THE RELATIONSHIP
BETWEEN A NEMATODE PARASITE
Trichostrongylus retortaeformis
AND ITS HOST OR ENVIRONMENT

by
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All work for the studies towards this thesis was performed entirely by me, except where specifically acknowledged.

(G.E. Ford)
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I should like to express sincere appreciation to Professor J.B. Smyth for arranging to have me in his department to study parasitology, for his liberal provision of resources for my experiments, and for his ready counsel and discussions on aspects of the habitat of the parasite in the host.

I am pleased to acknowledge my supervisor, Dr. W.L. Nicholas, for his interest and continual enthusiasm for new work, and the assistance provided in the laboratory (particularly with antibodies, cultures, and technical assistance with rabbits during my emergency hospitalization and convalescence).

I am indebted to him for the suggestion to use radiochemicals to examine growth of nematodes in culture.

In this department, Professor P.H. Silverman (while on sabatical leave from the University of Illinois, Urbana), and Dr. C. Bryant provided invaluable advice on techniques used to examine the viability of larvae in cultures. Dr. C.M. Tydale-Hickey discussed aspects of the physiology of the host animals. I am grateful to Dr. M.G. Howell for his assistance in commenting on the draft manuscript. Mr. A.A. Argyie has been most helpful in the provision of materials, Mr. I. Fox in the provision of photographic assistance, and Mrs. P.H. Proctor in carrying out the protein estimation on the parasite.

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All the studies were carried out during the tenure of an Australia Wool Board Scholarship. I am most appreciative of the support for this work by the Wool Board Production Research Committee.
A local isolate of the strongyle nematode *Trichostrongylus retortaeformis* from the small intestine was propagated in laboratory rabbits (*Oryctolagus cuniculus*).

After exsheathment of third-stage infective larvae, in the natural host, the individual parasites either developed to adults (up to 9 mm long) within a pre-patent period normally 10 - 13 days, or were inhibited in development at the late third-stage (ca. 560 µ long). After exsheathment, in artificial culture there was an accumulation of larvae arrested at the same late third-stage, and a few appeared to enter the third ecdysis at a pH ca. 6.5, but the cuticle was not cast. Radio-isotope labelled amino acids added to culture became adsorbed to killed larvae. The same level was retained by live exsheathed third-stage larvae which, however, showed evidence of activity, by catabolism of labelled l-isoleucine into volatile substances, and by fixation of labelled carbon dioxide in the medium.

Moderate levels of established populations (around 1,000 to 5,000 nematodes) in the rabbit remained stable until 4 or 7 weeks after infection, when some were expelled, leaving a residual population of less than 500 adults. Lower levels of infection persisted for much longer periods, and the adult nematode may live for a year. Mean egg production per female *T. retortaeformis* varied from 0 to 400 eggs per day, and a significant rhythm of egg output occurred, with a period of about 22 days between peaks from each rabbit. More eggs per female were passed at lower, than at higher, levels of established infection.

Infective larvae required a maturation period, after their first appearance in incubated faeces, to reach maximum infectivity. The infectivity declined with aging after this period, and with prolonged cold storage. Infective larvae exposed to cold caused a greater depression of body weight in rabbits, than fresh larvae. More surviving stored larvae became inhibited in development, than fresh larvae.

More nematodes were established and rabbits gained less weight in infections with multiple doses compared with a single dose of larvae. The number of nematodes established was depressed following a previous infection. Nematodes established in rabbits fed laboratory pellets passed more eggs, than for lucerne. In juvenile male rabbits more eggs were passed, than for females.

However, the greater the number, of nematodes established, or of eggs passed, the more likely was the subsequent expulsion of the parasite population. Loss of infection was invariably accompanied by a parallel loss of host body weight.

Lactation of rabbits was associated with a more rapid initial rise in egg count and with persistance of egg production in an established population, compared with control does. The number of nematodes remaining, and their egg production, was reduced in does weaned of their litters.
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GENERAL SUMMARY

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Trichostrongylus retortaeformis
with arrested development at the late third-stage

(This specimen was recovered from in vitro culture.)
The work described in this thesis was carried out to examine physiological host-parasite inter-relationships for *Trichostrongylus* spp. nematodes from the small intestine of grazing mammals. Responses of the parasite, to manipulations of the environment and of the physiology of the host, were observed to determine factors which control infection at moderate levels.

Laboratory studies with a local isolate of *T. retortaeformis* from rabbits (*Oryctolagus cuniculus*) were conducted with the nematode in two environments, and accordingly are reported in separate parts, following a General Introduction (Part I).

Part II deals with the nematode in the natural host environment. Following initial experiments on characterisation of *T. retortaeformis* infections in rabbits, the balance of this part was devoted to physiological responses of the infection. Moderate levels of infective larval doses were chosen to infect rabbits, with the object of avoiding levels which provoke gross pathological or immunological involvement and cause a labile host-parasite association, thus selecting levels at which the host-parasite association is in physiological balance.

Part III deals with the nematode in an artificial culture environment, where a more precise examination may be made of factors which influence its responses.
CHAPTER I
GENERAL INTRODUCTION

The family Trichostrongylidae comprises a group of strongyle nematodes, thread-like in appearance, with a well developed copulatory bursa in adult males, and a well developed uterine coil in adult females. The Russian classification of Vedenin, Shkhachalova, Schulz, Popova, Boev, and Beljanski (1946 pp.108) is followed in this study. It is shown in the following schedule, with the revised American classification of Chitwood (1953) as cited by Savage (1958 pp.43).

**Phylum**

- Nematoda

**Class**

- Trichostrongyloidea

**Order**

- Trichostrongylidae

**Superfamily**

- Trichostrongylidae

**Family**

- Trichostrongylidae

Characteristically, the members of the genus Trichostrongylus are economically important intestinal parasites of grazing mammals, and over 50 species have been named, some of these synonyms (Kröber, Shkhachalova, and Schulz 1954). The more well known species are T. columbiformis (syn. T. Anastubia) T. subtilis.
HISTORY OF THE PARASITE

Description of the Parasite

The family Trichostrongylidae comprises a group of strongyle nematodes, thread-like in appearance, with a well developed copulatory bursa in adult males, and a well developed uterine ovjector in adult females. The Russian classification of Skrjabin, Shikhobalova, Schulz, Popova, Boev, and Delyamure (1952) and used by Soulsby (1968 pp.148) is followed in this study. It is shown in the following schedule, with the revised American classification of Chitwood (1961) as cited by Levine (1968 pp.45).

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<th>American</th>
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Trichostrongylus retortaeformis was originally described as Strongylus retortaeformis (Zeder 1800), and is the type species for the genus Trichostrongylus (Looss 1905). It is typical, of the intestine species known, in its morphology, physiology, and relations with its host. 

T. retortaeformis has been described and redescribed several times (Zeder 1800; Dujardin 1845; Looss 1905; Hall 1916; Yorke and Mapleton 1926; Schulz 1931; Nagaty 1932; Travassos 1937; Clapham 1947; Skrjabin, Shikhobalova, and Schulz 1954; Yamaguti 1961). Adults may reach 9 mm in length, the males being a couple of mm shorter than the females. Grossly the adult nematode appears tapered at the head, with a blunt pointed tail in the female and a bursa at the caudal extremity of the male. The bursa has large lateral lobes and a poorly defined dorsal lobe. The spicules are stout and of similar size. The form of the spicules in the male is an important species characteristic.

Field Occurrence

Although T. retortaeformis is normally a parasite of the small intestine, it has been reported occasionally in the stomach (Railliet 1889; Hall 1916; Goodey 1922; Bull 1953b), and in the large intestine (Hesterman and Kogon 1963). T. retortaeformis appears to be widely distributed in Europe and northern Africa where it is commonly reported in samples of the helminth parasites of native lagomorphs (e.g. Dujardin 1845; Baudet 1928; Schulz 1931; Baylis 1939;
Evans 1940; Bernard 1965b; Novak, Dyk, and Zavadil 1966), but it apparently has not been verified from native lagomorphs in America (e.g. Harkema 1936; Dikmans 1937b; Erickson 1944, 1947; Moore and Moore 1947; Stringer, Harkema, and Miller 1969). Although not recorded in New Zealand and Australia in early reports (e.g. Gilruth 1906; Johnston 1909), it probably was introduced and became established with the introduced wild European rabbit (e.g. Bull 1953a; Mykytowycz 1956).

While T. retortaeformis appears closely adapted to the European rabbit (Oryctolagus cuniculus), its place is taken in the American rabbits (Sylvilagus spp.) by a similar species, T. calcaratus. Oryctolagus sp. released in North America by Beagle dog clubs has failed to become established, except in Washington state (Kirkpatrick 1959), and there are no published reports of its parasite, T. retortaeformis, infecting Sylvilagus spp. However, T. calcaratus has been established in Oryctolagus in the laboratory, both by natural infection (Stoll 1932) and artificial infection (Sarles 1932a). Both these "rabbit" species of Trichostrongylus occur in other lagomorphs, such as Lepus spp. (references as before), and have at times been recovered from rodents and ruminants (Leiper 1937; Sloan 1951; Oldham 1961; Dunn 1965). A third species, T. affinis, occurs normally in the large intestine of the rabbit (Graybill 1924).

Other species of Trichostrongylus have been recorded (especially T. colubriformis) from lagomorphs in the field (Hall 1916; Graybill 1924; Anon. 1931r; Tetley 1934; Roberts 1935; Dikmans 1937b; Erickson 1947; Bull 1953a; Hesterman and Kogon 1963; Bernard 1965a; Katiyar and Pande 1965; Samson 1970).

A second economically important small intestine trichostrongylid genus, Nematodirus, occurs naturally in rabbits in the field, but less commonly (e.g. Dikmans 1937a; Erickson 1947; Bull 1953a; Sommerville 1963; Bernard 1965b; Hansen, Bartel, Lyons, and El-Rawi 1965).
Life Cycle

The basic life cycle of members of the family Trichostrongylidae is direct. It was discovered for *T. retortaeformis* by Railliet (1889), and further outlined for other intestine *Trichostrongylus* spp. by Mönig (1926). A detailed life cycle for *T. retortaeformis* has never been published.

Eggs of *Trichostrongylus* spp. passed onto the pasture in the faeces of the host embryonate and hatch under suitable environmental conditions. The free-living stages feed and develop with rapid growth from the hatched first-stage larvae through the first ecdysis to the second-stage. When these develop to the third-stage, the second ecdysis does not occur, and these early "third-stage", protected by the unshed cuticle of the second-stage as a sheath, become the infective larvae. Depending on climatic conditions, these sheathed third-stage larvae (*SL*$_3$) survive on pasture for many months.

When infective larvae are ingested by a suitable grazing host, the second ecdysis (known as exsheathment) occurs under suitable environmental conditions. At the commencement of the parasitic-living stages, the released third-stage larvae (*XL*$_3$) metamorphose and develop through the third ecdysis to the early fourth-stage larvae (*EL*$_4$) in the small intestine. Another period of rapid growth, accompanied by sexual differentiation, ensues, with the development of the late fourth-stage larvae (*LL*$_4$). A fourth, and final, ecdysis (known as a moult) leads to the continued development of the fifth-stage or immature adult.

The duration of development varies widely at different stages, with environmental factors exerting a critical influence. With optimum conditions for development and survival, various *Trichostrongylus* species may progress from egg to infective larvae in about 4 to 8 days, and from infective larvae to patent infection in about 10 to 20 days.
Experimental Investigation

The free-living stages of *T. retortaeformis* have been studied as a model to investigate trichostrongylid egg hatching, and larval development and physiology, in the laboratory (Goodey 1922; Lapage 1933b; Crofton 1948b; Nnochiri 1950; Poole 1954; Wilson 1954, 1958; Prasad 1959; Gupta 1961, 1962; Bondy 1965), and to investigate ecology of infective larvae on pasture plots (Crofton 1948a, 1948b, 1954). Some laboratory cultures of *T. retortaeformis* used in North America were obtained from Britain (Prasad 1959).

The initiation of the parasitic stages of *T. retortaeformis* and *T. calcaratus* in laboratory rabbits was suggested in early work to occur with cutaneous infection as well as oral infection (Brumpt 1921; Sarles 1930, 1932a; Stoll 1932) but these reports did not mention whether the rabbits had been prevented ingesting the larvae from the skin. It has been shown with both species subsequently that skin penetration did not occur, compared with ancylostomid larvae as positive controls (Goodey 1922; Dixon 1963, 1965a). The infective larvae of *T. retortaeformis* have been studied as a model to investigate the second ecdysis of trichostrongylid nematodes (Lapage 1933a; Crofton 1947; Bailey 1968).

The parasitic stages of *T. retortaeformis* have received scant attention in the laboratory. Only Michel (1952a, 1952b, 1952c, 1963a, 1968) in Britain appears to have attempted to characterise the infection, and this work has "not been very fully written up" (J.F. Michel 1970 pers. comm.). In New Zealand and Australia the association with the rabbit has been used experimentally on a limited scale to investigate host-parasite reactions, both with artificial infections (Whitten 1948; Dunsmore 1966a), and with natural infections in enclosures (Dudzinski and Mykytowycz 1963; Dunsmore 1965b, 1966a,
Similarly, only limited laboratory studies in the rabbit have been carried out with *T. calcaratus* (Stoll 1932; Sarles 1930, 1932a, 1932b, 1934; Dixon 1965a), or with *T. affinis* (Dixon 1965a, 1965b). Davey (1938a) used *T. retortaeformis* taken from the rabbit in some nematode physiology studies.

The failure of research workers to utilise more fully either the normal host-parasite association, of the intestine *Trichostrongylus* spp., or of *Nematodirus* spp. (the other trichostrongylid genus which occurs naturally in the small intestine of rabbits), is surprising for three reasons as follows.

Firstly, the laboratory rabbit has been used experimentally as an abnormal host for other trichostrongylids occurring in the same habitat. Two small intestine species from domestic animals have commonly been transmitted to rabbits in the laboratory: *Trichostrongylus colubriformis* (Gazzigava 1929; Ortlepp 1939; Brackett and Bliznick 1949; Drudge, Leland, Wyant, and Elam 1955; Rohrbacher, Porter, and Herlich 1958; Rohrbacher 1958, 1960a; Wood 1958; El-Rawi 1960; Sommerville 1963; Williams and Palmer 1964; Gawad 1964; Dixon 1965a; Kates and Thompson 1965; Coirdia, Bizzell, and Porter 1965; Ciordia, Bizzell, Porter, and Dixon 1966; Ford 1968 preliminary studies); and *Cooperia punctata* (Alicata 1958; Wood 1958; Wood and Hansen 1960; Besch 1963, 1964, 1965; Leland and Wallace 1966). In addition, Sommerville (1963) established infections in some laboratory rabbits dosed with *Nematodirus spathiger* from ruminants, a species also found by Bernard (1965a) in domestic rabbits. A list of references to experimental studies with strongylate nematodes in rabbits is given in Appendix I.

Secondly, other less directly applicable models for small intestine trichostrongylid parasites of economic animals have been popular using laboratory rodents. The more extensively studied of these are the abnormal association of *T. colubriformis* in the guinea pig, *Cavia porcellus*, and the normal association of the heligmosomid,
Nippostrongylus brasiliensis (syn. N. muris), in the Norwegian rat, Rattus norvegicus. The latter (for which infection may take place through the skin) serves as a model for both ancylostomids and trichostrongylids in either medical or veterinary research.

Finally, the nematode Trichostrongylus retortaeformis is readily available in countries where its natural host, Oryctolagus cuniculus, is extensively used in research laboratories.

The Parasite

Trichostrongylus retortaeformis was used for all the experimental work outlined in this thesis.

Nematode infestations in wild rabbits on the New South Wales Southern Tablelands declined to unusually low levels associated with the 1967/68 drought in eastern Australia, and only slight contribution was made to the laboratory strain by nematodes from wild rabbits caught at three field sites in the geographic region of the laboratory: Ginninderra A. Valley, Snowy Plains, "Gungahlin", Canberra (both C.S.I.R.O. sites); and Flemish Valley, Canowoola. The major contribution to the laboratory strain came from wild rabbits caught on the N.S.W. South Coast at Mooy (C.S.I.R.O. site). Live rabbits were netted by spotlight or trapped in cages and brought to the laboratory.

Adult T. retortaeformis were recovered from the small intestine, and individually transferred to clean sodium bicarbonate to remove any eggs from the stomach nematode (Graphidium striatum) that may have been passed into the small intestine. After incubation at 30°C overnight, the eggs passed by female nematodes were pipetted onto plain agar plates, tightly seen the previous day with the bacterium Escherichia coli, and incubated at 35°C. Infective third-stage larvae were washed from the Petri dishes after 10
The Parasite

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Adult *T. retortaeformis* were recovered from the small intestine, and individually transferred to clean saline to remove any eggs from the stomach nematode (*Graphidium strigosum*) that may have been passed into the small intestine. After incubation at 38°C overnight, the eggs passed by female nematodes were pipetted onto plain agar plates, lightly sown the previous day with the bacterium *Escherichia coli*, and incubated at 25°C. Infective third-stage larvae were washed from the Petri dishes after 10
days, and used to dose the first laboratory rabbit with a pure infection of *T. retortaeformis*.

This laboratory strain of the parasite was passaged through "donor" rabbits ten consecutive times during the course of the studies. The record of the passages is compiled in Table 1. Usually a batch of four or five young male rabbits was used to propagate the nematode, but on two occasions (for Passages 5 and 6) single young male rabbits with reinfections were used as the source of larvae for the following passage.

Representative specimens of adult nematodes were sent to the taxonomist at The Commonwealth Bureau of Helminthology, St. Albans, Britain, who confirmed the identification as *Trichostrongylus retortaeformis* (L.F. Khalil 1970 pers. comm.). A general description of the parasite has already been given.

Purity of the laboratory strain of *T. retortaeformis* passaged in donor rabbits was continually checked by examination of the eggs in faeces, of third-stage larvae from faecal incubations, of adult nematodes from small intestines, and of stomachs to ensure there was no contamination by any other strongylate nematodes. No contaminants were found.

**The Host**

The natural host of the parasite is the European rabbit, *Oryctolagus cuniculus* (Linnaeus). A domestic type, New Zealand White breed, was used for all experimental infections, and for laboratory propagation of the parasite.

The rabbits were all bred and supplied by B.D. Everingham, "Tillside" Rabbit Stud, Yanderra, N.S.W., where a degree of inbreeding had been practiced, resulting in marked uniformity of batches. The only exceptions were the young rabbits used for Experiment 8, which were bred
as one batch in this laboratory from the same stock. Rabbits were obtained from "Tillside" as 5 to 8 week old male weaners for laboratory nematode propagation and Experiments 1 to 7, or as adult females for Experiments 9 to 12. (Mixed age rabbits were used in Experiment 3.)

The rabbits weighed 1 kg at about 5 weeks of age, 2 kg at about 10 weeks, and reached 3 kg at 15 to 20 weeks. Mature rabbits fed on laboratory pellets often exceeded 4 kg mature weight. Such animals were very fat, and lost weight for several weeks when transferred to a lucerne pellets diet. Stable adult weight was around 3.5 kg when fed lucerne pellets. Sexual maturity is reached around 5 months of age.

Examination of the gastro-intestinal tract and faeces of several rabbits from the "Tillside" colony showed them to be free from strongylate nematodes. In some rabbits, the oxyurate nematode *Passalurus ambiguus* was found in the large intestine, and occasional eggs of this parasite were seen, commonly in faeces from rabbits fed laboratory pellets, but very rarely in faeces from rabbits fed lucerne.
**Table 1**

Laboratory Strain of *Trichostrongylus retortaeformis*

Consecutive Passages of Pure Infections in Rabbits

<table>
<thead>
<tr>
<th>PASSAGE NUMBER</th>
<th>SOURCE OF INFECTIVE LARVAE (FROM PREVIOUS PASSAGE)*</th>
<th>Length of Infection in Donors</th>
<th>Length of Faecal Incubation at 25°C</th>
<th>Length of Faecal Incubation at 20°C</th>
<th>Length of Storage at 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>? (field)</td>
<td>10 days</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>16 days</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>32 days</td>
<td>-</td>
<td>8 days</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>104 days</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>29 days</td>
<td>-</td>
<td>7 days</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>31 days</td>
<td>-</td>
<td>12 days</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>79 days</td>
<td>-</td>
<td>13 days</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>58 days</td>
<td>-</td>
<td>26 days</td>
<td>17 days</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>83 days</td>
<td>-</td>
<td>10 days</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*sometimes animals were infected more than once. The times given are the examples which contributed the greatest number of infective larvae*

+ other passages were used for some experiments, but the line not continued on.
Studies on host-parasite relationships are conveniently considered in three phases:

1 - Characterisation of Infections

The characterisation is the description of the basic features observed during the course of an infection. Research on the characteristics of uncomplicated infections is directed at determining the parasitic life cycle in the field, supported by observations in the laboratory.

2 - Variations in Infections

Factors of diet, previous contact with the parasite, age, and environmental conditions may be considered to affect the parasite or the infected host. Effects of any given factor may be varied as outlined below.

3 - Mechanisms of Changes

In many occasions, experimental models may be substituted in place of economic animals or man as hosts. The rabbit forms an ideal laboratory animal to use in place of other grazing animals such as sheep, particularly for research on trichostrongylids, because of the availability of natural host-parasite associations. Secondly, the replacement of the host by laboratory culture of the parasite *in vitro* will allow studies isolated from influences which are uncontrolled within the normal habitat.
the development of the host, and in relationship to the responses of the animals to each other. The problems raised lead to other investigations, and in this phase of research the standards are determined on which to assess subsequent studies. For example, the rate of growth of a parasite in the normal host must be known in order to compare development in artificial culture.

Because infections with *Trichostrongylus retortaeformis* are inadequately characterised in the literature, some attention was given to this phase in a portion of Part II, Section A.

**Variations of Infections**

The physiological variations which are those considered in this study are differences in the basic features of infections, brought about by responses to changes in the host and the environment. Research on physiological variations may be directed to discover to what extent particular factors affect the parasite or the infected host. Effects of any given factor may be perspicuous, but more realistically the effects may vary due to interactions with other factors. Therefore experiments designed to examine more than one factor together may more readily explain field observations than examination of a single factor at a time, when some of the sources of variation may remain undiscovered.

Studies reported in this thesis were planned to examine the response of *T. retortaeformis* infections to manipulations of physiological influences from the host. Factors of diet, previous contact with the parasite, age, and endocrinological status are known to affect the establishment of the parasite population, which may then affect the effect of the parasite on the host.

Alternatively, manipulation of the parasite physiology may influence development of an infection - an approach relatively undeveloped in research in host-parasite relationships. Studies reported in this thesis also examined the influence, on the development of
T. retortaeformis, of variations in its environment prior to infection. A portion of Section B in Part II is devoted to observations on host-parasite interactions following different environmental treatment imposed on infective larvae.

Mechanisms of Changes

Research on mechanisms is directed at examining how variations are implemented at a cellular or molecular level. Effects may be direct or mediated by interacting mechanisms. For example, some physiological change in the host may control the immune system which in turn influences the parasite population. The effect of a final mediator on the parasite is particularly amenable to study in culture in vitro. Recent advances in biochemistry and biophysics - especially with regard to radio-active tracer labelling - have provided useful techniques for this type of investigation.
PART II

STUDIES IN A NATURAL HOST ENVIRONMENT

(1) Characterisation of Uncomplicated Infections

INTRODUCTION

A Review on Experimental Host-Parasite Relationships for Intestinal Infections of Trichostongylius spp. in Grazing Mammals.

Location of the Parasite in the Intestine

The location of the adult T. retortaeformis in the small intestine of naturally infected wild rabbits has been examined by Noll (1953b) and Sommerville (1953). Most commonly the incidence of the parasites along the length of the gut approached a normal frequency distribution, but with the mode close enough to the pylorus to cause a skewed effect, due to the absence of the proximal tail of the distribution which would have extended into the stomach. Nearly all the nematodes were situated in the proximal half of the small intestine, the duodenum. In a few rabbits the distribution was irregular.
INTRODUCTION

A Review on Experimental Host-Parasite Relationships for Intestinal Infections of Trichostrongylus spp. in Grazing Mammals.

(i) Characterisation of Uncomplicated Infections

Published reports on host-parasite relationships for Trichostrongylus retortaeformis are limited, as mentioned in the historical review of the experimental parasite. A great deal of scientific effort has been expended on the stomach trichostrongylids, especially Haemonchus spp., but considerably less appears to have been spent on any of the small intestine trichostrongylids.

Location of the Parasite in the Intestine

The location of the adult T. retortaeformis in the small intestine of naturally infected wild rabbits has been examined by Bull (1953b) and Sommerville (1963). Most commonly the incidence of the parasites along the length of the gut approached a normal frequency distribution, but with the mode close enough to the pylorus to cause a skewed effect, due to the absence of the proximal tail of the distribution which would have extended into the stomach. Nearly all the nematodes were situated in the proximal half of the small intestine, the duodenum. In a few rabbits the distribution was irregular.
Bull (1953b) considered that the frequency distribution may be modified by movement of material down the intestine or by the number of nematodes present, and Sommerville (1963) found a bimodal distribution with a concurrent Nematodirus sp. infection. Similar results were obtained with experimental infections of *T. colubriformis* in laboratory rabbits (Sommerville 1963), and with infections of *Trichostrongylus* spp. in sheep (Tetley 1937; Davey 1938a). More *T. colubriformis* were found in the posterior regions of the gut in the guinea pig, and related to poor adaptation of the parasite to that host (Herlich, Douvres, and Isenstein 1956; Sturrock 1963; Connan 1966a).

A stage of intimate contact with the host tissue, the "histotrophic phase" (Kotlan 1952), has not been clearly documented for *Trichostrongylus* spp. in the natural host, although Mönnig (1926) stated that larvae enter the wall of the intestine before the third ecdysis, and Michel (1952c) stated that the larvae burrow into the intestinal mucosa between the glands at this stage. Herlich (1969a) showed by histological section *T. colubriformis* larvae embedded in the lumina of intestinal glands in an abnormal host, the guinea pig.

**Development of Parasitic Larval Stages, and Pre-patent Period.**

There is no known data published on the period of time over which the parasitic larval stages of *T. retortaeformis* develop in the rabbit. The larval development of *T. colubriformis* in the gut of various hosts has been outlined (Mönnig 1926; Douvres 1957a; Fitzsimmons 1966a), but this species has a pre-patent period of about 18 days in each host (Andrews 1939; Herlich 1957; Bizzell and Ciordia 1965; El-Rawi 1960), whereas for *T. retortaeformis* Michel (1952b) described the pre-patent
period as a fortnight, which is similar to the period found in wild rabbits in the laboratory (J.D. Dunsmore 1968 pers. comm.).

The rate of development of the parasitic larval stages may be presumed to vary, because the pre-patent period is not constant. Thus, for *T. colubriformis*, Herlich (1957) reported a range of 15 - 23 days in calves, and Sturrock (1963) found a range of 16 - 25 days in guinea pigs. In lambs, Andrews (1939) reported 17 days, and Mönnig (1926) 25 days, as a pre-patent period.

### Size of Eggs

The egg of *T. retortaeformis* was illustrated by Mönnig (now see Soulsby 1968 pp. 795), and is characteristic of the genus. Eggs are passed in the host faeces in the blastomere stage of embryonic development. The dimensions given by various authors differ considerably as shown in the following schedule, and it seems they may vary between hosts.

<table>
<thead>
<tr>
<th>Length Range (µ)</th>
<th>Width Range (µ)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>78 - 85</td>
<td>45</td>
<td>Dujardin (1845)</td>
</tr>
<tr>
<td>76 - 80</td>
<td>40 - 42</td>
<td>Looss (1905)</td>
</tr>
<tr>
<td>75 - 80</td>
<td>40 - 45</td>
<td>Hall (1916)</td>
</tr>
<tr>
<td>86 - 87</td>
<td>41 - 46</td>
<td>Schulz (1931)</td>
</tr>
<tr>
<td>79 - 96</td>
<td>36 - 44</td>
<td>Nagaty (1932)</td>
</tr>
<tr>
<td>85 - 91</td>
<td>46 - 56</td>
<td>Mönnig (now see Soulsby 1968 p. 226)</td>
</tr>
<tr>
<td>86 - 87</td>
<td>41 - 46</td>
<td>Skrjabin, Shikhabalova, and Schulz (1954 p. 41)</td>
</tr>
<tr>
<td>68 - 90 (79*)</td>
<td>38 - 50 (44*)</td>
<td>Bull (1953a)</td>
</tr>
</tbody>
</table>

(* mean of 100 eggs)

Descriptions of the eggs of other intestine species of *Trichostrongylus* are given in texts (e.g. Mönnig) and by Shorb (1939). By plotting length against width, it has been shown that the different species have separate distributions.
(Wood 1931; Shorb 1940; Tetley 1941, 1949a; Cunliffe and Crofton 1953).

The size of eggs within a species assumes some importance, as smaller eggs develop more rapidly than larger eggs (Crofton and Whitlock 1965). Waller and Donald (1970) showed that the smaller *T. colubriformis* eggs survive dry conditions better.

**Survival of Parasite Population in the Host, and Primary Patent Period**

It is not known how long a population of *T. retortaeformis* may survive in the rabbit. Seasonal fluctuations from field data (Evans 1940; Bull 1964; Dunsmore 1966b) suggest a turnover at least once a year. With single dose laboratory infections, Michel (1963a) recorded a primary patent period of 3 or 5 weeks after the first positive egg count, terminated by elimination of adult nematodes. With doses of less than 5,000 infective larvae, J.D. Dunsmore (1968 pers. comm.) reported a patent period of about 4 weeks, occasionally up to 10 weeks, in wild rabbits under laboratory conditions.

Michel (1963a) represented graphically egg production in a laboratory infection lasting 40 weeks following a second infection with a large dose of larvae. He related this to the persistence of inhibited larvae (see below), with cycles of egg counts due to elimination of batches of adult worms soon after they reach maturity.

For *T. colubriformis*, Bizzell and Ciordia (1965) found a patent period of 7 weeks (range 3 to 10 weeks) in calves, and in guinea pigs infections have been shown to persist for about 2 weeks after the first positive egg count (Gordon, Mulligan, and Reinecke 1960; Wagland and Dineen 1965). Gordon (1950, 1967) discussed the conditions under which loss of *Trichostrongylus* spp. infection from the intestine occurred.
Inhibition of Parasite Larval Development

Information is also needed to verify whether infections are usually accompanied by suppression of development of a significant proportion of T. retortaeformis larvae. Such inhibition seems to be the main feature of laboratory infections described by Michel (1952a, 1952b, 1953, 1963a, 1968). Arrested stages of the intestine Trichostrongyulus species in wild rabbits and in sheep have not been detected in Australia (J.D. Dunsmore 1970 pers. comm.; N. Anderson 1970 pers. comm.; A.D. Donald 1971 pers. comm.).

The inhibition of nematode larvae at parasitic stages appears to be a common phenomenon (Taylor and Michel 1953) and occurs, at a histotrophic phase, in all those genera that have been examined. Sommerville (1960) pointed out that arrested development is often reported to precede or follow an ecdysis. The arrest in development of trichostrongylid species commonly occurs with fourth-stage larvae: after the third ecdysis for the stomach parasites Ostertagia (e.g. Sommerville 1953; Michel 1963b), Haemonchus (e.g. Bremner 1956), Obeliscoides (Sollod, Hayes, and Soulsby 1968), and probably Hyostrongylus (Kotlan 1949) and T. axei (Donald 1971 pers. comm.); or before the fourth ecdysis for the small intestine parasite Cooperia (Sommerville 1960). (Other authors have referred to inhibition of larvae, or of fourth-stage larvae, but have not stated at which step in development it has been found.)

Inhibition of the intestine species of Trichostrongyulus, however, occurs with the third-stage parasitic larvae (Michel 1952c). Bull (1955) reported that in the oldest (heaviest) rabbits sampled in the field, 12% had no T. retortaeformis in the intestine other than third-stage larvae. These may have been inhibited forms.

For some genera in other strongylate families, an arrest in parasitic larval development can occur at the third-stage, as in Ancylostoma (Scott 1927; Schwartz and Alicata 1934a; Stone and Willis 1967;) and Nippostrongylus (Taliaferro and Sarles 1939; Haley 1958b; Haley and
Schellberg 1966). In these genera the adults also occur in the small intestine, but the larvae may undertake a lymphovascular-pulmonary migration in the host, and the arrest at the third-stage occurs before the intestine is reached.

Migration in the Host

From lambs infected with intestine species of *Trichostrongylus*, Mönig (1926) examined samples of blood and pieces of liver and lung, without finding larvae that had migrated out of the intestine. No other investigations on migration of *Trichostrongylus* spp. are known to be published, and it is generally accepted that there is no migration outside the gastro-intestinal tract (Levine 1968 p.196).

Size of Parasite Population

The expression of characteristics in the host-parasite relationship is further dependent on the size of the nematode population in the host (and the stimulation of an immune or pathological response). Michel (1963a) illustrated the egg production from *T. retortaeformis* infections of different sizes in the rabbit, and showed the duration of the infection to bear an inverse relation to its size. A low level of infection apparently continued for at least 24 weeks, while higher levels were said to be short-lived and their termination fairly sudden. This was described as "self-cure" from the term coined by Stoll (1929) to refer to such a phenomenon in sheep. With very large doses of larvae the infection was terminated before reaching patency, but if the rabbit failed to eliminate the nematodes, it subsequently succumbed following a sharp rise in egg production by the parasite. Michel (1953, 1963a) reported that rabbits could tolerate otherwise lethal doses of *T. retortaeformis* without discernible effect if they had previously experienced lower levels of infection, by which they had developed
protection. The numbers of infective larvae used, or of nematodes recovered, by Michel are not available, but it was stated (Michel 1952c) that a dose of 100,000 larvae was used, when postulating relay development to adults from inhibited parasitic larvae. The preceding results have been previously summarised by Michel (1952a).

Increasing doses (up to 20,000) of *T. affinis* resulted in lower proportions recovered at post mortem examination, and higher body weight loss of rabbits (Dixon 1965b).

Other reports with graded infections of intestine *Trichostrongylus* are for *T. colubriformis* (mainly in an abnormal host, the guinea pig). In infections with low to moderate levels of infective larvae (up to 6,250) (Sturrock 1963; Herlich 1969a), the egg production and numbers of nematodes recovered increased with increasing larval dose. Host body weight showed erratic gains at different dose levels until the start of patency of the nematode populations, after which groups that had received more larvae lost more weight than others. In infections of guinea pigs with moderate to high levels of infective *T. colubriformis* larvae (from 5,000) (Gordon, Mulligan and Reinecke 1960; Poynter and Silverman 1962; Herlich 1969a), most animals receiving 15,000 or more died. The survivors were protected against subsequent challenge with otherwise lethal doses. Single infections from graded larval doses in goats (Fitzsimmons 1966b) gave similar results, with a greater and more rapid effect proportional to dose.
(ii) Physiological Variations through Responses of the Host

Prior Exposure to the Parasite

It has already been noted, that Michel (1953, 1963a) reported that prior exposure of rabbits to T. retortaeformis influenced the course of an infection with lethal doses. An effect of prior exposure was shown with lower doses - of the same species in groups of rabbits (Whitten 1948), of T. calcaratus in individual rabbits (Sarles 1930, 1932a), or of T. colubriformis in individual lambs (Andrews 1939) - as a decrease in egg counts, or as a decrease in the proportion of nematodes recovered relative to the accumulative dose of larvae.

Herlich (1966c) obtained the same result, and he showed (by terminating the first infection at different intervals with an anthelmintic) that the third-stage larvae of a prior infection were effective in depressing the numbers of T. colubriformis established in second infections of guinea pigs. However, with groups of four lambs, Kassai (1970) did not show a statistically significant effect of prior infections with T. colubriformis, on the total recovery of the accumulated infections.

Multiple Doses of Infective Larvae

Little attention appears to have been given, for intestine species of Trichostrongylus, to the effect of small multiple doses of infective larvae compared with the same number of larvae in a single experimental dose. Sarles (1932b, 1934) did examine infections with single and multiple doses of T. calcaratus in single rabbits, but did not compare equal numbers of larvae.

Nutrition of the Host

The host diet will directly effect the environment of the adult parasite in the lumen of the gut, as well as affecting other aspects of the physiology of the host. The host nutrition is one of the most complex factors to
investigate because it has a considerable number of possible variations, and it may act on the parasite directly, on the host directly, or on the host response and immunity to the parasite. Then, in a circular action, one or more of the effects may affect the dietary intake and utilisation.

Experimentally it has been found that a quantitative reduction in diet, or a qualitative supplement, may have no effect on the number of *T. colubriformis* established in lambs, from infections in the laboratory (Gordon 1964), or on pasture (White and Cushnie 1952). On the other hand, a dietary supplement depresses the level and persistence of egg production (White and Cushnie 1952; Stewart and Gordon 1953). The plane of nutrition during the course of an infection did not appreciably affect the establishment of a subsequent challenge infection of *T. colubriformis* (Stewart and Gordon 1953; Gordon 1964; Brunsdon 1964).

Effect of Infection on Host Physiology

The effects on host tissues of intestinal parasitism by *Trichostrongylus*, seem to arise from interactions with nutrition, as shown by experiments with *T. colubriformis* as follows,

in lambs:  
- Andrews (1939)  
- Andrews, Kauffman, and Davis (1944)  
- Franklin, Gordon, and Macgregor (1946)  
- Carter, Franklin, and Gordon (1946)  
- Gordon (1950)  
- Gordon (1958a)  
- Gordon (1960)  
- Gordon (1964)  
- Kates and Turner (1953)  
- Gallagher (1963)  
- Holder (1964)  
- Hiepe and Zimmerman (1966)  
- Graham and Searle (1967)  
- Horak, Clark, and Gray (1968)  
- Symons 1969a  
- Symons and Jones (1969)  
- Symons and Jones (1970b)  

in kids:  
- Fitzsimmons (1966b)  

in calves:  
- Herlich (1957)  

in guinea pigs:  
- Herlich (1958)  
- Herlich (1969b)  
- Symons and Jones (1970a)
The degree of effect was directly related to the size of the infective larval dose (r,s,t,u) or the number of nematodes established (b). Several authors used multiple doses (a,b,c,d,e,j). Total larval doses of 20,000 or more were used in ruminants, or around 5,000 or more in guinea pigs.

A primary effect of *T. colubriformis* infection appears to be a depression of voluntary food intake by the parasitised animals (a,b,e,f,h,i,n,o,r,s), which is reflected by decreased faecal output (b,h,n). Retention of ingesta in the proximal part of the gut has been shown (n). The proportion of water in the faeces of some animals increases during infection (b,e,h,i,n,o,s), but may be dependent on the nutrition, as diarrhoea is not necessary for fatal termination (j). A considerable retention of water in the gut contents, compared with a pair-fed control, was indicated in data for a lamb (n), although no statistically significant difference in the amount of water in the contents of the small intestine was found in guinea pigs (u).

A loss of water from the general body tissues was suggested (b) in lambs with diarrhoea, but when the plasma volume of infected lambs without diarrhoea was measured, variation in body water level was attributed (j) to body weight differences. Although an increased total weight of the intestine was recorded in lambs (n), no effect of *T. colubriformis* infection on the water content of the small intestine tissue was found in guinea pigs (u). Voluntary water intake was said (n) to closely follow the food intake in infected and control pair-fed lambs.

A depression of body weight gain parallels the depression of appetite in animals infected with *T. colubriformis* (b,e,f,g,h,i,j,m,q,r,s,t,u). Sarles (1934) also showed body weight loss in rabbits as an important effect of *T. calcaratus* infection, and Dixon (1965b) reported an increasing body weight loss in rabbits infected with increasing doses of *T. affinis*. The effects
are less marked when sheep are already on a low level of food intake from restricted pen feeding (h) or from grazing poor pastures (k).

The effect of *T. colubriformis* on the body weight of lambs is apparently due to some factors other than merely lowering the food intake, as it occurs with pair-fed animals (c, n), although Symons and Jones (p) reported no increased weight loss compared with pair-fed animals. Not only has a decreased digestion of protein been reported (b, c) but also a decreased retention of protein (b, m, n). Symons and Jones (q), however, did not find significantly reduced absorption of protein from the gut with radioisotope labelled material, but there was a significant depression of mucosal dipeptidase activity.

Infected animals show a depression of skeletal muscle protein synthesis (v), and a depression of protein albumin concentration (n, r). The same causative factors probably lead to the depressed wool growth reported (d, f, l, p).

Although Graham and Searle (m) reported, in work with Symons (see review 1969b), that they found no effect on metabolic rate in sheep due to intestine *Trichostrongylus* infection, Andrews, Kauffman and Davis (b) reported a decreased efficiency of food conversion into body weight gain, which they surmised was due to increased energy metabolism.

*T. colubriformis* infection has been shown (b, c, n, r) to lead to a drain of calcium and phosphorus levels in the host.

The effect of *T. colubriformis* infection on the physiology of the host has also been reviewed by Gordon (1950), Soulsby (1965 pp.427), and Fitzsimmons (1969). (These articles all cite Stewart (1933) who examined mixed infections, mainly with *Haemonchus*, in lambs.) Seddon (1950 pp.124) reviewed the infection in sheep in Australia.
Age (and Size) of the Host

Age commonly protects the host from parasitic infections, but it is a moot and vexed question whether age itself disposes to development of an innate resistance, enhances the ability to exhibit an acquired immunity, or merely results in an acquired immunity from previous exposure to infections. It is likely that the contribution of each of these varies with the particular situation and is influenced by environmental and other physiological interactions. It is readily predictable, therefore, that scientific observations will differ as to an age effect without necessarily being in error.

With rabbits exposed to *T. retortaeformis* in the field, Bull (1955, 1964) discovered that animals of all ages showed predominately high levels of infection, following an abrupt increase that he associated with commencement of grazing by younger rabbits. However, in the older (heavier) rabbits there was a tendency for low levels of infection to reappear. Bull and Taylor (1956) related the age of peak incidence to a succession of parasites, showing a peak of coccidia incidence in very young animals, then peaks of nematodes, and a maximum incidence of cestode cysts in the oldest animals.

The field results of Dunsmore (1966b) showed a tendency in male rabbits for younger animals at a particular sampling to have more *T. retortaeformis* than those a year older. Statistical analysis of pooled results for all samplings (Dunsmore and Dudzinski 1968) showed no clear relationship of nematode intensity and age of the host.

On examining natural infections in rabbits of known ages within enclosures, Dudzinski and Mykytowycz (1963) found a relatively constant level around 300 worms over the range of ages. They discovered that in the younger rabbits (1 to 3 months old) both male and female nematodes were longer, and the females contained more eggs.

Whitten (1948) infected groups of laboratory rabbits (aged 3, 6 and 12 months) with a single dose of
T. retortaeformis infective larvae. Although substantial infections became established in all animals, the egg production varied inversely with the age of the host. When subsequently given a challenge infection, each age group of rabbits exhibited a very low egg production. Sarles (1934) did not show any obvious age resistance to T. calcaratus by individual laboratory rabbits.

Adult sheep reared worm-free were shown to be susceptible to experimental infection with T. colubriformis (Stewart and Gordon 1953), and age resistance was not found in sheep grazed on infective pasture (Brunsdon 1962).

