%. There were significant differences ($p<0.05$) in the development success between the different host animals from which the faeces were sourced. The development at variable temperatures was higher than expected based on the average temperature and the results from the experiment using constant temperatures. In the field the number of recovered larvae declined rapidly during the experiment. If earthworms were inhibited from entering the plots the number of recovered larvae increased significantly ($p<0.05$). The development from egg to third stage larvae of *C. oncophora* was not only influenced by the ambient temperature but was also influenced by the host animal.

2.2 Introduction

Cooperia oncophora is one of the most common parasitic nematode species in cattle and is often abundant, especially during the first year of life. In New Zealand *Cooperia* has been reported on every cattle farm (Brunsdon, 1964; Waghorn et al., 2006a). Although considered one of the least pathogenic intestinal nematodes (Coop et al., 1979) there have been reports of loss of productivity by *C. oncophora* infections as a result of reduced appetite, lower weight gain and farming associated costs (Armour et al., 1987; Coop et al., 1979; Dimander et al., 2000; Forbes et al., 2000; Leathwick and Miller, 2013). In recent years there has been an increasing number of reports about anthelmintic resistance in *Cooperia* spp. in cattle (Demeler et al., 2009; Jackson, 2006) and in New Zealand almost every farm tested has resistance in this species (Mason and McKay, 2006; Waghorn et al., 2006a).

This has led to a requirement to develop a more detailed understanding of the dynamics of this parasite's lifecycle to develop sustainable control strategies. As for all trichostrongylid nematodes this life cycle is direct and can be divided into the non-parasitic free-living phase during which the egg develops to the infective third stage larvae and the parasitic phase where these larvae establish in the host, mature and produce eggs.

For the free-living phase the key parameters which influence development and survival are temperature and moisture. In field studies the rate of development and the proportion of eggs developing to third stage larvae have normally been investigated in combination (Banks et al., 1990; Fiel et al., 2012; Goldberg, 1968; Hertzberg et al., 1992; Smith, 1972; Waghorn et al., 2011; Young and Anderson, 1981). Most of the reports conclude that in temperate climates temperature plays a key role in both development and survival of the developing stages. For example, Reynecke et al. (2011) concluded that in the mild climate of New Zealand temperature has a greater impact than moisture. In contrast, in warmer Mediterranean and tropical climates temperature is less likely to be a factor limiting development (Aumont et al., 1989; Waruiru et al., 1998). Studies conducted under natural conditions, with their enormous variety of factors, often lack the precision to accurately investigate the

effect of individual parameters on larval survival and are difficult to extrapolate to other environments.

Other studies have investigated the effect of climate factors under more controlled conditions in the laboratory. It has been shown that for a variety of trichostrongylid nematodes both temperature and humidity have major effects for the development of the free-living stages and that temperature seems to be most restrictive when above or below the optimum temperature (Rossanigo and Gruner, 1995). For *Cooperia curticei* the effect of temperature on development has been examined in a comparison of laboratory and outdoor experiments (Ahluwalia and Charleston, 1974). In the case of the outdoor experiment, where temperatures were fluctuating, it was assessed that predictive estimations gave best results when using the average maximum temperatures. Nevertheless reports of the temperature effect on the development of *C. oncophora* are sparse.

The aim of this study was to investigate the effect temperature has on development from eggs to infective stage larvae. A series of experiments were conducted to explore the effect of constant and variable temperatures in the laboratory as well as under natural conditions in the field.

2.3 Materials and Methods

2.3.1 Experiments

Three experiments were conducted. In the first, development of *C. oncophora* eggs to infective stage larvae was assessed under constant temperatures in the laboratory. The second experiment assessed development in the laboratory with variable temperatures. In the third experiment, development was investigated under natural conditions in the field. The range of temperatures used in the second experiment replicated those of the third experiment.

2.3.1.1 Experiment 1 - Development at constant temperatures

Faeces from three of four naturally grazed donor animals (Animal A-D) were collected and maintained separately. These animals were kept on a farm with a known history of having ivermectin (IVM) resistant *C. oncophora*, but full efficacy of ivermectin against all other gastrointestinal nematodes. They were treated with ivermectin (0.2mg/kg) 7 to 10 days prior to collection of faeces. Only faeces with a minimum of 100 eggs per gram (epg) were used. For each source animal 100-135 pottles containing a vermiculite culture mixture of 10g of faeces were prepared (see Section 2.3.2.1, Appendix 1 SOP4). Because of the time needed to analyse the samples the Experiment was divided in two sequential parts. In the first part development at a range of temperatures was estimated by storing 30 pottles from each source animal (A, B and C) at 16, 20 and 24°C. In the second part this was then repeated at 8, 16, 28 and 32°C (Animals B, C and D). The use of 16°C in both experiments was to provide a benchmark for both experiments. The second part was conducted 3 months after the first part of this experiment. At specific times, with shorter intervals at higher temperatures, 3 pottles per source animal and temperature were examined using the Flotac® (Appendix 1 SOP2) flotation technique (Bauer et al., 2010; Cringoli, 2006). When the number of L3 reached a plateau over three consecutive sampling occasions the experiment was terminated for that particular temperature.

2.3.1.2 Experiment 2 - Development at variable temperatures

Five naturally grazed young calves were orally treated with ivermectin as above (Section 2.3.1.1) and after 7 days individually faecal sampled. Using FEC to ensure an equal number of eggs from each donor the samples were pooled and the FEC estimated again. A total of 40 pottles were prepared (see Section 2.3.2.1, Appendix 1 SOP4) and then placed in a programmable incubator (WTH-155, Daihan Scientific, Korea) where the temperature was changed on an hourly basis. The incubator was programmed using data gathered from the field study in Section 2.3.3. To simplify programming, each hour of each day over a 7 day period was averaged and used in the program to develop a 24 hour cycle over that week for a period of 20 weeks (Appendix 2). Thus, temperatures approximated those from the field without reproducing them

exactly. Based on the results of the first experiment 5 pottles were examined every 2 to 3 weeks using the Flotac® flotation technique (Appendix 1 SOP2).

2.3.1.3 Experiment 3 - Development under natural conditions

In March 2011 (autumn) an area of pasture not grazed for at least 2 years was used for this experiment. The pasture had been regularly mown over this period to maintain typical sward characteristics. Thirty-six PVC rings 20-25cm deep and 20cm in diameter were evenly distributed 100cm (centre) apart in a 3x12 grid (Appendix 1 SOP5). The rings were pressed 10cm deep into the soil and the herbage around and on the inside cut to a length of 3cm. The 36 rings were randomized into 7 groups of 5 plus one spare. Six additional rings of the same dimensions but with a bottom mesh to inhibit earthworms from entering were also buried in a separate line as above. The turf and soil inside these additional rings was removed, with any earthworms mechanically extracted after overnight soaking in water. Turf and soil were replaced inside the rings the following day.

Faeces from six donor animals treated as for Section 2.3.1.1 were thoroughly mixed and the FEC estimated. A manually formed faecal pat containing 200g faeces was placed inside each ring. One additional ring was set up in the same way and temperature probes were placed into the faecal pat, in the herbage 2-3cm above ground and 5cm deep into the soil. The temperatures were recorded on a 15 min basis (HOBO U12, Onset Computer Corp., MA, USA).

At intervals of 2 weeks for the first four occasions and 4 weeks for the last three occasions the remaining faeces, the herbage and the first 5cm of soil were sampled from all 5 rings of one group (Appendix 1 SOP6). The herbage included the leaves cut as close to the ground level as possible. The remaining turf was included with the first 5cm of soil. Each component was weighed and individually processed. If more than 20g faeces was recovered it was split, one half baermannized and the other half used for faecal egg counts. If less than 20g of faeces was recovered it was just baermannized. The herbage and soil samples were processed as described in Section 2.3.2.3 and the number of infective stage larvae determined. The 6 plots with mesh bottom rings were sampled at the same time as Group 4 in Week 8.

2.3.2 Parasitological techniques

2.3.2.1 Culturing faecal samples

Faecal samples were first weighed and then mixed with vermiculite until the mixture showed a crumbly texture to ensure adequate moisture and aeration (Appendix 1 SOP4). The culture mixture was weighed and the corresponding amount of mixture equivalent to 10g of faeces was placed into small pottles (75ml) without a lid. These were placed into larger pottles (250ml) with a ventilated lid and containing a thin water layer. This water was added in order to capture any escaping larvae and to maintain a high humidity. During incubation the water layer was regularly checked and the culture mixture sprayed with water if required. When counting larvae the faecal mixture in the small pottle as well as the water in the outer pottle was used.

2.3.2.2 Counting techniques

McMaster faecal egg count (Appendix 1 SOP1): The number of nematode eggs per gram faeces was estimated by mixing 2 grams of faeces with 28ml of saturated NaCl (sg=1.2). The mixture was sieved through a 200µm sieve and the retentate discarded. The permeate was examined by counting the eggs in four 0.15ml chambers using standard two-chamber McMaster slides (Chalex Corporation, 2x0.15ml). Each egg counted represented 25 eggs/g.

Flotac® technique (Appendix 1 SOP2): The Flotac® flotation technique was carried out as described by Bauer et al. (2010). The flotation solution used was magnesium sulphate ($MgSO_4$, sg=1.28) which will float both eggs and larvae. Two chambers per sample were counted such that each egg or larvae counted represented two per gram of faeces.

2.3.2.3 Recovering larvae from samples

To recover infective stage larvae from faecal or grass samples a Baermann technique was used (Appendix 1 SOP6). The sample was placed onto a single layer of tissue paper in a sieve. The sieve was positioned in a glass funnel closed at the bottom with a clamped rubber tube and water added to ensure the sample was fully submerged. For grass samples a drop of dish washing liquid was added to act as a surfactant. The larvae were collected with the first few millilitres of water from the tube the next day.

All grass samples were subsequently dried overnight at 80°C to determine dry matter weight.

Whitehead trays (Whitehead and Hemming, 1965) were used to extract larvae from soil samples. The soil was crumbled into small pieces and placed on a single layer of tissue paper in a mesh bottom tray. This tray was placed into another tray and water added to a level to fully cover the sample. The next day the inner tray containing the sample was carefully removed and the water in the outer tray transferred and sedimented overnight. After careful siphoning to remove the bulk of the water the precipitate was baermannized as for faecal samples.

2.3.3 Statistical methods

To investigate the effect of temperature and host on development logistic growth curves were fitted to the individual host data for each temperature. From these curves the maximum number of developed L3 was determined as the average development success as well as the point in time at which 50% (DT50) of these L3 had developed. To estimate the effect of temperature and host on the development success an ANOVA was performed on this data for each experiment separately with the host average L3 as the response and temperature and host as predictors subject to a significant F-test. The differences between temperatures and between hosts within each experiment were further compared using Tukey's method. To estimate the effect of temperature on development rate the DT50-values for each host and temperature were analysed using an ANOVA using temperature and experiment as fixed predictors, whereas host was used as a random factor. The differences in development rate between temperatures were also investigated using Tukey's method.

The period until the first L3 developed and the period until the number of L3 reached a plateau were used to calculate exponential regressions to describe the relationship between temperature and development times. These equations were subsequently used to estimate larval development with variable temperatures in Experiment 2 using one of the following: the overall average temperature, the weekly average temperatures and the hourly average temperatures. In Experiment 3 time and average

temperature from the start of the experiment up until each sampling occasion were fixed factors and plot was a random factor.

All manipulations of experimental animals were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

2.4 Results

2.4.1 Experiment 1 - Development under constant temperatures

For the temperatures used it was generally apparent that with increasing temperature there was a higher development rate and success (Figure 2.1, Table 2.1) although some variations were noted from this pattern. The overall pattern of development followed a cumulative normal distribution which can be divided into three phases. Initially there were no L3 as the individuals hatched from the eggs have to develop through the first two larval stages (L1 and L2), then a period of increasing numbers of developed L3 which finally reached a plateau. At 8°C the maximum number of developed L3 was 8% of the initial eggs after 60 days with a mean development success of 5.5%. At 32°C the percentage of developed L3 increased to a maximum of 35% after 9 days but did not maintain a stable plateau from this time and the mean development success was 26.4%. The results at 24°C were aberrant compared to the other temperatures as the developmental success rate was lower than expected. Similar results were observed at 32°C where the success rate peaked lower than found with 28°C but then rapidly decreased again. There was a significant ($p<0.05$) effect of temperature (Figure 2.1) and host on the proportion of eggs developing to L3 in both experiments (Figure 2.2). There was also a significant difference in developmental success between temperatures and between hosts within each experiment. Also, for development rate, represented by the analysis of the DT50 values, there was a significant ($p<0.05$) effect of temperature.

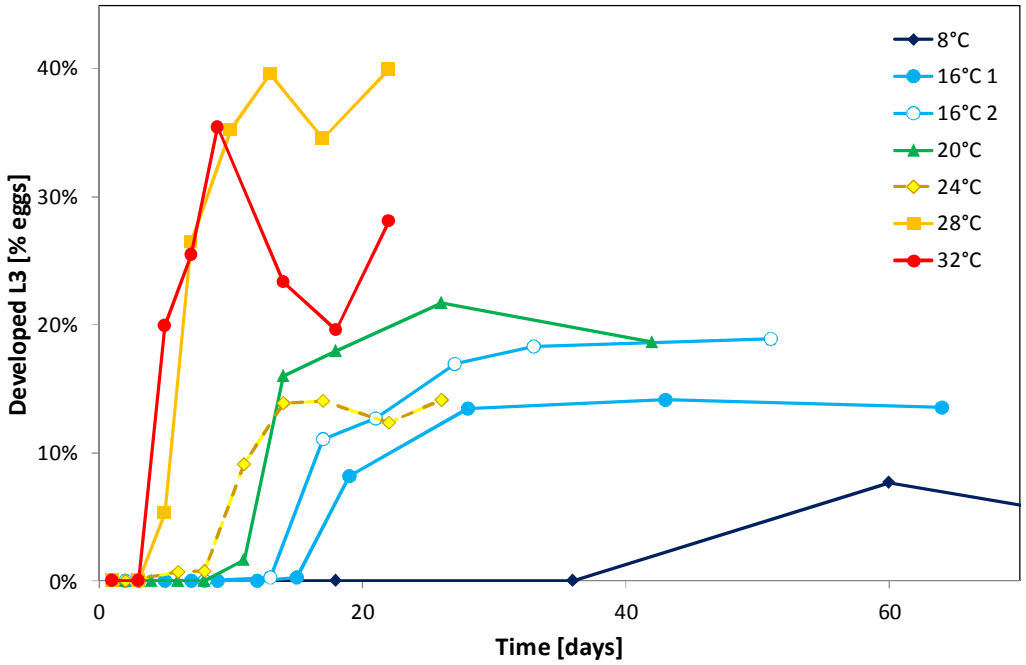

Figure 2.1 - The in-vitro percentage of *Cooperia oncophora* eggs developing to third stage larvae, at different constant temperatures over time.

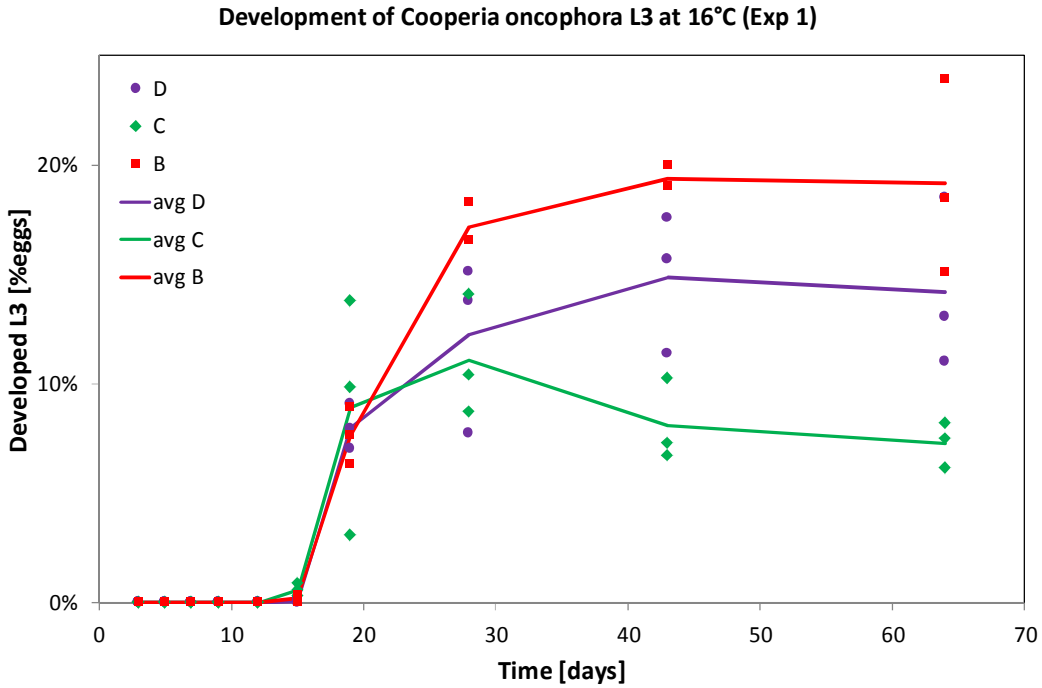

Figure 2.2 - The in-vitro percentage of *Cooperia oncophora* eggs developing to third stage larvae at a constant temperature of 16°C in faecal cultures prepared from 3 different cattle hosts (B, C, D).

Table 2.1 - Individual host and mean success rates (%) for development at different temperatures of *Cooperia oncophora* eggs to third stage larvae in faecal cultures prepared from 3 different calves. Hosts that do not share a letter within each experiment are significantly different for development success. Mean L3 numbers that do not share a letter within each experiment indicate a significant difference in development success between temperatures. DT50 that do not share a letter for both experiments indicate a significant difference in development rate for temperature.

Exp.		Host				Mean	DT50
part	Temp	A	B	C	D	L3	day
1	16	-	15.9	8.8	12.3	12.4^{b}	18^{b}
	20	-	21.1	14.0	20.6	18.6^{a}	13^{bc}
	24	-	12.9	11.3	13.9	12.7^{b}	10^{bc}
	Mean		16.6^{a}	11.4^{b}	15.6^{ab}		
2	8	9.3	0.2	7.0	-	5.5^{b}	56^{a}
	16	24.0	5.1	21.1	-	16.7^{ab}	20^{b}
	28	33.5	17.3	61.3	-	37.4^{a}	7^{bc}
	32	18.8	14.0	46.4	-	26.4^{ab}	5^{c}
	Mean	21.4^{ab}	9.1^{b}	34.0^{a}			

The time points on a $\log_{10}$ scale at which the first L3 were recovered and the maximum number of L3 was reached for each constant temperature is displayed in Figure 2.3. The regression equations together with the coefficient of determination are shown next to the corresponding regression line. The high values ($R^2 \geq 81\%$) indicate a good fit of the regression models to the data. Nevertheless, at 8°C the first recovered L3 and the maximum number were recorded at the same time point. This indicates that the interval between this time point and the previous had been too long, failing to record the increase in L3 numbers during this period.

2.4.2 Experiment 2 - Development under variable temperatures

The pattern of development was comparable to larval development at constant temperatures in showing a cumulative normal distribution. The first L3 were found after 14 days of incubation with an average temperature for this period of 11.4°C (Figure 2.4). The numbers of L3 plateaued at 42 days with an average temperature for this period of 10.9°C. The overall percentage of eggs which developed to L3 was 14.7%.

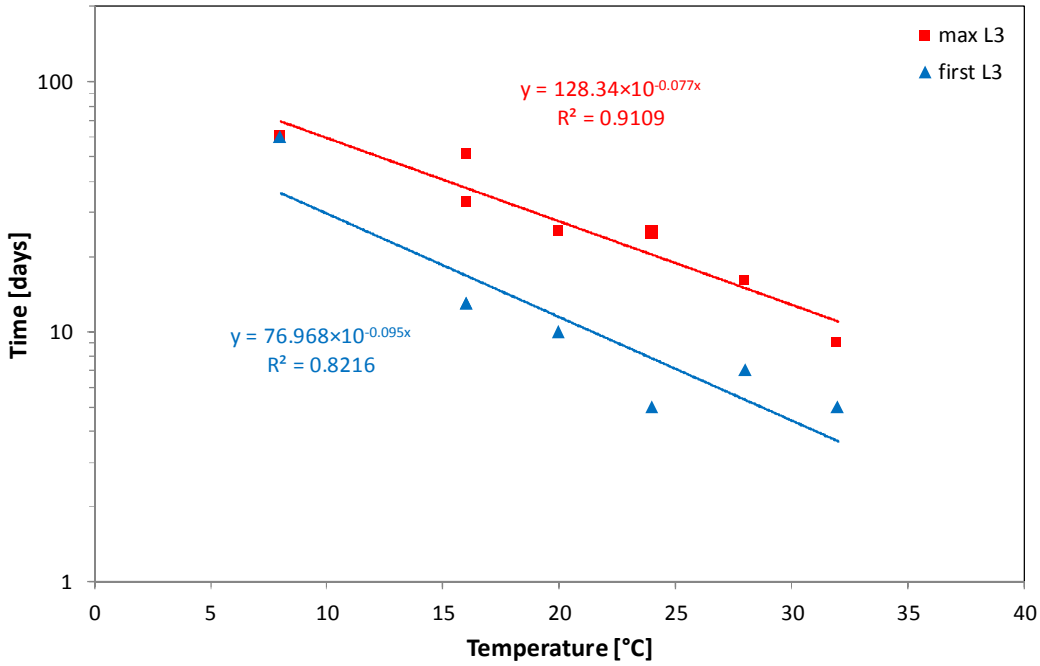

Figure 2.3 - Regression lines for the incubation period until first appearance of developed *Cooperia oncophora* third stage larvae and the period when numbers of developed third stage larvae reached a maximum at different constant temperatures.

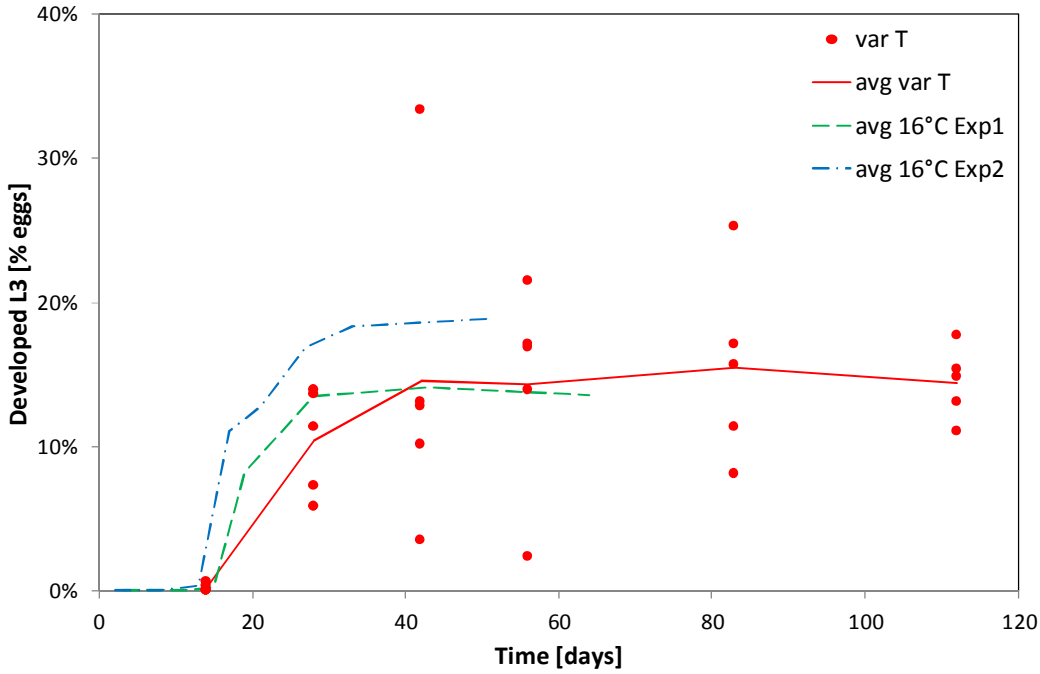

Figure 2.4 - Development of *Cooperia oncophora* third stage larvae under variable temperatures in the laboratory. Displayed are the individual sample counts and the mean for development under variable temperatures (var T and avg var T) as well as mean counts for development at 16°C (avg 16°C 1/2)

2.4.3 Experiment 3 - Development under natural conditions

The highest number of larvae was generally recovered from the faeces if any was left at the time of sampling and the mean number of larvae was highest at 28 days (Figure 2.5). Thereafter the amount of faeces remaining as well as the number of recovered L3 from the faeces rapidly declined. After Day 113 no faeces could be recovered, but a small number of larvae were still recovered up to Day 146. At the beginning of this study the number of larvae recovered from the herbage was higher than the number recovered from the soil. After 44 days 19.6 larvae were recovered from the herbage and 5.4 from the soil on average from each plot. This pattern changed during the study and after 86 days an average of 1.4 larvae were found in the herbage and 16.6 in the soil ($p<0.05$). The total number of larvae recovered from the meshed plots was significantly higher ($p<0.05$) compared to the unmeshed plots. The individual results for larvae recovered from the soil, the herbage or the larvae per gram faeces were however not significantly different.

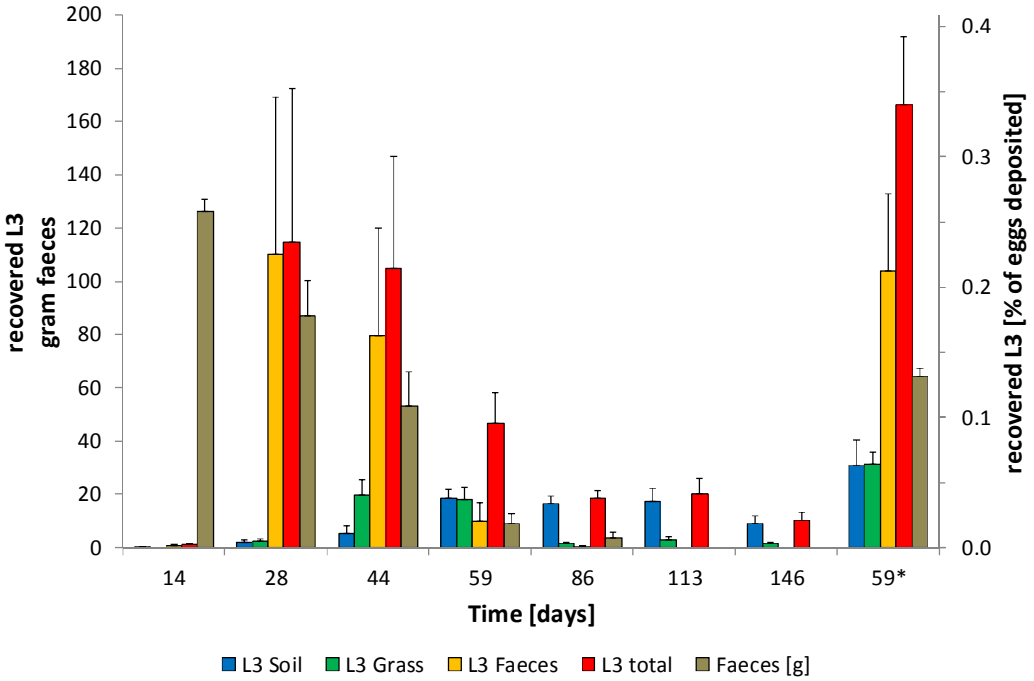

Figure 2.5 - Development of *Cooperia oncophora* third stage larvae under natural conditions in the field. Displayed are various mean raw total counts (+SEM) for 5 replicates on each sampling occasion after artificial faecal deposition (days). *meshed plots to inhibit earthworms from entering.

In plots not secured by a bottom mesh the amount of faeces was rapidly reduced to 8.9g (Range 0-19.7) after 59 days compared to 64.2g (Range 59.3-73.9) in the meshed plots ($p<0.05$). This indicates a high activity of earthworms feeding on the deposited faeces. On the following sampling dates no faeces could be recovered from the plots without the bottom mesh. This correlates with the amount of recovered larvae.

2.5 Discussion

The principal aim of this study was to investigate development of *C. oncophora* from eggs to infective stage larvae at constant and variable temperatures under laboratory conditions and under natural conditions in the field. A positive logarithmic relationship to temperature between development rate and success was evident at constant temperatures in the laboratory experiment. The development under natural conditions in the field experiment followed a pattern comparable to the laboratory experiment with variable temperatures. However, before reaching a stable plateau a decline in larval numbers was observed which is probably due to other factors in the natural environment killing or removing the larvae.

The main factors controlling development and success are temperature and the faecal moisture content. Regarding moisture during development, *C. oncophora* prefers a faecal moisture content of 60-70% as shown previously with sheep faeces (Rossanigo and Gruner, 1995). However, for New Zealand (North Island) conditions, temperature has been shown for some parasitic nematode species in sheep to have the most important effect on development of third stage larvae (Reynecke et al., 2011). Furthermore in a cattle pat compared to sheep pellets moisture is less likely to be the limiting factor for development (Young, 1983). In the current study under laboratory conditions moisture was controlled in order to exclusively investigate the effect of temperature on development.

The present results indicate that temperature influences development from eggs to third stage larvae in two ways. A rise in temperature increased the development rate to reach the maximum number of developed larvae from 60 days at 8°C to only 9 days at 32°C. This increase in development rate was expected as with higher temperatures

metabolic rate also increases. Similar results have been shown for a variety of trichostrongylid nematodes including *C. oncophora* (Ciordia and Bizzell, 1963). Secondly with rising temperatures development success, i.e. how many eggs fully developed to third stage larvae, increased from a mean success of 5.5% at 8°C to 37.4% at 28°C. In the present study there were anomalies in the development success for 2 of the 6 constant temperatures. At 24°C development initially commenced at the expected rate between 20 and 28°C but then slowed down and development success reached a maximum of only 12.7%, being similar to that observed at 16°C. This relatively low success was observed for all replicate samples incubated at 24°C. The most likely reason could only be an adverse event during the incubation, as samples from the same preparation were also used at 20°C where the development success was higher. The second unexpected result was the development success at 28°C, which resulted in a maximum of 40% compared to only 35% at 32°C. It is likely that 32°C is above the optimal temperature for development of *C. oncophora* larvae resulting in lower development success. Further, the pattern of recovered larvae showed a rapid increase to a development success of 35% followed by a decline to lower values on subsequent days. The decline can be an indication that earlier developed larvae are already dying due to the high temperature. For *Teladorsagia (Ostertagia) circumcincta* a decline in developmental success above an optimal temperature of 16°C has been shown previously (Pandey et al., 1989). Similarly with a variety of trichostrongylid nematodes Ciodia and Bizzell (1963) reported the proportion of recovered larvae at 25°C as 30% of the cultured eggs which was reduced to 5.3% at 32°C. However in the present study the optimal temperature for *Cooperia* appears to be higher with best development success at 28°C, which is supported by similar findings for *C. curticei* (Ahluwalia, 1970) in which the proportion of developed L3 at 27°C was 92% compared to only 7% at 37°C.

The development times at which the first L3 were recorded and the maximum numbers of L3 observed at different constant temperatures followed an exponential relationship (Figure 2.3). These results are similar to the development found for other trichostrongylid nematodes (Ahluwalia and Charleston, 1974; Pandey, 1972). Nevertheless, in the present study the values for the first observed third stage larvae

and the maximum number reached at 8°C were on the same time point. This is an indication that the time intervals for sample examination were too long for this temperature so that at the time of the previous examination no developed larvae were present, but numbers had already reached their maximum by the subsequent examination.

Although the effect of different isolates on development has been discussed previously (Pandey et al., 1989), the effect of the individual host animal has been rarely discussed. In the current study a significant host effect on development success could be demonstrated for all samples cultured at constant temperatures. This is consistent with one other previous report which indicated an effect of the host on larval development success of trichostrongylid nematodes in sheep (Jørgensen, 2000). The possibility of a host effect has been verified for the development of *Teladorsagia circumcincta* larvae cultured from sheep of two different age groups (Jørgensen et al., 1998), however in the study no individual differences between the animals within each group could be demonstrated. These results suggest that the effect is caused by the maturing immune response of the host animal. In the current study there was an interesting comparison between individual hosts, where for each temperature (Table 2.1) there was a difference in development success ($p<0.05$). The development at set temperatures was investigated over 2 consecutive parts with animals B and C used in both. In the first the development success of B was significantly lower than for C, whereas in the second part 5 months later the reverse was noted with development success for B significantly higher than for C. Whether this was due to a change in immune response or to other factors was not determined. The experimental design was intended to ensure similar moisture content in cultures thus removing this as a possible explanation.

Development at variable temperatures was similar to the findings at constant temperatures. However, when comparing to the results from the constant temperatures, the results are similar to the findings at a constant temperature of 16°C, although the average temperature was 10.9°C and therefore closer to 8°C. The most reasonable explanation is that the relationship between temperature and development rate is not linear as discussed above and shown previously for the

development of other trichostrongylid larvae (Leathwick, 2013a). Therefore short periods of higher temperatures during the experiment appeared to have a greater effect on larval development compared to similar periods of cooler temperatures. The use of an average temperature has been discussed before with the development of *C. curticei* (Ahluwalia and Charleston, 1974). In that report the authors concluded that the average daily maximum temperature was a better predictor than the average daily temperature and in the present study the average maximum temperature at day 42 where the number of L3 plateaued was 14°C.

Development under natural conditions in the field followed the results from the previous experiments. The maximum number of total third stage larvae was reached after 28 days with an average temperature of 11.3°C. However, as discussed above the relationship between temperature and development is not linear and therefore using the average temperature is unlikely to give correct predictions. Therefore the development observed in this experiment was more comparable to the development at a constant 16°C in the first experiment. The number of developed larvae in the field peaked after 28 days compared to 42 days in the laboratory experiment with variable temperatures. A possible reason for this could be that in the field study some of the earlier developed larvae could not be recovered after 44 days due to larval dispersal, e.g. by natural migration and/or earthworm activity, whereas in the laboratory experiment all escaping larvae were captured by the water in the outer pottle and recovered.

After 59 days more larvae were found in the soil than in the faeces or in the herbage and after 86 days 85-90% of the larvae were recovered from the soil samples. This is due to multiple reasons, primarily that during the first 59 days the amount of recovered faeces rapidly declined to low amounts, thereby reducing the main source of larvae. Secondly the soil seems to act as a reservoir for the third stage larvae in which they may be better protected against adverse effects compared to the herbage as seen for other trichostrongylids (Leathwick et al., 2011; Waghorn et al., 2002). Also the experiment took place during the winter, which in New Zealand is associated with a larger amount of rainfall. This could have washed larvae from the herbage off into the soil or dispersed them by splash droplets (Goldberg, 1968).

In the experiment some of the plots were secured with a bottom mesh to inhibit earthworm activity. These plots were sampled on day 59 along with the unmeshed ones. The results from this showed a significant effect on the amount of recovered faeces. However the number of recovered larvae from the herbage and the soil were not significantly different, indicating that the significantly greater number of total larvae recovered was due to the amount of faeces left. A significant effect of earthworm activity on the availability of third stage larvae in the herbage has been demonstrated before in various studies (Grønvold, 1987; Waghorn et al., 2002).

2.6 Conclusion

In the present study a significant effect of ambient temperature as well as host animal on the development of the third stage larvae of *C. oncophora* was observed. A more thorough investigation of the cause of the host effect could potentially facilitate different parasite control strategies or influence breeding selection.

The data from the experiment at constant temperature should be investigated using a more sophisticated modelling approach to enable a better understanding of the temperature effect on larval development.

2.7 Acknowledgements

The authors gratefully acknowledge the help provided by Anne Tunnicliffe for both laboratory and field assistance, Barbara Adlington for advice with laboratory techniques and nematode identification; Ben Bauer and various other Massey students for help with laboratory and field work as well as Tania Waghorn for support with the field experiment. Siva Ganesh for valuable help with the statistical analysis and the R-software.

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DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Christian W. Sauermann

Name/Title of Principal Supervisor: Prof. W. E. Pomroy

Name of Published Research Output and full reference:

Sauermann, C.W., Scott, I., Leathwick, D. M., Pomroy, W. E., due for submission, The effect of temperature on development of the free-living stages of Cooperia oncophora, Veterinary Parasitology

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or
- Describe the contribution that the candidate has made to the Published Work:

  Experimental design and planning in collaboration with supervisors, carry out experimental work and statistical analysis, preparing first draft of publication.

Candidate's Signature — 22 Jan 2014 (Date)

Principal Supervisor's signature — 22 Jan 2014 (Date)

GRS Version 3– 16 September 2011

Plate 1 – Adult gravid *Cooperia oncophora* female recovered from frozen small intestinal sample. The eggs in this section are inside the uterus and possess shells recognisable by their round shape. The stripes on the cuticle are part of multiple bands of similar surface patterns running on the cuticle from the anterior to the posterior region of the nematode.

This Chapter will be submitted to Veterinary Parasitology with the following headings and authors

The effect of temperature on the survival of *Cooperia oncophora* third stage larvae

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Chapter 3

The effect of temperature on the survival of *Cooperia oncophora* third stage larvae

3.1 Abstract

In a series of experiments the effect of temperature on the survival of *Cooperia oncophora* third stage larvae was investigated. Third stage *C. oncophora* larvae sourced from different host animals were kept in deionised water at constant temperatures (8, 16, 20, 24, 28, 37°C) and the number of living larvae determined on a regular basis. Additional experiments investigated survival as for the constant temperatures but at variable temperatures in the laboratory and under natural conditions in the field by contaminating herbage plots with third stage larvae suspended in water. For the constant temperatures an increase in temperature resulted in a decrease of survival. At 8°C the mean time to reach median survival was 512.2 days and decreased at 37°C to 6.38 days. There were significant differences of the median survival between the host animals the larvae were sourced from. At variable temperatures the survival decreased compared to expectations based on the results at constant temperatures. In the field experiment the number of larvae recovered from the herbage declined rapidly. An increase in temperature significantly decreased the survival time of the third stage larvae. The host animals had a significant effect on the median survival of the larvae.

3.2 Introduction

Cooperia oncophora has a widespread global distribution and although considered one of the least pathogenic parasitic nematodes in cattle (Coop et al., 1979) it is associated with loss of productivity and increased farming costs (Armour et al., 1987; Dimander et al., 2000; Forbes et al., 2000). In addition in New Zealand it has been found on every cattle farm studied (Brunsdon, 1964; Waghorn et al., 2006a) and reports of anthelmintic resistant *Cooperia* spp. have increased during the last few years (Mason and McKay, 2006; Waghorn et al., 2006b).

Better management of this parasite will require an understanding of the population dynamics of *C. oncophora*, and one aspect of this is the dynamics of the free-living stages outside the host. Development of the pre-infective stages has been reported in the first part of this series, whereas this chapter will focus on survival of the infective third stage (L3) on the herbage.

A small number of studies have examined survival of trichostrongylid larvae under controlled laboratory conditions (Boag and Thomas, 1985; Pandey et al., 1993). In one study with regards to temperature, when moisture was not limiting, the survival rates for *C. oncophora* were generally about the median for a range of trichostrongylid species including *C. curticei* (Boag and Thomas, 1985). In a separate study with *C. curticei* the optimum temperature for survival was shown to be 10°C with a rapid decline at both higher and lower temperatures (Ahluwalia, 1970). As *C. curticei* shares a similar geographical distribution to *C. oncophora* a similar trend might be expected.

The aim of the present study was to determine survival of *C. oncophora* infective stage larvae, initially at constant temperatures and then at variable temperatures under laboratory conditions, and finally under natural conditions in the field.

3.3 Materials and Methods

The effect of temperature on ivermectin-resistant *C. oncophora* larval survival was investigated in three different experiments. First the effects of constant temperatures were established by monitoring infective larvae (L3) kept in deionised water at constant temperatures. In the second experiment survival was monitored for larvae kept at variable temperatures that reproduced those of field conditions as observed in Section 2.3.1.3. In the third experiment survival under natural conditions was monitored on contaminated pasture plots.

3.3.1 Experiment 1 - Survival at constant temperatures

Individual faecal samples were taken from 5 young calves (8-10 months of age) which were kept on a farm with a known history of having ivermectin (IVM)-resistant *C.*

oncophora, but with full efficacy of ivermectin against all other gastrointestinal nematodes. They were treated with ivermectin (0.2mg/kg) 10 to 14 days prior to collection of faeces. The samples were mixed with vermiculite and cultured separately at 25-27°C. After 14 days the larvae were recovered using Baermann funnels, morphologically identified as *C. oncophora* and stored in cell culture flasks for 60 days at 8°C prior to the start of this experiment. For each donor animal 3 wells of a 24 well plate (Multidish, Nunc) were half filled with deionised water and 30-60 third stage larvae were added (Appendix 1 SOP7). The plate was closed with the lid but not sealed, then placed into a larger sealed container with regularly exchanged wet tissue paper to maintain a high humidity. Six plates were prepared this way and stored at 8, 16, 20, 24, 28 and 37°C. On a regular basis but with smaller intervals at higher temperatures, the plates were removed and the number of living larvae was determined by carefully shaking the plates or stirring the solution in the wells. All moving or coiled larvae were counted as alive. Larvae that were flaccid and did not respond to a touch with a soft probe (eyelash) were assumed to be dead. Counting for an individual plate was stopped when the proportion of living larvae compared to the initial number was below 3%.

3.3.2 Experiment 2 - Survival at variable temperatures

Individual faecal samples from 3 young calves (6-8 months of age) were collected and cultured as described for Experiment 1 (Section 3.3.1). The same number of larvae from each donor was pooled to obtain one mixed collection. Using a 24 well plate (Multidish, Nunc) 20-30 larvae from this mixed collection were placed into the 4 central wells. All remaining wells were filled with deionised water and otherwise prepared as for Experiment 1 (Section 3.3.1). The plate was then placed in a programmable incubator (WTH-155, Daihan Scientific, Seoul, Korea) where the temperature was changed on an hourly basis. The incubator was programmed using field data gathered from a larval development field study (Section 2.3.3). To facilitate programming, each hour of each day over a 7 day period was averaged to develop a 24 hour cycle for each week. This was then repeated for a total period of 20 weeks. This 20 week cycle was then repeated until the end of the experiment after about one year.

Every two weeks the viability of the larvae in the wells was assessed the same way as described for the constant temperatures.

3.3.3 Experiment 3 - Survival under natural conditions

To investigate survival under natural conditions, field plots with ryegrass dominant pasture were artificially contaminated with infective stage *C. oncophora* larvae (L3) suspended in water once per season over a 12 month period. The larvae were evenly pooled from 3 naturally grazed donor calves as for Experiment 2 (Section 3.3.2).

In one area 16 plots of pasture were marked, each 1.5x5m arranged in 4 quadrants each containing 4 plots side by side. One plot from each quadrant was randomly allocated to one season. Each season, *viz* 23 June 2009 (winter), 13 October 2009 (spring), 27 December 2009 (summer), 11 March 2010 (autumn), the four allocated plots were mowed to a residual height of 5cm using a domestic lawn mower, and then contaminated along the middle 20-30cm portion with 50,000 larvae per plot using a watering can (Appendix 1 SOP8).

Following contamination the herbage on the central 50 cm of the 4 plots was harvested on a monthly basis using a manual push mower to a height of 3cm (Appendix 1 SOP9). The remainder of the plot was mowed using a ride-on mower within 7 days and all clippings were removed. In case of severe wind or rain the harvesting was delayed by a few days to prevent losing clippings or larvae being washed off during mowing. This meant the subsequent sampling may have been slightly less than a month. The clippings from the central 50cm strip were split into 300g portions (by fresh weight). Larvae were recovered using modified Baermann funnels (30cm diameter) with PVC tubes attached to the top to increase the volume and a rubber tube attached to the bottom of the funnel. The funnels were filled with warm water (30°C), a drop of dishwashing detergent to act as a surfactant and left overnight. The first 200ml from the rubber tube was collected, pooled for each plot and concentrated to 100ml by sedimentation and stored at 8°C until counted. For counting this was further concentrated to 20-30 ml and examined in 0.2 to 0.3ml aliquots at 20X magnification. Prior to counting a drop of aqueous iodine was added to

kill larvae and to more easily allow discrimination between ensheathed larvae and free-living nematodes.

3.3.4 Statistical methods

For the experiments at constant temperatures the maximum number of larvae counted per host and temperature was used to represent the 100% value. This was not necessarily achieved at the commencement of the experiment.

To estimate the effect of temperature and host on the L3 survival, logistic survival curves were fitted to the individual host data for each temperature and the median survival or point in time at which 50% of the number of L3 had died (LT50) was determined. An ANOVA was performed on this data with temperature and host as fixed predictors. The differences between temperatures and between hosts were compared using the Tukey's method. All calculations were made with the software Minitab (Version 16, Minitab Inc., USA)

The period after which the number of surviving larvae fell below 95, 50 and 5% were used to calculate exponential regressions to describe the relationship between temperature and survival times. These equations were used to estimate larval survival with variable temperature in Experiment 2 for the overall average temperature, the weekly average temperature and hourly average temperatures. The results were then compared to the experimental data. In Experiment 3 time was used as a fixed factor together with average temperature and plot was a random factor. The difference between seasons was analysed with multiple comparison using Tukey's method.

3.4 Results

3.4.1 Experiment 1 - Survival at constant temperatures

The overall survival of *C. oncophora* larvae kept at constant temperatures (Figure 3.1) followed a cumulative normal distribution that can be divided into three phases. Initially survival showed a small but steady decline, then a period of more rapid decline and finally relatively prolonged survival of a few larvae. The time point between the

first and second phase was reached more rapidly at higher temperatures. When the number of surviving larvae reached 10-20% of the initial number the subsequent decline in survival of the remaining few larvae was prolonged. At 8°C the surviving larvae were normally inactive and coiled. At 16°C most larvae were also inactive and coiled with only a few being motile. At higher temperatures most surviving larvae were active.

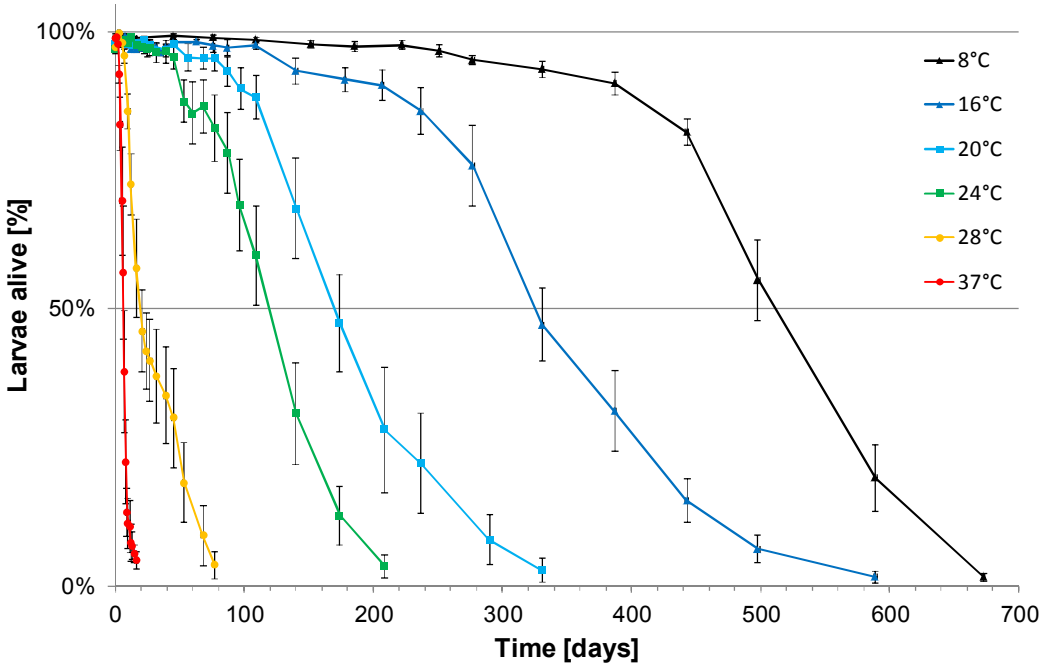

Figure 3.1 - Survival percentage (±SEM) of *Cooperia oncophora* third stage larvae kept in deionised water at different constant temperatures over time (days).

The survival period declined significantly with increasing temperature. At 8°C the calculated LT50 was 512 days but at 24°C this was reduced to only 118 days. At 28°C this had further reduced to 27 days and at 37°C to only 6 days indicating a marked reduction of survival time at these higher temperatures. The differences in median survival were significantly different ($p<0.05$) between all temperatures except for 28 and 37°C. Figure 3.2 displays the time points on a $\log_{10}$ scale at which the proportion of surviving larvae first fell below 95, 50 and 5% of the initial number of larvae for each constant temperature. The regression equations together with the coefficient of determination are shown in Table 3.1. The high values ($R^2 \geq 91\%$) indicate that the

models are a good fit to the data accounting for over 90% of the variation. However, despite the high R^2-values the residuals indicate the observed equations may not be the most appropriate models. For example the observed values from 8°C were lower than predicted in all three cases.

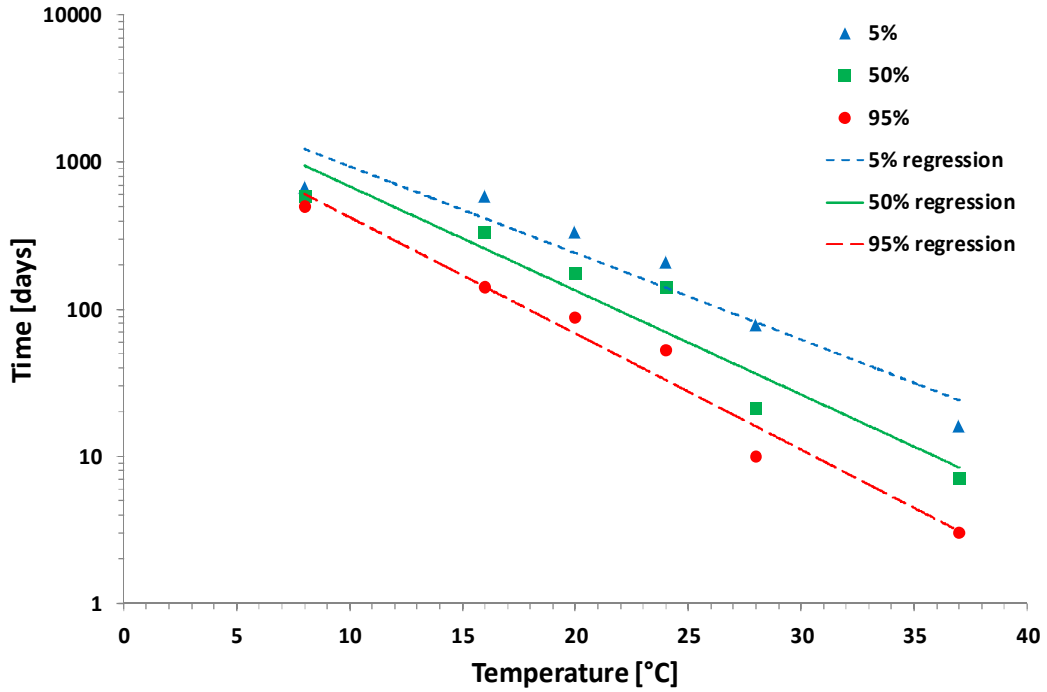

Figure 3.2 - Regression lines for 95, 50 and 5% survival of *Cooperia oncophora* third stage larvae kept in deionised water at different constant temperatures.

Table 3.1 - Regression equations for 95, 50 and 5% survival of *Cooperia oncophora* third stage larvae kept in deionised water at different constant temperatures. The estimated survival time in days are given for the overall mean temperature, the weekly mean temperature, hourly mean temperature and the observed larval survival at variable temperatures.

			Estimated survival time (days)			
	Regression Equation	**R^2**	**Overall**	**Weekly**	**Hourly**	**Observed**
95% L3	Days = $2604\times10^{-0.182\ temp}$	0.97	459	435	423	224
50% L3	Days = $3492\times10^{-0.163\ temp}$	0.92	737	711	694	327
5% L3	Days = $3629\times10^{-0.136\ temp}$	0.91	991	972	952	na

The larvae cultured from some host animals had a higher survival rate than from others, as shown for results at 24°C in Figure 3.3. As displayed in Table 3.2 the difference in survival of larvae cultured from different animals was significant ($p<0.05$). Overall the LT50 for larvae cultured from animal A was significantly lower than for animals D or E.

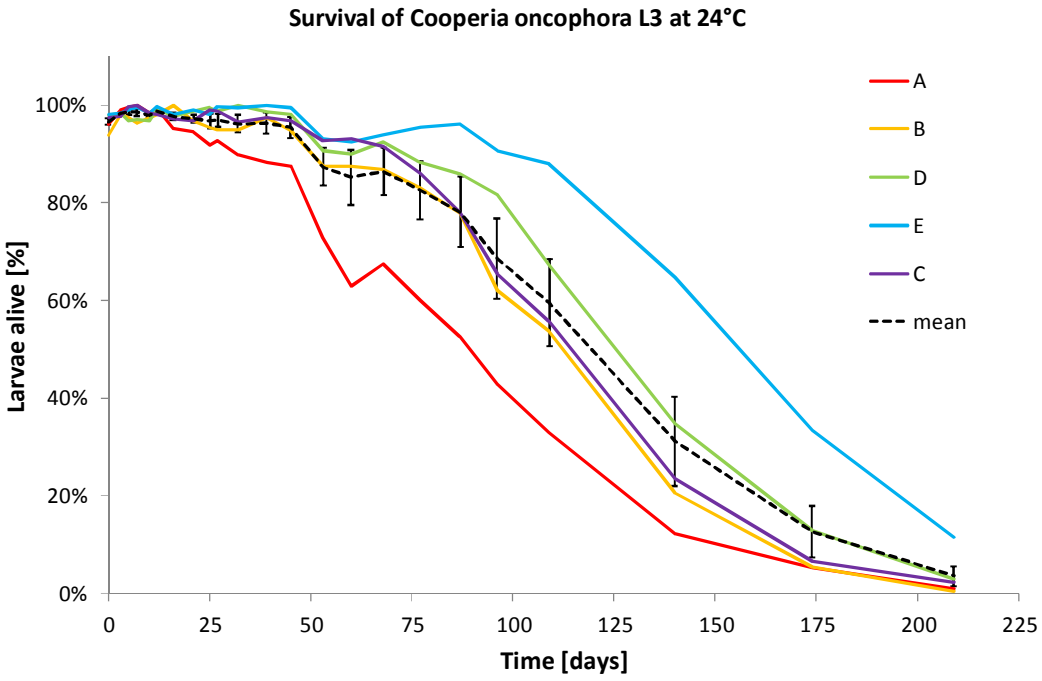

Figure 3.3 - Living *Cooperia oncophora* third stage larvae from different host animals (A-E) kept in deionised water at 24°C over time (days). The dashed line indicates the mean with error bars indicating the SEM.

Table 3.2 - The effect of the host animal (A-E) on survival of *Cooperia oncophora* L3 kept at constant temperatures. Time in days for median larval survival is given for each host per temperature. Different letters (a-c) within temperatures or host animals indicate significant ($p<0.05$) difference.

Temperature	Host animal					
[°C]	A	B	C	D	E	
8	506	497	469	551	538	a
16	293	296	325	363	388	b
20	136	161	160	183	244	c
24	87	111	113	126	155	d
28	17.7	21.8	23.9	21.9	49.8	e
37	4.7	5.9	5.8	6.9	8.6	e
	a	ab	ab	bc	c	

3.4.2 Experiment 2 - Survival at variable temperatures

At variable temperatures the pattern of larval survival was comparable to the larvae survival at constant temperatures in showing a sigmoid trend (Figure 3.4). The overall decline was faster when comparing the average weekly temperatures to the regression calculated for the constant temperatures in Table 3.1. The average overall temperature was 9.5°C, the weekly average temperature ranged from 6.8 to 11.7°C and average hourly temperature ranged from 4.9 to 15.2°C.

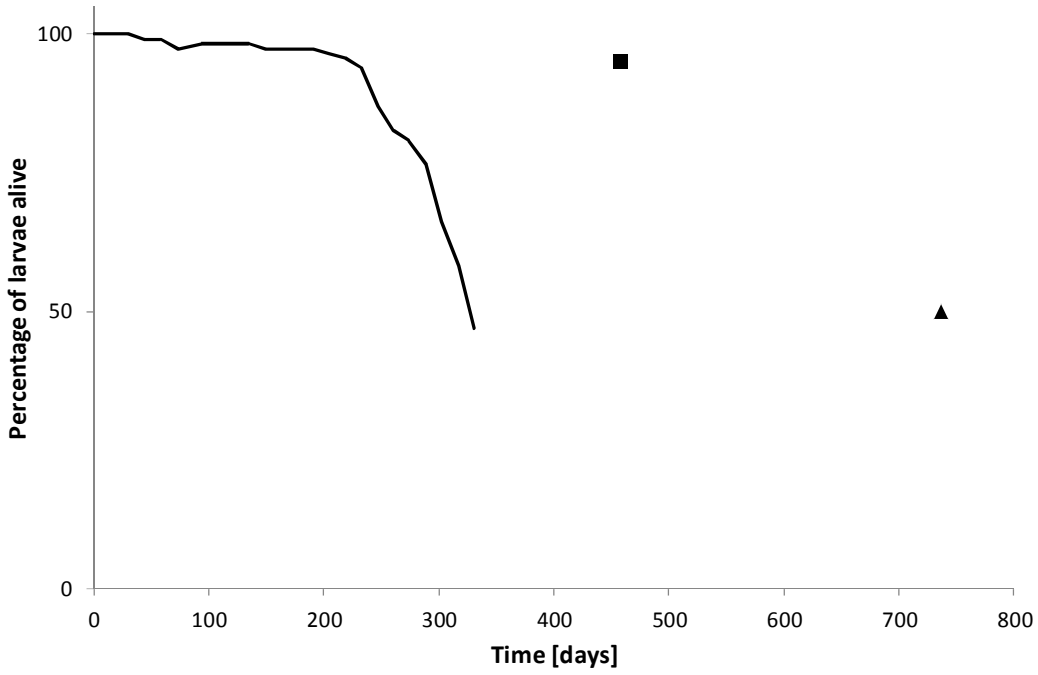

Figure 3.4 – A Comparison between the survival of *Cooperia oncophora* third stage larvae kept in deionised water with the calculated survival based on the exponential equations (Table 3.1) derived from larval survival at constant temperatures (■ for 95 % survival, ▲ for 50 % survival).

3.4.3 Experiment 3 - Survival under natural conditions

In the field larval recovery was low and the maximum number of recovered larvae was 250 on one plot contaminated in spring and harvested for the first time 41 days later. This represents only 0. 5% of the total larvae used for contamination (Figure 3.5, Table 3.3). In winter and spring, larvae could be detected for 4 months and only for 2 months in summer and autumn. The rate of the decline in recovered larvae was more rapid during spring and autumn and slower during summer and winter. However there

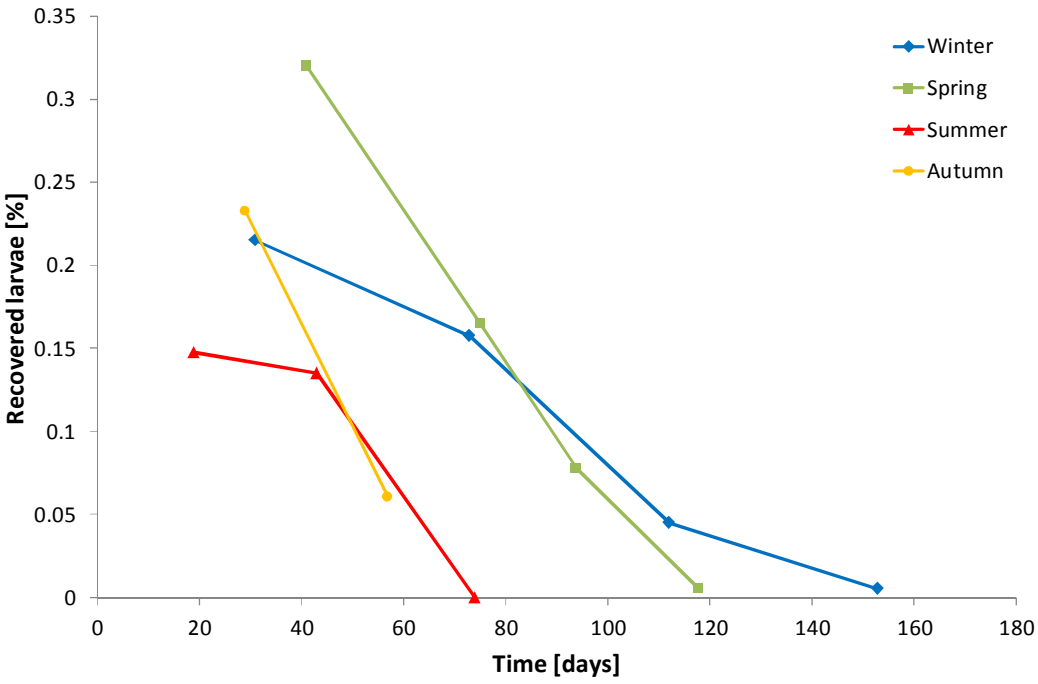

Figure 3.5 – The Percentage of recovered *Cooperia oncophora* third stage larvae from pasture plots over time following artificial contamination (L3 in water suspension).

Table 3.3 – The Percentage of *Cooperia oncophora* third stage larvae recovered from pasture plots collected over time following artificial contamination. Seasons which do not share a letter are significantly different (p<0.05) from each other.

Day	Winter	Day	Spring	Day	Summer	Day	Autumn
31	0.215	41	0.320	19	0.148	29	0.233
73	0.158	75	0.165	43	0.135	57	0.060
112	0.045	94	0.078				
153	0.005	118	0.005				
sum	0.423		0.568		0.283		0.293
	a, c		a		b		b ,c

were significant differences (p<0.05) in total recovery rates between the seasons (Table 3.3). Interestingly there was a trend for the total recovery for each season to be similar to the adjacent season (p>0.05) but different to the opposite season except between spring and summer which were different to each other.

3.5 Discussion

The principle aim of this study was to determine survival of *C. oncophora* infective stage larvae at constant temperatures, variable temperatures under laboratory conditions and under natural conditions in the field. When larvae were kept at constant and variable temperatures in the laboratory experiments there was clearly a negative relationship between the ambient temperature and larval survival. However the number of recovered larvae under natural conditions in the field followed a much more rapid decline in all seasons with highest recovery following contamination in spring.

The role of the L3 of trichostrongylid nematodes is to get ingested by a host and in many cases this requires an ability to survive long periods on pasture. While being ensheathed in the cuticle of the second stage and adapted to withstand a wide variety of adverse effects this stage lacks the ability to feed and has to rely on stored nutrients. For all organisms, including nematode larvae, the temperature has a strong effect on the metabolic rate (Gillooly et al., 2001). For example, with *C. punctata* larvae a positive relationship between the temperature and oxygen uptake as well as the depletion of stored nutrients has been observed (Eckert, 1967). As the L3 are unable to replenish this nutrient reservoir the larvae die once it is depleted. In the present study this trend was apparent in the short survival times at higher constant temperatures compared to the prolonged survival for nearly two years at 8°C. The median survival times with *C. oncophora* in the present study are generally lower than those previously reported (Boag and Thomas, 1985; Rossanigo and Gruner, 1996). Compared to the results from Boag and Thomas this difference became especially notable at higher temperatures where the survival time in the current study was less than 50% compared to the former report, predicted 118 days at 24°C to 295 days at 25°C respectively. A variation in methodology may partly explain these differences such as the age of the larvae at the beginning of the experiment. In the present study the water the larvae were kept in was not exchanged which may have caused additional stress for the larvae by accumulation of contaminants whereas it was partly exchanged regularly in the previous study. A comparison of the two studies with *C. oncophora* shows a steeper slope for the current study compared to that reported by Boag and

Thomas (1985) suggesting the *C. oncophora* isolate from New Zealand survives less well at high temperatures but is similar at low temperatures. At high temperatures around 30°C there is little difference between trichostrongylids but at lower temperatures the survival times from both studies are still indicating *C. oncophora* survives for shorter periods than *Teladorsagia circumcincta* or *Ostertagia ostertagi* but longer than *C. curticei* or *Haemonchus contortus*.

The survival times until the number of larvae fell below 95, 50 and 5% (Figure 3.2 and Table 3.1) at different constant temperatures follow an exponential relationship and the high coefficient of determination of the derived equations indicate that these had a high prediction value for the temperature range studied. However, at the lowest studied temperature of 8°C the experimental results were all lower than the respective regression lines especially for 50 and 5% survival. Ahluwalia (1970) reported an optimal temperature of 10°C for *C. curticei* larval survival with a potentially rapid decline with lower temperatures. In the present study it was not possible to determine what the optimal temperature would be as survival at 8°C was slightly longer than for 16°C. Further studies with larvae kept at closer temperature intervals would be required to determine if there was a similar rapid decline with lower temperatures as reported for *C. curticei*.

Few previous studies would appear to have shown an effect of the host on development and survival of free-living stages. In the present study larvae from different host animals showed significant differences in larvae survival as summarized in Table 3.2. A similar trend was observed for larval development success of *Teladorsagia circumcincta* in sheep (Jørgensen et al., 1998), where the age of the host animal had a significant effect on the development of the larvae. In that report it was suggested that the maturing immune response in the older animals was responsible for reducing development success. If such a reduced developmental rate influenced the accumulation of nutrients in larvae this may also have an effect on survival as well. The host animals in the present study were of comparable age but as no measure was made of their immune response it is not possible to determine if this may be an explanation for the difference between animals. How the host immune response might have influenced larval survival in this study is unclear but is worthy of further

investigation. Another factor which may influence lifespan is the composition of the faeces, but the culturing technique aimed to achieve similar water content for faeces from all animals, which should have negated this influence at least.

Larval survival showed the same sigmoid trend when kept at variable temperatures as observed with survival at constant temperatures (Figure 3.4). However, when compared to the predicted results from the regression equations using the mean overall temperature, the mean weekly temperatures or mean hourly temperatures, the number of observed surviving larvae declined much more rapidly. Clearly estimating expected survival rates using such a simple approach as average temperature is inappropriate. A possible explanation would be that the changing temperatures over the 24 hour period resulted in an increased metabolic rate. Eckert (1967) reported an increased oxygen uptake of infective larvae following a rise in temperature which was higher than required. The uptake then reduces after a short period to that normally expected at this temperature. Thus under varying temperatures such larvae will constantly have a higher metabolic rate and hence use nutrients at a faster rate. The expected effect of this would be for observed survival times with variable temperatures to be lower than that predicted for set temperatures. The relationship between survival rate and temperature is not linear and thus a higher temperature has a much greater impact compared to a lower temperature. However this is not accounted for when using average temperatures where a temperature above the average gets the same impact as a temperature of the same extent below the average.

Other studies on the survival of *C. oncophora* in the field have followed deposition of faeces containing eggs rather than just infective larvae as in the present study. These generally showed a decline that was not as rapid as in the present study but in these studies there was the opportunity for continued recruitment from the faecal pat (Rose, 1963; Smith et al., 1969). It was expected that the proportion of recovered larvae would be low as larvae would move into the soil and/or be killed by various organisms in the natural environment. Different fungi are known to trap nematodes and different studies have been conducted to investigate this influence (Grønvold et al., 1999; Waghorn et al., 2003). Nevertheless, the extent of the rapid decline of recovered

larvae in the field survival study was unexpected although a relatively fast decline for *C. oncophora* larvae on the herbage has been described before in the literature. On first principle it was expected that survival would be longer in winter and shorter in summer with spring and autumn being intermediate. In general this was the trend that was observed and a comparison of recovered larvae between seasons showed significant differences between opposite seasons. However, short term conditions such as the heavy rain after the summer contamination may also have influenced these results by washing larvae off the herbage even though plots were replicated on each occasion. In New Zealand different studies have demonstrated high numbers of larvae in soil and considered this to be a substantial reservoir for infective larvae (Leathwick et al., 2011; Waghorn et al., 2011). However no soil samples were taken in the present study to look for accumulated larvae. It is likely that most larvae would have moved downwards into the soil during the experiment which would mean that the number of larvae would actually represent disappearance rather than survival of larvae.

3.6 Conclusion

In the present study an increase of the ambient temperature significantly reduced the survival times of the third stage larvae. When comparing the median survival of third stage larvae sourced from different host animals a significant difference could be observed. A more thorough investigation of the cause of this effect could potentially facilitate different parasite control strategies or influence breeding selection.

The simplistic approach to calculate larval survival did not explain the observed results and clearly indicated a more sophisticated modelling approach is required.

3.7 Acknowledgements

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DRC 16

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Christian W. Sauermann

Name/Title of Principal Supervisor: Prof. W. E. Pomroy

Name of Published Research Output and full reference:

Sauermann, C.W., Scott, I., Leathwick, D. M., Pomroy, W. E., due for submission, The effect of temperature on the survival of Cooperia oncophora third stage larvae, Veterinary Parasitology

In which Chapter is the Published Work: Chapter 3

Please indicate either:

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  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Experimental design and planning in collaboration with supervisors, carry out experimental work and statistical analysis, preparing first draft of publication.

Candidate's Signature — 22 Jan 2014 — Date

Principal Supervisor's signature — 22 Jan 2014 — Date

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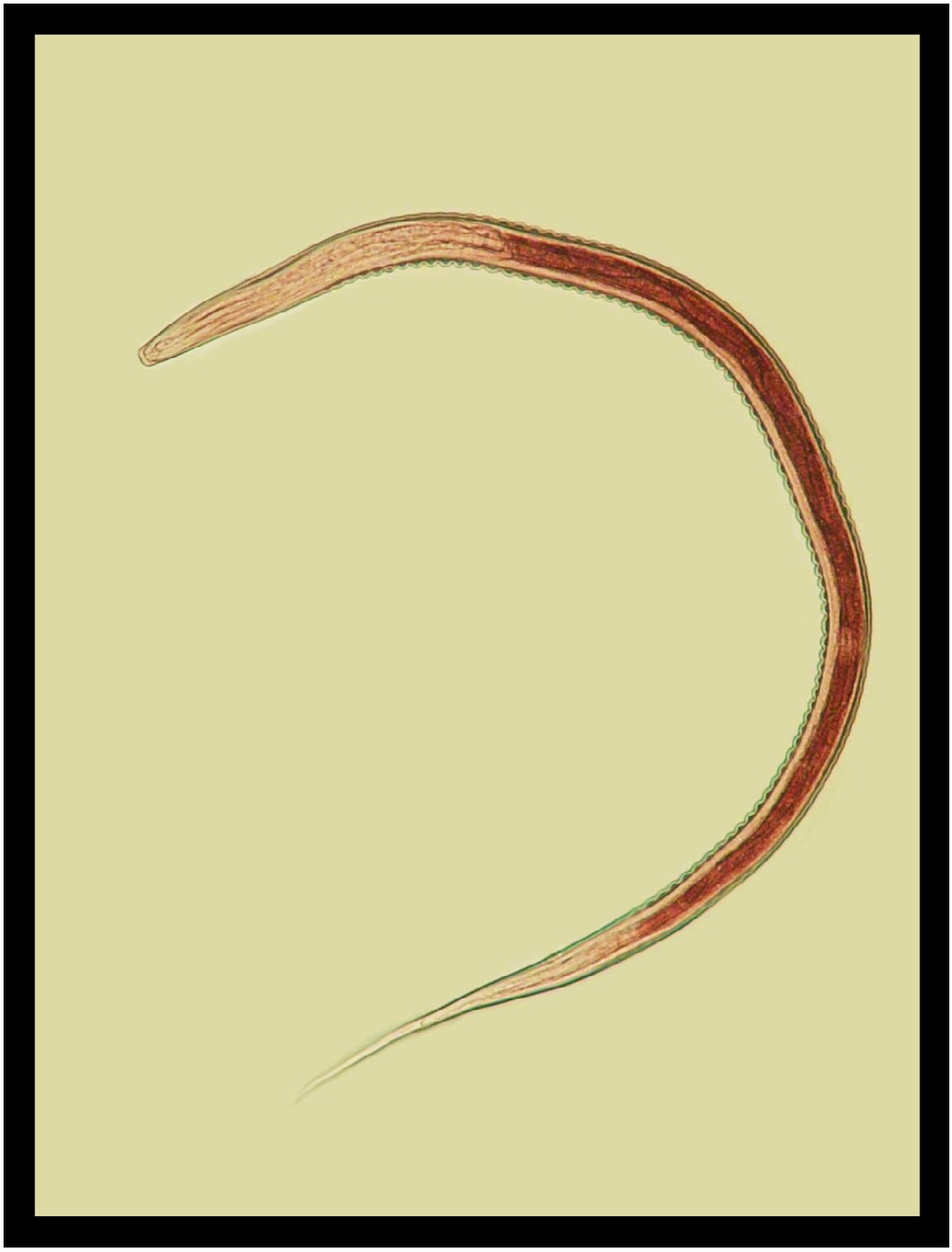

Plate 2 – Fully developed *Cooperia oncophora* third stage larvae ensheathed in the cuticle of the second stage. The sheath is visible as an uneven/corrugated skin and extends the larvae at the posterior end. The gut cells (16) which are used to store nutrients are clearly recognisable by their dark brown colour.

This Chapter will be submitted to Veterinary Parasitology with the following headings and authors

Modelling the development and ageing of *Cooperia oncophora* third stage larvae

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Chapter 4

Modelling the development and ageing of *Cooperia oncophora* third stage larvae

4.1 Abstract

To construct a model for the effect of temperature on the development and ageing of *Cooperia oncophora* third stage larvae (L3),data from previous experiments was used to estimate the hourly development and survival rates during egg to L3 and the hourly rate of L3 ageing in relation to temperature. These parameters were used in a model previously constructed for different nematode species and the model outputs were compared to results gathered in field and laboratory experiments. Further development and ageing with 12 hourly oscillating temperatures around constant temperatures of 14, 20 and 26°C were simulated using the model. Temperature data for a 10 year period from 2001 to 2011 was used to simulate the monthly L3 development for three different geographical locations in New Zealand. The variable temperatures encountered in the field and laboratory experiments resulted in a lower L3 development success and L3 ageing by the model simulations. An increase in the magnitude of oscillating temperatures always resulted in an increase of the L3 development rate and success. Using monthly climate data in the model indicated an increase in development during the summer compared to winter months but also an increase when moving to a warmer geographic location. The model provided a useful tool to gain a better understanding of the temperature effect on the development and ageing of *C. oncophora* L3. The results showed more experimental data is needed to sufficiently derive the parameters for the models especially at the lower and higher temperature limits.

4.2 Introduction

The widespread occurrence of anthelmintic resistance in *Cooperia oncophora* in New Zealand (Waghorn et al., 2006a) has resulted in different studies to investigate the population dynamics of this parasite (Chapters 2 & 3). Under New Zealand conditions temperature has been shown to be the major predictor for the development of different trichostrongylid larvae on the pasture (Reynecke et al., 2011). The results of these studies made it desirable to develop a model for the development and lifespan of the third stage larvae to gain a better understanding of the effect of temperature on this lifecycle phase. The reasons for modelling parasite population dynamics are different. Some are used as predictive models to forecast what happens in the field while others are created to gain a better understanding of the details of the parasite's biology (Smith, 2011). For a variety of parasitic nematodes the effect of temperature has been successfully modelled using the Escalator Boxcar Train technique (Leathwick, 2013a; Leathwick et al., unpublished). The aim of this study was to utilise these models in order to gain a better understanding of the effect of temperature on the development and lifespan of the third stage larvae of *C. oncophora.*

4.3 Materials and Methods

4.3.1 Egg to L3 Development Model (DM)

Data from the study on "Larval development under constant temperatures in the laboratory" (Section 2.3.1.1) was used to calculate the parameters required to model the development of *C. oncophora*. These parameters were then fitted to a model formerly constructed by Leathwick (2013a) for other parasitic nematodes. This model had been implemented in Excel (v14, Microsoft Corp., WA, USA) using the escalator boxcar train (EBT) method (de Roos, 1988) for hourly temperature data. The EBT method uses a queue ("train") of stages ("boxes") the individuals have to pass through ("escalator"). While in the EBT the individuals in the stages are subject to factors that define properties prior to progression to the next stage. When the eggs passed through the EBT they were assumed to have fully developed to the L3 stage. The model calculates the development from the egg to L3 stage as a continuous process

and therefore no statement can be made for the numbers of eggs developing and hatching to L1 or L1 moulting to L2 during development to L3 from this model.

In this study each EBT stage was subject to a development rate (d_t) which defines progression to the next stage and a survival rate (s_t) to account for mortality throughout the development from eggs to L3 (see Appendix 4, Section 9.4.1 for EBT schematics).

4.3.1.1 Development rate (d_t)

To estimate d_t the time in hours required for 50% of the maximum number of L3 to develop for each host and constant temperature was identified (DT50, Section 2.3.1.1). The reciprocals of these hours were then logit transformed and regressed against temperature using a linear model. To determine d_t the resulting regression was back transformed to hours as shown in Equation (1). As d_t was calculated for the development from egg to L3 as one step it also had to be adjusted for the 12 stages used in the EBT.

$$d_t = 12/(1/(\exp(a + bt))/(1 + \exp(a + bt))) \quad (1)$$

where d_t = hourly development rate of egg to L3 at temperature t

a and b = coefficients from the regression model

12 = number of stages in the L3 development EBT

4.3.1.2 Survival rate during development (s_t)

To estimate this rate, data from studies of development under constant temperatures were used. The rate was calculated for each host and constant temperature by rising the proportion off eggs to the power of the reciprocal number of hours required to reach the maximum number of L3. For example, at 20°C the proportion was 0.22 and

the time to maximum L3 was 600 hours so the survival rate per hour at this temperature equals: $s_t = p^{1/h} = 0.22^{1/600} \approx 0.997$ The results of s_t were then regressed against temperature with a quadratic model as described by Leathwick (2013a) and in Equation (2).

$$S_t = a + bt + ct^2 \quad (2)$$

where s_t = hourly survival rate during development to L3 at temperature *t*

a, b and c = coefficients of the regression model

Whereas the development rate (d_t) defines the speed the individuals move from one stage in the Development Model (DM) to the next and complete their development to L3, the survival rate (s_t) defines the developmental success which is the proportion of individuals successfully progressing from one stage in the DM to the next.

4.3.2 L3 Ageing Model (AM, ageing rate a_t)

Data from Experiment 1 in Chapter 3 ("Survival at constant temperatures", Section 3.3.1) was used to calculate the parameters required to model the lifespan of *C. oncophora* third stage larvae using the same approach as above (Section 4.3.1). In this context ageing refers to the fully developed L3 continuing to deplete stored nutrients which eventually leads to their death. As for the development of L3 (Section 4.3.1) the model was formerly constructed in Excel (v14, Microsoft Corp., WA, USA) using the EBT method by Leathwick et al. (unpublished). In this model each stage was subject to an ageing rate (a_t) which defined progression to the next stage. To estimate this rate the number of stages in the EBT was divided by the median L3 survival in hours for each host and constant temperature (LT50, Section 3.3.1) and then transformed by

calculating the negative reciprocal fourth root ($-1/[x^{-¼}]$). This transformation process enabled the fitting of a linear regression to the data. The resulting regression was back transformed and applied to the EBT as shown in Equation (3).

$$a_t = (-1/(a + bt))^4 \quad (3)$$

where a_t = hourly ageing rate of L3 at temperature *t*

a and b = coefficients from the regression model

A population of L3s is subject to a_t as a progression factor to determine how many progress from one model stage to the next model stage in the EBT. Moving through the EBT of the Ageing Model (AM) indicates progression towards death. Although the equation for a_t could be >1 above 43 °C for the purposes of this model it was set to a maximum value of 1 above this temperature. The higher a_t becomes with higher temperatures and approaches its maximum value of 1, the faster the individuals would pass through the EBT and thus be assumed to be dead and subtracted from the initial numbers of L3.

The two models DM and AM were also joined to a Combined Model in which the developed L3 from the DM were transferred as new recruits into the AM. In this way each L3 was immediately subject to ageing after its development.

4.3.3 Evaluating the model with experimental results

Results from field and laboratory experiments with variable temperatures were compared to the outcomes of the DM and AM. The models were run on the overall mean, daily maximum mean and hourly temperatures monitored or used during these experiments.

4.3.4 Application of the model

4.3.4.1 Monthly L3 development and ageing over a 10 year period

The monthly development from egg to L3 and L3 ageing was estimated using the DM and AM. Hourly herbage temperature data for January 2002 to December 2011 was obtained from the New Zealand National Climate Database for Warkworth (National Climate Database; Station 17838), Palmerston North (Station 21936) and Lincoln (Station 17603). The temperature data contained gaps of 3-24 hours which were ramped according to the last measurement before, to the first measurement after, the gap. The data for Palmerston North in October 2003 and Warkworth in 2010 had gaps > 24 hours and these were excluded from the calculations. From this temperature data 30 day blocks were taken from each location beginning on the first day of each month to create sets of temperature data for the model which were of similar length. For each of these 30 day blocks the proportion of eggs successfully developing to L3 was estimated using the DM. The AM was applied to this same dataset to estimate L3 ageing.

4.3.4.2 L3 development and lifespan with variable temperatures

The effect of variable temperature on L3 development and lifespan was estimated using 12 hourly rotations of 2 constant temperatures equally distributed around a mean temperature of 14, 20 and 26 °C using increments of ±0, 2, 4 and 6°C from this mean temperature. To estimate the proportion of eggs which successfully developed to L3 the respective model was used for each of the temperature pairs. Likewise the AM and the combined model were used for an estimation of L3 survival using the same temperature variations.

4.3.5 Statistical Methods

The calculations of the coefficient for the development rate (d_t) and survival rate (s_t) during development from egg to L3 as well as the ageing rate (a_t) for the L3 experimental results were transformed according to the rate (see model descriptions above) and regressed against temperature using Minitab (v16, Minitab Inc., USA).

4.4 Results

4.4.1 Model parameters

The parameters estimated with the regressions fitted to the experimental results on development and lifespan of *C. oncophora* L3 at constant temperatures are given in Table 4.1 and were used in Equations 1 to 3 respectively. The R^2 values for d_t and a_t were >93% whilst the R^2 for s_t was lower at 57%.

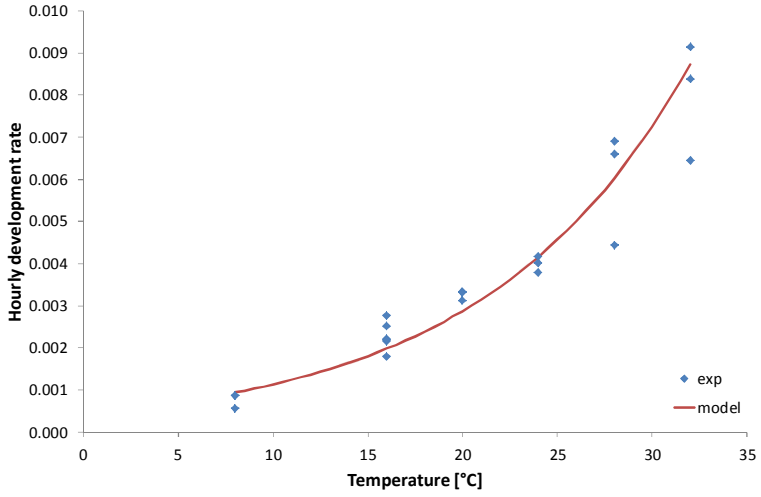
Table 4.1 - Parameters for L3 development (DM) and ageing (AM) models derived from regressions fitted to experimental data on egg to L3 development and L3 lifespan of *Cooperia oncophora* at constant temperatures.

		Model parameter			
		a	b	c	R^2
DM	Development rate (d_t)	-7.7152	0.09323		93.2%
	Survival rate (s_t)	0.99758	0.0000113	-0.000008	57.0%
AM	Ageing rate (a_t)	-7.02	0.1399		94.2%

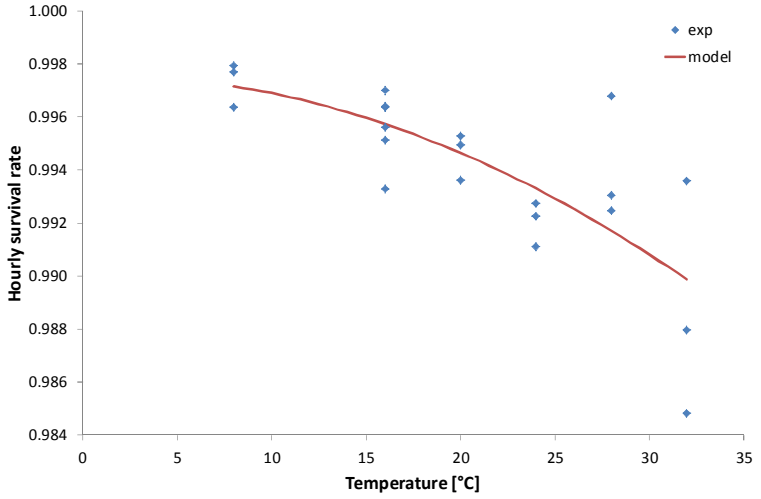
The estimations for d_t, and s_t used in the DM indicate that with rising temperature d_t increased compared to a decrease of s_t (Figure 4.1 a, b). For the AM the estimations of a_t increased with rising temperature and a_t exceeded 1 above 43°C (Figure 4.1 c). In the case that the temperature used during calculations was > 43°C the value for a_t was set to 1. This causes every individual in the model to be shifted to the next stage, causing a rapid progression. Thus over a 12 hour period, to account for all 12 stages in the EBT, all L3 will have died in terms of this model.

4.4.2 Evaluating the DM and AM

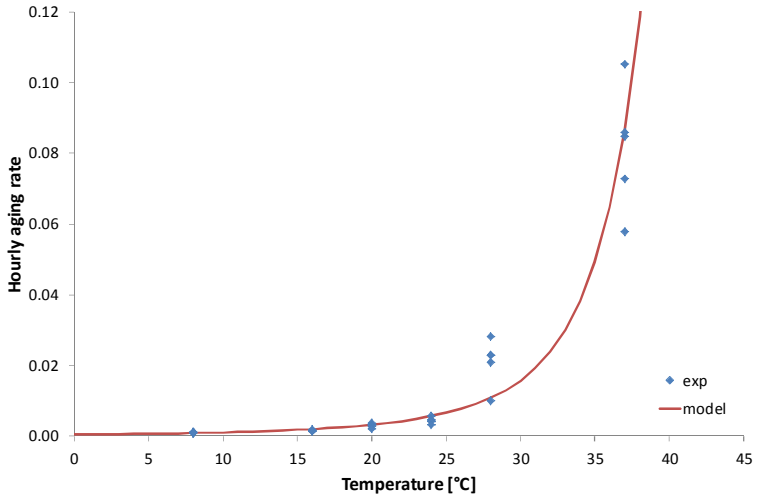
The best fit of the DM for development success using results for larval development with variable temperatures was accomplished using maximum daily temperatures (Figure 4.2 a). The utilisation of maximum daily temperatures in the DM predicted a developmental success of 13% which is similar to the experimentally observed mean developmental success of 14-15%. Model calculations using either the overall mean or hourly temperatures resulted in a developmental success of 8% and 10% respectively (Figure 4.2 a). Equally, calculations with the AM follow the experimental results more



(a)

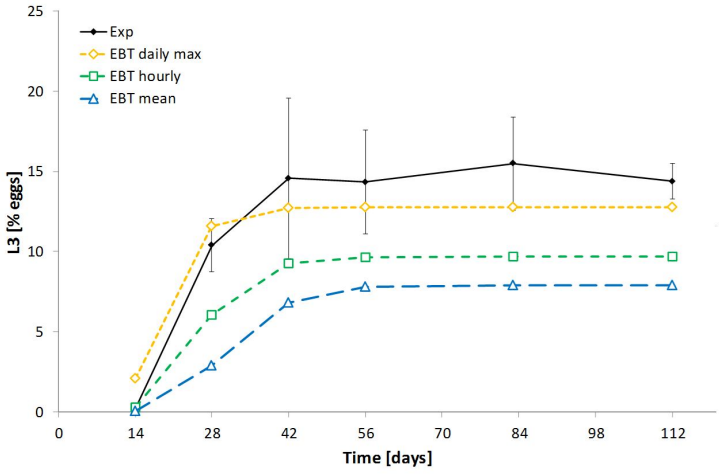


(b)

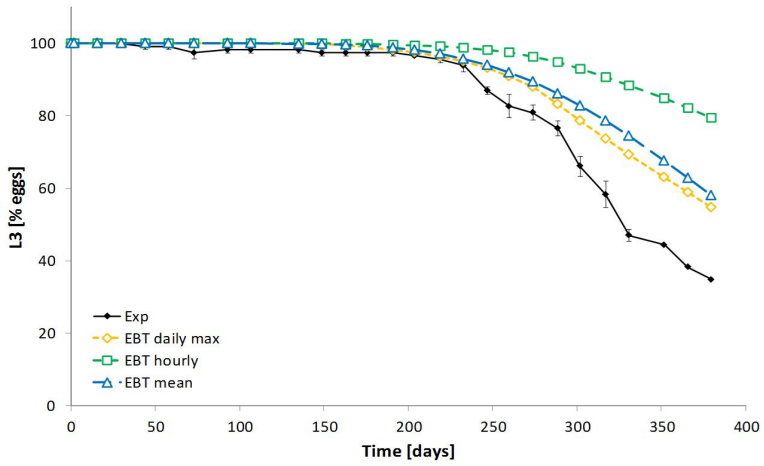


(c)

Figure 4.1 a, b and c – A comparison of estimated hourly development (a) and survival (b) rates during egg to L3 development and L3 ageing rate (c) of *Cooperia oncophora* with experimental results (exp) at constant temperatures (Chapter 2 and 3).



(a)



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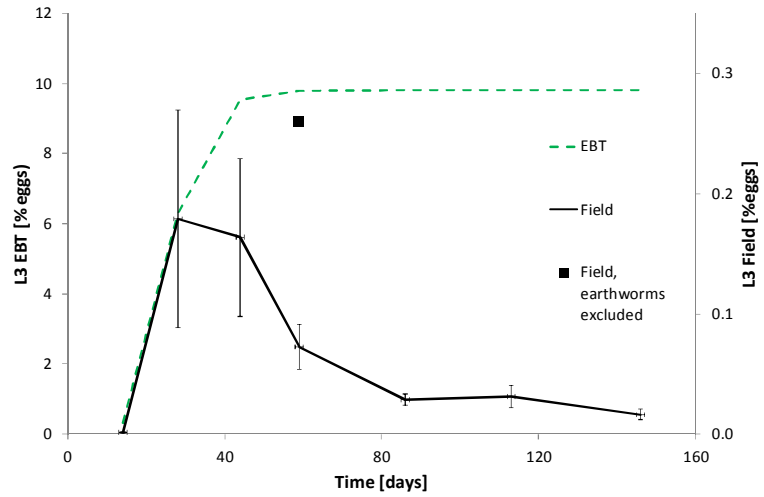


(c)

Figure 4.2 a, b and c – A comparison of model estimates (EBT) with experimental data (Exp, ±SEM) for *Cooperia oncophora* egg to L3 development (a), L3 ageing (b) and development in the field (c). For (a) and (b) different temperature settings have been used in the model: daily max = daily maximum temperatures; hourly = hourly temperatures; mean = overall mean temperature.

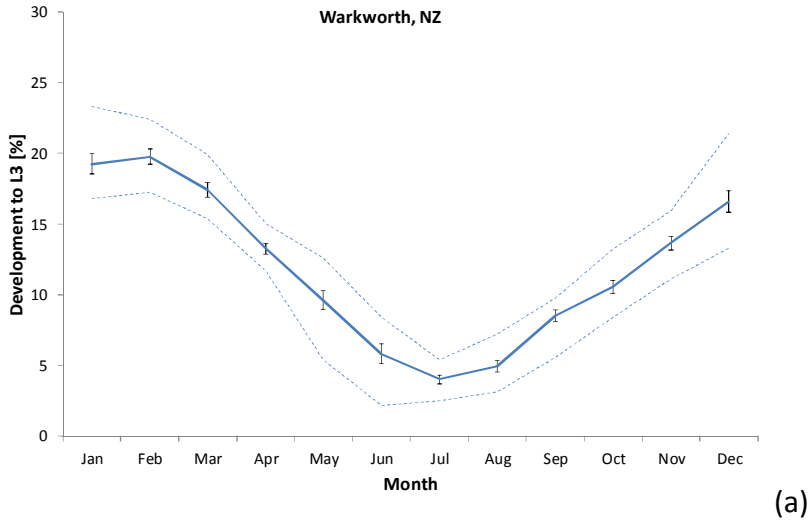
closely when using the maximum daily temperature compared to either the overall mean or hourly temperatures (Figure 4.2 b). When comparing DM results to development of L3 in the field there was a similar initial development rate but a dramatic reduction in the overall development success (Figure 4.2 c). The source of this dramatic reduction in terms of the model is unclear. There was no difference whether the comparison was with daily maximum, overall mean or hourly temperatures.

4.4.3 Monthly L3 development and ageing over a 10 year period

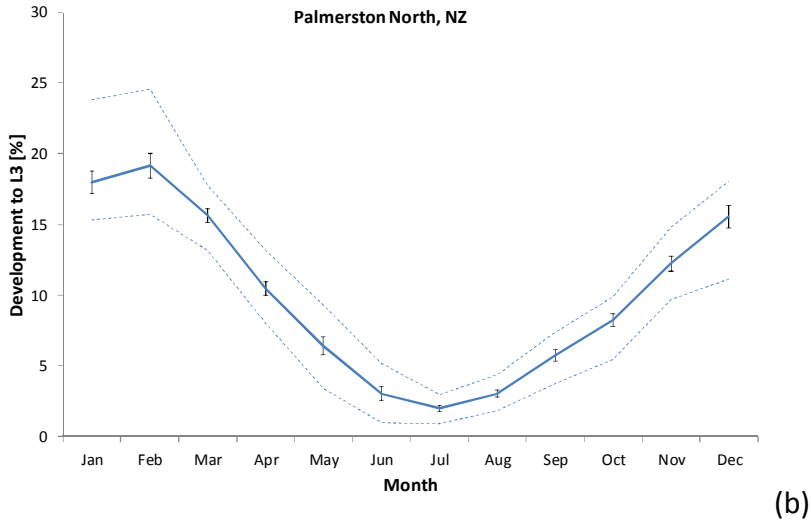
The results of the DM using 10 years of temperature data showed a comparable yearly trend (Figure 4.3). The highest/average/lowest temperatures during the 10 year period, were 44.6/13.8/-6.3°C for Warkworth, 39.7/12.2/-7.6°C for Palmerston North and 40.5/10.6/-9.1°C for Lincoln, respectively. The results indicate a consistent trend over a yearly cycle with high development during the summer and low during the winter. Model estimates indicated the highest average development success of 19.77% for Warkworth in February, 19.15% for Palmerston North in February and 17.85% for Lincoln in January. The estimated results from the AM followed a pattern of more rapid ageing during summer and slower ageing during winter. Neverthess, the average number of L3 alive during the summer months of January and February indicated that more than 99% of the larvae were still alive after 30 days for all 3 locations. In comparison the average number of L3 still alive after 30 days was 100% during April to October for Palmerston North and Lincoln whereas estimates for Warkworth showed 100% L3 survival for only May to September.

4.4.4 Egg to L3 development and L3 lifespan with variable temperatures

When using temperatures oscillating around a constant value in the DM the results showed an increase in the development rate and success with greater increments for all temperatures (Figure 4.4 a). For example, the time to reach 99.9% of maximum development at a constant temperature of 20°C was 3.8 weeks (17.6% development)



(a)



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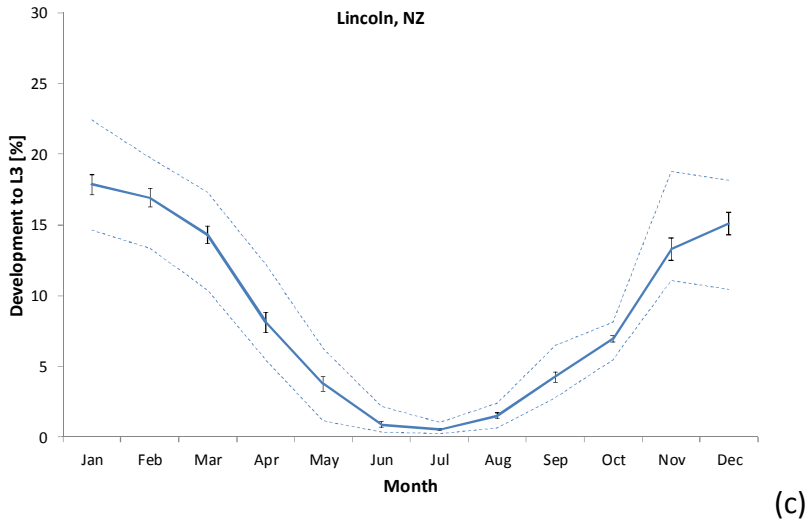


(c)

Figure 4.3 a, b and c - Development model estimates of mean development success (±SEM, minimum and maximum dashed lines) for *Cooperia oncophora* eggs to infective stage larvae over a 30 day period using temperature data from January 2002 to December 2011 for Warkworth (a), Palmerston North (b) and Lincoln (c).

compared to 3.3 weeks (20.3% development) with a mean temperature of 20°C but alternating between 14 and 26°C.

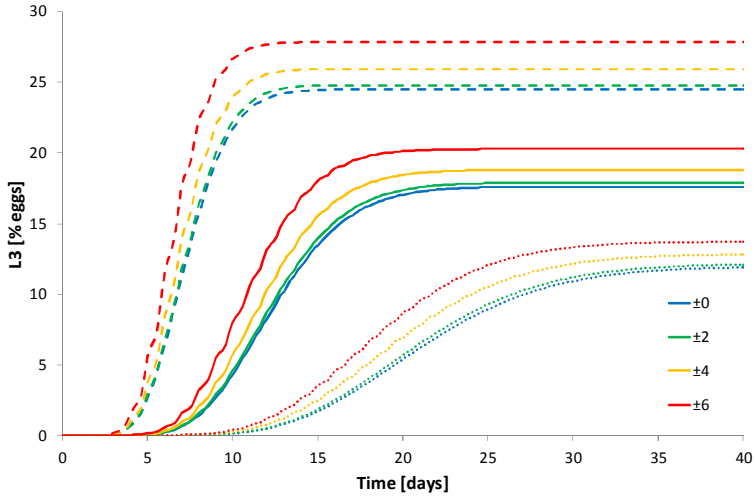
In contrast, the result from the AM using the same settings showed a rapid decrease of lifespan when the L3 were exposed to a higher range of temperatures (Figure 4.4 b). At a constant temperature of 20°C the median survival was between 23-24 weeks but was below 15 weeks when using a mean temperature of 20°C but alternating between 14 and 26°C.

Using the DM and AM in the combined model with the same temperature settings the maximum number of L3 occured with the largest temperature variation but the longest lifespan occurs at the set temperature. At a constant temperature of 20°C the number of L3 reached a maximum of 17.6% at 5.2 weeks with a medium survival at 22.9 weeks at 20°C (Figure 4.4 c). These compare to a maximum number of L3s of 20.3% at 4.2 weeks with a medium survival at 15 weeks when using alternating temperatures between 14 and 26° C.

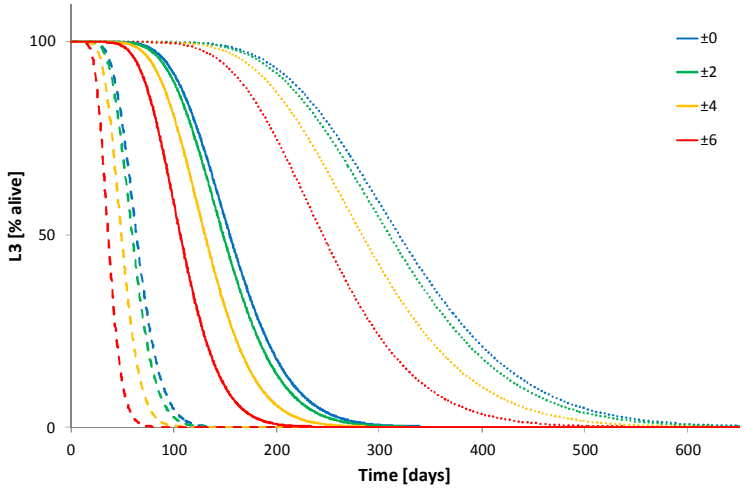
4.5 Discussion

In order to gain a better understanding of the effect of temperature on the development and lifespan of *C. oncophora* L3 two models were generated. Both models were constructed to allow for hourly changes in temperature and new recruitment of eggs or L3.

A variety of models have been used to estimate development and the lifespan of free-living stages of strongylid nematodes. Some models are species specific but most apply for a mix of species. None have been described specifically for *C. oncophora*. However, as discussed by Smith (2011), it has to be emphasised that the models in the current study were constructed to estimate larvae development and lifespan under near ideal laboratory conditions, so they do not account for effects found in the field other than temperature. The aim was not to give a tool for field prediction but to better understand the effect temperature has on the development and lifespan of *C. oncophora* infective larvae.



(a)



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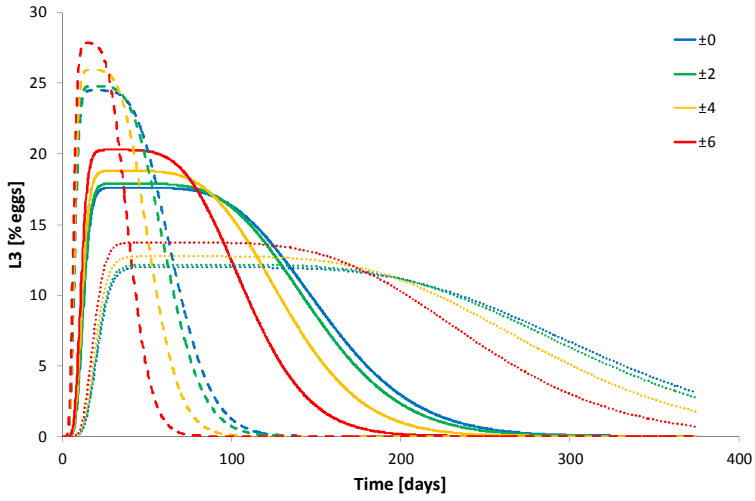

(c)

Figure 4.4 a, b and c - Model estimates for the development (a), lifespan (b) and a combination of both (c) for *Cooperia oncophora* L3 with oscillating temperatures. The temperature was changed on a 12 hourly basis around a mean of 14 (dotted), 20 (solid) and 26 (dashed) °C with fixed increments of ±0, 2, 4 and 6 °C.

The required parameters for d_t and s_t in the DM were calculated from data previously gathered during a development experiment under constant temperatures in the laboratory. Although each temperature was investigated using the same experimental procedure some variation in the results between temperatures was observed (Chapter 2) which resulted in a relatively low R^2 of 57% for the calculated regression to estimate the s_t but not for d_t (R^2=93.2%). However the estimates as displayed in Figure 4.1 (a) and (b) indicated that at temperatures above 32°C, the highest temperature experimental data was recorded (Chapter 2), the s_t may not be valid causing only a minor decrease with rising temperatures compared to a substantial increase in d_t. This would result in the model indicating a high development success as the individuals would rapidly progress through the EBT without being subject to an adequate mortality rate. Therefore additional data for a better prediction of d_t and s_t above 32°C is desirable especially towards the higher lethal temperature limit.

To model the development and survival of *C. oncophora* L3 the models for the development from egg to L3 and the lifespan of L3 were combined. The L3 emerging from the development model were transferred as new recruits into the lifespan model. This combined model used the same hourly temperatures for the development and the survival component. The combined model was built on the assumption that the process of developing and ageing could be described by this simple approach but no data was available to explicitly verify this. Nevertheless, the combined model made it possible to estimate the number of live L3 especially at higher temperatures where developed larvae were then subject to rapid ageing. Interestingly this approach indicated that for high temperatures some of the early developed L3 were already dying before the slower developing pre-infective stages finalized their development to L3, meaning the combined model gave lower maximum numbers of L3 at any time compared to the DM under these conditions.