Herlich (1960) found that adult cattle naturally infected by grazing acquired appreciable nematode burdens, but there was some age resistance apparent (for pooled trichostrongylid genera), because in the younger animals there were more nematodes established. With a single dose artificial infection in worm-free cattle, Herlich (1960) reported a clear age resistance and said adults appeared to be almost completely refractory to T. colubriformis, and to other intestinal genera.

In laboratory experiments with groups of guinea pigs of different ages, Herlich (1958) used moderate doses of T. colubriformis larvae. He reported that, in general, animals with the fewest worms established made the best weight gains. He stated that in an experiment younger females gained weight while older animals lost, and in another only older males lost weight while younger animals gained. This suggests that the younger animals may have been more resistant than the older.

Reproductive Status and Sex of the Host

It is particularly unfortunate that many scientific observations are reported without stating the reproductive status, or even the sex, of the animals concerned. It is clear that many of the foregoing responses are influenced by these factors. An extensive review (Ford 1968) into the influence of sex and reproduction on helminth parasitism
has been carried out, including about 150 references on experimental observations and about 500 references to field observations. From this and more recent information, the general conclusions may be summarised as follows. The influence of sex commonly appears to be slight in infant and senescent animals. From puberty, and during their sexually mature life, animals usually show resistance to parasitism with differences between the sexes, entire males generally appearing to be more susceptible. In animals that live in an environment where they experience a breeding season, an annual cycle is superimposed on the age pattern of resistance of the sexes to parasitism. The natural resistance of females tends to be lost during the period of reproduction, associated in mammals with pregnancy, and to a greater extent with lactation. This decline in resistance of breeding females is also apparent in species that may breed at any time of the year, such as man, and is most evident in populations for young adult females around the peak of their reproductive career. During this period adult females commonly become more susceptible than males. However, mature males too may show increased susceptibility during a period of sexual activity, but in a seasonally breeding population this tends to precede the increased susceptibility of the females.

The phenomenon of increased susceptibility of ewes to trichstrongylid nematodes (exhibited as a rise in nematode egg production) during lactation, was reviewed and discussed in depth by Ford (1968 pp.55), and has gained popularity as a research project since Dunsmore (1963, 1965a) postulated a mechanism related to reproductive hormones. The phenomenon was originally described from sheep for Haemonchus (Guberlet 1921; Veglia 1928), and for Trichostrongylus spp. where it was termed the "spring rise" (Veglia 1928). Later it was termed, correctly, the "lactation rise" by Tetley (1949b), but is now commonly known as the "post-parturient rise" (Crofton 1958).
Although frequently the parasites involved in the post-parturient rise in ewes have not been determined, intestinal Trichostrongylus spp. have been found to be present. No published work is known that particularly examines the contribution of intestine Trichostrongylus spp., although such experimental observations have been carried out by O'Sullivan (1970).

The most important contributions to the literature on the influence of sex and reproduction on natural trichostrongylid infections are for T. retortaeformis in wild rabbits by Bull (1959, 1964) and Dunsmore (1966b). This work has already been reviewed in detail, and effects discussed in relation to other published work, by Ford (1968 pp.69).

The influence of host reproduction may be illustrated by the New Zealand data of Bull (1964) and Watson (1957) shown in Figure 1 (taken from Ford 1968). Although there was some breeding of rabbits all year, a distinct breeding season was found from mid-winter to summer, and outside this period the young born were not reared. In the adult males there appeared to be an association between the rise in mean testes weight and the rise in mean nematode intensity. In the adult females the level of mean nematode intensity seems closely related to the percentage of females lactating, which was a couple of months behind the percentage pregnant.

In the N.S.W. Southern Tablelands data, Dunsmore (1965b, 1966b) recorded for 3 years a remarkably similar pattern of infection with T. retortaeformis to that found by Bull. The breeding season was much shorter, commencing later, and Dunsmore did not separate results for pregnant and lactating rabbits. Significantly greater parasite burdens occurred in adult females than in adult males during the breeding season. This difference followed about a ten-fold rise in the females, but was also contributed to by the spring drop in mean intensity of nematodes in the males. Outside the breeding season both authors found greater nematode intensities in male than in female rabbits.
Trichostrongylus retortaeformis in Rabbits at Gwavas Forest, New Zealand, 1950-51

Comparison of worm intensity and host reproductive status

Data from Bull (1964d) and Watson (1957)
Bull (1959, 1964) compared the mean intensity of *T. retortaeformis* in females of varying reproductive status. In young adult females the parasite burden was found to be higher in pregnant than non-pregnant animals. The number of nematodes increased progressively with the stage of pregnancy, and rabbits that were lactating but not pregnant had the most nematodes.

It is most likely that the same causative phenomenon is involved in both the rise in trichostrongylid faecal egg counts associated with reproduction in ewes, the so-called post-parturient rise, and the rise in *Trichostrongylus* adult nematode counts associated with reproduction in doe rabbits.

Support is given to the field observations by evidence from rabbits naturally infected in enclosures. In animals killed soon after the breeding season, Dudzinski and Mykytowycz (1963) reported a similar level of infection with *T. retortaeformis* in both sexes. However, in the counts of adult nematodes from rabbits killed during the breeding season, Dunsmore (1966a, 1966c) reported significantly greater intensities in breeding females than in males or ovariectomised females. The number of nematodes recovered from the breeding doe was related to the number of young (Dunsmore 1966a).

Dunsmore (1966a, 1968) administered luteinising hormone to breeding does and obtained the surprising result of increased resistance to infection. The implications of trophic hormones have previously been discussed (Ford 1968 pp.339), and it was considered that the somatotrophic hormones, such as prolactin, are more likely to have an independent effect, favouring the parasite, than the gonadotrophic hormones whose influence is integrated with the production of steroid hormones.

Following implants of an oestrogen in rabbits of both sexes, Rohrbacher (1958, 1960) found no apparent effect on the establishment of *T. colubriformis*, while Dunsmore (1968) found an unexpected result of increased natural infection of *T. retortaeformis* in enclosures.
Ciordia, Bizzell, Porter and Dixon (1966), with laboratory infections of *T. colubriformis*, recovered more nematodes from male than female rabbits following moderate doses of larvae, but recovered similar numbers of nematodes from both sexes of guinea pigs following low doses of larvae. Herlich (1958) in three experiments with *T. colubriformis* infections in guinea pigs consistently reported weight gains in groups of females and weight loss in groups of males. However he did not give the number of worms recovered and said that host sex apparently had little effect.

Photoperiod

Although seasonal cycles of parasitism in the field are common for intestinal *Trichostrongylus* spp., they have usually been related to exposure to differing levels of infective larvae on the pasture. No work appears to have been reported on whether a response to photoperiod, via a neural and hormonal link, plays a part in the host-parasite association. While the influence of photoperiod was not included in this study, it was examined for *T. retortaeformis* in the rabbit in concurrent work, as shown in Appendix II.

(iii) Physiological Variations through Responses of the Parasite

History of Infective Larvae

Influences acting on the host-parasite relationship derived from variations in host physiology have received a degree of experimental attention, but, by comparison, influences derived from variations in parasite physiology appear to have been largely ignored. Alterations in the physiology of trichostrongylid nematodes distinct from
interactions with the host, will be initiated in the free-living phase of the life cycle, or even in a previous generation.

The conduct and assessment of infection experiments has rarely taken into account the physiological status of the infective larvae, despite contentions that their history influences their infectivity (Rogers 1939) and their development at subsequent parasitic stages (Silverman 1963). Nor has it been considered in field studies, where it appears to be commonly assumed that surviving infective-stage larvae will transmit infection. Recently, however, Anderson, Armour, Jennings, Ritchie and Urquhart (1965, 1969) have suggested that physiological changes in the free-living stage of the parasite (as an alternative to changes in the host) are associated with a change in parasitic-stage development of trichostrongyloid nematodes in natural infections.

For *T. retortaeformis*, the development to the infective stage and the survival on pasture are mainly governed by climatic temperature and humidity (Crofton 1948b). This has been verified in the laboratory (Prasad 1959). With adequate moisture, optimum development occurs around 20°C to 25°C and the infective stage is reached in 3 to 4 days (Prasad 1959; Gupta 1961; Bondy 1965). At lower temperatures development is prolonged, at higher temperatures survival is shortened.

When aged at room temperature, *T. retortaeformis* infective larvae were found to survive for about 4 months (Prasad 1959), at 25°C to 20°C some survived for 4 to 6 months (Gupta 1961), and at 15°C for about 8 months (Nnochiri 1950; Gupta 1961).

When stored at a temperature of 5°C, some larvae survived for more than 12 months (Gupta 1961). Those that had been cultured at 20°C survived better than those cultured at 25°C.

Almost identical results were obtained for *T. colubriformis* with more extensive investigations (Andersen, Wang, and Levine 1966; Andersen and Levine 1968).
The subsequent infectivity of the *T. retortaeformis* larvae was not investigated, but Andersen, Wang and Levine (1966) recorded that an undetermined proportion of *T. colubriformis* larvae were infective to a sheep after 10 months storage at 4°C. Herlich (1966a) showed that the rate of infectivity of *T. colubriformis* larvae to guinea pigs showed no apparent fall during storage up to 12 months at 4°C.

However, the infectivity to guinea pigs of *T. colubriformis* larvae aged at 25°C fell between 10 and 30 days after the collection of donor faeces (Ciordia, Bizzell, and Porter 1965; Ciordia, Bizzell, Porter, and Dixon 1966). These authors also reported an optimum culture temperature around 25°C, from which the greatest recovery of nematodes from infected guinea pigs and rabbits was obtained, although cultures at a lower temperature had been carried on much longer before harvesting infective larvae.

Effect of Infection on Parasite Physiology

No work on examining changes in the physiology of trichostrongylid nematodes following exposure to host influences appears to have been carried out, apart from measurements of size and counts of worms and eggs as already mentioned. However, very recent interest has been taken in this approach, by an examination of effects, on a heligmosomid nematode, apparently resulting from host immune reactions (Edwards, Burt and Ogilvie 1971).
(1) Design and Judgement of Host Experiments

The investigations into host-parasite physiological reactions were planned with experimental designs leading to statistical analyses of variance. Judgement was then made on the biological consistency of mathematically significant results. This contrasts with Part III where Culture Trials were assessed on whether the parasite showed a particular feature.

However, in Part II Section A, concerning characterisation of uncomplicated infection, judgement also tended to be based on descriptive criteria. In Experiments 1, 2, and 3 the development of parasitic larvae was examined, and in Experiment 6 the survival of the nematode population in the intestine.

Although Experiment 4, on the development of patency with graded doses of infective larvae, was planned for statistical analysis of variance, it was found in subsequent work that different batches of infective larvae (Table 2), as used for different batches of rabbits (blocks), had a much greater influence on the development of the infection than the levels of the infective dose being examined. Subsequently the results were presented for absolute assessment. Within blocks it was seen that the results were biologically consistent, and the findings therefore were valid.
Statistical analyses for the rhythms of peaks or of troughs (attribute) in egg counts reported in Experiment 4 were made with the chi-square test for frequencies, using the basic formula:

\[ \chi^2 = \sum (n_k - pN_k)^2 / pN_k \]

where \( p = \frac{\sum n}{\sum N} \)

- \( n_k \) = number of samples with attributes
- \( N_k \) = total number of samples for period
- \( k \) = number of periods examined

Each part of Experiment 5 was planned to make direct comparisons between the sizes of eggs found in different samples. As explained in the Plan of the experiment, the results are presented for each observation as the incidences of eggs that are larger or smaller than the overall median size. Statistical comparison was made between pairs of observations, that is from a 2 x 2 contingency table, with the chi-square test for frequencies, using the adapted formula:

\[ \chi^2 = \frac{\sum n m}{\sum n m} \times \frac{(n_{1m} - n_{2m})^2}{N_1 N_2} \]

where
- \( n \) = number of larger eggs
- \( m \) = number of smaller eggs
- \( N \) = total number of eggs for observation

In Sections B and C of Part II judgement of the influence by the experimental factors (as below) complicating the infections was made on a statistical basis following analysis of the sources of variance. The estimate of population variance (\( s^2 \)) and its degrees of freedom (d.f.) was determined by Fisher's F test, and the significant difference between means calculated by "Student's" t test.
for the arbitrary probabilities (p) of 0.05 and 0.01 as conventionally used. Standard procedures were used for these tests. The calculation of missing plots was not required for any experiment.

Each of Experiments 7 to 12 was arranged as a factorial design for orthogonal factors (i.e. each represented equally) as follows, giving the experimental factors investigated or the factors on which rabbits were allocated to groups.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Rabbits (no.)</th>
<th>Factorial Design</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>8*</td>
<td>2A x 4*K</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>5A x 2H x 2B</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>5B x 2D x 2E</td>
</tr>
<tr>
<td>10</td>
<td>32</td>
<td>2B x 2C x 2D x 2E x 2I</td>
</tr>
<tr>
<td>11</td>
<td>18</td>
<td>3B x 2F x 3J</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>3G x 5J</td>
</tr>
</tbody>
</table>

* An additional two rabbits were used for some observations only

The factors were: A History of infective larvae
B Duration of infection to post mortem
C Multiple doses of infective larvae
D Previous infection
E Diet
F Reproductive status of adult female rabbits
G Lactation status of adult female rabbits
H Sex of rabbit
I Prior body weight
J Prior body weight gain
K Mixed treatment (as given in Plan of experiment)

The summaries of the pertinent analyses of variance, to which reference is made in the results, are given in Appendix III.

A logarithmic transformation of counts (e.g. Paver, Parnell, and Morgan 1955; Whitlock 1961) was carried out, to approximate to the normal distribution and stabilise the variance, so that Fisher's analysis of variance could be used. For total nematodes one tenth the number...
recovered (i.e. the number counted) was used.

Transformed value = \log_{10} (number + 1)

Geometric means are therefore given for nematode and for egg counts.

Allocation to groups was by use of a table of random numbers. The rabbits were previously identified at random on arrival, or ranked by body weight.

The Host

The rabbits have been described in the General Introduction.

(2) Housing and Feeding

All the rabbits were reared and individually maintained in suspended wire mesh cages. Faecal pellets passed through the floor so that self-augmentation of infection was prevented. In addition, steel trays under the cages at the laboratory were washed down daily. During the week approaching parturition of reproducing does, wooden fruit cases opened at the top were placed at one end of the cage and a handful of straw added. Any faecal pellets deposited in these nesting boxes were removed daily.

The adult female rabbits in Experiments 9 to 12 were accommodated in a roofed enclosure in the open air where they were subject to the fluctuations in seasonal environment. The other rabbits at the laboratory were held in an animal house room where the temperature was maintained at about 20°C to 25°C throughout the year.

For the various procedures, rabbits were restrained in a guillotine-type box (Leahy and Barrow 1953 p.256). From their arrival at the laboratory, most rabbits were fed on lucerne chaff, supplied in a pelleted form
(Red Bend Alfalfa Co.). For Experiments 9 and 10 only, some rabbits, as outlined in the Plan of the experiments, were fed on normal laboratory pellets (Drug Houses of Australia), in order to compare the two diets. The rabbits were bred at "Tillside" on these laboratory pellets.

The constituents of the pelleted foods are given in Table 3. The anti-oxidant, ethoxyquin, used to preserve vitamin activity, has been reported (Ciordia, Porter, and Bizzell 1967) to adversely affect the development of free-living stages of *T. colubriformis* from calves, when fed at 0.05% or more in the diet. However Gordon (1968) did not detect any effect at 0.125% in the diet in sheep, and there is no indication that the level of 0.008% present in the pelleted lucerne had any effect on *T. retortaeformis*. No anti-oxidant was used in the laboratory pellets.

The diet was supplemented with fresh carrots and cabbage leaves once or twice a week, except in experiments when the diet was being examined. Rabbits fed solely on laboratory pellets became cachectic and died after a few months. Lucerne alone maintained rabbits indefinitely (up to 1 year observed). Food hoppers were topped up daily so that feeding was *ad lib*. Water was supplied continuously through self-operated nipples.

(3) Inoculation of Rabbits with Infective Larvae

For oral inoculation, the rabbits were dosed, with the measured number of infective larvae, via a plastic stomach tube passed through a copper tube gag. The dose was administered from a water-repellant plastic syringe, rinsed with about 2 ml of water. Checks of syringes and tubes subsequent to dosing showed that if drops of water were retained, fewer than 50 larvae failed to be administered.

The intra-venous inoculations with exsheathed larvae in Experiment 2 were made into an ear vein. The larvae were exsheathed as in Methods of Part III.
Young rabbits were 6 to 8 weeks of age at the time of first inoculation, adult rabbits were more than 25 weeks of age. The time of the initial administration of infective larvae in an experiment is referred to as day 0 or week 0 for that experiment.

(4) Faecal Output and Growth

The faeces from each rabbit were collected on a 5 mm mesh wire slide placed under the respective cage. The weight of faeces passed per 24 hours was determined, and used to calculate the number of parasite eggs passed per day. The faecal output per 24 hours also provided an approximate assessment of appetite.

Negligible amounts of hard faecal pellets were passed during the day in the laboratory (when soft faecal pellets were refected). The 24 hour collections of hard pellets were therefore made from about 10 a.m. in winter, or from about 8 a.m. in summer.

The live body weight of all rabbits was measured at various intervals.

(5) Post Mortem Dissection

Rabbits were killed by cervical dislocation and severing the cervical spinal cord. The small intestine was immediately dissected out, and placed in an individual container. In some cases the proximal and distal halves were separated on length.

When live parasites were required (as for Part III observations), the intestine was not treated further. When a count of nematodes was required, the following procedure was adopted. Physiological saline (0.85% sodium chloride) was used.

The contents of the unopened small intestine were extruded, and the mucosa rinsed twice with saline. This constituted the small intestine wash. In some cases the weight of the washed small intestine was measured.
Table 2

Laboratory Strain of *Trichostrongylus retortaeformis*

Passages in Rabbits as used in Experiments

<table>
<thead>
<tr>
<th>EXPERIMENT NUMBER (AND SECT)</th>
<th>PASSAGE NUMBER</th>
<th>SOURCE OF INFECTIVE LARVAE (from previous passage)</th>
<th>Length of Infection in Donors at 25°C</th>
<th>Length of Faecal Incubation at 20°C</th>
<th>Length of Storage at 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>39 days</td>
<td>8 days</td>
<td>-</td>
<td>98 days</td>
</tr>
<tr>
<td>2*</td>
<td>9</td>
<td>14</td>
<td>-</td>
<td>15 days</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>46</td>
<td>-</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>4 (a)</td>
<td>3</td>
<td>29</td>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(b)</td>
<td>6</td>
<td>21</td>
<td>-</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>(c)</td>
<td>7</td>
<td>79</td>
<td>-</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>(d)</td>
<td>9</td>
<td>31</td>
<td>-</td>
<td>26</td>
<td>17</td>
</tr>
<tr>
<td>5*</td>
<td>8</td>
<td>58</td>
<td>-</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
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</tr>
<tr>
<td>7 - 1</td>
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<td>50</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
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<td>4</td>
<td>39</td>
<td>8</td>
<td>-</td>
<td>98</td>
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<td>4</td>
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</tr>
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<td>- 2</td>
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<td>36</td>
<td>-</td>
<td>8</td>
<td>-</td>
</tr>
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<td>21</td>
<td>9</td>
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<td>10*</td>
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<td>App.II</td>
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</tr>
<tr>
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<td>2</td>
</tr>
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</tr>
<tr>
<td>- 4</td>
<td>6</td>
<td>39</td>
<td>-</td>
<td>8</td>
<td>378</td>
</tr>
</tbody>
</table>

* source varies, main example(s) given

+ some rabbits dosed continuously
<table>
<thead>
<tr>
<th>Laboratory Pellets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pollard</td>
</tr>
<tr>
<td>Wheat Meal</td>
</tr>
<tr>
<td>Maize Meal</td>
</tr>
<tr>
<td>Coconut Meal</td>
</tr>
<tr>
<td>Lucerne Meal</td>
</tr>
<tr>
<td>Skim Milk Powder</td>
</tr>
<tr>
<td>Meat and Bone Meal</td>
</tr>
<tr>
<td>Bone Flour</td>
</tr>
<tr>
<td>Additives -</td>
</tr>
<tr>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>Magnesium Oxide</td>
</tr>
<tr>
<td>Vitamin A</td>
</tr>
<tr>
<td>Vitamin D</td>
</tr>
</tbody>
</table>

An anti-oxidant to preserve vitamin activity was not added.

<table>
<thead>
<tr>
<th>Pelleted Lucerne</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydrated Lucerne</td>
</tr>
<tr>
<td>Additives* - Tallow Ethoxyquin</td>
</tr>
</tbody>
</table>

* A small amount of tallow was added to help the pellet formation

* The anti-oxidant, ethoxyquin, was added to preserve vitamin activity.
The stomach was opened along the greater curvature for examination, and the bolus removed. This constituted the stomach contents. The mucosa was rinsed with saline, rubbing either side of the folds. This constituted the stomach wash.

Other viscera were retained for special observations. Within a few hours of starting to kill a batch of rabbits, each small intestine was opened along its full length, and digested for 4 to 6 hours at 40°C in 250 ml of 1% pepsin (1:2500 biochemical pepsin powder, British Drug Houses) and about 0.35% (i.e. 1% of concentrated) hydrochloric acid in saline (Sprent 1952). Digestion was terminated by adding formalin (40% formaldehyde solution) to give a final concentration of 10%, and raising the pH to around 7 with 15% sodium hydroxide solution. This constituted the small intestine digest.

Digestion of other organs and tissues was carried out by the same procedure. Lungs and liver were first homogenised in saline for 30 seconds in a Waring blender at a "Lo" speed setting.

All material retained was stored at 4°C in neutralised 10% formol saline.

The Parasite

The nematodes have been described in the General Introduction.

(6) Preparation and Recovery of Infective Larvae

Faecal incubations were set up two or three times weekly by adaptation of standard techniques, to provide a continual source of infective larvae for experimental work. The record of laboratory passages for each experiment described in the thesis is compiled in Table 2.
Hard faecal pellets collected from infected donor rabbits were moistened with distilled water, and incubated in 250 ml disposable plastic containers (St. Regis - A.C.I.). Incubations were carried out initially at 25°C up to the 5th passage, and then at 20°C. Infective larvae were harvested from the faeces after about a week (5 to 9 days) up to the 8th passage, or subsequently after 10 to 15 days. Other incubation times as given were used on some occasions.

To harvest infective larvae, a combination of standard techniques was adopted to maximise recovery of clean larvae.

The containers were filled with water and inverted onto disposable Petri dishes for 2 hours, for larvae to migrate out of the faecal pellets. The larvae from all the water were then collected on a filter paper in a Buchner funnel, and rinsed with clean water. The larvae were concentrated and further cleaned in a Baermann funnel by inverting the filter paper over a double layer of cellulose tissue. After 4 hours at 20°C, the larvae at the bottom of the funnel were drawn off. Further larvae were recovered after holding the funnel overnight at 4°C.

Harvested infective larvae which were not used immediately were held at 4°C (unless stated otherwise) in up to 1 cm distilled water in 250 ml Erlenmeyer flasks. When necessary, larvae were concentrated by centrifugation at 500 G for 1 minute.

(7) Counting Infective Larvae

A batch of clean infective larvae in water was continuously mixed in a 100 ml graduated Crowe receiver, by slowly bubbling air from the tapered bottom through a Pasteur pipette. Three or more aliquots of 0.1 ml were taken and the total number of live larvae in each counted under a microscope at 3.5 x 10 x magnification. The counts of stirred larvae were found to not deviate significantly from a random (Poisson) distribution.
When necessary, the volume of water in the cylinder was adjusted to give a suitable concentration, usually about 2,000 larvae per ml. The volume containing the required number of live larvae was pipetted into separate vials for each rabbit dose, or into centrifuge tubes for Part III trials.

(8) Counting Eggs

The number of parasite eggs passed in the faeces of infected rabbits was determined by adaptation of the standard McMaster flotation technique (Whitlock 1948a).

Samples of 3.0 gm of crushed faecal pellets from overnight or from 24 hour collections were weighed into 100 ml graduated disposable plastic containers, and soaked in 15 ml water at 4°C for 1 or more days. In a separate experiment, it was found that there was no appreciable effect of up to 3 weeks cold storage on the number of *T. retortaeformis* eggs counted. After this time there was a steady decline in the number of eggs recovered by salt flotation.

For counting, the soaked faeces were broken down with a plastic flail on a Braun homogeniser, and the containers topped up to the 90 ml graduation with saturated salt (sodium chloride) solution. While continuously mixing, samples of faecal suspension were taken with a McMaster sieve pipette and transferred to a modified McMaster counting slide (from H.V. Whitlock, J.A. Whitlock and Co.). For each chamber, the dilution factor to express the result in eggs per gram of faeces (e.p.g.) is 100. The eggs were counted under a microscope at 3.5 x by 10 x magnification. Two or more chambers were examined for each faecal sample, so that the minimum detectable level was never more than 50 e.p.g. The counts of eggs from stirred faecal suspensions were found to not deviate significantly from a random (Poisson) distribution. In some experiments the result was multiplied by the 24 hour faecal output to give the number of eggs passed per day (e.p.d.).
(9) Recovery of Parasitic Stage Nematodes

The Baermann funnel technique was used to obtain clean live nematodes from infected rabbits (as for Part III observations). The small intestine removed at post mortem dissection was slit open on a double layer of cellulose tissue supported on a gauze frame in a funnel filled with saline (0.85% sodium chloride) and held at 37°C. *Trichostrongylus* passed readily through the tissue filter, thus separating from the intestinal chyle which was retained for several hours. There was, however, a bias in the recovery of adults, as the larger nematodes were observed to take up to three times as long to pass through the filter as the first wave of smaller nematodes. Roughly 20% to 30% of the total number of nematodes in the infection could be recovered clean in 2 to 3 hours, after which the rate declined. Another about 5% could be collected in a further 6 hours, but the digesta began to diffuse throughout the saline during this period.

(10) Counting Parasitic Stage Nematodes

The total number of nematodes in the material collected at post mortem dissection was determined by the standard dilution counting technique. The stages of development (third-stage; early fourth-stage; late fourth-stage, male and female; and fifth-stage and adult, male (m) and female (f)) were differentiated.

Each preparation was made up to 250 ml, and continuously stirred with a Pasteur pipette through which air was slowly bubbling, while aliquots of 3.125 ml for digestes or 6.25 ml for washes were measured into sample beakers. Sufficient 10% aqueous iodine solution (double strength Lugol iodine solution) was added to each sample to give a final concentration of approximately 0.5% at counting. Although the iodine staining technique requires decolourisation of background material so that the nematodes stand out before their colour fades (Whitlock 1948b), this was not done because the smaller
larval stage forms decolourise so quickly they are overlooked. It was found that the larvae, particularly parasitic third-stage larvae in the digests, are more reliably visualised by retaining a background stain.

The whole of each sample was diluted with saline and examined in shallow layers, under a dissecting microscope at 1.6 x by 6.3 x magnification. Differentiation of larvae was done at higher magnification. Dummy runs were done from time to time with added third-stage larvae, because these are especially hard to detect amongst digests, as discussed later in relation to recovery of inhibited larvae. A minimum of one twentieth of each preparation was counted, and in some experiments a minimum of one tenth was examined. The counts of nematodes from stirred gut suspensions were found to not deviate significantly from a random (Poisson) distribution.

(11) Measurement of Nematodes and Eggs

All measurements were made under a microscope with a measuring eye-piece. The nematodes and eggs for measurement were recovered by the technique used for counting, as this is when they are routinely seen. The length of larvae and adults, and the length and width dimensions of eggs, are reported in this thesis.

In addition, the volume of eggs was calculated, by adopting the assumption of other workers that the egg approximates a prolate spheroid (Crofton and Whitlock 1965; Waller and Donald 1970), using the formula:

$$\text{volume} = \frac{\pi}{6} \times \text{length} \times (\text{width})^2$$

In results figures illustrating a frequency distribution of sizes, the moving average of adjacent points has been plotted.
EXPERIMENTS

SECTION A

DEVELOPMENT AND COURSE OF INFECTION

OBJECTS

The object of this section was to determine some of the characteristics of uncomplicated T. retortaeformis infections in laboratory rabbits, on which to base programmes for other \textit{in vivo} experiments in Part II, and \textit{in vitro} trials in Part III.

Experiment 1 was designed to provide knowledge of the rate at which the parasitic larval stages develop.

A lesser proportion of trichostrongylid larvae may be recovered from the gut for a few days after infection, than of adults subsequently. Although technical difficulties can result in some larvae not being detected amongst digesta and from mucosal crypts (usually after pepsin digestion of the gut wall), it can not yet be entirely refuted that the larvae may actually leave the alimentary tract for a short period. It is known that species from closely related families (e.g. ancylostomids and heligmosomids), such as \textit{Nipposrongylus brasiliensis}, may undergo a pulmonary migration before developing in the gut, although in these families, a migration is not essential for some species to develop after oral infection of normal hosts. An examination was therefore made in Experiment 2 of tissues, other than alimentary tract,
from rabbits killed in the first few hours and first few days after infection with *T. retortaeformis*. Other rabbits were injected intra-venously, with either sheathed infective larvae or with physiologically exsheathed third-stage larvae, to determine whether they would mature in the gut.

In Experiment 3, special conditions were imposed to detect the arrest or inhibition of development of parasitic larvae, to verify the stage at which arrest occurred in an intestine *Trichostongylus* species.

Although it appeared from the literature that some of a large population of *T. retortaeformis* may survive in the host for many months (apparently with continual replacement of adults by newly developing larval stages), it was not apparent whether mature adult nematodes can survive for a prolonged period. Also, it was not known whether a mature nematode population would be maintained in a host continually exposed to doses of infective larvae. Experiment 6 was planned to investigate these two points.

Experiment 4 was designed to examine levels of egg production by *T. retortaeformis* populations in rabbits following administration of graded doses of infective larvae. Five moderate levels were selected in a two-fold geometric progression.

In preliminary studies outside this thesis (on comparison with *Graphidium strigosum* infections) it was observed during egg counts that variations in the dimensions of *T. retortaeformis* eggs appeared to be associated with the duration of the infection. The proportion of larger eggs in a sample increased after reaching peak egg counts, compared with earlier in the patent period. Several comparisons were subsequently planned under various conditions to verify this observation, and if possible to characterise any change in egg size. The results form Experiment 5, which was carried out in a number of parts, as shown in the Observations.
(i) **Pre-patent Period and Early Development** (up to about 12 days)

**EXPERIMENT 1 ... EXAMINATION OF THE DEVELOPMENT OF PARASITIC LARVAL STAGES IN THE GUT**

**Plan**

Each rabbit in a uniform batch of young males was dosed with infective *T. retortaeformis* larvae at day 0, and *post mortem* dissection carried out to recover nematodes from individual rabbits at various times in the early course of the infection, as outlined in the following schedule.

<table>
<thead>
<tr>
<th>Group</th>
<th>Larval Dose (no.)</th>
<th>Duration of Infection (days to p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25,000</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

A moderately high dose of infective larvae was used to try and maximise the total number of developing larvae recoverable. In this experiment the stomach as well as the small intestine was routinely examined for larvae.

**Results**

The numbers, of each stage of developing larvae found, are given in Table 4. During examination of the material, it was noticed that there was a gap between the sizes of third-stage larvae and the earliest fourth-stage larvae recovered. Measurements of representative samples of each stage, as plotted in Figure 2, confirmed this observation. Each frequency curve is drawn proportional...
to the total number of larvae recovered at the time. Only one larva was found (at day 4) between third-stage and 1.04 mm.

The third-stage larvae from the gut were never more than 660 µ long, and in general were about 70 µ less than the free-living infective "third-stage" larvae inside the sheath (Table 4). Third-stage larvae can be readily distinguished by a notch near the end of the tail, which becomes more pronounced in the late third-stage. Early fourth-stage larvae have a bluntly pointed tail.

At day 2, an occasional larva appeared in the early fourth-stage, and 38.5% of those recovered at day 4 had reached this stage.

The size distribution was not spread out by day 2 or day 4 (Figure 2), and it seems that most of the larvae should have been around the smallest early fourth-stage at day 4. However only one larva of this size (as above) was found, and it seems they were missing, because the number of larvae recovered represented only 1.2% of the larval dose.

By day 6 most larvae had reached the late fourth-stage and the sexes were distinct in larvae greater than about 1.5 mm. An occasional male was found that had undergone the fourth ecdysis, and exceeded 2.8 mm in length. Other males, and females up to 3.2 mm, still retained the fourth-stage cuticle. In some cases this cuticle had separated, referred to by some authors as the moult fourth-stage, but observations in this study suggest that the separation may often be an artifact, occurring during the preparation for nematode counts.

By day 8 developing larvae, with few exceptions, had reached the fifth-stage. Their lengths varied widely, with females longer than the males. An estimate of the size is given by the mean of 25 larvae in Table 5.

The finding of young adult (fifth-stage) females on the 8th day, in which ova were beginning to form in the oviducts, is consistent with earlier observations on the
pre-patent period. In pilot studies with sixteen young male rabbits, eggs were first recovered in the faeces as indicated in the following schedule:

<table>
<thead>
<tr>
<th>Day after infection</th>
<th>Proportion of rabbits examined with positive egg counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>28.6%</td>
</tr>
<tr>
<td>12</td>
<td>83.3%</td>
</tr>
<tr>
<td>13</td>
<td>100%</td>
</tr>
<tr>
<td>14</td>
<td>100%</td>
</tr>
<tr>
<td>15</td>
<td>100%</td>
</tr>
</tbody>
</table>

The findings suggest that, from such infections, eggs will be released by the most advanced females around the 9th or 10th day, but eggs will not be present in detectable quantities in the faeces until a couple of days later.

Subsequently, positive egg counts have been obtained on the 10th day, but not earlier. These were infections derived from larvae cultured in faeces for longer than the customary 7 to 9 days after the collection of donor rabbits' faeces, and which never had been exposed to cold. These differences are consistent with results of later experiments.

As a bonus observation in Experiment 1, delayed development of approximately 2% of the larval dose was in evidence by day 4, and larvae were clearly separated into two populations by day 6. The inhibited larvae were arrested in development at the late third-stage. Inhibition at any other stage was not evident. An arrested third-stage larva is pictured in the Frontispiece.

Most larvae were recovered from the small intestine digest, and hardly any from the small intestine wash. None were found in the stomach. In previous pilot studies with young male rabbits, no T. retortaeformis were found in the stomach or large intestine. When the small
EXPERIMENT 1

Length Distribution of T. retortaeformis Parasitic Larval Stages Recovered at Intervals after Infection (from young male rabbits dosed with 25,000 infective larvae)

The length of the nematode is plotted with a moving average for intervals of 50 µ. The number of larvae recovered at day 2, and the infective larvae at day 0, are shown at 1/20 the scale of later times.
**TABLE 4**

**EXPERIMENT 1**

Recovery of *T. retortaeformis* Larval Stages at Intervals after Infection of Rabbits*

| DURATION OF INFECTION (days) | TOTAL NEMATODES (No.) (dose)* | L5 | LATE L4 | EARLY L4 | L3 |
|-----------------------------|-------------------------------|-----|---------|----------|-----|-----|
|                            | (No.) (% total)               | (No.) (% total) | (No.) (% total) | (No.) (% total) | (No.) (% total) | (dose)* |
| 2                           | 18,680 74.7                   | 0   | 0       | 20       | 18,660 (99.9%) | 74.6    |
| 4                           | 780 3.1                       | 0   | 0       | 300      | 480 (61.5%)    | 1.9     |
| 6                           | 6,340 25.4                    | 20 (0.3%) | 0       | 4,600 (72.6%) | 720 (11.4%) | 1,000 (15.8%) | 4.0 |
| 8                           | 4,240 17.0                    | 3,740 (88.2%) | 50.8 | 20 (0.5%) | 100 | 480 (11.3%) | 1.9 |

**TABLE 5**

**EXPERIMENT 1**

Mean Length Estimate of *T. retortaeformis* Larvae at Intervals after Infection of Rabbits*

<table>
<thead>
<tr>
<th>DURATION OF INFECTION (days)</th>
<th>DEVELOPING LARVAE (L5-L4)</th>
<th>L3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>females (mm)</td>
<td>males (mm)</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>2.42</td>
<td>2.12</td>
</tr>
<tr>
<td>8</td>
<td>5.55</td>
<td>4.44</td>
</tr>
</tbody>
</table>

* Young male rabbits given dose of 25,000 infective larvae.

+ Infective larvae - total mean length 660 µ including sheath.
intestine was split into proximal and distal halves for examination, a low, but appreciable, proportion of nematodes was found in the distal half. Subsequently, in adult rabbits killed after several doses of infective larvae, some T. retortaeformis larvae have been recovered from the stomach (see Experiment 3).

EXPERIMENT 2 ... EXAMINATION FOR MIGRATING PARASITIC LARVAE OUTSIDE THE GUT

Plan

Five rabbits were killed shortly after being infected, in an attempt to assess whether larvae could be detected in a hepatic or pulmonary migration during the early course of an infection with T. retortaeformis. Before intra-venous administration to three other rabbits, larvae were either surface sterilised and artificially exsheathed with sodium hypochlorite in Part 4), or in Part 5) stimulated to exsheath by carbon dioxide and hydrochloric acid, (as given in the Methods of Part III). The experiment was carried out in five parts as outlined for individual rabbits in the following schedule.

<table>
<thead>
<tr>
<th>Part</th>
<th>Larval Dose (no.)</th>
<th>Route of Administration</th>
<th>Duration of Infection (hours to P.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>10,000 oral</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>2)</td>
<td>100,000 oral</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>3)</td>
<td>25,000 oral</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>4)</td>
<td>5,000 intra-venous</td>
<td></td>
<td>66 (days observed)</td>
</tr>
<tr>
<td>5)</td>
<td></td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

* previously infected also
At post mortem dissection, the liver, lungs, and in Part 2) blood, were retained for examination. Following parenteral administration, rabbits were examined for development of patent infections.

**Results**

No larvae were recovered from the liver, lungs, or blood, of any of the rabbits, with the sole exception of a few live third-stage larvae found in freshly examined lungs of the rabbit killed 30 hours after infection. This rabbit had been given an extraordinarily large number of larvae, to facilitate their subsequent detection, and the larvae found in the lungs could have been regurgitated and inhaled at the time of dosing.

In Parts 4) and 5), the injected larvae apparently did not reach the intestine and grow to maturity, as a patent infection was not obtained.

No evidence was found of a parenteral migration.

**EXPERIMENT 3 ... EXAMINATION OF INHIBITED PARASITIC LARVAE**

**Plan**

An attempt was made to induce arrest or inhibition of developing *T. retortiformis* larvae, by incorporating three conditions under which other workers have reported the phenomenon. High larval doses were administered; adult rabbits were used; and they had previously been infected and shown some development of immunity (judged by faecal egg counts). The experiment was carried out in two parts as in the following schedule, with adult females in Part 1), and an adult male rabbit in Part 2).
Part Larval Dose Duration of Infection
(no.) (days to p.m.)
1) 25,000* 19
2) 25,000+ 3

* preceded by 9 doses of 25,000 larvae spread over 4 months,
total 250,000 infective larvae
+ preceded by 50 doses of 500 larvae spread over 12 months,
total 50,000 infective larvae

In addition to the small intestine, the stomach, lungs, and liver were also examined for the presence of larvae.

Results

Inhibited larvae were present in the small intestine and in the stomach of each rabbit, as shown in Table 6. No larvae were found in other organs. Only 1.5% of the third-stage larvae recovered in Part 2) were in the stomach, but 38.9% and 17.7% were in the stomach of the adult female rabbits killed at day 19.

These larvae did not differ in appearance from late third-stage larvae as recovered in Experiment 1. The appearance of an arrested larva is shown in the Frontispiece.

Although stored infective larvae were not used here to induce arrest, reference should be made to the results of Experiment 7, where up to 22.8% of the larval dose was recovered at 21 days as inhibited third-stage larvae in infections from larvae stored at 4°C for 14 weeks.

Part 2) was the sole occasion in this study when examination for inhibited larvae was made in less than the pre-patent period since the last dose of infective larvae. From the results of Experiment 1 a separation between arrested larvae, and developing larvae in the early fourth-stage, occurred by about day 3. However in the present experiment the expected proportion of developing
TABLE 6

EXPERIMENT 3

Recovery* of T. retortaeformis Larvae Inhibited in Development in Rabbits

<table>
<thead>
<tr>
<th>DURATION OF INFECTION (days)</th>
<th>TOTAL NEMATODES (No.)</th>
<th>L₂/ADULT (No.)</th>
<th>L₄ (No.)</th>
<th>L₃ (No.)</th>
<th>L₃ (% all doses)</th>
<th>stomach+ (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>8,160</td>
<td>0</td>
<td>0</td>
<td>8,160</td>
<td>16.3</td>
<td>120</td>
</tr>
<tr>
<td>19</td>
<td>720</td>
<td>0</td>
<td>0</td>
<td>720</td>
<td>0.3</td>
<td>280</td>
</tr>
<tr>
<td>19</td>
<td>5,960</td>
<td>1,220</td>
<td>0</td>
<td>4,740</td>
<td>1.9</td>
<td>840</td>
</tr>
</tbody>
</table>

* Special experimental conditions were imposed to induce inhibition of development.

+ All other nematodes were recovered from the small intestine.
larvae in the fourth-stage was not present at this time, and it is possible that the whole population was retarded rather than arrested in development.

(ii) Intermediate Development and Patent Infection (up to about 12 weeks)

EXPERIMENT 4 ... EXAMINATION OF PATENCY WITH DIFFERING LEVELS OF LARVAL DOSE

Plan

The experiment was carried out in twenty rabbits, with graded levels of *T. retortaeformis* infection as shown for the five groups in the following schedule. (The infective dose was varied around 2,500 larvae, which in pilot studies have produced a moderate level of infection with appreciable levels of egg production by the nematode population, and which was tolerated by the host.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Larval Dose (no.)</th>
<th>Duration of Infection (weeks observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>625</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>1,250</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>2,500</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>5,000</td>
<td>&quot;</td>
</tr>
<tr>
<td>5</td>
<td>10,000</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Each group consisted of four rabbits in separate blocks, (a), (b), (c), and (d). Each block was a uniform batch of young male rabbits, and was infected on a separate occasion with a different batch of larvae.

Patency was measured by weekly egg counts. For the first batch of rabbits (Block (a)) faecal samples could not be collected after week 7.
**EXPERIMENT 4**

Weekly Egg Counts from *T. retortaeformis* Infections at Different Levels of Infective Larvae (for four batches of infective larvae and of young male rabbits)

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose Level of Infective Larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>625</td>
</tr>
<tr>
<td>2</td>
<td>1,250</td>
</tr>
<tr>
<td>3</td>
<td>2,500</td>
</tr>
<tr>
<td>4</td>
<td>5,000</td>
</tr>
<tr>
<td>5</td>
<td>10,000</td>
</tr>
</tbody>
</table>
Results of the study are shown for each block in Figure 1. Although the results are not completely comparable, some conclusions can still be drawn. The graphs show the number of eggs per gram of faeces over a period of weeks. The data suggests a rapid increase in egg count followed by a decline in all groups. Further analysis is needed to determine the exact reasons for these patterns.
Results

The results are shown for each block in Figure 3, plotted on a logarithmic scale. Although the counts are presented as eggs per gram, they were comparable per day within blocks, as it was found that young rabbits in a batch together passed similar amounts of faeces, normally about 120 gms per day.

It was apparent from the figure that taking means for each larval dose level group would not have been useful. (Even within comparable patterns, taking means for each time of sampling has disadvantages, for example, a rapid fall in egg count may occur around say days 33 to 38, but while it may be apparent in some samples collected at week 7, in others it may not be detected until week 8.) Therefore the overall pattern assumed more importance than group means.

In the results there were greater differences within larval dose level groups than expected. (Work carried out since this experiment was designed has shown that the establishment and behaviour of an infection is largely dependent on the physiology of the pre-parasitic larvae (Section B(i)). The history of the infective larvae used here has been given in Table 2 in the Methods.) For example, similar egg count patterns were exhibited from a larval dose of 1,250 in Block (b) (infective larvae cultured 7 days at 20°C) and from a larval dose of 625 in Block (c) (12 days at 20°C). It is likely that different proportions of the infective dose became established from the different batches of larvae, so that there were similar numbers of adult nematodes in the above example.

However, two other clear results were shown by this experiment. Infections from lower dose levels were slower to reach peak egg production, but maintained an appreciable level much longer, than the infections from higher dose levels.
Secondly, certain features were repetitive. Nearly all the rabbits showed the first peak egg count at week 2 or week 3, and subsequent peaks occurred at weeks 5 or 6, and later at weeks 8 or 9. Negative egg counts occurred at weeks 4 or 5, and at weeks 7 or 8.

In order to examine this apparent cyclic phenomenon, weekly egg counts from 51 young male rabbits infected with *T. retortaeformis* were examined, and the times of peaks and troughs noted. Variations in both were statistically highly significant. The findings are shown in Figure 4. Some of the rabbits were killed during the period shown, with 12 remaining at 15 weeks, so the results are illustrated as the proportion of egg counts made at a particular week.

A distinct rhythm of egg counts emerged in Figure 4, with a frequency of peaks around 3 weeks. The irregularities, or overlapping of the occurrence of some peaks and troughs, shown in the histogram, would result if the frequency was not an exact multiple of the weekly sampling period. Further, the rhythm would be influenced by certain reactions, of which three were observed when assembling the results from all the rabbits.

Firstly, the initial peak egg count was delayed for about 2 weeks when the infective larvae were administered in multiple doses. Secondly, in a batch of rabbits, the timing of the first peak, in relation to the start of the infection, may be different to other batches. This is illustrated in Figure 3, where the timing in the rabbits of Block (a) seemed to be a sampling period behind that in the other rabbits.

Thirdly, a high magnitude or steep rate of increase of egg counts in an infection may precede a fairly precipitate fall which terminated the high count (and thus showed a peak) earlier than seen in the general pattern (and these were reflected two samplings later by a zero egg count). This brought the occurrence of some troughs closer to the preceding peaks. This effect
appeared less marked each cycle as the infection progressed. In Figure 3 this "displacement" is illustrated by the rabbits which showed a peak egg count at week 2 and a zero count at week 4, or those which showed a peak at week 5 and zero at week 7. The possible implication of a threshold parasite population influencing the rhythm of egg counts is mentioned in the Discussion.

With the larval dose levels up to 10,000 used in young male rabbits there was no evidence of inhibited larvae being present to contribute to a later maturation of nematodes and rise in egg counts.

From a consideration of the overall patterns of egg counts, it became apparent that egg production in susceptible hosts could be described by a finite number of basic patterns, which of course may merge by slight variations in magnitude. An attempt has been made in Figure 5 to show these as seven typical graphs, plotted on a logarithmic scale. The approximate number of nematodes present for each pattern could be predicted from the post mortem nematode counts carried out during these studies.

It seemed that the more precipitous falls were associated with a loss of the number of nematodes present. If this left a low number of adults (only a few hundred) remaining, the infection appeared to carry on as for the first or second pattern. If a severe fall was not precipitated, then a moderate number of adults (up to 2,000 or so) may still have been found after a couple of months.

Examples of the seven typical patterns described above are illustrated in Figure 3, excepting for the last, with a fatal parasite burden. (In Figure 3, one egg count pattern appeared anomalous: in the rabbit from Block (d) dosed with 1,250 infective larvae, the level of nematodes established appeared to be uncharacteristically low.)
EXPERIMENT 4

Incidence of Peaks and Troughs in Weekly Egg Counts from T. retortaeformis Infections
(in young male rabbits with varying doses of infective larvae)

Deviations in Peaks: $\chi^2_{(13)} = 61.65$ **

Deviations in Troughs: $\chi^2_{(13)} = 142.51$ **

(The deviations approach a regular periodicity. The interval of best fit is approx. 22 days between maxima.)

** $p \leq 0.01$
The proportions of eggs from different sources are depicted in the bar chart. The chart illustrates the incidence of feature in all egg counts against the duration of infection in weeks. The chart shows the distribution of peaks and troughs over the weeks of infection.
Figure 5

EXPERIMENT 4

Basic Patterns Selected as Typical of the Course of Weekly Egg Counts from *T. retortaeformis* Infections

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Estimated Level of Adult Nematodes Established (present at week 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>one</td>
<td>very low (less than 500)</td>
</tr>
<tr>
<td>two</td>
<td>low (less than 1,000)</td>
</tr>
<tr>
<td>three</td>
<td>moderate - around respective threshold in host</td>
</tr>
<tr>
<td>four</td>
<td></td>
</tr>
<tr>
<td>five</td>
<td></td>
</tr>
<tr>
<td>six</td>
<td>high (more than 5,000)</td>
</tr>
<tr>
<td>seven</td>
<td>very high (more than 10,000) - fatal</td>
</tr>
</tbody>
</table>
Part 3 - Comparative with Resistant Rabbits

The sites of eggs from two resistant rabbits, that showed transient egg counts (Table 9) 5 months after initially infected, were compared with eggs at the threshold that had been given single doses of larvae.

Pattern
one
two
three
four
five
six
seven

\[
\text{FOGGS PER GRAM OF FAECE}
\]

\[
(<10, 100, 1,000, 10,000)
\]

Days
1
2
3
4
5
6
7
8
9
10

\[
\text{DURATION OF INFECTION (weeks)}
\]

Many different comparisons were made in the course of this work, with measurements of 1,567 eggs made concurrently with a flotation egg count. The results here are based on the first ten eggs found in each faecal sample. The five parts listed show the association found with the stated factors.
EXPERIMENT 5 ... EXAMINATION OF CHANGES IN DIMENSIONS OF EGGS

Plan

In this experiment possible sources of variation in *T. retortaeformis* egg sizes were studied. Five parts are reported as follows.

Part 1) Comparison of Larval Dose Levels (2 replications)
The sizes of eggs were compared at week 5 between infections from five levels of infective larvae, progressing geometrically from 625 to 10,000.

Part 2) Comparison of Stages of Infection
The sizes of eggs were compared between week 5 and week 20 of the same infections, in three rabbits.

Part 3) Comparison with Resistant Rabbits
The sizes of eggs from two resistant rabbits, that showed transient egg counts (Table 9) 5 months after initially infected, were compared with eggs at the same duration of infection in young male rabbits that had received single doses of larvae.

Part 4) Progressive Comparison over Course of Infection (4 replications)
The sizes of eggs were determined weekly in four rabbits up to 12 weeks after infection.

Part 5) Progressive Comparison at Start of Patency
The sizes of eggs were determined every 2 days in three rabbits from the beginning of patency up to 30 days after infection.

Many different comparisons were made in the course of this work, with measurements of 1,567 eggs made concurrently with a flotation egg count. The reports here are based on the first ten eggs found in each faecal sample. The five parts listed show the association found with the stated factors.
Experiment 5

Scatter Distribution of the Dimensions of 200 T. retortaeformis Eggs (passed in faeces of various infected rabbits)

<table>
<thead>
<tr>
<th>Median values:</th>
<th>Length</th>
<th>86 µ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Width</td>
<td>47 µ</td>
</tr>
<tr>
<td></td>
<td>Volume</td>
<td>100 µ³ (x 10⁻³)</td>
</tr>
</tbody>
</table>
Preliminary assessments were made on three properties for each sample: mean egg volume, incidence of large eggs (on a volume basis), and proportion of eggs with small dimensions (width and length). Conditions of a rhythmic phenomenon in egg production, with the possibility of overlapping waves, in the sample could contain more than one population of eggs; possibly each with a different distribution of egg sizes, and show changes from one to another.

Therefore, the size observations were presented as the incidence of eggs in each sample greater than a given volume. The size selected was that for which approximately half of all eggs are greater, and half of all eggs less, as this is the most valid value to select for rational frequency testing. The median volume cut-off was found to be 100 thousand cubic micrometers; hence, the size distribution procedure was used to identify the different volume classes. Consequently, the volume size classes were reported on the scatterplot in the form of a factorial design.

As the beginning of the patent period, the length small but long eggs (approximately 85 \mu - 95 \mu) were
Preliminary assessments were made on three properties for each sample: mean egg volume; incidence of large eggs (on a volume basis); and proportion of eggs with small dimensions (width and length). Under conditions of a rhythmic phenomenon in egg production, with the possibility of overlapping waves, any one sample could contain more than one population of eggs, possibly each with a different distribution of egg sizes. Under this condition a mean value will not provide a valid description of a sample. Incidence figures will separate two populations of egg sizes, and show changes from one to another.

Therefore, the size observations were presented as the incidence of eggs in each sample greater than a given volume. The size selected was that for which approximately half of all eggs are greater, and half of all eggs less, as this is the most valid value to select for statistical frequency testing. The median volume to be used as a cut-off was found to be 100 thousand cubic µ (100,000 µ³).

(Similarly, the median dimensions provided a useful criterion for preliminary assessment. These were a width of 47 µ, and length of 86 µ. Both values were used to define for a sample the incidence of small eggs, classified as not exceeding either median dimension.)

In Figure 6 the distribution for the dimensions of 200 eggs from different samples is shown, and the volumes indicated, as well as the ratio between dimensions (length/width) which is a useful subjective criterion.

Results

It was found, from the measurements assembled in comprehensive tables and graphs, that the dimensions of eggs changed during the course of an infection, and interacted with progressive rises and falls in egg counts. This progress was influenced by such factors as larval dose, as outlined in Experiment 4.

At the beginning of the patent period, transient small but long eggs (approximately 85 µ - 95 µ) were
passed for the first few days, and shorter eggs (75 µ - 85 µ) appeared within 10 days. Early in the patent period, the width of eggs tended to increase (from 40 µ - 45 µ, to 45 µ - 50 µ), followed by an increase in length while not necessarily maintaining the increased width. Very wide eggs (more than 50 µ) did occur later, but the timing of their appearance seemed to be influenced by the course the infection was taking. It should be noted from the formula for the volume of a spheroid that small increases in width may have a significant effect on volume, while an increase in length alone may not be reflected by an appreciable increase in volume (Figure 6).

The results for Parts 1) to 5) are given for simplicity as the incidence of large eggs (volume greater than the median) in the sample. A proportion of 40% to 60% proved a strong indication of heterogeneity, indicating that two populations of eggs were present, when found subsequent to about 3 weeks after infection when the parasite population had become established. In the results given, all differences of 50% between proportions are statistically significant (p < 0.05) as calculated by the chi-square test, and differences of 60% are significant at the p < 0.01 level.

The results for Part 1) and Part 2), given in Table 7 and Table 8, show that egg size was not related per se to larval dose level, stage of infection, or the egg count. In Table 9, it is seen for Part 3) that there was a prima facie case for associating small eggs with resistance. (The experiment has not examined a causal relationship of immunity with egg size, and the result seen here may bear a relation to a new population of nematodes in the resistant rabbits.)

The results of Parts 4) and 5), presented in Figures 7 and 8, illustrate a relationship between egg size and the course of an infection. Initially in the patent period only small eggs were passed, with large eggs gradually appearing, until around week 3 of infection 50%
### TABLE 7

**EXPERIMENT 5**

Incidence of Large *T. retortaeformis* Eggs* from Infections with Different Doses of Larvae
(in faecal samples taken 5 weeks after infection of rabbits)

<table>
<thead>
<tr>
<th>LARVAL DOSE (No.)</th>
<th>1st replication</th>
<th>2nd replication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGG COUNT (e.p.g.)*</td>
<td>LARGE EGGS (%)*</td>
</tr>
<tr>
<td>625</td>
<td>250</td>
<td>40</td>
</tr>
<tr>
<td>1,250</td>
<td>1,700</td>
<td>60</td>
</tr>
<tr>
<td>2,500</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>5,000</td>
<td>850</td>
<td>70</td>
</tr>
<tr>
<td>10,000</td>
<td>2,950</td>
<td>40</td>
</tr>
</tbody>
</table>

### TABLE 8

**EXPERIMENT 5**

Incidence of Large *T. retortaeformis* Eggs* at Intermediate (week 5) and Late (week 20) Stages of Infection
(in faecal samples taken from the same rabbits)

<table>
<thead>
<tr>
<th>LARVAL DOSE (No.)</th>
<th>Week 5</th>
<th>Week 20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGG COUNT (e.p.g.)*</td>
<td>LARGE EGGS (%)*</td>
</tr>
<tr>
<td>625</td>
<td>250</td>
<td>40</td>
</tr>
<tr>
<td>1,250</td>
<td>1,700</td>
<td>60</td>
</tr>
<tr>
<td>2,500</td>
<td>150</td>
<td>50</td>
</tr>
</tbody>
</table>

* Incidence is the proportion of eggs in sample greater than 100,000 µ³ volume. A difference of 50% is required to be statistically significant (p ≤ 0.05).

+ Egg count in sample expressed as eggs per gram of faeces.
TABLE 9

EXPERIMENT 5

Incidence of Large *T. retortaeformis* Eggs* from Resistant and Susceptible Rabbits
(in faecal samples taken 5 months after the first infection)

LARVAL DOSE | EGG COUNTS* | LARGE EGGS | MEAN EGG VOLUME
(No.) | week-3 (epg)* | week-2 (epg)* | week-1 (epg)* | week 0 (epg)* | week+1 (epg)* | week+2 (epg)* | (%)* | ($\mu^3 \times 10^{-3}$)
--- | --- | --- | --- | --- | --- | --- | --- | ---
Susceptible Rabbits
625 | 150 | 0 | 200 | 350 | - | - | 70 | 99.3
1,250 | 650 | 500 | 950 | 500 | - | - | 80 | 106.3
2,500 | 200 | 250 | 100 | 150 | - | - | 60 | 101.8
Resistant Rabbits
8x25,000 | 0 | 0 | 0 | 550 | 0 | 0 | 0 | 88.9
8x25,000 | 0 | 0 | 0 | 650 | 0 | 0 | 10 | 95.8

* Incidence is the proportion of eggs in a sample greater than 100,000 $\mu^3$ volume. A difference of 50% is statistically significant ($p<0.05$).

* Weekly counts before and after egg measurements (week 0).

* Egg count in samples expressed as eggs per gram of faeces.

- Not counted.
EXPERIMENT 5

Changes in the Size of T. retortaeformis Eggs Produced during the Progression of Infections in Rabbits (compared with weekly counts of eggs in faeces)

A and B were adult, and C and D were juvenile, male rabbits.
Each rabbit was infected with 2,500 larvae of the same batch.

* The proportion of eggs greater than a volume of 100,000 µ³

A difference of 50% or more is significant (*p* < 0.05).
INCIDENCE OF LARGE EGGS (%) and NUMBER OF EGGS PER GRAM OF FAECES (log. scale)

DURATION OF INFECTION (weeks)

A

B

C

D

insufficient sample
EXPERIMENT 5

Changes in the Size of T. retortaeformis Eggs Passed at the Start of the Patent Period in Rabbits (compared with counts of eggs in faeces every 2 days)

Young male rabbits were infected with larvae of the same batch,
A received 625 larvae, B received 2,500 larvae, and C received 10,000 larvae.

* The proportion of eggs greater than a volume of 100,000 µ³.
  A difference of 50% or more is significant (p ≤ 0.05).
The rate of increase in egg counts varied, often accompanied by a reappearance of large eggs, which may be interpreted as two populations. If the finding was not absolute, then there was a heterogeneous population of large and small eggs, which may be interpreted as two populations.
or more of the eggs had a volume greater than 100 thousand \( \mu^3 \). The rate of increase in egg size was proportional to the rate of increase in egg counts. The incidence of large eggs reached a peak AFTER a peak in egg counts.

The incidence of large eggs declined, following a decline in egg counts, when a new rise in egg counts commenced accompanied by a reappearance of small eggs, and the cycle began again. If the fall in egg counts was not absolute, then with the start of another rise, there was a heterogeneous population of large and small eggs, which may be interpreted as two populations.

Reference should also be made to Experiment 11, where heterogenous populations of egg sizes are illustrated in Figure 23.

(iii) **Long Term Course and Patent Development** (up to about 12 months)

**EXPERIMENT 6** . . . EXAMINATION OF PATENCY WITH PROLONGED INFECTIONS

By comparison, in Group 2 the worm population was also maintained, but with a rhythmic rise and fall of egg counts which reached slightly higher levels. Despite each peak being higher, no overwhelming increase in egg counts occurred, and the recovery of inhibited larvae in other experiments with young male rabbits...

**Plan**

The experiment was carried out in two groups, infecting young male rabbits with varying doses of *T. retortaeformis* larvae, as shown in the schedule, on the next page.
<table>
<thead>
<tr>
<th>Group</th>
<th>Larval Dose (no.)</th>
<th>Time at which Dose given (weeks after first dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early Intermittent Larval Doses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(a) 100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3,000</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4,000</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>(b) 1,000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Continual Regular Larval Doses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(a) 500 each week</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td></td>
</tr>
</tbody>
</table>

Patency was measured by weekly egg counts for over a year. Larval doses of the magnitude used in Group 1 have not resulted in the recovery of inhibited larvae in other observations with young male rabbits.

**Results**

The results are summarised in Figure 9. From Group 1 it is evident that a patent nematode population may survive for at least 55 weeks without continual reinfection of the host. However, the egg production was low, and fluctuated around the minimum detectable level.

By comparison, in Group 2 a patent worm population was also maintained, but with a rhythmic rise and fall of egg counts which reached slightly higher levels. Despite each rabbit in Group 2 being given 25,000 infective larvae over this period, it was exceptional for negative egg counts to be recorded for more than two consecutive samples, so the rabbits apparently did not develop a solid immunity, or show a later clear resistance attributable to age.
EXPERIMENT 6

Weekly Egg Counts from *T. retortaeformis* Infections of More than 1 Year Duration in Rabbits:

A Doses of infective larvae given early in course of infection

B Doses of infective larvae given continually throughout infection
Influence of certain factors on the course of infection

In other work, it was found that infective larvas, recovered at 7 days from fecal cultures, showed an abnormally low infectivity to rabbits. As a consequence of this finding, experiment 7 was designed to include a test of the infectivity of infective larvas from the day after they first appeared in fecal culture. The effect of holding larvas for
SECTION B

INFLUENCE OF CERTAIN FACTORS ON THE COURSE OF INFECTION

OBJECTS

The object of this section was to determine reactions, of *T. retortaeformis* infections in laboratory rabbits, in response to selected treatments of the parasite or the host. Responses to female host reproduction are reported separately, in Section C.

Two single factor manipulations of infective larvae were examined, i.e. variations in the time held at two temperatures. These were selected as they are common laboratory variables in experimentation, although the time for which larvae are held before infection is usually not reported. Effects of holding larvae at a given temperature have normally been quantitated for trichostrongylid nematodes only by examining survival, and not by examining the effect on subsequent infectivity and parasitic development in the natural host.

The effect of holding infective larvae for a period at 4°C was investigated in Experiment 7. It has been shown that at this temperature there may be very little fall in survival rate for several months, and it is commonly used as a storage temperature in the laboratory. An infective dose of 25,000 larvae was used in Experiment 7, and the effect of cold storage of larvae was examined again with 2,500 larvae in Appendix II.

In other work, it was found that fresh *Trichostrongylus retortaeformis* infective larvae, recovered at 5 days from faecal cultures, showed an abnormally low infectivity in rabbits. As a consequence of this finding, Experiment 8 was designed to include a test of the infectivity of infective larvae from the day after they first appeared in faecal culture. The effect of holding larvae for
different periods at 20°C, the culture temperature, was investigated. Aging is used to refer to the procedure where larvae are held at an ambient temperature such as this.

Both sexes of young rabbits were evenly distributed in the groups for Experiment 8, and thus the factor of juvenile host sex was examined.

The course of infections in adult female rabbits was examined in two concomitant experiments, to provide information on which to prepare the designs for experiments in Section C. In Experiment 9 the progress was followed every 2 weeks, and in Experiment 10 the nematode population was sampled at two intervals.

Attempts were made to vary the environment of the parasite in the host by comparing two types of food, and by comparing infections between rabbits which had had previous exposure to infection and rabbits with primary infections. Further, the effect of administering the infective larvae as a single versus a multiple dose was investigated in Experiment 10.

Rabbits were separated according to weight prior to infection, and higher weight and lower weight animals evenly distributed in the groups for Experiment 10, and thus the factor of host initial body weight was examined.

Different intervals of photoperiod were studied in the experiment reported in Appendix II.
(i) Variations in the Physiology of the Parasite and the Environment at the Infective Stage

EXPERIMENT 7 ... INFLUENCE OF STORING INFECTIVE LARVAE AT 4°C

Plan

A group of four rabbits was dosed with freshly harvested *T. retortaeformis* infective larvae as normally used, and another similar group with larvae from a batch that had been stored at 4°C after harvesting, as given in the following schedule.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cold Storage of Larvae (weeks)</th>
<th>Larval Dose (no.)</th>
<th>Duration of Infection (weeks to post mortem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>25,000</td>
<td>3*</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* 6 weeks for Replicate (4)

Identical faecal cultures had been carried out for both batches of infective larvae. History of the larvae is given in Table 2 in the Methods. It should be noted that the larval dose number refers to live larvae. A high larval dose level was used to allow the phenomenon of inhibited development to be expressed.

Male rabbits were used (all first infected as young animals at about 2 months of age), and formed four replicates in each group. Replicates (1) and (2) had been infected with doses of 5,000 larvae previous to this experiment, as indicated in the next schedule. This
means that the rabbits were also different ages for this experiment.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Age of Rabbit (months)</th>
<th>Previous Infections (no.) (months before this infection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>6</td>
<td>2 : 4,1</td>
</tr>
<tr>
<td>(2)</td>
<td>4</td>
<td>1 : 2</td>
</tr>
<tr>
<td>(3)</td>
<td>2</td>
<td>0 : -</td>
</tr>
<tr>
<td>(4)</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Therefore Experiment 7 represented tertiary, secondary, and primary infections equally distributed in the groups. A fifth replicate, in which the rabbits were not killed, is shown for egg count results only. Replicate (5) was adult female rabbits with no previous infection.

Results

Weekly egg counts, and post mortem dissection nematode counts, are given in Tables 10 and 11. (For the rabbits with previous infections the egg counts for the 3 weeks proceeding this infection are shown.) Although the numbers of adult nematodes recovered in both groups are comparable (Figure 10), delayed development is apparent in the infections from stored larvae (Group 2), as evidenced by a slower rise in egg counts, and because a greater number of immature developing forms, fourth-stage larvae, persisted in the gut at the time of post mortem dissection.

It is notable that inhibited third-stage larvae were more apparent from stored larvae (Group 2) than from fresh larvae (Group 1). Even with so few animals and the great variation between replicates, this difference between groups is statistically significant (Figure 11).

The history of previous infections influenced the results, and created the greatest source of variation in the number of nematodes recovered (Appendix III). The rabbits receiving tertiary infections (Replicate (1)) were resistant, as no adult nematodes became established
(a statistically significant difference to the others, Figure 10), only inhibited third-stage larvae being recovered from the infection with stored larvae. In the rabbits receiving secondary infections (Replicate (2)) there were less adults and less eggs passed than in the primary infections, and also a significantly greater proportion of the larval dose became inhibited (Figure 11).

The consequences for the host were an important effect of this infection with a high larval dose. All rabbits showed a drop in faecal output within 1 week of infection. For the older animals (Replicates (1) and (2)) spot checks showed this drop to be from a previous normal about 80 gms per day, to daily levels of 10 gms to 30 gms. One only of these rabbits, given fresh larvae, showed transient diarrhoea which occurred around the fifth day of infection. The younger rabbits (Replicates (3) and (4)) all suffered with diarrhoea, from about the tenth day of infection. In three of these four rabbits this was exhibited as the classical disease of Intestinal Trichostrongyloysis referred to as "Black Scours".

Live body weight changes given in Table 12 illustrate a difference in pathogenicity of the parasite related to cold storage of infective larvae. Whereas all rabbits lost weight in the first week with infections by fresh larvae (Group 1), all but one of the rabbits infected by stored larvae (Group 2) continued to gain weight. This supports the finding of delayed development in infections from stored larvae. The older rabbit that showed transient diarrhoea lost considerably more weight in the first week than the other animals.

After 1 week the effect of the parasite was associated with the previous history of infections and age of the host. The older rabbits receiving secondary or tertiary infections recovered, but all the younger rabbits with a primary infection showed a visible loss of body weight, which amounted to approximately 1.5% of their total weight lost per day. Marked loss of appetite was noted for these rabbits, as observed by lack of interest in feeding and drop in faecal output.
## EXPERIMENT 7

**Table 10**

Egg Counts from *T. retortaeformis* Infections with Fresh and Stored* Infective Larvae (in rabbits receiving a primary, secondary\(^a\), or tertiary\(^b\), infection)

<table>
<thead>
<tr>
<th>GROUP AND INFECTIVE LARVAE(^b)</th>
<th>REPLICATE AND EXPERIMENTAL INFECTION</th>
<th>EGG COUNTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 FRESH LARVAE</td>
<td>(1) Tertiary(^b)</td>
<td>week-3</td>
</tr>
<tr>
<td></td>
<td>(a) Secondary(^a)</td>
<td>(epg)(^+)</td>
</tr>
<tr>
<td></td>
<td>(3) Primary</td>
<td>(epg)(^+)</td>
</tr>
<tr>
<td></td>
<td>(4) Primary</td>
<td>(epg)(^+)</td>
</tr>
<tr>
<td></td>
<td>(5) Primary</td>
<td>(epg)(^+)</td>
</tr>
<tr>
<td>2 STORED* LARVAE</td>
<td>(1) Tertiary(^b)</td>
<td>week-2</td>
</tr>
<tr>
<td></td>
<td>(2) Secondary(^a)</td>
<td>(epg)(^+)</td>
</tr>
<tr>
<td></td>
<td>(3) Primary</td>
<td>(epg)(^+)</td>
</tr>
<tr>
<td></td>
<td>(4) Primary</td>
<td>(epg)(^+)</td>
</tr>
<tr>
<td></td>
<td>(5) Primary</td>
<td>(epg)(^+)</td>
</tr>
</tbody>
</table>

\(^*\) Stored larvae were held at 4°C for 14 weeks.

\(^a\) Rabbits were once previously infected with 5,000 larvae.

\(^b\) Rabbits were twice previously infected with 5,000 larvae.

\(^+\) Egg count in samples expressed as eggs per gram of faeces.

\(-\) Not counted, rabbits killed at week 3.

\(^f\) All rabbits received an experimental dose of 25,000 live infective larvae at week 0.
### TABLE 11

**Exhibit 7**

Recovery† of *T. retortaeformis* from Infections§
with Fresh and Stored* Infective Larvae
(in rabbits receiving a primary, secondary, or tertiary, infection)

<table>
<thead>
<tr>
<th>GROUP AND INFECTIVE LARVAE*</th>
<th>REPPLICATE AND EXPERIMENTAL INFECTION</th>
<th>TOTAL NEMATODES</th>
<th>ADULTS</th>
<th>L₄</th>
<th>L₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(No) (% dose)</td>
<td>(No) (% adults)</td>
<td>(No) (%) total</td>
<td>(%) total</td>
<td>(%) dose</td>
</tr>
<tr>
<td>1 FRESH LARVAE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)  Tertiaryb</td>
<td>0 -</td>
<td>0 -</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>(2)  Secondarya</td>
<td>6,300 25.2</td>
<td>1,860 57.0</td>
<td>180 (2.9%)</td>
<td>4,260 (67.6%)</td>
<td>17.0</td>
</tr>
<tr>
<td>(3)  Primary</td>
<td>17,200 68.8</td>
<td>16,260 59.8</td>
<td>540 (3.1%)</td>
<td>400 (2.3%)</td>
<td>1.6</td>
</tr>
<tr>
<td>(4)  Primary</td>
<td>18,420 73.7</td>
<td>18,420 53.0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>2 STORED* LARVAE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)  Tertiaryb</td>
<td>460 1.8</td>
<td>0 -</td>
<td>0</td>
<td>460 (100%)</td>
<td>1.8</td>
</tr>
<tr>
<td>(2)  Secondarya</td>
<td>6,820 27.3</td>
<td>1,060 50.9</td>
<td>60 (0.9%)</td>
<td>5,700 (83.6%)</td>
<td>22.8</td>
</tr>
<tr>
<td>(3)  Primary</td>
<td>17,260 69.0</td>
<td>14,780 56.3</td>
<td>1,000 (5.8%)</td>
<td>1,480 (8.6%)</td>
<td>5.9</td>
</tr>
<tr>
<td>(4)  Primary</td>
<td>14,600 58.4</td>
<td>13,640 51.0</td>
<td>240 (1.6%)</td>
<td>720 (4.9%)</td>
<td>2.9</td>
</tr>
</tbody>
</table>

+ Nematodes recovered at week 3 (Replicates (1), (2), (3)) or week 6 (Replicate (4)).
§ All rabbits received an experimental dose of 25,000 live infective larvae at week 0.
* Stored larvae were held at 4°C for 14 weeks.
a Rabbits were once previously infected with 5,000 larvae.
b Rabbits were twice previously infected with 5,000 larvae.

(N.B. Normal weight gains when fed laboratory diet of about 350 g per week for 3 month old rabbits; declining to about 23 g per week for 6 month old rabbits.)
TABLE 12

EXPERIMENT 7

Live Body Weight Changes of Rabbits
with *T. retortaeformis* Infections from Fresh and Stored Infective Larvae
(for rabbits receiving a primary, secondary, or tertiary infection)

<table>
<thead>
<tr>
<th>GROUP AND INFECTIVE LARVAE</th>
<th>REPLICATE AND AGE OF RABBITS</th>
<th>INITIAL BODY WEIGHT AT WEEK 0 (gms)</th>
<th>BODY WEIGHT CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>weeks 0-1 (gms per wk)</td>
</tr>
<tr>
<td>1 FRESH LARVAE</td>
<td>(1) 6 months&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3,070</td>
<td>-20</td>
</tr>
<tr>
<td></td>
<td>(2) 4 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2,730</td>
<td>-170</td>
</tr>
<tr>
<td></td>
<td>(3) 2 months</td>
<td>2,670</td>
<td>-40</td>
</tr>
<tr>
<td></td>
<td>(4) 2 months</td>
<td>2,680</td>
<td>-10</td>
</tr>
<tr>
<td>2 STORED* LARVAE</td>
<td>(1) 6 months&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2,950</td>
<td>-20</td>
</tr>
<tr>
<td></td>
<td>(2) 4 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2,830</td>
<td>+170</td>
</tr>
<tr>
<td></td>
<td>(3) 2 months</td>
<td>2,350</td>
<td>+60</td>
</tr>
<tr>
<td></td>
<td>(4) 2 months</td>
<td>2,290</td>
<td>+240</td>
</tr>
</tbody>
</table>

<sup>Ø</sup> All rabbits received an experimental dose of 25,000 live infective larvae at week 0.

<sup>*</sup> Stored larvae were held at 4°C for 14 weeks.

<sup>a</sup> Rabbits were once previously infected with 5,000 larvae.

<sup>b</sup> Rabbits were twice previously infected with 5,000 larvae.

(N.B. Normal weight gains when fed lucerne:
about 250 gms per week for 2 month old rabbit
declining to about 25 gms per week for 6 month old rabbit.)
EXPERIMENT 7

Geometric Mean Numbers of Adult *T. retortaeformis*
Recovered from Rabbits, Infected with Stored Larvae, and with Primary, Secondary, or Tertiary Infections
(Each rabbit received an experimental infection of 25,000 larvae)
(See Plan of Experiment for details)

Significant differences between two means:

* $p \leq 0.05$
** $p \leq 0.01$
STORAGE OF INFECTIVE LARVAE

![Graph showing no significant difference between storage intervals of 0 and 14 weeks.]

INTERVAL OF STORAGE (weeks)

MEAN NUMBER OF ADULTS (log. scale)

EXPERIMENTAL INFECTION

![Graph showing recovery at week 3 of infection.]

(TERT SEC PRIM)

(Recovery at week 3 of infection)
EXPERIMENT 7

Geometric Mean Numbers of Inhibited Third-stage T. retortaeformis Recovered from Rabbits, Infected with Stored Larvae, and with Primary, Secondary, or Tertiary Infections

(Each rabbit received an experimental infection of 25,000 larvae)

(see Plan of Experiment for details)

Significant differences between two means:

* $p \leq 0.05$

** $p \leq 0.01$
STORAGE OF INFECTIVE LARVAE

INTERVAL OF STORAGE (weeks)

MEAN NUMBER OF LARVAE

EXPERIMENTAL INFECTION

(Recovery at week 3 of infection)

The conditions for faecal culture were the same for all batches of infective larvae. Batches 1 to 4 were held in shallow water, as outlined in the Methods, after harvesting at 9 days. History of the larvae is given in Table 2 in the Methods. It should be noted that the larval dose number refers to live larvae.
Progressively the effects were greatest in the young rabbits infected with stored larvae (Group 2), where the infection was fatal. One rabbit was killed at week 3 in extremis, and another died at 6 weeks after infection. This suggested that the stored infective larvae were more pathogenic than the infective larvae which had never been exposed to cold, but more results are required to be conclusive.

EXPERIMENT 8 ... INFLUENCE OF AGING INFECTIVE LARVAE AT 20°C

Plan

Fresh T. retortaeformis infective larvae, after harvesting from faeces cultured at 20°C, were aged at the same temperature in five batches. The "ages" of the larvae, from collection of donor faeces to time of infection for each batch, were varied geometrically with two-fold increments. Groups of four experimental rabbits were infected at the same time with the respective batch of larvae, as shown in the following schedule.

<table>
<thead>
<tr>
<th>Batch of Larvae</th>
<th>Group of Rabbits</th>
<th>&quot;Age&quot; of Larvae (days)</th>
<th>Larval Dose (no.)</th>
<th>Duration of Infection (days to p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>2,500</td>
<td>11+</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>8</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>15</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>30</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>60</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

+ 5 days for Replicates (1) and (2)

The conditions for faecal culture were the same for all batches of infective larvae. Batches 3 to 5 were held in shallow water, as outlined in the Methods, after harvesting at 9 days. History of the larvae is given in Table 2 in the Methods. It should be noted that the larval dose number refers to live larvae.
A comparison of the respective exsheathing ability of infective larvae from each batch was conducted at the time of infection. The technique for physiological exsheathment was used as outlined in the Methods of Part III of this thesis. Duplicate tests were carried out.

A single batch of young rabbits, half of each sex, was equally distributed in the five groups. Within each group, half of each sex were killed at day 5, and the remainder at day 11, forming the replicates as shown in the next schedule.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Sex of Rabbit</th>
<th>Duration of Infection (days to post mortem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>male</td>
<td>5</td>
</tr>
<tr>
<td>(2)</td>
<td>female</td>
<td>&quot;</td>
</tr>
<tr>
<td>(3)</td>
<td>male</td>
<td>11</td>
</tr>
<tr>
<td>(4)</td>
<td>female</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Therefore three factors were evenly represented in the plan for this experiment: Age of infective larvae (groups); Sex of young host; and Duration of infection.

In addition to post mortem nematode counts from all rabbits, egg counts and nematode measurements were made for each group from the rabbits killed 11 days after infection.

Results

The rate of in vitro exsheathing for each batch of infective larvae is shown in Figure 12. There was an increase in the exsheathing rate throughout the period of aging.

The results of nematode counts and egg counts are presented in Table 13. No inhibited larvae were recovered from rabbits in this experiment. No effect of the infection was seen on the body weight gain of the rabbits during infection (Table 13).
EXPERIMENT 8

In Vitro Exsheathment of T. retortaeformis Infective Larvae Held at 20°C for Various Intervals
(see Plan of Experiment for details)

* The proportion of live larvae with cast sheaths 20 hours after pseudo-gastric stimulus.
INTERVAL FROM COLLECTION OF DONOR FAECES TO USE OF INFECTIVE LARVAE (days)
## TABLE 13

**EXPERIMENT 8**

Recovery of *T. retortaeformis*, Eggs Passed, and Live Body Weight Changes of Rabbits during Infections by Larvae Incubated and Held at 20°C for Different Intervals (for male and female juvenile rabbits receiving a primary infection)

<table>
<thead>
<tr>
<th>GROUP AND INCUBATION INTERVAL*</th>
<th>REPL.</th>
<th>HOST SEX</th>
<th>DAY OF INFECTION</th>
<th>BODY WEIGHT CHANGE DURING INFECTION (gms per day)</th>
<th>TOTAL NEMATODES (No.)</th>
<th>(mean no.)</th>
<th>(%) female</th>
<th>(%) total</th>
<th>EGGS PASSED (per day) per female (thous.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (4 days)</td>
<td>(1)</td>
<td>male</td>
<td>5</td>
<td>+5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>female</td>
<td></td>
<td>+18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>male</td>
<td></td>
<td>+18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>female</td>
<td></td>
<td>+33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2 (8 days)</td>
<td>(1)</td>
<td>male</td>
<td>5</td>
<td>+32</td>
<td>190</td>
<td>7.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>female</td>
<td></td>
<td>+31</td>
<td>710</td>
<td>28.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>male</td>
<td></td>
<td>+26</td>
<td>1,560</td>
<td>62.4</td>
<td>48.7</td>
<td>52</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>female</td>
<td></td>
<td>+28</td>
<td>1,540</td>
<td>61.6</td>
<td>48.1</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>3 (15 days)</td>
<td>(1)</td>
<td>male</td>
<td>5</td>
<td>+24</td>
<td>480</td>
<td>19.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>female</td>
<td></td>
<td>+32</td>
<td>990</td>
<td>39.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>male</td>
<td></td>
<td>+22</td>
<td>1,980</td>
<td>79.2</td>
<td>49.5</td>
<td>94</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>female</td>
<td></td>
<td>+16</td>
<td>2,130</td>
<td>85.2</td>
<td>50.7</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>4 (30 days)</td>
<td>(1)</td>
<td>male</td>
<td>5</td>
<td>+23</td>
<td>760</td>
<td>30.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>female</td>
<td></td>
<td>+29</td>
<td>1,200</td>
<td>48.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>male</td>
<td></td>
<td>+22</td>
<td>1,880</td>
<td>73.2</td>
<td>43.6</td>
<td>98</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>female</td>
<td></td>
<td>+18</td>
<td>2,360</td>
<td>94.4</td>
<td>50.8</td>
<td>52</td>
<td>43</td>
</tr>
<tr>
<td>5 (60 days)</td>
<td>(1)</td>
<td>male</td>
<td>5</td>
<td>+24</td>
<td>500</td>
<td>20.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>female</td>
<td></td>
<td>+11</td>
<td>560</td>
<td>22.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>male</td>
<td></td>
<td>+21</td>
<td>910</td>
<td>36.4</td>
<td>52.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>female</td>
<td></td>
<td>+26</td>
<td>980</td>
<td>39.2</td>
<td>53.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* All rabbits received a dose of 2,500 live infective larvae at day 0.

* Interval at 20°C from collection of donor faeces until day 0.
Effect of Age of Infective Larvae:

The geometric mean nematode recoveries for the five larval age groups are illustrated in Figure 13, with the significant difference between two means indicated. Each of the four replicates showed the same difference between groups. There was a very low recovery of nematodes from infections with the youngest larvae, followed by a sharp rise in infectivity with aging. This rise was statistically significant between groups up to larvae aged for 15 days. Infectivity fell after aging for 30 days, with a highly significant drop in recovery when larvae were used at 60 days aging.

Insufficient nematodes were detected from Group 1 to examine for other effects, so this group is not included in the further assessments reported.

Differences between groups in the rate of development of the parasite populations were not obvious, as shown by the proportion at each larval stage. However, of the nematodes recovered at 11 days after infection, 3% were still late fourth-stage in Group 5, and 1% were early fourth-stage in Group 2. There were no fourth-stage larvae recovered from the other groups at this time.

The frequency distributions of sizes of 80 nematodes from each group at day 11 are compared in Figure 14. Each frequency curve is drawn proportional to the number of nematodes present. Although the mean lengths did not appreciably differ, the most advanced adult females, over 8 mm in length, which were present in Groups 2 to 4, were not found in Group 5. The apparent bimodal distribution of female nematodes occurred in every rabbit, although to a lesser extent in the infections of Group 5. It was not apparent in the distribution of male nematodes.

The pattern of faecal egg counts was similar to that described for nematode counts, with the important exception that no eggs were found from the infections with larvae aged for 60 days (Group 5). Despite this,
eggs were present at day 11 in the oviducts of some female nematodes from all groups including Group 5. This suggests that, at least in the development to fecundity, there was either some delay in the rate of development of all females, or the most rapidly developing females were missing, in the parasite populations derived from the infective larvae aged for the longest period.

Effect of Sex of the Young Host:

The recovery of nematodes from the female rabbits was consistently greater than from the male rabbits, and the difference between means of each sex was statistically significant (Figure 15). The effect was more marked at 5 days after infection than at 11 days, and this interaction of duration of infection with host sex provided a source of variation in the total nematode count as seen in the analysis (Appendix III). There was no apparent difference in the proportion of adult nematodes that were females.

On the other hand, there were always more eggs in the faeces of the young male rabbits than of the females. The difference in the geometric means for the animals with positive egg counts (Groups 2 - 4) was statistically highly significant. When the egg production by female nematodes was considered, the higher level in male rabbits than in female rabbits was particularly striking (Figure 15), and the difference between the geometric means of the host sexes was statistically highly significant (Appendix III).

Effect of Duration of Infection:

At 5 days after infection, fewer nematodes were recovered than at 11 days in all larval aging groups and for both host sexes. Therefore it appears that some of the developing parasitic larvae were missing a few days after infection, similar to the phenomenon reported in
Figure 13

EXPERIMENT 8

Geometric Mean Numbers of *T. retortaeformis* Recovered from Rabbits Infected with Larvae Held at 20°C for Various Intervals.
(Each rabbit received 2,500 infective larvae)
(see Plan of Experiment for details)

Significant differences between two means:

* p ≤ 0.05
** p ≤ 0.01
INTERVAL FROM COLLECTION OF DONOR FAECES TO USE OF INFECTIVE LARVAE (days)

MEAN NUMBER OF NEMATODES (log scale)

(groups 2 – 5)

(all groups)
EXPERIMENT 8

Length Distribution of *T. retortaeformis* Adults Recovered 11 Days after Infection of Rabbits Dosed with Larvae Held at 20°C for Various Intervals

(Each rabbit received 2,500 infective larvae)

(See Plan of Experiment for details).

<table>
<thead>
<tr>
<th>Group</th>
<th>Interval from Collection of Donor Faeces to Use of Infective Larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8 days</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
</tr>
</tbody>
</table>

The length of the nematode is plotted with a moving average for intervals of 0.25 mm.
EXPERIMENT 8

(i) Geometric Mean Numbers\(^a\) of, and (ii) Egg Production by Female\(^b\), *T. retortaformis* Recovered from Juvenile Male and Female Rabbits

(Each rabbit received 2,500 infective larvae.)