Different temperature settings were used when comparing the estimates from the DM and AM to the experimental data. The use of hourly temperature values resulted in a comparable development rate for the DM with that observed in the laboratory experiment (Figure 4.2). However, the development success was lower compared to the experimental data when using hourly temperature values and in this case the use

of the weekly maximum temperature provided a better estimate. Similarly, in another study with *C. curticei* the use of the mean maximum temperature resulted in better estimates than the average mean temperature (Ahluwalia and Charleston, 1974). In the present study, as the DM calculates development rate on an hourly basis the use of unmodified temperature data should provide better estimates and this differential indicates that more experimental data is required to fit more realistic curves. Alternatively, variation in the experimental data may be caused by the source of the larvae. The individual host has been shown to have an direct effect on the development success of the L3 (Jørgensen, 2000). The relationship between the estimates from the AM model and the experimental data reveal a similar behaviour where the use of the weekly maximum mean temperature resulted in improved estimates for the L3 lifespan compared to use of hourly temperatures. For both the DM and AM the data is based on a single experiment using a temperature range between 8-32 °C and 8-37 °C respectively. The difficulty in consistently fitting these models suggests that more data was required to more effectively calculate the parameters.

Recovery of L3 in the field experiment was very low compared to laboratory conditions, which was likely to be due to a heavy loss of larvae into the soil. Nevertheless, in general the DM should give an indication of the development rate as the appearance and increase in L3 numbers in the field samples should show a similar pattern. The only factor the model takes into account is the temperature compared to the huge variety of factors the larvae are exposed to under natural conditions.

The results of the DM using 10 years of temperature data showed a comparable yearly trend for development over a 30 day period (Figure 4.3). The DM model indicates the highest development success of 17-22% for February in Warkworth whereas in winter the model indicates a low development success. The DM also predicted a lower development success with decreasing temperatures when geographically moving south with the other two stations that were compared. This seasonal pattern of high development during the summer and low during the winter is consistent with previous results from field experiments with a variety of different trichostrongylid nematodes in New Zealand (Vlassoff, 1973; Waghorn et al., 2011) and Argentina (Fiel et al., 2012).

The model calculations were also generally more variable during the spring where the variation in temperature was normally higher compared to winter, especially for Warkworth. However, the general humidity or rainfall is not accounted for, which is typically higher during winter and lower during summer. This has to be acknowledged as the faecal moisture content has a proven effect on the development of the infective stage larvae (Rossanigo and Gruner, 1995). The results from the AM are contrary to those of the DM as in summer the lifespan of the L3 is normally reduced by the higher temperatures and during the cooler winter period the model indicates a longer lifespan. The average estimated proportion of living larvae was > 98% for all 3 climate stations. However, the period for calculation was only 30 days and if extended is likely to show a difference reflecting climatic differences. As for development of larvae other environmental factors such as the influence of humidity, will have an effect on the ageing and survival of larvae.

Fluctuations of temperatures around a mean temperature will potentially affect development and ageing of larvae. In the present study, increasing fluctuations around the same mean temperature generally resulted in an increased L3 development rate and success in the DM but also a reduced lifespan of the L3 in the AM. The increase in the L3 development rate and success with increasing fluctuations implies that the increase in the period of higher temperature outweighs the decrease during the similar period of cooler temperature. In the case of the AM the effect of the fluctuations was the same, but the higher temperatures in the larger oscillating range increases the ageing rate, in other words the larvae deplete their stored nutrients faster by an increase in metabolic rate. When the models were combined the two effects accumulate and resulted in increased development rate and success as well as faster decline of the developed L3 by ageing with larger oscillations. In the present study the temperatures used were neither very high temperatures where L3 could rapidly die or very low temperatures where little development would occur. Nevertheless, this supports the observation from 3 different climate stations over a 10 year period where the Warkworth climate station had larger temperature fluctuations over summer than the other two stations resulting in development success and rate being greater during summer, but where there was also a faster decline of developed L3 compared to the

other locations. These results are in contrast to the findings from Leathwick (2013a) who refined these assumptions and indicated that fluctuating temperatures at high temperatures were detrimental to development and ageing. The difference with the present study is the range of temperatures considered, as such high temperatures were not included here.

4.6 Conclusion

The DM and AM generally provide a good estimate for the development and lifespan of *C. oncophora* L3 and provide a useful tool to better understand the relationship between the ambient temperature and these life traits. However, a larger dataset for the calculation of the model parameters, especially for lower and higher temperatures, would better account for adverse temperature ranges and would have been preferable. The EBT modelling technique proved to be relatively straightforward and useful for this purpose. This approach allows individual components to be added (or removed) compared to some other modelling approaches based on field data where influences of separate factors are usually confounded. Additional components that could be added to the EBT include humidity, effects of freezing temperatures, infectivity of larvae as they age, herbage type and possibly soil type. Future work with this model should be to progressively include these additional components.

4.7 Acknowledgements

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DRC 16

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Christian W. Sauermann

Name/Title of Principal Supervisor: Prof. W. E. Pomroy

Name of Published Research Output and full reference:

Sauermann, C.W., Scott, I., Pomroy, W. E., Leathwick, D. M., due for submission, Modelling the development and aging of Cooperia oncophora third stage larvae, Veterinary Parasitology

In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 75%
  and / or
- Describe the contribution that the candidate has made to the Published Work:
  Parameterize the model, conduct model simulation, preparing first draft of publication.

Candidate's Signature — 22 Jan 2014 (Date)

Principal Supervisor's signature — 22 Jan 2014 (Date)

GRS Version 3– 16 September 2011

Chapter 5

Efficacy of ivermectin on two isolates of *Cooperia oncophora*

5.1 Abstract

To determine the efficacy of ivermectin on two isolates of *Cooperia oncophora*. A suspected resistant isolate was obtained from donor cattle which were naturally grazed and treated with ivermectin on a fortnightly schedule. The second isolate was identified as susceptible to ivermectin by a faecal egg count reduction test in 2005. For each isolate 12 calves were orally inoculated with 5,000 infective stage larvae on day 0, 1 and 4. On day 21 the animals were divided into control and treatment groups based on faecal egg counts. The animals in the treatment groups received the recommended dose of ivermectin (0.2 mg/kg). On days 27 and 28 all animals were euthanized and the small intestines taken for total worm counts. The efficacy of ivermectin based on arithmetic means of the worm counts was 10.5% for the resistant and 93.4% for the susceptible isolate. The experiment indicated that even though one isolate had originally been identified as susceptible to ivermectin based on faecal egg counts the efficacy was below 95% based on worm counts.

5.2 Introduction

Because of economic benefits anthelmintic drugs are regularly used to control parasitic worms in the farming industry. One active component developed during the 1970s was ivermectin (IVM) which belongs to the chemical group of avermectins, a fermentation product of the actinomycete *Streptomyces avermitilis* (Burg et al., 1979). Avermectins together with milbemycins form the group of macrocyclic lactones and all share the same mode of action. By binding to and opening the glutamate-activated chloride channels they increase the membrane permeability of nerve and muscle cells in nematodes and arthropods (Jagannathan et al., 1999). Nematodes were shown to have considerable variation in their susceptibility towards avermectins based on the amount of identified channel subunits (Campbell, 1985; Cully et al., 1994; Njue et al.,

2004; Njue and Prichard, 2004). *Cooperia oncophora* was identified as a dose limiting species for ivermectin before becoming commercially available (Egerton et al., 1979). In New Zealand farmers are dependent on anthelmintic use. A recent survey of beef cattle farms indicated a widespread ivermectin resistance problem in *Cooperia* (Waghorn et al., 2006a). It is therefore important to gain a better understanding of the population dynamics of this parasite to enable different control strategies to be developed. In the first chapters the development and the survival of the free-living third stage larvae have been investigated.

The aim of this study was to determine the efficacy of ivermectin against the two *C. oncophora* isolates, one suspected to be ivermectin-resistant and the other ivermectin-susceptible. These isolates were required for the study described in Chapter 6.

5.3 Materials and Methods

5.3.1 Cooperia oncophora isolates

Two different *C. oncophora* isolates were examined. The first was suspected to be resistant (RES) and the second susceptible (SUS) to ivermectin. The RES isolate was also the source of larvae for the study described in Chapters 2 and 3. The same protocol for the production of L3 was continued. In brief, to isolate the RES larvae 5 donor calves grazing pasture were, on a weekly basis, alternatively either drenched orally with ivermectin (0.2 mg/kg, IVOMEC® liquid for sheep and goats, Merial New Zealand Ltd) or superinfected with 15,000 infective stage larvae of their own isolate. This isolate had been exposed to this protocol for 20 months prior to the start of this particular experiment although the individual donor calves had been changed twice during this period. Between 7 to 12 days post drench, individual faecal samples were collected and cultured as described in Appendix 1 (SOP3). In brief faeces were aerated by mixing with vermiculite, cultured in trays at 25-27°C for 14 days and larvae recovered using modified Whitehead trays (Whitehead and Hemming, 1965), cleaned by repeated sedimentation and stored at 8°C until required. Larvae were identified morphologically as *C. oncophora* by examining at least 100 larvae from each separate culture.

The SUS larvae were originally obtained from a farm identified in a survey of North Island beef cattle (Waghorn et al., 2006a) where they were considered to be ivermectin susceptible although the original isolate contained 6% *Ostertagia* spp. They were subsequently passaged in two parasite-free and housed donor calves by AgResearch and then donated for this study. From 21 days after infection faeces were collected and cultured as for the resistant isolate. A species identification based on 300 SUS L3 indicated a contamination of 4% *Ostertagia* spp at this time.

5.3.2 Experimental animals

The experiment on testing the efficacy of ivermectin against the two *C. oncophora* isolates generally followed the WAAVP guidelines (Wood et al., 1995) and VICH guidelines (Vercruysse et al., 2001). On Day -14, eighteen 4-month-old Holstein-cross heifers were orally treated with a triple combination anthelmintic, delivering a dose of 0.2 mg/kg Abamectin, 4.54 mg/kg Oxfendazole and 8 mg/kg Levamisole (Matrix®-C, Merial Ancare NZ). They were housed and fed on commercial calf pellets and baleage made of Italian rye grass pasture that had never been grazed by livestock. They were randomized into two groups, one with 12 animals to test the IVM efficacy against the RES isolate and one with 6 animals to test the IVM efficacy against the SUS isolate. For the SUS isolate, the reduction in faecal egg count was 98.5% (McMurtry, pers. comm.) after treatment with IVM and it was determined to initially compare efficacy with only 3 animals per group. However the results from this initial study indicated it was necessary to repeat the efficacy study with a further 3 animals for each of the SUS treatment and control groups to give a total of 6 animals per group, as for the RES isolate. These additional 6 animals were bull calves but the same breed as the originals and at the same age at the start of the experiment.

5.3.3 Experimental Design

On Day 0 a FEC was performed to confirm the anthelmintic treatment when housed was effective for the original 18 animals. These animals were then orally inoculated three times with 5,000 infective stage larvae of the RES or SUS isolate on Day 0, 1 and

4 according to their group (Appendix 1 SOP13). Prior to ivermectin treatment on Day 21 animals were ranked by FEC within their group, allocated into successive pairs and then individuals within each pair were randomized into control and treatment groups. The animals in the treatment groups were then orally treated with the recommended dose of ivermectin (0.2mg/kg). Half the animals from each group were euthanized on Day 27 and the other half on Day 28. The small intestine was collected and frozen at -20°C for at least one week. After thawing the intestine was opened, washed and all contents gathered. Worm counts were undertaken as described in Appendix 1 (SOP 14, 15 and 16). In brief a 10% aliquot was sieved through a 38µm mesh with all worms in the retentate being counted. A second reserve 10% aliquot was preserved in 10% formalin. The experiment was repeated with another 6 animals using the same experimental design for further investigating the efficacy of ivermectin on the SUS isolate.

For each animal 100 male worms or the total number recovered if less than 100 were identified to species level.

5.3.4 Statistical analysis

The data for the SUS isolate from the two experiments was pooled following the VICH guidelines (Vercruysse et al., 2001). The results for the RES and SUS isolates were analysed by comparing the control groups with the corresponding treatment groups using the Kruskal-Wallis test with the software package R (R Core Team, 2012). The efficacy was calculated by comparing the arithmetic means of the control and treatment groups within the isolates. Geometric means have been calculated but were not taken into account further as this can provide biased efficacy results (Dobson et al., 2009).

5.4 Results

The egg counts prior to ivermectin treatment on Day 21 indicated that six animals infected with the RES isolate had a positive faecal egg count between 25 and 175

eggs/g. Only two animals infected with the SUS isolate had a positive faecal egg count of 25 eggs/g (see Appendix 5, Section 9.5.4). The worm count data for both isolates are summarized in Table 5.1. The absence of worms in one animal from the SUS control group and the high worm burden from another animal in the SUS treatment group made it necessary to repeat the experiment with another three animals in each of the two groups to produce a more meaningful interpretation of this isolate's resistance status.

Table 5.1 - Worm count data for determining the efficacy of ivermectin of two *Cooperia oncophora* isolates, one expected to be susceptible (SUS) and the other resistant (RES). For each isolate 12 calves were inoculated with 15,000 infective larvae over 3 days and divided into treatment or control group after 28 days when the treatment group animals were orally treated with ivermectin (0.2mg/kg). After a further 7 days animals were slaughtered. For the SUS isolate, data was pooled over two separate experiments comprising 6 animals in each. Efficacy is calculated for arithmetic mean. ‡animals in second experiment

	Cooperia oncophora isolate	
Group	resistant	susceptible
control	7270	12270
	5900	0
	5390	2670
	90	8990‡
	110	8750‡
	6390	350‡
mean (±SEM)	4192 (±1318)	5505 (±2108)
treatment	7030	20
	7640	1510
	6240	330
	160	210‡
	1440	20‡
	10	90‡
mean (±SEM)	3753 (±1464)	363 (±235)
efficacy	10.5%	93.4%
p-value	0.873	0.078

For both the RES and SUS infected groups identification of recovered male worms indicated on average 85% *C. oncophora* and 15% *Cooperia surnabada* (*mcmasteri*)

which is identical to the ratio reported for the United States previously (Burtner and Becklund, 1971). *C. surnabada* is considered a morphological variant of *C. oncophora* and consequently all identified male worms were counted as *C. oncophora* for both isolates.

The worm counts of the suspected SUS isolate indicated an efficacy of 93.4% based on arithmetic means. Although the reduction in worm count between the SUS control and treatment group was not significant there is an indication of an effect ($p<0.1$). When excluding the animal with the worm count of zero from the SUS control group the reduction fell below the significance level of 5% ($p=0.011$). The worm counts for the RES isolate indicated an efficacy of 10.5% based on arithmetic means. There was no significant difference between the RES control and treatment group ($p>0.05$).

5.5 Discussion

Planned experiments on the population-dynamics of *C. oncophora* made it necessary to identify two different isolates, one resistant (RES) and one susceptible (SUS) to ivermectin. The principle aim of the current study was to identify the resistance status for ivermectin of the two field isolates for use in a subsequent experiment.

During the development of ivermectin as an anthelmintic agent *C. oncophora* was recognised as a dose limiting species (Egerton et al., 1979). Nevertheless, the chosen dose rate of 0.2mg/kg for oral treatment was established to be effective against this species (Campbell, 1985; Egerton et al., 1981). Although the only oral formulations of ivermectin available are sheep formulations these have previously been available for cattle in the 1990s with a dose rate of 0.2mg/kg. In this study Ivomec® liquid for sheep and goats was used which is the same formulation and includes a claim for efficacy for *C. oncophora* in sheep.

In 1995 the first case of ivermectin resistant *C. oncophora* in New Zealand was reported (Vermunt et al., 1995) and by 2004/5 the survey of North Island beef farms indicated a prevalence of 92% (n=62) with resistance to ivermectin (Waghorn et al., 2006a). At that time it was difficult to find ivermectin-susceptible *C. oncophora* isolates

in New Zealand. However, 5 farms in that survey were considered to have above 95% efficacy and one of these was the source farm for the SUS isolate. Initially it was stored at °8C and then cycled through 2 calves. Treatment of these calves indicated a 98.5% reduction in egg count. Based on these previous results the number of animals in the control and treatment groups for testing the ivermectin efficacy on this isolate was reduced from 6 to 3. However, a worm count for one of the animals in the SUS control group was zero using both the primary and the reserve sample, whereas one of the ivermectin treated animals still showed a relatively high worm count of 1500. This made it difficult to estimate efficacy for the SUS isolate in this initial study. Consequently the decision was made to repeat the experiment with another 3 calves in each of the two SUS groups.

Although there was a slight reduction in RES worm burdens the final results indicated no significant difference ($p = 0.873$) in worm burden between the control and ivermectin treated groups. The efficacy of ivermectin on the RES isolate was estimated to be 10.5%. For the SUS isolate a difference between the combined treatment and control groups was indicative but not significant ($p = 0.078$), but became significant ($p = 0.011$) when the control animal with the worm count of zero was excluded. For the SUS isolate including the control animal with zero worm burden the efficacy of ivermectin was 93.4%. Comparing these results to Waghorn (2006a) indicates that the efficacy of ivermectin on the current SUS isolate is still high but not fully susceptible as originally expected. The survey results were based on a faecal egg count reduction and there is evidence that the egg output does not always correlate to the actual worm burden. Previous reports have indicated reduced fecundity with the developing immune response and the possibility of density-dependent effects on fecundity (Brunsdon, 1971; Eysker and Ploeger, 2000). However, the current study is based on actual worm count and should not be affected by such effects.

When using these two isolates in additional experiments it has to be emphasized that there still is an indication of a minor efficacy of ivermectin on the RES isolate as well as slightly reduced efficacy against the SUS isolate. In this regard approximately 10% RES worms were killed and 10% of the SUS worms survived the treatment with a recommended dose of ivermectin.

The identification of recovered male worms revealed a proportion of approximately 15% *C. surnabada* which is now considered to be a morphological variant of *C. oncophora* (Newton et al., 1998). The proportion of the smaller *C. surnabada* males was not different between the two isolates in the control groups ($p<0.05$) nor between treatment groups where enough males could be recovered. For the RES isolate this was all animals but for the SUS isolate it was only 1 animal. This gives an indication that the resistance status does not influence the proportion of morphological types.

It was intended that for subsequent experiments a totally resistant and fully susceptible isolate would be available. These results indicate that neither of these isolates totally fulfilled this desire. At the time of the experiment the SUS isolate was considered to be the most susceptible *C. oncophora* locally available. It is clear if a more susceptible isolate than this one is identified it should be preserved to be available for further scientific use.

5.6 Acknowledgements

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Establishment of *Cooperia oncophora* in calves

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Chapter 6

Establishment of *Cooperia oncophora* in calves

6.1 Abstract

The establishment rate of *Cooperia oncophora* related to host age and previous infection was investigated in young calves. Three groups, each of 16 similarly aged calves, were kept on a feed pad. Two groups received trickle infections with an ivermectin-susceptible *C. oncophora* isolate of 2,000 or 10,000 infective stage larvae per week respectively, one group was kept as an uninfected control. At intervals over a period of 11 months two animals from each group were challenged with 15,000 infective stage larvae of an ivermectin-resistant isolate, treated orally 25 days later with ivermectin (0.2mg/kg; Ivomec®) and 5 days later slaughtered for worm counts. On 3 occasions additional calves (n=2) were included with the high trickle infection rate group but also received an ivermectin treatment before challenge to remove the existing worm burden. Two further groups (n=4) of similar aged calves were introduced at the beginning and the end of the experiment to determine the effect of larval age on establishment rate. The establishment rate was significantly different between the control group and the two trickle infection groups. The establishment in the two trickle infection groups declined to <10% within the first three months. In the animals receiving the high trickle infection but an anthelmintic treatment before challenge the establishment rate was not significantly different to the control group. The establishment rate in the control group declined over time from 53% at the beginning to <20% at the end of the experiment which was similar to the decrease recorded in the groups to determine the effect of larval age at the beginning and end of the experiment. The findings indicate that an existing nematode burden has a strong effect on the establishment of incoming larvae in the trickle infected groups but this effect was not observed if the existing burden was removed before the final challenge. The decline in establishment rate in the control group was due to the age of the larvae and not the age of the calves *per se*.

6.2 Introduction

Cooperia oncophora is one of the most common parasitic nematode species in cattle and is often abundant in calves during the first and second year of life. Although considered one of the least pathogenic intestinal nematodes (Coop et al., 1979) there have been reports of reduced productivity with *C. oncophora* infections by loss of appetite, lower weight gain and farming associated costs (Armour et al., 1987; Dimander et al., 2000; Forbes et al., 2000; Grønvold, 1989). These reports have often been related to anthelmintic resistant strains with resistance to the macrocyclic lactone (ML) class of anthelmintics now being common (Demeler et al., 2009; Jackson et al., 2006; Mason and McKay, 2006; Waghorn et al., 2006a; Waller, 1997). These problems indicate a need for a more integrated approach to parasite control in the future, which in turn will rely on a more detailed understanding of the dynamics of the parasitic lifecycles (Barger, 1999). It is known that during their first two years calves develop a natural immunity towards *C. oncophora* and the age of the host as well as exposure to the parasite are thought to play an important role in this process (Armour, 1989; Kloosterman et al., 1991; Ploeger et al., 1995). Kanobana (2004) investigated the development of immunity in calves based on establishment of *C. oncophora* in previously primed calves given a previous infection and found a significant reduction compared to the naïve control animals. However detailed data on the dynamics of establishment related to host age and different levels of previous experience is sparse. The presence of widespread AR has made this species of greater interest than its pathogenicity might indicate. However, little is known about the population dynamics of this species.

The aim of the current study was to investigate the establishment rate of *C. oncophora* in calves with a focus on determining the effect of the age and previous infection of the host.

6.3 Materials and Methods

6.3.1 Experimental overview

For the main study 58 young steers (3.9 – 5.7 months of age) were restrictively randomized initially by age then by live weight into 5 groups (A-E) including an uninfected control group (B) and two groups receiving either a low (C) or high (D) rate of trickle infection with an ivermectin-susceptible *C. oncophora* isolate (SUS) (Appendix 1 SOP12). Over a period of 10 months, commencing on 6th September 2010, animals from each group were challenged with an ML resistant *C. oncophora* isolate (RES) (Appendix 1 SOP13), treated with ivermectin (IVM) and then slaughtered to allow total worm burdens to be determined. An additional 3 animals were allocated to Groups B-D as spares and were treated as for the remainder of their group except they were not slaughtered. The infectiveness of RES larvae was tested early during the study and at the completion of the main study with two groups (F1 and F2) of similarly aged calves.

Group A (n=4) received a challenge dose of 15,000 ivermectin-resistant *C. oncophora* larvae on Day 0, were treated 25 days later with an oral dose of IVM (0.2mg/kg; Ivomec® liquid for sheep and goats, Merial New Zealand Ltd) and were slaughtered for total worm counts 5 days after IVM treatment

Group B (n=16) were the uninfected control animals. At intervals of 3-7 weeks apart over 10 months (Table 6.1) 2 animals were challenged with RES larvae, treated with IVM and slaughtered for total worm counts following the same schedule as for Group A. If any Group B animal was positive following regular faecal egg counts (FECs) all animals in this group received an oral treatment with a combination of oxfendazole 4.53 mg/kg and levamisole 8.0 mg/kg (Scanda®, Coopers Animal Health, New Zealand) according to individual body weight except for those animals that were to be challenged with RES larvae within one week.

Group C (n=16) received a trickle infection of 1000 SUS larvae twice a week whereas Group D (n=16) received 5000 SUS larvae twice a week. Following the same timetable as for Group B, two animals per group received a challenge with RES larvae, followed by IVM treatment and were subsequently slaughtered for total worm counts.

Group E (n=6) animals received the same schedule of trickle infection as for Group D except they were treated with an effective oral anthelmintic containing oxfendazole and levamisole as for Group B animals 10 days prior to challenge with RES larvae to remove the existing SUS worm burden. They received no further SUS larvae after this treatment. This group included two animals that received the same challenge with RES larvae on Days 91, 112 and 140 as for respective Group D animals with subsequent IVM treatment and slaughter following the same schedule.

Table 6.1 - Infection schedule with susceptible and ivermectin-resistant *Cooperia oncophora* for treatment groups A to E. SUS indicates the period when animals in groups C-E received a trickle infection with an ivermectin-susceptible *C. oncophora* isolate; group C received 1000 L3 twice per week, group D and E 5000 L3 twice per week over the period indicated. Group A and control group B received no SUS trickle infection. RES indicates challenge with 15,000 ivermectin-resistant L3. These animals were orally treated with ivermectin 25 days post-challenge and slaughtered for total worm burden determination 30 days post-challenge. Animals in group E were treated with an effective oral anthelmintic 10 days before RES.

	Days								
Group	**0**	**42**	**91**	**112**	**140**	**168**	**210**	**252**	**294**
A	RES								
B		RES							
C/D	SUS	RES							
B			RES						
C/D/E	SUS		RES						
B				RES					
C/D/E		SUS		RES					
B					RES				
C/D/E			SUS		RES				
B						RES			
C/D			SUS			RES			
B							RES		
C/D				SUS			RES		
B								RES	
C/D				SUS				RES	
B									RES
C/D					SUS				RES

Group F1 and Group F2 each contained 4 young calves which were used to test the infectivity of the RES larvae over the course of the experiment. Group F1 was challenged with 15,000 RES larvae on Day 63 and Group F2 on Day 344. Both groups of

calves were of the same age at the time of their respective challenge with RES larvae. As for Groups A-D they were treated with IVM on day 25 and slaughtered 5 days later for total worm counts.

Nematode egg viability (=egg hatch) was assessed using faeces from the animals in Groups C and D (plus respective spare animals) in the last two challenge groups. Every 8 weeks over the whole study FECs were estimated from each animal and the proportion of eggs developing to L3 was calculated for the SUS isolate.

6.3.2 Experimental animals and Husbandry

The calves in groups A-E were spring-born and commercially reared under standard husbandry conditions. They were initially reared indoors on wood shavings and were moved outside to pasture with supplementary calf pellets for approximately 2-3 weeks prior to transfer to the experimental site. This was a feed pad located in an outdoor setting with most of the yard comprising bedding material of untreated wood shavings with the area immediately adjacent to the baleage troughs being clear concrete with no bedding. This clear area allowed spilt feed to be removed as necessary. On arrival the animals were treated with an effective oral triple combination anthelmintic comprising abamectin 0.2mg/kg + oxfendazole 5mg/kg + levamisole 8mg/kg (Matrix® C, Merial New Zealand Ltd) and vaccinated against Leptospirosis (Leptoshield®, Pfizer). Animals arrived in 2 groups and were allowed at least 2 weeks to acclimate to the feed pad. Seven days before the start of the experiment the animals were orally treated with Scanda® at the recommended dose rate. At the start of the experiment the average animal age was 4.8 months, ranging from 3.9 to 5.7 months. All animals were cross-bred New Zealand dairy cattle with a dominance of the Holstein-Friesen breed and some Jersey component.

All animals had continuous access to water and were fed with annual ryegrass baleage plus 20% protein pellets. This baleage was taken from paddocks that had not been grazed by ruminants for at least one year. Analysis of the baleage showed it contained only 6.4% crude protein which is insufficient for growing calves and this was

supplemented with 20% protein calf pellets to ensure a minimum of 12.5% crude protein feed intake overall .

Animals in Groups F1 and F2 were raised under helminth free conditions on wood shavings with no access to pasture. During the experimental period they continued to be housed indoors under helminth-free conditions and were fed a ration comprising 20% calf pellets and a commercial baleage ration (FibreEzy®/ FibreStart®,Fibre Fresh Feeds Ltd., Reporoa, New Zealand) with some access to aged hay. Both groups were about 3-4 months of age at the time of their respective challenge with Group F2 born out-of-season in autumn.

6.3.3 Infective larvae

Two different isolates of *C. oncophora* were used for the experiment. The efficacy of IVM for both isolates was determined in a separate experiment following VICH and WAAVP guidelines (Vercruysse et al., 2001; Wood et al., 1995). The efficacy of IVM for the SUS isolate was estimated to be 93% and for the RES isolate 10% (Chapter 5).

The SUS larvae were from a farm, identified in a survey of anthelmintic resistance in cattle parasites, on which the efficacy of IVM against *C. oncophora* was high (Waghorn et al 2006a). Larvae were collected by infecting donor cattle and culturing the faeces with vermiculite at 23°C for 9 to 14 days. Larvae were accumulated 270-290 days prior to the start of the experiment and stored at 8°C. The same pool of larvae was used throughout the experiment as well as for the preceding study to determine the efficacy of IVM for both isolates. Each week the viability of the larvae was monitored and the infective dose modified to account for any deaths. Morphological identification indicated the culture contained 4% *Ostertagia* larvae and all further calculations for SUS larvae were corrected for this factor.

The RES larvae were obtained by culturing faeces from naturally grazed donor calves on the same farm where severe IVM resistance was identified in 1995 (Vermunt et al., 1995). These were on an alternate weekly IVM drench and super-infection regime. In one week the calves were orally treated with IVM and 7 days later in the following

week were infected with 15,000 RES larvae. Eggs were only collected during this second week. This regimen had been operating, using different calves, for 18 months prior to the start of the experiment. The RES larvae were accumulated 195-210 days prior to the start of the experiment and this same pool of larvae was used throughout the experiment. On each occasion the viability of the larvae was monitored and the infective dose modified to account for any deaths.

6.3.4 Parasitological techniques

Individual FECs for every animal were determined fortnightly using a modified McMaster technique (Stafford et al., 1994) but double the number of chambers was counted so that each egg represented 25 eggs/g (Appendix 1 SOP1).

At necropsy the small intestines were collected and stored frozen at -18°C for at least one week before further processing. Intestines were subsequently thawed, thoroughly washed and 10% aliquots were sieved over a 38µm sieve and all worms in the retentate were counted to estimate the total worm count (Appendix 1 SOP14, 15 and 16). For size measurements up to 30 female worms were collected directly from separate washings and transferred into normal saline. . To confirm the efficacy of the intestinal washing the small intestines of 10 animals with high worm counts were also subjected to a pepsin/HCl digest (Appendix 1 SOP19). An aliquot of 10% of the digested intestines was then processed as for the intestinal washings and any worms seen were counted.

To assess the developmental success of eggs developing to infective larvae the individual FEC was estimated three times for each host animal in Groups C and D on each occasion. The faecal samples were then weighed and then mixed with vermiculite until the mixture showed a crumbly texture to ensure adequate moisture and aeration (Appendix 1 SOP4). The culture mixture was weighed and the corresponding amount of mixture equivalent to 10g of faeces was placed into small pottles (75ml) without a lid. These were placed into larger pottles (250ml) with a ventilated lid and containing a thin water layer. The purpose of this water was to capture any escaping larvae and maintain a high humidity. After culturing at 25°C for two weeks the larvae were

recovered by baermannization at room temperature and counted. To estimate how many *Ostertagia* larvae were present a similar number of larvae from each animal were pooled and 100 were identified to genus using morphological criteria (Anonymous, Moredun lab manual).

Baermannization was undertaken by placing the sample onto a single layer of tissue paper in a sieve. The sieve was positioned in a glass funnel closed with a clamped rubber tube and water added to ensure the sample was fully submerged. The larvae were collected with the first few millilitres of water from the tube the next day and counted.

6.3.5 Regular measurements and samples

The live weight for each animal was recorded on a weekly basis during the experiment. Every 8 weeks the shavings on the feed pad were examined for parasitic infective stage larvae. Samples were taken from different locations of the feed pad, bulked and baermannized to recover any infective stage larvae. The baleage was examined for possible parasite contamination on a four weekly basis by taking a sample of the opened bale at the time and examined as for the feed pad samples.

6.3.6 Worm length and fecundity

For each animal all non-damaged adult female worms to a maximum of 30 per animal were recovered from the intestinal washings and transferred into normal saline. For each worm a photographic image was taken and the individual length measured by manually fitting segmented lines with the software ImageJ (National Institute of Health, USA). Calibration was achieved by comparison with a micrometre slide (Appendix 1 SOP17).

The fecundity was estimated by counting eggs in up to 30 non-damaged female worms per animal using a differential interference contrast microscope (Olympus BH2 DIC) after worms had been cleared in lactophenol (Appendix 1 SOP18). An egg was counted if an obvious shell was evident.

6.3.7 Statistical analysis

The worm burdens in groups B-D animals were analysed by using non-linear regression. Differences between groups were compared by calculating the confidence intervals. Results from Group E were compared to Groups D separately using ANOVA on square-root transformed worm count with group and time as fixed factors. Similarly results from Group E were compared separately to Groups B to D including a multiple comparison using the Tukey method. A possible difference in worm counts between Groups B and F was analysed by comparing the slopes of linear regressions against time.

The ratio between recovered female to male worms, the length of adult female worms and the fecundity of adult female worms was analysed with one factor ANOVA for all groups using Group as the fixed effect. For the female to male ratio the data was limited to host animals from which at least 10 nematodes had been recovered from the aliquot counted. Fisher's LSD method was used to compare the differences between Groups. The number of recovered nematodes decreased over time, especially in Groups C-E and the opportunity to undertake statistical analysis was limited.

The weight gain data for the calves in Groups B to D over the course of the experiment was analysed to investigate a possible effect of the trickle-infection. The individual weight data per animal from the beginning of the experiment until the animal was challenged was used to calculate the weight gain per day. This was analysed with an ANOVA using Group, time till challenge and the interaction as fixed factors. The weight at the commencement of the experiment was used as a covariate.

All statistical analysis was done using the software package R (R Core Team, 2012)

6.4 Results

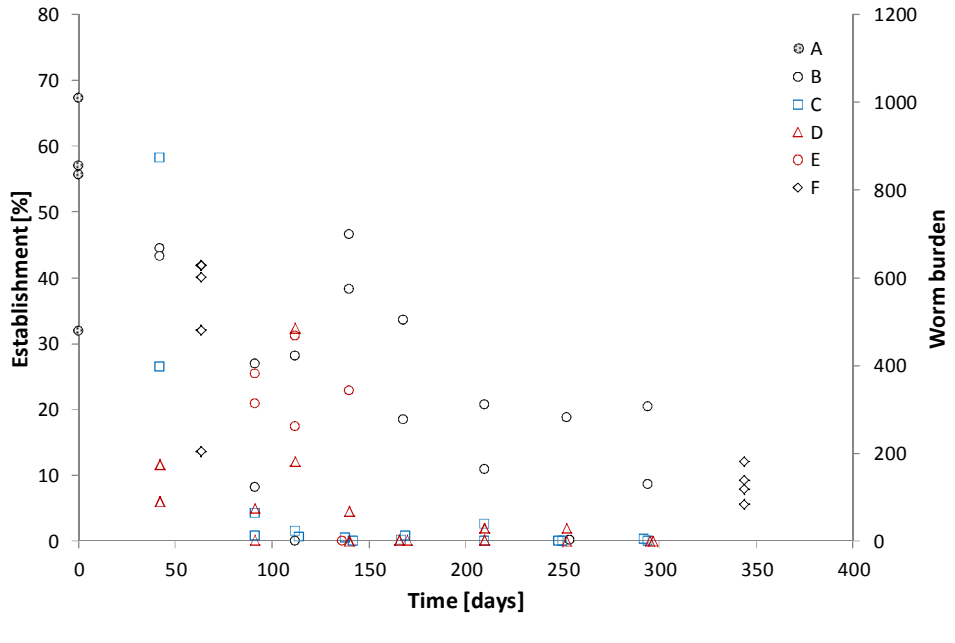
6.4.1 Baleage and shaving samples

The analysed baleage samples showed no contamination with nematodes. The samples taken from the shavings in the feed pad tested positive for contamination with infective stage larvae from Day 70 till the end of the experiment.

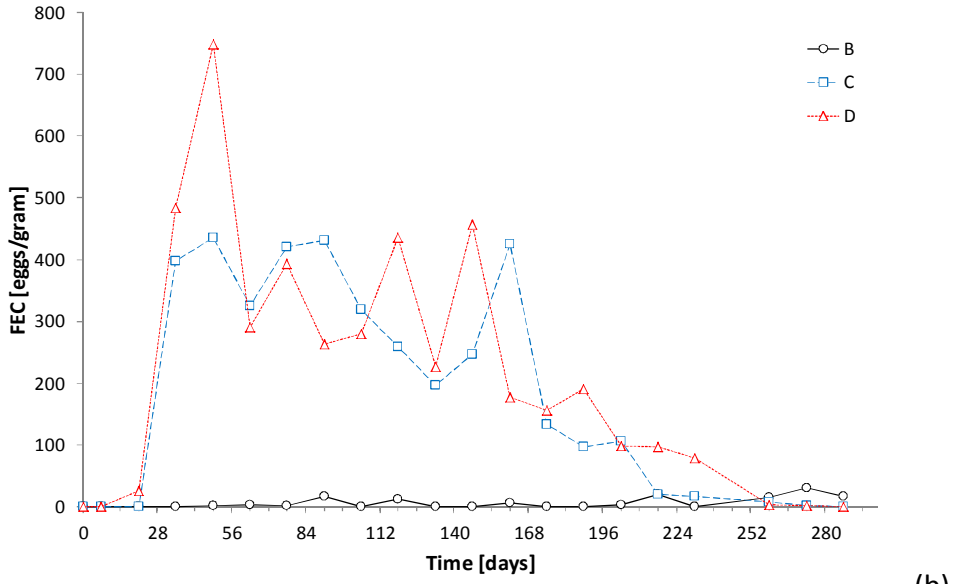
6.4.2 Establishment

The proportion of the challenge infection recovered as adult worms decreased in groups B-D over the course of the experiment (Figure 6.1.a). Group A which was challenged at the start of the experiment prior to any trickle infection had an average establishment of 53%. This declined in the control animals (Group B) over the course of the experiment to be only 10-20% after 294 days. A more rapid reduction was observed in Group D which had declined to <12% by 42 days and in Group C by 91 days. In both Groups C and D the establishment rate then remained low for the remainder of the experiment, with the exception of Group D at 112 days where both experimental animals had slightly higher establishment rates of 12% and 31% respectively. The results from the non-linear regression therefore indicated that from Day 112 onwards the worm counts from Group D were significantly lower than for Group B ($p<0.05$) whereas the worm counts for Group C were significantly lower compared to Group B by Day 91 ($p<0.05$). The worm counts at Days 91, 112 and 140 were not significantly different ($p>0.05$) between Group E (establishment rate 23.1%, 24.2% and 11.4% respectively) and B (17.6%, 14.1% and 42.4% respectively) or Group D (2.6%, 22.3%, 2.3% respectively). However, there were apparently aberrant high worm counts for Group D at Day 112. The worm counts for Group E were significantly higher ($p<0.05$) than for Group C animals with 2.5%, 1.1%, 0.2% establishment rate respectively at these same time points. The mean worm count for Group F1 on Day 63 was 4790 (31.9% establishment rate) which was significantly higher ($p<0.05$) than that for Group F2 on Day 344 of 1310 (8.7% establishment rate) indicating the infectivity of the RES larvae had declined over time. The worm counts at both time points were not different ($p>0.05$) from those of the control animals in Group B.

No fourth stage larvae were detected in any of the samples except for one animal in group F1 from which 1 larva was recovered. No nematodes were detected in the pepsin digests of the 12 animals from Group D indicating these digests were not necessary.



(a)



(b)

Figure 6.1 - a) *Cooperia oncophora* establishment and worm burden in young calves kept in different treatment groups (A-F) (superimposed points have been spread horizontally). Calves in Groups C-E received a trickle infection with an ivermectin-susceptible *C. oncophora* isolate; Group C received 1000 L3 and Group D/E 5000 L3 2x/week. Group A/B received no trickle infection. Time indicates the days individual calves were challenged with 15,000 ivermectin-resistant L3 (RES). Animals were orally treated with ivermectin 25 days post-challenge and slaughtered for total worm burden determination 30 days post-challenge. Group E animals received effective oral anthelmintic treatment 10 days prior to challenge. Group F animals were of similar age at the time of their respective challenge to test the infectivity of the RES larvae over the course of the experiment. b) Mean faecal egg counts (FEC) for Groups B to D. FEC results from individual animals are included until RES challenge.

6.4.3 Sex ratio

The proportion of females increased in groups receiving a trickle infection (Group C and D compared to all others; $p<0.05$) otherwise there were no differences in sex ratio (Table 6.2; Appendix 6, Section 9.6.2).

6.4.4 Worm length

The overall mean lengths (Table 6.2; Appendix 6 – Section 9.6.4) were shorter for those animals receiving a trickle infection with those in Group D being shorter than for those in Groups C and E ($p<0.05$) which in turn were shorter than those of Groups B and F ($p<0.05$). Lengths for Group A were only different to those of Group D ($p<0.05$).

Table 6.2 - The proportion, length and fecundity of female worms recovered from calves 28 days after challenge with 15,000 infective stage larvae of an ivermectin-resistant *Cooperia oncophora* isolate. Group A were challanged at the commencement of the experiment and were initially nematode-free; Group B were uninfected control animals; Groups C and D received a trickle infection with susceptible larvae of 1,000 and 5,000 L3 2x/week, respectively; Group E were trickle-infected as for Group D but treated with an anthelmintic 10 days before challenge; Groups F1 and F2 were helminth-free raised calves of comparable age challenged on two occasions during the experiment. Letters next to the values indicate significance levels and groups that do not share a letter are significantly different from each other, n = the number of available host animals.

Group	Females (%), n			Length (mm), n			Fecundity (eggs), n		
A	56	a	4	10.9	bc	4	50.5	abc	4
B	61	a	14	11.4	c	11	80.8	d	11
C	72	b	8	10.1	b	4	37.4	ab	9
D	76	b	8	8.9	a	4	30.6	a	9
E	60	a	5	10.2	b	4	57.5	bc	5
F1	57	a	4	11.8	c	3	74.4	cd	2
F2	61	a	4	11.6	c	3	65.9	bcd	2

6.4.5 Fecundity

A comparison of the number of eggs in utero of female nematodes recovered from Group A with 50.5, to the overall means of the other groups indicated that there were no significant differences ($p>0.05$) except compared to Group B (Table 6.2; Appendix 6 – Section 9.6.3). The recovered females from Group B contained the highest number of

eggs with a mean of 80.8 which was not significantly different ($p>0.05$) from F1 and F2 (74.4, 65.9), but significantly higher ($p<0.05$) than Groups C, D and E (37.4, 30.6 and 57.5) respectively. However, the results from Group B were also significantly higher compared to Group A. Females from Group E in which the calves had been treated with an anthelmintic before challenge had significantly more eggs ($p<0.05$) compared to those recovered from Group D which were not treated before challenge.

6.4.6 FEC

At the beginning of the experiment no eggs were seen in faeces from any animal (Figure 6.1.b). Animals from Group E were included together with Group D until the pre-challenge treatment for Group E. In Groups C and D/E there were 0 and 11 animals with a positive *Cooperia* FEC by Day 21 which increased to 16 and 23 by Day 35, respectively. From this time there was an increase in the number of excreted *Cooperia* eggs by animals in these groups reaching a peak of the group mean egg count at Day 49 of 750 for Group D/E. The highest mean *Cooperia* FEC for Group C was 483 eggs/g on Day 161. Subsequent to these days the FEC in these groups gradually decreased to below 50 eggs/g by 260-280 days. For group B, the positive counts from Day 49 on indicated repeatedly low infections with parasitic nematodes. These probably had their origin in contaminated shavings on the feed pad reported above. If any counts for Group B were positive all the animals in the group were treated (Appendix 6) with Scanda® according to individual live weight with the exception of animals that were scheduled for RES infection within the next week (Appendix 6).

6.4.7 Egg to [L3] development rate

Culture of faeces from Groups C and D showed there was a decreasing number of *Cooperia* but an increasing number of *Ostertagia* eggs being shed during the experiment to a stage at Day 273 where only *Ostertagia* larvae were recovered from the cultures. The egg to third stage larval development rate and FEC results have been corrected by this ratio

The egg to [L3] development rate did not change over the course of the experiment ($p>0.05$) with the average development rate being between 30 to 50% for both Groups C and D.

6.4.8 Weight gain

The weight gain of the animals was small at the beginning of the experiment and actually decreased over Week 3. An analysis of the baleage revealed a crude protein content of only 6.4%. A complete ruminant ration should have a minimum protein level of 12.5% and to modify the ration it was necessary to feed additional calf pellets containing 20% protein fed throughout the experiment from Week 6 onwards. After this alteration to their diet the animals increased in weight. Overall, the daily weight gains for the three main groups were 0.62 kg for Group B, 0.61 kg for Group C and 0.56 for Group D with no significant difference between the control and two treatment groups ($p>0.05$).

6.5 Discussion

6.5.1 Establishment

In this experiment, establishment in the control group (Group B) declined from 53% at the start of the study to 10-20% at the end. Interestingly this decline in establishment rate appeared to be due to the age of the RES larvae used throughout the experiment and not to the age of the calves *per se* (see discussion below). In contrast the establishment rate declined rapidly in calves that were under constant trickle infection with SUS third stage larvae and therefore had an established parasite population at the time of challenge with RES larvae (Groups C and D). At the beginning of the experiment, the calves which received the higher weekly larval dose (Group D) showed a more rapid decrease in establishment of the challenge dose than those with the lower weekly larval dose (Group C). At Day 42 the establishment rate in the calves receiving the higher number of larvae (Group D) had already notably declined compared to the control group (Group B) which received no trickle infection. At this first slaughter date there was no apparent effect on establishment in calves receiving

the lower number of larvae (Group C). After this initial difference the establishment rates were similar in these two trickle-infected groups and remained at levels of 0-5% until the end of the experiment. However, establishment of RES on Day 112 for the calves which received the high weekly larval dose (Group D) appear to be aberrant in that the establishment was surprisingly high by comparison with these calves slaughtered prior and after this time, especially the ones immediately before and after Day 112.

If the existing nematode population was eliminated before the challenge dose was given (Group E) the establishment rate increased to levels between control (Group B) and the trickle infected groups (Groups C and D). These values were not significantly different to those of the control group (Group B) but were different ($p<0.05$) to those of the calves receiving the lower weekly larval dose (Group C). The two aberrant results for calves receiving the high weekly larval dose (Group D) overlap on Day 112 and hence the overall Group results are not different ($p>0.05$). These results indicate that presence of an existing population of adult nematodes had a significant effect on the establishment of incoming larvae. For *Haemonchus contortus* the existence of an existing population reduced the establishment rate in goats and young sheep (Barger et al., 1985; Watson and Hosking, 1993). In the sheep example lambs aged only 4 months effectively resisted the establishment of *Haemonchus* larvae whereas drenched lambs of the same age did not develop an effective immune response until they were 8 months of age. A threshold or minimum accumulated dose of larval infection needed to trigger a host immune response has been proposed for *H. contortus*, *Nematodirus spathiger* and *T. colubriformis* (Dineen et al., 1965a; Dineen et al., 1965b; Dobson et al., 1990a; Windon et al., 1984). In these reports the threshold required for each species was relatively low and would have been exceeded in the present study early during the trickle infection phase. Under normal farming conditions when calves are grazing they are under a constant trickle infection with larvae picked up with the herbage. Under such circumstances the threshold for *C. oncophora* is likely reached during the first grazing season when a development of immunity can be observed generally protecting the animal in the second season (Bisset and Marshall, 1987; Brunsdon, 1971). With trickle infections as with Group D in the

current study the host should develop an immune response to *C. oncophora* and start to expel the nematodes 2 to 3 months after the first infection (Kloosterman et al., 1991). If the existing worm burden in the calves was removed before challenge (Group E) the establishment rate in these calves indicated no effective immune response. The first of these animals (Group E) were challenged on Day 91 by which time they had received at least 100,000 L3 over 8 weeks. Thus, in the present study this development of the immune response was not evident when the existing worm burden was removed.

The declining establishment rate with animals that were trickle infected in the current study was also similar to those for lambs infected with *Trichostrongylus colubriformis* (Dobson et al., 1990c). A similar experimental approach of trickle infecting with a susceptible isolate then challenging with a resistant isolate was followed. These authors reported an initial establishment rate for *T. colubriformis* of 50-60% in 5 month old lambs which rapidly decreased to 0-5% at 49 days in a group given a high dose trickle infection (similar magnitude to Group D) whereas a group given a lower dose trickle infection (similar magnitude to Group C) was slightly slower in developing apparent resistance. There was no group in the study with *Trichostrongylus* in sheep in which an existing worm burden had been eliminated as in the current study (Group E) so in this respect no comparison is possible. However, the results of Group E stand in contrast to those of Kanobana et al. (2004), who reported a significant reduction in subsequent establishment after the *C. oncophora* worm burden of a priming parasite dose was removed prior to challenge. In this present study experimental animals were not raised under entirely helminth free conditions before the study but had been on pasture for about two weeks. It is possible that all animals had been exposed to the parasite before the experiment and during this time acquired some degree of immunity, although this exposure did occur at about 3 months of age. However, if this was the case the establishment rates in the helminth free raised calves (Groups F1 and F2) should have been similar to the previous findings of Kanobana et al (2004) but were instead not significantly different to that of the control (Group B).

There was a significant decline ($p<0.05$) in establishment in the two groups (Groups F1 and F2) which were challenged to compare the infectivity of the RES larvae over the

time of the experiment. The calves were of similar age for both these groups and were raised under helminth free conditions with no prior exposure to *C. oncophora.* Interestingly, this decline in establishment for Group F overall was not different ($p>0.05$) to that of Group B indicating an effect of the age of larvae on establishment rate rather than some innate effect of age of animals in Group B affecting establishment rate. The RES larvae in the current study had been cultured and stored 180 days before the start of the experiment. At the time Group F1 and F2 were infected with 15,000 viable larvae which had been stored at 8C for 243 and 524 days respectively. During this time the larvae would have depleted stored nutrition which is likely to have influenced their ability to successfully establish in the host. A related study (Chapter 3) has shown that at least 50% of *C. oncophora* larvae remained alive for 510 days with a decline from 90% at 180 days to 50% at 500 days. This study did not examine the ability of those larvae to infect a host, only to remain visibly alive. In the present study the number of larvae given to the animals was corrected for those which had died and the dose indicated was only of live larvae. Few studies have followed the ability of larvae to infect their host after varying periods of storage. Eckert (1967) investigated several physiological parameters of *C. punctata* third stage larvae and reported a reduction of both protein and lipids in ageing larvae, especially the latter. Such a reduction may affect the capability of the larvae to successfully establish in the host. A decrease of infection rate in the intermediate host in relation to larval age has been shown for the first stage larvae of *Arianta arbustorum* (Skorping, 1982), a parasite of reindeer. In this study the infection rate decreased steadily over time whereas the motility of the larvae was nearly unaffected for most of this period until eventually declining rapidly. The results for Group F indicate a decline in infection rate for the RES isolate over time with *C. oncophora*. Thus the decline in establishment in Group B is likely to be at least partially due to the age of the RES larvae and not due to an effect of the host age per se. However, even though Group B received no intentional trickle-infection during the study, regular egg counts indicated they did pick up low numbers of infective larvae while being on the feed pad and were also likely to have been infected during the short period they spent on pasture prior to the start of the experiment. Nevertheless, the results indicate the reduction in establishment rate was due to the declining infectivity of larvae rather than the developing immune

response due to this small exposure. It has been argued that immunity towards *C. oncophora* develops faster as the age of the host animals increases (Kloosterman et al., 1991). For *Trichostrongylus axei* in calves Herlich (Herlich, 1979) found that establishment in calves decreased rapidly by 310 days. The results of the present study indicate that the amount of previous exposure to *Cooperia* was not sufficient to trigger the development of an immune response which could affect the establishment of *C. oncophora* larvae in the challenge dose.