(See Plan of Experiment for details.)

\(^a\) recovered at days 5 and 11 (Groups 2 - 5)
\(^b\) recovered at day 11 (Groups 2 - 4)

**Significant differences between two means:**

* \( p \leq 0.05 \)
** \( p \leq 0.01 \)
Experiment i. All larvae recovered in each rabbit were fourth-stage. "Larval" and "Juvenile" were synonymous with fourth-stage larvae. Larvae had reached their mature stage between fourth-stage larvae and fifth-stage juveniles by 5 days. But none were recognized as adults. Sex was very early fourth-stage larvae. After the third analysis found that although 15% of the larvae were still sexually undifferentiated even at that stage.

(ii) Variation in the infection of the host and the environment of the parasitic stages

**Graphs**

**Experiment ii.** Progressive development of an infection in the adult female rabbit. The influence of two factors: previous infection and diet plan. The progressive development of *F. heterosomia* infections was examined in five groups as shown in the following schedule, with each 6-month-old female rabbits. Group 1: Normal Diet. Group 2: Normal Diet + 10% (w/v) *F. heterosomia* infective larvae. This infection was allowed to reach maturity, and checked by rectal egg count after 19 days when all dosed rabbits showed a patent infection. On May 30, this infection was terminated when all animals were
Experiment 1. All larvae recovered in each rabbit were fourth-stage. A proportion of the *T. retortaeformis* larvae had reached the moult between fourth-stage larvae and fifth-stage young adult by 5 days, but none were recognised as having moulted. Nor were very early fourth-stage larvae just after the third ecdysis found, although 15% of the larvae present were still sexually undifferentiated early fourth-stage.

(ii) Variations in the Physiology of the Host and the Environment of the Parasitic Stages

EXPERIMENT 9 . . . PROGRESSIVE DEVELOPMENT OF AN INFECTION IN THE ADULT FEMALE HOST, AND INFLUENCE OF TWO FACTORS: PREVIOUS INFECTION AND DIET

Plan

The progressive development of *T. retortaeformis* infections was examined in five groups as shown in the following schedule, each of four 6 month old female rabbits.

<table>
<thead>
<tr>
<th>Group</th>
<th>Larval Dose (no.)</th>
<th>Duration of Infection (weeks to p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,500</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>8</td>
</tr>
</tbody>
</table>

At 1 month prior to the experimental infection, half the rabbits were dosed with 1,000 *T. retortaeformis* infective larvae. This infection was allowed to reach maturity, and checked by faecal egg count after 19 days, when all dosed rabbits showed a patent infection. On day 20 this infection was terminated when all animals were
given the anthelmintic laevo-tetramisole ("Nilverm" - Imperial Chemical Industries) sub-cutaneously at the level of 15 mg per kg for each respective rabbit. Four days later all rabbits were treated with l-tetramisole again at the level of 22.5 mg per kg. (The standard therapeutic dose rate is 7.5 mg per kg.) Thus half the rabbits had had a previous infection, and received a secondary experimental infection, and the remainder a primary infection.

Three days before the previous infection just described, the rabbits were allocated equally to two different diets, laboratory pellets and lucerne chaff, as described in the Methods.

Both of these factors, previous infection and diet, were represented equally in each of the five groups, according to the replicate as given in the next schedule.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Infection</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>Primary</td>
<td>Lucerne Chaff</td>
</tr>
<tr>
<td>(2)</td>
<td>&quot;</td>
<td>Laboratory Pellets</td>
</tr>
<tr>
<td>(3)</td>
<td>Secondary</td>
<td>Lucerne Chaff</td>
</tr>
<tr>
<td>(4)</td>
<td>&quot;</td>
<td>Laboratory Pellets</td>
</tr>
</tbody>
</table>

The parasite populations were examined post mortem at the five intervals as shown by groups, and egg counts were made weekly.

**Results**

The numbers of nematodes and egg counts at post mortem examination are given in Table 14, and the geometric means for Groups 2 - 5, and for both factors examined, are illustrated in Figures 16 and 17. The weekly egg counts are presented later in Figure 18, with those of Experiment 10. There were no recoveries of early fourth-stage larvae, and insignificant recovery overall of inhibited third-stage larvae, only 1.04% of the total nematode population in one rabbit. The proportion of females in the adult nematode population did not appear to be influenced by any of the factors considered.
Effect of Duration of Infection:

Although there was a decline in the geometric mean number of nematodes recovered from the rabbits killed during the course of the infection (Figure 16), this was largely influenced by the loss of infection in two rabbits in which less than 500 nematodes persisted at the time of post mortem, and the decline is not statistically significant for the experiment. In one rabbit no nematodes were recovered at week 4, and in another there were only 80 stunted fifth-stage (immature adult) nematodes present at week 8. Both these rabbits had had positive egg counts.

In this experiment a population around 1,270 nematodes (the grand geometric mean excluding the two rabbits showing loss of infection) was maintained in most rabbits for the duration of 8 weeks.

Changes were observed for the egg output from the rabbits killed at 2 week intervals. A fall after week 2 was not statistically significant due to the wide variation, but there was a significant decline in the geometric mean egg production per female nematode at week 8 (Figure 17).

Effect of Previous Infection:

More nematodes were consistently recovered from the rabbits with primary infections than from those previously infected with 1,000 larvae. The difference between geometric means was statistically significant.

The egg production per female nematode for the 24 hours preceding post mortem was depressed by the previous infection, but the difference in geometric means was not statistically significant.

Effect of Diet:

In this experiment the feeding of lucerne or laboratory pellets made no contribution to the variance in mean number of nematodes recovered (Appendix III), but
### TABLE 14

Recovery of *T. retortaforma* and Eggs Passed at Intervals during the Progress of Infection* in Adult Female Rabbits (that received a primary or secondary* infection and fed laboratory pellets or lucerne)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>REPL.</th>
<th>WEEK OF P.M.</th>
<th>EXPERIMENTAL INFECTION</th>
<th>DIET</th>
<th>TOTAL NEMATODES (no.)</th>
<th>ADULTS (no.):</th>
<th>L₄ (no.)</th>
<th>L₃ (no.)</th>
<th>EGGS PASSED per day (thous.)</th>
<th>per female (mean no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(1)</td>
<td>1</td>
<td>Prim.</td>
<td>Luc.</td>
<td>1,800 (72.0)</td>
<td>1,040 (55.8)</td>
<td>760 (0)</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>1,140 (45.6)</td>
<td>580 (48.3)</td>
<td>560 (0)</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>Sec.*</td>
<td>Luc.</td>
<td>1,300 (52.0)</td>
<td>800 (52.5)</td>
<td>500 (0)</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>1,360 (54.4)</td>
<td>1,020 (51.0)</td>
<td>340 (0)</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>(1)</td>
<td>2</td>
<td>Prim.</td>
<td>Luc.</td>
<td>1,920 (76.8)</td>
<td>1,900 (50.5)</td>
<td>0 (20)</td>
<td>207</td>
<td>216</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>2,220 (88.8)</td>
<td>2,220 (51.4)</td>
<td>0 (0)</td>
<td>173</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>Sec.*</td>
<td>Luc.</td>
<td>1,760 (70.4)</td>
<td>1,760 (48.9)</td>
<td>0 (0)</td>
<td>180</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>1,280 (51.2)</td>
<td>1,280 (48.4)</td>
<td>0 (0)</td>
<td>215</td>
<td>347</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(1)</td>
<td>4</td>
<td>Prim.</td>
<td>Luc.</td>
<td>1,220 (48.8)</td>
<td>1,220 (55.7)</td>
<td>0 (0)</td>
<td>23</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>1,140 (45.6)</td>
<td>1,140 (47.4)</td>
<td>0 (0)</td>
<td>87</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>Sec.*</td>
<td>Luc.</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>880 (35.2)</td>
<td>880 (52.3)</td>
<td>0 (0)</td>
<td>48</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(1)</td>
<td>6</td>
<td>Prim.</td>
<td>Luc.</td>
<td>1,240 (49.6)</td>
<td>1,240 (40.3)</td>
<td>0 (0)</td>
<td>25</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>960 (38.4)</td>
<td>960 (47.9)</td>
<td>0 (0)</td>
<td>53</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>Sec.*</td>
<td>Luc.</td>
<td>900 (36.0)</td>
<td>900 (51.1)</td>
<td>0 (0)</td>
<td>10</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>600 (24.0)</td>
<td>600 (53.3)</td>
<td>0 (0)</td>
<td>98</td>
<td>306</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(1)</td>
<td>8</td>
<td>Prim.</td>
<td>Luc.</td>
<td>1,860 (74.4)</td>
<td>1,860 (49.5)</td>
<td>0 (0)</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>1,280 (51.2)</td>
<td>1,280 (57.8)</td>
<td>0 (0)</td>
<td>92</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>Sec.*</td>
<td>Luc.</td>
<td>1,160 (46.4)</td>
<td>1,160 (44.8)</td>
<td>0 (0)</td>
<td>41</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot;</td>
<td>Lab.</td>
<td>80 (3.2)</td>
<td>80 (0)</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* All rabbits received an experimental dose of 2,500 infective larvae at week 0.

* Rabbits were previously infected with 1,000 larvae, removed by anthelmintic after 20 days.
EXPERIMENT 9

Geometric Mean Numbers of Adult *T. retortaeformis* Recovered at Intervals During the Progress of Infection in Rabbits with Primary or Secondary Infections, and Fed Laboratory Pellets or Lucerne

(Each rabbit received an experimental infection of 2,500 larvae)

(See Plan of Experiment for details).

PRIM = primary infection
SEC = secondary infection
LUC = fed lucerne
LAB = fed laboratory pellets

This mean is depressed by a single value of zero which is uncharacteristic of the result at that time in this experiment. The mean number without this count is shown by the dotted line.

Significant differences between two means:

* $p \leq 0.05$
** $p \leq 0.01$
DURATION OF INFECTION (weeks)

- no significant difference

EXPERIMENTAL INFECTION

- DIET

* * *

- no significant difference
EXPERIMENT 9

Geometric Mean Egg Production by Female T. retortaeformis Recovered at Intervals During the Progress of Infection in Rabbits with Primary or Secondary Infections, and Fed Laboratory Pellets or Lucerne

(Each rabbit received an experimental infection of 2,500 larvae)

(See Plan of Experiment for details).

PRIM = primary infection
SEC = secondary infection
LUC = fed lucerne
LAB = fed laboratory pellets

Significant differences between two means:

* p ≤ 0.05
** p ≤ 0.01
the number of rabbits was too small to show significant interactions in this experiment. An interaction with the duration of infection was not detected clearly in Experiment I.

The geometric mean egg production per female nematode was significantly higher in the rabbits fed laboratory pellets than in those fed lucerne (Figure 17).

**EXPERIMENTAL INFECTION**

**DIET**

The persistence of *T. reesus* infection at 8 weeks was examined in two groups of adult female rabbits. Each group at each sampling interval was of rabbits infected with a single dose of 2,500 larvae at day 0. One group was of rabbits fed a diet by multiple single doses of 250 larvae each, given every seventh day and dosing at day 0 and day 8. The other group was fed a diet at day 1 and day 16. The results were used to study establishment of the infection, as shown in the following schedule.

Each group consisted of eight 6-month-old female rabbits, each allocated to a replicate.
the number of rabbits was too small to show significant interactions in this experiment. An interaction with the duration of infection is demonstrated clearly in Experiment 10.

The geometric mean egg production per female nematode was significantly higher in the rabbits fed laboratory pellets than in those fed lucerne (Figure 17).

EXPERIMENT 10 ... PERSISTANCE OF AN INFECTION IN THE ADULT FEMALE HOST, AND INFLUENCE OF THREE FACTORS: MULTIPLE DOSING, PREVIOUS INFECTION, AND DIET

Plan

The persistence of *T. retortaeformis* infection at 8 weeks was examined in two groups of adult female rabbits, and another two groups were sampled at half this interval to examine establishment of the infection, as shown in the following schedule.

One group at each sampling interval was of rabbits infected with a single dose of 2,500 larvae at day 0. The other group was of rabbits infected by multiple dosing, which received 10 doses of 250 larvae each given every second day commencing at day 0 and finishing at day 18. Fresh larval harvests were used for each dose, and none of the infective larvae were stored in the cold.

<table>
<thead>
<tr>
<th>Group</th>
<th>Larval Dose (no.)</th>
<th>Duration of Infection (weeks to P.m.)</th>
<th>Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,500</td>
<td>4</td>
<td>Single</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Multiple</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>8</td>
<td>Single</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Multiple</td>
</tr>
</tbody>
</table>

Each group consisted of eight 6 month old female rabbits, each allocated to a replicate.
A single batch of 44 rabbits was used in Experiments 9 and 10. Prior to infection the body weight was determined, and the 16 heaviest rabbits selected to be used as Replicates (5) to (8) as in the next schedule. The lower initial body weight rabbits were used as Replicates (1) to (4) in both Experiment 9 and Experiment 10.

The effects of previous infection and type of food were considered exactly as described in the Plan for Experiment 9. Each factor was allocated equally with every other factor, as set out in the next schedule.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Initial Body Weight</th>
<th>Infection</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>Lower</td>
<td>Primary</td>
<td>Lucerne Chaff</td>
</tr>
<tr>
<td>(2)</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Laboratory Pellets</td>
</tr>
<tr>
<td>(3)</td>
<td>&quot;</td>
<td>Secondary</td>
<td>Lucerne Chaff</td>
</tr>
<tr>
<td>(4)</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Laboratory Pellets</td>
</tr>
<tr>
<td>(5)</td>
<td>Higher</td>
<td>Primary</td>
<td>Lucerne Chaff</td>
</tr>
<tr>
<td>(6)</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Laboratory Pellets</td>
</tr>
<tr>
<td>(7)</td>
<td>&quot;</td>
<td>Secondary</td>
<td>Lucerne Chaff</td>
</tr>
<tr>
<td>(8)</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Laboratory Pellets</td>
</tr>
</tbody>
</table>

With the experimental design used for this experiment, each mean result was calculated on 16 rabbits for the main effects (single factors), or on 8 animals for the interaction effects (between two factors). (Interactions between three factors were not analysed, as more animals would probably be required to achieve statistical significance.)

Post mortem examinations of the parasite populations were carried out at the two intervals shown by groups, and egg counts were made weekly with Experiment 9.

Results (Prologue)

The numbers of nematodes and egg counts at post mortem examination, and the body weight gain of rabbits from the start of the experimental infection, are given in Table 15. The weekly egg counts throughout Experiments 9 and 10 are illustrated in Figure 18.
TABLE 15

EXPERIMENT 10

Recovery of *T. retortaeformis* and Eggs Passed in Established and Persistent Infections, (that were administered as single or multiple doses) and Live Body Weight Changes, in Adult Female Rabbits (that received a primary or secondary* infection and fed laboratory pellets or lucerne)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>REPL.</th>
<th>DOING</th>
<th>INITIAL BODY WEIGHT LEVEL</th>
<th>EXPERIMENTAL INFECTION</th>
<th>BODY WEIGHT CHANGE (gms per wk)</th>
<th>TOTAL NEMATODES</th>
<th>ADULTS</th>
<th>L4</th>
<th>L3</th>
<th>EGGS PASSED per day per female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(no.) (% dose)</td>
<td>(no.)</td>
<td>(no.)</td>
<td></td>
<td></td>
<td>(thous.) (mean no.)</td>
</tr>
<tr>
<td>1</td>
<td>(1)</td>
<td>4</td>
<td>Sing. Lower Prim. Luc.</td>
<td>+ 4</td>
<td>1,220 48.8 1,220 55.7</td>
<td>0 0</td>
<td>0 0</td>
<td>23</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot; Lab. + 44</td>
<td>- 15</td>
<td>1,140 45.6 1,140 47.4</td>
<td>0 0</td>
<td>0 0</td>
<td>87</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>&quot; Sec.* Luc. + 12</td>
<td>- 0</td>
<td>880 35.2 880 52.3</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot; Lab. + 100</td>
<td>- 8</td>
<td>1,120 44.8 1,120 57.1</td>
<td>0 0</td>
<td>0 0</td>
<td>48</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Higher Prim. Luc.</td>
<td>- 8</td>
<td>1,120 44.8 1,120 57.1</td>
<td>0 0</td>
<td>0 0</td>
<td>29</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>&quot; Lab. + 66</td>
<td>- 3</td>
<td>1,180 47.2 1,180 54.2</td>
<td>0 0</td>
<td>0 0</td>
<td>21</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td></td>
<td>&quot; Sec.* Luc. + 86</td>
<td>- 3</td>
<td>1,120 47.2 1,120 54.2</td>
<td>0 0</td>
<td>0 0</td>
<td>237</td>
<td>238</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td></td>
<td>&quot; Lab. + 100</td>
<td>- 12</td>
<td>1,140 45.6 1,140 51.9</td>
<td>0 0</td>
<td>0 0</td>
<td>63</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td></td>
<td>&quot; Sec.* Luc. + 86</td>
<td>- 9</td>
<td>1,120 47.2 1,120 54.2</td>
<td>0 0</td>
<td>0 0</td>
<td>21</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(1)</td>
<td>4</td>
<td>Sing. Lower Prim. Luc.</td>
<td>- 30</td>
<td>1,440 57.6 1,440 55.6</td>
<td>0 0</td>
<td>0 0</td>
<td>50</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot; Lab. + 27</td>
<td>- 40</td>
<td>2,140 85.6 2,140 51.9</td>
<td>0 0</td>
<td>0 0</td>
<td>123</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>&quot; Sec.* Luc. + 96</td>
<td>- 52</td>
<td>1,500 60.0 1,420 59.2</td>
<td>0 0</td>
<td>0 0</td>
<td>248</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot; Lab. + 100</td>
<td>- 8</td>
<td>2,420 96.8 2,420 50.4</td>
<td>0 0</td>
<td>0 0</td>
<td>43</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>Higher Prim. Luc.</td>
<td>- 8</td>
<td>2,320 92.8 2,300 51.3</td>
<td>20 0</td>
<td>0 0</td>
<td>23</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td></td>
<td>&quot; Lab. + 51</td>
<td>- 51</td>
<td>2,420 96.8 2,420 50.4</td>
<td>0 0</td>
<td>0 0</td>
<td>208</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td></td>
<td>&quot; Sec.* Luc. + 3</td>
<td>- 8</td>
<td>2,320 92.8 2,300 51.3</td>
<td>20 0</td>
<td>0 0</td>
<td>23</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td></td>
<td>&quot; Lab. + 100</td>
<td>- 8</td>
<td>2,420 96.8 2,420 50.4</td>
<td>0 0</td>
<td>0 0</td>
<td>208</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(1)</td>
<td>8</td>
<td>Sing. Lower Prim. Luc.</td>
<td>- 34</td>
<td>1,860 74.4 1,860 49.5</td>
<td>0 0</td>
<td>0 0</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot; Lab. + 34</td>
<td>- 34</td>
<td>1,280 51.2 1,280 57.8</td>
<td>0 0</td>
<td>0 0</td>
<td>92</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>&quot; Sec.* Luc. + 72</td>
<td>- 5</td>
<td>1,160 46.4 1,160 44.8</td>
<td>0 0</td>
<td>0 0</td>
<td>41</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot; Lab. + 72</td>
<td>- 3</td>
<td>3 8 32 80</td>
<td>0 0</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>Higher Prim. Luc.</td>
<td>- 14</td>
<td>1,660 66.4 1,660 61.4</td>
<td>0 0</td>
<td>0 0</td>
<td>74</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td></td>
<td>&quot; Lab. + 66</td>
<td>- 4</td>
<td>1,220 48.8 1,220 59.0</td>
<td>0 0</td>
<td>0 0</td>
<td>60</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td></td>
<td>&quot; Sec.* Luc. + 49</td>
<td>- 4</td>
<td>880 35.2 880 47.7</td>
<td>0 0</td>
<td>0 0</td>
<td>54</td>
<td>129</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td></td>
<td>&quot; Lab. + 49</td>
<td>- 4</td>
<td>880 35.2 880 47.7</td>
<td>0 0</td>
<td>0 0</td>
<td>54</td>
<td>129</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(1)</td>
<td>8</td>
<td>Sing. Lower Prim. Luc.</td>
<td>- 3</td>
<td>60 2.4 60 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>&quot; Lab. + 4</td>
<td>- 4</td>
<td>140 5.6 140 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>&quot; Sec.* Luc. + 2</td>
<td>- 3</td>
<td>2,040 81.6 2,040 52.0</td>
<td>0 0</td>
<td>0 0</td>
<td>87</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>&quot; Lab. + 2</td>
<td>- 8</td>
<td>260 10.4 260 0</td>
<td>0 0</td>
<td>0 0</td>
<td>24</td>
<td>201</td>
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</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>Higher Prim. Luc.</td>
<td>- 18</td>
<td>20 0.8 20 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td></td>
<td>&quot; Lab. + 18</td>
<td>- 33</td>
<td>40 1.6 40 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td></td>
<td>&quot; Sec.* Luc. + 1</td>
<td>- 1</td>
<td>2,050 82.4 2,060 55.3</td>
<td>0 0</td>
<td>0 0</td>
<td>85</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td></td>
<td>&quot; Lab. + 18</td>
<td>- 19</td>
<td>0 0 0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* All rabbits received a total experimental dose of 2,500 live larvae commencing at week 0.

a Multiple doses of 250 larvae were given every second day on ten occasions.

* Rabbits were previously infected with 1,000 larvae, removed by anthelmintic after 20 days.

a 3.2% of dose.
Some inhibited third-stage larvae were recovered at week 4 from four rabbits, although not found at this time in Experiment 9. Of these rabbits, three were from higher initial body weight replicates which did not appear in Experiment 9, and also three had received multiple doses of infective larvae which were not used in Experiment 9. The incidence, and numbers, of inhibited third-stage larvae found were not great enough for statistical testing.

The proportion of females in the adult nematode population did not appear to be influenced by any of the factors considered.

The geometric means of total number of adult nematodes, egg production per female, and body weight gain, are illustrated (for the five factors examined) in Figures 19, 20 and 21 respectively. The interactions between these factors are shown in the figures. For each figure it would be possible to make 95 direct, and 200 indirect, statistical comparisons. Alternatively, each of the 15 histograms of means for each feature may be taken to represent a simple experiment with thirty two animals. The latter approach is followed as much as possible in the protracted outline of the results.

The results of this multi factor experiment provide an illustration of the confusion in trying to extrapolate simple laboratory observations to a natural field situation with a complexity of uncontrolled factors. It is seen that with the introduction of a factor there is interaction with other effects, so that in its presence there may be quite different results to those obtained in its absence.

**Protracted Results - Weekly Data**

The weekly egg counts are plotted in eight blocks (Figure 18) as in the following schedule. There was no difference apparent between lower and higher initial body weight, so this factor was not separated.
<table>
<thead>
<tr>
<th>Block</th>
<th>Dosing</th>
<th>Infection</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Single</td>
<td>Primary</td>
<td>Lucerne</td>
</tr>
<tr>
<td>(b)</td>
<td>Single</td>
<td>Primary</td>
<td>Lab. Pellets</td>
</tr>
<tr>
<td>(c)</td>
<td>Multiple</td>
<td>Primary</td>
<td>Lucerne</td>
</tr>
<tr>
<td>(d)</td>
<td>Multiple</td>
<td>Primary</td>
<td>Lab. Pellets</td>
</tr>
<tr>
<td>(e)</td>
<td>Single</td>
<td>Secondary</td>
<td>Lucerne</td>
</tr>
<tr>
<td>(f)</td>
<td>Single</td>
<td>Secondary</td>
<td>Lab. Pellets</td>
</tr>
<tr>
<td>(g)</td>
<td>Multiple</td>
<td>Secondary</td>
<td>Lucerne</td>
</tr>
<tr>
<td>(h)</td>
<td>Multiple</td>
<td>Secondary</td>
<td>Lab. Pellets</td>
</tr>
</tbody>
</table>

A resume of the most salient points exhibited is given. The statistical analysis of variance for the two major sampling times, week 4 and week 8, is summarised in Appendix III, and shows the occurrence of statistically significant interactions between some of the factors considered.

The infection of rabbits by multiple dosing resulted in a slower rise of egg production (Blocks (c), (d), (g), (h)) compared to a single dose, and was followed by a complete decline in egg production in all the animals with primary infections (Blocks (c), (d)). It was concluded that multiple dosing depressed the egg production as compared with infections from the same number of larvae given in one dose.

In the rabbits with secondary infections, only 2/8 showed zero egg counts towards the end of the experiment (Blocks (e), (f), (g), (h)), while 6/8 of those with primary infections showed zero egg counts after week 6. It was concluded that previous infection interfered with the depression of egg counts that followed multiple dosing.

Although egg counts per gram of faeces were higher from rabbits fed laboratory pellets, these rabbits generally produced less faeces, about 50 gms per day at this season of the year, than rabbits fed lucerne, about 90 gms per day. The results show there was no consistent difference in magnitude of egg production per day between the two food types. The rabbits fed lucerne and with previous infections (Blocks (e), (g)) showed the most
EXPERIMENT 10

Weekly Egg Counts from *T. retortaeformis* Infections in Rabbits with Primary or Secondary Infections, Receiving Multiple or Single Doses of Larvae and Fed Laboratory Pellets or Lucerne

(Each rabbit received a total experimental infection of 2,500 larvae)

(See Plan of Experiment for details).

MULT = multiple doses
SING = single dose
PRIM = primary infection
SEC = secondary infection
LUC = fed lucerne
LAB = fed laboratory pellets

(Samples at week 3 were lost)
persistent egg output through the experiment. It was concluded that interrace feed promoted total egg production of infection.

**Results**

The graph shows the duration of infection in weeks for different experiments.

1. **SING - PRIM - LUC**
2. **SING - SEC - LUC**
3. **SING - PRIM - LAB**
4. **SING - SEC - LAB**
5. **MULT - PRIM - LUC**
6. **MULT - SEC - LUC**
7. **MULT - PRIM - LAB**
8. **MULT - SEC - LAB**

The y-axis represents the number of eggs passed per day, and the x-axis represents the duration of infection in weeks.
persistent egg output through the experiment. It was concluded that lucerne feed promoted total egg production over 8 weeks of infection.

Protracted Results - Post Mortem Data

For the results from the post mortem examination, only the more important effects of the factors considered are outlined for the principle parameters: number of adult nematodes (Figure 19); egg production per female for 24 hours (Figure 20); and body weight gain of the host (Figure 21). The figures are inserted after page 85.

In this report, "loss of infection" is defined as occurring when less than 500 nematodes are found in a rabbit from which normal egg counts had earlier been made.

Effect of Duration of Infection:

There was a statistically significant fall in the number of nematodes recovered between week 4 (geometric mean 1,120) and week 8 (geometric mean 270). By week 8 half the rabbits showed an appreciable loss of infection, while at week 4 this decline was observed in only one rabbit.

Overall there were significantly less eggs produced per female nematode remaining at week 8, than per female recovered at week 4. This experiment, with two sampling times, did not provide any information on a rhythm of egg production.

When examining the rate of host body weight gain during the infection, an important phenomenon was observed. Although the rabbits killed at week 8 had significantly less parasites remaining, they showed significantly less body weight gain per week than the rabbits killed at week 4. This finding was consistently repeated throughout the results for different factors and interactions of factors.
At week 4 it was observed that there was a positive relationship between those rabbits that failed to gain weight and their resistance to the infection as measured by the egg production. At the same time, the number of parasites present was not related to the rate of weight gain overall.

Effect of Multiple Dosing:

The recovery of nematodes at week 4 from the rabbits given multiple doses of larvae approached 100% of the total number of infective larvae administered. (The infective larvae used in the multiple doses were from cultures incubated for 7, 9, or 11 days at 25°C.) The recovery was only 10 days after the last larval dose, and insignificant numbers of late fourth-stage larvae (less than 1% of the total) were found in two rabbits, which indicates no delay in development.

Overall there was no difference in the mean number of nematodes recovered from single or multiple doses. However, this was because at week 4 there were significantly more present following multiple dosing, but by week 8 there had been a substantial fall in number in those cases, to levels significantly less than remaining in the single dose infections (Figure 19). (It was noticed that the only rabbits with multiple doses in which there was not a loss of infection at week 8 were those with a previous infection and fed lucerne (Table 15), a finding consistent with egg production previously reported.)

The results for eggs per female with multiple dosing were related to the findings for the number of adult nematodes recovered.

The mean rate of host body weight gain during infection showed highly significant effects of multiple versus single dosing. Rabbits with single dose infections gained weight at a mean of 37 gms per week throughout the experiment. On the other hand, the rabbits with multiple
dose infections gained significantly less during the first 4 weeks, and over 8 weeks of infection lost weight. The loss of body weight was directly associated with the loss of infection.

Effect of Previous Infection:

At week 4 there were more nematodes recovered from primary infections than from rabbits that had experienced a previous infection, as found in Experiment 9 for the mean of samples at weeks 2, 4, 6, and 8. However, at week 8 there was a highly significant fall in the mean number of nematodes recovered from primary infections in Experiment 10, but a smaller non significant fall from secondary infections, so that more nematodes were recovered from the secondary infections. This reversal was apparently related to an interaction with multiple doses, because the highest mean number of nematodes was in secondary infections from multiple doses, and the lowest in primary infections from multiple doses. (Multiple doses were not used in Experiment 9.)

The influence of previous infection on mean egg production per female resulted in more eggs in secondary infections than in primary infections. The interactions with other factors were similar to those described as acting on mean numbers of nematodes recovered.

Overall, rabbits which had experienced a previous infection gained more weight per week than those with a primary infection. This was due to the effect within the first 4 weeks of experimental infection, when rabbits with primary infections gained weight at only half the rate of those with secondary infections. Thus the body weight gain early in the infection appeared for this factor to be inversely associated with the number of nematodes established in the first 4 weeks.

The overall body weight change was also influenced by an interaction with multiple dosing. There was a
significant mean loss of weight in the rabbits with multiple dose primary infections compared to the other combinations. However, experience of a previous infection did not completely protect rabbits from a depression of weight gain, as there was still a highly significant difference in weight gain per week between multiple dose secondary infections (less) and the single dose infections (more).

**Effect of Diet:**

There was no statistically significant difference at week 4 between the number of adult nematodes counted from rabbits fed lucerne, and those fed laboratory pellets. However, at this time, inhibited third-stage larvae (up to 5.33% of the total nematode population counted) were found in half of the rabbits fed laboratory pellets. None were found in the rabbits on lucerne chaff. While for lucerne feeding only 2/8 rabbits showed a loss of infection at week 8 (no significant mean difference to week 4), on laboratory pellets 6/8 rabbits showed loss of infection, and the difference in means of number of nematodes counted, was highly significant between food types at week 8.

Overall, the rabbits fed lucerne that had had a previous infection had the greatest number of nematodes, and those with a primary infection fed laboratory pellets had least, suggesting that feeding lucerne may have moderated any resistance associated with the previous infection.

The highest level of egg production per female *T. retortaeformis* over 24 hours for this experiment was recorded 4 weeks after infection from rabbits fed laboratory pellets, with a geometric mean of 148 e.p.f., and range of 105 to 258. (In Experiment 9 values over 300 e.p.f. were obtained at the peak periods of week 2 and week 6 which were not sampled in Experiment 10.)
The difference in the mean egg output for the two food types at week 4 was statistically significant.

However, while there was only a non significant fall at week 8 in rabbits fed lucerne, there was a highly significant drop in egg production in the rabbits fed laboratory pellets. This drop was associated with the loss of infection already described. If the persistent infections at week 8 are considered, then there was still greater egg production from the female nematodes in laboratory pellet fed rabbits (geometric mean 126), than in lucerne fed rabbits (50). This is an example of the association between depression of egg output by the female parasites and the exhibition of host resistance to established infection.

Contrary to the report already given for the number of nematodes, the greatest egg production was from rabbits fed laboratory pellets that had had a previous infection, and least from those with a primary infection fed lucerne.

The rabbits fed laboratory pellets continued to gain weight rapidly at an average of 71 gms per week during the first 4 weeks of infection. However, all the rabbits fed lucerne failed to gain weight over this period (average 19 gms per week loss), although they had been gaining weight immediately prior to the experimental infection. But over 8 weeks of infection, rabbits on laboratory pellets gained only an average of 20 gms per week, meaning that they lost weight at 31 gms per week between weeks 4 and 8. The rabbits fed lucerne, on the other hand, gained weight at 19 gms per week between weeks 4 and 8 to give a net gain of 5 gms per week over 8 weeks of infection.

An apparent association of failure to gain weight and resistance to the parasite is exhibited by comparison of Figures 19, 20, and 21. In the first 4 weeks of infection, loss of body weight (on lucerne) was associated with fewer worms established and the lowest mean production of eggs per female of any factor examined,
as opposed to a big increase in body weight (on laboratory pellets) associated with more nematodes established and the highest egg production. In the second 4 weeks of infection, loss of body weight (on laboratory pellets) was associated with removal of the established nematodes, as opposed to an increase in body weight (on lucerne) associated with a tolerance of the nematodes established.

The type of food modified the effect on body weight gain of a previous infection which has already been reported. While on laboratory pellets there was a significantly greater mean rate of body weight gain overall by the rabbits with secondary infections than by the rabbits with primary infections, while on lucerne feed the previous experience of infection had no overall effect on body-weight gain.

Effect of Initial Host Body Weight:

The initial host body weight factor was not expected to influence the infection, and no single effects were found in the results. But several significant interactions with other factors were found. In general, when differences in a parameter due to other experimental factors occurred, they were more pronounced for the initial higher body weight rabbits than for the initial lower body weight rabbits.

For example, there were more nematodes present at week 4, and a greater fall in the number recovered at week 8, from the higher than from the lower body weight rabbits. Over the whole experiment, the greatest mean difference in nematodes recovered between primary (less) and secondary (more) infections occurred in the higher body weight rabbits, with a non significant mean difference in the lower body weight rabbits.

Contracted Results

To gather the same information as in this experiment without the factorial design, 10 experiments each with the two times of post mortem examination, would have had to be
EXPERIMENT 10

Geometric Mean Number of Adult *T. retortaeformis* Established (Week 4) and Persisting (Week 8) in Rabbits with Primary or Secondary Infections Receiving Multiple or Single Doses of Larvae and Fed Laboratory Pellets or Lucerne

(Each rabbit received a total experimental infection of 2,500 larvae)

(See Plan of Experiment for details).

MULT = multiple doses
SING = single dose
PRIM = primary infection
SEC = secondary infection
LUC = fed lucerne
LAB = fed laboratory pellets
HBW = higher initial body weight
LBW = lower initial body weight

Significant differences between two means:

* p ≤ 0.05
** p ≤ 0.01
EXPERIMENT 10

Geometric Mean Egg Production by Female T. retortaeformis Established (Week 4) and Persisting (Week 8) in Rabbits with Primary or Secondary Infections Receiving Multiple or Single Doses of Larvae and Fed Laboratory Pellets or Lucerne

(Each rabbit received a total experimental infection of 2,000 larvae)

(See Plan of Experiment for details).

MULT = multiple doses
SING = single dose
PRIM = primary infection
SEC = secondary infection
LUC = fed lucerne
LAB = fed laboratory pellets
HBW = higher initial body weight
LBW = lower initial body weight

Significant differences between two means:

* $p < 0.05$
** $p < 0.01$
EXPERIMENT 10

Mean Body Weight Gain of Rabbits during 4 weeks, or 8 weeks, of *T. retortaeformis* Infection for Rabbits with Primary or Secondary Infections Receiving Multiple or Single Doses of Larvae and Fed Laboratory Pellets or Lucerne

(Each rabbit received a total experimental infection of 2,500 larvae)

(See Plan of Experiment for details)

MULT = multiple doses  
SING = single dose  
PRIM = primary infection  
SEC = secondary infection  
LUC = fed lucerne  
LAB = fed laboratory pellets  
HBW = higher initial body weight  
LBW = lower initial body weight

Significant differences between two means:

* p ≤ 0.05  
** p ≤ 0.01
carried out. While the complete results outlined cannot be retracted, a compendium of results for the main effects follows.

(i) Establishment of infection (0 to 4 weeks)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Rise in Establishment Egg Count of Nematodes</th>
<th>Egg Production by Females</th>
<th>Host Body Weight Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple Dosing</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Previous Infection</td>
<td>0</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Lab. Pellets Diet</td>
<td>+</td>
<td>+</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Bigger Rabbits</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

(ii) Persistence of infection (4 to 8 weeks)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Maintenance of Egg Count</th>
<th>Survival of Nematodes</th>
<th>Egg Production by Females</th>
<th>Host Body Weight Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple Dosing</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Previous Infection</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Lab. Pellets Diet</td>
<td>-</td>
<td>- - -</td>
<td>-</td>
<td>- - -</td>
</tr>
<tr>
<td>Bigger Rabbits</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

Comparison with alternative factor: + greater
- depressed
0 nil effect

The host susceptibility to establishment of infection was reflected by depressed weight gain, except for the feeding of laboratory pellets when there were more nematodes and a better weight gain.

The host resistance to an established infection was first shown as a depression of egg production, followed by a loss of infection leaving only 0% to 10% of the infective larval dose, and associated with a marked loss of body weight.
In general, the greater the infection with T. retortaeformis established, the more extreme the reaction some weeks later, and the greater the resistance shown, the worse for the host.

The object of this section was to investigate whether it could be shown in the laboratory that the course and development of T. retortaeformis infection in the female rabbit is affected by reproduction and lactation. Reports of such an effect on natural infections in the wild rabbit are mentioned in the Introduction to Part II. In view of the importance of parasite egg production from reproducing female hosts providing pasture contamination to infect the next host generation, it was desired to see if a relationship could be confirmed between infection in rabbits and the post-partum rise in egg output for trichostongylid infections in other grazing animals described in the Introduction.

The experimental infection regime was selected from the results of Section B(1). Single doses of larvae, without previous infection, were used. A dose of 2,500 infective larvae had been found to be tolerated by young rabbits, and it was decided to use a higher dose of 7,500 in the adult rabbits. All rabbits were fed pelleted lucerne chaff from 6 months prior to the experiments.

In Experiment II sampling intervals were designed with the object of examining the course of the infection at mid-gestation, at parturition, and at mid-lactation; to observe if the infection differed from that in control rabbits. Secondly, in order to examine the effect of lactation on the development of infection, Experiment 12 was designed to sample infections in groups of rabbits when lactating (infected after parturition), and when weaned of their litter (but infected before parturition).
SECTION C

IMPACT OF FEMALE HOST REPRODUCTION ON THE COURSE OF INFECTION

OBJECTS

The object of this section was to investigate whether it could be shown in the laboratory that the course and development of *T. retortaeformis* infection in the female rabbit is affected by reproduction and lactation. Reports of such an effect to natural infections in the wild rabbit are mentioned in the Introduction to Part II. In view of the importance of parasite egg production from reproducing female hosts providing pasture contamination to infect the next host generation, it was desired to see if a relationship could be confirmed between infection in rabbits and the post-parturient rise in egg output for trichostrongyloid infections in other grazing animals described in the Introduction.

The experimental infection regime was selected from the results of Section B(ii). Single doses of larvae, without previous infection, were used. A dose of 2,500 infective larvae had been found to be tolerated by young rabbits, and it was decided to use a higher dose of 5,000 in the adult rabbits. All rabbits were fed pelleted lucerne chaff from 4 months prior to the experiments.

In Experiment 11 sampling intervals were designed with the object of examining the course of the infection at mid-gestation, at parturition, and at mid-lactation, to observe if the infection differed from that in control rabbits. Secondly, in order to examine the impact of lactation on the development of infection, Experiment 12 was designed to sample infections in groups of rabbits when lactating (infected after parturition), and when weaned of their litter (but infected before parturition).
EXPERIMENT 11 ... IMPACT OF GESTATION, PARTURITION, AND LACTATION ON PERSISTANCE OF INFECTION IN THE ADULT FEMALE HOST

Plan

Three groups of three adult female rabbits, infected with *T. retortaeformis* soon after copulation, were killed at 15 day intervals to correspond with mid-gestation, parturition, and lactation, as in the following schedule. An equivalent three groups of non-mated control rabbits were examined at the same sampling times. All other factors were kept constant.

<table>
<thead>
<tr>
<th>Group</th>
<th>Larval Dose (no.)</th>
<th>Reproduction Status at p.m.</th>
<th>Duration of Infection (days to p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5,000</td>
<td>+ Gestation</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>- Control</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>+ Parturition</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>- Control</td>
<td>&quot;</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>+ Lactation</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>- Control</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

The doe rabbits used for Experiments 11 and 12 were of a single uniform batch, from which the three lightest animals were discarded to reduce variation. For the random allocation for the two experiments, the rabbits were ranked in order of body weight gain over the month preceding mating or infection. The replicates in the results are given in this order, greatest gain first.

Experiment 11 was designed with six replicates in each group, but staggered into two series 7 days apart for handling requirements. Two periods were needed to synchronise mating, between which the bucks were rested. However, infections were not established in one half of
the rabbits, dosed with fresh infective larvae harvested from a 5 day faecal culture. (The problem of the failure of fresh larvae to infect has since been examined in Experiment 8.) Thus three replicates remained.

At mating, the does were 8 months old. In a mating period, six bucks were used so that all does were joined within a 56 hour interval. To maximise conception, double mating was practised, and the does with which more than one buck did not copulate were discarded. Extra does were mated as reserves, but only 2 out of 26 does mated were non-pregnant. The rabbits littered at 28, 29, or 30 days after dosing with infective larvae, close to the nominated 30 days in the course of infection. The does in Group 3 were killed within 12 hours of parturition, with the corresponding replicate from Group 4.

Faecal egg counts were carried out every 5 days. Post mortem examinations of the parasite populations were carried out at the three intervals already stated during the course of reproduction and of infection. Samples of eggs passed were measured from each of these groups.

Results

The data from the time of post mortem examination are given in Table 16 with information on the number of foetuses or kittens born and suckled. The egg counts every 5 days throughout the experiment are presented in Figure 24, and the frequency distribution of egg volumes at the post mortem sampling times are shown in Figure 26.

At 15 days after infection, nematode numbers were comparable between rabbits in mid-gestation (Group 1) and control does (Group 2). Although delayed development was recognised in two gestation rabbits by the detection of late fourth-stage larvae, these represented less than 1% of the total nematode count. Inhibited third-stage larvae were recovered in two control rabbits, representing up to 2.3% of the total nematode count in a rabbit. More inhibited larvae were expected, since inhibited larvae
TABLE 16

EXPERIMENT 11

Recovery of *T. retortaeformis* and its Egg Production from Infections in Reproducing* and Control Adult Female Rabbits

<table>
<thead>
<tr>
<th>GROUP</th>
<th>REPL.</th>
<th>DAY* OF F.M.</th>
<th>REPROD. STATUS*</th>
<th>KITTENS</th>
<th>TOTAL NEMATODES</th>
<th>ADULTS</th>
<th>L4</th>
<th>L3</th>
<th>EGG PRODUCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Foetus (%)</td>
<td>Suckled (no.)</td>
<td>all (no.)</td>
<td>female (no.)</td>
<td>% adults</td>
<td>(no.)</td>
</tr>
<tr>
<td>1</td>
<td>(1)</td>
<td>15 Gestn.</td>
<td>-</td>
<td>10 -</td>
<td>4,140 82.8</td>
<td>4,140 57.5</td>
<td>0 0</td>
<td>0 0</td>
<td>440 185</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>-</td>
<td>-</td>
<td>9 -</td>
<td>3,220 64.4</td>
<td>3,200 58.1</td>
<td>0 0</td>
<td>0 0</td>
<td>248 133</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>-</td>
<td>-</td>
<td>6 -</td>
<td>2,060 41.2</td>
<td>2,040 47.1</td>
<td>0 0</td>
<td>0 0</td>
<td>66 69</td>
</tr>
<tr>
<td>2</td>
<td>(1)</td>
<td>Control</td>
<td>-</td>
<td>- -</td>
<td>4,600 92.0</td>
<td>4,560 47.8</td>
<td>0 40</td>
<td>0 0</td>
<td>98 45</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>-</td>
<td>-</td>
<td>- -</td>
<td>4,420 88.4</td>
<td>4,320 57.4</td>
<td>0 100</td>
<td>0 0</td>
<td>160 67</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>-</td>
<td>-</td>
<td>- -</td>
<td>2,640 52.8</td>
<td>2,640 53.0</td>
<td>0 391</td>
<td>0 0</td>
<td>297 11.6</td>
</tr>
<tr>
<td>3</td>
<td>(1)</td>
<td>Partn.</td>
<td>7</td>
<td>180 63.6</td>
<td>3,180 54.1</td>
<td>0 0</td>
<td>0 0</td>
<td>504 293</td>
<td>6.15</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>8</td>
<td>3,480 69.6</td>
<td>3,480 50.6</td>
<td>0 0</td>
<td>0 0</td>
<td>36 20</td>
<td>3.35</td>
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</tr>
<tr>
<td></td>
<td>(3)</td>
<td>7</td>
<td>4,680 93.6</td>
<td>4,420 52.5</td>
<td>260</td>
<td>5 2</td>
<td>32.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(1)</td>
<td>Control</td>
<td>-</td>
<td>- -</td>
<td>100 2.0</td>
<td>100 -</td>
<td>0 0</td>
<td>0 0</td>
<td>299 176</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>-</td>
<td>-</td>
<td>- -</td>
<td>3,000 60.0</td>
<td>3,000 56.7</td>
<td>0 0</td>
<td>0 0</td>
<td>260 160</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>-</td>
<td>-</td>
<td>- -</td>
<td>80 4.0</td>
<td>60 -</td>
<td>0 0</td>
<td>0 0</td>
<td>120 60</td>
</tr>
<tr>
<td>5</td>
<td>(1)</td>
<td>Lactn.</td>
<td>5</td>
<td>2</td>
<td>240 4.8</td>
<td>240 -</td>
<td>0 0</td>
<td>0 0</td>
<td>34 211</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>2</td>
<td>2,440 48.8</td>
<td>2,420 50.5</td>
<td>0 0</td>
<td>0 0</td>
<td>359 249</td>
<td>2.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>6</td>
<td>3,960 79.2</td>
<td>3,720 60.2</td>
<td>100</td>
<td>140</td>
<td>1199</td>
<td>5.25</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(1)</td>
<td>Control</td>
<td>-</td>
<td>- -</td>
<td>120 2.4</td>
<td>120 -</td>
<td>0 0</td>
<td>0 0</td>
<td>240 22</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>-</td>
<td>-</td>
<td>200 4.0</td>
<td>100 -</td>
<td>0 0</td>
<td>0 0</td>
<td>120 123</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>-</td>
<td>-</td>
<td>980 15.6</td>
<td>420 57.1</td>
<td>0 0</td>
<td>0 0</td>
<td>23 97</td>
<td>1.95</td>
</tr>
</tbody>
</table>

* All rabbits received an experimental dose of 5,000 infective larvae at day 0.
* Reproducing does were mated during the 3 days before infection.
* Prior body weight gain:
  (1) greatest
  (2) medium
  (3) least
* fourth-stage larvae:
  + early (L4)
  # late (L4)
* Sum of egg production for preceding 15 days.
* 7.2% of larval dose
were more common from rabbits killed later in the duration of the infection. Inhibited larvae recovered at day 15 represented only 0.5% of the total number of infective larvae administered to both groups of rabbits, while at day 45 the inhibited larvae recovered represented 2.0% of the larval doses.

At 30 days after infection, the parturient rabbits (Group 3) had all maintained their high nematode population in excess of 3,000 adults. In one of these rabbits, from which 93.6% of the larval dose was recovered, 1/5 of the adults were immature fifth-stage, and late fourth-stage larvae were recovered representing 5.6% of the total nematode count. The comparable control rabbits (Group 4) had lost their nematode infection, with less than 500 adult nematodes persisting, except one rabbit. The geometric mean fall in nematode numbers was statistically highly significant, compared with the mean recoveries from the parturient does, and from the groups killed earlier at 15 days after infection.

At 45 days after infection, only rabbits in mid-lactation (Group 5) retained persistent infections over 500 adult nematodes. The mean difference with the control rabbits (Group 6) was statistically highly significant. Developing larvae, as late fourth-stage, were present in the reproducing rabbits again, but were not found in the control rabbits. However, inhibited third-stage larvae were recovered from the intestine of rabbits from both groups, with up to 7.2% of the larval dose from a control rabbit, and 2.8% of the larval dose from a reproducing rabbit. From another rabbit, which had a litter of seven kittens but weaned herself immediately, only 40 adult nematodes were recovered.

The proportion of female nematodes did not show any difference between groups at any of the three times of sampling.

There was no appreciable difference in total egg output per day (Figure 24) between groups at the beginning of infection. At 25 days after infection, most of the
control rabbits showed a trough in egg output, while in the rabbits approaching parturition the egg count tended to be maintained. In the lactating does the egg count was maintained at, or rose to, a high level of 34,000 to 445,000 eggs per day, while in the control rabbits the egg count did not exceed a maximum of 23,000 eggs per day at this period.

Not only were the counts per gram of faeces generally higher in the reproducing rabbits than in the control rabbits during the experiment, but also the reproducing rabbits tended to pass a greater weight of faeces after parturition than the usual level of about 120 gms per day at this season of the year. The faecal pellets from lactating does were much wetter than others, and during mid-lactation, the rabbit suckling four kittens passed 300 to 400 gms faeces per day.

The histogram (Figure 25) giving mean sum of the egg production, for each group, during each 15 day period in the course of the experiment, clearly shows the persistence at day 30 to day 45 of a high level of egg output in lactation, compared with a statistically highly significant fall in egg output from non-reproducing rabbits with the same duration of infection.

There was no difference in the egg production by female worms over 24 hours (Figure 23) between the groups at day 15. Both the parturient and the control groups showed a decline at day 30, presumably associated (in the rabbits with high parasite burdens) with a surge of developing adult nematodes, which were not laying eggs. The highest mean egg production per female nematode was found at day 45 in the mid-lactation group, and the difference with the control group was statistically significant.

From the measurement of 30 eggs per group at the end of each 15 day period, one parameter, that of egg volume, is displayed as a frequency distribution in Figure 26. Each frequency curve was drawn proportional
to the egg output per day (logarithmic scale) at the
time. Bimodal egg size populations were evident, and at
the first and second periods there were more small eggs
from the non-reproducing rabbits, which was particularly
marked at day 30. Two of these control rabbits had
negative egg counts 5 days before at day 25, while none
of the pregnant rabbits killed at the second period had had
negative counts. By the end of the third period 45 days
after infection, all of the control rabbits, but only one
of the reproducing rabbits, had experienced negative egg
counts. There were more small eggs at this time, however,
from the lactating rabbits, presumably associated with
the development of immature adults as suggested above.
In this case the appearance of small eggs would be
related to a rise in egg output associated with
parturition and lactation, rather than with a cycle
following a depressed egg count as seen in the controls
and in Experiment 5. Statistical testing for expected
frequencies of eggs less than 100,000 µ3 (as for Experiment
5), showed a significant variation in the incidence of
small eggs between groups during the experiment
\( \chi^2 (4) = 9.84, p \leq 0.05 \).

Although the separation of rabbits (into replicates)
by body weight gain prior to infection did not affect
the number of nematodes recovered, it did affect the egg
production of the female nematodes. Overall there was
significantly greater egg production in the reproducing
rabbits with the greatest prior body weight gain than with
the least, but the reverse occurred in the control rabbits
(Figure 23). This interaction provided a significant
source of variation in the egg production by female
nematodes (analysis Appendix III).

The sum of egg output over all the experiment did not
differ in the reproducing rabbits in relation to prior
body weight gain, but there were significantly fewer eggs
passed from the infections in the control rabbits with the
greatest rate of body weight gain prior to infection
(Figure 25).
EXPERIMENT 11

Geometric Mean Number of Adult *T. retortaeformis* Recovered at Mid-gestation, Parturition, and Mid-lactation, from Rabbits Mated Shortly before Infection, and Their Controls.

(Each rabbit received an infection of 5,000 larvae)

(See Plan of Experiment for details).

Significant differences between two means:

* $p \leq 0.05$

** $p \leq 0.01$
MEAN NUMBER OF NEMATODES (log. scale)

DURATION OF INFECTION (days)

15  30  45

**copulation**  **parturition**

*  **

REPRODUCING DOES

CONTROL DOES
EXPERIMENT 11

Geometric Mean Egg Production by Female T. retortaeformis Recovered at Mid-gestation, Parturition, and Mid-lactation, from Rabbits Mated shortly before Infection, and Their Controls.

(Each rabbit received an infection of 5,000 larvae)

(See Plan of Experiment for details).

<table>
<thead>
<tr>
<th>Replicates</th>
<th>Prior Body Weight Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>Greatest</td>
</tr>
<tr>
<td>(2)</td>
<td>Medium</td>
</tr>
<tr>
<td>(3)</td>
<td>Least</td>
</tr>
</tbody>
</table>

Significant differences between two means:

* p 0.05
** p 0.01
REPRODUCING DOES ■

CONTROL DOES □

copulation

parturition

1000

100

10

1

15 30 45

DURATION OF INFECTION (days)

MEAN DAILY EGG PRODUCTION PER FEMALE (log. scale)

(1) (2) (3) (1) (2) (3)

REPLICATE

(signif. as above)
EXPERIMENT 11

Egg Counts at 5-day Intervals from T. retortaeformis Infections during Gestation, Parturition, and Lactation in Rabbits Mated shortly before Infection, and Their Controls.

(Each rabbit received an infection of 5,000 larvae).

(See Plan of Experiment for details).
The diagrams illustrate the geometric means of the mean number of eggs passed per day by reproducing does and control does over the duration of infection (days). The graphs show the increase and decrease in the number of eggs passed, with peaks indicating the period of copulation and parturition. The control rabbits (solid line) show a lower peak compared to the reproducing does (dashed line) after parturition.
EXPERIMENT 11

Geometric Mean of the Sum Egg Output by *T. retortaeformis* Infections for a 15-day Period up to Mid-gestation, Parturition, or Mid-Lactation in Rabbits Mated shortly before Infection, and their Controls. (Each rabbit received an infection of 5,000 larvae).

(See Plan of Experiment for details).

<table>
<thead>
<tr>
<th>Replicates</th>
<th>Prior Body Weight Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>Greatest</td>
</tr>
<tr>
<td>(2)</td>
<td>Medium</td>
</tr>
<tr>
<td>(3)</td>
<td>Least</td>
</tr>
</tbody>
</table>

Significant differences between two means:

* \( p \leq 0.05 \)

** \( p \leq 0.01 \)
The figure shows the mean sum of egg output for different periods of infection (0-15, 15-30, and 30-45 days) and replicates. The bars represent the data for reproductive does compared to control does. The significance is indicated by asterisks (* for p < 0.05, ** for p < 0.01).

The graph indicates that there is a significant difference (as above) between the reproductive and control groups in the mean sum of egg output during the 15-30 and 30-45 day periods.
EXPERIMENT 11

Size Distribution of T. retortaeformis Eggs Passed at Mid-gestation, Parturition, or Mid-lactation in Rabbits Mated shortly before Infection, and their Controls (Each rabbit received an infection of 5,000 larvae).
(See Plan of Experiment for details).

The volumes of eggs are plotted with a moving average for intervals of 1,000 µ³

Deviations in the incidence of eggs greater or smaller than the median volume (100 µ³ x 10⁻³):

\[ \chi^2(4) = 9.84 \]

* \( p \leq 0.05 \)
GESTATION 193*

CONTROL 185*

PARTURITION 45*

CONTROL 17*

LACTATION 176*

CONTROL 7*

VOLUME OF EGGS (μ^3 \times 10^{-3})

- Geometric mean egg count (thous. eggs per day)
EXPERIMENT 12... IMPACT OF LACTATION OR WEANING ON DEVELOPMENT OF INFECTION IN THE ADULT FEMALE HOST

Plan

The development of *T. retortaeformis* infection was compared in three groups of does with differing lactational status as in the following schedule. The rabbits in Groups 1 and 2 were joined with bucks over a 60 hour interval. When half the does had littered, all rabbits were dosed with infective larvae. As the remainder of the does littered, their kittens were removed to wean the does.

<table>
<thead>
<tr>
<th>Group</th>
<th>Larval Dose (no.)</th>
<th>Lactation Status</th>
<th>Duration of Infection (days to p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5,000</td>
<td>Parturient &amp; Weaned</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Lactating</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Control</td>
<td></td>
</tr>
</tbody>
</table>

The doe rabbits and plan for mating have already been described with Experiment 11. Rabbits were allocated in five replicates per group for Experiment 12. Group 1 experienced parturition but not lactation during the development of the infection, and Group 2 experienced lactation but not parturition while infected.

Faecal egg counts were carried out at 5 day intervals, and post mortem examination made at a time soon after infections reached maturity.

Results

The nematode count and egg count data are given in Table 17, with parturient information.

The mean number of adult nematodes recovered from the weaned does (Group 1) at day 15 was significantly less than from the other rabbits (Figure 27). There was also a statistically highly significant depression of egg...
production per female nematode from the weaned does at day 15 (Figure 28). The lactating and control groups did not differ at day 15.

The typical level of infection as seen in Experiments 11 and 12 is more than 2,000 adult nematodes recovered at 15 days after dosing with 5,000 infective larvae. On this basis normal nematode numbers were established in all the lactating rabbits, but recovered from only two of the weaned rabbits. In addition, inhibited third-stage larvae representing up to 7.7% of the total nematode number were recovered from the weaned rabbits which were parturient 12 to 24 hours after dosing with infective larvae.

The typical egg count at day 10 was 12,000 to 24,000 eggs per day. In the lactating group 3/5 rabbits were considerably higher, and in the other two groups 1/5 lower, indicating a faster rise in egg production from lactating rabbits, than from control rabbits.

The weaned rabbits from which less than 500 adult nematodes were recovered at day 15 had shown normal egg production at day 10, indicating that the depression of infection in weaned rabbits occurred after the pre-patent period. Only one weaned rabbit failed to show severe depression of egg production at day 15, and this animal was parturient and weaned 36 hours after dosing with infective larvae.
TABLE 17

Recovery of *T. retortaeformis* and its Egg Production from 15-day Infections (in lactating, weaned, or control adult female rabbits)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>REPL.</th>
<th>LACTATION STATUS</th>
<th>KITTENS INTERVAL* TO DOSING (no.) (hrs.)</th>
<th>TOTAL NEMATODES (no.) (% dose)</th>
<th>ADULTS all (no.) female (% adults)</th>
<th>L4 per female (thous.)</th>
<th>L3 per female (thous.)</th>
<th>EGG PRODUCTION per female (mean no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(1)</td>
<td>Weaned</td>
<td>9 0 24-36</td>
<td>3,200 64.0 3,200 47.5</td>
<td>0 0 12 242 159</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>8 0 12-24</td>
<td>1,380 37.6 1,320 43.9</td>
<td>0 0 0 27 46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>6 0</td>
<td>520 10.4 480 58.3</td>
<td>0 0 0 24 7 23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>7 0</td>
<td>2,020 40.4 2,000 54.5</td>
<td>0 0 0 12 78 71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(1)</td>
<td>Lactating</td>
<td>10 4 0-12</td>
<td>2,520 50.4 2,520 43.7</td>
<td>0 0 100 292 266</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>4 4</td>
<td>2,280 45.6 2,280 57.9</td>
<td>0 0 0 16 188 142</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>8 5</td>
<td>2,180 43.6 2,180 50.5</td>
<td>0 0 0 106 170 155</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>6 2</td>
<td>4,740 94.8 4,740 55.7</td>
<td>0 0 0 153 525 199</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>8 2</td>
<td>2,960 58.2 2,960 46.6</td>
<td>0 0 0 15 239 173</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(1)</td>
<td>Control</td>
<td>- -</td>
<td>520 10.4 520 53.9</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td>- -</td>
<td>3,480 69.6 3,480 51.1</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>- -</td>
<td>3,640 72.8 3,640 48.9</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td>- -</td>
<td>3,520 70.4 3,520 43.8</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>- -</td>
<td>3,140 62.8 3,140 45.2</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All rabbits received an experimental dose of 5,000 infective larvae at day 0.

* Group 1 dosed with larvae at interval before parturition.
  Group 2 dosed with larvae at interval after parturition.

* A 1.2% of larval dose.
EXPERIMENT 12

Geometric Mean Number of Adult *T. retortaeformis*
Recovered 15 days after Infection from Lactating, Weaned, or Control Rabbits.
(Each rabbit received an infection of 5,000 larvae)
(See Plan of Experiment for details).

**WEAN** = parturient, and weaned of litter, shortly after infection
**LACT** = parturient before, and lactating during, infection
**CONT** = control does

Significant differences between two means:
*  $p \leq 0.05$
**  $p \leq 0.01$
MEAN NUMBER OF NEMATODES

(log. scale)

WEAN LACT CONT

10000

1000

100

10

*    **
EXPERIMENT 12

Geometric Mean Egg Production for Female T. retortaeformis Recovered 15 days after Infection from Lactating, Weaned, or Control Rabbits.

(Each rabbit received an infection of 5,000 larvae).

(See Plan of Experiment for details).

WEAN = parturient, and weaned of litter, shortly after infection
LACT = parturient before, and lactating during, infection
CONT = control does

Significant differences between two means:

* $p \leq 0.05$
** $p \leq 0.01$
MEAN DAILY EGG PRODUCTION PER FEMALE (log. scale)
DISCUSSION

Prologue

The characteristics of Trichostrongylus retortaeformis infections in rabbits, and the effects on the characteristics of the various reactions studied, are considered in this portion of the thesis. The results of all the experiments are discussed together for respective features so that the various factors examined can be compared in the same section. Gordon (1957) reviewed the effects of various factors as manifestations of immunity. A general review of different features in nematode parasites has been published by Rogers (1962), and Haley (1962b) outlined effects of host relationships.

The pattern of infection of nematodes in their hosts has been described by Jarrett, Jarrett, and Urquhart (1968) as consisting of four successive phases: an initial loss of larvae on infection (Loss Phase 1); an established level of nematodes (Plateau Phase); a subsequent loss of adults (Loss Phase 2); and a level of persistent infection (Residual Phase). They showed for Nippostrongylus in the rat, that effects due to host resistance occurred at Loss Phase 1 within 1 day, so that variations in infectivity of larvae cannot be distinguished from the variations in susceptibility of the host. In uncomplicated infections of Trichostrongylus retortaeformis in laboratory rabbits, the Loss Phase 2 appeared to usually occur around week 4, so that nematode recoveries up to this time were considered to relate to establishment. Recoveries after week 4 were considered as persistent infections.
EFFECTS ON PARASITE MEASUREMENTS

(i) Infectivity of Larvae, and Establishment

Definition

The infectivity of infective larvae (the ability to infect) is described as the proportion taken in by a susceptible host that survives and becomes established. It is measured by the recovery of mature nematodes (or inhibited forms surviving the same period) after the pre-patent period, but before a loss of parasite population due to some reaction in their parasitic environment. (A different measurement may be made by the recovery of developing larval stages in the host before the Plateau Phase.) The establishment of infection in the host (the capacity to be infected) is also measured by the recovery of nematodes at the Plateau Phase. It is considered that infectivity is determined by the physiology of the infective stage of the parasite, but establishment is influenced by the physiology of the host. Thus, variations in infectivity must be assessed in uniform groups of hosts, and host variations in establishment with uniform batches of larvae.

Infectivity

The physiology of the infective stages and the process of infection have been reviewed by Rogers and Sommerville (1963, 1968), who considered that infection by strongylate nematodes is initiated by the response of the infective larvae to a stimulus for exsheathment. It was found (Experiment 8) that exsheathment of *T. retortaeformis* was
facilitated by aging. Augustine (1923) and Lapage (1935a) similarly noted that older larvae exsheath more readily than young third-stage larvae. Trichostrongylid larvae stored at 4°C for more than 3 months showed a tendency to increased rate of exsheathment (Silverman and Podger 1964) but there was no appreciable change for Haemonchus up to 3 months (Slocombe and Whitlock 1970).

While the rate of exsheathment (Experiment 8) was directly related to the establishment of infection for larvae which were aged at 20°C up to 30 days from the collection of donor faeces, this relationship was not followed for the oldest larvae (aged 60 days). It appears that the ability to exsheath readily on ingestion by the host does not necessarily mean an ability to infect and develop in that host.

The fate of the oldest larvae may be explained by the observations that live exsheathed undeveloped larvae may be recovered from the faeces for a few days after dosing previously uninfected hosts (Scott 1928; Stoll 1941; Thatcher and Scott 1962b). Paradoxically, Stoll (1941) reported that these larvae are still infective. On the other hand, T.B. Stewart (1958) and Daskalov (1966) found third-stage larvae passed in the faeces were still sheathed. These may not have received an exsheathing stimulus, or may have been immature larvae.

Some of the larvae in the oldest batch (aged 60 days) were found to stick to the side of the glass tube on washing with balanced salt solution after 2 hours exposure to hydrochloric acid (Experiment 8). Crofton (1947) found a similar occurrence with "fresh" T. retortaeformis larvae. This phenomenon may be related to infectivity, and it is noted that Leland (1967b) obtained better development of Cooperia to adults when derived from eggs that adhered to the side of a tube.

Two opposing effects on the infectivity of survivors were seen when infective larvae were held at 20°C
(Experiment 8) - up to 30 days, an increasing infectivity ("maturation"), followed by a fall in infectivity ("aging"). A hypothesis to distinguish between the effects is illustrated in Figure 29. In an earlier observation (preceding Experiment 8), very poor development was found from young *T. retortaeformis* infective larvae compared with larvae aged at 20°C. In two groups, of 22 adult female rabbits each, 41% had positive egg counts (mean 40 e.p.g.) 15 days after infection with larvae from a 5 day faecal incubation, compared to 100% (mean 1,910 e.p.g.) with 25 day incubation larvae. A similar effect to aging was seen when infective larvae were held at 4°C ("storage") over a prolonged period. The declines in the recovery of adult *T. retortaeformis* when infective larvae were stored for 8 weeks or 14 weeks were not statistically significant (Appendix II and Experiment 7), but storage for 54 weeks before infection (Appendix II) caused a marked reduction in infectivity.

It is clear that infective stage larvae from different periods of faecal incubation and storage were not equally infective, and differences in the number of nematodes becoming established have occurred independently of the host. (It should not be overlooked that a very young batch of larvae may represent a selected population - the first to reach the sheathed third-stage, and an older batch of larvae may represent a selected population - the survivors.)

A sole report of increasing larval infectivity with age in strongylates has been found. Durie (1957) reported a higher infectivity of *Haemonchus* larvae aged at 26°C for 20 days from collection of faeces, than of those recovered at 5 days. Analogous improving development in *vitro* has been shown for *Dictyocaulus* aged at 15°C up to 19 days (Silverman 1963), and for *Haemonchus* aged in aerated water up to 16 days (Silverman, Alger, and Hansen 1966).
VARYING INFECTIVITY, OF THE SURVIVING INFECTIVE-STAGE LARVAE, WITH TIME

(A general hypothesis)

The initial increase in infectivity from the appearance of infective larvae may be a common phenomenon, as a time course has been reported for Bucephalidae in host blood and in egg (Wakelin 1968) and Ancylostoma (Moore 1933). Todd, Hansen, Kelly, and Wyke (1960) also found increasing establishment of Ascaridia after aging in the egg, and they suggested that differing behaviors of the developing parasitic stage larvae stimulate different degrees of host resistance. A decline in observed infectivity for infective larvae has been reported, although it is not always observed in other genera of nematodes, for example, Trichostrongylus (Keesling 1966a, data adapted from Peterson 1964), Trichuris (Peterson 1964 and Cornell and Jones 1966), Ascaris (Keesling 1966b), and Dipetalonema (Tucker et al. 1954, 1955) in environments such as moisture and temperature were held constant. The numerical value of the scale will vary with environmental conditions.

The numerical value of the scale will vary with environmental conditions.
The initial increase in infectivity from the appearance of infective larvae may be a common phenomenon, as a time requirement for "maturation" appears in other groups of nematodes. It has been reported for Dirofilaria in host blood (Kartman 1953), Trichuris in the egg (Wakelin 1969), and Ascaris in the egg (Stoll 1933). Todd, Hansen, Kelly, and Wyant (1950) also found increasing establishment of Ascaridia after aging in the egg, and they suggested that differing behaviour of the developing parasitic stage larvae stimulated different degrees of host resistance.

A decline in infectivity with time has been reported for infective larvae (presumably of survivors) of other strongylates, for example: Necator (Payne 1923; Ackert 1924); Ancylostoma (Payne 1923; Rogers 1939); Haemonchus (Rogers 1940); T. colubriformis (Herlich 1966a; data adapted from Ciordia, Bizzell, Porter, and Dixon 1966) and T. axei (Herlich and Ryan 1970); Nematospiroides (K. M. Dash 1970 pers. comm.); Nippostrongylus (Haley and Clifford 1958, 1960; Simaren 1970); and Dictyocaulus (Cornwell and Jones 1970).

A decrease in infectivity with age has also been reported in other groups of nematodes, for example: Strongyloides (Barrett 1969); Ascaris (Jaskoski 1960); and Ascaridia (Ackert, Cooper, and Dewhirst 1947a, 1947b; Ameel, Elliott, and Ackert 1950; Elliott 1954; Todd, Hansen, Kelly, and Wyant 1950).

The degree and rapidity of known physiological changes associated with age are determined by environmental factors such as moisture and temperature. These factors were held constant in the present study. It is likely that at higher temperatures, which favour faster development and faster mortality of free-living stages (see Levine 1963), the changes found in infectivity would occur more rapidly. The depth of water and oxygen tension in which the larvae were held may also have been important.
Radiation is another factor that has been shown to affect larvae, with enhancement of infectivity (maturation process?) at low levels, followed by a decline of infectivity (aging process?) as the levels of X-irradiation were increased (Ciordia and Bizzell 1960; Mulligan, Gordon, Stewart, and Wagland 1961; Kassai, Fitzpatrick, and Mulligan 1966).

Constant conditions in the laboratory do not have direct application, but do have direct implications to the field situation. Thus studies which assess only development and survival of infective stages (see Kates 1965) are incomplete without infectivity data. Studies with "tracer" grazing animals have shown that some strongylate larvae may retain infectivity when aged on the pasture for prolonged periods (see Crofton 1963), and a few laboratory studies have also checked infectivity of aged larvae (for example: Mönning 1930; Durie 1961; Andersen, Wang, and Levine 1966; Andersen and Levine 1968; and also Levine 1937 for Ascaridia), but such studies have yet to assess the proportion of surviving larvae that can become established in the host.

The nature of changes in the infective larvae which effect their parasitic development are unknown. Silverman (1963, 1965b) stated that the physiological state and the prior history of infective larvae were important. It was considered that aging to a "ready state" due to an "intrinsic maturation" was necessary for later development or for exsheathment of strongylates (Silverman, Alger, and Hansen 1966; Meza-Ruiz and Alger 1968), and separation from inside the sheath was used to determine when infective larvae were "mature" (Hansen, Silverman, and Buecher 1966).

Sprent (1963) found that the ascaridate Amplicae cum larvae may not develop beyond the infective third-stage in its definitive host, if it did not reach an apparent "threshold of maturity" before ingestion. He later suggested that one feature involved in failure to
subsequently develop might be lack of availability to the larvae of an unknown growth factor (Sprent and McKeown 1967). Some feature other than the ability to exsheath rapidly as suggested for strongylates must be invoked to account for the maturation period required for the ascaridate and rhabditate species cited, in which the infective larvae are non-sheathed, or may still be in the egg.

Changes in a number of metabolic parameters during aging of infective larvae have been measured (see Rogers and Sommerville 1963; Eckert 1967a; and especially Barrett 1969, for earlier references; Clark 1969). For example, the rate of oxygen consumption decreases in infective larvae with age (Schwabe 1967). The loss in lipid from free-living infective larvae with time has been examined by several authors as an index of physiological age, although Rogers (1939) first reported that it fell more rapidly than the loss in infectivity. Contrarily, Durie (1957) found the increase in infectivity of Haemonchus larvae occurred after the loss of all detectable fat. In ascaridates, which do not feed during their free-living development, loss of lipid in the larva occurs during maturation, and thus during the phase of increasing infectivity. The fall in lipid content is more likely to be directly related to the activity of larvae, which itself is not a measure of infectivity in an individual larva.

If parasitic development is initiated by chemical reactions that may become inhibited (Rogers 1966, review Rogers and Sommerville 1968), then holding infective stage larvae may lead to the formation ("maturation"), and later the alteration ("aging"), of the chemical substrate or mechanism.

In order to monitor the varying infectivity of a population of strongylate larvae (as in Figure 29), it appears to date that exsheathment rate provides the best simple index of the gain of infectivity during maturation, and that loss of viability amongst a
population probably provides the best sign of the fall in virulence of the survivors during aging.

The other factors examined in this thesis which affected the establishment of *Trichostrongylus* in the host seem less startling.

Establishment

Higher recoveries of adult *T. retortaeformis* were found at 4 weeks after infection, with a moderate level of infection, when the larvae were administered in ten multiple doses, rather than the same number of larvae in a single dose (Experiment 10). It appeared that the multiple doses did not continue long enough to reach a maximum infection of the rabbits (after which the proportion of larvae established would decline), because many of the rabbits had retained 90% to 100% of the total of the ten infective doses. Michel (1963b, 1969a), working with *Ostertagia* in calves, concluded that daily dosing resulted in a plateau of establishment at about 4 weeks, after which there was a turnover of adult nematodes. The magnitude and timing of this maximum was dependent on the dose level of infective larvae.

On the other hand, with a low level of infective larvae, Ackert, Cooper, and Dewhirst (1947b) found for *Ascaridia* no effect on the number of nematodes established when the infection was given as ten multiple doses, compared with a single dose of the same total size.

The results suggest competition between potentially infective larvae to become established in the host, due to some requirement, the availability of which is depleted or altered in response to those larvae that first develop in sufficient numbers. It is not unlikely that immune features may play a part in a response. D.F. Stewart (1958) pointed out that the size of an initial dose of trichostrongyloid larvae was important in the development of immunity, and suggested that following a reduction in the rate of larval intake, an increased establishment of
infection related to a lower build up of immunity. Gibson (1952) showed a greater acquired immunity, to a challenge infection of *T. axeii* in lambs, from a previous infection when given as ten multiple doses, than when given as a single dose.

More *T. retortaeformis* were established with a primary infection in adult female rabbits (Experiments 9 and 10), than in rabbits that had a previous infection (which was at a low level and terminated with an anthelmintic soon after patency). This protection of the host, by initial low level infection, occurs commonly in trichostrongyloid infections (e.g. Gibson 1952; Michel 1953; Gibson and Everett 1963) and may be considered as a vaccination effect. Urquhart, Jarrett, Jennings, McIntyre, and Mulligan (1966) reported that protection following a vaccination dose of infective larvae was more successful in adults than in young animals.

Host responses against the establishment of *Trichostrongylus*, acquired from a previous infection, are apparently expressed during the development of the immature parasite. For *T. colubriformis* in guinea pigs, Herlich (1966c) showed that there was a response to third-stage larvae, and Dineen and Wagland (1966b) reported immunological attack against the developing fourth-stage larvae. From other work (e.g. Chandler 1932b; Ross 1963) smaller adult nematodes could be expected to develop in secondary infections.

It appears to be unusual for nutrition per se to affect the numbers of nematodes which become established, although the development and fecundity of the parasite may be affected as discussed later. Apparent effects of nutrition on establishment of infection may be associated with other factors such as an effect on growth of the host (see Bawden 1969a, 1969b).

The type of food (laboratory pellets or lucerne) given to the rabbits was not shown to have a direct effect on the numbers of *T. retortaeformis* established, although
there was evidence that it modified the effect of previous infection (Experiments 9 and 10).

Rohrbacher (1957, 1960b) reported no statistical difference in the total number of *T. axei* established in rabbits fed on laboratory pellets, laboratory pellets plus lucerne, or on fresh grass. In young rabbit kittens, Rohrbacher, Porter, and Herlich (1958) recovered a significantly smaller number of *T. colubriformis* from those sucking the doe compared to weaned litter mates. However, sucking did not interfere with the establishment of this parasite in calves (Rohrbacher, Porter, and Herlich 1958) or in lambs (Gordon 1960; Connan 1968c).

The influence of host sex on the numbers of nematode parasites recovered has received several brief reviews (e.g. Culbertson 1941 pp.24; Sadun 1951; von Brand 1952 pp.277; Haley 1958a; Mathies 1959; Dobson 1961; Solomon 1966), and recently a more extensive review has been published by Solomon (1969), with about 140 references to the influence of sex and reproduction on helminth parasites.

The establishment of *T. retortaeformis* in juvenile female rabbits was significantly greater than in juvenile male rabbits (Experiment 8). This appears contrary to the general theory advanced by Ford (1968), and outlined in the Introduction to Part II of this thesis. However, it is readily explained by the conclusion that low levels of oestrogen may favour the parasite (Ford 1968 pp. 334). There is evidence of a reciprocal relationship between the pituitary and the gonad in juvenile animals (Donovan and Harris 1966), and the effect seen in Experiment 8 may arise as females approach puberty at an earlier age than males.

In the adult female rabbits, pregnancy made no difference to the number of *T. retortaeformis* established (Experiment 11). Similar observations have been made for *Nippostrongylus* (Haley and Sansone 1962; Haley and Lassner 1965), and for *Ostertagia* in the field (Connan 1968b).
Further, lactation did not cause a significant difference in the number of *T. retortaeformis* recovered 15 days after infection (Experiment 12). There do not appear to be any published reports on the number of nematodes established from infection during lactation. Connan (1968a) treated non-breeding ewes with a tranquilizer to raise prolactin levels, and recovered significantly more *Ostertagia* from them than from controls following daily doses of larvae. The author did not consider an effect of the tranquilizer independent of a prolactin effect in his report. Work by Oshima (1961) showed an effect on recovery of *Toxocara* due to lactation and prolactin. However, his experiments involved a paratenic host, in which development of the parasite meant resumed tracheal migration and loss of nematodes in the faeces.

There was a significant depression of the *T. retortaeformis* recovered at day 15, when doe rabbits were weaned of their litters soon after infection (Experiment 12). It appears that there was a sudden expulsion of adult nematodes associated with drying-off, because at day 10 the egg counts were normal. Published reports on the establishment of nematodes in weaned parturient animals are not known, but Jacobs (1966) put forward a similar view to explain a sudden decline in egg count following weaning of sows.

(ii) Development and Growth of the Parasite

Rate of Growth

The growth of *Trichostrongylus retortaeformis* larval stages in the small intestine of the young male rabbit was characterised in Experiment 1. Rose (1969) illustrated the parasitic development of a species of a closely related genus, *Ostertagia*, which appears to be applicable...
to the growth of *Trichostrongylus retortaeformis*, allowing for the slower maturation time and greater length of *O. ostertagi*.

Developing *T. retortaeformis* third-stage larvae recovered at day 4 were longer than at day 2, as distinct from a residual population of third-stage larvae in which growth was inhibited (Experiment 1). No inhibited larvae were recovered that were greater than 600 µ in length.

Immature larval forms persisted longer in young rabbits infected with larvae stored 14 weeks at 4°C, than with fresh infective larvae, thus indicating a delay in development following cold storage (Experiment 7). A similar retardation of development has been found for *Nematospirooides* when the infective larvae were stored at 4°C for 2 to 3 months (K.M. Dash 1970 pers. comm.).

There also appeared to be a delay in population development following aging at 20°C for 60 days (Experiment 8). The frequency distribution of measurements of nematodes recovered at day 11 suggested that the most advanced adult females, seen in the other infections from younger larvae, were not present, resulting in a mean delay in the population of nematodes present. From this it may be hypothesised that the infective larvae with the potential for the fastest parasitic development are either the first to die, or to become uninfected, on aging or storage. They probably use up their energy reserves more rapidly than the survivors.

Such a feature could provide a useful mechanism in the perpetuation of the parasite species. If infective larvae have the opportunity to age on the pasture, then the likelihood of ingestion by the host must be small, and the environment may be unsuitable for further immediate transmission. Therefore there would be no advantage for the parasite to mature and produce eggs to recontaminate the pasture in the minimum possible time, and a delay in development would be advantageous. This possibility is reinforced by the finding of a greater delay in egg production with storage or aging, as discussed later.
When a larval dose was administered in several multiples (Experiment 10), there was no evidence of a delay in development.

In the results for the recoveries of parasitic larvae, the terms "moult third-stage" or "moult fourth-stage" as used by some other authors have been avoided. The description is based on a visible separation of the cuticle from underlying cuticle of the succeeding stage. It has been observed that separation of an inner newly forming cuticle prior to an ecdysis, and a cuticular dilatation at other stages, may be artificially caused by some of the preparation methods used in the recovery and examination of nematodes. Principle causes are heating, and a low pH solution such as formalin (pH ca.4), or for pepsin digest of the gut (pH 1 - 2). It should be noted that with careful examination of individual larvae (as in Part III of this thesis), the moult third-stage and moult fourth-stage can be identified without separation of the two cuticles. Because this identification was not always carried out in Part II, the terms have not been used, and the larvae are classified as late third-stage or late fourth-stage respectively.

Missing Larvae

In Experiment 1, it was reported that at day 4 after infection, a considerable number of larvae were apparently not accounted for (there being only about 10% of the number recovered at day 6). The missing larvae were the population expected in the earliest fourth-stage and at the third ecdysis. At day 2 there was a similar, less noticeable, gap in the size distribution. Also, there was a gap, for each sex, in the size distribution at days 6 and 8, for larvae expected in the earliest fifth-stage and at the fourth ecdysis.

In addition it was noted in Experiment 8 that many larvae were missing at day 5, compared to the recovery at day 11. The growth of larvae was approaching the
distribution at day 6 in Experiment 1. All the larvae found were in between, and none at, either the earliest fourth-stage or the earliest fifth-stage.

Three possibilities could explain the disappearance of developing larvae. First, it is technically difficult to recognise larvae less than 3 mm long amongst digesta. However, special steps were taken in the present study to minimise this difficulty, and their success was indicated by the frequent recovery from the gut of third-stage larvae less than 1 mm long. Second, the larvae may not be in the gut, but this was not supported experimentally.

Third, it was considered that larvae, immediately after moulting in the gut wall (third and fourth ecdysis), were susceptible to the action of the acid pepsin digestion used for the recovery of larval stages in the gut wall. When *T. retortaeformis* larvae which had reached the moult third-stage in culture (Part III Section A) were incubated in pepsin-hydrochloric acid, it was found that the outer third-stage cuticle remained intact while the pre-fourth-stage larva became digested. Similarly, dead nematodes placed in a pepsin digestion are destroyed. Further, the earliest fourth-stage larvae were recovered at 3 days without digest of the gut wall (Part III Section A).

The hypothesis suggested, therefore, is that following ecdysis the nematode cuticle undergoes a hardening process, possibly tanning (review: Fairbairn 1960), which protects the larva from an unfavourable environment. Before this hardening is complete, the developing larva is susceptible to chemical attack, and intestine species of *Trichostrongylus* will be destroyed by pepsin digestion. The tanning process may continue during development (Bird 1958). Extra protection is achieved by larvae of members of other strongylate families by a digest-resistant cyst formed around the developing larval stages in the gut wall (Dash 1970). Hobson (1948) reviewed the concept of protective anti-enzymes, as alternative protection to the
cuticle. Some larvae evade digestion, as exhibited by the pulmonary migration of Nippostrongylus, which cannot survive in the gut until after the second ecdysis (Schwartz and Alicata 1934b).

Migration of Larvae

Evidence of migration by T. retortaeformis developing larvae in the rabbit was not established (Experiment 2), supporting the conclusion that the parasitic larvae of intestine Trichostrongylus species do not leave the intestine (Männig 1926).

When T. retortaeformis larvae were exsheathed and introduced into the circulation of rabbits (Experiment 2), it appeared that they did not reach the intestine and mature. The result indicates a lack of ability to escape from the vascular system and establish in the gut, because control larvae treated in the same ways for exsheathing and then administered to other rabbits by stomach tube developed into patent infections.

In other work, attempts were made to recover T. colubriformis larvae from the intestinal lymphatic trunk of a sheep. Lymph was examined for 100 hours after infection. In the first 24 hours after injection of infective larvae into the abomasum, 26 larvae were recognised from collected lymph, representing 0.05% of those administered.

It is generally held for trichostrongylids that there is no migration outside the gastrointestinal tract (Levine 1968 p.196), although species in closely related families (such as ancylostomids and heligmosomids) may undergo a pulmonary migration before developing in the gut. However, even for some ancylostomids, a migration is not necessary for development after oral infection of normal hosts (Yokogawa and Oiso 1926; Foster and Cross 1934).

If trichostrongylid larvae were to migrate from the gut it is likely that some would pass through to the
lymphatic drainage vessels, and thence to the circulation, as this is a route followed by *Nippostrongylus* (Gharib 1961; Clarke 1967), and some of the other strongylate species (Mahaffey and Adam 1963; Machida 1965; Waddell 1969) which finally mature in the gut. However, it has been reported that parasitic larvae lack orientation in migratory behaviour, and may be carried at random around the body, so that the more larvae, the more likely that various routes would be used (Fülleborn 1929; Waddell 1969; McCully, Kruger, Basson, Ebedes and van Niekerk 1969).

Larvae of a trichostrongylid, *Haemonchus contortus*, were recovered by Ransom (1920) from the lungs of a guinea pig 2 days after being fed the larvae, and later Ransom (1923) reported that *H. contortus* "grow to a considerable size" in the guinea pig. This suggestion of a trichostrongylid migration appears never to have been confirmed. Indeed, Wood (1958; Wood and Hansen 1960) and Ford (1966 unpublished) were not able to infect either rabbits or guinea pigs with *H. contortus*. Although Wood recovered 12 nematodes from two doses of 100,000 infective larvae which were exsheathed before dosing, a repeat experiment failed. Stoll (1943) found that *H. contortus* failed to reach the abomasum after sub-cutaneous or intra-peritoneal injection in sheep.