6.5.2 Length, fecundity and proportion of female worms

The evaluation of the proportion of recovered female nematodes, the body length of these and their fecundity indicated a change in Group D compared to Group B which was partly reversed in Group E. Whereas the proportion of females of the recovered worms increased in Group D the length of these and their fecundity decreased compared to Group B. This is consistent with previously published findings on the sex-ratio and length of recovered female nematodes found for *C. punctata* in trickle infected calves (Yatsuda et al., 2002). However, if the pre-existing worm burden was eliminated before challenge as in Group E the sex-ratio returned to levels comparable to Group B, which is an indication that the ratio was influenced by an existing nematode population at the time of infection. The length of recovered female nematodes from Group E also showed an increase compared to Group D but only to levels similar to those seen in Group C, indicating that the growth of the nematodes was not only influenced by an already existing nematode population but also by another factor. Interestingly, the fecundity of the recovered female nematodes in Group E increased from levels seen in Group D to levels similar to that seen in Group C. Previous studies with *C. oncophora* in cattle and *T. circumcincta* in sheep (Kanobana et al., 2004; Stear et al., 1995) found that worms in previously exposed animals had both a reduced fecundity and length. Although the length of female *C. oncophora* recovered from Group B in the current study were longer when compared to those reported by Kanobana et al. (2004) they correspond closely to the lengths reported by Albers et al. (1982). Overall, in the current study there appears to be two factors affecting fecundity and worm length; the presence of an existing burden as well as previous exposure,

implying a developing immune response which has been seen in a number of other host-parasite systems with a variety of different types of nematode parasites (Poulin, 1997).

6.5.3 Egg viability

The investigation of the development rate of eggs in the current study showed inconclusive outcomes. Previous studies with *T. circumcincta* and *O. ostertagi* have shown an effect of the developing immune response being able to reduce the developmental success of eggs to L3 (Jørgensen et al., 1998; Sutherland et al., 1999). In the present study there were 4% *Ostertagia* larvae in the SUS larvae supply used for the trickle-infection, and this apparently resulted in an increasing number of *Ostertagia* establishing in the cattle as measured by the increasing proportion of *Ostertagia* larvae in the faecal cultures. This could have obscured changes in the development success of *C. oncophora* eggs as it was only possible to calculate the proportion of *Cooperia* based on the identification of larvae which actually developed. It was assumed that both *Ostertagia* and *Cooperia* had similar development success rates. Using this criteria there was no effect of time or previous exposure ($p>0.05$) demonstrated in this experiment on development of eggs to L3 for *Cooperia*.

6.5.4 Weight gain

Previous studies have shown an indication of reduced weight gain in young cattle infected with *C. oncophora* (Armour et al., 1987; Coop et al., 1979). Both these studies were challenging young cattle with ≥70,000L3/week which is higher than the trickle infection in the present study. Although there was a short period of reduced weight gain early in the present experiment this was considered to be due to inadequate protein in the diet. After this was corrected in Week 6 the daily weight gain increased to expectations in all groups. Interestingly, no significant difference in weight gain between Group B and either of the trickle-infected Groups (C and D) could be identified in this experiment. Even Group D with the highest trickle infection of 10,000 *Cooperia* larvae per week, which also included 400 *Ostertagia* larvae per week, did not

show a reduction in growth rate. This is consistent with the conclusions of Coop et al (1979) indicating *C. oncophora* was of minimal pathogenicity. Armour et al (1987) did find a difference in weight gain with 70,000L3/week for 6 weeks in young calves but also acknowledged there was a small proportion of *Ostertagia* in the larval challenge which may have contributed to this reduced growth rate although the *Ostertagia* burdens were <2500. Nevertheless, both these two reports and the current study are consistently indicating *C. oncophora* has minimal effects on growth rates of young cattle.

6.6 Conclusion

In this study there was a slow decrease in establishment rate in the control animals over time. However, this decrease was due to the ageing of the RES larvae used throughout the experiment. Furthermore there is an indication that in animals which received a trickle-infection during the study the decrease in establishment rate was primarily stimulated by the presence of a pre-existing worm burden. Removal of this before the challenge resulted in an establishment rate similar to that seen in the control group. Challenging animals with a pre-existing worm burden also resulted in a higher proportion of females, a shorter body length and reduced fecundity of recovered adult female worms. The interesting results on the influence of an existing worm burden at the time of challenge need to be examined in more detail.

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DRC 16

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Christian W. Sauermann

Name/Title of Principal Supervisor: Prof. W. E. Pomroy

Name of Published Research Output and full reference:

Sauermann, C.W., Ganesh, S., Scott, I., Leathwick, D. M., Pomroy, W. E., due for submission, Establishment of Cooperia oncophora in calves, Veterinary Parasitology

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Experimental design and planning in collaboration with supervisors, conducting animal work (not including daily husbandry), laboratory work processing samples (worm counts partly done by Paul Mason), preparing first draft of publication.

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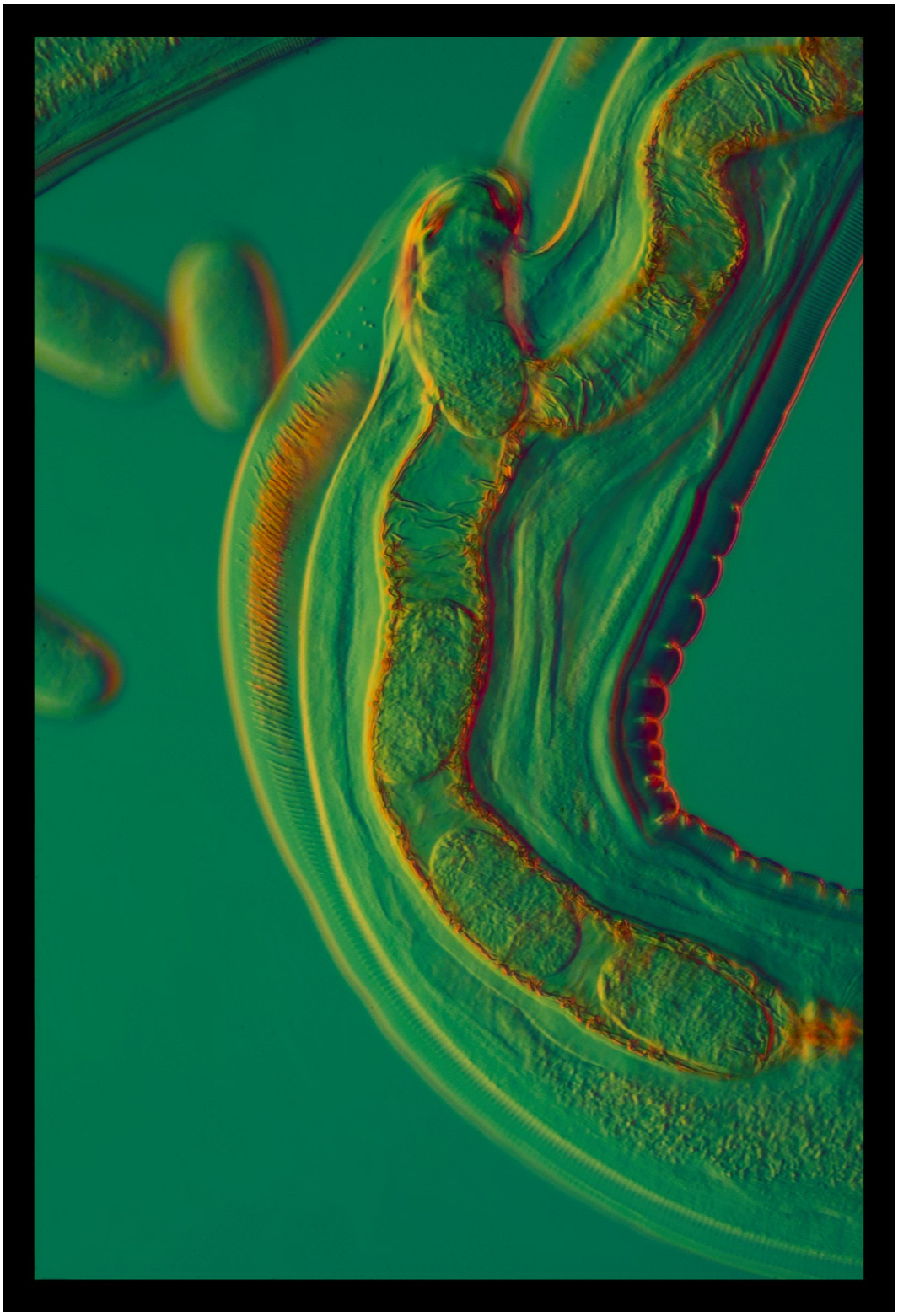

Plate 3 - Adult gravid *Cooperia oncophora* female recovered from a frozen small intestinal sample showing the vulva and part of the ovijector with eggs. The ovijector (orange tube) lies between the end of the uterus and the vulva to aid in the expulsion of the eggs.

Chapter 7

General Discussion

The general aim of this thesis was to investigate the population dynamics of *Cooperia oncophora* and to use the data generated to facilitate the construction of models describing the life cycle. One of the main reasons for this investigation was the rapidly developing problem of anthelmintic resistance in *C. oncophora*. It was apparent that little focus had been given to better understand the general dynamics of this species, yet for cattle in New Zealand it was likely to be the first nematode species where total failure of all available anthelmintics occurs. An important tool to compare the effects of different treatment and control strategies is to model the life cycle of a parasite. To facilitate modelling of the free-living stages of *C. oncophora*, a series of experiments were conducted to quantify the effect of the ambient temperature on their development and survival (Chapter 2 and 3). This data was then incorporated into EBT models (Chapter 4) to simulate the effects of temperature on the dynamics of the free-living phase.

The experiments on the free-living stages including the model simulations showed that:

- The third stage larvae of *C. oncophora* survived for a long period at lower temperatures.
- The Development Model indicated that at low temperatures development was decreased to a point where most individuals died before reaching the third larval stage.
- The Ageing Model indicated that at high temperatures the first larvae to reach the third stage were already dying while development of larvae was still continuing for the remainder.
- The host animal of the *C. oncophora* population had a significant effect on the development and survival of the free-living stages.

To explore the parasitic phase an experiment investigated the establishment rate of the third stage larvae in calves (Chapter 6). This included the effect of host age and different levels of *C. oncophora* trickle infection prior to challenge.

The results from the experiment on the establishment rate indicated that:

- Larval age influenced the infectivity of the larvae.
- The establishment rate was reduced by an existing worm burden at the time of challenge.
- The weight gain of the calves was not significantly influenced by the presence of a *C. oncophora* infection regardless of the dose of the trickle infection given.

These key findings are further discussed in the following sections.

The effect of temperature on the development and survival of the free-living third stage larva

For larvae it has been reported that lower temperatures reduce the metabolic rate resulting in a longer period to develop but also a lower consumption of stored nutrients by the third stage larvae (Eckert, 1967) leading to longer survival times at lower temperatures. These general trends have been observed for a range of trichostrongylid nematodes (Boag and Thomas, 1985; Ciordia and Bizzell, 1963). The proportion of eggs successfully developing to third stage larvae in the present experiments was decreased at lower temperatures which was consistent with the findings of Ciordia and Bizzell (1963) for a variety of trichostrongylid species. This was not simply due to delayed development at these lower temperatures. The higher mortality rate during larval development was a result of the decrease in development rate but still with a relatively similar survival rate during development meaning the pre-infective stages are subject to a similar probability of dying per unit of time but as development occurs over a longer period fewer reach the infective stage.

Development success estimated from the model (Chapter 4) indicated that the survival of larvae at higher temperatures (>32C) decreased to a point where the first larvae to

reach the infective third stage had already died before the last larvae to successfully finish their development, resulting in a lower recorded development success. However, the temperatures used in the model were above the temperature range investigated experimentally and need to be confirmed. In one previous study with *C. oncophora* it was noted there was a sharp reduction of development success above 32°C (Ciordia and Bizzell, 1963) which is generally consistent with present model predictions.

The median survival times at a range of temperatures for *C. oncophora* third stage larvae were observed to be mid-way between those of other trichostrongylid species, being lower compared to *Ostertagia* but higher compared to *Haemonchus* (Boag and Thomas, 1985). The present experiment confirmed the general trend of longer survival times at lower temperatures but the median survival times were lower than those previously reported (Boag and Thomas, 1985). As there were no confidence intervals given in this earlier study it is not possible to assess whether these are substantially different. Provenance of the isolate and/or variations in methodology may explain this difference.

The practical consequences of these trends is still consistent with general observations made by many authors which indicate that during the cooler winter months fewer larvae will develop and do so more slowly but L3 will survive for a prolonged period. This will change in the summer to a higher success rate at a faster rate but also a shorter survival period of the larvae in the absence of temperatures greater than 32°C.

Host effect on development and survival

The development success and survival of *C. oncophora* third stage larvae was significantly influenced by the individual host animal from which the faeces for culture were collected. This has previously been reported for the developmental success by Jørgensen (1998) for *T. circumcincta* in sheep, but the results here are the first reported indication of such an effect with *C. oncophora* in cattle. The possible cause for the observed difference and which other traits of the larvae are predisposed by the

host is unclear at this stage and need to be addressed in additional experiments. Interestingly, only a few other previous reports suggest an effect of host on these parameters. However, many studies do not give sufficient information to enable the reader to determine if the source of eggs was from one animal or a pooled collection from several animals and whether this varied over the course of an experiment. The results from the present experiment would indicate this may have influenced their observations. Future experiments should consider this factor and carefully pool faeces over a number of animals such that each animal contributes a similar number of eggs or include replications by host animal.

The development and survival model

There have been two major motivations to describe the dynamics of nematode parasites with the help of models. The first is to forecast changes and predict occurrence, whereas the second is to use model simulations in order to gain a better understanding of the biology of the nematode. To predict the occurrence of the parasite a wide range of factors have to be incorporated which makes it more difficult to investigate the effect of an individual factor based on the model. The principle aim of the present experiments on larval development and ageing have been to study the effect of temperature; therefore the development of a model was focused on further exploration of this particular effect. The Escalator Boxcar Train approach makes it possible to theoretically investigate a structured population, e.g. the individual larvae in the different stages of the ageing model differ by age. This easily allows for later integration of new traits such as the effect of age of larvae on their infectivity as described in Chapter 6. It could also be used to describe the development and survival of nematodes within the host. Furthermore, this approach allows without difficulty models on the different life-cycle segments to be combined, as seen in Chapter 4 with models on development and ageing of third-stage larvae. Nevertheless, the models in Chapter 4 require further work to identify the effect of lower and higher temperatures than those investigated in Chapter 2 and 3.

Many models on parasitic nematodes are created to simulate dynamics on pasture, in the host animals or a combination of both (Dobson et al., 1990a; Laurenson et al., 2012; Leathwick et al., 1992; Leathwick et al., 1995). None of these models partition the various factors that contribute to the outcome in detail. Nevertheless, these models deliver a research tool for studying the parasites on pasture, in the hosts and the interaction of both. In contrast, the model reported in Chapter 4 only explains the changes caused by temperature on the free-living stages of one nematode species. As such this model provides a better understanding of this single parameter than a model incorporating a greater variety of parameters would allow. However, as indicated in Chapter 4 the model needs to be validated with supplementary experiments for lower and higher temperatures.

Larval age and establishment in the host

In the study investigating the establishment rate of *C. oncophora* the RES larvae used for infections were cultured 195-210 days before the commencement of the experiment and stored at 8°C till used. The decrease in establishment over time in Group B, which did not receive any trickle infection and served as a control group, followed the same trend as seen in Group F, which was included to account for a change in the infectivity of the larvae caused by their age, indicating that the decrease in establishment was indeed due to the age of the larvae and not to the developing immune response in older calves. For each challenge infection the number of larvae given was adjusted for the proportion of viable larvae. After 293 days, the time the last animals were challenged, more than 90% of the larvae were still viable but the proportion of successfully establishing larvae decreased from >50% at the start down to only 10-20% at the end of the experiment. Taking into account that the larvae were stored before the experiment commenced their true age would have been up to 500 days. By comparison with the regression line for survival of larvae in Chapter 3 the proportion of >90% viable larvae observed in this experiment was slightly higher than that predicted. Indeed the survival experiment indicated that some larvae still survive for another 200 days but it is likely that the infectivity of these larvae in a host would

be very low. This is of practical consequence when investigating larval populations of unknown origin and age as normally encountered in the field. Even though the contamination of herbage with infective stage larvae can be estimated the ability of these to successfully establish in a host animal can vary greatly. It is, however, unclear if this is caused by the true age of the larvae or their nutritional status and should be studied in more detail. The results could potentially be incorporated into the larval ageing model and be of further advantage towards being a useful tool.

Existing worm burden affects establishment

The establishment experiment (Chapter 6) indicated that an existing *C. oncophora* burden in the host animal influences the establishment rate of newly incoming larvae. The rapid decrease of establishment rate in the two trickle-infected groups (Groups C and D) compared to the establishment rate in animals (Group E) which were trickle infected with the highest dose of larvae but effectively treated before challenge, indicated that an existing worm burden had a negative effect on the ability of the incoming larvae to successfully establish. This is in accordance with similar findings with other nematodes and hosts including *Trichostrongylus colubriformis* in sheep, *Teladorsagia circumcincta* in goats and *Haemonchus contortus* in sheep (Dobson et al., 1990c; Watson and Hosking, 1993). Such a phenomenon could have a variety of effects on the dynamics of nematode populations in the field. For example this may influence development of anthelmintic resistance in this species. If some nematodes survive a treatment this remaining population may reduce the establishment of subsequently ingested larvae thus reducing the influence of the *refugia* population. With an anthelmintic with a prolonged period of activity, the possibility that after treatment nematodes with a higher resistance to anthelmintics are the first to establish in the host as the drug concentration allows, which may then limit the establishment of fully susceptible larvae at a later time.

Live weight gain

In the establishment rate experiment (Chapter 6) there was no apparent effect on weight gain of the calves in Groups C and D which were trickle-infected with 2000 and 10,000 *C. oncophora* third stage larvae per week respectively, compared to the control animals (Group B) which were not trickle infected. Due to the housing conditions in this experiment with all cattle on the same feed pad it was observed that the control group animals did develop small infections presumably due to access to some larvae on the wood shavings or spilled feed picked up by the animals from the ground, although the baleage used was tested negative for contamination with parasitic nematodes. The lack of difference in weight gain was observed even though these trickle infections also added another 80 or 400 infective larvae/week of *Ostertagia* for Group C and D, respectively. In the design of the experiment no provision was made to measure the *Ostertagia* burden so their cumulative effect cannot be realistically estimated. Nevertheless, indications for a decrease in weight gain relating to gastrointestinal nematode infections can be found in numerous published studies. However, very few have specifically investigated the effect of *Cooperia* spp., especially *C oncophora*. Most publications infer that *C. oncophora* is not very pathogenic compared to other bovine gastrointestinal nematodes (Coop et al., 1979). In a study investigating use of sustained release boluses compared to single treatments with short-acting anthelmintics (Armour et al., 1987), the live weight gain was only significantly different between the trickle-infected animal group and the one treated with a sustained release bolus, but not between the trickle-infected group and the uninfected control. Other studies indicate that the reduction in weight gain may be caused by inappetence of calves suffering from an infection with gastrointestinal nematodes (Forbes et al., 2000). The same has also been reported for other nematodes in lambs (Sykes and Coop, 1977). In the establishment experiment in this thesis the animals were given supplementary pellets to increase the low protein content of the baleage. It is therefore possible that the nutritional value of the feed in the establishment experiment (Chapter 6) still had an overall limiting effect on the weight gain disguising a negative effect of the parasite infections. Nevertheless, this

experiment did not show a significant effect of the trickle infections on the live weight gain of the calves.

Future Prospects

There are two reasonable approaches to investigate population dynamics, one is to start with general observations and break these down into segments ("top-down") the other is the opposite ("bottom-up"). The approach followed in this thesis was to study the population dynamics for *C. oncophora* solely using the "bottom-up" process by just investigating the effects of temperature on larvae. Hence, from here on further experiments need to be conducted to enable further factors to be included as well as to investigate the life-cycle of *C. oncophora* in full, including the parasitic phase. The first would include studies to examine lower and higher temperatures for development and survival of the free-living stages. This would allow the incorporation of the adverse effects of these temperatures into models for the free-living phase. The second would be to study the death rate of established nematodes in the host which would enhance modelling the parasitic phase of the life-cycle. However, these experiments would only describe a small part of the population dynamics of *C. oncophora* and future work also needs to investigate other climate factors like humidity and biological factors such as the infectivity of aged larvae. At some stage this would allow the results of studies on *C. oncophora* from both approaches to be merged to better understand the population dynamics and exploit this knowledge for alternative control mechanisms.

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Chapter 9 Appendices

Appendix 1

Standard Operating Procedures

SOP 1 - Faecal Egg Count

1. Purpose

This SOP describes the procedure for performing faecal egg counts on animal faeces in order to calculate the number of nematode eggs or protozoal oocysts per gram.

2. Responsibility

This SOP must be followed by all staff in the Parasitology Diagnostic Laboratory, IV ABS.

3. Materials and Equipment

- Workbook to record results
- Scales to weigh faecal material (accuracy ± 0.1g)
- Small sieve (tea strainer)
- Small round bowl approx. 100ml capacity
- Teaspoon
- Pasteur pipette and rubber bulb
- Saturated NaCl solution
- Universal bottle (28ml capacity)
- McMaster Egg counting slide
- Microscope
- Slide tray
- Disposable rubber gloves
- Paper towels or toilet tissue
- Hydrometer
- Beaker for salt solution
- Household salt
- Mechanical mixer in salt container

4. Safety

Good hygiene is an important safety feature with disposable gloves providing this protection.

5. Definitions

FEC = Faecal Egg Count; epg = Eggs per gram; NaCI = Sodium chloride (table salt);
s.g. = Specific gravity

6. Procedure

6.1. To make a saturated salt solution add salt to the blue plastic container until V. full and then fill to near the top with hot water. Tum mixer on full and leave on until when tested with a hydrometer the solution reads 1.2.s.g. More salt may be added as necessary.

6.2. Place the required number of sets of utensils on the bench (sets consist of a bowl, sieve and spoon).

6.3. Place each set in tum on the scales and press tare then weigh out 2 grams of faeces.

6.4. Fill a universal bottle with saturated salt solution and pour it into the tea strainer. Work the faeces through the sieve using the teaspoon until just plant residue remains in the strainer. Ensure the sieve is in the liquid whilst stirring. Discard the strainer and rinse any lumps off the spoon.
6.5. Place the required number of McMaster slides onto a slide tray.
6.6. Mix the contents of the bowl thoroughly with the teaspoon using a to and fro action and at the same time remove a sample with a pipette. For diagnostic purposes the pipette can be rinsed with water between samples and reused.
6.7. Place the pipette at the opening of a chamber on the McMaster slide and quickly fill the chamber. Expel the remaining contents of the pipette back into the bowl.
6.8. Repeat steps 6.6 and 6.7 for the other chamber on the slide and then dispose of the pipette. Repeat these steps until all have been sampled.
6.9. Allow the slide to sit for 1-2 minutes to allow the eggs to float to the surface. This will not be necessary when doing 5-10 samples at one time.
6.10. Using the 10 X objective with 10 X eyepieces or 4 X objective with 15 X eyepieces, focus on the gridlines and air bubbles so that the eggs to be counted are on the same viewing level (never use the 40x objective).
6.11. Start at one comer of each counting grid and count eggs proceeding up and down the sections of the grid of both chambers. Count all eggs touching the top and left lines of each section but not the bottom or right hand lines. Multiply the total number of eggs by 50 to give the number of eggs per gram of faeces. This should be entered in the workbook.
6.12. Thoroughly clean all utensils under running water to remove all traces of faeces and replace in storage. Discard faecal samples in the bin for hazardous waste disposal.
6.13. If the sample weighs less than 2.0g record the weight in the workbook and use the formula: Eggs x 100 + weight to work out the epg.

7. Notes

Faecal egg counts are a useful diagnostic tool for ruminants. Faecal floats are more useful for small animals.

The counting system relies on 28 faeces displacing 2ml fluid, which together with 28ml NaCI totals 30ml. The volume under each set of gridlines is 0.15ml (1cm x 1cm x 0.15cm) for a total of 0.3ml for a slide. This represents an aliquot of 0.01 of the original implying a multiplication factor of 100 X. As there were 2g of faeces each egg counted represents 50 eggs/g.

If two slides (4 chambers) are counted for each egg/counted represents 25 eggs/g.

8. History

The original SOP was written by SM Calder and W.E. Pomroy 25.11.98.

Addition of 2 slide counting for 25 eggs/g C.W. Sauermann 2009.

9. Appendices

Manual of veterinary Parasitological Laboratory Techniques. Ministry of Agriculture. Fisheries & Food, UK, 1986.

SOP 2 - Egg & larval counting using the Flotac technique

1. Purpose

This SOP describes for counting eggs and larvae with Flotac.

2. Background

Flotac was developed by Guiseppe Cringoli (2006), University of Naples as a means to more accurately count eggs in faecal material. It uses floatation to recover eggs with a unique apparatus and its principle benefit is its ability to enable counting down to an accuracy of one egg or larva/g of faecal material. The technique has been described in a series of publications including (Bauer et al., 2010; Gaglio et al., 2008; Utzinger et al., 2008)

3. Materials and Equipment

- Gloves
- Centrifuge tubes (15ml) & rack
- Plastic pipettes
- Permanent marker (fine, best to use different colours as well)
- Beakers 150-250ml, 2x per sample (2 samples can be processed at a time)
- Small kitchen sieves/tea strainers (0.25mm aperture)
- Plastic spoons (good quality, not brittle)
- Sponge cloth
- Facial tissues
- Flotation solution (in this case $MgSO_4$, d=1.28)
- Deionised water
- Flotacs (at least 2)
- Microscope
- Centrifuge (Flotacs need to fit into buckets)
- Scale

Warning!

Flotacs are made of plastic and can get scratched easily! Always use soft tissues/sponges for cleaning (do not use paper towels – too rough!). The salt solutions will build up crystals which can scratch the plastic. Avoid this by using lots of water when cleaning.

4. Procedure (2 samples can be processed at the same time)

a) <u>!!! Soak grid, rubber and chamber of Flotac for 24 hours in deionised water before using !!!</u>

b) Label 2 set of following material, one with A the other B (best to use different colours) : 1x Flotac chamber one side, 2x beaker, 10x centrifuge tube

c) Soak grid, rubber and chamber of Flotac for 24 hours in deionised water (like for McMaster slides)

d) Weigh 10g of sample into beaker, fill up with to 100ml with deionised water and stir with spoon till no lumps are left. If sample is dry (e.g. contains crust from faecal pad) cover with water and let soak for 15 min.

e) Sieve sample through kitchen sieve (0.5mm aperture) into new beaker

f) Stir sample back and forth and fill set of 10 centrifuge tube evenly with solution using a pipette

g) Prepare second sample if needed and proceed simultaneously from here

h) Spin centrifuge tubes at 470g for 3min (set centrifuge breaks to medium/low)

i) Take Flotacs out of water bath, dry with wet sponge cloth and assemble (see Flotac manual for description and (4+5) for additional info)

j) Take 4 centrifuge tubes and discard the supernatant. Discard the remaining 6 centrifuge tubes at this stage

k) Keep 2 of these 4 tubes as a reserve until the sample has been counted

l) Fill the other 2 tubes up to 4ml with flotation solution The flotation solution has a strong osmotic effect so proceed quickly after this point

m) Vortex tubes until pellet has dissolved (hold tube at 10-12ml mark)

n) Fill one chamber with the solution from one tube and carefully top up with flotation solution (if you get bubbles in this or the following steps see *Tips*)

o) Close Flotac and prepare second (if you examine just one sample fill second with flotation solution to counterbalance the centrifuge)

p) Place Flotacs in centrifuge buckets and spin at 200g for 5min (set centrifuge breaks to medium/low)

Handle Flotacs carefully from now on! Keep them level and try not to rotate them (e.g. when you turn!) Otherwise the surface can be disturbed which will results in unreadable samples or counting failures!

q) Take Flotacs out, place on bench and rotated to reading position

r) Unscrew Flotacs and **carefully** clean surface with moist/wet(!) cloth from salt crystals if needed, dry surface with facial tissue (see *Tips*)

s) Put positioning plate on microscope stage and place Flotac on top

t) Read both chambers as for McMaster slides

u) Disassemble Flotac under running tap, clean and put back into water if used again.

5. Additional Information

Prior to assembling Flotacs they should have been soaked in water. Take parts out of water and get rid of excessive water with the use of a moist sponge cloth, never rub surface with dry cloth/paper to prevent scratching. Follow the guidelines in the Flotac manual for assembly but the following procedure is recommended to reduce the risk of getting bubbles:

- Put some flotation solution on the chamber plate and the rubber as well as between the rubber and the grid plate, excessive solution will be pressed into chambers or through gaps in reading plate. After assembling wipe excess with a moist cloth and empty the chambers with a pipette.
- Each chamber will hold 5ml. It is important to stay below this volume when you add the flotation solution to your sample so the whole sample can fit into one chamber. Top up the chamber after filling in the sample with flotation solution.

Calculating centrifuge rotor speed (RPM):

You need to measure the radius from the rotor nave to the bucket/tube holders for spinning the centrifuge tubes & the radius from the rotor nave to the bottom of the buckets you use for spinning the Flotacs – flip the buckets horizontally for measurement. Use the attached table or following formula for calculating the RPM:

$$RPM = \sqrt{\frac{g}{0.00001118 \cdot R}}$$ g=centrifugal force / R=radius in cm

6. Tips

If you get bubbles in the chambers after spinning use more flotation solution while assembling the Flotacs and try not to tighten the screw too hard

If the flotation layer under the grids is too disturbed, check if you can set the breaks of the centrifuge to a lower value. Also handle Flotacs after spinning very carefully!!

If material is very dirty after centrifuging it can be difficult to see eggs etc. In this case the sample should be diluted by using half/quarter the sample but multiply by 2/4 when calculating the epg etc..

To clean the Flotacs use plenty of running water and only soft/wet materials especially when some flotation solution has crystallised otherwise you risk scratching the Flotacs.

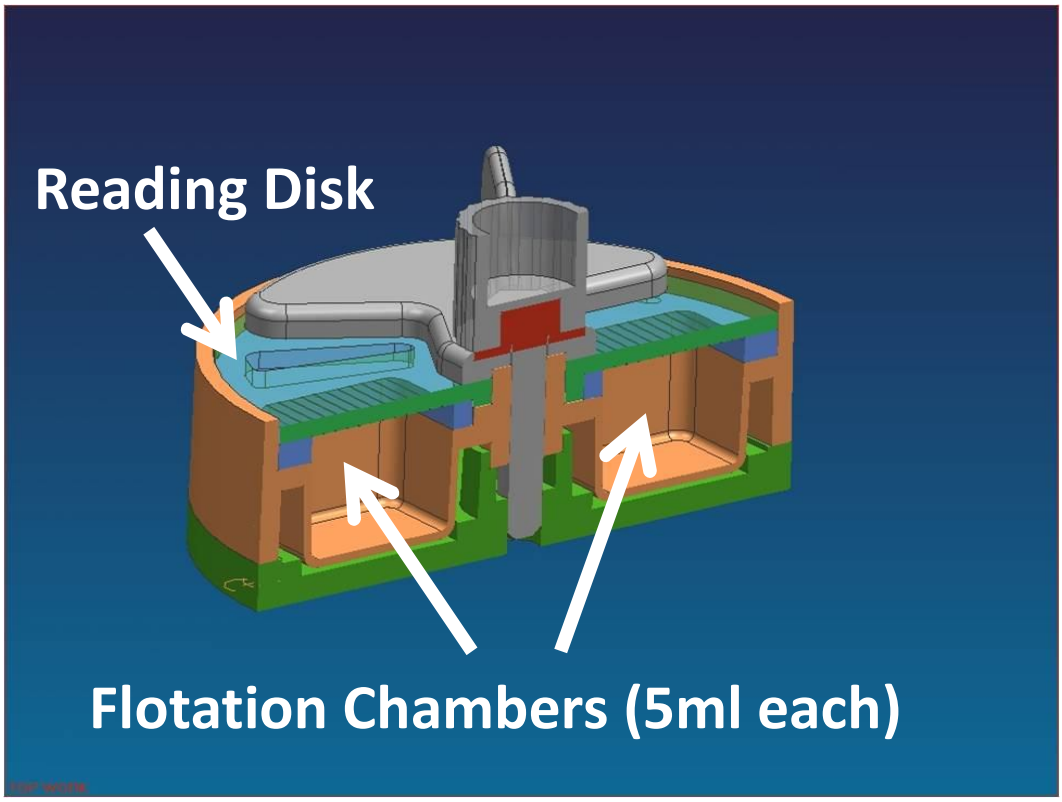
7. References

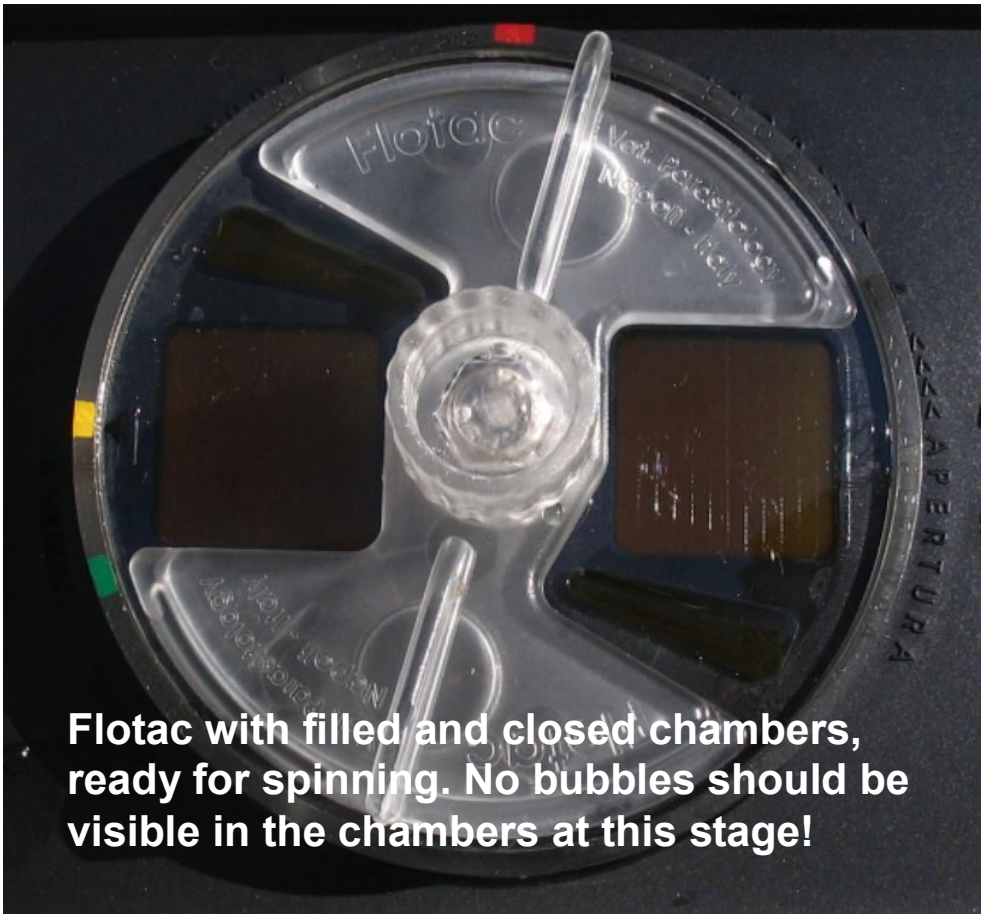
Bauer, B.U., Pomroy, W.E., Gueydon, J., Gannac, S., Scott, I., Pfister, K., 2010, Comparison of the FLOTAC technique with the McMaster method and the Baermann technique to determine counts of *Dictyocaulus eckerti* L1 and strongylid eggs in faeces of red deer (*Cervus elaphus*). Parasitology Research 107, 555-560.

Cringoli, G., 2006, FLOTAC®, a novel apparatus for a multivalent faecal egg count technique. Parassitologia 48, 381-384.

Gaglio, G., Cringoli, G., Rinaldi, L., Brianti, E., Giannetto, S., 2008, Use of the FLOTAC technique for the diagnosis of *Aelurostrongylus abstrusus* in the cat. Parasitology Research 103, 1055-1057.

Utzinger, J., Rinaldi, L., Lohourignon, L.K., Rohner, F., Zimmermann, M.B., Tschannen, A.B., N'Goran, E.K., Cringoli, G., 2008, FLOTAC: a new sensitive technique for the diagnosis of hookworm infections in humans. Transactions of the Royal Society of Tropical Medicine and Hygiene 102, 84-90.


Reading Disk
Flotation Chambers (5ml each)


Flotac
Flotac with filled and closed chambers, ready for spinning. No bubbles should be visible in the chambers at this stage!

	Radius										
RPM	10	11	12	13	14	15	16	17	18	19	20
1000	111.8	123	134.2	145.3	156.5	167.7	178.9	190.1	201.2	212.4	223.6
1100	135.3	148.8	162.3	175.9	189.4	202.9	216.4	230	243.5	257	270.6
1200	161	177.1	193.2	209.3	225.4	241.5	257.6	273.7	289.8	305.9	322
1300	188.9	207.8	226.7	245.6	264.5	283.4	302.3	321.2	340.1	359	377.9
1400	219.1	241	263	284.9	306.8	328.7	350.6	372.5	394.4	416.3	438.3
1500	251.6	276.7	301.9	327	352.2	377.3	402.5	427.6	452.8	477.9	503.1
1600	286.2	314.8	343.4	372.1	400.7	429.3	457.9	486.6	515.2	543.8	572.4
1700	323.1	355.4	387.7	420	452.3	484.7	517	549.3	581.6	613.9	646.2
1800	362.2	398.5	434.7	470.9	507.1	543.3	579.6	615.8	652	688.2	724.5
1900	403.6	444	484.3	524.7	565	605.4	645.8	686.1	726.5	766.8	807.2
2000	447.2	491.9	536.6	581.4	626.1	670.8	715.5	760.2	805	849.7	894.4
2100	493	542.3	591.6	640.9	690.3	739.6	788.9	838.2	887.5	936.8	986.1
2200	541.1	595.2	649.3	703.4	757.6	811.7	865.8	919.9	974	1028	1082
2300	591.4	650.6	709.7	768.8	828	887.1	946.3	1005	1065	1124	1183
2400	644	708.4	772.8	837.2	901.6	966	1030	1095	1159	1224	1288
2500	698.8	768.6	838.5	908.4	978.3	1048	1118	1188	1258	1328	1398
2600	755.8	831.3	906.9	982.5	1058	1134	1209	1285	1360	1436	1512
2700	815	896.5	978	1060	1141	1223	1304	1386	1467	1549	1630
2800	876.5	964.2	1052	1139	1227	1315	1402	1490	1578	1665	1753
2900	940.2	1034	1128	1222	1316	1410	1504	1598	1692	1786	1880
3000	1006	1107	1207	1308	1409	1509	1610	1711	1811	1912	2012
3100	1074	1182	1289	1397	1504	1612	1719	1826	1934	2041	2149
3200	1145	1259	1374	1488	1603	1717	1832	1946	2061	2175	2290
3300	1218	1339	1461	1583	1705	1826	1948	2070	2192	2313	2435
3400	1292	1422	1551	1680	1809	1939	2068	2197	2326	2456	2585
3500	1370	1507	1643	1780	1917	2054	2191	2328	2465	2602	2739
3600	1449	1594	1739	1884	2028	2173	2318	2463	2608	2753	2898
3700	1531	1684	1837	1990	2143	2296	2449	2602	2755	2908	3061
3800	1614	1776	1937	2099	2260	2422	2583	2744	2906	3067	3229
3900	1700	1871	2041	2211	2381	2551	2721	2891	3061	3231	3401
4000	1789	1968	2147	2325	2504	2683	2862	3041	3220	3399	3578
4100	1879	2067	2255	2443	2631	2819	3007	3195	3383	3571	3759
4200	1972	2169	2367	2564	2761	2958	3155	3353	3550	3747	3944
4300	2067	2274	2481	2687	2894	3101	3307	3514	3721	3928	4134
4400	2164	2381	2597	2814	3030	3247	3463	3680	3896	4112	4329
4500	2264	2490	2717	2943	3170	3396	3622	3849	4075	4302	4528

SOP 3- Strongylid Larval Culture and Identification SOP

1. Purpose

This SOP details the procedure for preparing larval cultures and identifying infective larvae from cattle, goat, sheep, horse and deer faeces.

2. Responsibility

This SOP must be followed by all staff in the Parasitology Diagnostic Laboratory, IVABS.

3. Materials and Equipment

3.1 Larval culture:

- Nuplex fine grade vermiculite
- scoop
- mortar and pestle or spatula
- deionised water
- glass jars and lids or plastic trays with glass lids
- 27°C incubator
- rubber gloves
- marker pen and masking tape

3.2 Baermann's apparatus:

- Either 25 cm diameter glass funnels with rubber tubing and clamps attached or plastic bowls (15 cm diameter, 10 cm high with sloping slides)
- kitchen sieve approximately 22 cm diameter with an aperture of 2 mm
- clamp
- stand for glass funnel
- culture bottles
- 10°C incubator
- suction pump
- measuring cylinder (1L or 2L)
- Snowtex tissue or equivalent

3.3 Identifying Larvae:

- Slides
- Coverslips
- aqueous or Lugol's iodine
- pipettes
- bulb
- multicounter
- eyepiece micrometer

4. Safety

Disposable gloves and lab coat should be worn when mixing faeces. If there is fungal growth in the cultures it is advisable to wear a protective face mask during mixing and recovery procedures.

5. Procedure

5.1 Cleanliness is of utmost importance. Extreme awareness of not contaminating (introducing any foreign nematodes to the culture ie change gloves with each new sample).

5.2 Vermiculite container: Do not use scoop with dirty hands or gloves. Always remove gloves when needing more vermiculite in sample being cultured.

5.3 For a bulk culture, all the faecal material is mixed with Vermiculite and water with a spatula or gloved hands depending on which is more convenient, until a consistency is achieved whereby squeezing the culture results in excess moisture being expressed. If the faeces are pelletised they can be left to soak in deionised water until they are soft enough to breakup.

5.4 For diagnostic cultures a representative sample of faeces is taken from each animal in each group and mixed together (the groups being kept separate).

5.5 The culture is either placed loosely in glass jars until they are about half full, with the lid loosely applied, or placed in trays to a depth of about 4cm with a glass lid placed on top. The jars or trays must clearly show the date the egg counts were performed, the date the cultures were put up if different from the previous and identification of the sample.

5.6 The cultures are placed in a 26-27°C incubator for 10 days. The cultures require stirring at 1-2 day intervals to ensure adequate aeration and to inhibit fungal growth. Deionised water should be added if the cultures start to dry out. They should be kept moist not wet.

5.7 After 10 days the culture can be transferred to a Baermann's apparatus. A glass funnel is placed in a stand and a clamp is placed on the rubber tubing. A kitchen sieve is then lined with a single layer of tissue paper and then the faecal culture is added to a maximum depth of 3cm. More deionised water is then added to cover the faeces. Alternatively the sieve can be placed in a plastic bowl with deionised water instead of the glass funnel.

5.8 The culture is left in the Baermann's funnel for at least 6 hours, preferably overnight. The bottom 100-200ml is tapped off by opening the clamp and allowing the fluid to be collected in a measuring cylinder or beaker. If a bowl has been used the contents are gently siphoned off from the top of the solution until 2-3cm of fluid is left

in the bowl. The sieve may be removed once the level of the solution is below the bottom of the sieve. If the sieve is removed before siphoning the bowl must be left to stand for an hour before siphoning as the sediment will have been disturbed.

5.9 The solution is then transferred to a 1-2L measuring cylinder, filled up with deionised water and then left to sediment for 2 hours. The supernatant is then carefully removed from the top of the solution with a suction pump until 100-200ml remains. If the fluid is still dirty it should be re-sedimented until the supernatant is clear. This is most important for the storage of bulk cultures but not essential for diagnostic cultures.

5.10 Cultures are stored in plastic tissue culture bottles, on their side at a depth of approximately 1 cm in a 10°C incubator. The bottles are clearly labelled with the date and identification. For bulk cultures use the date they were recovered, for other cultures use the date of FEC

5.11 To identify the larvae they are concentrated by standing the culture bottle upright for %hour. A subsample is removed from the bottom with a pipette and placed on a glass slide with a small drop of Lugol's or aqueous iodine to kill the larvae. Alternatively the slide can be flamed for approximately 3 seconds as this relaxes the larvae and causes them to straighten which aids measuring them. A coverslip is placed on top.

5.12 The slide is placed under the microscope and examined systematically. A total of 100 larvae are identified if present. The results are recorded in the workbook. Identification is made by reference to a standard text such as that mentioned in the Appendix.

6. History

This SOP is the original document and was written by SM Calder and W.E.Pomroy 25.11.98.

7. Appendix

Manual of Veterinary Parasitological Laboratory Techniques reference book 418. Pages 37 & 38.

SOP 4- Culturing individual faecal samples using subsamples

To investigate the development rate and success from eggs to third stage larvae

1. Purpose

The purpose of this SOP is to describe in detail the procedure of individually culturing faeces to investigate the development success of eggs to third stage larvae.

2. Safety

Disposable gloves and lab coat should be worn when mixing faeces. If there is fungal growth in the culture it is advisable to wear a protective mask during mixing and recovery procedures.

3. Culturing faecal samples

3.1. Materials and Equipment

- Gloves
- Scales (accuracy <0.01g)
- Bowl/bucket to mix culture
- Vermiculite (fine)
- Pottles for culturing (BS-4/100ml and BS-10/300ml, foodgrade)
- Lids for big pottles (BS-20L, foodgrade)
- Faeces for culturing

3.2. Procedure

a) All weights measured to 0.1g.
b) Change gloves for each new sample.
c) Record weight of empty bucket *(B_e)*.
d) Thoroughly mix faeces from one animal in bucket and determine FEC on three samples with an accuracy of 25epg (2 slides).
e) Record weight of bucket *(B_f)* and weight of faeces: $F = B_e - B_f$
f) Thoroughly mix faeces with vermiculite. The faeces should be crumbly but still sufficiently moist so that when squeezed some water exudes from the mixture
g) Record weight of bucket *(B_m)* to enable calculation of the weight of vermiculite added to the faeces.
h) Weigh empty 50ml pottles *(P_e)* and add the equivalent of 10g faeces to each by allowing for the weight of the additional vermiculite that had been added to the faeces and weigh *(P_m)*. The number of reps per faecal sample varied between experiments.
i) The lids of the larger 150ml pottles had 3 holes punched in them with a hot wire.

j) The small pottles without lids were then placed inside the larger 150ml pottles in which 1cm of deionised water was added. The lids of the larger 150ml pottles were then closed.
k) Place the cultures in culture room/incubator and check every 2-3 days. Open each pottle to exchange air and add deionised water if volume gets too low.
l) At time of sampling count the number of third stage larvae *($L3_p$)* individually for each pottle using Bearmann technique following SOP 3 - Appendix 1 or use the Flotac technique as in SOP 2 - Appendix 1
m) Calculate the development success using the equations below.

4. Calculating egg to L3 development success

- Amount of faeces in each pottle: $F_p = F \cdot P_m / B_m$

- Development success = $\frac{L3_p}{F_p \cdot epg}$

SOP 5 - Setting up field experiment for development of third stage larvae

1. Purpose

The purpose of this SOP is to describe in detail the procedure of setting up a field experiment on the development of third stage larvae using artificial faecal pats.

2. Materials and Equipment

- Gloves
- Faeces
- PVC-rings (20cm diameter, 20-25cm long)
- Paper towels
- Water
- Sturdy knife (blunted)
- 2x plastic pottles (200ml, 15cm inner diameter)
- Spoon
- Scissors
- Scale (battery powered, accuracy <0.1 gram)
- Tape measure (and string)
- Black permanent waterproof pen (Vivid)
- Plastic bags (sturdy freezer bags)
- Rubbish bag

3. Procedure

a) Mix faecal sample thoroughly to ensure an even distribution of eggs. Take 1 pottle and cut out bottom. Cut the side of this pottle so it can be opened. This will be the mould for the faecal pats.

Prepare rings:

b) Lay out tape measure in straight line according to first line of rings.
c) Take first ring and press down on ground at beginning of tape measure. Cut into ground along the ring using the knife.
d) Press ring down into ground until only 10cm stays above ground and mark using a permanent marker (row & number).
e) Repeat steps (c) + (d) at each full metre mark along the tape measure.
f) For determine position of next line of rings measure 1m sideways from first and last ring in previous line and repeat steps b to e.

Prepare rings with bottom mesh:

g) Cut ground for ring as in step c and put ring aside.
h) Adjust depth of cut so that only 10cm of ring will be above ground.
i) Lift up sward and soil to fit the ring into ground. Put sward and soil into a plastic bag.
j) Put ring into hole.
k) Mechanically remove earthworms from sward/soil.
l) Submerge sward and soil in water overnight. Remove earthworms next morning.
m) Refill rings with soil and sward on top.

Bring out faecal pats:

n) Cut grass in and around each ring with scissors to a height of 3 cm.
o) Put whole pottle on scale and zero. Fill pottle with 200 gram of faeces (approx. full to capacity).
p) Lay down cut pottle onto ground in middle of ring and fill with measured sample.
q) Open cut pottle carefully on side and lift away.
r) Repeat steps (o) to (r) for each ring.

4. Additional Information

- Rings are easier to push into ground if the lower end is “sharpened” using a file or sand paper.
- Tilting ring slightly from side to side when pressing down makes the process easier.
- Mark rings on shade side (south in NZ) as even black marker ink may not be photo-stable.

SOP 6 - Processing samples for development in the field experiment

1. Purpose

The purpose of this SOP is to describe in detail the procedure for taking samples for the experiment on larval development in the field and processing these samples.

2. Materials and Equipment

- Gloves
- Freezer bags (medium, 250 x 350 mm)
- Freezer bags (10L)
- Knife (sturdy, blunt)
- Scissors
- Permanent waterproof marker
- Glass funnel (20cm diameter) with rubber tube on their base and tube clamp
- Coarse kitchen sieves (1mm mesh) that will sit within the top of the funnel
- Whitehead&Hemming trays – plastic trays (20 x 30 x 6 cm) with bottom removed and a layer of 1mm mesh glued with silicon sealant over the entire base. These trays are placed inside a similar unmodified tray.
- Small buckets (5L)
- Tissue paper
- Dishwashing detergent
- Ruler
- Scale
- Drying cabinet with stainless steel trays (for grass)
- Tissue culture Flasks (100mls) for sample storage
- 1ml counting chambers – perspex ring (outer diameter 1cm, inner diameter 0.8cm) glued to standard microscope slide.
- coverslips
- Compound microscope

3. Procedure

Taking samples in the field

a) Identify plots to sample.
b) Transfer any faecal material from plot into freezer bag and label.
c) Cut grass with scissors, collect cuttings in freezer bag and label.
d) Take top 5cm of soil including the turf and place on large freezer bag. It is often possible to carefully lift the PVC ring up on one side so it will normally still contain the soil inside. Otherwise first cut the turf & soil away outside one side of the ring to enable a horizontal cut on the underside of the ring using the knife. Rings with bottom mesh can be sampled carefully using the first method.
e) Cut off top 5cm (use ruler), transfer into large freezer bag and label remainder is discarded.

Processing faecal samples using the Baermann technique

a) Weigh sample. If sample is >20g use half of the sample for FEC with a McMaster slide.
b) Put one layer of tissue paper over the sieve, suspend the sieve in the funnel and place faeces onto the tissue paper.
c) Fill funnel with tap water to cover the faeces
d) Leave at room temperature for overnight (16-24 hours)
e) Transfer fluid in lower part of rubber tube into flask, label and store in fridge until counting

Processing grass samples

f) Weigh sample and use the Baermann technique overnight as for the faecal samples.
g) Transfer fluid into flask and label.
h) Dry grass from funnel in incubator at 70-80°C overnight and weigh.

Processing soil samples using Whitehead and Hemming Tray Method

i) Weigh sample and break up into small pieces.
j) Place a single layer of tissue paper into the tray with sieve bottom, place soil sample into this tray, place this tray into second entire tray, cover with water and leave overnight.
k) The next day slowly lift the inner tray. Let inner the tray sit on the rim of the lower tray and leave for 10-15min.
l) Get rid of any earthworms in the lower tray (and on the lab floor).
m) Transfer fluid in lower tray into small bucket and leave overnight.
n) Carefully siphon off the supernatant fluid and keep the bottom 4-5cm.
o) Transfer fluid into flask and let sediment overnight in the fridge at 4°C.
p) Siphon down to 3cm (sample can still be dirty).
q) Transfer sediment to 1ml counting chamber, add water to shallow positive meniscus, apply coverslip
r) Count larvae using compound microscope (100X).