The only other known reports of a trichostrongylid recovered outside the lumen of the gut or its crypts are for *T. axei* said to be in lymph nodes (Parnell 1962), and for *Cooperia* spp. in the sub-serosa (Bailey 1949) and pancreas (Ivanova 1969). The text reports, without references, that the larvae of intestine *Trichostrongylus* species "penetrate" or "burrow" into the mucosa "underneath the epithelium" (Lapage 1956 p.65; Levine 1968 p.218, p.262; Soulsby 1968 p.224, p.226) are presumably carried on from the original text (Mönning 1934), and from the report that they "burrow into the intestinal mucosa" (Michel 1952a).
In histological sections of the small intestine of guinea pigs infected with *T. colubriformis*, Herlich (1969a) showed that the larvae which had infiltrated the mucosa were still in the lumina of the crypts. He pointed out (Herlich 1969a, 1969b), that it is still unanswered whether similar involvement with the tissues of the small intestine occurs in the normal host. In the present study, *T. retortaeformis* adults were usually recovered in the small intestine wash, but larvae were almost invariably found in the small intestine digest.

The experiments described indicate that migration, at least through the intestinal lymphatic drainage and into the anterior vena cava, and probably through the intestinal portal drainage, is not a common or essential part of the life cycle for *Trichostrongylus* spp. The very small proportion of larvae in the sheep that passed through the intestinal wall and through the mesenteric lymph nodes probably represent aberrant forms.

Such aberrant larvae may have an immunological importance in carrying specific antigen to the host immunologically receptive cells in the lymph. In small infections (less than 1,000 larvae), none may penetrate the gut wall and there may be little if any immediate stimulus of a host response. After a time a small adult nematode infection may liberate sufficient material to stimulate an immune reaction (related to the duration of the infection). However, it is possible that to provide a more immediate antigenic stimulus (related to the size of the infection), sufficient to exceed a threshold of immunity (Dineen 1963), the ingestion of a large number of larvae is required for a minimum number of aberrant forms to penetrate the host mucosa.

*Trichostrongylus* larvae show a remnant of a former migratory process in their penetration amongst the villi of the small intestinal mucosa to pass the third ecdysis (Mönnig 1926; Föllleborn 1929). In comparison, species from other strongylate families still migrate. In
support of this explanation, a parallel situation is noted amongst ascaridate families. Members of the family Ascaridae migrate, while members of Ascaridiidae do not, except for the occasional finding of aberrant larvae outside the gastro-intestinal tract (Guberlet 1924; Ackert 1931; Roberts 1937).

For *Trichostrongylus* in lagomorphs, and certain rodents, another mechanism could replace an ancestral pulmonary migration. Refection occurs daily in the rabbit (Taylor 1940; Eden 1940), and third-stage larvae, which have passed through the gut alive as already mentioned, may be returned to the stomach by coprophagy. This possibility has not been established for *Trichostrongylus*, but a precedent has been established for *Toxocara*, whereby undeveloped larvae passed in the faeces resume development to mature infections when reingested (Sprent 1961).

More intensive study is required to specifically determine if *Trichostrongylus* spp. leave the lumen of the gut and its crypts for a brief period during development.

Location of the Nematode

Tetley (1937) and Davey (1938a) found that intestine *Trichostrongylus* species can withstand high levels of acidity as found in the stomach. In addition, the pH of the soft faecal pellets excreted is relatively low (Griffiths and Davies 1963), and similar to the pH 6.5 expected in the duodenum (Robinson 1935). In the present study, *T. retortaeformis* were not found in the stomach in primary infections, but appreciable numbers of inhibited third-stage larvae were recovered (Experiment 3) from the stomach digests of adult rabbits infected several times, and which had received a large number of larvae.

Davey (1938b) related the usual location of intestinal *Trichostrongylus* species, including *T. retortaeformis*, at the proximal end of the duodenum, to the finding that
they could withstand high levels of bile salts, as found near the bile duct opening, better than other trichostrongylids. The location of strongylate nematodes in the small intestine is also influenced by the presence of the other sex of the parasite (Drum 1965; Roche 1966), and varies with the duration of the infection (Brambell 1965; Alphay 1970).

In the observations for this thesis it was confirmed that in the rabbit, *T. retortaeformis* are mainly located in the proximal part of the small intestine. The movement of *T. vitrinus* and *T. colubriformis* towards the distal part of the small intestine and the large intestine has been related to their expulsion from sheep (Tetley 1937) and guinea pigs (Herlich, Douvres, and Isenstein 1956; Gordon, Mulligan and Reinecke 1960; Sturrock 1963; Connan 1966a). The adult *Trichostrongylus* spp. recovered from the large intestine of the rabbit (Hesterman and Kogan 1963) may have been on the way out. Wood (1958) reported that the *T. colubriformis* found in the large intestine of the rabbit were moribund.

(iii) Inhibition of Larvae

Although the phenomenon of arrested development ("inhibition") of strongylate nematodes has received a lot of attention since a brief review by Taylor and Michel (1953), and since Sommerville (1953, 1954) drew attention to its occurrence in *Ostertagia*, it has not always been clearly defined. Michel (1963b) and Dunsmore (1963) emphasised that arrested (inhibited) development meant a specific stop in the process of maturation, and must be distinguished from retarded (delayed) development, when growth to the adult stage is continuous but the nematode does not mature in the usual pre-patent period. The two phenomena were separated in the present study by
examining the nematode population after the pre-patent period, when inhibition was evident as an accumulation of arrested larvae at a particular step in development.

This work confirmed that *T. retortaeformis* may become inhibited in the small intestine at the third-stage (Michel 1952c), when the nematode is less than 1 mm long, and very difficult to discover and identify amongst digesta during nematode counts. It is not surprising that there are fewer findings of inhibited *Trichostrongylus* spp., than of other genera which are larger when inhibited, especially as it has not been uncommon in some laboratories to wash gut samples before counting in a sieve with an aperture greater than 200 µ, through which immature forms pass readily. Bull (1953b) stated that while counting "immature" *Trichostrongylus* in the rabbit, third-stage larvae seen were not counted. It is possible that many workers have not appreciated that *Trichostrongylus* spp. third-stage larvae could have been inhibited parasitic forms, and regarded them as recently ingested infective larvae as did Bull (1955, 1964).

A significantly greater number of inhibited third-stage *T. retortaeformis* larvae were found in older rabbits which had been previously infected, than were found in primary infections in younger rabbits, when dosed with 25,000 larvae (Experiment 7).

When infective larvae were aged at 20°C up to 60 days, inhibited larvae were not recovered from infections with doses of 2,500 larvae (Experiment 8).

Storage of infective larvae in the cold for 14 weeks caused a significantly greater number of *T. retortaeformis* to be inhibited than in infections with fresh larvae, when rabbits were dosed with 25,000 live larvae (Experiment 7). The effect was only detected at a high dose level, because when the experiment was repeated with doses of 2,500 live larvae (Appendix II), no inhibited larvae were recovered in infections from larvae stored at 4°C up to 54 weeks.
Further, it is worth noting that there may be an effect (as yet unproven) of storage of infective larvae leading to an elevated immunity and different antibody response to infection. The adult female rabbit from which only 0.3% of the total larval doses were recovered (all inhibited third-stage) (Experiment 3), was first infected with larvae stored 14 weeks in the cold (Experiment 7). In contrast, eight times as many nematodes were recovered (a fifth of them adults) from the comparative rabbit first infected with fresh larvae. It was also discovered in work not reported in this thesis (Ford 1971b), that 3 weeks after the primary infection, these rabbits had produced different reaginic antibodies to separate fractions of third-stage larvae.

In the other experiments, when doses of 2,500 or 5,000 infective larvae were used, the incidence of inhibited larvae recovered was not great enough for statistical testing. However, the effects found over all experiments to be most important, are summarised (for primary infections with fresh larvae) by the results in the following schedule, which shows the maximum recoveries (+) of inhibited larvae, as the proportion of the larval dose.

<table>
<thead>
<tr>
<th>Larval Dose</th>
<th>Age of Rabbit</th>
<th>larval dose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>juvenile</td>
<td>adult</td>
</tr>
<tr>
<td>2,500</td>
<td>0⁺</td>
<td>3%</td>
</tr>
<tr>
<td>25,000</td>
<td>2%</td>
<td>17%</td>
</tr>
</tbody>
</table>

Thus, the principle requirements for the detection of inhibited larvae appear to be: (a) A sufficient number of infective larvae susceptible to conditions causing inhibition must be taken in by the host; (b) An environment that will induce inhibition of such susceptible larvae must exist in the host.

Inhibition of some larvae was anticipated from the different batches of *T. retortaeformis* used following aging or prolonged storage (Experiment 8 and Appendix II).
as the proportion of other larvae inhibited has been reported to vary from batch to batch (e.g. Dunsmore 1963; Michel and MacKenzie 1965; Parfitt and Sinclair 1967), and for *Ostertagia* the proportion has been shown to increase with aging of infective larvae (Armour, Jennings, and Urquhart 1969b; Armour 1970). Three factors are suggested to contribute to the presence or absence of inhibited larvae.

Firstly, inhibited larvae were recovered at a high level of infection, while at a lower level inhibited larvae were not detected. This difference is outlined by the hypothesis illustrated in Figure 30, which summarises the following discussion of the literature. A dose of 2,500 larvae given to young rabbits may have been insufficient for the expression of inhibition and the subsequent detection of arrested larvae. Thus, Michel (1952c) used doses of 100,000 infective *Trichostrongylus* larvae and reported up to 35% of the dose inhibited, while for *Ostertagia* Armour (1970 pers. comm.) used 100,000 dose with up to 19% inhibited, and Connan (1969) used 75,000 dose with up to 10% inhibited, in individual host animals.

In addition to a greater number of inhibited individuals being available for detection with a greater number in an infective dose, it has been shown for several trichostrongylids that a smaller proportion of moderate infective doses becomes inhibited than of large infective doses (Thomas and Urquhart 1956; Dunsmore 1960; Russell, Baker, and Raizes 1966).

However, Stockdale, Fernando, and Lee (1970) reported a high level of inhibition of *Obeliscoides* in young male rabbits with doses of only 2,500 infective larvae that had been stored in the cold. The proportion of the larval dose that exhibited the characteristic is not available. At post mortem examination in the pre-patent period they found some nematodes still at the fourth-stage, but it is not clear from their report whether these larvae were actually arrested, or were retarded as reported for stored *Trichostrongylus* in the present study.
Michel (1969a) has shown that with continuing larval intake, the apparent rise in the number of inhibited larvae after a plateau of mature nematodes is reached (Michel 1963b), may be described by a constant proportion of each larval dose being added to the number of existing inhibited forms. (Note a different impression is given by reporting the arrested larvae as a proportion of the number of established parasites remaining in the host, than by reporting them as a proportion of the infective larval dose – see Figure 30.)

The second factor possibly contributing to inhibition of development is that the larvae ingested by the host may have to be "inhibition prone" (Armour 1970).

Seasonal climatic conditions may select against non-inhibiting larvae, or may alter the physiology of surviving infective larvae, to cause variations in the proportion of larvae becoming inhibited in the host (Armour 1970).

The likelihood of larvae becoming inhibited appears to be genetically controlled. Several laboratory passages of Ostertagia have been found to drastically reduce the ability of the resultant laboratory strain to display inhibition in calves, compared with a freshly isolated field strain (Armour, Jennings, and Urquhart 1967, 1969b; Armour 1970), and Denham (1969b) did not recover inhibited larvae in lambs with a laboratory strain. Similarly, Ostertagia even when stored at 4°C has failed to become inhibited in lambs following several laboratory passages (Connan 1968b, 1969), but showed inhibition when the parent nematode population was that participating in the post-parturient rise in the field (Connan 1969). Michel is said (1971 private communication) to have been able to select a laboratory strain with an extended maturation time and increased predisposition for inhibition. Thus the scale on the hypothetical Figure 30 would be compressed or expanded depending on the strain of parasite.
Figure 30

VARYING INHIBITION, OF ESTABLISHED
PARASITIC-STAGE LARVAE, WITH SIZE OF INFECTIVE DOSE

(A general hypothesis)

- Total Number of Nematodes
- Adults
- Inhibited Larvae

\* % of Total

The numerical value of the scale will vary with
(a) the proportion of 'inhibition-prone' larvae
and (b) the conditions met in the host environment.

There may initially be a latent period in the wild strain of *G. rostecseforma* in Australia, and
the passage speed of the larva in the intestine could also
have tended to increase in the absence of eruptive
inhibition. The effect of a behavioural advantage in unusual
laboratory practices made from those
nematodes that mature in the minimum time.

A third reason possibly contributing to low levels
of inhibition in these experiments is the effect
the environment to influence the growth of the
inhibited. It has already been suggested under
the intensity of larvae and establishment, that the most
rapidly developing larvae may alter the requirements for
which larvae compete in the host environment. It is now
also suggested that if sufficient larvae arrive and
develop together; the availability to each larva of space
in the environment will be altered. Of those
larvae not already past the critical point will become inhibited. Donohue (1963) found
larvae larvae were inhibited and those in the dose placed
in the abomasum were not inhibited.
An early concept related to larval development was
the threshold, an immune mechanism caused the development of
larvae to be inhibited by a temporary immune response in the
host. The theory of immune reaction has been reviewed
by Urquhart, Darrell, and Halliday (1961), Meaden (1963)
and Michel (1965).
There may initially be a low potential for inhibition in the wild strain of *T. retortaeformis* in Australia, and the passages carried out in this laboratory would also have tended to select against the characteristic. Inhibition of development confers no advantage in usual laboratory practice, and passages are made from those nematodes that mature in the minimum time.

A third reason possibly contributing to low levels of inhibition in these experiments is an unsuitable environment in the host.

The number of the infective larval dose could affect the environment to influence the proportion becoming inhibited. It has already been suggested under Infectivity of Larvae and Establishment, that the most rapidly developing larvae may alter a requirement for which larvae compete in the host environment. It is now further suggested that if sufficient larvae arrive and develop together, the availability to each larva of some factor in the environment will be altered so that those established larvae not already past the critical phase will become inhibited. Dunsmore (1963) found that when *Ostertagia* larvae were exsheathed and the whole dose placed in the abomasum of sheep, almost all became inhibited.

An earlier concept related the number of larvae taken in to an immune response causing inhibition. For example, Dineen, Donald, Wagland, and Offner (1965) considered that when the number of larvae reached an antigenic threshold, an immune mechanism caused the development of further larvae to be arrested. Schwartz, Alicata, and Lucker (1931) suggested that inhibited development was due to elaboration of a specific growth inhibiting substance by a host that had had a previous infection. Taliaferro and Sarles (1939) considered arrested larvae to be immobilised by a temporary immune response in the host. The theory of immune reaction has been reviewed by Urquhart, Jarrett, and Mulligan (1962), Madsen (1962), and Michel (1968).
A significantly greater number of inhibited third-stage larvae were found in older rabbits which had been previously infected, than were found in primary infections in younger rabbits, when dosed with 25,000 *T. retortaeformis* larvae (Experiment 7). This could be due to the previous dose (an immune reaction), or to an effect of host age. No experiments were designed in this thesis to examine the specific effect of either of these factors on inhibition, but it does appear from the overall results summarised earlier that a greater proportion of the larval dose is likely to be inhibited in adults than in young rabbits.

Diet and reproduction had no significant effect on the level of inhibition found in adult female rabbits given moderate levels of infection (Experiments 9 to 12). However, Rohrbacher (1957, 1960b) reported an increased proportion of "immature" *T. axei* in rabbits at 20 or 30 days when they were given lucerne or green feed instead of laboratory pellets. Secondly, he found less "immature" larvae when lucerne was fed in doe rabbits mated after infection, than in non-pregnant rabbits. There are no known effects of sex on inhibition.

A failure to observe inhibition of intestine species of *Trichostrongylus* has been noted under field conditions in which *Ostertagia* larvae were inhibited (e.g. O'Sullivan and Donald 1970). Early fourth-stage *T. axei* were found to accumulate in the stomach of sheep (Benz and Todd 1969; Anderson 1971 in press; A.D. Donald 1971 pers. comm.), although Vegors (1957, 1958) did not obtain inhibition of *T. axei* when *Ostertagia* larvae were inhibited.

Unfortunately many authors have reported inhibited larvae as "immature" forms, a term used to describe fourth-stage or the immature fifth-stage. Michel (1963b) discussed the inhibition of nine genera, and Muller (1968) also briefly reviewed the stage at which inhibition of gastro-intestinal nematodes occurs in grazing animals.
Bull (1953a, 1964) reported that many rabbits contained immature forms, and (third-stage) larvae, of *T. retortaeformis*. The possibility is not excluded in reports for immature larvae that the intestine *Trichostrongylus* species may be susceptible under certain conditions to an additional or alternative latent phase at the early fifth-stage, such as has been reported for *Dictyocaulus* (Taylor and Michel 1953). Based on field survey data, Muller (1968) stated that in the case of *Trichostrongylus* spp. the accumulation occurred at the fifth-stage, compared with fourth-stage in other genera. He did not record any third-stage larvae, but did say that larvae in the third ecdysis were classified as fourth-stage. No evidence was obtained in the present study to suggest arrest of *Trichostrongylus* in the intestine at other than the third-stage, and it is possible that the fifth-stage larvae reported by Muller (1968) were developing rather than dormant.

Dineen and Wagland (1966b) assumed the experimental susceptibility of *T. colubriformis* to immune attack at the fourth-stage was relevant to inhibition at this stage as noted for *Haemonchus* and *Ostertagia*. Denham (1969a) suggested that inhibition of *T. colubriformis* did not occur on the basis of a failure to recover fourth-stage larvae.

The dormant stage of *Trichostrongylus* is further considered in Part III Discussion.

(iv) Persistence of the Parasite Population

Although experiments for this thesis were generally planned to examine stable infections of *T. retortaeformis*, the infection level adopted appeared to have been around an immunological threshold, and loss of infection commonly occurred at about 4 to 5 weeks after dosing with infective
larvae. In rabbits given moderate doses of larvae, about 1,000 to 5,000 nematodes matured. A level of approximately 1,000 to 2,000 adults was found could survive in some rabbits beyond the first critical time as above, but otherwise there was a substantial loss of infection leaving a residual level of 0 to about 500 adult nematodes.

The expulsion of established nematodes from the gut is described as Loss Phase 2 in the pattern of infection (Jarrett, Jarrett, and Urquhart 1968) as mentioned on p.96. Even following single dose primary infections, the loss of nematodes is considered to be a self-cure phenomenon (e.g. Mulligan, Urquhart, Jennings, and Neilson 1965). The self-cure reaction induced by a challenge dose of larvae has been shown to cause a change of the parasite's environment in the gut, associated with some immunological changes (Stewart 1953, 1955). For *T. colubriformis*, the time of the loss of infection may be delayed by adaptation (in an abnormal host), or may be precipitated by transfer of isogenic immune cells in the guinea pig (Wagland and Dineen 1965). Gordon (1967) reviewed the self-cure reaction for intestine *Trichostrongylus* species in relation to other trichostrongylids.

Several factors were found to influence the loss or survival of *T. retortaeformis* beyond week 4 at moderate levels of infection.

Rabbits which had received the infective larvae split into ten multiple doses showed a highly significant loss of this infection at week 8, compared with the rabbits given the same number of larvae in a single dose, in which the infection persisted (Experiment 10). On the other hand, self-cure of *T. colubriformis* at a challenge infection was reduced when the previous infection was given in multiple doses (Gordon 1967).

The rabbits with a primary infection showed a highly significant spontaneous loss of *T. retortaeformis* after 4 weeks, compared to the rabbits which had received a previous infection, and in which the loss was non significant.
The most persistent *T. retortaeformis* were found in rabbits with secondary infections that were fed on lucerne.

The single factor with the greatest effect on persistence of the infection was diet (Experiment 10). There was no apparent loss of infection in the rabbits fed lucerne, while least nematodes were recovered at week 8 from the rabbits fed laboratory pellets. Gordon (1950) reported that 9/10 sheep fed a better ration showed loss of *T. colubriformis* infection, but only 3/10 on a poorer ration did so. Similarly, Bawden (1969b) reported that nutrition affected the survival of *Nematospiroides* infection in mice.

The three influences on *T. retortaeformis* outlined above all had one property in common. The greater the number of nematodes established and the greater the egg production in the Plateau Phase of establishment, then the higher the likelihood of, and the more complete, the expulsion of the nematodes at the Loss Phase 2.

Lactation was discovered to promote persistence of *T. retortaeformis* infection (Experiment 11). Around day 30, loss of infection was detected in the control rabbits, associated with zero egg counts 5 days earlier, but the parasite population persisted in the parturient does. At the post mortem examination 45 days after infection, only the lactating rabbits maintained their infection. Some late fourth-stage larvae were present at this time in lactating does (presumably from previously inhibited larvae, that were undiscovered in the rabbits killed earlier in the course of the infection).

Bull (1964) suggested that "some factor" prevented self-cure of *T. retortaeformis* in breeding female rabbits. It is shown above that this factor is a result of lactation. The finding was confirmed by Connan (1970), who showed that lactation in rats prevented the self-cure of *Nippostrongylus*. 
(v) Egg Production by the Parasite

A most important facet of the life cycle is the output of eggs to provide a source of contamination to perpetuate the parasite. Variations are found in the duration of the pre-patent period, the rate of the initial rise in egg count, the magnitude of the peak egg production, the duration of the initial patent period, and the egg output during the residual period of infection. Most of the published data on experimental T. retortaeformis infections of laboratory rabbits are based on faecal egg counts (Whitten 1948; Michel 1963a).

Pre-patent Period and Initial Rise in Egg Count

Faecal egg counts at 1 week after infection with T. retortaeformis were always negative, and at 2 weeks were usually positive, but there appeared to be some variation in the pre-patent period between these times. In Experiment 1 a pre-patent period of 11 to 13 days was reported in young male rabbits, but this was from infections with larvae harvested at 7 to 9 days of faecal incubation. With larvae recovered at 12 or more days of incubation, the pre-patent period was subsequently found to more commonly be only 10 days (e.g. seen in Experiments 11 and 12 with adult female rabbits).

It is clear that the pre-patent period does vary with the larval batch.

The pre-patent period was prolonged in rabbits dosed with infective larvae aged for several weeks (Experiment 8). When the infective larvae had been exposed to several months cold storage before dosing, the pre-patent period was also delayed (Experiment 7 and Appendix II). This is contrary to the report that storage of Cooperia larvae had no influence on the pre-patent period in rabbits given massive doses (Besch 1963, 1964).

Although there was a delay in development of T. retortaeformis following long storage of the infective
larvae (as discussed earlier), a second effect appears to have influenced the detected pre-patent period. It was noticed in individual rabbits on several occasions that the pre-patent period was prolonged when only low levels of nematodes were established (e.g. seen in Experiments 4, 8, 11, and Appendix II). Sturrock (1963) reported the same effect for *T. colubriformis* in guinea pigs given graded doses of infective larvae, and Herlich (1957) showed a shortened pre-patent period in calves given high levels of larvae. On the other hand, Besch (1963) stated that the pre-patent period was not influenced by the size of the larval dose, but it appeared that only large doses were used.

From Sturrock's (1963) illustration it can be seen that the rate of the initial rise in egg count is much slower with lower levels of infection and thus detectable levels appear later. This effect was seen for *T. retortaeformis* in Experiment 4, and was also noticed with multiple dosing in Experiment 10. With a higher minimum detectable level of eggs counted in faeces, the pre-patent period with a low level of infection would appear to be even longer.

However, a depressed rate of the initial rise in egg count was observed following cold storage of the infective larvae, even when similar numbers of nematodes were established (Experiment 7).

While pregnancy had no influence on the rate of rise of egg counts (Experiment 11), a faster rise was found in the rabbits infected when lactating, compared to controls (Experiment 12).

Food type was not discovered to effect the beginning of patency (Experiment 10), although host nutrition has been reported to effect the pre-patent period of another trichostrongylid (Weir, Bahler, Pope, Phillips, Herrick, and Bohstedt 1948).

*T. colubriformis* was shown in the laboratory to have a longer pre-patent period in resistant sheep (6 - 8 weeks)
than in non-resistant animals (15 - 20 days) (Gordon 1958b).

Egg Production per Female Nematode

The quantity of eggs produced by females is probably the parameter examined which is the most susceptible to environmental influences - particularly to those related to reactions by the host following previous experience of the parasite. Chandler (1932b) showed that even after contact of the host with one nematode, the egg production by females in subsequent infections of a different, related, genus of nematode, was reduced.

A daily output of about 200 eggs per female is the "usual" level for *T. colubriformis* (Gordon 1950), and a similar level was found during the initial patent period for *T. retortaeformis*.

The magnitude of egg production by *T. retortaeformis* females was depressed in rabbits that had been exposed to a previous infection (Experiments 9 and 10). The same effect has been reported for *T. colubriformis* in sheep (Gordon 1957, 1958b; Stewart and Gordon 1958; Mulligan, Gordon, Stewart, and Wagland 1961; Denham 1969a), and appears to be of wide occurrence. It is especially well known for *Nippostrongylus* (e.g. Chandler 1932b; Spindler 1936; Sarles and Taliaferro 1936; Ogilvie 1965; Jarrett, Jarrett, and Urquhart 1968).

It was observed for *T. retortaeformis* that the egg production per female also became reduced during the course of a current primary infection (Experiment 10). That is, depression of egg production followed experience of the parasite by the host, whether the experience came from a previous or from a current infection.

It appeared that the changes in the intestinal environment, which caused expulsion of some of the parasites a few weeks after infection, also depressed the egg production of the survivors of the same infection. The greater the loss of infection, the greater the suppression
of egg output by the residual infection (Experiment 10). Host experience of a previous infection thus protected the parasites that developed - because there were fewer established, there were fewer expelled and greater fecundity of those present, compared with a primary infection after 4 weeks.

Suppression of egg production that results from development in an unfavourable intestinal environment may not be permanent, because when the parasite is transferred to a fresh host environment fecundity rises to a normal level (Chandler 1936; Ogilvie and Hockley 1968). But other effects that suppress egg production may be permanent when acting on fully developed adult nematodes (Ogilvie and Hockley 1968).

With the lower levels of multiple doses of infective larvae in Experiment 10, the egg production per female at week 4 was higher than from a single infection, but was suppressed after week 5 or 6. Sarles (1929a) and Krupp (1961) reported that the egg production per female Ancylostoma was reduced with increasing levels of infection, over a moderate level.

Michel (1963b) showed that as the total number of Ostertagia larvae taken in with daily doses increased arithmetically, the egg production from calves decreased exponentially, irrespective of the number of adults remaining (which declined, and reached a trough after several months). The number of eggs found in the uteri of females declined during the course of the experiment.

From subsequent experiments, Michel (1967) advanced the theory that the total egg production was limited by the host, and later presented more details in an important series of papers (e.g. Michel 1969a, 1969b, 1969c, and several others). With single dose Ostertagia infections, Michel (1969b) found an exponential decrease in total egg production during the experiment, similar to the decrease observed with multiple dose infections (Michel 1969a). He counted more eggs in the uteri of females when lower
numbers of nematodes were present, but showed that during the course of an infection the total number of eggs released was depressed more rapidly than the decline in eggs present in the uterus per female.

The observations for *T. retortaeformis* and their gradual decline in egg counts (Experiments 4 and 6) are comparable to Michel's findings. It was calculated that the commencement of the decline (Appendix II) could be described by an exponential fall which was similar to that reported by Michel for *Ostertagia*, and by Sarles (1929b) for *Ancylostomum*. Sarles (1932) also showed an exponential decrease in egg count with a moderate infection from 2,500 *T. calcaratus* larvae in the laboratory rabbit.

It was demonstrated that the onset of egg output by *T. retortaeformis* infections from aged or stored larvae was delayed at the beginning of the initial patent period (Experiments 7 and 8, Appendix II).

When egg production per female was examined at a later stage, there was found to be a direct association with the time of cold storage of the infective larvae (Appendix II). At this time, 29 days after infection, it appeared that the total egg output from the higher levels of infection (derived from fresher larvae) was being limited by the host (cf. Michel 1967 cited above), with a correspondingly higher output of eggs per female at the lower level of infection (derived from stored larvae).

It has also been shown for *T. colubriformis*, that the egg output is suppressed in females derived from larvae subjected to unfavourable conditions before infection (Jarrett, Jennings, McIntyre, and Sharp 1960; Mulligan, Gordon, Stewart, and Wagland 1961).

It is apparent that the initial rate of egg production is dependent on the history of the infective larvae, but the host response controls the peak egg output and determines the subsequent fall in egg output. Host suppression of nematodes already in the gut affects their cytological structure (Ogilvie and Hockley 1968; Lee 1969),
and it seems that a delay in egg production may have some survival value for the parasite. The female nematodes with a delay in egg output may make a major contribution to a subsequent post-parturient rise in egg counts, as mentioned later.

The type of food eaten by the host has been shown to influence the level of egg production per female nematode. There was a highly significant increase in the *T. retortaeformis* female egg output during the initial patent period from rabbits fed laboratory pellets compared with lucerne (Experiments 9 and 10). The influence of diet on egg production was not the same as the effect of diet on the establishment of infection. Subsequently, at week 8, the reverse effect of the two foods on egg production was seen, but this was directly associated with the loss of infection. In a pilot study, in young male rabbits given 2,500 larvae, no effect was detected on the development and decline of *T. retortaeformis* egg production due to feeding fresh carrots *ad lib.* from 4 days before until 6 days after infection.

Most studies on diet and egg production of trichostrongylids have been carried out with *Haemonchus,* where it has been shown for example that egg production is increased by feeding laboratory pellet type food instead of cereal and lucerne (Vetter, Hoekstra, Todd, and Pope 1963), by feeding pelleted food with cereal instead of lucerne (Kates, Allen, and Wilson 1962), and by feeding cereal chaff instead of lucerne (Roberts 1957). Bawden (1969a) reported greater egg output for female *Oesophagostomum* on feeding cereal chaff than on feeding lucerne. On diets with reduced fibre egg production was found to be depressed for *Haemonchus* (Theuer, Vetter, Hoekstra, Pope, and Todd 1965), and for *Nematospirooides* (de Witt and Weinstein 1964).

These findings are consistent with the results for *T. retortaeformis* at the beginning of egg production, except for the influence of fibre (which was lower in
laboratory pellets than in lucerne). On the other hand, egg production persisted much longer in the rabbits fed lucerne.

No direct comparisons of host age with primary infections of *T. retortaeformis* are reported in this thesis, but comparable egg counts were obtained from juvenile and adult rabbits given similar doses of larvae in different experiments. In a pilot study when young and adult male rabbits were infected with 2,500 larvae, no difference in egg count pattern (as in Figure 7 from Experiment 5) was found related to age of the host.

It has not been established whether egg production per female *Trichostrongylus* is influenced by host age. Gibson and Everett (1963) found a depression of egg production by female *Nematodirus* with age resistance in sheep.

It was discovered that the egg production by female *T. retortaeformis* in young male rabbits was significantly higher than in young female rabbits (Experiment 8). This is consistent with the experimental findings and review conclusions by Ford (1968) that androgens in the host favour the egg production early in infection, and the influence is independent of the effect on the establishment of infection. The reversal of effects on establishment and female egg production in Experiment 8 may be related to an interaction in the juvenile host with levels of growth hormone, which have been shown to influence the immune response (see Pierpaoli, Fabris, and Sorkin 1970).

Two effects for lactation, on egg output per female nematode, were seen in Experiments 11 and 12. No effect of lactation was observed for nematodes recovered 15 days after infection (Experiment 12). The rabbits received only a moderate dose of infective larvae, and a rise in initial egg production greater than the rise in control rabbits may not be exhibited with less than a threshold dose of larvae. In the field, the availability of larvae may exceed the threshold at which nematodes are suppressed in non-lactating animals. The level of increased egg
count with lactation in ewes in the field is complicated by a relationship to nutrition and larval intake, and at times a rise in egg count may not be seen (e.g. Tetley and Langford 1965).

The first effect for lactation was a clearly persistent egg production of _T. retortaeformis_ in the lactating does, which included a rise in egg output after parturition in those in which the egg count had fallen to zero. There were significantly more eggs per female passed by the nematodes recovered from rabbits in mid-lactation than from the non-lactating rabbits (Experiment 11).

Although Taylor in 1935 expressed the view that the post-parturient egg count rise in ewes may be referable to a greater egg production of nematodes already present, it has not received experimental investigation in published work. Connan (1968b) for example, showed an increase in faecal egg count and also did _post mortem_ nematode counts, but did not give the relationship of the increased egg production to the egg output by female nematodes. For _Trichostrongylus_ in sheep, O'Sullivan (1970) and O'Sullivan and Donald (1970) supported the view that an increase in egg production by mature nematodes, as seen here in rabbits, provided the major source of the rise in lactation.

Further comparisons with the literature are not made because more than seventy references to increased egg output with reproduction are known, of which the latest published review available (Gibbs 1967) mentions about half. The literature up to 1967 has already been discussed by Ford (1968 pp.55).

It is noted that there are marked effects on the habitat of the nematode, associated with lactation: For example, an increased voluntary food intake (see Forbes 1970), an increased blood flow to the intestine (Chatwin, Linzell, and Setchell 1969), and an enlargement of the intestines as observed in this study.

The second effect for lactation was a marked drop in egg production of _T. retortaeformis_ when the parturient
does were weaned of their litter (Experiment 12). The suppression of egg output per female nematode recovered 15 days after infection was highly significant, and much more marked than the fall in the number of nematodes recovered. The sharp fall in egg count after weaning has been reported to occur in sows (Jacobs 1966; Connan 1966b, 1967b) and in ewes (Connan 1966b, 1967a, 1968b; Arundel and Ford 1969; Salisbury and Arundel 1970; O'Sullivan and Donald 1970), with mixed strongylate infections.

In an approach to investigate the mechanism of the post-parturient rise, O'Sullivan (1970) showed that the higher egg production during lactation could be reproduced with *T. colubriformis* in the guinea pig. He found that the transfer of isogenic immune cells resulted in a similar suppression of the parasite during lactation as seen when transferred to non-lactating guinea pigs. He concluded at that time (1970) that the post-parturient rise follows an immunological depression during lactation. However, O'Sullivan showed that immune cell transfer suppressed the parasite irrespective of whether the host was lactating or not, and therefore the result has not eliminated the interpretation that an additional cause (due to lactation) may account for the different levels in *Trichostrongylus* egg output between non-immune lactating and non-immune non-lactating guinea pigs.

It is suggested that the host hormones associated with lactation (e.g. prolactin) exert an advantageous effect on the development and the egg output of a parasite (growth hormone or anabolic influence). This effect may be additional to a raising of the (immune) threshold of nematode production that is tolerated by the host. The relative importance of each mechanism in a given situation requires further study. The (immune) expulsion of *T. retortaeformis* adults already present did not occur during lactation, as it did before lactation or in the control rabbits (Experiment 11). Connan (1970) also found that lactation in rats prevented the self-cure of *Nippostrongylus*. 
The more *Trichostrongylus* present the greater the potential egg output in lactation to contaminate the pasture and infect the next host generation. Thus it is to the parasite's advantage not to precipitate self-cure, when there is both a loss of infection as discussed earlier, and the residual parasite population may be damaged (Ogilvie and Hockley 1968). Because inhibition of *Trichonstrongylus* larvae is not as prevalent as in other trichostrongylid genera, the presence of more adult nematodes is needed to contribute to the post-parturient rise than for e.g. *Ostertagia* or *Haemonchus*, where inhibited larvae have been shown to play the major role.

Slow development of *Trichostrongylus* at establishment has been seen to lead to greater persistence of the infection, and is hypothesised to be a useful mechanism in the perpetuation of the parasite species (especially when the environment was unfavourable such that free-living stages aged before ingestion, as discussed earlier). It is suggested that the more slowly developing populations of *Trichostrongylus* may play the major role in a post-parturient rise, when environmental conditions become more favourable for transmission on pasture.

Populations of *T. retortaeformis*, with mature females containing eggs in the uterus, but from which no eggs were detected in the faeces, were found associated with aging of the infective larvae (Experiment 8). The same thing presumably occurs in other cases when zero egg counts are followed by a rise in egg production that cannot be accounted for by maturation of inhibited larvae. Denham (1969a) noted the phenomenon for *T. colubriformis* recovered from "immune" sheep.

**Egg Size**

It was demonstrated in Experiment 5 that the dimensions of *T. retortaeformis* eggs vary between samples, and the frequency of eggs of different sizes shows significant changes during the course of infection. These changes may
explain to some extent the different egg sizes for
*T. retortaeformis* in the literature, as reported in the
Introduction to Part II.

The scatter diagram of 100 *T. retortaeformis* eggs
from a wild rabbit in New Zealand (Bull 1953a) shows smaller
dimensions than those found here, including from natural
infections in wild rabbits. Dunsmore (1970 pers. comm.)
also noticed eggs from Australian wild rabbits to be
larger than shown by Bull. All these eggs were examined
in faecal samples. The discrepancy may indicate different
strains of the nematode present in New Zealand and southern
New South Wales. It is noted that two intestinal types
of *Trichostrongylus* in sheep, *T. vitrinus* and
*T. colubriformis*, from the same locality show different
dimensions (Shorb 1940; Tetley 1941).

A regular sequence of egg sizes was found (Experiment
5). At the beginning of a patent period, transient small
elongated eggs appeared. The eggs passed early when egg
counts were rising were smaller than the eggs which appeared
later in each patent period when egg counts were falling.
The size of eggs on the pasture has some developmental
and survival importance (Waller and Donald 1970).

The increase in size during an egg laying period has
remarkable parallels in other animals. The changing egg
size in birds is an accepted fact, and related not so much
with body size as with physiological development (C.G. Payne
1970 pers. comm.). The oocyst size of coccidia also
increases during development of infection, and has been
shown to be influenced by the number of the infective dose,
the age of the host, and the immune response (Rommel 1970).

The smaller *T. retortaeformis* eggs obtained from
resistant rabbits (Experiment 5), may indicate an actual
depression of egg size, or alternatively show that a fresh
nematode population in an immune animal may not develop
past the early stage when small eggs are passed.

It was noted (Experiment 5) that after establishment
of *T. retortaeformis* infection, a heterogeneic population
of eggs may appear in a faecal sample. Similar heterogeneic
size distributions have been shown for other intestinal nematode eggs (Piatkowska 1966). It does not follow that there is a change in the size of the female nematode population, as Bull (1953a) found no correlation between the length of *T. retortaeformis* females and the dimensions of eggs recovered from the uterus.

When infection of groups of reproducing and control adult female rabbits was compared (Experiment 11), significant changes in the incidence of larger versus smaller eggs occurred. At days 15 and 30 there were more smaller eggs from the control, than from the pregnant, rabbits, suggesting that individual nematodes had not reached peak egg production in the former at the time of sampling. More smaller eggs appeared from the lactating rabbits at day 45, suggesting renewed egg production, compared with the non-lactating rabbits.

**Periodicity of Egg Output**

A rhythm of *T. retortaeformis* egg count peaks and troughs was described with Experiment 4. It was distinct from circadian cycles (of light or feeding activity) and seasonal cycles (of climatic, nutritional, or reproductive nature) which have been reported for parasitic nematodes. In young male rabbits given single doses of infective larvae periodicity of egg production appeared, with peaks at intervals of a little more than 3 weeks, which bears no relation to any routine in the animal house. The rhythm appeared to be delayed in onset with continuous low level dosing with infective larvae. In the post mortem examination of infections from non-reproducing adult female rabbits every 2 weeks (Experiment 9), peaks of egg production greater than 300 eggs per female per 24 hours were found at week 2 and week 6. Michel's (1963a) sketches of egg counts from *T. retortaeformis* in rabbits showed a periodicity with peaks occurring roughly every 4½ weeks. It is noted that a similar periodicity, with an interval of roughly 3 weeks, appears to fit the individual
egg counts for Ostertagia given by Michel (e.g. 1963b, 1969a).

Seven typical basic patterns of T. retortaeformis egg counts are shown with a logarithmic scale in Figure 31. They are taken from actual counts (as for Figure 5, Experiment 4). Peaks at lower levels (patterns one and two) exhibit the general periodicity, which is suggested to be a natural rhythm.

Higher peaks (which are followed by steep falls) in egg counts, tend to be detected at the faecal sampling time preceding the general periodicity (patterns five and six). The steep falls are assumed to be due to a host immune response to the parasite biomass and/or rate of egg output, and represent a loss of infection. Pattern four is suggested to represent a threshold where the host response may not be exhibited until after the natural peak. If the egg output begins to rise to a threshold level again there is another host immune response associated with a subsequent peak.

Pattern three appears to represent a special case of a labile host-parasite equilibrium: as the egg production rises to a relatively high level it is suppressed before the natural peak, but there is no loss phase, so that the same effect is repeated. It is from these rabbits (in which the peak egg counts appear out of phase with the hypothetical natural periodicity) that more than 2,000 adult nematodes are recovered several weeks after infection.

Wykoff (1959) also showed a periodicity in laboratory rabbits, again of 22 days, for the egg output of a trematode parasite, with a major peak in egg count every three periods (10 weeks).

What appears to be the same periodicity has been observed in the field from lambs. Crofton (1955) reported rhythmical fluctuations in egg counts, with a relatively constant interval of 2 to 3 weeks between peaks for individual hosts. Because the time of rise and fall of egg count was not the same for different individuals at
Figure 31

RHYTHMS IN THE EGG PRODUCTION
FROM T. retortaeformis INFECTION IN INDIVIDUAL RABBITS
(The Basic Patterns compared with the General Periodicity)

Pattern one
Level of established nematode burden (at 2 weeks)

- Pattern one, very low (less than 500)
- Pattern two, low (less than 1,000)
- Pattern three, moderate - just below threshold
- Pattern four, moderate - around threshold
- Pattern five, moderate - just above threshold
- Pattern six, high (more than 5,000)
- Pattern seven, very high (more than 10,000)

Pattern one (log. scale) with approx. 100 gms fesses per day

Pattern two

Pattern three

Pattern four

Pattern five

Pattern six

Pattern seven

DURATION OF INFECTION (weeks) AND GENERAL PERIODICITY (O)

This shows the rhythm was not determined by the continuous source of third-stage larvae. The presence of a new batch of nematodes developing for each rise in egg counts cannot apply to the moderate levels of single dose T. retortaeformis infections. In this study, amount of the nematodes matured before the first egg was seen as the reservoir of inhibited larvae. Therefore, at least...
pasture, the periodicity could not be recognised in mean data. This shows the rhythm was not determined by the availability of feed or of infective larvae. Crofton's observations were for mixed trichostrongylid egg counts, mainly T. vitrinus and Ostertagia, therefore the rhythm was not separate for nematode species.

Previously Tetley (1949c) compared the faecal egg count with the elimination of gastro-intestinal trichostrongylids in the faeces of a lamb at pasture. He showed coincidental cycles of elimination and falls in egg count which appeared to be synchronised for many species, and reported a rhythm of 15 to 20 days. T. colubriformis and T. vitrinus together were the most abundant species at post mortem examination, but were least expelled during the observations, in summer-autumn.

Britton (1938) showed cycles of peak Strongylus egg count at a little over 3 week intervals, synchronised for two horses running together. Major troughs occurred after every third peak (but at different times for the separate infections, which commenced at different times).

Palmer (1941) found a rhythm of Necator egg production in man, which was directly associated with a cycle in faecal output of about 2 weeks.

Crofton (1955) suggested the termination of egg count peaks by a host immune response to a new larval intake, with loss of nematodes and replacement by a new infection, similar to Michel's theory (1952c, 1963a) that rhythms of T. retortaeformis egg counts were due to relays of third-stage larvae developing to adults in batches. Subsequently Michel (1963b) suggested that development of Ostertagia to adults was continuous with a continuous source of third-stage larvae. The theory of a new batch of nematodes developing for each rise in egg counts cannot apply to the moderate levels of single dose T. retortaeformis infections in this study, as most of the nematodes matured before the first peak and there was no reservoir of inhibited larvae. Therefore, at least
for *Trichostrongylus*, changes in the egg output per female were the most important source of cyclic variation.