4. Additional Information

Be very careful and deliberate setting up the whitehead trays as it will reduce the amount of debris in the samples which can speed up the counting process significantly!

SOP 7 - Larval survival experiment

1. Purpose

The purpose of this SOP is to describe in detail the procedure preparing and conducting a laboratory experiment on survival of third stage larvae.

2. Materials and Equipment

- Gloves
- Recovered nematodes
- Deionised water
- Microscopic probe (heat treated fishing line or eyelash glued to toothpick)
- Pipette
- Microscopic slides/petri dish
- Stereo microscope
- 24 well plates (Multidish 24 wells, Nunc), one for each temperature
- Empty 2L ice cream container (for experiments at constant temperatures, 1 for each well plate)
- Temperature logger (Onset)
- Laboratory counter
- Permanent marker

3. Procedure

Preparing experiment

a) For each larval sample mark clearly mark the identity of each well on the corresponding lid (3 wells for each sample for the set temperature experiments, 4 wells for each sample for variable temperature experiments). Also label multiwall plate with the desired temperature, date, name and “Do NOT touch!”
b) Transfer 30-50 larvae from each sample into each of the corresponding wells.
c) Top-up water level in each well (also empty wells) to ½ to ¾.
d) Prepare one multi well plate for each temperature following steps a-c.
e) Put each multi well plate into an ice cream container if larvae will be stored at constant temperatures.
f) Store multiwell plates at the desired temperature settings. Check temperatures with logger placed next to plate (in ice cream container).

Conducting experiment

a) For counting larvae take plate and slightly tap on hard surface (table).
b) Place under stereo microscope and carefully stir each well with a soft probe. This will cause live larvae to coil or move.
c) Count each larvae that is curled up or moving. If larvae are straightened (banana shaped) and show no movement then gentle touch with the soft probe and look for a reaction – if the larvae does not react assume they are dead and do not count.
d) After plate is counted repeat steps g-I twice more to give a total of 3 counts per plate.
e) Check water level in each well and top-up if required.

4. Additional Information

- Counting larvae will get exponentially harder with the number of larvae per well. A useful number to enable effective counting is 30 larvae per well.
- Especially at higher temperatures the larvae are very active which in addition to previous point will make counting difficult. Repeated counting will minimize counting errors.
- Larvae can lie in the lower rim between bottom and walls of the well. Because of the refraction the light at the water surface they might not be seen and counted, especially when the water level in the well is too high (!).

SOP 8 - Setting up for the field experiment for survival of third stage larvae

1. Purpose

The purpose of this SOP is to describe in detail the procedure of setting up a field experiment to measure the survival of third stage larvae. The contamination of the herbage uses a watering can and third stage larvae in water.

2. Materials and Equipment

- Gloves
- Third stage larvae
- Watering can (5L) with sprinkler
- Water (not chlorinated!)
- Long plastic ruler
- String + 2 x pegs
- Tape measure

3. Procedure

Preparing larvae and plots

a) Prepare one flask containing an adequate number of larvae for each experimental plot.

b) The herbage height on the plots should be 3 to 5cm.

Contaminating experimental plots

c) Identify location of 5m X 1m experimental plots

d) Place a string along the middle (use tape measure) of the 5m long plot and secure ends with pegs.

e) Transfer contents of one flask into watering can, fill flask with water and tip into watering can.

f) Fill watering can with water to the 5L mark. After this step proceed quickly or use the ruler to stir the contents of the watering can back and forth just before contamination.

g) Pour contents of the watering can inside the borders of the experimental plot along the string. Make sure not to hold the watering can too low or too high, the water should touch the herbage within 10-15cm on each side of the string.

h) Repeat steps c to g for each plot due for contamination.

4. Additional Information

Practice the contamination with the watering can on a different piece of paddock. Lay out a 5m long piece of the string and use just water. Practice how high the watering can needs to be above ground and how fast to move along the string.

SOP 9 - Processing samples for survival in the field experiment

1. Purpose

The purpose of this SOP is to describe in detail the procedure of taking samples for the experiment on larval development in the field and the following processing of these samples.

2. Materials and Equipment

- Gloves
- Big plastic bags
- Permanent waterproof marker
- Tape measure and string
- Mechanical push mower with catch
- Modified Baermann funnels for grass samples
- Scale
- Dishwashing detergent
- Drying cabinet (for grass)
- Flasks to store samples

3. Procedure

a) Measure out middle line for each plot to be sampled.
b) In each plot cut grass in the 50cm centre line using push mower, transfer cuttings into plastic bag and label.
c) In the laboratory weigh each sample and divide into 300g sub samples.
d) Transfer each sub sample into Baermann funnels, fill with hand warm water, add one drop of dishwashing detergent and leave for 24h.
e) Fill fluid in lower tube into labelled flask. Samples from the sub samples can be pooled for each plot. Store the flasks upright in fridge overnight and siphon down to 3cm.
f) Combine grass samples for each plot, dry overnight in cabinet and weigh.

4. Additional Information

Dirt in the sample is normally sand and cannot be removed using further sedimentation.

SOP 10 - Preparing infective stage larvae for trickle infection in the Establishment Trial

1. Purpose

The purpose of this SOP is to describe in detail the procedure of preparing a solution of infective stage larvae to orally infect animals in the Establishment Trial.

2. Materials and Equipment

- Gloves
- Beaker
- Canister to store solution
- Deionised water
- Larvae from fridge
- Pipette
- Microscope

3. Procedure (susceptible strain for Establishment trial: concentration=34 L3/ml)

a) Fill the canister with 8-8.5L deionised water and leave in fridge over night
b) Take flask with pre counted susceptible strain larvae solution and add to canister
c) Tighten canister lids!
d) Shake well 6-10 times
e) Thoroughly clean beaker with deionised water!
f) Fill beaker with solution (app. 50ml)
g) Stir solution in beaker and take 1ml sample
h) Count worms and repeat 5 times (5x1ml samples)
i) Calculate the average of the counts and multiply by 0.96 (solution contains 96% Cooperia) to give number of Cooperia larvae/ml. Calculate the volume required to deliver 1000 larvae
j) The concentration of Cooperia larvae should be 34 L3/ml which will deliver 1020 L3 in a full drench gun dose (max. 30ml). If concentration is too low, carefully add a few drops from SUS reserve flash (marked with red X) and repeat steps c-i.
k) The drench gun can only be set to even numbers of ml (24-26-28-30). Choose the nearest to infect animals. 1 dose for the group receiving 1000 larvae (blue ear-tag) and 5 doses for the group receiving 5000 (red ear-tag).
l) For infecting animals follow SOP 12– Appendix 1

SOP 11 - Artificial infection of donor calves with infective stage larvae

1. Purpose

The purpose of this SOP is to describe in detail the procedure for infecting calves with parasitic nematode infective stage larvae for subsequent harvesting of nematode eggs. All infections are applied per os (oral).

2. Safety

For safety reasons the number of animals does not matter, however if infecting large groups of animals extra care should be taken. Follow the guidelines given by Massey University regarding work clothes and large animals handling. When handling large animals make sure **not to work alone**. If animals are big or nervous consider the use of a head bail.

3. Infecting animals

This method is used for infecting donor calves with an approximate number of infective stage larvae. If the number of calves to infect is larger refer to SOP "Artificial infection of large calve numbers with infective stage larvae" or for precise control of larval numbers "L3Inf_RES Artificial challenge of animals with parasitic nematode infective larvae"

4. Materials and Equipment

- Gloves
- 2x Beaker (for fieldwork preferable plastic)
- 2x Syringe (50ml) with 10cm rubber tube attached
- Water
- Larvae solution (adjust larvae concentration to 40-50ml solution per animal)

5. Procedure

a) Fill one beaker with larvae solution and toughly stir back and forth every time before filling syringe. Fill second beaker with water.
b) Fill syringe with appropriate volume of solution, get rid of any air bubbles and refill syringe to needed volume if necessary. Fill second syringe with water.
c) Secure animal head with your arm or head bale and elevate the mouth.
d) Open animal mouth and insert tip of tube/syringe into cheek pouch or behind the dorsal prominence of the tongue. Empty syringe slowly and make sure animal has swallowed before releasing.
e) Repeat step d) with the water filled syringe.

6. Tips

Beware – cattle have strong necks and especially when bailed are quite stressed. Do not get caught/squashed between the cattle head and fixed objects! If you cannot secure the cattle head by hand use a halter and a rope to restrain the head in a head bail pulled 90° sideways.

SOP 12 - Artificial infection of large numbers of calves with infective stage larvae

1. Purpose

The purpose of this SOP is to describe in detail the procedure of infecting large groups of animals (cattle) with parasitic nematode infective stage larvae. All infections are applied per os (oral).

2. Responsibility

This SOP must be followed by all staff in the animal experiments carried out by Christian Sauermann

3. Safety

For safety reasons the number of animals does not matter, however if infecting large groups of animals extra care should be taken. Follow the guidelines given by Massey University regarding work cloth and large animals handling. When handling large animals make sure **not to work alone**. If animals are big or nervous consider the use of a head bail.

4. Infecting animals

This method is used for infecting large calve numbers with infective stage larvae. For the infection of donor calves refer to SOP "Artificial infection of donor calves with infective stage larvae" or for precise control of larval numbers "L3Inf_RES Artificial challenge of animals with parasitic nematode infective larvae"

5. Materials and Equipment

- Gloves
- Drench gun (20-30ml capacity, new/never been used with drench!!!)
- 5 litre container for drench gun (new/never been used with drench!!!)
- Sample tubes
- Larvae in water

6. Procedure

a) Connect drench gun to container. Make sure the tube connecting the canister and the drench gun is secured firmly.
b) Gently rock the container whilst filling the drench gun. Make sure the lids are closed tightly before going to next step.
c) Prime drench gun.
d) Secure animal head with your arm or head bale and elevate the head.
e) Open animal mouth and dose the animal. The tip of the drench gun should be inserted into the cheek pouch or behind the dorsal prominence of the tongue. Before releasing animal or if more than one squirt has to be applied make sure the animal swallows the solution (putting the thumb of the hand holding the cattle under the tip of the tongue can trigger swallowing).
f) After dosing each animal gently rock the container to keep larvae from settling whilst refilling the drench gun.
g) After each pause (e.g. sampling, getting new animals in etc.) the solution has to be remixed by gently rocking the container. The fluid in the tube to the drench gun has to be replaced by squirting the drench gun 3-4 (solution can be put back into container).
h) Periodically take samples of the larvae solution: At specific times while infecting the animals fill a sample tube with a squirt of the drench gun. These samples should be analysed in the lab for checking larvae concentration and viability.

7. Tips

Beware – cattle have strong necks and especially when bailed are quite stressed. Do not get caught/squashed between the cattle head and fixed objects! If you cannot secure the cattle head by hand use a halter and a rope to restrain the head in a head bail pulled 90° sideways.

Safety First!

SOP 13 - Challenge calves with a defined number of infective stage nematode larvae

1. Purpose

The purpose of this SOP is to describe in detail the procedure of infecting calves with defined numbers of parasitic nematode infective stage larvae. For the infection of donor calves refer to SOP "Artificial infection of donor calves with infective stage larvae" or for the infection of large calves numbers with infective stage larvae refer to SOP "Artificial infection of large calve numbers with infective stage larvae"

2. Safety

For safety reasons the number of animals does not matter, however if infecting large groups of animals extra care should be taken. Follow the guidelines given by Massey University regarding work cloth and large animals handling. **Never work alone** (!) when handling large animals. If animals are big or nervous consider the use of a head bail and/or pony halter.

3. Materials and Equipment

- Gloves
- Sample tubes with larvae solution
- Water
- Single dose drench gun (never been used with drench!!!). These are filled dose by dose through the delivery nozzle

Or

- 60ml Syringe with 10cm rubber tube attached

4. Procedure

a) Prepare one sample tube with larvae solution containing the exact amount of larvae for each animal.
b) Shake tube with solution and fill drench gun/syringe.
c) Secure animal head with your arm or head bale and elevate the head.
d) Open animal mouth and apply larvae solution. The tip of the drench gun should be inserted into the cheek pouch or behind the dorsal prominence of the tongue. Make sure the animal swallowed. If the tube still contains solution refill and re-dose.
e) Rinse tube twice with a little bit of water and re-dose the animal as above.

5. Tips

Beware – cattle have strong necks and especially when bailed are quite stressed. Do not get caught/squashed between the cattle head and fixed objects! If you cannot secure the cattle head by hand use a halter and a rope to restrain the head in a head bail pulled 90° sideways.

Safety First!

SOP 14 - Washing and sieving small intestinal contents

1. Purpose

The purpose of this SOP is to describe in detail the procedure for recovering the desired aliquot of intestinal contents from a bovine small intestine.

2. Materials and Equipment

- Gloves
- Plastic apron
- 2x Bucket (one with volume scale and up to 20L for big animals)
- Tray
- Sink/basin
- Scissors
- Sieve 38µm (+ backup sieve!)
- Stirrer (plastic ruler or the like)
- Scoop (50ml)
- Beaker (100-200ml)
- 2 pottles (250-500ml)
- Garden sprinkler (see picture)
- Plastic bucket to hold 38 µm sieve with lower half removed (sieve must fit 2-3 cm into the top)
- Hose and connector (for sprinkler)
- Knife, scissors
- Ruler

3. Procedure to obtain 10% aliquot of intestinal contents

a) Defrost small intestine if frozen (should be the same throughout an experiment)

b) Strip contents manually into a bucket by gently squeezing the intestine through one hand and pull with other so the contents remain in the part of the gut still in the bucket. Be careful not to rip intestine. It may be necessary to cut the intestine of bigger animals if the amount of contents gets too much.

c) Fill first 20-30cm of intestine with water and repeat step (b) above This will flush the inside of the intestine. Repeat this process for a second time.

d) Cut open the whole intestine using scissors.

e) Over a tray wash the inside surface of intestine thoroughly under a low water flow by rubbing with fingers.

f) Combine all contents collected this far in one bucket

g) Fill content of bucket up with water to next volume scale mark. While thoroughly stirring back and forth take out 10% aliquot by removing portions with a scoop and filling a measured container to the required volume. Repeat this step for a second 10% aliquot. Place one washed sample in fridge for counting (can be stored up to a week if kept cool.

Preserve second sample as a reserve with formalin to achieve a final concentration of 10%.

h) Wash and sieve each 10% sample using the 38µm sieve. This was either undertaken manually or with the use of a sprinkler situated under the sieve

i) Manual Sieving
- small amounts of contents at a time makes it easier to avoid clogging the sieve

j) Sieving with a sprinkler
- Connect hose to sprinkler using the connector. Place sprinkler in sink and cut hose to a length that 20cm of the hose rest on bottom of the sink and the end can be easily connected to tap.
- Place sieve into bucket over the sprinkler so that sieve sits 5 cm above the top of the sprinkler. Cut 4 holes into lower end of bucket to allow water to escape (see picture).
- Slowly turn on water and, If possible, change angle of sprinkler nozzles to allow for slow turning when sieving. This may need some adjustments the first time you use it with samples.
- When samples are placed into sieve, the water from the sprinkler should just gently push through the sieve in order to inhibit blockage by sample. If too much water pressure is too high it may lift sample partly out of sieve or may damage nematodes.
- Time to sieve varies but varies from 20-60 minutes

k) If required the washed small intestine may be refrozen for potential digestion at a later time.

SOP 15 - Sieving small intestinal or abomasal contents utilising a garden sprinkler

1. Purpose

The purpose of this SOP is to describe in detail the procedure of preparing a garden sprinkler to be used when sieving gastrointestinal content samples.

2. Materials and Equipments

- Garden sprinkler (see picture)
- Bucket (sieve must fit 2-3 cm into the top)
- Hose and connector (for sprinkler)
- Knife, scissors, waterproof marker, ruler
- 10% aliquot of small intestinal or abomasal contents

3. Procedure

a) Connect hose to sprinkler using the connector. Place sprinkler in sink and cut hose to a length that 20cm of the hose rest on bottom of the sink and the end can be easily connected to tap.
b) Measure height of sprinkler, how deep the sieve sits in the bucket and add 5 cm (distance between sprinkler and sieve). Cut the bucket to this height. Cut 4 holes into lower end of bucket (see picture).
c) Place bucket onto sprinkler with hose going through one of the holes. Place sieve inside bucket and slowly turn on water.
d) If possible, change angle of sprinkler nozzles to allow for slow turning when sieving. This may need some adjustments the first time you use it with samples.
e) When samples are placed into sieve, the water from the sprinkler should just gently push through the sieve in order to inhibit blockage by sample. If water pressure is too high it may lift sample partly out of sieve or may damage nematodes.
f) Time to sieve varies but normally takes between 20-60 minutes.

SOP 16 - Counting worms in intestinal samples

1. Purpose

The purpose of this SOP is to describe in detail the procedure for counting worms in intestinal samples

2. Materials and Equipment

- Gloves
- Normal saline solution
- 70% alcohol
- Water
- Sample to process. Normally a 10% aliquot of intestinal washings already sieved through a 38µm sieve.
- Petri dish – marked with lines 10mm apart
- Spoon
- Microscopic probe (needle or glass)
- Labelled pottles
- Small bucket (waste)
- Multi-counter
- Dissecting microscope

3. Procedure

a) Warning! When nematodes are needed for further processing use the following solutions for storage:
   - Normal saline if size measurements are required – store in fridge.
   - 70% alcohol if nematodes may be used for PCR.
   - 70% alcohol or 10% formalin otherwise.

b) Transfer ½ spoon of sample into petri dish and place under dissecting microscope at a magnification so that two 10mm channels were visible in a field of view.

c) Systematically examine contents

d) Transfer each nematode to a prepared pottle and add 1 on counter for gender, respectively.

e) Dispose of sample from petri dish into waste bucket when finished.

f) Repeat steps b to f until whole sample is finished.

4. Tips

- Before commencing a worm count get some spare nematodes and place them into a petri dish sample to practice. Recognizing nematodes in samples gets faster and easier with experience.
- Get someone with experience to check some of the waste for missed nematodes at the beginning.
- When encountering dirty samples or samples containing lots of mucus (sticks contents together) reduce amount of sample transferred into petri dish.

SOP 17 - Measuring the body length of adult nematodes

1. Purpose

The purpose of this SOP is to describe in detail the procedure for measuring the body length of adult nematodes.

2. Materials and Equipment

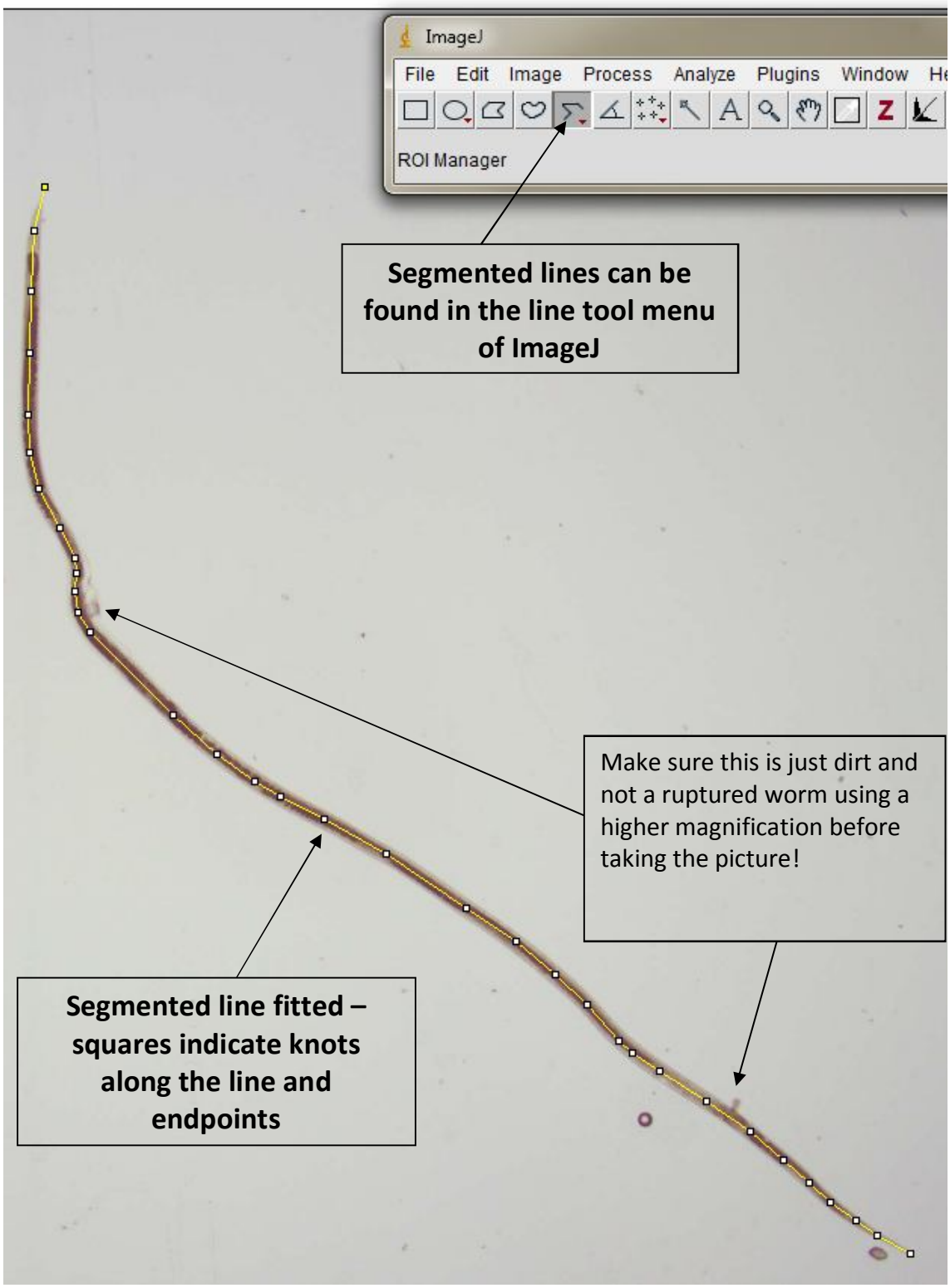
- Gloves
- Normal saline solution
- Recovered nematodes (in normal saline)
- Petri dish
- Microscopic probe (needle or glass)
- Microscope slide with measurement scale (Objective Micrometer – Olympus, Tokyo) in µm to calibrate with the software
- Pipette
- Microscope slides and cover slips
- Plasticine (modelling clay)
- Black rubber mat (10x10cm) or other dark sheet
- Dissecting microscope
- Compound microscope with 1.25X objective and camera
- PC and an image analysing software (ImageJ for Microscopy)

3. Procedure

a) Warning! Storage of nematodes in formalin or alcohol can potentially lead to shrinkage, compromising measurement results. Only use water for processing sampled intestines and normal saline to store nematodes.
b) Transfer nematodes into the petri dish and place under the dissecting microscope.
c) Put 1 drop of normal saline onto a microscope slide and place onto black rubber (nematodes will be whitish).
d) Transfer nematodes to prepared slide using probe. Multiple nematodes can be arranged side by side.
e) Under the dissecting microscope check if all nematodes are undamaged and are apart from each other.
f) Knead a small amount of plasticine till soft and apply a small amount at each corner on one side of a cover slip (foots). These will prevent the coverslip squeezing the nematodes, leading to false measurements or damage.
g) Carefully place cover slip on the slide – one side first and gently lower other side (using the probe may help).
h) Recheck slide under dissecting microscope again.
i) Place slide under compound microscope using 1.25X objective and take a picture of every nematode on the slide.
j) Carefully remove cover slip and wash nematodes off cover slip and slide with normal saline. The nematodes will then be used for egg counting as a measure of fecundity.
k) Transfer pictures to analysing software and use the segmented line tool to measure total length for each nematode in each picture.
l) Copy measurements from ImageJ to Excel, the values from ImageJ will by default be pixel. Measure pixel per mm with grid/measurement slide to transfer measurement into mm.

4. Additional Information

- Get some spare nematodes and get familiarized with the procedure and the software before the experiment.
- The raw measurements with ImageJ are in numbers of pixels. ImageJ offers a function to transfer these pixel counts into other units such as µm. For this the software needs to be calibrated using an image of the measured slide with known dimensions.
- Even slightly damaged nematodes cannot be used for measurements as the length of the animal might have been affected.



Screenshot of nematode with overlaid segmented line as measured by ImageJ.

SOP 18 - Counting eggs in adult female nematodes

1. Purpose

The purpose of this SOP is to describe in detail the procedure for counting the eggs in adult female nematodes.

2. Materials and Equipment

- Gloves
- Recovered nematodes
- Petri dish
- Microscopic probe (needle or glass)
- Pipette
- Microscopic slides and cover slips
- Plasticine (modelling clay)
- Lactophenol (see below)
- Black rubber mat (10x10cm) or other dark sheet
- Dissecting microscope
- Compound microscope with Differential Interference Contrast (Olympus BH2 DIC)

3. Procedure

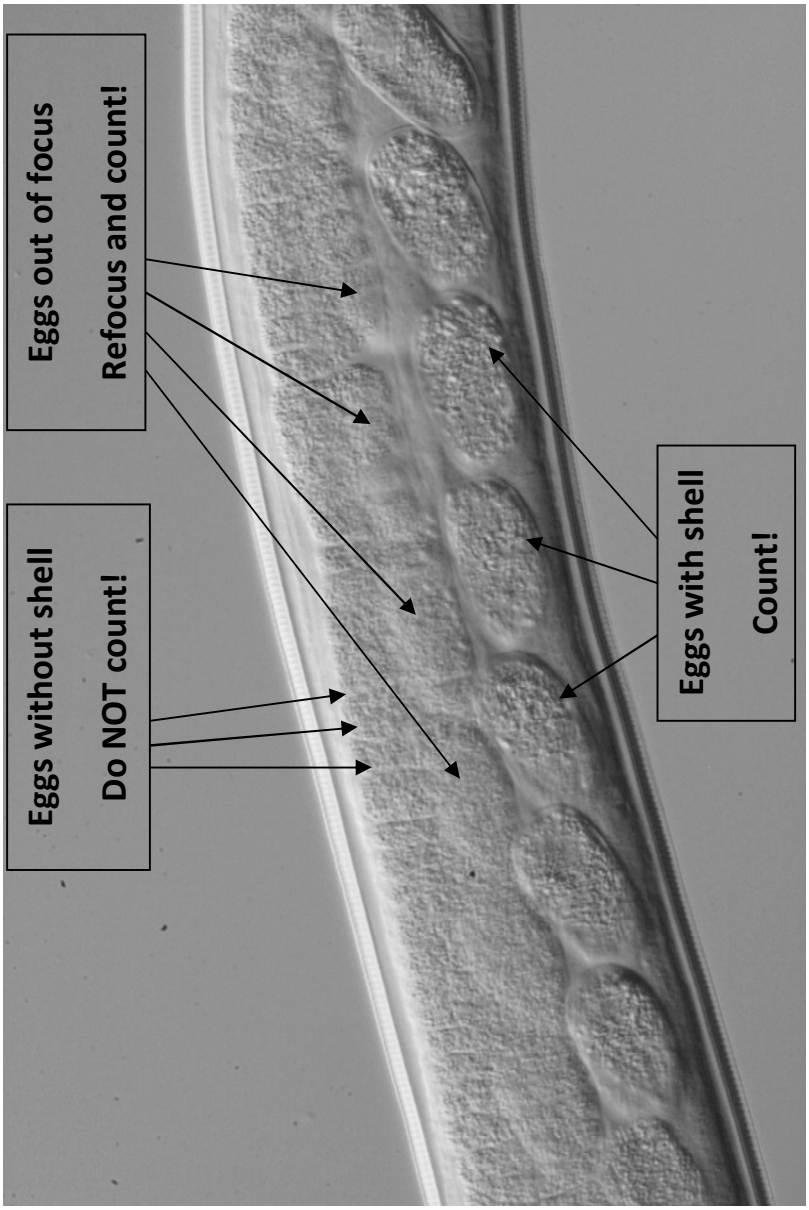
a) Beware! Nematodes that have been stored in formalin or alcohol are sometimes brittle and can break, rendering them unusable for egg counting purpose.
b) Transfer nematodes into petri dish and place under dissecting microscope.
c) Put 1 drop of lactophenol onto a microscope slide and place onto rubber (nematodes will be whitish at first). Lactophenol tends to spread on glass, practice before using!
d) Transfer nematodes to prepared slide using probe. Multiple nematodes can be arranged on one slide.
e) Under the dissecting microscope check if all nematodes are undamaged and are apart from each other.
f) Knead a small amount of plasticine till soft and apply a very small amount at each corner on one side of a cover slip (foots). These will prevent the coverslip to squeeze and damage the nematodes.
g) Carefully place cover slip on slide – one side first and gently lower other side (using the probe may help).
h) Recheck slide under dissecting microscope again. Add additional lactophenol if necessary.
i) Place slide under compound microscope and turn up lightning to maximum. Nearly close aperture diaphragm and move stage to a position that the light hits the back side of the slide. This will then allow the light to pass through the slide from the edge.
j) Nematodes will now be visible by eye on the slide as white lines. Sketch the position of the nematodes – this will help you to find them later.
k) Move stage to first nematode. Adjust light to comfortable level and turn on Differential interference contrast.
l) Count eggs and record results next to nematode in sketch. If nematode was found to be damaged, strike out on sketch. Count eggs if an obvious oval shape is apparent.
m) Continue with the remaining nematodes following a systematically pattern.
n) Dispose of slide and transfer results.

4. Additional Information

Get some spare nematodes and become get familiarized with the procedure before counting for the experiment.

5. Preparing lactophenol:

a) Dissolve 15g polyvinyl alcohol in 100ml distilled water (using a water bath (80°C) or incubator to dissolve).
b) Add 40ml lactic acid
c) Add 40ml phenol crystals
d) Keep in a dark glass bottle!



Picture of adult female nematode indicating the difference between eggs *in utero* with and without shells. Only count eggs with shell for a measure of fecundity.

SOP 19 - Small intestinal pepsin digest

1. Purpose

The purpose of this SOP is to describe in detail the procedure of undertaking a pepsin digest on a previously washed bovine small intestine.

2. Materials and Equipment

- Gloves
- Plastic apron
- Glass jars
- Aluminium foil
- Trays (2x)
- Sink/basin
- Bucket (with scale)
- Scissors
- Scoop
- Beaker (100ml with scale)
- Sieve 250 µm (kitchen, +backup)
- Sieve 38 µm (+ backup sieve)
- Stirrer (plastic ruler or the like)
- 2 pottles (50-100ml)
- Permanent marker
- Heated water bath
- Sponge cloth/paper towels
- Pepsin (Merck)
- 33% Hydrochloric acid (Merck)

3. Preparing pepsin solution

In the following order (!) add:

1. 600ml H O
2. 10g Pepsin (Merck)
3. 10ml conc. HCl (always add acid to water)

4. Procedure to obtain 10% aliquot of intestinal contents

a) Defrost small intestine if frozen (use same storage process throughout an experiment).
b) Turn on water bath and set to 37°C.
c) Cut small intestine into 5-10cm pieces.
d) Evenly fill glass jars with cuttings (up to ½ by volume) and add pepsin solution to ¾.
e) Close jars with aluminium foil and label with marker.
f) Put jars into water bath. The water needs to be able to freely flow around jars. The water should be level with the fluid level within the jars.
g) Leave jars in water bath for 2 hours.
h) For each jar separately sieve contents through kitchen sieve (1mm apertures) into a bucket. Wash sieve contents with tap water 2x.
i) Fill bucket with water to next scale mark.
j) Transfer 10% of contents into a beaker.
k) Wash this 10% aliquot over a 38 µm sieve using warm tap water and transfer into a labelled pottle.
l) Repeat steps j+k for a reserve sample.
m) Store both samples at -20°C in different locations.
n) Dispose of digested intestine and bucket contents
o) Count as for intestinal contents (see Appendix x)

5. Additional Information

When washing digested contents use warm water. The fat in the contents will solidify in cold water clogging up the sieve.

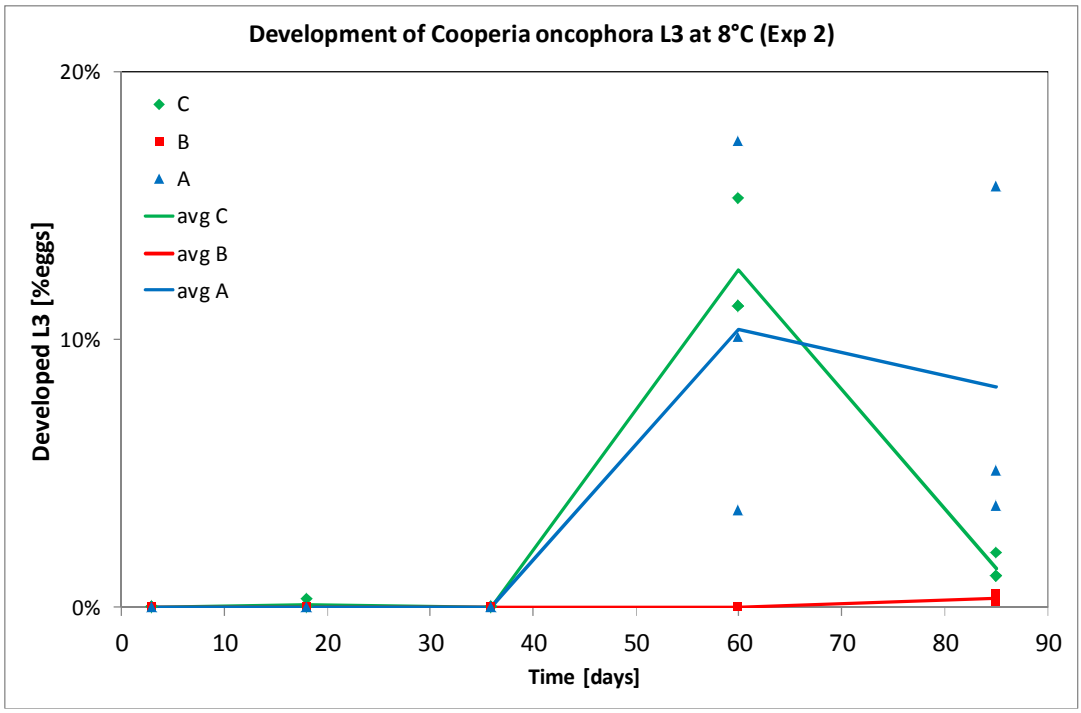
Appendix 2

Supplementary information for Chapter 2

The effect of temperature on development of the free-living stages of *Cooperia oncophora*

9.2 Chapter 2 - Development of egg to third stage larvae

9.2.1 Supplementary figures for development at constant temperatures



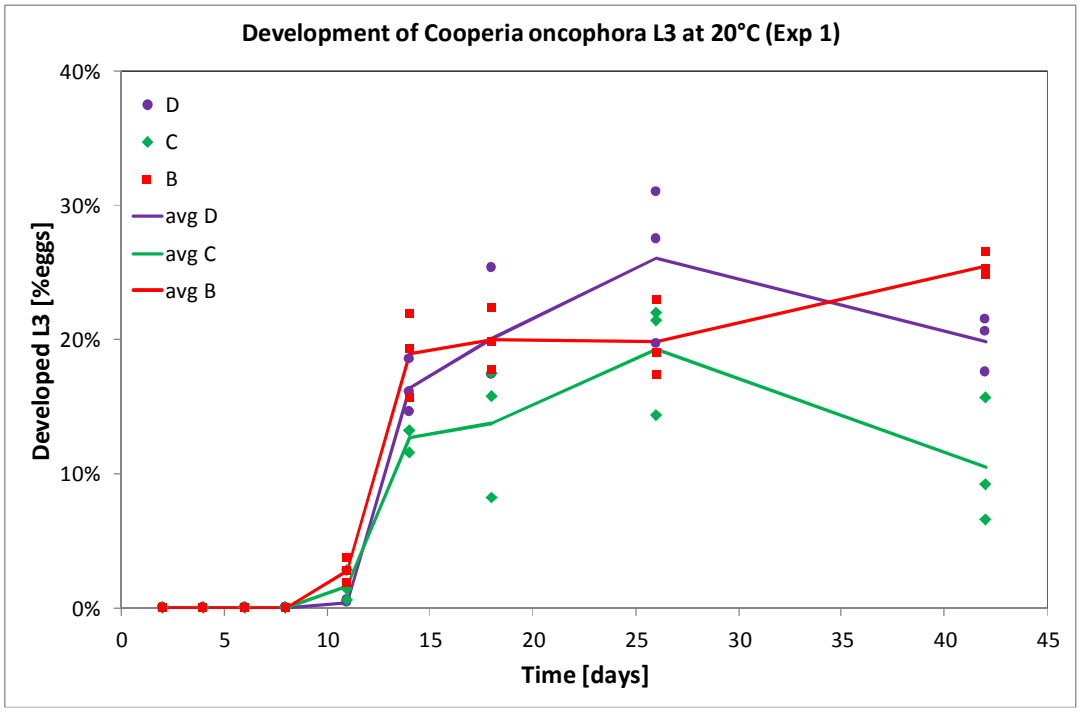


Figure 9.2.1 - *Cooperia oncophora* egg to third stage larvae development at 8 and 20°C in faecal cultures prepared from 3 different hosts.

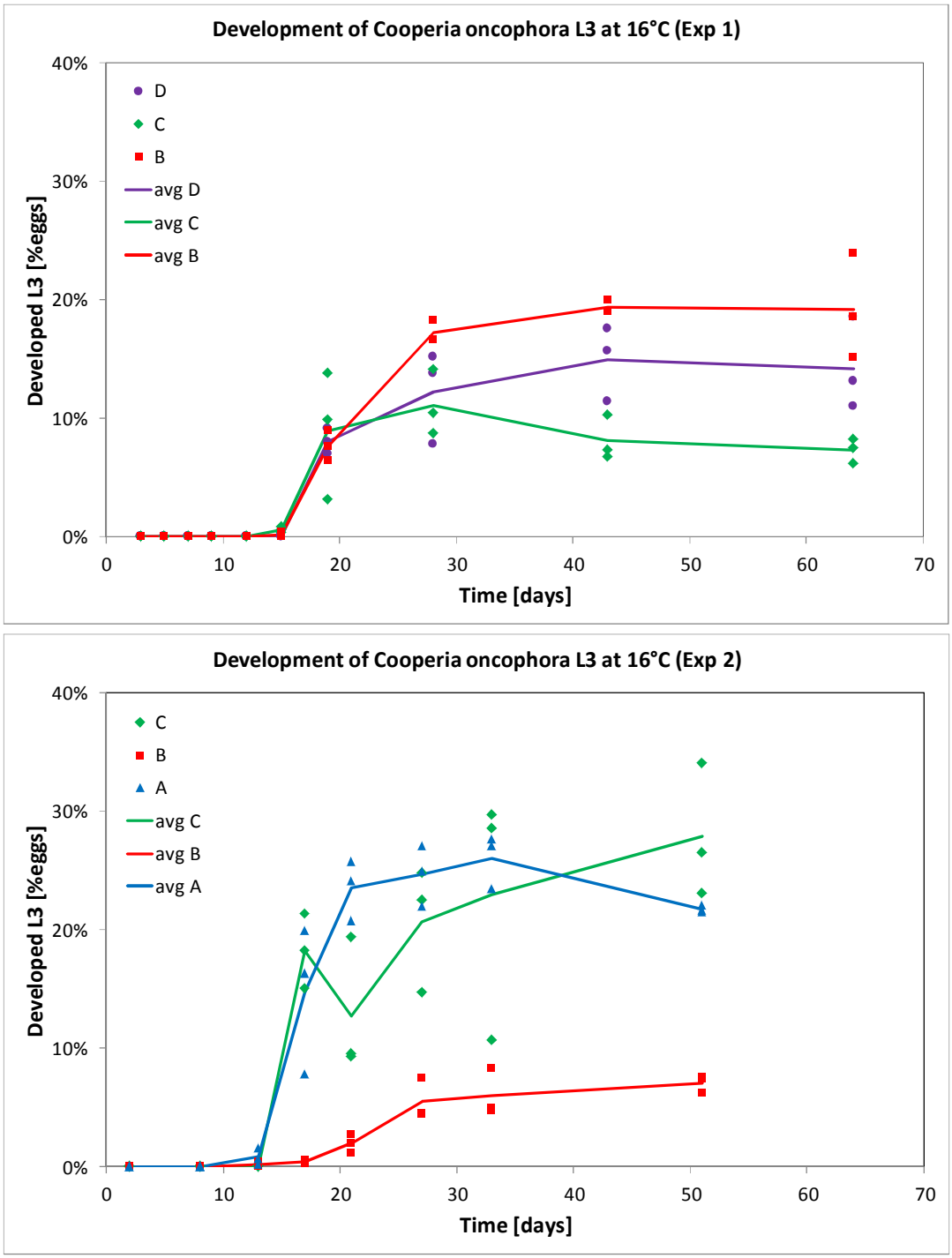


Figure 9.2.2 - *Cooperia oncophora* egg to third stage larvae development at 16°C for both experiments in faecal cultures prepared from 3 different hosts.

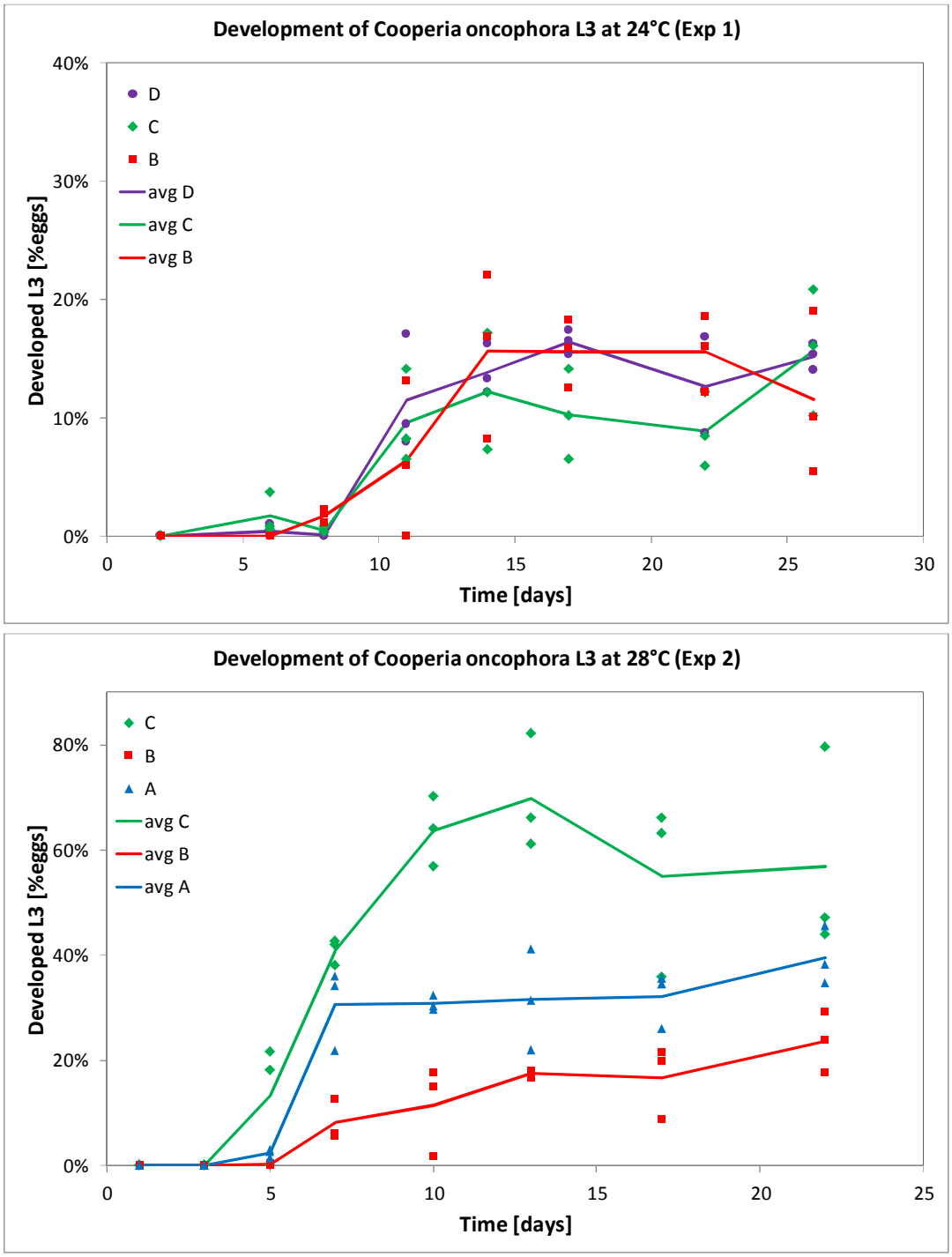


Figure 9.2.3 - *Cooperia oncophora* egg to third stage larvae development at 24 and 28°C in faecal cultures prepared from 3 different hosts.

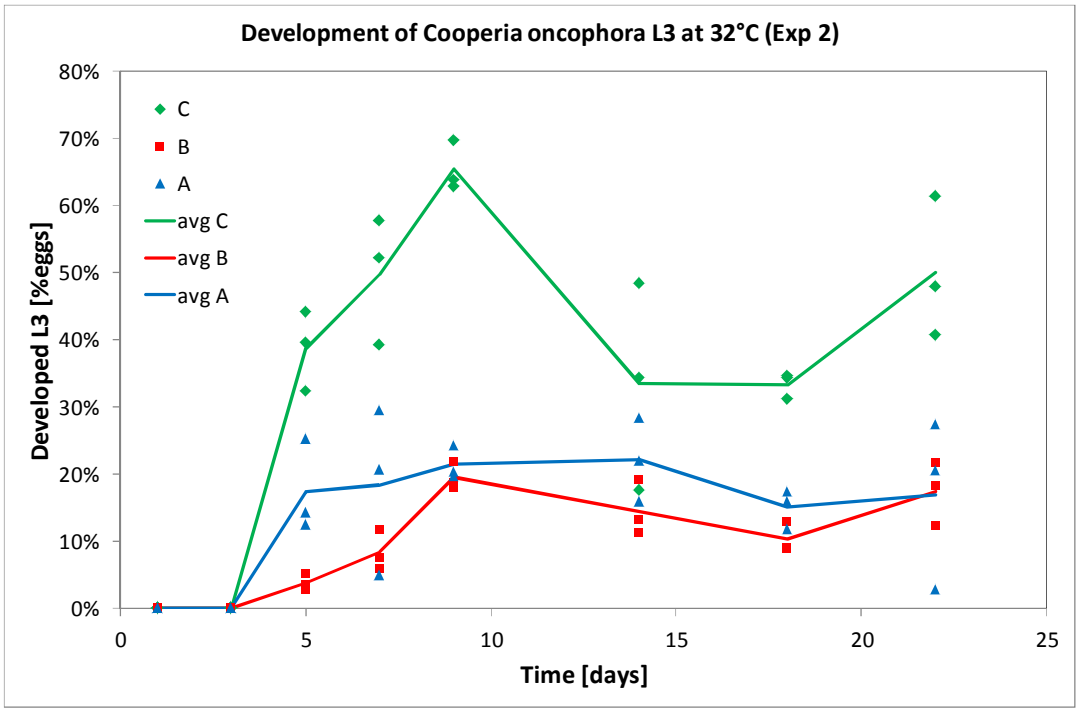


Figure 9.2.4 - *Cooperia oncophora* egg to third stage larvae development at 32°C in faecal cultures prepared from 3 different hosts.

9.2.2 Data - Development at constant temperatures

Temp = temperature, Exp = experiment 1 or 2, Days = time after experimental start in days, Host = host animal the faeces was sourced from, Host-ID = host ear tag number, Eggs = number of eggs counted, L1/L2/L3 = number of first/second/third stage larvae counted.