However, a heterogeneic population of mature nematodes may contribute to successive rises in egg production. Tetley (1937) found that it was possible for a large elimination of *Trichostrongylus* to take place from the small intestine, and yet an appreciable population to remain. It has already been discussed that large and small *T. retortaeformis* eggs may occur at the same time, apparently representing the tail and the start of successive egg count cycles. It is not known how the reappearance of smaller eggs comes about in the absence of a source of larvae, infective or inhibited. It seems unlikely that the nematodes get smaller during the time of depression of egg production, and Michel (1963b) dismissed this possibility. Perhaps the amount of material a nematode is able to acquire for egg production is less at the beginning of a laying period.

Figure 32 illustrates the hypothetical natural periodicity due to alternating cycles of egg production. The sum of the successive cycles is the typical observed egg count pattern, with the magnitude of each rise dependent on the level of infection and host response as already discussed. A trough below the minimum detectable level is observed as a zero count. The supposition that mature nematodes from a single larval dose may form a heterogeneic population is supported not only by the egg size information, but also by the spread in development (Experiment 1), and by the finding of bimodal populations of female (but not of male) *T. retortaeformis* 11 days after infection (Experiment 8).

Palmer (1941) suggested that periods spent by the nematodes in copulation could cause cycles in egg release.

The control of a rhythm of egg production by the parasite has two obvious possibilities: an innate periodicity of the nematode; or a response to an innate cycle in the host. It is suggested that they may be the
RHYTHMS IN THE EGG PRODUCTION FROM *T. retortaeformis* INFECTION IN AN INDIVIDUAL RABBIT

(The General Case)

There is ample evidence that host reproductive hormone may control the parasite and influence the response of the host (Ford 1968, pp. 33). Pierpont and Jorick 1968, review some of the work of Frederiksen and van Doort 1968. 

There is ample evidence that host reproductive hormone may control the parasite and influence the response of the host (Ford 1968, pp.33, Pierpont and Jorick 1968, review some of the work of Frederiksen and van Doort 1968). In a man, the regulatory hormones of the cycle of a few weeks may be expected to be measured, as reported for total nematode egg output (Pierpont and Jorick 1968). In other host species, the periodicity of parasite egg output approximates the interval of an annual cycle. If values (1976) suggested that peaks of nematode egg output at 3-week intervals in the ewe were dependent on natural activity.

The numerical value of the scale will vary with the level of infection and response of the host (see previous figure).
same, with an integration of rhythms during a host-parasite evolutionary association. Integration of parasite and host life cycles are common (Ford 1968 pp.364; Solomon 1969). Further, sub-cycles of strongylate egg laying activity with a periodicity of a few days have been correlated with physiological activity in the host (Tetley 1949c; Rep, Vetter, Eysker, and van Joost 1968).

There is ample evidence that host reproductive hormones may control the parasite (Ford 1968 pp.333) or influence the response in the host (Ford 1968 pp.347; Pierpaoli and Sorkin 1968; review by Kappas and Palmer 1963), and it seems that a periodicity in activity by the parasite of a few weeks may be associated with a sexual cycle. In a man practising the "rhythm method" of contraception, a peak activity every 2 weeks would not be unexpected, as reported for total nematode egg output (Palmer 1941). In other host species, the periodicity of parasite egg output approximates the interval of an oestrus cycle. Jacobs (1966) suggested that peaks of *Oesophagostomum* egg output at 3 week intervals in the sow were dependent on hormonal activity.

Other hormonal rhythms could occur with the same periodicity as oestrus cycles, and they could be adrenocortical, gonadal, or pituitary - all of which are known to influence the host-parasite relationship (be it directly, or through immunological stimulation or depression), or they could be neural or unknown in origin.

The influence of various hormones on the parasite's habitat, the small intestine, has been reviewed by Levin (1969), and the importance of faecal excretion, intestinal absorption, and the entero-hepatic circulation, of steroids is well known (reviews: e.g. Wright 1958; Glover and Morton 1958; Aldercreutz 1970).

The possibility that host hormonal cycles cause periodicity in the nematode egg output is suggested. Nematode egg production is stimulated by host hormones (Ford 1968). It is hypothesised that the egg count of the
earliest maturing nematodes rises until a stage in the host cycle when the stimulus is withdrawn, or an immunological suppression is triggered, and the egg counts decline. (With a more severe suppression, there is expulsion of part of the nematode population as well.) In a heterogeneous population, the nematodes not affected by this decline may begin another cycle of egg production. Administration of a single dose of infective larvae to the host at different phases of its cycle may be reflected in variations in the timing of the first egg count peak, as noted in Experiment 4.

It is seen that in animals freely grazing at pasture the timing of the egg count rhythm differs between hosts (Crofton 1955). In the laboratory, the timing of the egg count periods from young male rabbits showed a significant synchronisation (Experiment 4). The rabbits were housed together in open mesh cages in the same room, and subject to the influences, surmised of pheromones, which lead to synchrony of hormonal cycles in animals closely associated with each other (Whitten 1957; Whitten, Bronson, and Greenstein 1968; McClintock 1971; discussion in Sadlier 1969 pp.252).

Refection and Egg Output

Coprophagy has long been known in the rabbit as a pseudo-rumination (Moses ca.1500BC; Madsen 1939), and has been associated with the daily resting period in wild rabbits in Australia (Myers 1955). In this laboratory, when rabbits were fed ad lib., the soft faecal pellets reingested directly from the anus were passed during the middle of the day. The observation by Mykytowycz and Hesterman (1958), that T. retortaeformis eggs are passed in the same concentration in both soft and hard faecal pellets from infected rabbits, was confirmed.

The total faecal output measured from ten rabbits, fitted with leather Elizabethan collars (Leahy and Barrow 1953 p.176) to prevent refection, showed 22% (range
18% - 28%) of the daily output to be soft pellets. This suggests that under these laboratory conditions approximately 1/5 of the *T. retortaeformis* eggs passed in any day will be reingested.

Besch (1965) stated that *Cooperia* eggs from a calf were passed in the faeces of rabbits 8 to 10 hours after feeding them, and these eggs were still viable. However, these rabbits were on a severely restricted diet. Reingested soft material in adequately fed rabbits remains in the gut for much longer than 8 to 10 hours (Thacker and Brandt 1955). If the eggs ingested with soft pellets were reexcreted, there would be a net balance over a period.

*T. retortaeformis* eggs recovered from faeces by sugar flotation (Whitlock 1959) were used to dose worm-free rabbits, and their faeces collected and examined for eggs several times a day for 4 to 6 days. In three trials eggs were recovered once, representing only 0.8% of the eggs administered, and spread out over 6 to 48 hours after dosing. It is apparent from the data of Elliott and Barclay-Smith (1904) with coloured beads, that by 3 days more than 50% of the eggs should have been excreted. It appeared from this observation that such eggs do not survive re-passage through the alimentary tract, and are probably digested. However, it is possible that eggs may not be destroyed when reingested directly from the anus, if they have not commenced development past the blastula stage at which they are normally excreted. Approximately 30% of the eggs used in the above observation became larvated during the harvesting procedures before administration.

Development of eggs may be triggered by a lowered temperature or a higher oxygen concentration when eggs have passed from the rectum, but delayed while the eggs are still in the host. Prasad (1959) found that *T. retortaeformis* eggs survived less than 24 hours at 40°C, but some survived at 35°C, and their development was delayed compared with eggs at 20°C to 30°C. Development
of ancylostomid eggs was inhibited at low oxygen concentration (Bandyopadhyay and Chowdhury 1964), and the development of Strongyloides eggs was inhibited by lactic acid (Supperer 1963). Lactic acid is formed in soft faecal pellets of the rabbit (Griffiths and Davies 1963). Further, Gibson and Everett (1961) recovered Nematodirus eggs passed through the alimentary canal of a sheep, although there was a loss of viability of eggs in the gut, or at 37°C in the laboratory, for more than 24 hours.

Hence, while the daily egg output in hard faeces from the rabbit is an accurate measurement of pasture contamination potential, the possibility remains that as an estimate for the daily egg production per female T. retortaeformis it may be up to about 20% too low.

EFFECTS ON HOST MEASUREMENTS

(vi) Body Weight Gain

Prologue

Measurement of host parameters was not a part of the design of experiments in this thesis, thus non-infected control animals were not kept. However, some striking differences in body weight gain between groups of infected rabbits were observed related to planned experimental variation.

None of the moderate levels of T. retortaeformis infection as used had any clinically observable effects on any rabbits, and the important sub-clinical effects were only detected by measurement. (Experiment 7 is excepted, where a higher level of infection was used, that was lethal in young rabbits.) Spedding (1953) drew
attention to the depression of live weight gain in lambs, due to *T. axei* infections that were not clinically detectable.

The host body weight gain was found to be affected by the history of the infective larvae. In Experiment 7 there was less depression of body weight at the beginning of the infection with the larvae stored in the cold for 14 weeks, than with the fresh larvae. Subsequently, the stored larvae appeared to be more pathogenic in the young rabbits that had no previous experience of infection, although a similar number of nematodes developed from both batches of larvae.

When *T. retortaeformis* larvae were exposed to cold for only 2 days before dosing, at moderate levels of primary infection (Appendix II) they caused lower body weight gain (and bigger spleens) during establishment of infection, than larvae never exposed to cold, although the same number of nematodes were recovered. If the effect on the larvae due to cold was continued in batches of larvae stored for longer intervals, then it was obscured by decreasing infectivity with storage (although the decrease between larvae stored 2 days (82.6% recovered) and 57 days (67.7% recovered) was not statistically significant). In this experiment there was a highly significant linear regression between body weight gain and faecal output (reflecting appetite). No reason is known for increasing pathogenicity of *T. retortaeformis* larvae after exposure to cold, but a change in immunogenicity possibly occurs also (Ford 1971b).

Anomalous results of the presence of parasites and effect on host body weight gain occurred in the experiments where the rabbit weights were compared. In Experiment 7 it was seen that loss of body weight in young rabbits was associated with a high level of nematode burden, while loss of body weight in the older rabbits that had been previously infected was associated with the expression of resistance to establishment of the nematode.
Chandler (1932a), in a review of resistance, pointed out that the development of resistance to establishment of a parasite infection is quite distinct to the development of resistance to results of a parasite infection. The two manifestations may be exhibited in harmony (direct effect) or in antipathy (paradoxical effect) as discussed separately below. In Experiment 10 the common observation, of the more nematodes recovered the poorer the body weight gain, was found during establishment of infection up to week 4. However, when the observations were continued to compare the persistance of infection up to week 8, only the reverse was found.

**Direct Effect on Body Weight**

A highly significant depression of body weight gain was found following multiple dosing, associated with a greater number of nematodes established, as compared with the same number of *T. reortaeformis* given in a single dose. In the previously infected rabbits there were less nematodes established, and a significantly greater body weight gain up to week 4, compared with primary infections. In contrast to Experiment 7 (with high larval dose) as commented on above, it appears that a state of merely being resistant (to a moderate larval dose) does not result in a depression of body weight gain.

It has been shown for *Ancylostoma* that the direct effect on the host bears a linear relationship to the total biomass of established nematodes (Georgi, le Jambre, and Ratcliffe 1969), and the level of *Haemonchus* egg production has been assumed to cause the severity of its effect on the host (Whitlock, Georgi, Robson, and Federer 1966).

The direct effect, for depression of host body weight gain with increasing levels of nematode parasite burden or egg production, and vice versa, is taken to be the normal, as reviewed for intestinal *Trichostrongylus* infections in the Introduction to Part II. It is therefore not discussed further.
Paradoxical Effect on Body Weight

The first anomaly appeared as an interaction with nutrition during establishment of *T. retortaeformis* (Experiment 10). The rabbits fed laboratory pellets made large weight gains during the first 4 weeks of infection, but also had slightly more nematodes, and in particular a highly significant higher egg output per female, than the infections in the other rabbits. A further significant experimental observation seems to be pertinent to the situation. The rabbits with higher body weight, or higher body weight gain, prior to infection showed a greater establishment of nematodes when fed laboratory pellets (Experiment 10) or when pregnant (Experiment 11), compared with non-pregnant control rabbits fed lucerne, in which the higher body weight rabbits had fewer nematodes.

This appears the same as the finding that more *T. axei* developed to adults in rabbits fed laboratory pellets (without lucerne) or when pregnant (with or without laboratory pellets), compared with non-pregnant control rabbits fed lucerne (*ad lib.* with laboratory pellets) (Rohrbacher 1960b). Such a nutritional involvement has been reported for other groups of helminth parasites, and has been discussed by Ford (1968 pp.352), who suggested a mechanism related to an anabolic influence stimulated by factors from the food or by reproductive hormones.

Four explanations have been suggested for the contrary situation of a higher parasite burden and greater host growth:

1. Thriving host (Fraser, Robinson, and Ritchie 1936)
2. Acquired tolerance by the host (Taylor 1934)
3. Enhancement by the parasite (Lincicome 1963)
4. Depressed expression of resistance by the host (Todd and Hansen 1961).
Fraser, Robsinon, and Ritchie (1936) found a positive association between trichostrongyloid burden and body weight gain in grazing lambs, and suggested that thriving lambs ingested more infective larvae. While a greater intake of larvae is no longer tenable (D.F. Stewart 1958), it is clear that an innate or nutritional factor is acceptable, as an explanation. For example, both an Haemonchus infection and the host were favoured by administration of testosterone (Ford 1968 p.315) or of a dietary supplement (Weir, Bahler, Pope, Phillips, Herrick, and Bohstedt 1948).

Gordon (1948) found a higher rise in Trichostrongylus egg counts and higher body weight gain in sheep grazing a winter crop, compared with sheep on a poorer pasture. In later discussion, (e.g. Gordon 1960) he pointed out that sheep with adequate nutrition may be resistant to the effects, but not to the establishment, of T. colubriformis.

Taylor (1934) gave a detailed consideration to experimental trichostrongyloid infections in grazing lambs. He suggested that the lambs with better body weight gains that had more parasites had acquired a tolerance to the parasites' harmful action, that is, a resistance to the effects of infection. The conclusions of Egerton and Hansen (1955) from experiments with Ascaridia support this concept. They suggested that serum transferred from a resistant host carried antitoxins that lead to better growth of parasitised hosts and better parasite establishment. Further investigation is required to distinguish between observations favouring this explanation, or supporting the former theory.

Lincicome (1963) briefly reviewed reports of enhanced growth in parasitised animals, and later (Lincicome 1971) put forward a mass of data to support the hypothesis that parasitism is good for the host. He noted, for example, elevated vitamin levels in infected hosts. Mueller has investigated the phenomenon for a cestode, in a series of papers, and suggested (e.g. Mueller 1968) that there is stimulation of the growth mechanism in the host. The
phenomenon, at least at low levels of infection, could be related to the first theory above. Weatherly (1970) described enhanced survival of mice with *Trichinella* infections.

The experimental results relating to the fourth explanation are outlined as follows.

The second anomaly occurred in association with the residual *T. retortaeformis* infection detected 8 weeks after dosing. The fewer nematodes present, the poorer the body weight gain (Experiment 10).

A loss of infection was invariably associated with a loss of body weight, and a highly significant fall in body weight gain occurred whenever there was a significant fall in nematode numbers, irrespective of the factor examined. The severity of the effect was clearly related to the level of the infection established at week 4, and is therefore taken to be a host immune response to the parasite.

The reaction causing loss of body weight with expulsion of the nematode burden over-ruled all other effects. In particular, it showed no moderation in the rabbits fed laboratory pellets which were most severely affected, thus reversing the favourable effect discussed above. The rabbits fed lucerne, which retained the most nematodes, did not suffer, and this was the only factor examined for which the host made a weight gain during this period.

Andrews, Kauffman, and Davis (1944) observed the same phenomenon for *T. colubriformis* when they showed the most depressed weight gain (of survivors) in lambs which expelled their infections. Loss of body weight was also observed with a challenge infection of *T. colubriformis*, when egg production of mature nematodes was completely suppressed (Stewart and Gordon 1958). Sturrock (1963) showed that, within groups, the guinea pigs infected with *T. colubriformis* which lost the most weight had the least nematodes.
Todd and Hansen (1951) found a positive association between the burden of *Ascaridia* and the body weight gain in chickens, and suggested that the energy used in resisting infection prevents maximum host weight gain. Thus the host achieves greater weight gains if it does not express a resistance to a parasite, present at low levels. This explanation may be extended to include a depression of body weight, associated with a self-cure type of immune response, in which it is more likely that the mechanism of expulsion itself interferes with the host well-being.

This situation has been noticed for trichostrongylid infection in the field by Anderson (1970a, 1970b), who discovered with prolonged observations of grazing sheep, that wool production was depressed in immune hosts which were continually rejecting new infections, compared with other groups of sheep which did not have the same exposure to infective larvae or which were protected by anthelmintic treatment.

As outlined above, it was disadvantageous to the host to reject moderate or low levels of infection. However, it is illustrated by Experiment 7, that the older rabbits which expelled a high level of infection - although they lost weight doing so - did survive, whereas the younger rabbits which did not express immunity lost weight anyway, due to the pathogenic level of the infection. Secondly, from an epidemiological point of view, Gordon (1948) pointed out that hosts which withstand the effects of many parasites are dangerous carriers.
PART III

STUDIES IN AN ARTIFICIAL CULTURE ENVIRONMENT

Initiation of Parasitic Stages

The initiation of the second ecysis, by a stimulus from the host on ingestion of the sheathed infective larva, is regarded as beginning the parasitic development of trichostrongyloid nematodes. Both chemical and temperature stimuli are needed for optimum rate of physiological exsheathment (review: Rogers and Sommerville 1969, 1968). Although development of third-stage larvae may continue in culture after a temperature elevation, without a known chemical stimulus (Weinstein and Jones 1957; Leland 1968b).

Lepage (1933a) obtained the first parasitic stage of T. retortaeformis by artificial exsheathment in vitro with short exposures to a sodium hypochlorite solution. It is a passive technique which exsheaths killed larvae (Glauser and Stoll 1940). It apparently acts by dissolving
INTRODUCTION


Following a consideration of the characteristics of intestine Trichostrongylus species in culture, this section deals with applications of culture to examine the physiology of the parasite. The main theme of this thesis is a study of variations in the features of the nematode, in response to factors which influence its environment. The aims are outlined with the Trials in Section A and Section B.

Initiation of Parasitic Stages

The initiation of the second ecdysis, by a stimulus from the host on ingestion of the sheathed infective larva, is regarded as beginning the parasitic development of trichostrongylid nematodes. Both chemical and temperature stimuli are needed for optimum rate of physiological exsheathment (review: Rogers and Sommerville 1963, 1968), although development of third-stage larvae may continue in culture after a temperature elevation, without a known chemical stimulus (Weinstein and Jones 1957; Leland 1967b). Lapage (1933a) obtained the first parasitic stage of T. retortaeformis by artificial exsheathment in vitro with short exposures to a sodium hypochlorite solution. It is a passive technique which exsheaths killed larvae (Glaser and Stoll 1940). It apparently acts by dissolving...
collagenous material of the cuticle, and ultimately dissolves the whole larva over a period of time (personal observations). The method has been used for other species successfully grown to the fifth-stage in culture (e.g. Weinstein and Jones 1956; Leland 1963). Crofton (1947) discovered that *T. retortaeformis* infective larvae exsheathed when incubated for 48 hours with 0.2% pepsin in hydrochloric acid at a pH of 4 or less. Subsequently, Sommerville (1957) found that *T. colubriformis* exsheathed rapidly in ingesta taken from the abomasum, and more slowly in ingesta from the small intestine, of a sheep. Rogers (1960) found that culturing in hydrochloric acid did not stimulate the exsheathment of *T. colubriformis* without the presence of undissociated carbonic acid, supplied as carbon dioxide in the gas phase. Silverman and Podger (1964) confirmed these results, and in addition reported that the effects of 1% pepsin and carbon dioxide at low pH enhanced each other. Optimum conditions for an exsheathment stimulus to *T. retortaeformis* were reported as hydrochloric acid at pH 3 under 40% or more carbon dioxide, and a temperature of 40°C (Bailey 1968). After 1 hour in the stimulus solution, larvae were transferred to saline. Bailey reported no additional influence of 0.2% pepsin on the rate of exsheathment for 4 hours after larvae were first exposed to the stimulus. Rogers (1965) reported that infective *T. colubriformis* larvae, when stimulated to exsheath, released an enzyme into the "exsheathing fluid".

**Development of Parasitic Stages**

In this introduction reference is only made to salient points in the history of attempts to culture strongylate nematodes occurring in the intestine, because a comprehensive review has recently been published by Silverman (1965b), and briefly revised by Nicholas (in Rothstein and Nicholas 1969). Taylor and Baker (1968 pp.272) summarised some of the published techniques.
Very few authors have referred to *Trichostrongylus* species in journal publications, and to date it has been a most recalcitrant organism to attempts to culture it in vitro.

Lapage (1933a, 1935b) cultured exsheathed third-stage larvae of *Trichostrongylus* species from the rabbit and sheep in more than two hundred sterile media, but could not get larvae to go through the next ecdysis to the early fourth-stage, although larvae of mixed species usually lived for 18 to 30 days in culture. The larvae did not respond to fragments of intestine in the media. Lapage suggested that for normal development there is a possible association with micro-organisms carried in the lumen of the nematode intestine. While working with Lapage, Davey (1938a) examined the effect of various culture media and conditions on the survival of adult *Trichostrongylus* recovered from the intestine of sheep and rabbits, and of other gastro-intestinal trichostrongylids from sheep, but did not report any development.

In work aimed at the recovery of metabolic antigens from cultures, Silverman (1962; Silverman, Poynter, and Podger 1962) incubated exsheathed third-stage larvae of *T. colubriformis* in balanced salt solution and liver extracts, and obtained development to the fourth-stage. The maximum proportion of *T. colubriformis* developing was obtained between pH5 and pH6 (Silverman, Alger, and Hansen 1966). Silverman (1965b) reported considerable variation, between different sources, in the efficacy of liver extracts used in his laboratory. This confirmed the report of Stoll (1940) on the variability in potency of liver extract used in the culture of *Haemonchus*. Both authors reported preparations from the same company (DIFCO) to be the most successful. Neilson (1967) reported that no satisfactory medium which would promote growth and development of *T. colubriformis* was found. There are no reports of fourth-stage development for intestine *Trichostrongylus* species.
A similar problem was found with the stomach species, *T. axeii*. Leland (1963) was unsuccessful in extensive efforts, with many different media, to obtain the third ecdysis and growth of *T. axeii* in culture. Similarly, Silverman (1965b) found that *T. axeii* larvae failed to undergo development in a variety of solutions, but did develop to the early fourth-stage in a chemically defined medium, Parker 199 (Silverman 1962).

It is surprising that growth of *T. colubriformis* and *T. axeii* in culture has not been obtained, as these species are able to infect a wide range of hosts.

In contrast, advanced development of other species of strongylata in culture has been obtained. The principle examples are the successful development to the fifth-stage of two nematodes that mature in the intestine. Weinstein and Jones (1956, reviewed 1959) cultured *Nippostrongylus* to the immature fifth-stage, and Leland (1963, reviewed 1970) has reported maturation and egg production by *Cooperia* cultured from infective larvae, and later cultured from the egg. Some infective larvae of the gastric trichostrongylids *Haemonchus* and *Ostertagia* also developed successfully to the immature fifth-stage in culture (Silverman 1959, reviewed 1965b).

All successful cultures of strongylate parasites have contained undefined tissue extracts from liver, serum, or embryos in the medium.

The greatest advances in the development and definition of media for the axenic culture of nematodes have come from studies with the free-living organism *Caenorhabditis briggsae*, which can be continuously grown throughout its life cycle in culture (reviews: Dougherty, Hansen, Nicholas, Mollett, and Yarwood 1959; Nicholas, Dougherty, and Hansen 1959). A chemically defined maintenance medium was developed in Hansen's laboratory (Sayre, Hansen, and Yarwood 1963) based on the amino acids found in the bacterium *Escherichia coli*. Originally referred to as *E. coli* medium (EM) this is now referred to
as *C. briggsae* maintenance medium (Medium CbM). The medium has been slightly simplified (Nicholas 1962). An undefined factor from a tissue extract is still required for growth and reproduction, but Hansen's group now appears close to replacing this factor with a defined component (Buecher, Hansen, and Gottfried 1970; Buecher, Hansen, and Yarwood 1970).

The CbM Medium has been found to support the trichostrongylid parasite, *Haemonchus*, in culture (Hansen, Silverman, and Buecher 1966). Similarly, Berntzen (1965) was able to culture a parasitic nematode, *Trichinella*, throughout its life cycle in chemically defined media adapted from those developed for cestodes, with the addition of a tissue extract. Later, Berntzen (1966) was successful with his Medium 126 (plus enzymes for exsheathment), in which the only substance not chemically defined was a plasma fraction.

**Physiological Applications in Culture**

In perspective, the major fields of investigation of parasites in culture are:

1. **Physiological Requirements**
   - (a) replacement of the host environment
   - (b) nutrition

2. **Biochemical Reactions**
   - (a) metabolism
   - (b) pharmacology, and activity of anthelmintics

3. **Immunological and Pathological Responses**
   - (a) elaboration of toxins, allergens, and other antigens
   - (b) response to host immune products

In a discussion of projected uses for axenic culture, Weinstein (1958) added another major field:

4. **Morphogenesis and Behaviour of life cycle stages**

Various applications of parasites in culture as biological models have been discussed by Smyth (1969). The physiology of the environment in the host intestine has been reviewed by Hobson (1948), and Read (1950, 1955), and related to culture conditions by Smyth (1968). Rogers (1962) discussed environmental factors in culture experiments
in his review of the life of parasites. The nutrition and metabolism of nematode parasites have been reviewed by Fairbairn (1960, 1970) and von Brand (1966). The use of cultures to collect antigens has been discussed by Silverman (1963, 1965a). In a classic experiment, Sarles (1938) examined the effect of immune serum in vitro, and this technique has been extended by other authors (e.g. Douvres 1962). Weinstein (1966) reviewed morphological studies in culture, and Sommerville and Weinstein (1964) examined reproductive behaviour in culture.

This thesis is concerned with the physiological control and responses of the intact nematode in its parasitic stage habitat, and studies of free-living stages or of nematode extracts were omitted. Literature on investigations with the parasitic stages of *Trichostrongylus* in culture is meagre, although some other trichostrongylid species and heligmosomoid species have been investigated. In general, the much larger *Ascaris* has been the main nematode laboratory model used in vitro.

Davey (1938a) studied the range of salt concentrations, the pH range, and the bile salt concentration tolerated by *T. colubriformis, T. vitrinus* and *T. retortaeformis*, and other trichostrongylid nematodes taken from the gastrointestinal tract of sheep. He was able to correlate the findings with the locality of the parasite in the host.

The synthesis of haemoglobin in trichostrongylids was reported by Davey (1938a), and he examined (Davey 1938b) the maximum period of survival without oxygen. The interval was much shorter for *Trichostrongylus* and *Ostertagia* than for *Cooperia* and *Nematodirus*. Rogers (1948, 1949a, 1949b) studied the oxygen uptake and haemoglobins in *Nippostrongylus*, *Nematodirus*, and *Haemonchus*. He concluded that the haemoglobin was not important in oxygen transport when oxygen tension was not limiting as in the host.

Rogers (1952, 1955) suggested that a high level of nitrogen excretion found for the intestinal trichostrongylid, *Nematodirus*, in culture was due to the utilisation of protein as a source of energy. The excreted nitrogen was
found mainly in free amino acids and peptides, ammonia, and lesser amounts of urea.

Trace labelling with radio-isotopes provides a sensitive technique to investigate the submorphological physiological reactions of nematodes in culture. Early work on the incorporation of phosphorus-32 into the strongylates Nippostrongylus, Trichostrongylus, Haemonchus, and Oesophagostomum was conducted to examine their nutrition in the host (Rogers and Lazarus 1949; Esserman and Sambell 1951). The use of carbon-14 in cultures of nematodes appears to have been introduced to examine the nutrition of Caenorhabditis (Nicholas, Dougherty, Hansen, Holm-Hansen, and Moses 1960), and has had little application to trichostrongylid parasites.

The uptake and metabolism of glucose labelled with carbon-14 has been examined for some strongylate species in culture (e.g. Dickson, Dunlap, and Gordon 1960; Fernando and Wong 1964; Roberts and Fairbairn 1965; Wong and Fernando 1970).

The uptake of amino acids labelled with carbon-14 was observed by Ball and Bartlett (1969) for some strongylate species, but in the natural environment rather than in artificial culture.

Chromatographic evidence has been obtained of metabolism by T. retortaeformis larvae of carbon-14 labelled steroids added to culture (W.L. Nicholas 1971 pers. comm.).

A further use of carbon-14 tracing in culture has been to investigate conditions influencing the uptake of a labelled anthelmintic by adult Haemonchus (Davis, Wescott, and Musgrave 1969).
GENERAL METHODS AND MATERIALS

(1) Design and Judgement of Culture Trials

The studies of features in vitro environments were not intended to provide measurements of comparative significance, in contrast to Part II, so that experimental designs leading to statistical analyses were not required. In these trials judgement was based on whether the parasite showed, or did not show, a particular property under the conditions being tested. The principle features assessed were survival, development, and radiochemical uptake.

Individual trials were planned so that a number of conditions were examined together. A base situation that had been observed in other trials was included to provide a control on new circumstances. For example, a basic, or simple, medium of balanced salt solution was included in trials on selected complex media and conditions.

Within any one trial, the design catered for two or more levels (including zero) and combinations of various media components. The object was to observe whether the situation had a stimulatory or suppressive effect on the criteria being assessed.

Any storage of components prior to a trial was carried out in a deep freeze. A batch of medium was freshly mixed for each trial.
(2) Incubation Conditions

Two basic types of culture vessels were used. For larger volume observations, and for incubation of adult nematodes, flat disposable plastic 30 ml capacity culture flasks (Falcon) were used. For many incubations of larvae, glass 20 ml capacity tubes (Pyrex) were used. Smaller disposable plastic 9 ml capacity tubes (Medos) were used for smaller volume studies, such as the trials with radiochemicals.

All incubations were carried out in the dark at 38°C (range 36°C to 39°C), except during sampling.

Three types of movement were studied. The stationary mode was always used with flat flasks. A slow roll (1 r.p.m.) was used in a few trials with the larger round tubes. A simultaneous rock (20 r.p.m.) and roll (25 r.p.m.) mode was used in several trials, particularly for radiochemical studies and for the pseudo-gastric exsheathing stimulus before culture.

(3) Fundamental Media Components

A list of the salt solutions, chemically defined nutrient solutions, and tissue extracts used as the liquid phase in various trials is given in Table 18 with their abbreviation and the source of the preparation. The formulations and preparations are given by the suppliers’ manuals and by standard texts (e.g. Anon. 1968mb, 1969m; Parker 1962; Taylor and Baker 1968).

Any storage of components prior to a trial was carried out in a deep freeze. A batch of medium was freshly mixed for each trial.

The Earle BSS, no dextrose, had the sugar excluded to compare the observation that related genera of trichostrongylids may develop from third-stage to fourth-stage larvae without an exogenous energy source. Ferric
Table 18

Fundamental Media Components

<table>
<thead>
<tr>
<th>Category</th>
<th>Component (abbreviation)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt Solution</td>
<td>Saline</td>
<td>φ</td>
</tr>
<tr>
<td></td>
<td>Hank Balanced Salt Solution</td>
<td>φ</td>
</tr>
<tr>
<td></td>
<td>Earle Balanced Salt Solution, no dextrose (BSS nod)</td>
<td>φ</td>
</tr>
<tr>
<td></td>
<td>Earle Balanced Salt Solution, compl. (BSS com)</td>
<td>φ</td>
</tr>
<tr>
<td>Chemically Defined Nutrient Solution</td>
<td>Eagle Basal Medium (BM)</td>
<td>GIBCO</td>
</tr>
<tr>
<td></td>
<td>Parker Medium 199</td>
<td>GIBCO</td>
</tr>
<tr>
<td></td>
<td>Parker Medium 858</td>
<td>CSL</td>
</tr>
<tr>
<td></td>
<td>Hansen C. briggsae Maintenance Medium* (CbM)</td>
<td>GIBCO</td>
</tr>
<tr>
<td></td>
<td>Modif. C. briggsae Medium*</td>
<td>φ</td>
</tr>
<tr>
<td>Tissue Extract</td>
<td>... Sera</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Foetal Calf Serum (FCS)</td>
<td>CSL</td>
</tr>
<tr>
<td></td>
<td>Normal Bovine Serum (NBS)</td>
<td>CSL</td>
</tr>
<tr>
<td></td>
<td>Normal Rabbit Serum (NRS)</td>
<td>CSL</td>
</tr>
<tr>
<td></td>
<td>Normal Rabbit Serum (NRS)</td>
<td>φ</td>
</tr>
<tr>
<td></td>
<td>Bovine Embryo Extract, filt. (CEE)</td>
<td>CSL</td>
</tr>
<tr>
<td></td>
<td>Chick Embryo Extract, centr. (CEE)</td>
<td>CSL</td>
</tr>
<tr>
<td></td>
<td>Chick Embryo Extract, partic. (CEE)</td>
<td>φ</td>
</tr>
<tr>
<td></td>
<td>10% &quot;Bacto&quot; Peptone (PT)</td>
<td>DIFCO</td>
</tr>
<tr>
<td></td>
<td>Nicholas Autoclaved Liver Extract (ALE)</td>
<td>φ</td>
</tr>
</tbody>
</table>

φ prepared in this laboratory.

GIBCO  Grand Island Biological Co., New York
DIFCO  DIFCO Laboratories, Detroit.
CSL    Commonwealth Serum Laboratories, Parkville.
* previously known as E. coli Medium (EM).
+ Embryo extracts were prepared in Hank BSS.
nitrate was included in Earle BSS, complemented, (at the same final concentration as in Medium 199; one tenth of the concentration in Medium 858) following the observation that ferric ions may be required for certain cell development (Daniel and Millward 1969).

The pH was controlled by the addition of concentrate bicarbonate (CBC: 2.8% sodium bicarbonate) in combination with carbon dioxide in the gas phase (Umbreit, Burris, and Stauffer 1957). Phenol red (pH 6 to 8) and in some cases congo red (pH 3 to 6) were used as indicators.

The selection of media components is considered in the Objects of Section A Trials.

From the observations in Section A, two standard media were chosen for the Section B Trials, as follows.

<table>
<thead>
<tr>
<th>Simple Medium</th>
<th>Complex Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Earle BSS, compl. (with 1% CBC)</td>
<td>Earle BSS, compl. (with 1% CBC)</td>
</tr>
<tr>
<td>100%</td>
<td>50%</td>
</tr>
<tr>
<td>Medium CbM</td>
<td>CEE</td>
</tr>
<tr>
<td>20%</td>
<td>20%</td>
</tr>
<tr>
<td>NRS</td>
<td>NRS</td>
</tr>
<tr>
<td>10%</td>
<td></td>
</tr>
</tbody>
</table>

(4) Discretional Media Components

The following substrates, variable gas phase, and special additives were used as indicated in the results of Section A Trials.

Substrate Phase

Three readily available types of substrate were selected. Larvae were inoculated into 75% monolayer HeLa cell cultures (CSL) in Medium 199, and transferred to fresh cell cultures after 3 days. A solid serum base (Smyth 1967) was prepared by heat coagulation of NRS on the floor of culture flasks, and media added as usual. Glass wool loosely packed into culture tubes (Hansen and Cryan 1966) was used as a base to provide a thin film of medium.

Gas Phase

The levels of carbon dioxide (0 to 8%) and oxygen (5% to 100%) equilibrated with the medium were varied as shown in the results. The gas mixtures (Commonwealth
Industrial Gases) were directed onto the liquid through a Pasteur pipette.

Special Additives

1) Reducing Chemicals

Reducing solutions that are advantageous for the development of diverse helminths in culture, for example in Hansen Medium CbM and in Berntzen Medium 126 (Berntzen 1966), are based on the following substances. The final concentrations used in culture were glutathione (reduced) 40 mg%, l-glutamic acid 200 mg%, and l-cysteine 40 mg% (all Nutritional Biochemicals Corporation). A stock solution was mixed immediately before use in pre-boiled distilled water brought to pH 7.4 with sodium hydroxide. The reducing solution was added to culture with 5% CBC and regassed with 5% CO₂ plus 5% O₂ in N₂.

2) Secretions and Excretions

Crude rabbit bile collected with sterile technique was used at a final concentration of 2 ml% (extracted bile salt preparations are considered under steroids). Pancreatin (porcine: Sigma) was added to media to give a final concentration of 100 mg%, with a further 5% CBC to raise the pH. Pepsin digest was examined with biochemical pepsin (1:2500, British Drug Houses) at a final concentration of 1 gm% activated in 0.35% hydrochloric acid.

3) Steroids and Lipids

Dilutions of hydrocortisone (= cortisol) from a stock solution of hydrocortisone acetate at 25 mg per ml (Roussel) were added to give the final concentrations in media shown in the results. Cholesterol (Sigma) was added as shown in the results from a 0.5 gm% stock solution, prepared by dissolving in polysorbate which was then dissolved in water (see solubising substances following). The conjugated bile salt, sodium glycocholate (Nutritional Biochemicals Corporation), was added to media, as shown in the results, from a 10 gm% stock solution. Linseed oil was used as a fourth source of sterols and lipids. It was added to media as a saturated aqueous solution at 1 ml% (prepared by
dissolving in chloroform, mixing this with water, and dispersing the chloroform with nitrogen), or as a fine emulsion to give a final concentration of 0.01% (prepared by mixing with an equal volume of polysorbate and adding to water to give a stock solution of 1 ml%).

4) Growth Hormones

Stock solutions of prolactin (ovine: Sigma) were prepared at 100 x concentration to be used at 100 µg% and 1 µg% in media as shown in the results. Although said to be practically insoluble in water (Merck Index 1968 p.629), it has been used in aqueous solution up to 20 mg% (Bern, Nicoll, and Strohman 1967) or up to 1,250 mg% (Yansi and Nagasawa 1969).

5) Solubising Substances

Four solvents compatible with water, in which special additives not readily soluble in water may be dissolved, were considered. Each was examined between 0.1 and 5.0 ml% in culture. Polyoxyethylene sorbitan mono-oleate, polysorbate (PSB), was premixed as a workable solution in water of 10 ml% PSB ("Tween-80", Sigma). Dimethyl formamide (DMF) (Unilab) was added to media from a 50 ml% solution. Dimethyl sulphoxide (DMSO) (supplied by Dr. G. Subba Rao of the Organic Chemistry Department) could be added directly to media. Ethanol was considered separately as a solvent (levels up to 5.0 ml% as above), and as a fixative to terminate activity (levels up to 50 ml%).

(5) Control of Micro-organism Contamination

Nematodes were treated in at least two of the following three ways to eliminate micro-organisms before inoculation into culture. Infective larvae were treated with the surface antiseptic, sodium hypochlorite, as outlined under exsheathment stimulus (or with hydrochloric acid to pH 3). Secondly, larvae or nematodes recovered from the intestine were repeatedly washed in sterile saline - this was most effective. Thirdly, the nematodes were exposed at 38°C for 2 to 3 hours to high levels of the antibiotics sodium
penicillin G ("Crystapen", Glaxo-Allenburys), streptomycin sulphate (Drug Houses of Australia), and nystatin ("Mycostatin", Squibb), each at 1,000 units per ml. During culture, each of the three antibiotics above was routinely added to the medium at around 250 units per ml. These treatments suppressed micro-organisms in the observations reported in this thesis. The presence of contamination was assessed by check of visible growth, subculture into thioglycollate broth (Brewer), or detectable uptake of radio-isotope labelled protein hydrolysate in control cultures.

All instruments, glass and disposable plastic ware were sterilised by autoclaving, dry heat or gamma radiation. All solutions were treated by autoclaving, ultracentrifugation, or ultrafiltration through 0.22 µ pore (Millipore). Gasses were sterilised by passage through autoclaved cotton wool wads.

(6) Inoculation of Cultures with Nematodes

The calculated inoculum of larval stages (less than about 5 mm length) was transferred, in a predetermined volume of sterile salt solution with a Pasteur pipette, to the culture vessel containing the balance of the medium. Larger nematodes were transferred from salt solution by lifting with a mounted needle.

The number of nematodes in each culture is given with the Observations.

(7) Sampling Cultures for Visible Examination

For examination of maximum development of larvae, culture vessels were stood on end, and a small sample taken from the bottom with a Pasteur pipette and transferred to a microscope slide. Developing larvae tend to accumulate on the bottom more rapidly than the more active undeveloped larvae, while ghosts tend to drift longer, and cast sheaths to float.
For assessments of proportions of nematodes, examinations were made directly on an inverted microscope, as there were too few nematodes per ml to sample mixed cultures, and settled nematodes were not representative.

(8) Incubation with Radichemicals ("Pulse" Incubation)

Radio-isotope labelled substrates (Radio Chemical Centre) as follows were added to warm cultures. The studies on uptake used either a complex of carbon-14 labelled amino acids (Table 19) supplied as protein hydrolysate from the alga *Chlorella vulgaris*, or used the single essential amino acid l-isoleucine. The labelled substrates were added in 100 µl of BSS. Where sufficient material was available, the concentration was adjusted to give $10^6$ disintegrations per minute (DPM) (0.45045 µc) for each sample of medium and nematodes taken for counting. For the l-isoleucine this was approximately 50 µg% in the culture medium.

The studies on turnover of carbon-14 from labelled l-isoleucine used a higher concentration of $2.2 \times 10^6$ DPM (1.0 µc) for each sample taken for counting. The studies on the utilisation of carbon dioxide from labelled sodium bicarbonate used a concentration to make available of the order of 10 µc per sample taken for counting.

The concentration of larvae was usually 10,000 per sample for the radio-isotope pulse incubations. The duration of the incubations is given with the results.

(9) Sampling Cultures for Radiochemical Examination

To check the total radio-activity in culture, 25 µl of the medium were taken by micro-pipette, and transferred directly to scintillation counting vials.

Precise samples of nematodes were taken by one of two methods. In trials where pulses of larvae were carried out for a progression of times, the culture was mixed and immediately a sample of 100 µl, 250 µl, or 500 µl (for respective trial) was taken by micro-pipette. In other trials larvae or adult nematodes were sampled at a terminal time by taking the whole culture from a tube.
### Amino Acid Components of Carbon-14 Labelled Algal Protein Hydrolysate

#### Percentage composition by radio-activity:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Radio-activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-leucine</td>
<td>11.4%</td>
</tr>
<tr>
<td>l-glutamic acid</td>
<td>8.7%</td>
</tr>
<tr>
<td>l-alanine</td>
<td>7.9%</td>
</tr>
<tr>
<td>l-arginine</td>
<td>7.7%</td>
</tr>
<tr>
<td>l-phenylalanine</td>
<td>7.7%</td>
</tr>
<tr>
<td>l-lysine</td>
<td>7.6%</td>
</tr>
<tr>
<td>l-valine</td>
<td>7.6%</td>
</tr>
<tr>
<td>l-aspartic acid</td>
<td>7.3%</td>
</tr>
<tr>
<td>l-proline</td>
<td>5.2%</td>
</tr>
<tr>
<td>l-isoleucine</td>
<td>5.1%</td>
</tr>
<tr>
<td>l-threonine</td>
<td>4.5%</td>
</tr>
<tr>
<td>glycine</td>
<td>4.4%</td>
</tr>
<tr>
<td>l-serine</td>
<td>4.2%</td>
</tr>
<tr>
<td>l-tyrosine</td>
<td>2.4%</td>
</tr>
<tr>
<td>l-histidine</td>
<td>1.8%</td>
</tr>
<tr>
<td>l-methionine</td>
<td>1.6%</td>
</tr>
</tbody>
</table>

Analysis from supplier, Radio-Chemical Centre, Amersham.
The Parasite

The parasite has been described in the General Introduction.