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
8	exp2	3	A	132	0.37434	0.00088	0	0
8	exp2	3	A	132	0.41327	0	0	0
8	exp2	3	A	132	0.37788	0	0	0
8	exp2	3	B	137	0.54707	0	0	0
8	exp2	3	B	137	0.55955	0	0	0
8	exp2	3	B	137	0.54707	0	0	0
8	exp2	3	C	146	0.99519	0	0	0
8	exp2	3	C	146	1.07885	0.00577	0	0
8	exp2	3	C	146	0.83654	0	0	0
8	exp2	18	A	132	0.00354	0.00265	0	0
8	exp2	18	A	132	0.00619	0.05575	0	0
8	exp2	18	A	132	0.00619	0.03009	0	0
8	exp2	18	B	137	0.00718	0.07977	0	0
8	exp2	18	B	137	0.00983	0.21777	0	0
8	exp2	18	B	137	0.02004	0.26805	0	0
8	exp2	18	C	146	0.02596	0.36058	0	0.00288
8	exp2	18	C	146	0.02019	0.73846	0	0
8	exp2	18	C	146	0.04327	0.00577	0	0
8	exp2	36	A	132	0	0.19735	0.00088	0
8	exp2	36	A	132	0.00354	0.19292	0	0
8	exp2	36	A	132	0.00177	0.12478	0.00177	0
8	exp2	36	B	137	0.00189	0.18374	0	0
8	exp2	36	B	137	0.00227	0.06503	0	0
8	exp2	36	B	137	0.00227	0.05898	0	0
8	exp2	36	C	146	0	0.06058	0	0
8	exp2	36	C	146	0.00865	0.19327	0	0
8	exp2	36	C	146	0	0.04904	0	0
8	exp2	60	A	132	0	0.01062	0.0177	0.17434
8	exp2	60	A	132	0	0.08673	0.04956	0.03628
8	exp2	60	A	132	0.00177	0.03097	0.0177	0.10088
8	exp2	60	B	137	0.00113	0.00794	0.00227	0
8	exp2	60	B	137	0.00038	0.01966	0.00265	0
8	exp2	60	B	137	0	0.03365	0.00454	0
8	exp2	60	C	146	0.00288	0.00865	0.00865	0.15288
8	exp2	60	C	146	0	0.00577	0	0.1125
8	exp2	60	C	146	0.00288	0.02019	0.02308	0.1125
8	exp2	85	A	132	0	0.09204	0.05929	0.03805
8	exp2	85	A	132	0.00088	0.00885	0.01593	0.15752
8	exp2	85	A	132	0	0.02743	0.02566	0.05133

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
8	exp2	85	B	137	0	0.03138	0.00832	0.00227
8	exp2	85	B	137	0.00038	0.01323	0.00491	0.00491
8	exp2	85	B	137	0	0.01021	0.0034	0.00302
8	exp2	85	C	146	0	0.18173	0.02308	0.01154
8	exp2	85	C	146	0	0.21923	0.05769	0.02019
8	exp2	85	C	146	0	0.08365	0.02308	0.01154
16	exp1	3	B	137	0.00374	0.11028	0	0
16	exp1	3	B	137	0.00935	0.09533	0	0
16	exp1	3	B	137	0.00935	0.1271	0	0
16	exp1	3	C	146	0.00563	0.11549	0	0
16	exp1	3	C	146	0.00563	0.11549	0	0
16	exp1	3	C	146	0.00563	0.10986	0	0
16	exp1	3	D	306	0.00568	0.19117	0	0
16	exp1	3	D	306	0.00568	0.37855	0	0
16	exp1	3	D	306	0.00757	0.16278	0.00189	0
16	exp1	5	B	137	0.00187	0.01308	0	0
16	exp1	5	B	137	0	0.02056	0	0
16	exp1	5	B	137	0	0.01121	0	0
16	exp1	5	C	146	0.01127	0.16338	0	0
16	exp1	5	C	146	0.00563	0.07606	0	0
16	exp1	5	C	146	0	0.09577	0	0
16	exp1	5	D	306	0.00189	0.00946	0	0
16	exp1	5	D	306	0.00757	0.0265	0	0
16	exp1	5	D	306	0.00189	0.02461	0	0
16	exp1	7	B	137	0	0.01308	0	0
16	exp1	7	B	137	0.00187	0.02243	0	0
16	exp1	7	B	137	0	0.01308	0	0
16	exp1	7	C	146	0.00282	0.02535	0	0
16	exp1	7	C	146	0.00282	0.02254	0	0
16	exp1	7	C	146	0	0.05352	0	0
16	exp1	7	D	306	0.00189	0.0511	0	0
16	exp1	7	D	306	0	0.04164	0	0
16	exp1	7	D	306	0	0.053	0	0
16	exp1	9	B	137	0	0.10093	0	0
16	exp1	9	B	137	0	0.0972	0	0
16	exp1	9	B	137	0.00748	0.16449	0.01495	0
16	exp1	9	C	146	0.00282	0.01972	0	0
16	exp1	9	C	146	0	0.06761	0	0
16	exp1	9	C	146	0	0.04225	0	0
16	exp1	9	D	306	0	0.03975	0	0
16	exp1	9	D	306	0.00379	0.10221	0.00379	0
16	exp1	9	D	306	0.00189	0.03218	0	0
16	exp1	12	B	137	0	0.08972	0.01495	0
16	exp1	12	B	137	0	0.13832	0.0486	0

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
16	exp1	12	B	137	0	0.09159	0.04486	0
16	exp1	12	C	146	0	0.01127	0.00563	0
16	exp1	12	C	146	0	0.08451	0.0169	0
16	exp1	12	C	146	0.00282	0.10704	0.01408	0
16	exp1	12	D	306	0	0.03975	0.00189	0
16	exp1	12	D	306	0.00189	0.11356	0.00757	0
16	exp1	12	D	306	0	0.02271	0.00189	0
16	exp1	15	B	137	0	0.10467	0.04112	0.00187
16	exp1	15	B	137	0	0.09346	0.02617	0
16	exp1	15	B	137	0	0.12897	0.06542	0.00374
16	exp1	15	C	146	0	0.08169	0.04507	0.00563
16	exp1	15	C	146	0.00282	0.07887	0.0338	0.00845
16	exp1	15	C	146	0	0.10141	0.04507	0.00282
16	exp1	15	D	306	0	0.05868	0.03785	0.00189
16	exp1	15	D	306	0.00189	0.0511	0.01893	0
16	exp1	15	D	306	0	0.07003	0.04353	0
16	exp1	19	B	137	0.00187	0.05234	0.02804	0.08972
16	exp1	19	B	137	0	0.05421	0.02243	0.06355
16	exp1	19	B	137	0	0.05981	0.03551	0.07664
16	exp1	19	C	146	0	0.05915	0.00845	0.09859
16	exp1	19	C	146	0	0.02254	0.03662	0.13803
16	exp1	19	C	146	0	0.03662	0.02817	0.03099
16	exp1	19	D	306	0	0.06435	0.03028	0.07003
16	exp1	19	D	306	0.00189	0.09653	0.0511	0.0795
16	exp1	19	D	306	0	0.08707	0.03218	0.09085
16	exp1	28	B	137	0	0.00374	0.01121	0.18318
16	exp1	28	B	137	0	0.00374	0.00374	0.16636
16	exp1	28	B	137	0	0	0.01682	0.16636
16	exp1	28	C	146	0	0	0.02535	0.14085
16	exp1	28	C	146	0	0.00563	0.0169	0.10423
16	exp1	28	C	146	0	0.00563	0.01127	0.08732
16	exp1	28	D	306	0	0.01136	0.02271	0.0776
16	exp1	28	D	306	0	0.02271	0.03028	0.13817
16	exp1	28	D	306	0	0.01136	0.01136	0.15142
16	exp1	43	B	137	0	0	0.00374	0.2
16	exp1	43	B	137	0	0	0.00187	0.19065
16	exp1	43	B	137	0	0	0.00187	0.19065
16	exp1	43	C	146	0	0.00187	0.00187	0.1028
16	exp1	43	C	146	0	0	0	0.06729
16	exp1	43	C	146	0	0.00187	0.00187	0.0729
16	exp1	43	D	306	0	0	0.00935	0.15701
16	exp1	43	D	306	0	0	0.00748	0.11402
16	exp1	43	D	306	0	0.00187	0.00374	0.1757
16	exp1	64	B	137	0	0	0	0.23925

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
16	exp1	64	B	137	0	0	0	0.1514
16	exp1	64	B	137	0	0	0	0.18505
16	exp1	64	C	146	0	0	0	0.08224
16	exp1	64	C	146	0	0	0	0.07477
16	exp1	64	C	146	0	0	0	0.06168
16	exp1	64	D	306	0	0	0	0.13084
16	exp1	64	D	306	0	0	0	0.11028
16	exp1	64	D	306	0	0	0	0.18505
16	exp2	2	A	132	0.45664	0.00708	0	0
16	exp2	2	A	132	0.47434	0.0177	0	0
16	exp2	2	A	132	0.54336	0.01947	0	0
16	exp2	2	B	137	0.74405	0.00076	0	0
16	exp2	2	B	137	0.57164	0.01437	0	0
16	exp2	2	B	137	0.52779	0.01815	0	0
16	exp2	2	C	146	1.22308	0.01154	0	0
16	exp2	2	C	146	0.89423	0.01154	0	0
16	exp2	2	C	146	0.81923	0.01154	0	0
16	exp2	8	A	132	0.00442	0.22389	0.00619	0
16	exp2	8	A	132	0.00708	0.31416	0.03451	0
16	exp2	8	A	132	0.00354	0.26283	0.05929	0
16	exp2	8	B	137	0.00529	0.20643	0.00832	0
16	exp2	8	B	137	0.00416	0.10057	0.00265	0
16	exp2	8	B	137	0.0034	0.13535	0.00265	0
16	exp2	8	C	146	0.02019	0.08077	0	0
16	exp2	8	C	146	0.01154	0.15288	0	0
16	exp2	8	C	146	0.01731	0.38654	0	0
16	exp2	13	A	132	0	0.07876	0.03717	0.01593
16	exp2	13	A	132	0.00354	0.09558	0.0354	0.00177
16	exp2	13	A	132	0.00354	0.18319	0.05044	0.00619
16	exp2	13	B	137	0.00076	0.04499	0.00718	0
16	exp2	13	B	137	0.00189	0.06238	0.00945	0
16	exp2	13	B	137	0.00113	0.11531	0.02873	0.00378
16	exp2	13	C	146	0.00577	0.075	0.02019	0
16	exp2	13	C	146	0.01731	0.05769	0.02596	0
16	exp2	13	C	146	0.00288	0.09231	0.03173	0
16	exp2	17	A	132	0.00177	0.02389	0.05752	0.07788
16	exp2	17	A	132	0.00354	0.02389	0.04336	0.19912
16	exp2	17	A	132	0.00265	0.04425	0.03894	0.16283
16	exp2	17	B	137	0.00113	0.03894	0.00756	0.00302
16	exp2	17	B	137	0	0.01853	0.00529	0.00227
16	exp2	17	B	137	0.00076	0.04575	0.01059	0.00605
16	exp2	17	C	146	0.01154	0.10385	0.09231	0.18173
16	exp2	17	C	146	0.02019	0.19904	0.06923	0.15
16	exp2	17	C	146	0.00577	0.11827	0.07788	0.21346

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
16	exp2	21	A	132	0	0.03451	0.05841	0.25752
16	exp2	21	A	132	0	0.04956	0.06018	0.20796
16	exp2	21	A	132	0.00088	0.01681	0.03097	0.24159
16	exp2	21	B	137	0.00076	0.11493	0.01059	0.01172
16	exp2	21	B	137	0	0.04499	0.01588	0.01966
16	exp2	21	B	137	0	0.06087	0.02193	0.02684
16	exp2	21	C	146	0.01154	0.03462	0.02885	0.09519
16	exp2	21	C	146	0	0.01731	0.02308	0.09231
16	exp2	21	C	146	0	0.02308	0.03173	0.19327
16	exp2	27	A	132	0.00088	0.00265	0.01681	0.24956
16	exp2	27	A	132	0	0.0115	0.02566	0.21947
16	exp2	27	A	132	0.00177	0.00442	0.0177	0.2708
16	exp2	27	B	137	0.00076	0.01701	0.02647	0.04423
16	exp2	27	B	137	0	0.01172	0.01664	0.04499
16	exp2	27	B	137	0	0.03025	0.031	0.07486
16	exp2	27	C	146	0	0.00288	0.04904	0.225
16	exp2	27	C	146	0	0	0.02019	0.24808
16	exp2	27	C	146	0	0.00577	0.01442	0.14712
16	exp2	33	A	132	0	0	0.01593	0.2708
16	exp2	33	A	132	0	0.00088	0.03009	0.23451
16	exp2	33	A	132	0	0.00088	0.01593	0.27611
16	exp2	33	B	137	0	0.01021	0.03667	0.08318
16	exp2	33	B	137	0.00038	0.00038	0.02609	0.04688
16	exp2	33	B	137	0	0.00605	0.02949	0.04877
16	exp2	33	C	146	0	0	0	0.10673
16	exp2	33	C	146	0	0	0.01154	0.28558
16	exp2	33	C	146	0	0	0.01442	0.29712
16	exp2	51	A	132	0	0.00088	0.0115	0.22035
16	exp2	51	A	132	0	0	0.01062	0.21681
16	exp2	51	A	132	0	0	0.0115	0.21504
16	exp2	51	B	137	0	0.00113	0.01021	0.062
16	exp2	51	B	137	0	0.00076	0.01172	0.07524
16	exp2	51	B	137	0	0.00113	0.01626	0.07372
16	exp2	51	C	146	0	0	0.00288	0.26538
16	exp2	51	C	146	0	0	0.00577	0.34038
16	exp2	51	C	146	0	0	0	0.23077
20	exp1	2	B	137	0.02243	0.15327	0	0
20	exp1	2	B	137	0.00561	0.09907	0	0
20	exp1	2	B	137	0.01495	0.26542	0	0
20	exp1	2	C	146	0.00845	0.06479	0	0
20	exp1	2	C	146	0.00845	0.0507	0	0
20	exp1	2	C	146	0.00845	0.06479	0	0
20	exp1	2	D	306	0.01325	0.21388	0	0
20	exp1	2	D	306	0.01325	0.21199	0	0

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
20	exp1	2	D	306	0.00757	0.053	0	0
20	exp1	4	B	137	0.00187	0	0	0
20	exp1	4	B	137	0.00187	0.00748	0	0
20	exp1	4	B	137	0	0.01869	0	0
20	exp1	4	C	146	0	0.0169	0	0
20	exp1	4	C	146	0.00282	0.00563	0	0
20	exp1	4	C	146	0.00282	0.02817	0	0
20	exp1	4	D	306	0.00379	0.00568	0	0
20	exp1	4	D	306	0.00189	0.04921	0	0
20	exp1	4	D	306	0.00189	0.0265	0	0
20	exp1	6	B	137	0.00374	0.08972	0	0
20	exp1	6	B	137	0.00187	0.06355	0	0
20	exp1	6	B	137	0.00187	0.1028	0	0
20	exp1	6	C	146	0	0.06479	0	0
20	exp1	6	C	146	0	0.10704	0	0
20	exp1	6	C	146	0	0.10141	0	0
20	exp1	6	D	306	0.08139	0.2858	0.00568	0
20	exp1	6	D	306	0.00189	0.08517	0.00189	0
20	exp1	6	D	306	0	0.13438	0.00189	0
20	exp1	8	B	137	0.00187	0.1514	0.00187	0
20	exp1	8	B	137	0	0.10093	0.00187	0
20	exp1	8	B	137	0.00561	0.17757	0.00187	0
20	exp1	8	C	146	0.00282	0.09577	0	0
20	exp1	8	C	146	0	0.04789	0	0
20	exp1	8	C	146	0	0.12958	0	0
20	exp1	8	D	306	0	0.11167	0.00568	0
20	exp1	8	D	306	0	0.12492	0.00379	0
20	exp1	8	D	306	0.00189	0.20063	0.00379	0
20	exp1	11	B	137	0	0.08972	0.00935	0.02804
20	exp1	11	B	137	0	0.0972	0.02056	0.03738
20	exp1	11	B	137	0	0.08785	0.03738	0.01869
20	exp1	11	C	146	0	0.06197	0.0169	0.02817
20	exp1	11	C	146	0	0.07324	0.00282	0.00563
20	exp1	11	C	146	0	0.05352	0.01408	0.01408
20	exp1	11	D	306	0	0.10978	0.01893	0.00379
20	exp1	11	D	306	0	0.053	0.01514	0.00568
20	exp1	11	D	306	0	0.08139	0.03785	0.00379
20	exp1	14	B	137	0	0.02991	0.04299	0.19252
20	exp1	14	B	137	0	0.03178	0.0243	0.21869
20	exp1	14	B	137	0	0.03178	0.02804	0.15701
20	exp1	14	C	146	0	0.00845	0.0169	0.11549
20	exp1	14	C	146	0	0.01127	0	0.13239
20	exp1	14	C	146	0	0.03099	0.01127	0.13239
20	exp1	14	D	306	0	0.03975	0.01136	0.14574

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
20	exp1	14	D	306	0	0.03596	0.00568	0.18549
20	exp1	14	D	306	0	0.03218	0.01514	0.16088
20	exp1	18	B	137	0	0.01308	0.02056	0.17757
20	exp1	18	B	137	0.00187	0.01121	0.02804	0.2243
20	exp1	18	B	137	0	0.01308	0.03551	0.19813
20	exp1	18	C	146	0	0.00282	0.0169	0.08169
20	exp1	18	C	146	0	0	0.0169	0.17465
20	exp1	18	C	146	0	0.00845	0.01408	0.15775
20	exp1	18	D	306	0	0.00379	0.01514	0.17413
20	exp1	18	D	306	0	0.00379	0.00189	0.17413
20	exp1	18	D	306	0	0.00568	0.02839	0.25363
20	exp1	26	B	137	0	0	0.00935	0.17383
20	exp1	26	B	137	0	0	0.01682	0.19065
20	exp1	26	B	137	0	0.00374	0.02804	0.22991
20	exp1	26	C	146	0	0	0.00563	0.21972
20	exp1	26	C	146	0	0	0.00845	0.21408
20	exp1	26	C	146	0	0	0	0.14366
20	exp1	26	D	306	0	0	0.01893	0.19685
20	exp1	26	D	306	0	0	0.01325	0.31041
20	exp1	26	D	306	0	0.00189	0.02839	0.27445
20	exp1	42	B	137	0	0	0.00187	0.26542
20	exp1	42	B	137	0	0	0	0.2486
20	exp1	42	B	137	0	0	0	0.25234
20	exp1	42	C	146	0	0	0.00187	0.06542
20	exp1	42	C	146	0	0	0	0.09159
20	exp1	42	C	146	0	0	0.00374	0.15701
20	exp1	42	D	306	0	0	0.00374	0.1757
20	exp1	42	D	306	0	0	0.00374	0.21495
20	exp1	42	D	306	0	0	0.01121	0.20561
24	exp1	2	B	137	0.00935	0.04486	0	0
24	exp1	2	B	137	0.00374	0.06729	0	0
24	exp1	2	B	137	0.01308	0.06542	0	0
24	exp1	2	C	146	0.00986	0.07887	0	0
24	exp1	2	C	146	0.01643	0.06573	0	0
24	exp1	2	C	146	0.00329	0.09531	0	0
24	exp1	2	D	306	0.01546	0.11041	0	0
24	exp1	2	D	306	0.00505	0.06562	0	0
24	exp1	2	D	306	0.00883	0.05079	0	0
24	exp1	6	B	137	0.00187	0.11028	0.00187	0
24	exp1	6	B	137	0	0.16449	0	0
24	exp1	6	B	137	0	0.0972	0.00374	0
24	exp1	6	C	146	0.09859	0.0169	0.07042	0.00845
24	exp1	6	C	146	0.11549	0.03944	0.08169	0.03662
24	exp1	6	C	146	0.17183	0.01972	0.06197	0.00563

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
24	exp1	6	D	306	0.053	0.02082	0.03596	0.00946
24	exp1	6	D	306	0.03218	0.02082	0.03407	0.00379
24	exp1	6	D	306	0.06814	0.03596	0.04353	0
24	exp1	8	B	137	0	0.04486	0.01308	0.02243
24	exp1	8	B	137	0.00187	0.03551	0.00748	0.01121
24	exp1	8	B	137	0	0.04112	0.03364	0.01869
24	exp1	8	C	146	0	0.04507	0.01972	0.00282
24	exp1	8	C	146	0	0.01972	0.01408	0.00845
24	exp1	8	C	146	0	0.02254	0.00845	0.00282
24	exp1	8	D	306	0	0.03218	0.00379	0.00189
24	exp1	8	D	306	0	0.03596	0.00189	0
24	exp1	8	D	306	0	0.09085	0.00189	0
24	exp1	11	B	137	0	0.02056	0.00748	0.05981
24	exp1	11	B	137	0.01869	0.01308	0.0972	0
24	exp1	11	B	137	0	0.01495	0.01308	0.13084
24	exp1	11	C	146	0	0.01972	0.00845	0.06479
24	exp1	11	C	146	0	0.02535	0.01972	0.08169
24	exp1	11	C	146	0	0.0169	0.00563	0.14085
24	exp1	11	D	306	0	0.0511	0.03785	0.17035
24	exp1	11	D	306	0.00189	0.03596	0.0265	0.0795
24	exp1	11	D	306	0	0.03028	0.03218	0.09464
24	exp1	14	B	137	0	0.01121	0.02056	0.16822
24	exp1	14	B	137	0	0.0243	0.01495	0.22056
24	exp1	14	B	137	0	0.01308	0.00374	0.08224
24	exp1	14	C	146	0	0.0169	0	0.07324
24	exp1	14	C	146	0.18873	0	0.00282	0.12113
24	exp1	14	C	146	0.16338	0.00845	0	0.17183
24	exp1	14	D	306	0	0.01325	0.00379	0.16278
24	exp1	14	D	306	0	0.00757	0.00946	0.12114
24	exp1	14	D	306	0	0.04164	0.01325	0.13249
24	exp1	17	B	137	0	0.00374	0.02056	0.12523
24	exp1	17	B	137	0	0.00748	0.02243	0.18318
24	exp1	17	B	137	0	0.01495	0.03551	0.15888
24	exp1	17	C	146	0	0	0.01127	0.10141
24	exp1	17	C	146	0	0.00282	0.0169	0.14085
24	exp1	17	C	146	0	0.00845	0.00845	0.06479
24	exp1	17	D	306	0	0.00568	0.00946	0.17413
24	exp1	17	D	306	0	0.02082	0.00568	0.15331
24	exp1	17	D	306	0	0.00757	0.01325	0.16467
24	exp1	22	B	137	0	0	0.00935	0.18505
24	exp1	22	B	137	0	0.00187	0.00935	0.1215
24	exp1	22	B	137	0	0	0.02056	0.16075
24	exp1	22	C	146	0	0	0.02535	0.08451
24	exp1	22	C	146	0	0	0.00282	0.05915

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
24	exp1	22	C	146	0	0	0.01972	0.12113
24	exp1	22	D	306	0	0	0.01136	0.08707
24	exp1	22	D	306	0	0.00189	0.00946	0.12303
24	exp1	22	D	306	0	0	0.01325	0.16845
24	exp1	26	B	137	0	0	0.00187	0.05421
24	exp1	26	B	137	0	0	0	0.10093
24	exp1	26	B	137	0	0	0.00187	0.19065
24	exp1	26	C	146	0	0	0	0.20845
24	exp1	26	C	146	0	0	0	0.16056
24	exp1	26	C	146	0	0	0.00845	0.10141
24	exp1	26	D	306	0	0	0.00189	0.16278
24	exp1	26	D	306	0	0.00189	0	0.15331
24	exp1	26	D	306	0	0.00189	0	0.14006
28	exp2	1	A	132	0.13451	0	0	0
28	exp2	1	A	132	0.34956	0	0	0
28	exp2	1	A	132	0.3469	0	0	0
28	exp2	1	B	137	0.23138	0.00038	0	0
28	exp2	1	B	137	0.18374	0	0	0
28	exp2	1	B	137	0.24575	0	0	0
28	exp2	1	C	146	0.92308	0	0	0
28	exp2	1	C	146	0.55673	0.00577	0	0
28	exp2	1	C	146	0.65481	0.00577	0	0
28	exp2	3	A	132	0.00442	0.42124	0	0
28	exp2	3	A	132	0.00619	0.11062	0	0
28	exp2	3	A	132	0.00619	0.16106	0	0
28	exp2	3	B	137	0.00983	0.11229	0	0
28	exp2	3	B	137	0.01021	0.25898	0	0
28	exp2	3	B	137	0.00529	0.2586	0	0
28	exp2	3	C	146	0.19327	0.3	0	0
28	exp2	3	C	146	0.11538	0.39808	0	0
28	exp2	3	C	146	0.08365	0.75288	0	0
28	exp2	5	A	132	0.00177	0.15044	0.03628	0.01504
28	exp2	5	A	132	0.00354	0.12655	0.05841	0.02655
28	exp2	5	A	132	0.00088	0.18142	0.10885	0.03009
28	exp2	5	B	137	0.00454	0.06314	0.00983	0.00416
28	exp2	5	B	137	0.00189	0.07297	0.00567	0.00038
28	exp2	5	B	137	0.00227	0.09868	0.00643	0.00189
28	exp2	5	C	146	0.08654	0.06058	0.11538	0
28	exp2	5	C	146	0.02596	0.10962	0.09808	0.21635
28	exp2	5	C	146	0.05192	0.1125	0.075	0.18173
28	exp2	7	A	132	0.00265	0.00885	0.04867	0.34159
28	exp2	7	A	132	0.00265	0.01504	0.06195	0.35929
28	exp2	7	A	132	0.00088	0.03274	0.08407	0.21858
28	exp2	7	B	137	0.00038	0.02911	0.09792	0.12476

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
28	exp2	7	B	137	0	0.0344	0.11229	0.0552
28	exp2	7	B	137	0.00038	0.03554	0.09149	0.06163
28	exp2	7	C	146	0.03173	0.06346	0.06923	0.41827
28	exp2	7	C	146	0.02596	0.04615	0.08654	0.42692
28	exp2	7	C	146	0.01731	0.0375	0.09808	0.38077
28	exp2	10	A	132	0.00088	0.00885	0.08673	0.30177
28	exp2	10	A	132	0	0.01858	0.09735	0.32389
28	exp2	10	A	132	0.00177	0.00442	0.0292	0.29646
28	exp2	10	B	137	0.00038	0.04499	0.08318	0.17732
28	exp2	10	B	137	0	0.01815	0.08658	0.14858
28	exp2	10	B	137	0.00038	0.0189	0.00302	0.01664
28	exp2	10	C	146	0.00577	0.00288	0.00865	0.56827
28	exp2	10	C	146	0.00577	0.02596	0.02885	0.70096
28	exp2	10	C	146	0.00577	0.02885	0.02596	0.64038
28	exp2	13	A	132	0	0.00531	0.03628	0.4115
28	exp2	13	A	132	0	0.00796	0.07168	0.21947
28	exp2	13	A	132	0	0	0.03982	0.31327
28	exp2	13	B	137	0	0.00302	0.10548	0.17921
28	exp2	13	B	137	0	0.00302	0.12023	0.16597
28	exp2	13	B	137	0	0.01096	0.11796	0.17958
28	exp2	13	C	146	0.01442	0.00577	0.02885	0.66058
28	exp2	13	C	146	0	0.00577	0.03173	0.61154
28	exp2	13	C	146	0	0.03173	0.02308	0.82212
28	exp2	17	A	132	0	0.00354	0.00796	0.35664
28	exp2	17	A	132	0	0	0.01327	0.26106
28	exp2	17	A	132	0	0.00088	0.01062	0.34513
28	exp2	17	B	137	0	0.02079	0.02079	0.21361
28	exp2	17	B	137	0.00038	0.00529	0.00756	0.08696
28	exp2	17	B	137	0	0.00189	0.04575	0.19811
28	exp2	17	C	146	0	0	0.00577	0.63173
28	exp2	17	C	146	0	0.00288	0.00865	0.66058
28	exp2	17	C	146	0	0.00288	0.00865	0.35769
28	exp2	22	A	132	0	0	0.0115	0.45664
28	exp2	22	A	132	0	0	0.03451	0.38142
28	exp2	22	A	132	0	0	0.0469	0.34779
28	exp2	22	B	137	0	0	0.01399	0.17732
28	exp2	22	B	137	0	0	0.02193	0.23894
28	exp2	22	B	137	0	0	0.01474	0.29112
28	exp2	22	C	146	0	0	0.00865	0.43846
28	exp2	22	C	146	0	0	0.00288	0.47019
28	exp2	22	C	146	0	0	0.00577	0.79615
32	exp2	1	A	132	0.44602	0.17168	0	0
32	exp2	1	A	132	0.3823	0.1	0	0
32	exp2	1	A	132	0.33274	0.1292	0	0

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
32	exp2	1	B	137	0.24726	0.2155	0	0
32	exp2	1	B	137	0.27448	0.11493	0	0
32	exp2	1	B	137	0.18979	0.38563	0	0
32	exp2	1	C	146	0.11538	0.18462	0	0
32	exp2	1	C	146	0.34038	0.525	0	0
32	exp2	1	C	146	0.24231	0.54808	0	0
32	exp2	3	A	132	0.00796	0.16991	0	0
32	exp2	3	A	132	0.00442	0.34071	0	0
32	exp2	3	A	132	0.00531	0.09558	0	0
32	exp2	3	B	137	0.03176	0.74556	0	0
32	exp2	3	B	137	0.02042	0.11758	0	0
32	exp2	3	B	137	0.00718	0.15198	0	0
32	exp2	3	C	146	0.20192	0.43846	0	0
32	exp2	3	C	146	0.09808	0.55385	0	0
32	exp2	3	C	146	0.21346	0.70962	0	0
32	exp2	5	A	132	0.00088	0.05664	0.09558	0.25221
32	exp2	5	A	132	0	0.12389	0.06814	0.12478
32	exp2	5	A	132	0.00088	0.03186	0.04956	0.14336
32	exp2	5	B	137	0.00227	0.11531	0.02987	0.0344
32	exp2	5	B	137	0.00265	0.05217	0.01928	0.05104
32	exp2	5	B	137	0.00038	0.07599	0.03025	0.0276
32	exp2	5	C	146	0.03462	0.07212	0.06635	0.39519
32	exp2	5	C	146	0.01731	0.05481	0.05769	0.44135
32	exp2	5	C	146	0.03173	0.05481	0.02596	0.32308
32	exp2	7	A	132	0	0.00796	0.09469	0.29469
32	exp2	7	A	132	0	0.02124	0.14867	0.20708
32	exp2	7	A	132	0	0.00708	0.01062	0.04867
32	exp2	7	B	137	0	0.05936	0.0983	0.05936
32	exp2	7	B	137	0.00076	0.01588	0.10397	0.11607
32	exp2	7	B	137	0.00076	0.02042	0.1138	0.07561
32	exp2	7	C	146	0.01442	0.01154	0.00865	0.57692
32	exp2	7	C	146	0.01154	0.00288	0.03462	0.52212
32	exp2	7	C	146	0.02308	0.02596	0.02019	0.39231
32	exp2	9	A	132	0	0.00619	0.04779	0.19735
32	exp2	9	A	132	0.00088	0.01239	0.02301	0.20354
32	exp2	9	A	132	0	0.00531	0.01504	0.24248
32	exp2	9	B	137	0	0.05633	0.07977	0.18715
32	exp2	9	B	137	0	0.01059	0.06503	0.21739
32	exp2	9	B	137	0	0.01285	0.08242	0.17921
32	exp2	9	C	146	0.00577	0.00577	0.00865	0.62885
32	exp2	9	C	146	0.00577	0.00577	0.01731	0.69808
32	exp2	9	C	146	0	0.02308	0.00865	0.6375
32	exp2	14	A	132	0	0	0.02124	0.15929
32	exp2	14	A	132	0	0.00265	0.0177	0.21947

Temp	Exp	Days	Host	Host-ID	Eggs	L1	L2	L3
32	exp2	14	A	132	0	0.00354	0.02743	0.28319
32	exp2	14	B	137	0	0.01021	0.04083	0.13119
32	exp2	14	B	137	0	0.00302	0.03781	0.19055
32	exp2	14	B	137	0	0.00189	0.03516	0.11229
32	exp2	14	C	146	0.00288	0	0.00865	0.48462
32	exp2	14	C	146	0	0	0.00288	0.34327
32	exp2	14	C	146	0	0	0.00288	0.17596
32	exp2	18	A	132	0	0	0.01416	0.17345
32	exp2	18	A	132	0	0	0.02212	0.11858
32	exp2	18	A	132	0.00088	0.00177	0.02301	0.15929
32	exp2	18	B	137	0	0.00151	0.03478	0.08922
32	exp2	18	B	137	0	0.00038	0.03365	0.09074
32	exp2	18	B	137	0	0.00038	0.03289	0.12779
32	exp2	18	C	146	0	0.04904	0.00865	0.34615
32	exp2	18	C	146	0	0	0	0.34327
32	exp2	18	C	146	0	0.00288	0.00577	0.31154
32	exp2	22	A	132	0	0	0.00442	0.27434
32	exp2	22	A	132	0	0	0.00177	0.02832
32	exp2	22	A	132	0	0	0.00973	0.20531
32	exp2	22	B	137	0	0.00113	0.00983	0.1225
32	exp2	22	B	137	0	0.00038	0.01739	0.18147
32	exp2	22	B	137	0	0	0.00681	0.21626
32	exp2	22	C	146	0	0	0.00288	0.61442
32	exp2	22	C	146	0	0	0.00288	0.40673
32	exp2	22	C	146	0	0	0.00288	0.47885

9.2.3 Data - Development at variable temperatures

Day = time after experimental start in days, Eggs = number of eggs counted, L1/L2/L3 = number of first/second/third stage larvae counted, %L3 = proportion of eggs developed to L3

Day	Eggs	L1	L2	L3	%L3
14	31	28	2	0	0
14	4	30	0	0	0
14	1	48	1	0	0
14	1	46	5	2	0.005804
14	0	42	12	1	0.002902
28	0	6	9	39	0.113175
28	0	18	18	20	0.058038
28	0	10	9	25	0.072548
28	0	5	9	48	0.139292
28	0	3	10	47	0.13639
42	0	1	10	44	0.127684
42	0	2	9	35	0.101567
42	0	1	7	45	0.130586
42	0	3	5	115	0.33372
42	3	4	3	12	0.034823
56	0	0	7	48	0.139292
56	0	0	7	74	0.214742
56	0	0	5	59	0.171213
56	0	0	4	58	0.168311
56	2	0	0	8	0.023215
83	0	0	0	39	0.113175
83	0	0	1	87	0.252467
83	0	0	2	54	0.156703
83	0	0	0	59	0.171213
83	0	0	1	28	0.081254
112	0	0	1	61	0.177017
112	0	0	0	53	0.153802
112	0	0	0	51	0.147998
112	0	0	0	45	0.130586
112	0	0	0	38	0.110273

9.2.4 Data – Incubator temperature settings for development at variable temperatures

Week = week of experiment, hour = hour of day for temperature, temp = temperature setting for incubator

week	hour	temp
1	1	13.5
1	2	12.2
1	3	11.0
1	4	10.2
1	5	9.8
1	6	9.5
1	7	9.4
1	8	9.4
1	9	9.2
1	10	9.4
1	11	9.7
1	12	9.5
1	13	9.2
1	14	9.2
1	15	9.4
1	16	9.3
1	17	9.7
1	18	11.0
1	19	12.6
1	20	13.9
1	21	14.8
1	22	15.2
1	23	14.9
1	24	14.4
2	1	13.3
2	2	12.5
2	3	11.9
2	4	11.5
2	5	11.2
2	6	10.9
2	7	10.8
2	8	10.5
2	9	10.4
2	10	10.4
2	11	10.2
2	12	10.1
2	13	10.1
2	14	10.0
2	15	10.0
2	16	10.1
2	17	10.4
2	18	11.7
2	19	13.0
2	20	14.3
2	21	14.8
2	22	14.9
2	23	14.7
2	24	14.1
3	1	13.1
3	2	12.1
3	3	11.6
3	4	11.3
3	5	11.0
3	6	10.8
3	7	10.6
3	8	10.7
3	9	10.6
3	10	10.4
3	11	10.3
3	12	10.1
3	13	9.9
3	14	9.7
3	15	9.6
3	16	9.7
3	17	9.9
3	18	11.2
3	19	12.7
3	20	14.0
3	21	14.6
3	22	14.9
3	23	14.8
3	24	14.4
4	1	12.4
4	2	11.7
4	3	11.2
4	4	10.9
4	5	10.5
4	6	10.4
4	7	10.2
4	8	10.0
4	9	9.9
4	10	9.8
4	11	9.9
4	12	9.9
4	13	9.7
4	14	9.4
4	15	9.3
4	16	9.1
4	17	9.1
4	18	10.2
4	19	11.6
4	20	12.8
4	21	13.5
4	22	13.5
4	23	13.4
4	24	12.9
5	1	11.5
5	2	10.6
5	3	10.1
5	4	9.8
5	5	9.7
5	6	9.4
5	7	9.0
5	8	9.0
5	9	8.8
5	10	9.0
5	11	8.9
5	12	8.8
5	13	8.8
5	14	8.9
5	15	8.9
5	16	8.7
5	17	8.9
5	18	9.6
5	19	10.7
5	20	11.7
5	21	12.6
5	22	13.0
5	23	12.8
5	24	12.5
6	1	11.5
6	2	10.7
6	3	10.3
6	4	10.1
6	5	9.9
6	6	9.7
6	7	9.4
6	8	9.4
6	9	9.4
6	10	9.2
6	11	8.9
6	12	8.5
6	13	8.4
6	14	8.4
6	15	8.6
6	16	8.5
6	17	8.5
6	18	9.3
6	19	10.4
6	20	11.6
6	21	12.4
6	22	12.5
6	23	12.5
6	24	12.1
7	1	10.1
7	2	9.0
7	3	8.1
7	4	7.7
7	5	7.4
7	6	7.2
7	7	7.0
7	8	7.1
7	9	7.2
7	10	7.0
7	11	6.8
7	12	6.8

week	hour	temp
7	13	7.0
7	14	7.0
7	15	7.0
7	16	7.0
7	17	7.1
7	18	7.8
7	19	9.0
7	20	10.2
7	21	11.0
7	22	11.5
7	23	11.2
7	24	10.6
8	1	10.4
8	2	10.1
8	3	9.9
8	4	9.7
8	5	9.5
8	6	9.5
8	7	9.4
8	8	9.4
8	9	9.2
8	10	9.1
8	11	9.0
8	12	9.0
8	13	9.0
8	14	8.9
8	15	8.6
8	16	8.6
8	17	8.8
8	18	9.2
8	19	9.9
8	20	10.6
8	21	11.0
8	22	11.4
8	23	11.2
8	24	10.8
9	1	9.3
9	2	8.2
9	3	7.5
9	4	6.9
9	5	6.4
9	6	6.0
9	7	5.9
9	8	5.6
9	9	5.3

week	hour	temp
9	10	5.1
9	11	5.1
9	12	5.1
9	13	5.2
9	14	5.2
9	15	5.2
9	16	5.3
9	17	5.5
9	18	6.3
9	19	7.6
9	20	8.5
9	21	9.3
9	22	10.0
9	23	10.1
9	24	9.9
10	1	8.6
10	2	7.7
10	3	7.0
10	4	6.6
10	5	6.4
10	6	6.2
10	7	6.0
10	8	5.7
10	9	5.5
10	10	5.3
10	11	5.2
10	12	5.2
10	13	5.2
10	14	5.3
10	15	5.4
10	16	5.4
10	17	5.5
10	18	6.1
10	19	7.3
10	20	8.5
10	21	9.3
10	22	9.8
10	23	9.7
10	24	9.1
11	1	9.5
11	2	8.9
11	3	8.4
11	4	8.0
11	5	7.8
11	6	7.8

week	hour	temp
11	7	7.8
11	8	7.7
11	9	7.7
11	10	7.6
11	11	7.6
11	12	7.5
11	13	7.4
11	14	7.4
11	15	7.3
11	16	7.2
11	17	7.3
11	18	8.0
11	19	9.1
11	20	10.0
11	21	10.7
11	22	10.9
11	23	10.9
11	24	10.6
12	1	10.1
12	2	9.5
12	3	8.9
12	4	8.4
12	5	8.0
12	6	7.8
12	7	7.5
12	8	7.3
12	9	7.3
12	10	7.3
12	11	7.3
12	12	7.2
12	13	7.2
12	14	7.4
12	15	7.2
12	16	7.0
12	17	7.2
12	18	7.9
12	19	9.0
12	20	9.6
12	21	10.3
12	22	10.5
12	23	10.6
12	24	10.5
13	1	10.0
13	2	9.3
13	3	8.5

week	hour	temp
13	4	7.7
13	5	7.4
13	6	7.3
13	7	7.4
13	8	7.4
13	9	7.3
13	10	7.1
13	11	6.9
13	12	6.9
13	13	6.9
13	14	7.0
13	15	7.0
13	16	7.1
13	17	7.4
13	18	7.9
13	19	8.6
13	20	9.2
13	21	9.5
13	22	9.8
13	23	9.9
13	24	9.8
14	1	8.6
14	2	8.0
14	3	7.3
14	4	6.9
14	5	6.6
14	6	6.3
14	7	6.1
14	8	5.9
14	9	5.8
14	10	5.6
14	11	5.4
14	12	5.3
14	13	5.2
14	14	5.0
14	15	5.0
14	16	4.9
14	17	5.4
14	18	6.3
14	19	7.4
14	20	8.3
14	21	9.0
14	22	9.5
14	23	9.8
14	24	9.6

week	hour	temp
15	1	11.6
15	2	11.1
15	3	10.5
15	4	10.0
15	5	9.7
15	6	9.6
15	7	9.4
15	8	9.2
15	9	9.1
15	10	9.1
15	11	9.1
15	12	9.0
15	13	8.9
15	14	9.0
15	15	9.1
15	16	9.2
15	17	9.6
15	18	10.2
15	19	11.0
15	20	11.5
15	21	12.0
15	22	12.2
15	23	12.2
15	24	12.1
16	1	12.0
16	2	11.4
16	3	10.3
16	4	9.6
16	5	9.6
16	6	9.5
16	7	9.3
16	8	9.2
16	9	9.1
16	10	9.0
16	11	8.8
16	12	8.7
16	13	8.5
16	14	8.5
16	15	8.4

week	hour	temp
16	16	8.4
16	17	8.8
16	18	9.7
16	19	10.6
16	20	11.5
16	21	12.1
16	22	12.4
16	23	12.5
16	24	12.3
17	1	12.0
17	2	11.6
17	3	11.1
17	4	10.8
17	5	10.5
17	6	10.1
17	7	10.1
17	8	10.0
17	9	9.9
17	10	9.8
17	11	9.6
17	12	9.6
17	13	9.5
17	14	9.3
17	15	9.2
17	16	9.3
17	17	9.9
17	18	10.4
17	19	11.0
17	20	11.4
17	21	11.9
17	22	12.1
17	23	12.1
17	24	12.1
18	1	11.7
18	2	11.3
18	3	10.6
18	4	10.1
18	5	9.9
18	6	9.6

week	hour	temp
18	7	9.4
18	8	9.2
18	9	9.2
18	10	9.1
18	11	9.1
18	12	9.1
18	13	8.9
18	14	8.9
18	15	8.7
18	16	8.9
18	17	9.5
18	18	10.0
18	19	10.6
18	20	11.1
18	21	11.6
18	22	12.0
18	23	12.1
18	24	12.1
19	1	12.1
19	2	11.6
19	3	10.4
19	4	9.4
19	5	8.7
19	6	8.4
19	7	8.5
19	8	8.3
19	9	8.0
19	10	8.0
19	11	8.0
19	12	8.1
19	13	8.1
19	14	7.9
19	15	7.7
19	16	8.0
19	17	8.8
19	18	9.7
19	19	10.6
19	20	11.4
19	21	12.1

week	hour	temp
19	22	12.4
19	23	12.6
19	24	12.5
20	1	13.7
20	2	13.2
20	3	12.5
20	4	11.9
20	5	11.3
20	6	11.0
20	7	10.9
20	8	10.7
20	9	10.6
20	10	10.3
20	11	9.9
20	12	9.6
20	13	9.5
20	14	9.3
20	15	9.2
20	16	9.6
20	17	10.6
20	18	11.6
20	19	12.6
20	20	13.6
20	21	14.3
20	22	14.8
20	23	14.9
20	24	14.6

9.2.5 Data - Development in the field (part 1 of 2)

Plot = field plot, mesh = mesh to inhibit earthworm entry into plot (no/yes), day = time after experimental start in days, avg_Temp = average Temperature since start, Soil_wgt = weight of soil sample in gram, Soil_L3 = number of third stage larvae recovered from soil sample, Grass/Grass_dried = weight of grass recovered fresh/dried in gram, Grass_L3 = number of third stage larvae recovered from grass sample

Plot	mesh	day	avg_Temp	Soil_wgt	Soil_L3	Grass	Grass_dried	Grass_L3
C8	n	14	11.419944	2201.3	0	11.2	1.8	0
A4	n	14	11.419944	1824.7	0	12.2	1.9	0
A8	n	14	11.419944	1830.4	0	19.9	4.4	0
A12	n	14	11.419944	2147.9	1	17.1	2.8	0
C4	n	14	11.419944	2061.7	0	10.3	2.2	0
C12	n	28	11.329606	2086.09	5	10.4	2.2	4
B3	n	28	11.329606	2247.79	3	29.3	10.8	1
C7	n	28	11.329606	2237.19	1	12.6	4.3	0
A11	n	28	11.329606	2020.59	0	12.3	3.5	2
B12	n	28	11.329606	2069.49	0	19.6	5.9	5
C3	n	44	10.801776	1815.9	16	23.9	8.9	38
C11	n	44	10.801776	2229.2	6	27.6	12.5	27
C6	n	44	10.801776	1986.8	1	34.3	12.9	14
B4	n	44	10.801776	1579.7	0	18.7	9.1	4
C1	n	44	10.801776	1623.9	4	19.2	10.3	15
C9	n	59	10.325705	2036.7	19	19.9	15.3	12
B8	n	59	10.325705	1677.4	24	31	17.6	5
B2	n	59	10.325705	1742.3	17	34	17.5	23
A7	n	59	10.325705	2008.7	8	22.5	15.9	19
A3	n	59	10.325705	2185.2	25	36.8	16.8	31
A5	n	86	9.4312611	1681.5	13	31.8	17.4	1
B10	n	86	9.4312611	2025.6	21	30.7	16.9	0
A6	n	86	9.4312611	1476.2	16	27.5	16.5	1
C5	n	86	9.4312611	2593.8	9	20.7	15.7	1
C2	n	86	9.4312611	1714.8	24	35.4	17.8	4
B5	n	113	9.3148591	1782.2	26	43.7	20.6	7
B11	n	113	9.3148591	2179.4	8	26.3	17.8	0
C10	n	113	9.3148591	1707.6	31	24.1	17.2	2
A2	n	113	9.3148591	2763.9	6	26.9	18.2	0
A9	n	113	9.3148591	2129.8	15	44.6	23.3	5
A10	n	146	9.6716147	2899	17	32.6	22.1	0
B6	n	146	9.6716147	3376.6	2	44.6	37.2	2
B7	n	146	9.6716147	2764.7	14	48.2	39.1	2
B9	n	146	9.6716147	3278.8	9	46.7	38.7	2
A1	n	146	9.6716147	3290	3	51.6	43.1	1
D3	y	59	10.325705	1952.9	25	21.6	15.7	28
D2	y	59	10.325705	2112.7	9	16.1	14.5	31
D6	y	59	10.325705	2126.1	64	17.8	15.1	17
D5	y	59	10.325705	2007.7	20	17.3	14.5	38
D1	y	59	10.325705	1936.6	36	22.6	16.1	43

9.2.6 Data - Development in the field (part 2 of 2)

Plot = field plot, mesh = mesh to inhibit earthworm entry into plot (no/yes), day = time after experimental start in days, avg_Temp = average Temperature since start, Faeces_total = weight of recovered faeces in gram, Faeces_Baermann = weight of faeces placed into Bearmann funnel, Baermann_L3 = number of third stage larvae recovered with Baermann technique from faeces sample

Plot	mesh	day	avg_Temp	Faeces_total	Faeces_Baermann	Baermann_L3
C8	n	14	11.41994	113.74	53.7	0
A4	n	14	11.41994	128.01	65.6	1
A8	n	14	11.41994	120.12	47.5	0
A12	n	14	11.41994	139.05	73.6	0
C4	n	14	11.41994	131.04	63.2	1
C12	n	28	11.32961	43.88	22.02	4
B3	n	28	11.32961	70.04	37.05	48
C7	n	28	11.32961	99.49	54.79	187
A11	n	28	11.32961	100.91	51.12	27
B12	n	28	11.32961	120.09	56.3	28
C3	n	44	10.80178	25	13	6
C11	n	44	10.80178	79.3	40.8	118
C6	n	44	10.80178	46.2	22.3	42
B4	n	44	10.80178	27.15	9.41	3
C1	n	44	10.80178	87.6	35.6	25
C9	n	59	10.3257	12.91	12.91	8
B8	n	59	10.3257	0	0	0
B2	n	59	10.3257	0	0	0
A7	n	59	10.3257	11.71	11.71	4
A3	n	59	10.3257	19.7	13.91	26
A5	n	86	9.431261	0	0	0
B10	n	86	9.431261	9.4	5.7	0
A6	n	86	9.431261	2.5	1.4	1
C5	n	86	9.431261	7.1	4.5	0
C2	n	86	9.431261	0	0	0
B5	n	113	9.314859	0	0	0
B11	n	113	9.314859	0	0	0
C10	n	113	9.314859	0	0	0
A2	n	113	9.314859	0	0	0
A9	n	113	9.314859	0	0	0
A10	n	146	9.671615	0	0	0
B6	n	146	9.671615	0	0	0
B7	n	146	9.671615	0	0	0
B9	n	146	9.671615	0	0	0
A1	n	146	9.671615	0	0	0
D3	y	59	10.3257	68.01	46.5	26
D2	y	59	10.3257	59.31	41.8	142
D6	y	59	10.3257	73.91	52.6	75
D5	y	59	10.3257	60.81	45.3	91
D1	y	59	10.3257	59.11	35	31

9.2.7 Statistical Analysis for development at constant temperatures - Minitab output (for DT50 values see Appendix 4)

temp =constant temperature used; Host letter/number = donor calf ID/Experimental number; exp = experiment 1 or 2

9.2.7.1 Results for: EXP1 max L3 mean

General Linear Model: max L3 mean versus temp, Host

```
Factor  Type   Levels  Values
temp    fixed       3  16, 20, 24
Host    fixed       3  Host_B1, Host_C1, Host_D1

Analysis of Variance for max L3 mean, using Adjusted SS for Tests


Source  DF     Seq SS     Adj SS     Adj MS      F      P
temp     2  0.0073156  0.0073156  0.0036578  11.45  0.022
Host     2  0.0046115  0.0046115  0.0023057   7.22  0.047
Error    4  0.0012775  0.0012775  0.0003194
Total    8  0.0132045

S = 0.0178708   R-Sq = 90.33%   R-Sq(adj) = 80.65%

Grouping Information Using Tukey Method and 95.0% Confidence


temp  N    Mean  Grouping
20    3  0.1857  A
24    3  0.1272    B
16    3  0.1235    B

Means that do not share a letter are significantly different.


Grouping Information Using Tukey Method and 95.0% Confidence


Host     N    Mean  Grouping
Host_B1  3  0.1663  A
Host_D1  3  0.1562  A B
Host_C1  3  0.1140    B

Means that do not share a letter are significantly different.
```

9.2.7.2 Results for: EXP2 max L3 mean

General Linear Model: max L3 mean versus temp, Host

```
Factor  Type   Levels  Values
temp    fixed       4  8, 16, 28, 32
Host    fixed       3  Host_A2, Host_B2, Host_C2

Analysis of Variance for max L3 mean, using Adjusted SS for Tests


Source  DF   Seq SS   Adj SS   Adj MS     F      P
temp     3  0.16621  0.16621  0.05540  5.31  0.040
Host     2  0.12320  0.12320  0.06160  5.91  0.038
Error    6  0.06255  0.06255  0.01042
Total   11  0.35196

S = 0.102101   R-Sq = 82.23%   R-Sq(adj) = 67.42%
```

```
Grouping Information Using Tukey Method and 95.0% Confidence

temp  N     Mean  Grouping
28    3  0.37353  A
32    3  0.26383  A B
16    3  0.16721  A B
 8    3  0.05499    B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Host     N     Mean  Grouping
Host_C2  4  0.33951  A
Host_A2  4  0.21384  A B
Host_B2  4  0.09132    B

Means that do not share a letter are significantly different.
```

9.2.7.3 Results for: DT50

General Linear Model: DT50 versus temp, exp, Host

```
Factor  Type   Levels  Values
temp    fixed       6  8, 16, 20, 24, 28, 32
exp     fixed       2  exp1, exp2
Host    fixed       4  A, B, C, D

Analysis of Variance for DT50, using Adjusted SS for Tests

Source  DF   Seq SS   Adj SS   Adj MS      F      P
temp     5  5486.64  5122.81  1024.56  39.81  0.000
exp      1     5.38    19.06    19.06   0.74  0.408
Host     3   177.73   177.73    59.24   2.30  0.134
Error   11   283.12   283.12    25.74
Total   20  5952.87

S = 5.07328   R-Sq = 95.24%   R-Sq(adj) = 91.35%

Unusual Observations for DT50

Obs     DT50      Fit  SE Fit  Residual  St Resid
  2  73.0000  60.8024  3.3897   12.1976      3.23 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

temp  N    Mean  Grouping
 8    3  55.029  A
16    6  18.308    B
20    3  13.392    B C
24    3  11.040    B C
28    3   5.939    B C
32    3   4.025      C

Means that do not share a letter are significantly different.
```

9.2.8 Statistical Analysis for development under natural conditions - Minitab output

9.2.8.1 Results for: L3 recovered from locations herbage (grass) vs. soil

One-way ANOVA: L3 versus location Day 14

```
Source    DF     SS     MS     F      P
location   1  0.100  0.100  1.00  0.347
Error      8  0.800  0.100
Total      9  0.900

S = 0.3162   R-Sq = 11.11%   R-Sq(adj) = 0.00%

                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level     N    Mean   StDev  ---+---------+---------+---------+------
Grass_L3  5  0.0000  0.0000  (------------*------------)
Soil_L3   5  0.2000  0.4472          (------------*------------)
                            ---+---------+---------+---------+------
                            -0.25      0.00      0.25      0.50

Pooled StDev = 0.3162
```

One-way ANOVA: L3 versus location Day 28

```
Source    DF     SS    MS     F      P
location   1   0.90  0.90  0.20  0.667
Error      8  36.00  4.50
Total      9  36.90

S = 2.121   R-Sq = 2.44%   R-Sq(adj) = 0.00%

                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level     N   Mean  StDev  ---+---------+---------+---------+------
Grass_L3  5  2.400  2.074      (--------------*--------------)
Soil_L3   5  1.800  2.168  (--------------*--------------)
                           ---+---------+---------+---------+------
                            0.0       1.5       3.0       4.5

Pooled StDev = 2.121
```

One-way ANOVA: L3 versus location Day 44

```
Source    DF    SS   MS     F      P
location   1   504  504  4.73  0.061
Error      8   852  107
Total      9  1357

S = 10.32   R-Sq = 37.16%   R-Sq(adj) = 29.31%

                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level     N   Mean  StDev  -----+---------+---------+---------+----
Grass_L3  5  19.60  13.13                (----------*----------)
Soil_L3   5   5.40   6.39  (----------*----------)
                           -----+---------+---------+---------+----
                                0        10        20        30

Pooled StDev = 10.32
```

One-way ANOVA: L3 versus location Day 59

```
Source    DF     SS    MS     F      P
location   1    0.9   0.9  0.01  0.914
Error      8  585.2  73.2
Total      9  586.1

S = 8.553   R-Sq = 0.15%   R-Sq(adj) = 0.00%

                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level     N    Mean   StDev   --+---------+---------+---------+-------
Grass_L3  5  18.000  10.000   (-----------------*-----------------)
Soil_L3   5  18.600   6.804     (-----------------*-----------------)
                             --+---------+---------+---------+-------
                            10.0      15.0      20.0      25.0

Pooled StDev = 8.553
```

One-way ANOVA: L3 versus location Day 86

```
Source    DF     SS     MS      F      P
location   1  577.6  577.6  29.93  0.001
Error      8  154.4   19.3
Total      9  732.0

S = 4.393   R-Sq = 78.91%   R-Sq(adj) = 76.27%

                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level     N    Mean  StDev  ----+---------+---------+---------+-----
Grass_L3  5   1.400  1.517  (-----*-----)
Soil_L3   5  16.600  6.025                      (------*-----)
                            ----+---------+---------+---------+-----
                              0.0       7.0      14.0      21.0

Pooled StDev = 4.393
```

One-way ANOVA: L3 versus location Day 113

```
Source    DF      SS     MS     F      P
location   1   518.4  518.4  7.95  0.023
Error      8   521.6   65.2
Total      9  1040.0

S = 8.075   R-Sq = 49.85%   R-Sq(adj) = 43.58%

                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level     N    Mean   StDev  -------+---------+---------+---------+--
Grass_L3  5   2.800   3.114  (----------*---------)
Soil_L3   5  17.200  10.986                   (---------*----------)
                             -------+---------+---------+---------+--
                                  0.0       8.0      16.0      24.0

Pooled StDev = 8.075
```

One-way ANOVA: L3 versus location Day 146

```
Source    DF     SS     MS     F      P
location   1  144.4  144.4  6.52  0.034
Error      8  177.2   22.1
Total      9  321.6

S = 4.706   R-Sq = 44.90%   R-Sq(adj) = 38.01%

                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level     N   Mean  StDev  -------+---------+---------+---------+--
Grass_L3  5  1.400  0.894  (---------*---------)
Soil_L3   5  9.000  6.595                (---------*---------)
                           -------+---------+---------+---------+--
                                0.0       5.0      10.0      15.0

Pooled StDev = 4.706
```

9.2.8.2 Results for: Unmeshed vs. Meshed plots (mesh: n=no, y=yes)

One-way ANOVA: L3 Total versus mesh

```
Source  DF     SS     MS      F      P
mesh     1  35830  35830  18.00  0.003
Error    8  15926   1991
Total    9  51756

S = 44.62   R-Sq = 69.23%   R-Sq(adj) = 65.38%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level  N    Mean  StDev  +---------+---------+---------+---------
n      5   46.36  26.41  (-------*------)
y      5  166.08  57.30                    (-------*------)
                          +---------+---------+---------+---------
                          0        60       120       180

Pooled StDev = 44.62
```

One-way ANOVA: Faeces_total versus mesh

```
Source  DF      SS      MS       F      P
mesh     1  7663.5  7663.5  130.68  0.000
Error    8   469.1    58.6
Total    9  8132.6

S = 7.658   R-Sq = 94.23%   R-Sq(adj) = 93.51%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level  N    Mean  StDev  +---------+---------+---------+---------
n      5   8.864  8.646  (---*---)
y      5  64.230  6.521                            (---*---)
                          +---------+---------+---------+---------
                          0        20        40        60

Pooled StDev = 7.658
```

Appendix 3

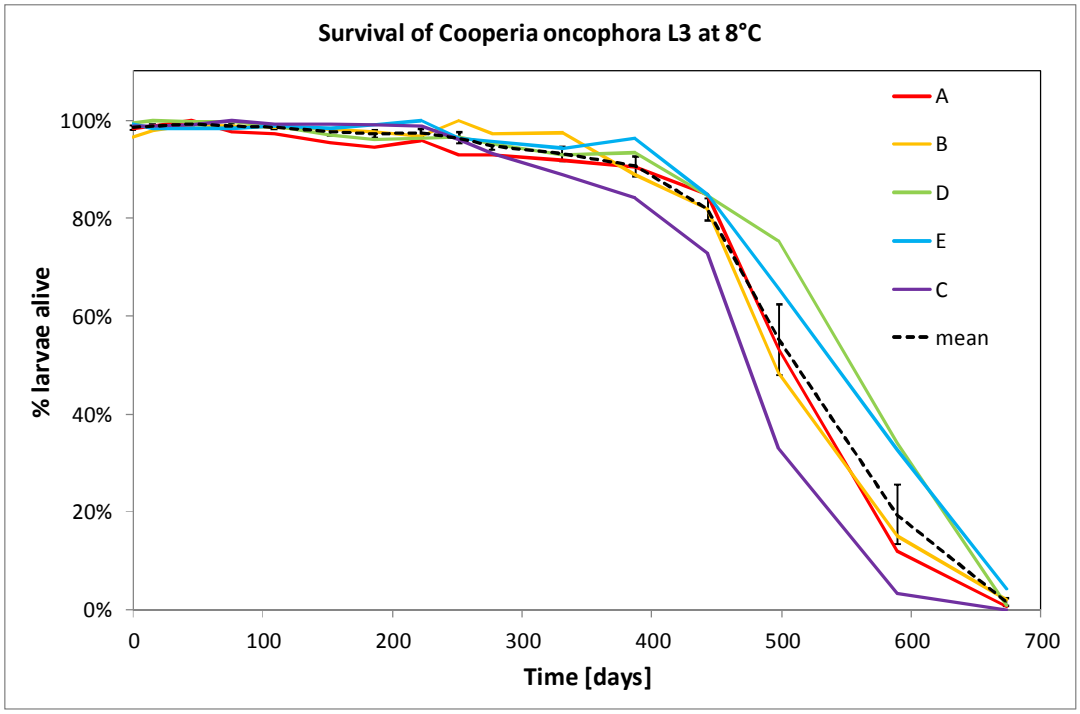
Supplementary information for Chapter 3

The effect of temperature on the survival of

Cooperia oncophora third stage larvae

9.3 Chapter 3 - Survival of *Cooperia oncophora* third stage larvae

9.3.1 Supplementary figures for survival of third stage larvae at constant temperatures



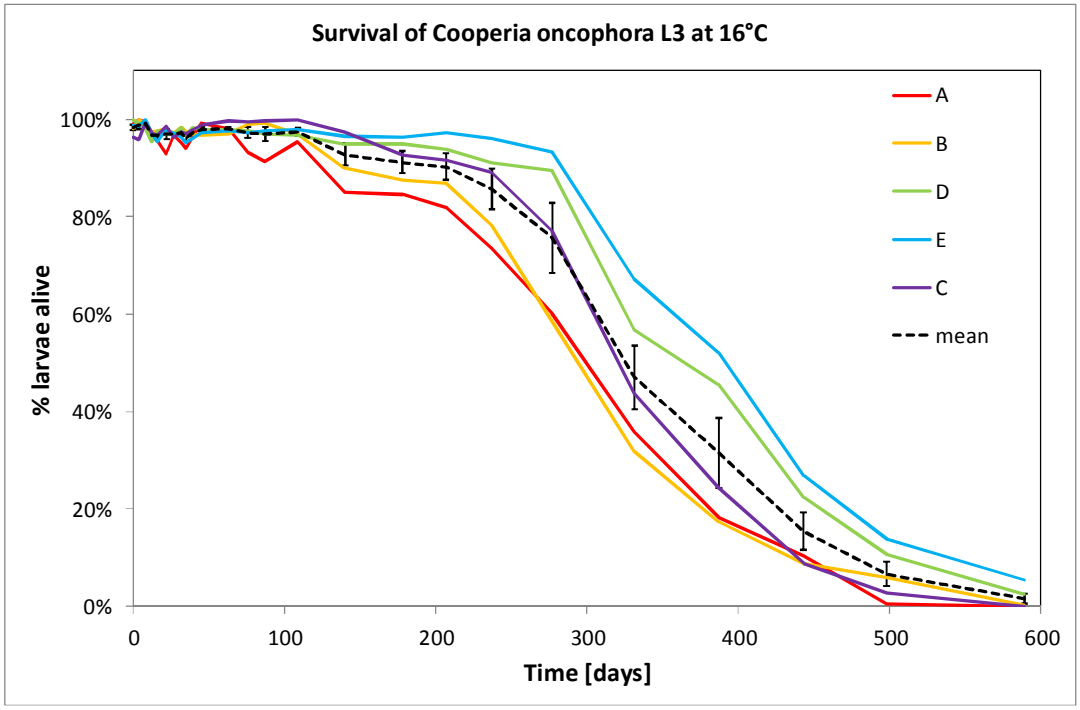
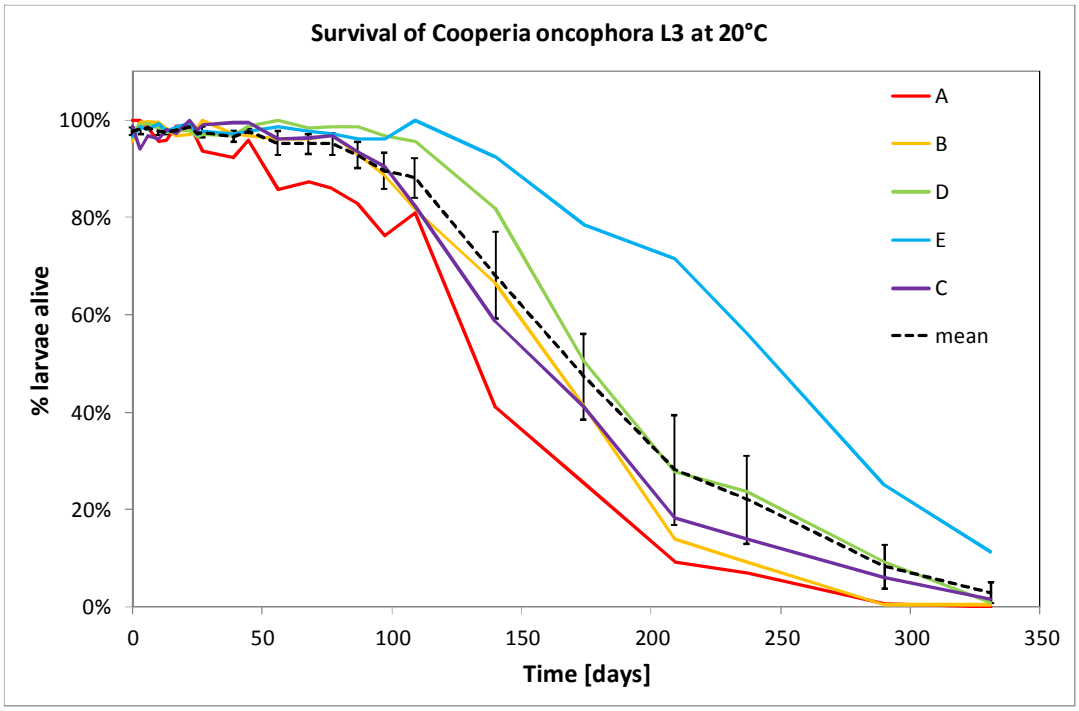


Figure 9.3.1 - Percentage of living *Cooperia oncophora* third stage larvae from different host animals and the mean (±SEM) kept in deionised water at 8 and 16 °C over time (days).



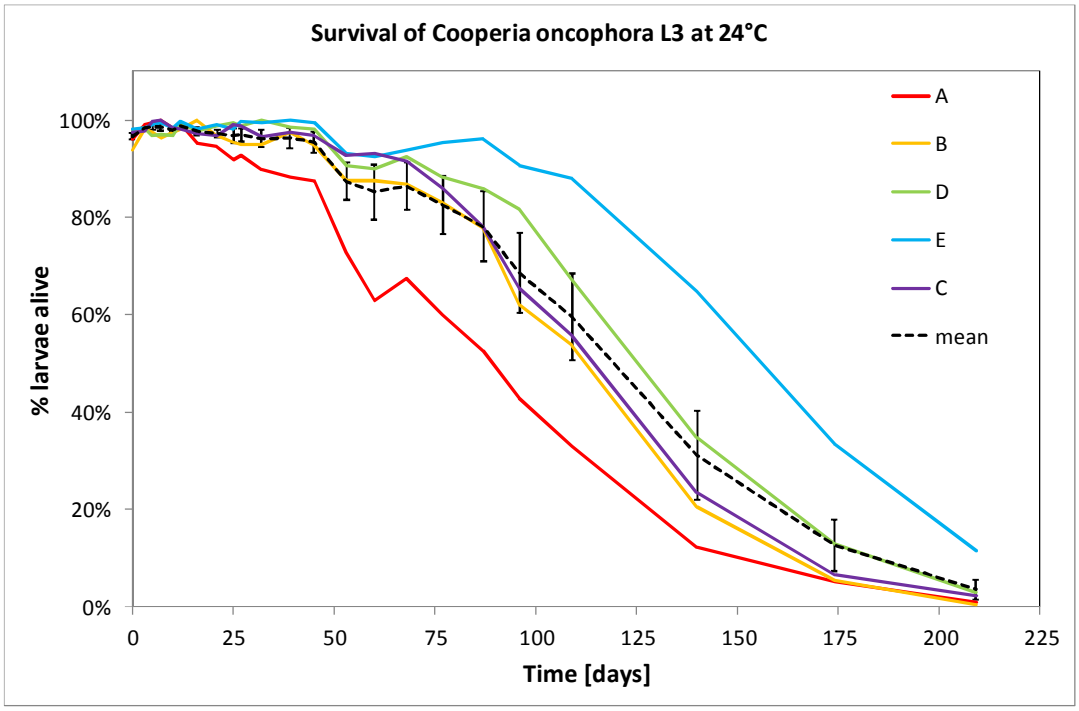
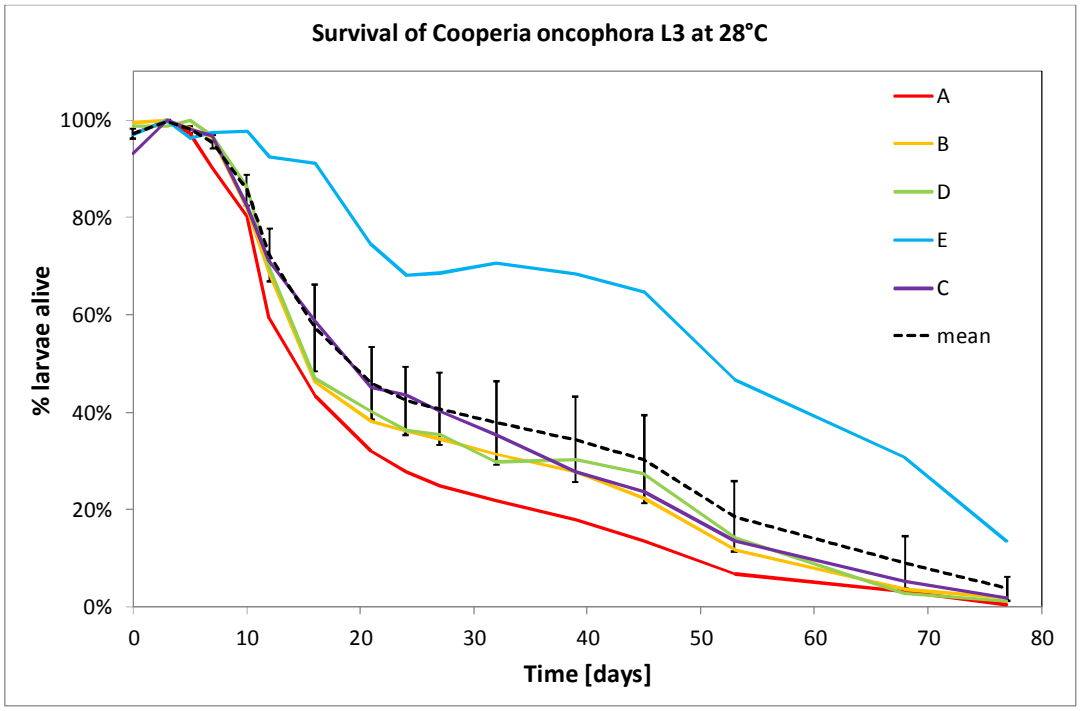


Figure 9.3.2 - Percentage of living *Cooperia oncophora* third stage larvae from different host animals and the mean (±SEM) kept in deionised water at 20 and 24 °C over time (days).



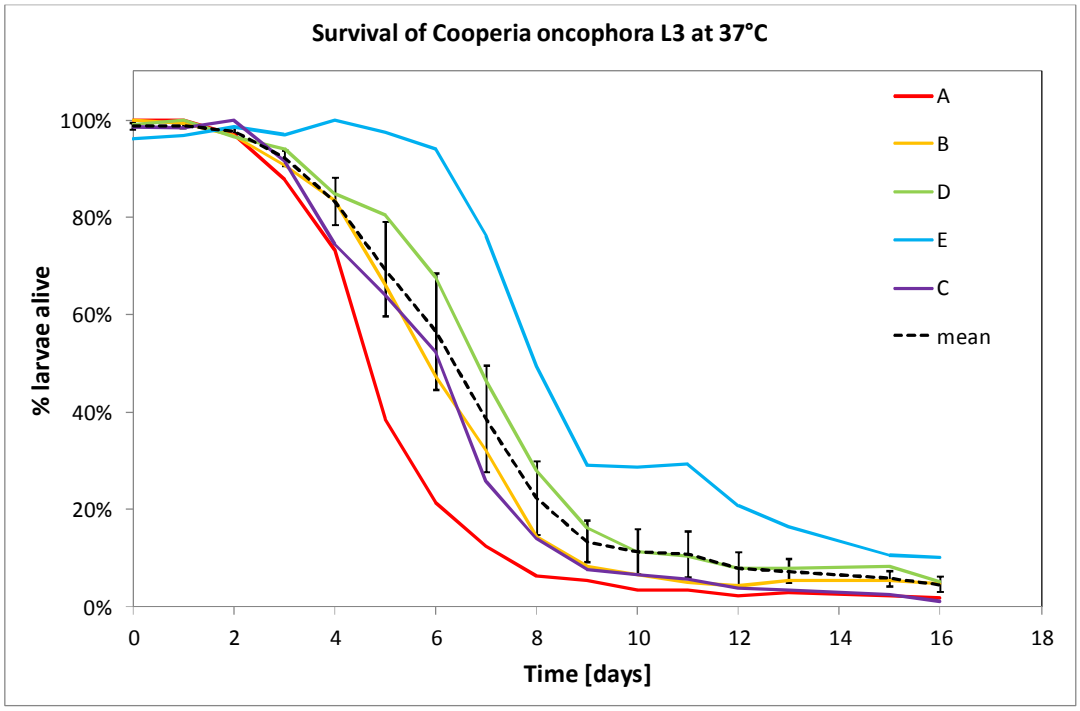


Figure 9.3.3 - Percentage of living *Cooperia oncophora* third stage larvae from different host animals and the mean (±SEM) kept in deionised water at 28 and 37 °C over time (days).

9.3.2 Data - Survival at constant temperatures

Temp = temperature, Day = time after experimental start in days, L3_% = average proportion of L3 alive per host, Host = host animal the faeces was sourced from.

Temp	Day	L3_%	Host A	Host B	Host D	Host E	Host C
8	0	0.985	347	192.5	177.5	238	157
8	15	0.9879	348.5	195.5	178.5	235.5	157
8	45	0.9922	352.5	197.5	178	235.5	157.5
8	76	0.9888	344	197	178	235.5	159
8	109	0.9853	342.5	196.5	176.5	236.5	158
8	152	0.9767	336.5	196	173	235.5	158
8	186	0.9729	333.5	195	171.5	237	157.5
8	222	0.9755	338	193	172	239.5	157
8	251	0.9643	328	199.5	172.5	230.5	153
8	277	0.9485	328	194	170	229	148
8	331	0.9315	324	194.5	166	226	141.5
8	387	0.9063	318.9	177.3	166.7	230.7	133.7
8	443	0.8182	299	163.5	151	203	116
8	498	0.5517	188	96.5	134.5	157.5	52.5
8	589	0.195	42.5	30	61	78.5	5.5
8	673	0.0159	2.5	4	1.5	10.5	0
16	0	0.9847	187.5	213.5	223.5	242	177
16	4	0.9876	189.5	217.5	222	241.5	176
16	8	0.9919	188.5	215.5	219.5	245	182
16	12	0.9679	182.5	212	213.5	238.5	178.5
16	16	0.967	181.5	212.5	218.5	234	177.5
16	22	0.9695	176	213.5	217	240	181
16	27	0.9699	183.5	211.5	217	238.5	177
16	32	0.9719	180.5	214	220	236	179
16	35	0.9617	178.5	212	216.5	233.5	178
16	39	0.9714	182.5	211	220	235.5	179.5
16	45	0.9801	188	210.5	219	238	181.5
16	63	0.9813	186	211	219	239.5	183
16	76	0.9736	176.5	215.5	218.5	238.5	182.5
16	87	0.9704	173	216	217	239.5	183
16	109	0.9747	181	211	216.5	240	183.5
16	140	0.9285	161	196	212.5	236.5	179
16	178	0.9127	160.5	190.5	212.5	236	170
16	207	0.9027	155	189	210	238	168
16	237	0.8566	139.5	170.5	203.5	235.5	163.5
16	277	0.7577	114	127.5	200	229	141.5
16	331	0.4709	68	69	127	164.5	80.5
16	387	0.3152	34.5	38	102	127.5	44.5
16	443	0.1541	19.5	19	50	66	16

16	498	0.0664	1	12.5	23.5	33.5	5
16	589	0.016	0	0.5	5.5	13	0
20	0	0.9778	157.5	196	199	262	165
20	3	0.9825	157.5	204	201.5	267.5	157
20	6	0.9846	155	204.5	202	266	161.5
20	10	0.9774	150.5	204	200	269	160.5
20	13	0.9749	151	201	198.5	265	164
20	17	0.9788	155.5	198.5	199.5	267	162.5
20	22	0.9862	155.5	199	199.5	269	167
20	25	0.9714	151	201.5	196.5	263	163.5
20	27	0.9752	147.5	205	197.5	265	165.5
20	39	0.9663	145.5	199.5	197	263.5	166
20	45	0.9773	151	198.5	201	265	166
20	56	0.9529	135	197	203.5	267	160.5
20	68	0.9514	137.5	197	200	264.5	161
20	77	0.9511	135.5	199	200.5	263.5	161.5
20	87	0.9277	130.5	190.5	200.5	260.5	156
20	97	0.8966	120	182	197	260.5	151
20	109	0.8811	127.5	167.5	194.5	271	137.5
20	140	0.6813	64.5	136.5	166.5	251	98
20	174	0.4737	40	84.5	103	213	68.5
20	209	0.2814	14.5	28.5	56.5	194	30.5
20	237	0.2204	11	19	48	152.5	23.5
20	290	0.0831	1	1	19	68	10
20	331	0.0284	0	1	2	30.5	2.5
24	0	0.9668	285.5	222	203	253	155.5
24	3	0.9833	294	232	204	254	156
24	5	0.9851	295.5	230.5	201	255.5	159
24	7	0.9859	297	228	201.5	256.5	159.5
24	10	0.9794	292.5	230.5	201	254	157
24	12	0.9893	295	232	206	257.5	156.5
24	16	0.9762	283	236.5	202.5	253	155
24	21	0.9719	280.5	229	205	255.5	154.5
24	25	0.9682	273	225.5	206.5	253.5	158
24	27	0.9701	275.5	224.5	205	257.5	157.5
24	32	0.9616	267	224.5	207.5	256.5	154
24	39	0.9634	262	230.5	204.5	258	155.5
24	45	0.9536	260	224	203.5	257	154.5
24	53	0.8741	216.5	207	188	240.5	148
24	60	0.8523	187	207	187	238.5	148.5
24	68	0.8645	200.5	205.5	192	242	146
24	77	0.8253	178	196.5	183	246.5	137
24	87	0.7811	156	184	178.5	248	124.5
24	96	0.686	127.5	147	169.5	234	104.5

24	109	0.5954	98	127	139.5	227	89
24	140	0.3111	36	48.5	72	167	37.5
24	174	0.1268	15.5	13	26.5	86	10.5
24	209	0.0356	2.5	1	6	29.5	3.5
28	0	0.972	216.5	270.5	181.5	250	159.5
28	3	0.9978	222.5	272	181.5	257.5	171
28	5	0.9804	216.5	267.5	183.5	248	168
28	7	0.9553	201	262	177.5	251	165.5
28	10	0.8564	178	223	158	251.5	141
28	12	0.7236	132	187	128.5	238.5	121.5
28	16	0.5732	96.5	126	86	235	100.5
28	21	0.4597	71.5	103.5	73.5	192	77
28	24	0.4233	61.5	98	66.5	175.5	74.5
28	27	0.4066	55	94	65	176.5	68.5
28	32	0.378	48.5	85.5	54.5	182	60.5
28	39	0.3438	39.5	75.5	55.5	176	47.5
28	45	0.3034	30	61	50	167	40.5
28	53	0.1858	15	32	26	120.5	23
28	68	0.091	7	10	5	79	9
28	77	0.0374	1	5	2	35	3
37	0	0.9878	275.5	243	161	258	191.5
37	1	0.9889	275.5	241	162.5	260	191
37	2	0.9775	267	235	157	264.5	194
37	3	0.9221	241.5	220.5	153	260.5	177.5
37	4	0.8325	202	203	138	268.5	144.5
37	5	0.6938	106.5	160.5	131	261.5	124.5
37	6	0.5656	59	115	110	252.5	101.5
37	7	0.386	34	78	75.5	205	50
37	8	0.224	17.5	35	45.5	132.5	27
37	9	0.1335	15	20	26.5	78	15
37	10	0.1127	9	16	18.5	77	12.5
37	11	0.1071	9	12	17	78.5	11
37	12	0.0781	6	10.5	13	55.5	7.5
37	13	0.0724	8	13	13	44.5	6.5
37	15	0.0581	6	13	13.5	28.5	5
37	16	0.0457	5	11.5	8.5	27	2

9.3.3 Data - Survival at variable temperatures

Day/Hours = time after experimental start in days/hours, Well1-4 = number of alive L3 in well 1-4, Sum = sum of alive L3 in well 1-4, % alive = proportion of alive L3.

Day	Hours	Well1	Well2	Well3	Well4	Sum	% alive
0	0	29	27	28	31	115	100
2	48	29	27	28	31	115	100
16	384	29	27	28	31	115	100
30	720	29	27	28	31	115	100
44	1056	29	27	28	30	114	99
58	1392	29	27	28	30	114	99
73	1752	29	25	28	30	112	97
93	2232	29	26	28	30	113	98
107	2568	29	26	28	30	113	98
135	3240	29	26	28	30	113	98
149	3576	29	26	27	30	112	97
163	3912	29	26	27	30	112	97
176	4224	29	26	27	30	112	97
191	4584	29	26	27	30	112	97
204	4896	28	26	27	30	111	97
219	5256	28	26	26	30	110	96
233	5592	26	26	26	30	108	94
247	5928	26	23	24	27	100	87
260	6240	26	20	23	26	95	83
274	6576	25	21	23	24	93	81
289	6936	23	19	22	24	88	77
302	7248	21	16	19	20	76	66
317	7608	19	13	17	18	67	58
331	7944	15	12	13	14	54	47
352	8448	14	12	12	13	51	44
366	8784	12	10	10	12	44	38
380	9120	11	9	10	10	40	35

9.3.4 Data - Survival in the field (part 1 of 2)

Day = time after experimental start in days, Season = season the plot was contaminated, Plot = plot the sample was sourced from, Block = the block 1-4 the plot belonged to, L3 = number of recovered L3.

Day	Season	Plot	Block	L3
31	1_winter	p2	b1	175
73	1_winter	p2	b1	105
112	1_winter	p2	b1	40
153	1_winter	p2	b1	0
31	1_winter	p7	b2	105
73	1_winter	p7	b2	40
112	1_winter	p7	b2	0
153	1_winter	p7	b2	10
31	1_winter	p11	b3	90
73	1_winter	p11	b3	90
112	1_winter	p11	b3	50
153	1_winter	p11	b3	0
31	1_winter	p15	b4	60
73	1_winter	p15	b4	80
112	1_winter	p15	b4	0
153	1_winter	p15	b4	0
41	2_spring	p4	b1	150
75	2_spring	p4	b1	120
94	2_spring	p4	b1	45
118	2_spring	p4	b1	0
41	2_spring	p5	b2	250
75	2_spring	p5	b2	110
94	2_spring	p5	b2	80
118	2_spring	p5	b2	10
41	2_spring	p9	b3	80
75	2_spring	p9	b3	90
94	2_spring	p9	b3	0
118	2_spring	p9	b3	0
41	2_spring	p16	b4	160
75	2_spring	p16	b4	10
94	2_spring	p16	b4	30
118	2_spring	p16	b4	0

9.3.5 Data - Survival in the field (part 2 of 2)

Day = time after experimental start in days, Season = season the plot was contaminated, Plot = plot the sample was sourced from, Block = the block 1-4 the plot belonged to, L3 = number of recovered L3.

Day	Season	Plot	Block	L3
19	3_summer	p3	b1	80
43	3_summer	p3	b1	0
74	3_summer	p3	b1	0
19	3_summer	p8	b2	60
43	3_summer	p8	b2	80
74	3_summer	p8	b2	0
19	3_summer	p12	b3	75
43	3_summer	p12	b3	100
74	3_summer	p12	b3	0
19	3_summer	p14	b4	80
43	3_summer	p14	b4	90
74	3_summer	p14	b4	0
29	4_autumn	p1	b1	130
57	4_autumn	p1	b1	0
29	4_autumn	p6	b2	165
57	4_autumn	p6	b2	0
29	4_autumn	p10	b3	100
57	4_autumn	p10	b3	40
29	4_autumn	p13	b4	70
57	4_autumn	p13	b4	80

9.3.6 Statistical Analysis for survival at constant temperatures - Minitab output (for LT50 values see Appendix 4)

temp = constant temperature used; Host letter = donor calf ID

General Linear Model: LT50 versus Temp, Host

```
Factor  Type   Levels  Values
Temp    fixed       6  8, 16, 20, 24, 28, 37
Host    fixed       5  A, B, C, D, E

Analysis of Variance for LT50, using Adjusted SS for Tests

Source  DF  Seq SS  Adj SS  Adj MS       F      P
Temp     5  948924  948924  189785  477.07  0.000
Host     4   13299   13299    3325    8.36  0.000
Error   20    7956    7956     398
Total   29  970179

S = 19.9452   R-Sq = 99.18%   R-Sq(adj) = 98.81%

Unusual Observations for LT50

Obs   LT50     Fit  SE Fit  Residual  St Resid
 30  8.626  41.435  11.515   -32.809     -2.01 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method and 95.0% Confidence

Temp  N     Mean  Grouping
 8    5  512.384  A
16    5  332.990    B
20    5  176.506      C
24    5  118.271        D
28    5   27.044          E
37    5    6.388          E

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Host  N     Mean  Grouping
E     6  230.644  A
D     6  208.616  A B
C     6  182.781    B C
B     6  182.044    B C
A     6  173.901      C

Means that do not share a letter are significantly different.
```

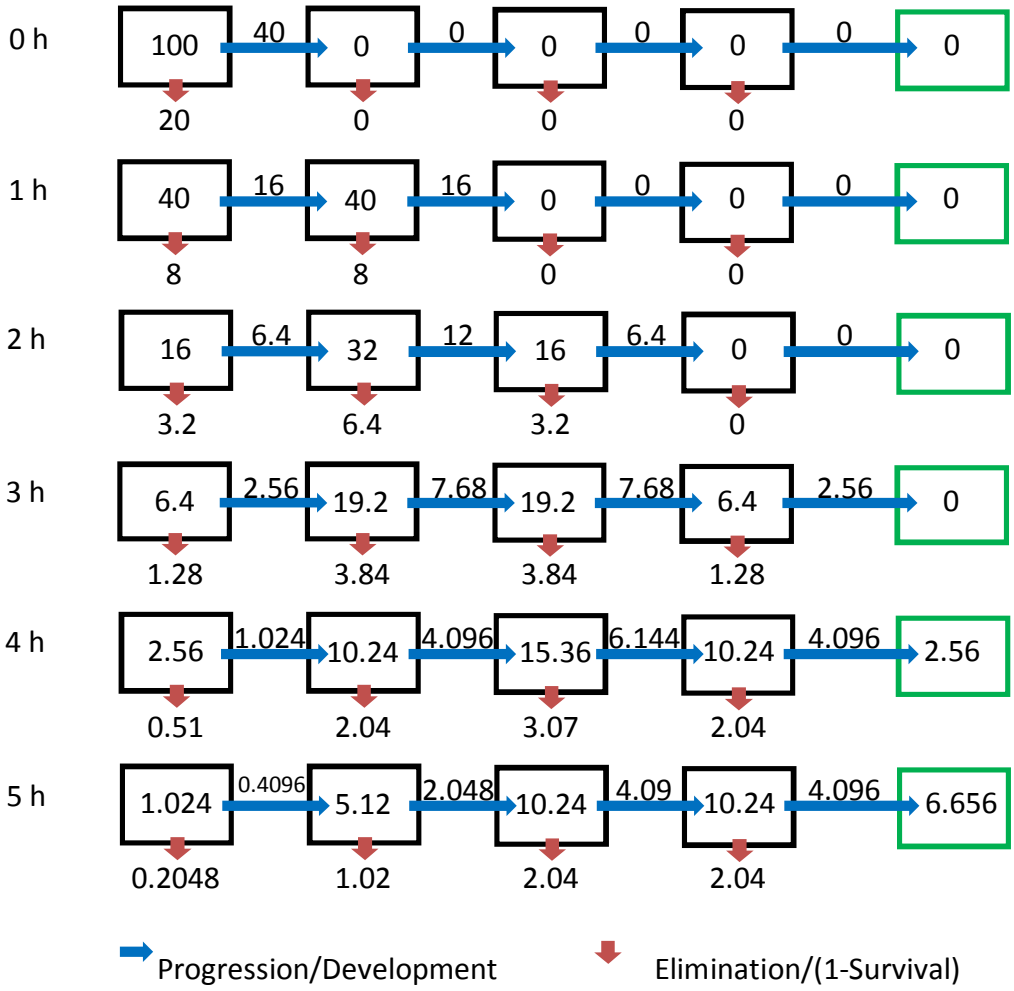
Appendix 4

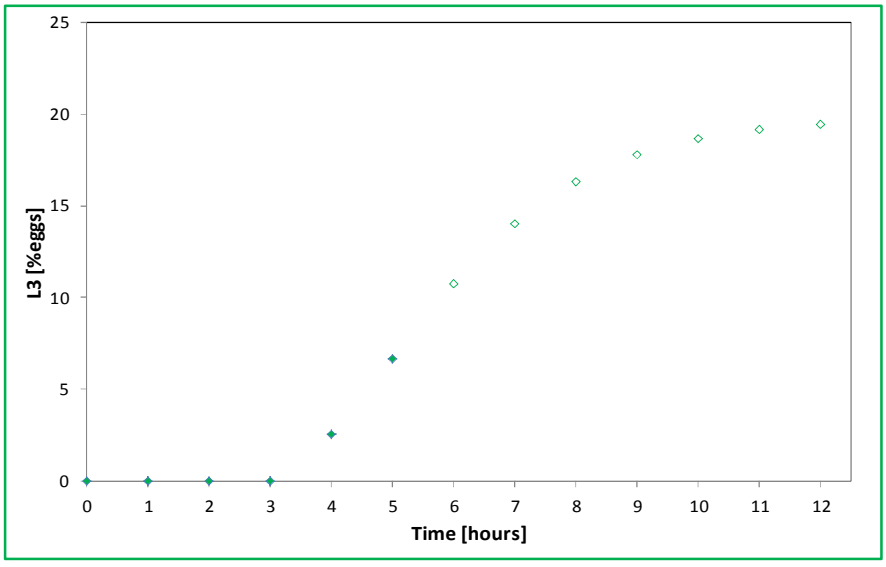
Supplementary information for Chapter 4

Modelling the development and ageing of *Cooperia oncophora* third stage larvae

9.4 Chapter 4 – Modelling the development and Survival of *Cooperia oncophora* third stage larvae

9.4.1 Schematics of the calculations by a 4 stage EBT with a progression rate (blue) of 0.4 and an elimination rate (red) of 0.2 for the first 6 hours





9.4.2 Data - Predicted time to 50% development (DT50) and proportion of L3s developed from eggs from the development experiments at constant temperatures (Chapter 2)

temp = temperature, exp = experiment 1 or 2, Host = host animal the faeces was sourced from, Dt50 [hours] = time in hours at which 50% L3 developed, L3 plateau = the average proportion of development success.

temp	exp	Host	DT50 [hours]	L3 plateau
8	exp2	A	1152	0.093068
8	exp2	B	1752	0.001701
8	exp2	C	1152	0.070192
16	exp1	B	464.4576729	0.185891
16	exp1	C	360.7567907	0.088231
16	exp1	D	450.447087	0.137788
16	exp2	A	396.7517138	0.240999
16	exp2	B	558.6143209	0.066101
16	exp2	C	456.6845022	0.256175
20	exp1	B	300.288132	0.218027
20	exp1	C	301.4460054	0.145124
20	exp1	D	320.0064756	0.220056
24	exp1	B	264.3137667	0.145793
24	exp1	C	239.6914074	0.117453
24	exp1	D	248.3707791	0.145299
28	exp2	A	144.8565954	0.334602
28	exp2	B	225.3814377	0.207629
28	exp2	C	151.2545405	0.614738
32	exp2	A	109.3804859	0.187670
32	exp2	B	155.1059865	0.152625
32	exp2	C	119.2203552	0.464034

9.4.3 Data - Predicted median survival time (LT50) values from survival experiment at constant temperatures (Chapter 3)

temp = temperature, Host = host animal the faeces was sourced from, LT50 = median survival time in days.

Temp	Host	LT50
8	A	505.7311
8	B	497.4118
8	C	469.0487
8	D	551.384
8	E	538.3448
16	A	292.8163
16	B	296.0439
16	C	325.2948
16	D	362.809
16	E	387.9843
20	A	135.5198
20	B	160.5634
20	C	159.8632
20	D	182.7943
20	E	243.7915
24	A	86.87779
24	B	110.5099
24	C	112.7288
24	D	125.9195
24	E	155.3211
28	A	17.71394
28	B	21.8435
28	C	23.9406
28	D	21.92421
28	E	49.79648
37	A	4.749832
37	B	5.890626
37	C	5.809322
37	D	6.863585
37	E	8.625985

9.4.4 Data – Development Model simulation of egg to L3 development (%) for 30 day periods using hourly temperature data commencing on the 1st day of each month from Jan 2002 to Dec 2011

month	mean	SEM	max	min
DM Lincoln, NZ				
Jan	17.85	0.73	22.41	14.62
Feb	16.92	0.64	19.74	13.32
Mar	14.29	0.61	17.33	10.37
Apr	8.09	0.72	12.19	5.44
May	3.72	0.52	6.25	1.14
Jun	0.86	0.19	2.18	0.32
Jul	0.50	0.07	1.03	0.24
Aug	1.48	0.20	2.38	0.59
Sep	4.23	0.37	6.47	2.78
Oct	6.93	0.24	8.11	5.42
Nov	13.28	0.78	18.76	11.07
Dec	15.09	0.78	18.14	10.41
DM Palmerston North, NZ				
Jan	17.97	0.81	23.83	15.31
Feb	19.15	0.87	24.54	15.69
Mar	15.64	0.47	17.78	13.18
Apr	10.46	0.47	13.19	8.00
May	6.43	0.62	9.31	3.39
Jun	3.02	0.47	5.14	0.99
Jul	1.98	0.21	2.98	0.93
Aug	3.02	0.27	4.35	1.82
Sep	5.73	0.41	7.38	3.75
Oct	8.23	0.46	9.90	5.43
Nov	12.23	0.56	14.78	9.72
Dec	15.54	0.78	18.02	11.10
DM Warkworth, NZ				
Jan	19.26	0.73	23.34	16.78
Feb	19.77	0.53	22.43	17.24
Mar	17.42	0.49	19.90	15.35
Apr	13.25	0.35	15.03	11.71
May	9.60	0.67	12.59	5.36
Jun	5.80	0.71	8.42	2.15
Jul	4.01	0.31	5.37	2.50
Aug	4.95	0.40	7.18	3.10
Sep	8.51	0.37	9.75	5.56
Oct	10.55	0.45	13.23	8.42
Nov	13.66	0.49	15.95	11.11
Dec	16.59	0.75	21.41	13.26

9.4.5 Data – Ageing Model simulation on [L3] survival (%) for 30 day period using hourly temperature data commencing on the 1st day of each month from Jan 2002 to Dec 2011

month	mean	SEM	max	min
AM Lincoln, NZ				
Jan	99.98681	0.006673	99.99996	99.9436
Feb	99.99854	0.000545	100	99.99581
Mar	99.99995	2.55E-05	100	99.99974
Apr	100	4.73E-07	100	100
May	100	9.92E-10	100	100
Jun	100	1.14E-10	100	100
Jul	100	1.91E-11	100	100
Aug	100	1.31E-10	100	100
Sep	100	2.92E-09	100	100
Oct	100	7.79E-09	100	100
Nov	99.99759	0.001723	100	99.98371
Dec	99.99838	0.0013	100	99.98676
AM Palmerston North, NZ				
Jan	99.97936	0.014388	99.99984	99.8514
Feb	99.95583	0.03186	99.99999	99.68131
Mar	99.99851	0.001243	100	99.98744
Apr	100	3.49E-07	100	100
May	100	5.74E-09	100	100
Jun	100	4.95E-10	100	100
Jul	100	1E-10	100	100
Aug	100	4.32E-10	100	100
Sep	100	3.72E-09	100	100
Oct	100	3.5E-08	100	100
Nov	99.99997	1.71E-05	100	99.99984
Dec	99.99873	0.000912	100	99.99063
AM Warkworth, NZ				
Jan	99.72883	0.164948	99.99993	98.36782
Feb	99.97383	0.013829	99.99988	99.88154
Mar	99.98467	0.014116	99.99999	99.85777
Apr	100	8.85E-07	100	99.99999
May	100	5.98E-08	100	100
Jun	100	4.47E-08	100	100
Jul	100	8.98E-10	100	100
Aug	100	1.74E-09	100	100
Sep	100	2.07E-08	100	100
Oct	100	7.11E-07	100	99.99999

Nov	99.99317	0.006802	100	99.93195
Dec	99.89866	0.100709	100	98.99228

9.4.6 Data – Combined model simulation of egg to L3 development (%) and [L3] survival (%) for 30 day periods using hourly temperature data commencing on the 1st day of each month from Jan 2002 to Dec 2011

month	mean	SEM	max	min
CM Lincoln, NZ				
Jan	17.81	0.72	22.35	14.62
Feb	16.91	0.64	19.71	13.32
Mar	14.29	0.61	17.32	10.37
Apr	8.09	0.72	12.19	5.44
May	3.72	0.52	6.25	1.14
Jun	0.86	0.19	2.18	0.32
Jul	0.50	0.07	1.03	0.24
Aug	1.48	0.20	2.38	0.59
Sep	4.23	0.37	6.47	2.78
Oct	6.93	0.24	8.11	5.42
Nov	13.27	0.77	18.74	11.07
Dec	15.08	0.78	18.09	10.41
CM Palmerston North, NZ				
Jan	17.92	0.80	23.74	15.30
Feb	19.08	0.84	24.41	15.69
Mar	15.62	0.46	17.76	13.18
Apr	10.46	0.47	13.19	8.00
May	6.43	0.62	9.31	3.39
Jun	3.02	0.47	5.14	0.99
Jul	1.98	0.21	2.98	0.93
Aug	3.02	0.27	4.35	1.82
Sep	5.73	0.41	7.38	3.75
Oct	8.23	0.46	9.90	5.43
Nov	12.23	0.56	14.78	9.72
Dec	15.53	0.78	18.00	11.10
CM Warkworth, NZ				
Jan	18.98	0.68	22.87	16.76
Feb	19.71	0.51	22.33	17.24
Mar	17.38	0.47	19.62	15.35
Apr	13.25	0.35	15.03	11.71
May	9.60	0.67	12.59	5.36
Jun	5.80	0.71	8.42	2.15
Jul	4.01	0.31	5.37	2.50
Aug	4.95	0.40	7.18	3.10

Sep	8.51	0.37	9.75	5.56
Oct	10.55	0.45	13.23	8.42
Nov	13.62	0.48	15.94	11.11
Dec	16.52	0.71	20.74	13.25

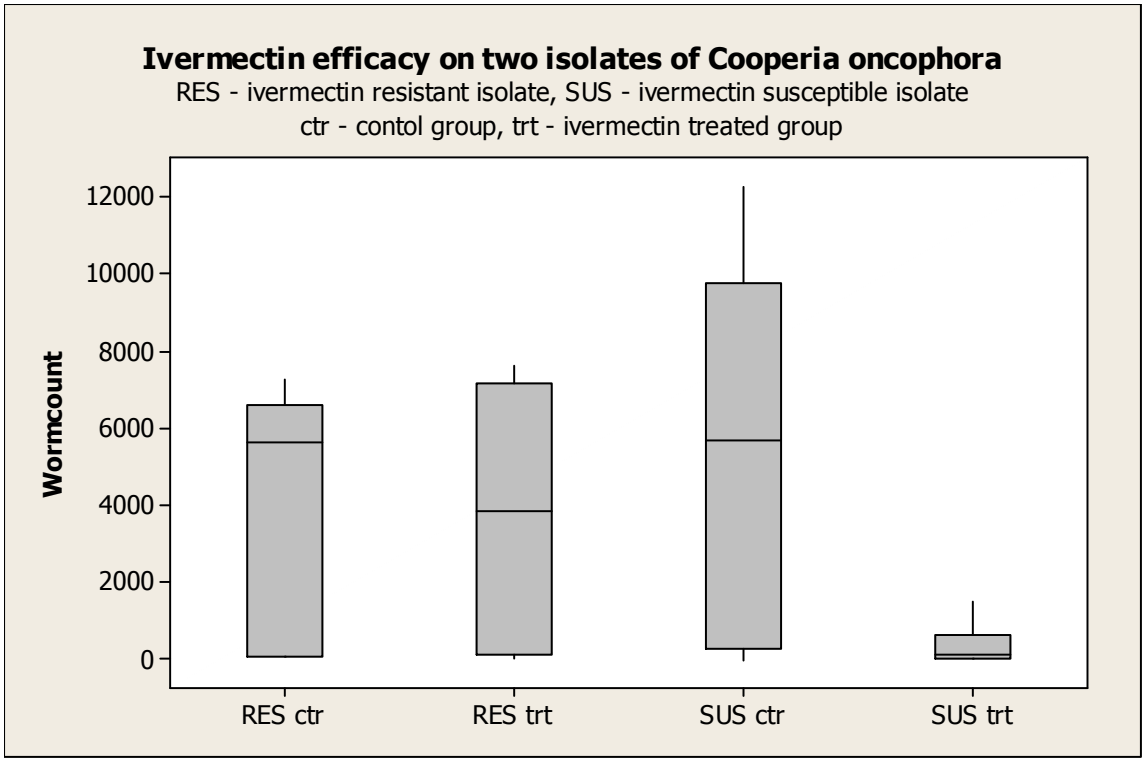
Appendix 5

Supplementary information for Chapter 5

Efficacy of ivermectin on two isolates of *Cooperia oncophora*

9.5 Efficacy of ivermectin for two isolates (susceptible (SUS) & resistant (RES)) of *Cooperia oncophora*

9.5.1 Supplementary figure for ivermectin-efficacy experiment



Boxplot for the worm count data for two *Cooperia oncophora* isolates one ivermectin-susceptible (SUS) and one ivermectin-resistant (RES). The control groups (ctr) were untreated the treatment groups (trt) received the recommended oral dose of ivermectin

9.5.2 Timetable Experiment 1

day	weekday	susceptible (n=3) SUS crt	(n=3) SUS treat	resistant (n=6) RES ctr	(n=6) RES treat
0	Monday	Exp start	Exp start	Exp start	Exp start
0	Monday	drench	drench	drench	drench
10	Thursday	infection	infection	infection	infection
11	Friday	infection	infection	infection	infection
14	Monday	infection	infection	infection	infection
31	Thursday		drench		drench
37	Wednesday	kill (1x)	kill (2x)	kill (3x)	kill (3x)
38	Thursday	kill (2x)	kill (1x)	kill (3x)	kill (3x)

9.5.3 Timetable Experiment 2

day	weekday	susceptible (n=3) SUS crt	(n=3) SUS treat
0	Monday	Exp start	Exp start
0	Monday	drench	drench
10	Thursday	infection	infection
11	Friday	infection	infection
14	Monday	infection	infection
31	Thursday		drench
37	Wednesday	kill	kill

9.5.4 Randomization of calves in Exp1 based on faecal egg count (epg)

group	id	epg	group	id	epg
sus contr	651	25	re contr	596	175
sus contr	607	0	re contr	582	25
sus contr	110	0	re contr	130	25
sus treat	662	25	re contr	658	0
sus treat	631	0	re contr	664	0
sus treat	569	0	re contr	581	0
			re treat	526	100
			re treat	586	75
			re treat	595	25
			re treat	648	0
			re treat	90	0
			re treat	594	0

Appendix 6

Supplementary information for Chapter 6

Establishment of *Cooperia oncophora* in calves

9.6 Establishment of *Cooperia oncophora* in calves

9.6.1 Timetable (Groups A-E)

Infection/Drench/Kill-Timetable

Farm days	Monday and Friday
days infection-drench	25
days drench-kill	5
days infection-kill	30

# of susceptible L3/week+group B: zero = 0 // C: low = 2x1000 // D: high = 2x5000

infection oral infection with resistant C. oncophora [L3]/individually prepared for each animal

drench recommended dose of IVOMEC liquid

Week	interval [weeks]	Days	Day		Task	Animals involved	Group					Total kills	Notes	
			5/07/2010	Monday	arrival	all	all							
					weigh	all	all							
					taging	all	all							
					initial drench	all	all		Tag #				recom. dose Scanda-C/individual weight	
		0	6/09/2010	Monday	infection	4	A	1011	1013	1014	1015		7 days	
		25	1/10/2010	Friday	drench	4	A							
0	0	30	6/10/2010	Wednesday	kill	4	A	B	C	D	E	4		
		42	18/10/2010	Monday	infection	6	B/C/D	1507	1761	2200				
		67	12/11/2010	Friday	drench	6	B/C/D	1518	1717	2364			!!!	
6	6	72	17/11/2010	Wednesday	kill	6	B/C/D					10		
		91	6/12/2010	Monday	infection	8	B/C/D/E	1527	1715	2396	2347			26/11/2010 Friday
		116	31/12/2010	Friday	drench	8	B/C/D/E	1513	1720	2102	2141		group E animals must be drenched 10 days before infection	
12	7	121	5/01/2011	Wednesday	kill	8	B/C/D/E					18		
		112	27/12/2010	Monday	infection	8	B/C/D/E	1556	1703	2110	2315			17/12/2010 Friday
		137	21/01/2011	Friday	drench	8	B/C/D/E	1511	1708	2185	2187			
16	3	142	26/01/2011	Wednesday	kill	8	B/C/D/E					26		
		140	24/01/2011	Monday	infection	8	B/C/D/E	1517	1729	2440	2202			14/01/2011 Friday
		165	18/02/2011	Friday	drench	8	B/C/D/E	1553	1714	2435	2511			
20	4	170	23/02/2011	Wednesday	kill	8	B/C/D/E					34		
		168	21/02/2011	Monday	infection	6	B/C/D	1520	1707	2112				
		193	18/03/2011	Friday	drench	6	B/C/D	1510	1724	2535				
24	4	198	23/03/2011	Wednesday	kill	6	B/C/D					40		
		210	4/04/2011	Monday	infection	6	B/C/D	1503	1725	2321				
		235	29/04/2011	Friday	drench	6	B/C/D	1524	1710	2646				
30	6	240	4/05/2011	Wednesday	kill	6	B/C/D					46		
		252	16/05/2011	Monday	infection	6	B/C/D	1525	1758	2318				
		277	10/06/2011	Friday	drench	6	B/C/D	1528	1722	2443				
36	6	282	15/06/2011	Wednesday	kill	6	B/C/D					52		
		294	27/06/2011	Monday	infection	6	B/C/D	1516	1721	2345				
		319	22/07/2011	Friday	drench	6	B/C/D	1505	1709	2355				
42	6	324	27/07/2011	Wednesday	kill	6	B/C/D	1514	1716			58		
							subtotal					58		
							spare animals					3		
							total animals					61		

9.6.2 Group B Scanda treatments (per individual liveweight)

Date = date treatment was given, Animals excluded = animals excluded from treatment – either challenged or scheduled for challenge within 14days

Date	Animals excluded
10/12/2010	1527, 1513
07/01/2011	1556, 1511
18/02/2011	1517, 1553, 1520, 1510
15/04/2011	1503, 1524

9.6.3 Data - Establishment – worm count results

FID = Ear tag number of calve, group = treatment group, challenge = date the calves were challenged, day = time of challenge after experimental start in days, age = age of calve at challenge, male/female = number of male/female *Cooperia oncophora* recovered from 10% small intestinal sample, total = sum of male and female, Establishment = established proportion of 15,000 *Cooperia oncophora* given as challange

FID	group	challenge	day	age	male	female	total	Establishment
1011	A	6/09/2010	0	167	472	537	10090	0.67
1013	A	6/09/2010	0	151	400	455	8550	0.57
1014	A	6/09/2010	0	145	357	478	8350	0.56
1015	A	6/09/2010	0	120	184	294	4780	0.32
1507	B	18/10/2010	42	201	313	354	6670	0.44
1518	B	18/10/2010	42	180	286	363	6490	0.43
1513	B	6/12/2010	91	221	71	52	1230	0.08
1527	B	6/12/2010	91	240	136	268	4040	0.27
1511	B	27/12/2010	112	235	193	229	4220	0.28
1556	B	27/12/2010	112	271	0	1	10	0.00
1517	B	24/01/2011	140	290	299	400	6990	0.47
1553	B	24/01/2011	140	272	207	367	5740	0.38
1510	B	21/02/2011	168	307	193	310	5030	0.34
1520	B	21/02/2011	168	317	111	165	2760	0.18
1503	B	4/04/2011	210	374	51	112	1630	0.11
1524	B	4/04/2011	210	354	100	210	3100	0.21
1525	B	16/05/2011	252	406	93	188	2810	0.19
1528	B	16/05/2011	252	382	1	2	30	0.00
1505	B	27/06/2011	294	436	105	202	3070	0.20
1514	B	27/06/2011	294	437	44	86	1300	0.09
1717	C	18/10/2010	42	179	181	217	3980	0.27
1761	C	18/10/2010	42	191	380	493	8730	0.58
1715	C	6/12/2010	91	248	16	47	630	0.04
1720	C	6/12/2010	91	221	3	8	110	0.01
1703	C	27/12/2010	112	282	0	10	100	0.01
1708	C	27/12/2010	112	250	7	15	220	0.01
1714	C	24/01/2011	140	282	0	0	0	0.00
1729	C	24/01/2011	140	305	1	5	60	0.00
1707	C	21/02/2011	168	316	0	3	30	0.00
1724	C	21/02/2011	168	297	1	11	120	0.01
1710	C	4/04/2011	210	356	6	32	380	0.03
1725	C	4/04/2011	210	363	0	0	0	0.00
1722	C	16/05/2011	252	392	0	0	0	0.00
1758	C	16/05/2011	252	403	1	0	10	0.00
1716	C	27/06/2011	294	411	0	0	0	0.00
1721	C	27/06/2011	294	457	0	4	40	0.00
2200	D	18/10/2010	42	209	43	132	1750	0.12

FID	group	challenge	day	age	male	female	total	Establishment
2364	D	18/10/2010	42	177	21	70	910	0.06
2102	D	6/12/2010	91	229	19	56	750	0.05
2396	D	6/12/2010	91	246	0	2	20	0.00
2110	D	27/12/2010	112	276	44	138	1820	0.12
2185	D	27/12/2010	112	254	144	342	4860	0.32
2435	D	24/01/2011	140	285	0	0	0	0.00
2440	D	24/01/2011	140	287	15	54	690	0.05
2112	D	21/02/2011	168	317	0	2	20	0.00
2535	D	21/02/2011	168	311	0	3	30	0.00
2321	D	4/04/2011	210	364	5	25	300	0.02
2646	D	4/04/2011	210	337	0	2	20	0.00
2318	D	16/05/2011	252	416	8	21	290	0.02
2443	D	16/05/2011	252	391	0	0	0	0.00
2345	D	27/06/2011	294	447	0	0	0	0.00
2355	D	27/06/2011	294	425	0	0	0	0.00
2141	E	6/12/2010	91	218	146	236	3820	0.25
2347	E	6/12/2010	91	218	135	177	3120	0.21
2187	E	27/12/2010	112	239	188	279	4670	0.31
2315	E	27/12/2010	112	239	96	164	2600	0.17
2202	E	24/01/2011	140	267	138	204	3420	0.23
2511	E	24/01/2011	140	267	0	1	10	0.00
1345	F1	8/11/2010	63		87	118	2050	0.14
1344	F1	8/11/2010	63		203	277	4800	0.32
1343	F1	8/11/2010	63		283	346	6290	0.42
1342	F1	8/11/2010	63		261	341	6020	0.40
505	F2	16/08/2011	344		36	49	850	0.06
501	F2	16/08/2011	344		55	83	1380	0.09
509	F2	16/08/2011	344		38	80	1180	0.08
508	F2	16/08/2011	344		74	108	1820	0.12

9.6.4 Egg counts *in utero* in recovered adult female *Cooperia oncophora*

FID = Ear tag number of calf, group = treatment group, day = time of challenge after experimental start in days, 1-30 = individual egg count in one recovered female, mean = mean egg count.

FID	1011	1013	1014	1015	1507	1518	1513	1527	1511	1517	1520
group	A	A	A	A	B	B	B	B	B	B	B
day	0	0	0	0	42	42	91	91	112	140	168
1	47	79	50	47	80	56	83	100	75	131	107
2	50	52	51	29	122	81	49	70	107	156	67
3	33	67	34	12	57	58	45	75	82	50	75
4	44	51	48	35	96	62	65	116	98	230	90
5	53	33	47	31	124	63	0	110	65	115	105
6	54	80	48	4	102	90	72	99	65	94	68
7	47	89	40	37	124	46	39	66	40	102	73
8	38	55	60	48	86	40	0	119	56	139	56
9	31	66	49	48	101	77	49	52	34	147	77
10	53	55	58	34	84	2	78	68	49	136	92
11	52	40	70	42	166	69	67	57	73	148	69
12	47	63	78	28	93	58	71	58	80	168	46
13	49	78	56	23	52	66	53	75	79	109	24
14	51	76	58	42	56	22	79	134	70	94	57
15	53	49	59	1	110	127	50		23	67	81
16	31	28	58	33	115	72	29		61	126	56
17	73	88	79	33	116	38	65		70	135	79
18	19	86	62	37	112	114	69		64	83	59
19	80	60	72	28	117	77	64		91	82	47
20	44	106	44	18	78	61	57		55	110	85
21	57	65	76	43	72	45			83		77
22	29	70	29	43	95	75			51		62
23	48	110	31	21	111	51			42		49
24	56	74	81	43	94	30			77		56
25	44	43	9	51	128	73			99		47
26	57	38	39	37	124	42			58		99
27	76	89	45	33	99	46			65		67
28	44	98	46	50	100	45			76		101
29	57	72	58	6	102	105			73		53
30	54	67	53	36	84	63			49		82
mean	49	68	53	32	100	62	54	86	67	121	70

Data - Establishment – Egg counts in recovered adult female *Cooperia oncophora*

FID	1510	1524	1525	1516	1717	1761	1720	1703	1708	1707	1724
group	B	B	B	B	C	C	C	C	C	C	C
day	193	210	252	294	42	42	91	112	112	168	168
1	54	60	126	84	10	70	52	18	44	1	31
2	30	63	113	99	90	45	43		28	25	
3	96	110	93	117	96	92	53		37	0	
4	54	70	83	109	88	91	38		38	3	
5	71	72	104	69	103	64			35	23	
6	72	84	108	71	46	91			15	4	
7	43	69	82	83	104	97			42	20	
8	52	76	86		81	40			26		
9	48	102	91		4	46			77		
10	55	60	105		11	69			27		
11	55	77	110		71	65			49		
12	59	66	137		26	97			20		
13	66	73	79		19	81			52		
14	80	106	89		98	81			34		
15	71	51	91		65	82			16		
16	55	75	97		64	36			12		
17	61	74	121		53	71			48		
18	59	108	89		38	66			57		
19		89	90		74	81			51		
20		84	86		113	97			48		
21		100	99		40	48			51		
22		84			48	19			45		
23		92			27	25			51		
24		55			72	71			48		
25					84	65			57		
26					80				38		
27					19				28		
28					60				45		
29					13						
30					56						
mean	60	79	99	90	58	68	47	18	40	11	31

Data - Establishment – Egg counts in recovered adult female *Cooperia oncophora*

FID	1710	1721	2200	2364	2102	2110	2185	2440	2535	2321	2318
group	C	C	D	D	D	D	D	D	D	D	D
day	210	294	42	42	91	112	112	140	168	210	252
1	34	17	67	7	60	50	20	34	30	34	41
2	17	65	9	8	8	43	4	38		32	42
3	24		9	10	16	48	38	39		9	25
4	13		47	5	26	57	2	50		29	48
5	28		16	0	64	39	3	21		39	
6			11	0	27	36	1	39		36	
7			10	6		42	10	50		31	
8			42	3		49	0	52		26	
9			33	0		33	6			39	
10			55	5		33	12			24	
11			27	4		48	34				
12			53	0			15				
13			23	9			36				
14			60	0			33				
15			66	11			42				
16			62	4			0				
17			26	1			14				
18			58	8			55				
19			21	1			0				
20			31	0			0				
21			61	10			2				
22			50	5			8				
23			27	3							
24			47	8							
25			54	4							
26			59	2							
27			52	10							
28				0							
29											
30											
mean	23	41	40	4	34	43	15	40	30	30	39

Data - Establishment – Egg counts in recovered adult female *Cooperia oncophora*

FID	2141	2347	2187	2315	2511	1344	1343	501	505
group	E	E	E	E	E	F1	F1	F2	F2
day	91	91	112	112	140	63	63	344	344
1	30	81	66	47	72	98	39	91	49
2	59	64	61	37		95	23	95	52
3	103	76	37	51		103	10	91	37
4	37	79	74	41		126	57	105	23
5	62	57	5	40		123	42	67	19
6	56	66	51	33		120	47	85	37
7	47	82	63	44		97	19	80	
8	74	108	58	36		127	29	113	
9	76	37	75	28		84	21	73	
10	70		57	40		133	55	108	
11	68		37	35		106	33	92	
12	86		66	9		112	39	104	
13	57		77	35		133	31	106	
14	52		29	34		111	21	109	
15	54		49	48		109	38	111	
16	60		40	30			27	116	
17	55		48	0			77	107	
18	64		62	8			68	86	
19	69		48	38			15	90	
20			41	24			27	69	
21			26				43	111	
22			65				52		
23			25						
24			40						
25			21						
26			34						
27			49						
28			43						
29			51						
30									
mean	62	72	48	33	72	112	37	96	36

9.6.5 Length in mm of recovered adult female *Cooperia oncophora*

FID = Ear tag number of calf, group = treatment group, day = time of challenge after experimental start in days, 1-30 = individual length of one recovered female (mm), mean = mean length.

FID	1011	1013	1014	1015	1505	1507	1510	1511	1513	1517
group	A	A	A	A	B	B	B	B	B	B
day	0	0	0	0	294	42	193	112	91	140
1	11.37	10.70	10.83	11.77	10.95	11.48	9.98	12.35	10.38	13.22
2	10.62	12.72	10.38	11.37	11.08	11.49	11.03	13.91	10.29	12.53
3	11.14	11.34	10.66	11.51	10.18	12.31	10.66	12.99	9.96	13.08
4	12.18	11.75	9.81	9.84	10.52	12.96	11.53	12.35	8.14	13.48
5	12.64	12.09	9.89	9.46	10.72	11.64	10.49	12.05	9.37	12.04
6	12.04	10.89	10.29	9.70	11.20	11.49	11.16	10.87	10.32	12.60
7	11.53	12.53	10.62	11.06	9.84	11.51	10.78	10.33	10.25	12.51
8	12.93	11.89	10.34	10.04	10.23	11.09	12.16	12.48	10.17	13.13
9	11.51	11.48	10.60	9.74	11.55	12.59	10.90	11.19	10.55	12.93
10	12.61	10.51	10.09	10.62	10.13	12.43	11.62	9.89	10.78	14.05
11	11.28	12.79	9.89	11.42	11.41	12.80	11.53	10.62	11.20	12.76
12	11.64	10.57	9.31	10.56	11.35	12.70	10.66	11.18	10.22	13.47
13	11.66	12.83	10.30	10.28	10.67	10.52	11.21	10.83	10.10	13.70
14	11.31	11.66	10.43	10.72	12.39	12.75	10.69	11.26	8.94	12.55
15	11.66	10.83	10.55	10.29	11.67	11.75	10.94	10.71	10.33	12.12
16	10.57	12.27	11.06	10.07	11.53	12.99		10.81	10.97	12.27
17	10.13	12.47	10.36	9.85	11.01	11.27		12.40	10.88	14.13
18	11.96	11.90	10.92	10.31	12.35	12.32		11.13	10.32	12.37
19	11.00	10.87	9.84	11.90	11.65	10.87		11.60	10.35	13.74
20	11.93	12.56	9.71	8.84	11.66	11.90		11.22	10.46	13.28
21	11.27	10.39	10.30	9.28	12.08	10.40		11.76		13.08
22	11.53	11.26	10.40	10.00	10.82	11.75		11.11		12.31
23	12.39	10.78	10.61	10.53	11.69	12.88		11.75		13.60
24	10.31	10.78	11.31	9.20	9.73	12.20		11.13		
25	12.32	11.84	10.39	9.13	11.45	10.96		11.55		
26	10.89	11.17	10.10	10.43	11.87	11.80		11.84		
27	10.52	11.49	9.97	9.99	10.23	12.40		10.72		
28		12.62	10.94		10.79			12.40		
29		11.44	10.41		10.65			12.13		
30			10.03							
mean	11.52	11.60	10.34	10.29	11.08	11.90	11.02	11.54	10.20	13.00

Data - Establishment – Length in mm of recovered adult female *Cooperia oncophora*

FID	1518	1520	1525	1527	1553	1707	1708	1724	1761	2112
group	B	B	B	B	B	C	C	C	C	D
day	42	168	252	91	140	168	112	168	42	168
1	9.92	11.11	10.80	10.88	11.79	9.19	8.67	10.28	12.93	8.57
2	10.48	10.54	12.05	11.24	12.92	8.81	8.18	10.73	12.04	
3	11.72	11.11	12.01	10.75	12.57	8.53	8.45	11.30	11.50	
4	12.69	11.34	12.04	11.50	12.30	9.73	8.34		11.99	
5	12.72	10.85	11.79	11.40	10.97	8.72	9.27		11.71	
6	11.93	11.18	11.45	12.19	12.01	8.59	8.70		12.20	
7	11.17	9.71	11.14	10.92	11.05		8.31		12.24	
8	11.61	9.85	12.75	11.79	12.32		8.51		12.14	
9	10.70	11.18	11.60	10.43	11.86		8.67		12.35	
10	12.58	9.60	12.81	11.97	12.23		8.55		11.94	
11	11.79	10.98	11.10	11.93	11.83		10.24		10.27	
12	12.63	11.15	11.06	10.91	11.85		8.80		11.39	
13	13.49	9.52	12.35	9.41	11.31		9.05		12.28	
14	11.81	10.37	10.83	11.54	11.26		8.57		12.63	
15	12.02	9.59	11.28	11.07	11.19		8.46		12.95	
16	12.01	10.08	11.62	11.93	11.72		8.74		12.85	
17	13.38	9.81	11.68	11.53	11.70		8.55		11.55	
18	12.09	10.73	10.85	11.27	11.50				12.20	
19	11.32	11.75	11.20	11.05	10.79				11.81	
20	11.86	11.73	13.21	11.22	11.29				12.13	
21	12.15	10.27	11.57	10.39	11.31				11.99	
22	12.87	10.37	12.03		11.95				12.33	
23	11.82	11.29	11.18		12.31				13.23	
24	12.84	10.76			11.73				11.55	
25	10.86	10.96			11.02				13.28	
26	12.31	9.71			11.53				12.41	
27	12.40	10.89			11.68					
28	12.00	10.42			11.20					
29	11.70	10.83			12.36					
30	11.29	11.51			11.55					
mean	11.94	10.64	11.67	11.21	11.70	8.93	8.71	10.77	12.15	8.57

Data - Establishment – Length in mm of recovered adult female *Cooperia oncophora*

FID	2200	2321	2364	32141	32187	32315	32347	1343	1344	1345
group	D	D	D	E	E	E	E	F1	F1	F1
day	42	210	42	91	112	112	91	63	63	63
1	9.44	7.72	10.45	10.03	10.66	7.69	10.37	12.84	12.32	12.50
2	9.09	8.41	8.58	10.48	10.43	9.02	10.22	12.77	12.89	11.50
3	10.71	8.84	6.62	11.57	10.61	9.39	10.00	11.79	12.26	12.07
4	9.98	8.64	9.57	11.83	10.65	7.97	10.39	12.62	11.50	11.21
5	8.64	8.34	7.15	10.87	10.71	8.99		13.22	11.77	10.85
6	8.90	8.97	7.53	9.64	10.74	8.96		11.60	12.61	11.50
7	9.23	9.31	8.77	9.54	10.60	9.19		12.32	12.16	10.84
8	9.57	9.15	10.24		10.45	9.54		12.55	11.97	11.70
9	9.61		6.97		10.06	9.12		11.57	12.00	10.31
10	8.83		9.80		10.74	9.00		11.87	12.65	11.60
11	9.15		9.88		10.70	9.64		11.15	12.42	9.91
12	9.37		9.60		10.81	9.47		11.97	12.52	11.12
13	10.54		7.74		11.50	8.77		12.19	11.59	11.39
14	9.27		8.15		10.86	9.69		10.50	11.11	11.38
15	10.44		8.80		11.34	9.27		11.84	11.40	11.92
16	9.62		8.69		11.18	8.74		13.00	11.41	11.45
17	9.77		8.07		11.16	9.32		12.09	9.65	10.94
18	9.92		9.75		11.25	9.93		13.06		11.90
19	9.13		9.21		11.26	9.39		11.75		10.02
20	8.36		8.82		9.38	9.27		12.11		11.32
21	9.93		10.96		10.10	8.90		12.29		11.92
22	9.19		8.47		10.72	8.79		12.14		11.19
23	8.50		7.86		11.88	9.57		11.93		10.95
24	10.22		7.46		11.34	10.62				10.51
25	10.01		9.94		11.18	8.73				11.39
26	9.39		8.29		10.90	9.07				12.58
27	9.15		9.63		11.51					12.08
28	8.79				11.92					10.86
29					11.41					10.86
30										
mean	9.46	8.67	8.78	10.56	10.90	9.15	10.24	12.14	11.90	11.30

Data - Establishment – Length in mm of recovered adult female *Cooperia oncophora*

FID	501	505	509
group	F2	F2	F2
day	344	344	344
1	11.84	11.20	10.92
2	11.83	12.90	10.98
3	11.44	10.81	10.68
4	12.20	12.05	12.73
5	11.36	10.83	11.85
6	12.22	11.45	11.90
7	11.00	11.51	11.55
8	10.71	10.66	11.86
9	11.20	10.25	12.29
10	11.19	11.79	12.50
11	9.57	11.88	11.71
12	11.78	11.74	11.29
13	11.62	10.46	11.24
14	11.74	11.13	12.20
15	10.40	10.54	11.91
16	12.02	11.19	11.99
17	11.68	12.52	11.79
18	10.86		12.46
19	11.61		12.31
20	11.72		10.69
21	12.76		12.13
22	12.78		11.91
23	12.51		10.61
24	12.43		9.92
25	13.36		10.45
26	13.44		
27	11.71		
28	11.75		
29	12.59		
30	10.42		
mean	11.72	11.35	11.59

9.6.6 Data - Establishment – Establishment of *Cooperia oncophora* in calves as calculated for statistical analysis

Host = Host animal (group&FID) each group including the animals from A, Group = treatment group, Treatment = treatment the animal received (group), day = time of challenge after experimental start in days, Wormcount = total worm count from 10% sample multiplied by 10, FitCI = fit for fitted value; lwr for lower confidence limit; upr for upper confidence limit, FitWC = value for FitCI

Host	Group	Treatment	Day	Wormcount	FitCI	FitWC
A_1011	B	Ctr	0	10090	fit	5598.832
A_1013	B	Ctr	0	8550	fit	5598.832
A_1014	B	Ctr	0	8350	fit	5598.832
A_1015	B	Ctr	0	4780	fit	5598.832
B_1507	B	Ctr	42	6670	fit	4254.576
B_1518	B	Ctr	42	6490	fit	4254.576
B_1527	B	Ctr	91	4040	fit	3088.457
B_1513	B	Ctr	91	1230	fit	3088.457
B_1556	B	Ctr	112	10	fit	2692.285
B_1511	B	Ctr	112	4220	fit	2692.285
B_1517	B	Ctr	140	6990	fit	2241.955
B_1553	B	Ctr	140	5740	fit	2241.955
B_1520	B	Ctr	168	2760	fit	1866.95
B_1510	B	Ctr	168	5030	fit	1866.95
B_1503	B	Ctr	210	1630	fit	1418.703
B_1524	B	Ctr	210	3100	fit	1418.703
B_1525	B	Ctr	252	2810	fit	1078.079
B_1528	B	Ctr	252	30	fit	1078.079
B_1516	B	Ctr	294	1300	fit	819.2364
B_1505	B	Ctr	294	3070	fit	819.2364
A_1011	B	Ctr	0	10090	lwr	1459.907
A_1013	B	Ctr	0	8550	lwr	1459.907
A_1014	B	Ctr	0	8350	lwr	1459.907
A_1015	B	Ctr	0	4780	lwr	1459.907
B_1507	B	Ctr	42	6670	lwr	1438.375
B_1518	B	Ctr	42	6490	lwr	1438.375
B_1527	B	Ctr	91	4040	lwr	1302.907
B_1513	B	Ctr	91	1230	lwr	1302.907
B_1556	B	Ctr	112	10	lwr	1194.419
B_1511	B	Ctr	112	4220	lwr	1194.419
B_1517	B	Ctr	140	6990	lwr	1006.254
B_1553	B	Ctr	140	5740	lwr	1006.254
B_1520	B	Ctr	168	2760	lwr	794.3816
B_1510	B	Ctr	168	5030	lwr	794.3816
B_1503	B	Ctr	210	1630	lwr	505.8211
B_1524	B	Ctr	210	3100	lwr	505.8211
B_1525	B	Ctr	252	2810	lwr	299.7809

Host	Group	Treatment	Day	Wormcount	FitCI	FitWC
B_1528	B	Ctr	252	30	lwr	299.7809
B_1516	B	Ctr	294	1300	lwr	171.0512
B_1505	B	Ctr	294	3070	lwr	171.0512
A_1011	B	Ctr	0	10090	upr	21471.86
A_1013	B	Ctr	0	8550	upr	21471.86
A_1014	B	Ctr	0	8350	upr	21471.86
A_1015	B	Ctr	0	4780	upr	21471.86
B_1507	B	Ctr	42	6670	upr	12584.63
B_1518	B	Ctr	42	6490	upr	12584.63
B_1527	B	Ctr	91	4040	upr	7320.989
B_1513	B	Ctr	91	1230	upr	7320.989
B_1556	B	Ctr	112	10	upr	6068.555
B_1511	B	Ctr	112	4220	upr	6068.555
B_1517	B	Ctr	140	6990	upr	4995.121
B_1553	B	Ctr	140	5740	upr	4995.121
B_1520	B	Ctr	168	2760	upr	4387.694
B_1510	B	Ctr	168	5030	upr	4387.694
B_1503	B	Ctr	210	1630	upr	3979.113
B_1524	B	Ctr	210	3100	upr	3979.113
B_1525	B	Ctr	252	2810	upr	3877.01
B_1528	B	Ctr	252	30	upr	3877.01
B_1516	B	Ctr	294	1300	upr	3923.668
B_1505	B	Ctr	294	3070	upr	3923.668
A_1011	C	Low	0	10090	fit	5203.57
A_1013	C	Low	0	8550	fit	5203.57
A_1014	C	Low	0	8350	fit	5203.57
A_1015	C	Low	0	4780	fit	5203.57
C_1761	C	Low	42	8730	fit	1627.832
C_1717	C	Low	42	3980	fit	1627.832
C_1715	C	Low	91	630	fit	419.5682
C_1720	C	Low	91	110	fit	419.5682
C_1703	C	Low	112	100	fit	234.6695
C_1708	C	Low	112	220	fit	234.6695
C_1729	C	Low	140	60	fit	108.1423
C_1714	C	Low	140	0	fit	108.1423
C_1707	C	Low	168	30	fit	49.83499
C_1724	C	Low	168	120	fit	49.83499
C_1725	C	Low	210	0	fit	15.58988
C_1710	C	Low	210	380	fit	15.58988
C_1758	C	Low	252	10	fit	4.876979
C_1722	C	Low	252	0	fit	4.876979
C_1721	C	Low	294	40	fit	1.525665
C_1716	C	Low	294	0	fit	1.525665
A_1011	C	Low	0	10090	lwr	1191.45
A_1013	C	Low	0	8550	lwr	1191.45

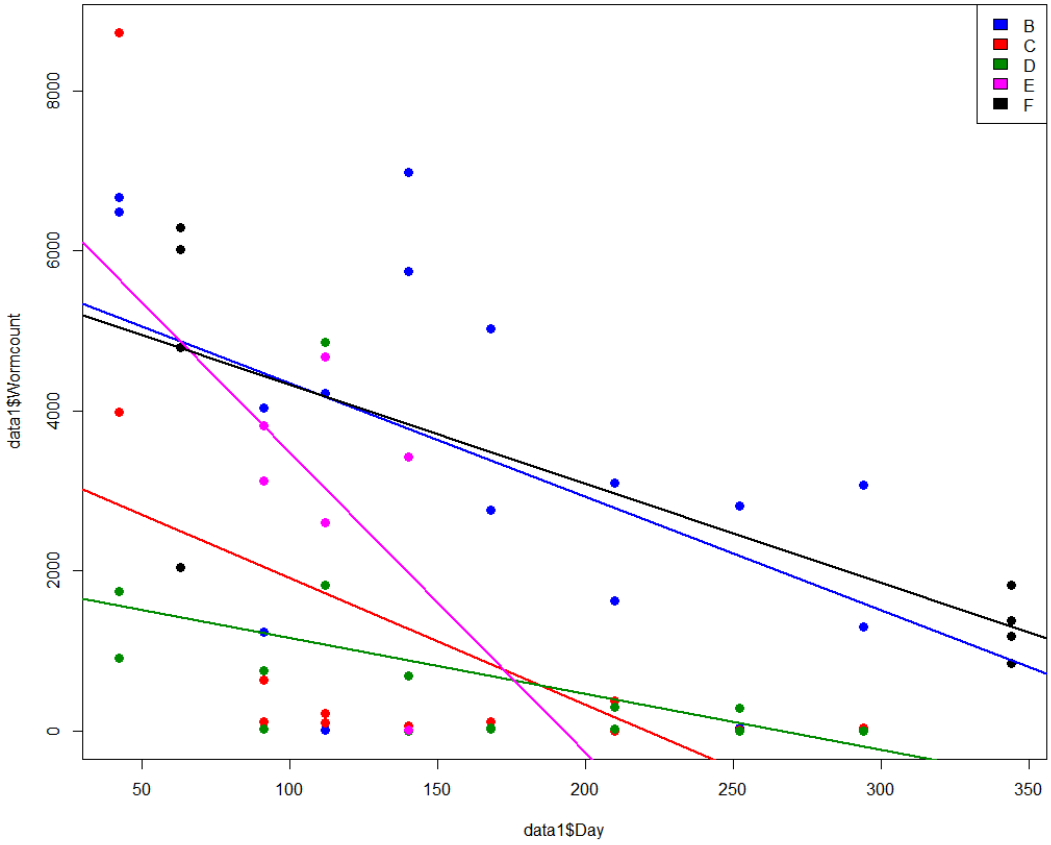
Host	Group	Treatment	Day	Wormcount	FitCI	FitWC
A_1014	C	Low	0	8350	lwr	1191.45
A_1015	C	Low	0	4780	lwr	1191.45
C_1761	C	Low	42	8730	lwr	495.5406
C_1717	C	Low	42	3980	lwr	495.5406
C_1715	C	Low	91	630	lwr	162.8272
C_1720	C	Low	91	110	lwr	162.8272
C_1703	C	Low	112	100	lwr	96.24081
C_1708	C	Low	112	220	lwr	96.24081
C_1729	C	Low	140	60	lwr	44.91911
C_1714	C	Low	140	0	lwr	44.91911
C_1707	C	Low	168	30	lwr	19.52287
C_1724	C	Low	168	120	lwr	19.52287
C_1725	C	Low	210	0	lwr	5.030773
C_1710	C	Low	210	380	lwr	5.030773
C_1758	C	Low	252	10	lwr	1.198262
C_1722	C	Low	252	0	lwr	1.198262
C_1721	C	Low	294	40	lwr	0.273773
C_1716	C	Low	294	0	lwr	0.273773
A_1011	C	Low	0	10090	upr	22726.2
A_1013	C	Low	0	8550	upr	22726.2
A_1014	C	Low	0	8350	upr	22726.2
A_1015	C	Low	0	4780	upr	22726.2
C_1761	C	Low	42	8730	upr	5347.367
C_1717	C	Low	42	3980	upr	5347.367
C_1715	C	Low	91	630	upr	1081.131
C_1720	C	Low	91	110	upr	1081.131
C_1703	C	Low	112	100	upr	572.2083
C_1708	C	Low	112	220	upr	572.2083
C_1729	C	Low	140	60	upr	260.3514
C_1714	C	Low	140	0	upr	260.3514
C_1707	C	Low	168	30	upr	127.2112
C_1724	C	Low	168	120	upr	127.2112
C_1725	C	Low	210	0	upr	48.31151
C_1710	C	Low	210	380	upr	48.31151
C_1758	C	Low	252	10	upr	19.84952
C_1722	C	Low	252	0	upr	19.84952
C_1721	C	Low	294	40	upr	8.502143
C_1716	C	Low	294	0	upr	8.502143
A_1011	D	High	0	10090	fit	5687.137
A_1013	D	High	0	8550	fit	5687.137
A_1014	D	High	0	8350	fit	5687.137
A_1015	D	High	0	4780	fit	5687.137
D_2200	D	High	42	1750	fit	1857.265
D_2364	D	High	42	910	fit	1857.265
D_2396	D	High	91	20	fit	503.3279

Host	Group	Treatment	Day	Wormcount	FitCI	FitWC
D_2102	D	High	91	750	fit	503.3279
D_2110	D	High	112	1820	fit	287.6346
D_2185	D	High	112	4860	fit	287.6346
D_2440	D	High	140	690	fit	136.4043
D_2435	D	High	140	0	fit	136.4043
D_2112	D	High	168	20	fit	64.68668
D_2535	D	High	168	30	fit	64.68668
D_2321	D	High	210	300	fit	21.12492
D_2646	D	High	210	20	fit	21.12492
D_2318	D	High	252	290	fit	6.89883
D_2443	D	High	252	0	fit	6.89883
D_2345	D	High	294	0	fit	2.252972
D_2355	D	High	294	0	fit	2.252972
A_1011	D	High	0	10090	lwr	1099.502
A_1013	D	High	0	8550	lwr	1099.502
A_1014	D	High	0	8350	lwr	1099.502
A_1015	D	High	0	4780	lwr	1099.502
D_2200	D	High	42	1750	lwr	493.2496
D_2364	D	High	42	910	lwr	493.2496
D_2396	D	High	91	20	lwr	175.2267
D_2102	D	High	91	750	lwr	175.2267
D_2110	D	High	112	1820	lwr	106.4929
D_2185	D	High	112	4860	lwr	106.4929
D_2440	D	High	140	690	lwr	51.22422
D_2435	D	High	140	0	lwr	51.22422
D_2112	D	High	168	20	lwr	22.75715
D_2535	D	High	168	30	lwr	22.75715
D_2321	D	High	210	300	lwr	5.987097
D_2646	D	High	210	20	lwr	5.987097
D_2318	D	High	252	290	lwr	1.442842
D_2443	D	High	252	0	lwr	1.442842
D_2345	D	High	294	0	lwr	0.331946
D_2355	D	High	294	0	lwr	0.331946
A_1011	D	High	0	10090	upr	29416.52
A_1013	D	High	0	8550	upr	29416.52
A_1014	D	High	0	8350	upr	29416.52
A_1015	D	High	0	4780	upr	29416.52
D_2200	D	High	42	1750	upr	6993.285
D_2364	D	High	42	910	upr	6993.285
D_2396	D	High	91	20	upr	1445.778
D_2102	D	High	91	750	upr	1445.778
D_2110	D	High	112	1820	upr	776.8941
D_2185	D	High	112	4860	upr	776.8941
D_2440	D	High	140	690	upr	363.2291
D_2435	D	High	140	0	upr	363.2291

Host	Group	Treatment	Day	Wormcount	FitCI	FitWC
D_2112	D	High	168	20	upr	183.8704
D_2535	D	High	168	30	upr	183.8704
D_2321	D	High	210	300	upr	74.53736
D_2646	D	High	210	20	upr	74.53736
D_2318	D	High	252	290	upr	32.98619
D_2443	D	High	252	0	upr	32.98619
D_2345	D	High	294	0	upr	15.29128
D_2355	D	High	294	0	upr	15.29128

9.6.7 Statistical Analysis for establisment in calves

Regression lines (Straight Line Fit) of worm count by day for the various groups (B-F) plotted with raw data



Comparison of the slope of the line fitted from values for Group B with that of Group F: (based on data for the two groups combined)

```
Coefficients:

            Estimate Std. Error t value Pr(>|t|)

(Intercept) 5769.569   1071.546   5.384 2.86e-05 ***
Day          -14.207      5.894  -2.411   0.0257 *
GroupF      -198.795   1581.283  -0.126   0.9012
Day:GroupF     1.813      7.540   0.241   0.8124
```

A comparison of the regression lines for Groups B and F are shown. *Day* is significantly different (p=0.0257) indicating change with time. The *(Intercept)* is significantly different indicating neither regression line passes through 0 (p=2.86e-05). *GroupF* is not significantly different (p=0.9012) indicating the intercepts are not different from each other. *Day:GroupF* is also not significantly different (p=0.8124) indicating the slopes of the regression lines for Group B and Group F and not different from each other.

Worm count – female/male: (using column 'Female' in spreadsheet)

Comparing Groups B, C & D ignoring Days

Only data satisfying 'Worm count' > 99 used…

```
Number of hosts per treatment:
 B  C  D
14  8  8
```

One-Factor ANOVA: (assumptions not fully verified…; permutation test gave very similar p-value)

```
          Df Sum Sq Mean Sq F value  Pr(>F)   Perm_p_value
Group      2 0.1660 0.08299   8.508 0.00136         0.0011
Residuals 27 0.2633 0.00975
```

Overall Group effect is significant …;
and the pairwise comparisons are shown here →

```
$`Pairwise p-value`
        B       C
C 0.0033
D 0.0016  0.9081
```

Comparing Groups A - E

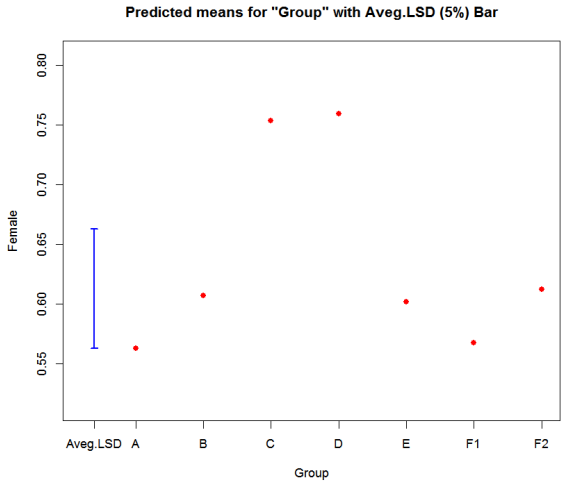
Only data satisfying 'Worm count' > 99 used…

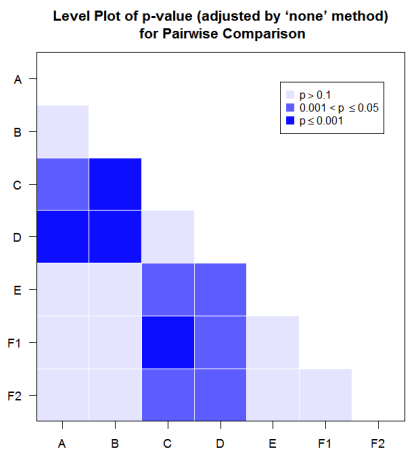
```
Number of hosts per treatment:
 A  B  C  D  E F1 F2
 4 14  8  8  5  4  4
```

One-Factor ANOVA: (assumptions not fully verified…; permutation test gave very similar p-value)

```
          Df Sum Sq Mean Sq F value    Pr(>F)  Perm_p_value
Group      6 0.2807 0.04678   6.758 0.0000535        0.0001
Residuals 40 0.2769 0.00692
```

Overall Group effect is significant …; and the pairwise comparisons are shown below:





"These are permutation p-values"

Total Length of Female worms

Comparing Groups A - E

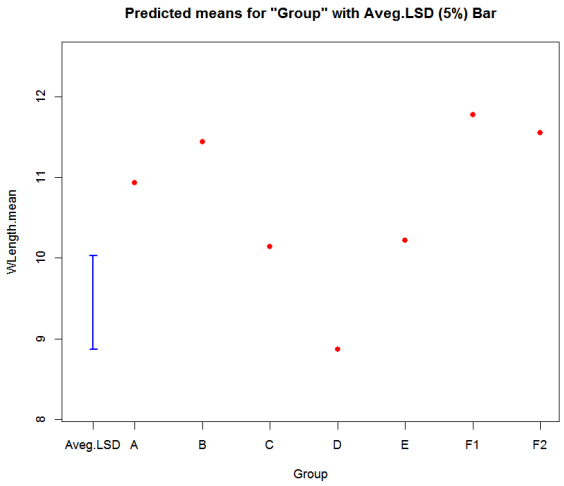
Mean length of worms per host is used…

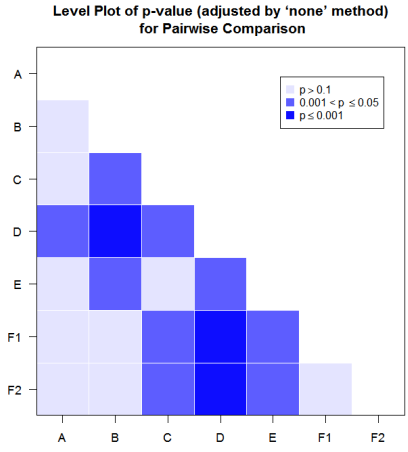
```
Number of hosts per treatment:
  Group:      A  B  C  D  E F1 F2
 n/group:     4 11  4  4  4  3  3
```

One-Factor ANOVA: (assumptions not fully verified…; permutation test gave very similar p-value)

```
          Df Sum Sq Mean Sq F value  Pr(>F)    Perm_p_value
Group      6  27.26   4.543   6.677 0.00023          0.0006
Residuals 26  17.69   0.680
```

Overall Group effect is significant …; and the pairwise comparisons are shown below:





"These are permutation p-values"

Fecundity of Female worms

Comparing Groups A – E

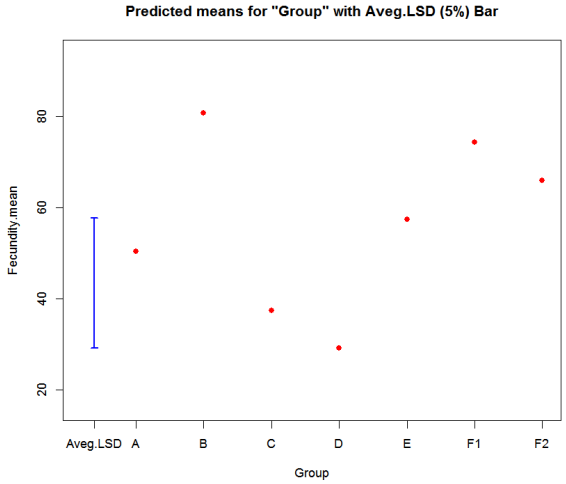
Mean egg count of worms per host is used…

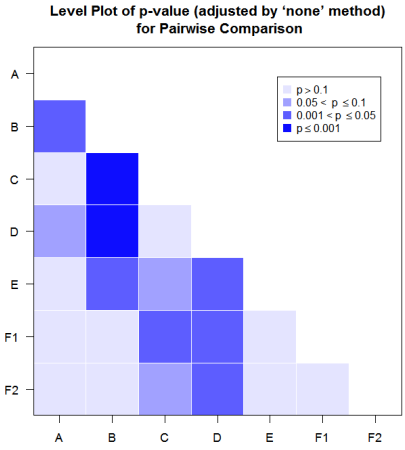
```
Number of hosts per treatment:
 A  B  C  D  E F1 F2
 4 11  9  9  5  2  2     Note: sample size of 2 is bare minimum!
```

One-Factor ANOVA: (assumptions not fully verified…; permutation test gave very similar p-value)

```
          Df Sum Sq Mean Sq F value    Pr(>F)    Perm_p_value
Group      6  17099  2849.8   6.752 0.0000816          0.0001
Residuals 35  14773   422.1
```

Overall Group effect is significant …; and the pairwise comparisons are shown below:





"These are permutation p-values"

Graphs of live weight data

Animals were grouped by length of treatment (i.e. when they were removed from the trial for killing/measurement)

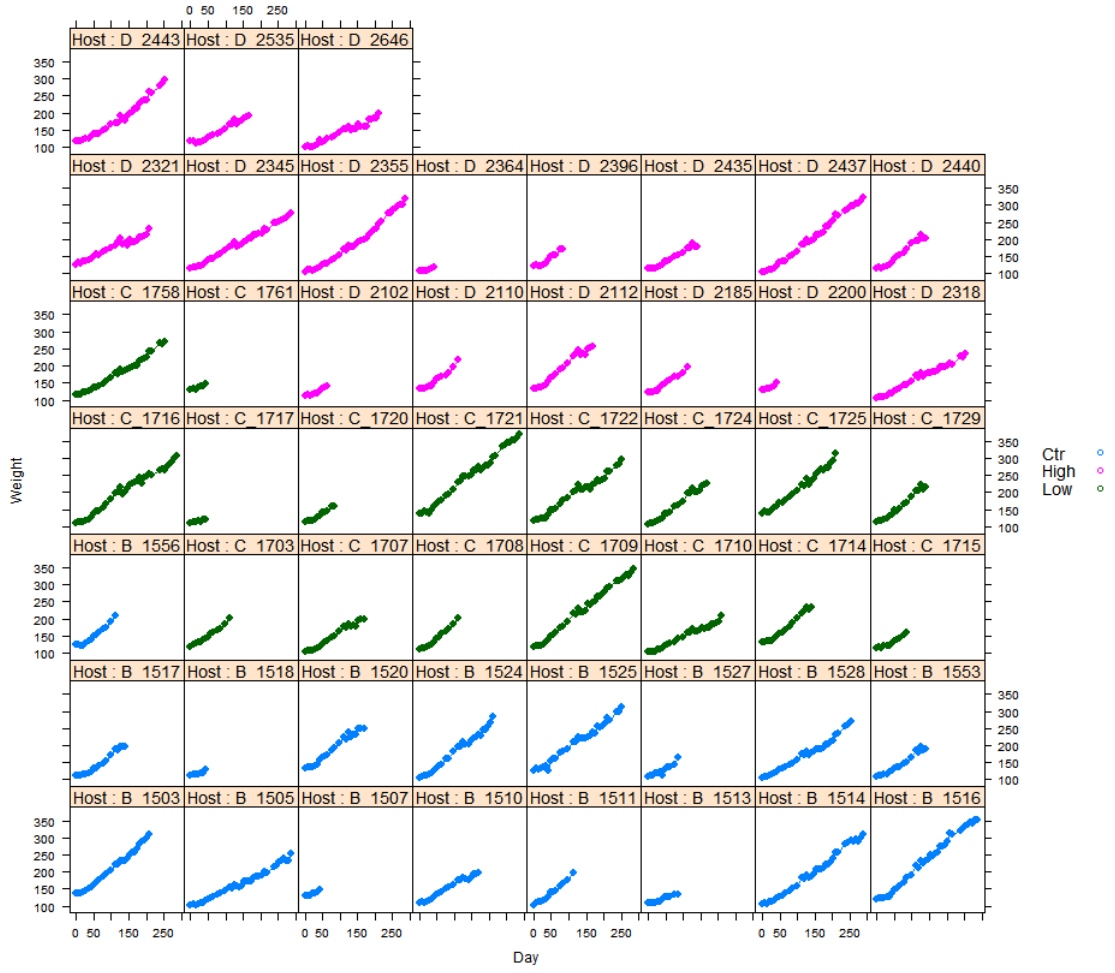


Table below shows the no. of hosts and the length of treatment:

Day

Group	0	7	14	21	28	35	42	49	56	63	77	84	98	112	119	126	133	140	147	154	161	168	175	182	189
B	17	17	17	17	17	17	17	15	15	15	15	15	13	13	11	11	11	11	9	9	9	9	7	7	7
C	17	17	17	17	17	17	17	15	15	15	15	15	13	13	11	11	11	11	9	9	9	9	7	7	7
D	17	17	17	17	17	17	17	15	15	15	14	14	13	13	11	11	11	11	9	9	9	9	7	7	7

Day

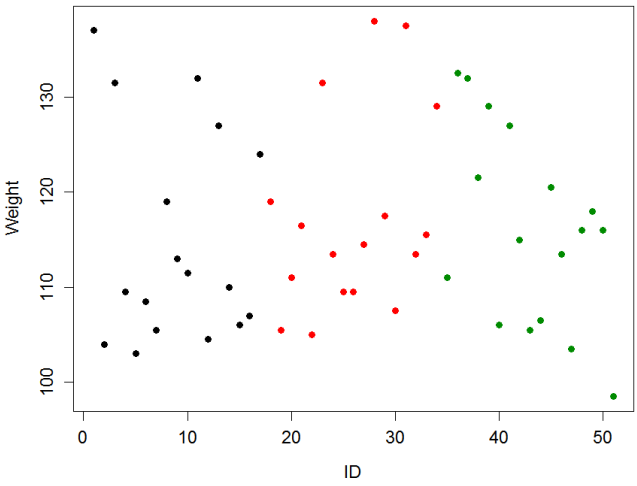
Group	196	203	210	217	238	245	252	259	266	273	280	287
B	7	7	7	5	5	5	5	3	3	3	3	3
C	7	7	7	5	5	5	5	3	3	3	3	3
D	7	7	7	5	5	5	5	3	3	3	3	3

6 (=17-15=2 in each trt) hosts for 42 days; 1 host for 63 days in trt D; 5 for 84 days (2 in trts B & C, 1 in D; 6 each for 112, 140, 168, 210 & 252 days; 9 for 287 days.

Note: Hosts B_1513, B_1527, C_1715, C_1720 and D_2396 have TrtLnth 84, but Host D_2102 has TrtLnth 63. So, the following analyses consider TrtLnth 63 days for all 6 Host here, ignoring the growth data for the extra days for the first 5 Hosts.

Graph above indicates that weight growth is linear…

However, initial weight (at day 0) varies a great deal within each of the treatment (see graph below)




```
Summary(Weight)
Min.    1st Qu.  Median   Mean    3rd Qu.  Max.
98.5    107.2    113.5    116.1   122.8    138.0
```

Analyse rate of growth

Average weight gain per day analysed using starting weight (*LW_Start*) as a covariate

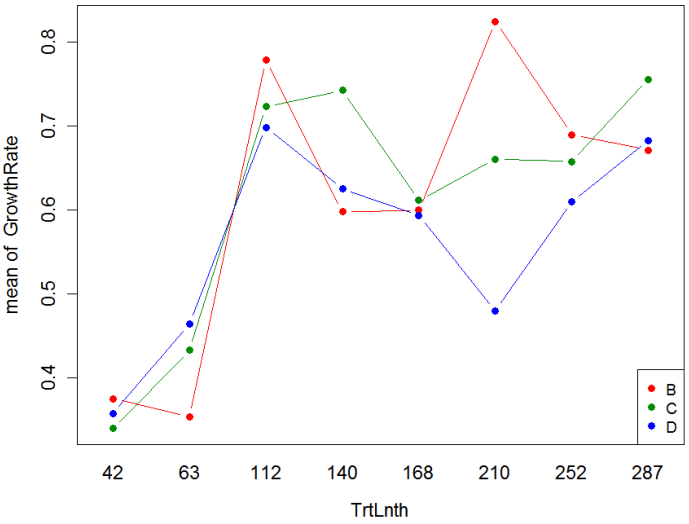
WgtGain/day = (max weight-min weight)/length of treatment (experimental time), noting that "Weight gain per day" & "growth rate" have equivalent interpretation here – assuming linearity of weight growth for each host.
Group: animals in Groups B, C and D.
TrtLnth: categorical variable for challenge time with the RES isolate

ANOVA: (assumptions not fully verified…; permutation test gave very similar p-value)

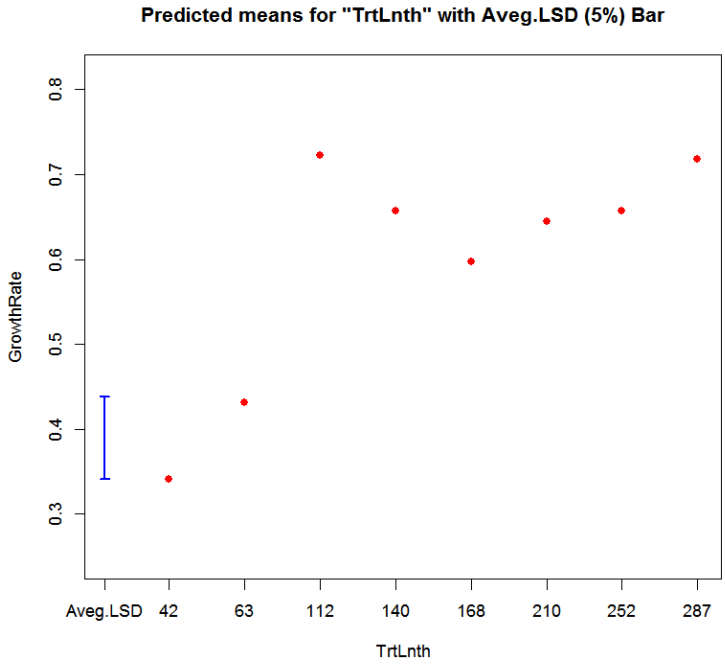
```
               numDF  denDF   F-value  p-value  Perm_p_value
LW_Start           1     26   12.6804   0.0015        0.0017
Group              2     26    1.7796   0.1886        0.1883
TrtLnth            7     26   16.2976   0.0000        0.0001
Group:TrtLnth     14     26    1.1906   0.3381        0.3357
```

```
Note: Perm_p_value of F test is obtained by '10000' times permutation
```

The interaction effect between *Group* and *TrtLnth* isn't significant (see graph below); the overall differences between groups is also not significant (see table below); however, the overall *TrtLnth*s differ significantly (see graph and table below); all with respect to average growth rates.



Although some differences appear to be large, they are not significant.




```
Pairwise p-value (based on 10000 permutations)
        42      63     112     140     168     210     252

63  0.0906
112 0.0001  0.0001
140 0.0001  0.0002  0.1954
168 0.0001  0.0026  0.0180  0.2395
210 0.0001  0.0002  0.1288  0.8121  0.3368
252 0.0001  0.0003  0.2012  0.9914  0.2385  0.8055
287 0.0001  0.0001  0.9217  0.1903  0.0127  0.1168  0.1845
```

Recall that *TrtLnth* factor has 7 levels, corresponding to the days at which a group of animals was taken out of the trial and killed to measure worms. It appears that there is an increasing trend in growth rates except for an anomaly at Days 112 and 140 (see graph above), i.e. the longer the animals are in the trial, the higher their daily weight gain.
The anomaly indicates that the max. weight gain is achieved after 112 days, instead of 287 days.

Predicted Group Means

```
B      C      D
0.6152 0.6110 0.5619
```

The average growth rates for Groups B and C are similar, but Group D has a lower growth rate. However, the difference isn't significant.

Note:
The predicted means are estimated at covariate LW_Start = 116.0588 (i.e. average live weight at Day 0 = *TrtLnth* 0)