(10) Source of Nematodes for Inoculation

Infective larvae were recovered clean in distilled water from the incubations of rabbit faeces already described in the Methods of Part II. The number of larvae for inoculation was determined by either direct counting of samples (as in Part II Methods), or by estimation from sedimented volume (centrifuged at 500 G for 1 minute), where 0.25 ml is equivalent to 100,000 fresh infective larvae.

Parasitic stage nematodes were recovered from the rabbit intestine as already described in the Methods of Part II, and gathered clean in saline. Larval forms (less than about 5 mm long) were transferred by Pasteur pipette into a centrifuge tube and handled similarly to exsheathed infective larvae, and following antiseptic treatments the volume adjusted to give the calculated concentration for inoculation. Adult forms were transferred to another dish and counted with a mounted needle, ready for inoculation into culture following antiseptic treatments.

(11) Exsheathment Stimulus to Infective Larvae

Pilot studies were carried out to determine suitable conditions for exsheathment (second ecdysis), and the following standard procedures adopted. Either technique was used, as given in the Observations.

Infective larvae were artifically exsheathed by mixing with 0.05% sodium hypochlorite in 0.8% sodium chloride, pH 8.5 (a 1 in 20 dilution of "Milton", Richardson-Merrell Ltd.), at room temperature for about 15 minutes, as recommended for *T. retortaeformis* by Lapage (1933a). This treatment dissolved all the sheaths, contrary to earlier reports, but comparable to the effect of 5% sodium hydroxide reported by Goodey (1922).
Physiological exsheathment was initiated by a pseudo-gastric stimulus of hydrochloric acid and carbon dioxide, as recommended for *T. colubriformis* by (Rogers 1960). Infective larvae were incubated (for 2 hours at 38°C in a closed tube on the roller rocker) in $10^{-3}$N hydrochloric acid (pH 3) saturated with 100% carbon dioxide bubbled through from a cylinder (Commonwealth Industrial Gases). They were then washed in 3 rinses of saline, and reincubated in BSS overnight to complete exsheathment. This standard procedure consistently gave exsheathment rates (sheaths cast at 24 hours) in excess of 90%, irrespective of the batch of larvae. Therefore a sub-optimal stimulus of $10^{-4}$N (instead of $10^{-3}$N) hydrochloric acid with 5% (instead of 100%) carbon dioxide was used when required to make comparative assessments between batches of larvae, as in Part II, Section B, Experiment 8. The standard procedure for *T. retortaeformis* published by Bailey (1968) during this study, which was $10^{-4}$N hydrochloric acid under 40% carbon dioxide, was a less efficient stimulus than that outlined above.

(12) Gross Assessment for Survival and Visible Activity

A daily quantitative assessment of activity and survival was made for each culture. Larvae were mixed, and 50 or more over several microscope fields allocated to one of the following criteria.

1) Alive  
2) "  
3) "  
4) Dead  

Vigorous larvae showed threshing movements, and were almost invariably those not developed. Slow moving larvae were those exhibiting either leisurely sinusoidal movement, or questing motions of the head, sometimes with the posterior end coiled. Coiled larvae usually show tight coils of the whole body or of the posterior end, and are believed to be
at a latent phase, as these larvae became active on changes in conditions. Stiff larvae were classed as dead.

(13) Gross Assessment for Visible Development and Growth

Four criteria were considered, ecdysis (development to next larval stage), size (growth within a stage), metamorphosis (differentiation within a stage), and in adults, mating and fecundity. However, for _T. retortaeformis_ only metamorphosis, and only in the parasitic third-stage, needed to be assessed, because this was the only criterion that occurred.

For the results of this study, development of the third-stage is divided into three steps, early (EL₃), late (LL₃), and moult (ML₃). Although buccal cavity development has been used for a trichostrongylid to distinguish such steps (Sommerville 1966), the easiest appraisal was found to be the development of the tail notch present in intestine _Trichostrongylus_ species. This development is described in the Results of Section A.

Changes in several other features were not as consistently observed.

Samples of cultures were examined under a compound microscope at 40 x by 10 x magnification to determine the step reached by the most advanced larvae.

(14) Precise Assessment for Radiochemical Activity

Several pilot trials were carried out on carbon-14 isotope labelling of nematodes in culture, and the following standard procedures adopted for the treatment of samples taken at the end of a "pulse" period.

Treatment of Samples for Uptake of Labelled Substrate

In studies on amino acid uptake, the sample of nematode plus medium was pipetted into cold saline, and the nematodes given four 10 ml washes (centrifuging at 1,250 G for 5 minutes and removing the supernatant through a Pasteur pipette with a J end). The first wash in cold saline (with or without unlabelled substrate in solution
e.g. l-isoleucine at 5 mg%) removed the medium, the intermediate washes in cold 5% trichlor-acetic acid (TCA) solution removed free substrate, and the final wash with cold distilled water removed solutes (which caused adherence of nematode material to glassware). Negligible radio-activity, less than background, was removed in rinses subsequent to the first two washes.

After washing, the nematode residue was transferred with a Pasteur pipette directly to a scintillation counting vial, and the tube rinsed with the scintillation cocktail.

Treatment of Samples for Metabolism of Labelled Substrate

In studies on labelled substrate metabolism, the sample of nematode plus medium was pipetted into ethanol to give an 80% solution which was held at 4°C for treatment. (For incubations in complex medium 20% ethanol was used to avoid precipitation of the medium.)

For the labelled l-isoleucine pulsed samples, the volume was reduced and the whole sample spotted on chromatography paper (Whatman No. 1, medium flow rate). The material was subjected to two way descending chromatography with phenol water, and then butanol-propionic acid. Separated spots were located by autoradiography onto medical X-ray film (Kodak) exposed in the dark for 16 days. The positions representing labelled material were excised and the pieces transferred to scintillation counting vials.

For the labelled sodium bicarbonate pulsed samples, the free labelled carbon dioxide was removed by slowly bubbling through 100% carbon dioxide from a cylinder (Bryant and Janssens 1969) for 4 hours. (Checks showed the radio-activity was stable within 3 hours.) Each tube was reconstituted to a volume of 5 ml, and the solution separated from the larvae by centrifugation. Samples of the extracted media were transferred to scintillation counting vials. The extracted larvae were washed twice in ethanol solution, and transferred to scintillation counting vials.
Radiochemical Incubation and Treatment Controls

Controls and comparisons examined in radio-isotope uptake and metabolism studies included blank vials of scintillation cocktail, samples of wash solutions before or after use, samples of media with or without labelled substrate added, invisible residue treated as if nematodes were present, nematodes not pulsed, and killed nematodes pulsed. The killed larvae controls were heated in a water bath of 60°C for 5 minutes immediately before pulse incubation. Checks on the use of formalin showed that larvae could survive this treatment, so that it was not reliable.

Scintillation Counting

The water miscible dioxane-based scintillation cocktail solution as described by Bray (1960) was used in all of these trials. (The secondary fluor, POPOP, was omitted as it is not needed for the counter.) Bulk cocktail batches were mixed from scintillant grade reagents for several trials at a time, filtered through a grade 3 glass sinter funnel, and held in the dark. Quantities of 10 ml of cocktail (15 ml for chromatography paper) were measured into clean glass counting vials (Beckman), and the mean blank background counts determined, which were around 60 counts per minute (CPM).

Experimental radio-activity counts were made in a three-channel liquid scintillation spectrometer for soft beta counting ("Model CPM-200 TM", Beckman Instruments), following the procedures in the manual (Anon. 1966m). The counts reported were made at a gain calibration adjustment setting of 350, with a fixed window width in a factory calibrated plug-in isoset, to give a discriminator measuring the whole carbon-14 spectrum peak ("P-32 isoset"). The mean cocktail scintillant and machine background was subtracted automatically.

The scintillation counts were automatically made to $10^4$ counts, which with 95% statistical confidence limits provides a counting resolution of ± 2% error due to the random timing of disintegrations. For samples of lower
radio-activity, counts were made for a fixed time, commonly 10 minutes, with consequent greater error. For example, a counting rate of 100 CPM measured for 10 minutes ($10^3$ counts) has a resolution error of ± 6% with the above confidence limits (Anon. 1968ma).

Photoluminescence, chemiluminescence, and quenching related to the treatment of experimental material, were examined in preliminary studies, and the problems found avoided in the trials reported.

Normal quenching and counting efficiency was monitored by two methods. Checks were made during trials with an internal standard of $C^{14}$-glycine in water, or of $C^{14}$-benzoic acid in ethanol. For the scintillant cocktail and added material used, the counting efficiency was reasonably constant around 80% to 90%. Secondly, an automatic external standard channels ratio is provided in the machine (Anon. 1966m). In the trials reported, an external standard ratio of 1.0 to 1.5 was recorded (depending on the batch of cocktail). This is within the optimum range of 1.0 to 1.6 (Anon. 1966m), and indicated slight quenching only.
The aim of this section was to select a culture medium and conditions for the optimal support of *T. retortaeformis* parasitic stages, particularly of the exsheathed third-stage. Three objectives were sought: a simple convenient method with readily procurable components of constant quality; maximum development achieved in the closest time to that observed in the host; and optimal survival of active nematodes.

The major groupings of media components were as follows. Selection of individual materials was based on known work with other parasitic nematodes (review: Silverman 1965b).

**Fundamental Components** - Salt solution
- Chemically defined nutrient solution
- Tissue extract

**Discretionary Components** - Substrate
- Gas Phase
- Special Additives

The individual components examined are given in the Methods.
In addition to selecting materials on their previous use for gastro-intestinal nematodes in culture, consideration was given to two wider selective bases, environmental and phylogenetic.

The salt concentrations and most chemically defined nutrient solutions used were those considered to be physiological to the environment of tissues of the mammalian host. Although concentrations in the lumen of the host digestive tract change from time to time, parasite larvae which are in close apposition with host cells in the gastro-intestinal crypts are probably in contact with an environment comparable to interstitial fluid. Components were selected by precedent of successful use in culture for other mammalian gastro-intestinal helminths (review for non-nematode parasites: Clegg and Smyth 1968), or for mammalian tissue culture (Parker 1962).

The other components used were selected on a phylogenetic basis from successful use in culture for other nematodes (review for non-parasitic nematodes: Rothstein and Nicholas 1969).

The factors reported in this study as special additives fall into five types as follows.

1) Reducing chemicals
2) Secretions and Excretions
3) Steroids and Lipids
4) Growth Hormones
5) Solubising Substances

The function of this section was to determine a medium for the ultimate examination of the influence of particular factors on the nematode, and so a peculiar quandary was created, a factor to be tested in a viable culture system may be the factor required for a viable system.
TRIALS ON DEVELOPMENT OF STAGES FROM INFECTIVE LARVAE

Plan

In these trials, a large number of combinations of media components and conditions for culture were examined, of which 141 are described in Table 20, together with the pre-culture treatment of the larval inoculum.

Concentrations of third-stage larvae, from 100 per ml to 10,000 per ml were used in various trials as shown in the table.

The larvae are designated as fresh when used soon after they were harvested from faecal incubations. The stored larvae used had been held for 6 to 21 weeks at 4°C. The larvae designated as aged had been held at 20°C for 3 weeks after harvesting.

The infective larvae were treated for exsheathing and the initiation of parasitic stages, before inoculation into the culture, as shown in the table.

Special additives included at the start of a culture are shown in the table. Reducing chemicals or host secretions were added to particular cultures after 3 days to examine for an influence on the progress or inducement of ecdysis to the fourth-stage. The use of solutions after 3 days is shown as footnotes to the table.

The more successful combinations of components and conditions were repeated in subsequent trials with other batches of infective larvae.

Results

The assessment of each culture, as the proportions of larvae active or surviving and the maximum development detected, is shown in Table 20. Representative results are shown once in the table for the culture combinations tested in duplicate or in triplicate.
Table 20

TRIALS ON DEVELOPMENT IN CULTURE

The Activity, Survival, and Development of *T. retortaeformis* Third-Stage Larvae
when Cultured
in Various Combinations of Media and Conditions.

(twelve parts of Table 20 follow)

* Maximum Development step of third-stage larvae reached:

  - E = early (EL₃), i.e. no development
  - L = late (LL₃), with accumulation of developed larvae
  - M = moult (ML₃), i.e. pre-fourth-stage larvae, before third ecdysis

Footnotes:

- a  CO₂ only, nil HCl, stimulus
- b  nil CBC added in BSS
- c  Fe not in this BSS com
- d  after 3 days, added glutathione reducing solution, and changed gas phase to 5% CO₂ + 5% O₂/N₂
- e  after 6 days, changed gas phase to 100% O₂
- f  after 3 days, added pancreatin solution, and rabbit bile
- g  after 3 days, added pepsin - HCl solution

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| **CULTURE**               |        |        |        |        |        |        |        |        |        |        |
| - Fundamental Medium      |        |        |        |        |        |        |        |        |        |        |
| - Salt Soln.              |        |        |        |        |        |        |        |        |        |        |
| - Saline, %               | 100    | 6      | 6      | 6      | 2      | 2      | 2      | 2      | 2      | 2      |
| - BSS Hank, %             | 94     |        |        |        |        |        |        |        |        |        |
| - BSS Earle, nod %        |        |        |        |        |        |        |        |        |        |        |
| - BSS Earle, com %        |        |        |        |        |        |        |        |        |        |        |
| - Defined Nutrient Soln.  |        |        |        |        |        |        |        |        |        |        |
| - BM Eagle, %             | 87     | 86     | 86     | 44     | 53     | 53     | 25     | 25     | 23     | 98     |
| - 199 Parker, %           |        |        |        |        |        |        |        |        |        |        |
| - BSS Parker, %           |        |        |        |        |        |        |        |        |        |        |
| - CbM (Modif.), %         |        |        |        |        |        |        |        |        |        |        |
| - CbM Hansen, %           |        |        |        |        |        |        |        |        |        |        |
| - Tissue - Sera           |        |        |        |        |        |        |        |        |        |        |
| - PBS, %                  | 7      | 6      | 6      | 2      | 54     | 25     | 25     | 20     | 23     | 98     |
| - NBS, %                  |        |        |        |        |        |        |        |        |        |        |
| - NBS (Local), %          |        |        |        |        |        |        |        |        |        |        |
| - NBS (Bought), %         |        |        |        |        |        |        |        |        |        |        |
| - Embryo - BEE, %         | 2      |        |        |        |        |        |        |        |        |        |
| - CEE, Filtr., %          |        |        |        |        |        |        |        |        |        |        |
| - CEE, Centr., %          |        |        |        |        |        |        |        |        |        |        |
| - CEE, Partic., %         |        |        |        |        |        |        |        |        |        |        |
| - Liver - 10% Peptone, %  |        |        |        |        |        |        |        |        |        |        |
| - ALE, %                  |        |        |        |        |        |        |        |        |        |        |
| - Gas                     |        |        |        |        |        |        |        |        |        |        |
| - Air                     | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| - 5% CO₂/Air              |        |        |        |        |        |        |        |        |        |        |
| - 5% CO₂ + 10% O₂ / N₂    |        |        |        |        |        |        |        |        |        |        |
| - 8% CO₂ / N₂             |        |        |        |        |        |        |        |        |        |        |
| - Substrate               |        |        |        |        |        |        |        |        |        |        |
| - HeLa Cell               |        |        |        |        |        |        |        |        |        |        |
| - Solid Serum             |        |        |        |        |        |        |        |        |        |        |
| - Glass Wool              |        |        |        |        |        |        |        |        |        |        |
| - Special Additives       |        |        |        |        |        |        |        |        |        |        |
| - Hydrocortisone, mg %    |        |        |        |        |        |        |        |        |        |        |
| - Cholesterol, mg %       |        |        |        |        |        |        |        |        |        |        |
| - Na Glycocholate, mg %   |        |        |        |        |        |        |        |        |        |        |
| - Prolactin, µg           |        |        |        |        |        |        |        |        |        |        |
| - Polysorbate (PSB), ml%  |        |        |        |        |        |        |        |        |        |        |
| - Ethanol, ml%            |        |        |        |        |        |        |        |        |        |        |
| - DMF, ml%                |        |        |        |        |        |        |        |        |        |        |
| - DMSO, ml%               |        |        |        |        |        |        |        |        |        |        |
| - pH Estimate             | 5.8    | 7.6    | 7.6    | 7.6    | 7.6    | 7.6    | 7.6    | 7.6    | 7.6    | 7.6    |
| - Movement                |        |        |        |        |        |        |        |        |        |        |
| - Stationary              | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| - Slow Roll               |        |        |        |        |        |        |        |        |        |        |
| - Rock & Roll             |        |        |        |        |        |        |        |        |        |        |

| **LARVAL ASSESSMENT AT 3 DAYS** |        |        |        |        |        |        |        |        |        |        |
| - Activity (%)            | 35     | 45     | 75     | 75     | 75     | 80     | 80     | 80     | 80     | 40     | 40     |
| - Survival (%)            | 80     | 80     | 80     | 80     | 85     | 85     | 85     | 85     | 50     | 50     |
| - Development Step*       | E      | L      | L      | L      | L      | L      | L      | L      | L      | L      |

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- **Activity (%)**
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- **Survival (%)**
  - 95 95 95 85 95 85 95 95 95 95 95 95
- **Development Step**
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# Pathology pronounced. See text.

## Separation of L₄ inside L₃ cuticle.
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OTHER FACTORS

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| - Fundamental Medium - Salt Soln.|        |        |        |        |        |        |        |        |
| - Saline, %                      |        |        |        |        |        |        |        |        |
| - BSS Hank, %                    |        |        |        |        |        |        |        |        |
| - BSS Earle, mod %               |        |        |        |        |        |        |        |        |
| - BSS Earle, com %               |        |        |        |        |        |        |        |        |
| - Defined Nutrient Soln.         |        |        |        |        |        |        |        |        |
| - HB Eagle, %                    |        |        |        |        |        |        |        |        |
| - 199 Parker, %                  |        |        |        |        |        |        |        |        |
| - BSS Parker, %                  |        |        |        |        |        |        |        |        |
| - CMM (Modif.), %                |        |        |        |        |        |        |        |        |
| - CMM Hansen, %                  |        |        |        |        |        |        |        |        |
| - Tissue - Sera - FCS, %         |        |        |        |        |        |        |        |        |
| - NSS, %                         |        |        |        |        |        |        |        |        |
| - NSS (Local), %                 |        |        |        |        |        |        |        |        |
| - NSS (Bought), %                |        |        |        |        |        |        |        |        |
| - Embryo - BEE, %                |        |        |        |        |        |        |        |        |
| - CEE, Flit., %                  |        |        |        |        |        |        |        |        |
| - CEE, Centr., %                 |        |        |        |        |        |        |        |        |
| - CEE, Partic., %                |        |        |        |        |        |        |        |        |
| - Liver - ICM Peptone, %         |        |        |        |        |        |        |        |        |
| - ALB, %                         |        |        |        |        |        |        |        |        |
| - Gas - Air                      |        |        |        |        |        |        |        |        |
| - 1% CO₂/Air                     |        |        |        |        |        |        |        |        |
| - 5% CO₂ + 1% O₂/N₂              |        |        |        |        |        |        |        |        |
| - 5% CO₂/N₂                     |        |        |        |        |        |        |        |        |
| - Substrate - Hela Cell          |        |        |        |        |        |        |        |        |
| - Solid Serum                    |        |        |        |        |        |        |        |        |
| - Glass Wool                     |        |        |        |        |        |        |        |        |
| - Special Additives              |        |        |        |        |        |        |        |        |
| - Hydrocortisone, mg %           |        |        |        |        |        |        |        |        |
| - Cholesterol, mg %              |        |        |        |        |        |        |        |        |
| - Na Glycocholate, mg %          |        |        |        |        |        |        |        |        |
| - Prolactin, µg µg %             |        |        |        |        |        |        |        |        |
| - Polyethylene (PSB), ml %       |        |        |        |        |        |        |        |        |
| - Ethanol, ml %                  |        |        |        |        |        |        |        |        |
| - DMF, ml %                      |        |        |        |        |        |        |        |        |
| - DMSO, ml %                     |        |        |        |        |        |        |        |        |
| - pH Estimate                    | 6.5    | 6.8    | ?      | 6.8    | ?      | 6.8    | ?      | 7.6    | ?      | 6.6    | ?      |
| - Movement                       | Stationary | Slow Roll | Rock & Roll |        |        |        |        |        |        |        |        |
| - Activity (%)                   | 30     | 65     | 60     | 60     | 65     | 60     | 55     | 75     | 85     | 25     | 85     | 95     |
| - Survival (%)                   | 60     | 80     | 80     | 80     | 70     | 85     | 90     | 90     | 85     | 90     | 85     | 95     |
| - Development Step*              | M      | L      | L      | L      | L      | L      | L      | L      | L      | M      | L      |

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<tr>
<td>- Stationary</td>
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<tr>
<td>- Slow Roll</td>
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<tr>
<td>- Rock &amp; Roll</td>
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<tr>
<td><strong>LARVAL ASSESSMENT AT 3 DAYS</strong></td>
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<td></td>
</tr>
<tr>
<td>- Activity (%)</td>
<td>85</td>
<td>80</td>
<td>85</td>
<td>95</td>
<td>0</td>
<td>0</td>
<td>85</td>
<td>90</td>
<td>85</td>
<td>90</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
<td>- Survival (%)</td>
<td></td>
<td></td>
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<td>- L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>E</td>
<td>E</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>M</td>
<td>M</td>
</tr>
</tbody>
</table>

**OTHER FACTORS**
A greater proportion of larvae showed development in some tubes than others, but this finding is not included in the assessment reported because it is not sufficiently objective with the use of a non-random sampling technique.

Development of the early third-stage (EL$_3$) inocula was observed to the late third-stage (LL$_3$) and up to the metamorphosed moult third-stage (ML$_3$) but not beyond. In the early third-stage, a faint notch could be detected near the end of the tail. At the late third-stage this had apparently deepened, so that there was a distinct knob (approximately 10 µ in diameter) at the end of the tail, and some thickening of the cuticle was detectable. For the moult third-stage, the thickened cuticle was sometimes apparent as two layers, causing the knob to appear as a terminal bud, because it was beyond the new pre-fourth-stage tail being formed. Although separation of this pre-fourth-stage from its sheath (the third-stage cuticle) was obtained under certain circumstances as outlined below, ecdysis to the free early fourth-stage did not occur. Occasionally larvae reached the moult third-stage while still in the earlier sheath (the second-stage cuticle).

An appraisal of the overall results indicated that the most significant influences were exerted by the presence of bicarbonate and by acidity. The effect of exposure to carbon dioxide or to bicarbonate in five different combinations is summarised in the following schedule.

<table>
<thead>
<tr>
<th>Source</th>
<th>Combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-culture Stimulus:</td>
<td></td>
</tr>
<tr>
<td>CO$_2$</td>
<td>- + - + -</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>- - - - -</td>
</tr>
<tr>
<td>Culture Status:</td>
<td></td>
</tr>
<tr>
<td>CO$_2$</td>
<td>- - - - +</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>- - + + +</td>
</tr>
<tr>
<td>L$_3$ Development by day 3</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>E* E L M L M</td>
</tr>
<tr>
<td>+ present</td>
<td></td>
</tr>
<tr>
<td>(* only tested stored infective larvae)</td>
<td></td>
</tr>
</tbody>
</table>
Those larvae which reached the moult third-stage without carbon dioxide pre-culture stimulus (third and fifth columns above) were found to all have one factor in common, which was that the ten culture media involved contained Medium CbM (20% to 100%). Nine had serum (15% to 20%), and seven had embryo extract (10% to 50%) also.

Larvae exposed to carbon dioxide and hydrochloric acid as a pre-culture stimulus, and then cultured in a salt solution (in fourth column above), only reached the moult third-stage when at a pH up to 6.6, and not when the pH was 6.8 or more. The acid pH resulted from the addition of antibiotics or dextrose without neutralisation, or from the addition of hydrochloric acid to the media. It is possible that the above apparent benefit of Medium CbM which had a measured pH of 6.3, may be related also to acidity. (The pH of double strength Medium CbM given by the suppliers is 5.9.)

The development of larvae to the moult step of the third-stage requires the visible formation of the cuticle of the pre-fourth-stage. The separation of the third-stage and the fourth-stage cuticles was observed, but only when the larvae were incubated at a pH of less than 6.0. When the pH was around 5, or less as in the hydrochloric acid activated pepsin treatment, then it appeared that separation may have been pathological rather than physiological. The larvae seemed mutilated, flattened and twisted, and at very low pH the entire larva was shrivelled, shrinking to a length of about 400 µ, inside an intact sheath about 600 µ long. This suggests a vulnerability of larvae with new unhardened cuticle to digestive effects in an acid medium. The survival of larvae at pH 6 or less was very poor, around 25% at the third day of culture.

There was no clear evidence obtained for an influence of any of the defined nutrient solutions or of the tissue extracts. However, larvae incubated in complex media appeared different to those incubated in simple, salt solution, media. Describing subjectively, they appeared
in better "condition", with fuller bodies and more expanded intestinal cells. There was greater tendency for browsing-like, or head probing, movement to take place in complex media. Pulsing, or sucking activity, of the pharynx was observed.

In general, larvae showed better survival in the presence of mixtures of nutrient solutions and tissue extracts than without. A drop in survival from roughly 75% to 25% occurred 1 or 2 days later than for salt solutions where it occurred on the third day of culture.

Both Medium CbM and NRS gave good survival when present at 20%, but were poorer at 50% and towards 100%. In Medium 858 at 20% or 50%, larval survival was better than at 80% or 100%. The inclusion of CEE, used up to 50%, always seemed to enhance development to the late third-stage, and survival.

However, it appears that the better the development, the poorer the activity and survival subsequently. In a medium of 80% Medium CbM and 20% NRS nearly all larvae survived to 3 days, and some of these developed to the moult third-stage, but in the next 2 days survival in these tubes was poorer (down to 30% all coiled), than in comparable tubes containing some CEE (70% active and 10% coiled).

The inclusion of a solid substrate, or of particulate matter, in the culture did not endow any detected benefit. The special additives included in cultures appeared to make no contribution to development or survival of larvae. It is noted that some of them or derivatives were present in tissue extracts anyway.

The use of reducing solution and lowered oxygen tension after 3 days did not enhance progress to the fourth-stage, and no effect of the change to the culture was evident.

After the addition of pancreatin at 3 days most larvae showed vigorous activity, and even some dead nematodes showed spasmic movement. Within 12 hours incubation many dead nematodes were being digested. No effect on the survival, or development, of live larvae was noted.
The effect of treating 3 day cultures with pepsin and hydrochloric acid has been reported above with the results of low pH.

Of the four solubising substances, at the levels examined, polysorbate was non-toxic and may even have enhanced development in a complex medium; dimethyl sulphoxide apparently enhanced survival of larvae in salt solution but its presence had no detected effect on development; ethanol was non-toxic at 0.5% and below but fatal over several hours at 5%; and dimethyl formamide was harmful even at concentrations as low as 0.25%.

Movement of the culture vessel showed a slight enhancing effect on the survival and activity of larvae. The degree of development was about the same whether cultures were moving or stationary.

The concentration of larvae in media had no detectable influence in these cultures. No obvious effect due to storage of infective larvae before culturing was observed.

In general, except in those cultures where no development of the exsheathed early third-stage larva was noted, some larvae developed to the late third-stage as shown in the Frontispiece. There was an accumulation of larvae reaching this step in cultures, often associated with inactivity and coiling of such larvae. The size of these developed larvae was about 560 µ, slightly less than undeveloped larvae.

Standard media, selected from this work to use in other observations, are given in the Methods.

TRIALS ON STAGES RECOVERED FROM THE HOST INTESTINE

Plan

In these trials several media and conditions based on those described for infective larvae were tested. Six cultures for parasitic third-stage larvae recovered 1 day after removal from the host intestine. Opening and grasping movements of the male burse was common, but copulation was not observed in any culture. In media with 0.5% CSE, development was maintained, even up to 6 days, in media with 10% CSE was not as good. In cultures with 30% CSE, development was best observed in media with 10% CSE, while 10% CSE was not as good.
after infecting rabbits, and six cultures for early fourth-stage larvae recovered at 3 days, were examined for progress in development following natural stimuli in the host. Twenty seven cultures of adult worms were examined for survival and maintenance of activity and ova production.

Concentrations of 500 per ml for third-stage and 300 per ml for fourth-stage larvae were used, and of between one and ten adults per ml. Both sexes were placed in each culture vessel for incubation of adult worms.

Special additives used with complex media included linseed oil, polysorbate, the steroids hydrocortisone (25 mg%) and sodium glycocholate (200 mg%), and the growth hormone prolactin (100 ug%).

Results

For both third-stage and early fourth-stage larvae, up to 85% survived for eight days in complex media which contained 20% to 30% Medium CbM, 15% to 20% embryo extract, and 15% to 20% serum. No growth was obtained in this period. Most third-stage larvae were around 600 µ long, with a maximum about 700 µ. The maximum length of the fourth-stage cultured was 1.5 mm. These larvae were shorter than those recovered after pepsin digestion of the gut wall (Experiment 1).

Adult Trichostrongylus invariably survived for 5 days in all media, including in Earle BSS complete with dextrose. Approximately 1% of worms survived up to 15 to 17 days - these were females. Eggs were passed only for 1 or 2 days after removal from the host intestine. Opening and grasping movements of the male bursa was common, but copulation was not observed in vitro.

"Normal" wandering movements of most worms were best maintained, even up to 8 days, in media with 20% CEE present, while 10% CEE was not as good. In cultures with 20% Medium CbM movement was no better than in BSS alone. After nine days, only slow movements were recorded.
Variations of the following factors were judged to have no influence: Transferring to fresh medium (at 3 days); pH (around 6.6 to 7.6); The gas phases (air, 5% CO$_2$ in air, and 5% CO$_2$ with 5% O$_2$); Movement (a slow roll or stationary mode); Concentration of worms; and Inclusion of the special additives given above.

The aim of this section was to examine in culture techniques to give a critical measure of the physiological status of *T. rerio* in response to its environment. The function of such a technique was for the ultimate examination of the influence of particular factors on the nematode, and to possibly examine the host.

This work was intended to use the uptake of radioisotope-labelled substances to examine the development of nematodes in culture, in place of the older visual histochemical and manometric techniques. Biochemical studies on the fate and incorporation of substances by *Trichostrongylus* are not reported in this thesis. Complementary research in this facet has been undertaken by W.J. Nicholas, in examination of contrast with *Caenorhabditis*.

The quantitative uptake of amino acids, trace labelled with carbon-14, was investigated as a sensitive means of determining growth and development. In pilot triple nucleotides provided similar information to the work with amino acids, so are not recorded here.

Principle culture media were selected from results in Section A. Variations in the culture techniques, particularly the factors involved in the initiation of parasitic stages, were examined for enhancement or suppression of amino acid uptake.

Secondly, the gross utilization of labelled amino acid, and later of labelled carbon dioxide in the medium, was investigated to determine whether uptake and turnover of isotope labelled substances was a vital process in the culture conditions.
SECTION B

UPTAKE AND TURNOVER OF SUBSTANCES BY PARASITIC STAGES

OBJECTS

The aim of this section was to examine in culture, techniques to give a critical measure of the physiological status of T. retortaeformis in response to its environment. The function of such a technique was for the ultimate examination of the influence of particular factors on the nematode, and its possible extension to use in the host. This work was intended to use the uptake of radio-isotope labelled substances to examine the development of nematodes in culture, in place of the older visual histochemical and manometric techniques. (Biochemical studies on the fate and incorporation of substances by Trichostrongylus are not reported in this thesis. Complementary research in this facet has been undertaken by W.L. Nicholas, in examination of contrast with Caenorhabditis.)

The quantitative uptake of amino acids, trace labelled with carbon-14, was investigated as a sensitive means of determining growth and development. In pilot trials nucleosides provided similar information to the work with amino acids, so are not recorded here.

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Secondly, the gross utilisation of labelled amino acid, and later of labelled carbon dioxide in the medium, was investigated to determine whether uptake and turnover of isotope labelled substances was a vital process in the culture conditions.
OBSERVATIONS

(i) Examination for Growth and Development

TRIALS ON AMINO ACID UPTAKE

Plan

In these trials various samples totalling four million larvae were incubated ("pulsed") with carbon-14 labelled amino acids, and the radio-activity retained by the nematode counted. About one tenth of these samples are described to illustrate the uptake of protein hydrolysate or l-isoleucine during a short period of time. Series of samples were taken for examination during 5 or 6 hours pulse incubation, or a single time was examined, usually at 3 hours. The pulse incubations were carried out in simple medium based on BSS, or in complex medium containing nutrient solution and tissue extracts, as described in the Methods. The handling of samples and controls is given in the Methods.

Variations in the conditions and treatment of *T. retortaeformis* before pulsing which were examined included: different exsheathment stimuli for varying times of exposure; various times of temperature adjustment at 20°C after storage at 4°C; incubation of larvae at 38°C prior to pulsing; and development of larvae in the host prior to pulsing.

Results

All the results cited are expressed as counts per minute (CPM) retained per $10^4$ third-stage larvae (that is, approximately per 1 mg dry matter or per 0.5 mg
Lowry protein). For comparisons in the text, the level of uptake per hour has been calculated. The concentration of radio-activity in the culture varied with the batch of radiochemical used, so the activity available per each sample of $10^4$ larvae is also stated below. The level of radio-activity in the medium did not, however, appear to affect the amount retained, within the range of levels used.

The retention of radio-activity from amino acids is illustrated by four examples as follows. The results of all trials were consistent, with slight differences only in the magnitude of activity.

1) The Uptake of $^{14}C$ from Labelled Protein Hydrolysate by Exsheathed Larvae

A gradual increase in amino acid uptake over 6 hours is shown in Figure 33. The repeatability of the results in three cultures of both simple and complex media is shown, and secondly, a comparison is given of exsheathment with carbon dioxide and hydrochloric acid, exsheathment with sodium hypochlorite, and killing the larvae before pulsing. In all cases the uptake by $10^4$ larvae was less than 150 CPM per hour. (There was 800,000 CPM per $10^4$ larvae available in the medium.) The uptake in complex medium was consistently lower than in simple medium. Another consistent feature was a higher uptake by larvae whose sheaths had been dissolved away in sodium hypochlorite, than by live larvae plus sheaths cast under the stimulus of carbon dioxide and hydrochloric acid. It is notable that the killed larvae took up amino acids to the same order of magnitude as the live larvae. The amount of amino acid retained was incompatible with protein synthesis and growth.

2) The Uptake of $^{14}C$ from Labelled L-isoleucine by Exsheathed Larvae

Similar observations are shown for L-isoleucine in Figure 34 as found with the protein hydrolysate mixture
of amino acids. For larvae exsheathed by carbon dioxide and hydrochloric acid stimulus, a low uptake of around 75 CPM per hour per $10^4$ larvae was achieved in simple medium, and half this in complex medium. (Each sample of $10^4$ larvae was exposed to 2,000,000 CPM of labelled L-isoleucine in the medium.) Larvae killed before pulsing had a similar uptake at 5 hours to live larvae.

3) The Uptake of C$^{14}$ from Labelled Protein Hydrolysate by Larvae after Different Exsheathment Stimuli

A comparison is given in Figure 35 of the amino acid uptake in simple medium, by larvae stimulated with carbon dioxide and hydrochloric acid, treated with sodium hypochlorite, or with no treatment before incubation at 38°C. (There was 1,000,000 CPM per $10^4$ larvae in the medium.) The larvae treated with hypochlorite retained about four times as much activity (around 200 CPM per $10^4$ larvae in an hour) as the physiologically exsheathed larvae. Sheathed larvae took up a considerably greater amount of the radio-active label. This effect was confirmed in other trials, but it was found that sheathed larvae did not take up more than exsheathed larvae in complex medium.

4) The Uptake of C$^{14}$ from Labelled L-isoleucine by Stages Recovered from the Intestine

When samples of the parasitic stages of T. retortaeformis recovered from rabbits were pulsed for 2½ hours, low levels of L-isoleucine uptake, still incompatable with tissue synthesis were obtained. The levels were, however, greater than those of exsheathed infective larvae, as shown in Table 21.

Other Factors)

No other treatment (as in the Plan), or combination of treatments, before incubation with the labelled amino acid, was found to have any effect on the retention of the radio-isotope by the nematode.
TRIALS ON AMINO ACID UPTAKE

Progressive Uptake of Carbon-14 from Labelled Protein Hydrolysate by Third-stage T. retortaeformis in Culture.

(There was approx. 800,000 CPM in medium available for each sample of $10^4$ larvae.)

Exsheathment stimulus:

A - NaOCl (live larvae)
B - $CO_2$/HCl (live larvae)
C - $CO_2$/HCl (larvae killed before incubation)
Protein Culture

(CPM above background x 10^{-3})

Simple Medium
Complex Medium

C-14 UPTAKE PER 10,000 LARVAE

INTERVAL OF INCUBATION (hours)

A
B
C

SIMPLE MEDIUM

COMPLEX MEDIUM
TRIALS ON AMINO ACID UPTAKE

Progressive Uptake of Carbon-14 from Labelled L-isoleucine by Third-Stage T. retortaeformis in Culture.

(There was approx. 2,000,000 CPM in medium available for each sample of $10^4$ larvae.)

Exsheathment stimulus:

$\text{CO}_2/\text{HCl}$

A - live larvae
B - larvae killed before incubation
TRIALS ON AMINO ACID UPTAKE

Uptake (over 1 Hour) of Carbon-14 from Labelled Protein Hydrolysate by Third-stage *T. retortaeformis* in Culture following Different Exsheathment Stimuli.

(The larvae were incubated in simple medium.)

(There was approx. 100,000 CPM in medium available for each sample of 10^4 larvae.)

Exsheathment stimulus:

A - Nil (live larvae)
B - NaOCl (live larvae)
C - CO₂/HCl (live larvae)
D - CO₂/HCl (larvae killed before incubation)
C-14 UPTAKE PER 10,000 LARVAE

<table>
<thead>
<tr>
<th>Stage of Nematode</th>
<th>Simple Medium (thous CPM)</th>
<th>Complex Medium (thous CPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheathed L₂</td>
<td>1.12</td>
<td>0.04</td>
</tr>
<tr>
<td>Exsheathed</td>
<td>0.96</td>
<td>0.03</td>
</tr>
<tr>
<td>1 day L₂</td>
<td>1.45</td>
<td>0.43</td>
</tr>
<tr>
<td>3 day L₄</td>
<td>3.53</td>
<td>0.43</td>
</tr>
<tr>
<td>Adults</td>
<td>2.08</td>
<td>0.58</td>
</tr>
</tbody>
</table>
Table 21

TRIALS ON AMINO ACID UPTAKE IN CULTURE

The Radio-activity Uptake per Hour by Samples of Different *T. retortaeformis* Stages Cultured for 2.5 hours with l-isoleucine-\(^{14}\)C

<table>
<thead>
<tr>
<th>STAGE OF NEMATODE</th>
<th>RADIO-ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Available per Sample(^0) in Medium (thous CPM)</td>
</tr>
<tr>
<td></td>
<td>Simple Medium (thous CPM)</td>
</tr>
<tr>
<td>Sheathed L(_3)</td>
<td>800</td>
</tr>
<tr>
<td>Exsheathed L(_3)</td>
<td>2,000</td>
</tr>
<tr>
<td>1 day L(_3) *</td>
<td>6,000</td>
</tr>
<tr>
<td>3 day L(_4) *</td>
<td>6,000</td>
</tr>
<tr>
<td>Adults *</td>
<td>3,000</td>
</tr>
</tbody>
</table>

\* Samples compared with biomass approximately equivalent to 10\(^4\) third-stage larvae (about 1 mg dry matter).

Results

Overall, the results showed that there was a slow uptake of amino acid, lasting at least 2.5 hours, by both live and killed larvae, and that this uptake was reduced in the presence of non-labelled amino acids. The results are apparently a measure of absorption, and not of growth or development in culture. Physiologically, sheathed infective larvae, despite the presence of cast sheaths in the sample also.

Samples of nematodes taken at intervals to 48 hours (and chromatographed and autoradiographed together) showed diminishing intensity of the isoleucine spot with time of incubation. The activity of these spots for a trial in simple medium is shown in Figure 36. This loss of substrate activity was not replaced by the appearance of alternative substances on the chromatograms, so it is likely that feeding.
Overall, the results showed that there was a slow uptake of amino acid, for a few hours at least, by both live and killed larvae, and that this uptake was reduced in the presence of complex media which contained non-labelled amino acids. The results are apparently a measure of absorption, and not of growth or development in culture of T. retortaeformis. Of the stages examined, the retention of the label was lowest in physiologically exsheathed infective larvae, despite the presence of cast sheaths in the sample also.

(ii) Examination for Viability

TRIALS ON AMINO ACID TURNOVER

Plan

Infective T. retortaeformis larvae exsheathed by carbon dioxide and hydrochloric acid stimulation were cultured with the addition to the medium of 1 µc of carbon-14 labelled l-isoleucine per sample of 10⁴ larvae. Samples of larvae were taken at series of intervals to examine for changes to the labelled substrate, as outlined in the Methods.

Results

Samples of nematodes taken at intervals to 48 hours (and chromatographed and autoradiographed together) showed diminishing intensity of the isoleucine spot with time of incubation. The activity of these spots for a trial in simple medium is shown in Figure 36. This loss of substrate activity was not replaced by the appearance of alternative substances on the chromatograms, so it is likely prima facie
TRIALS ON AMINO ACID TURNOVER

Loss of Carbon-14 Labelled L-isoleucine during Incubation of Exsheathed Third-stage T. retortaeformis.

(The larvae were exsheathed with CO₂/HCl, and cultured in simple medium.)

(The labelled isoleucine lost was not replaced by other substances detected with chromatography.)
that the larvae were metabolizing the amino acid to form non-volatile products. Under these circumstances it may be proposed that labeled substrate will not accumulate in the medium.

Larvae were starved with radio-active carbon dioxide in the medium supplied as carbon-14 labeled sodium bicarbonate. Three groups of five larvae were incubated, each group of five larvae. A sample of larvae and media was taken at each point of intervals to examine for the incorporation of labeled carbon dioxide, as outlined in Methods.

The radio-activity remaining after removal of the carbon dioxide is due to substances formed by metabolism of the larvae, as the treated medium was examined for non-volatile material and that retained by the larvae, by extracting with ethanol. The radio-activity incorporated directly by, or extracted from, the larvae, when the carbon dioxide retention level reached about 15,000 CPM per 10^3 larvae, the activity incorporated showed a plateau. This occurred at about 1 to 2 hours in simple...
that the larvae were metabolising the amino acid to volatile products. Under this circumstance it may be deduced that labelled substrate will not accumulate in viable larvae.

TRIALS ON CARBON DIOXIDE UTILISATION

Plan

Larvae were cultured with radio-active carbon dioxide in the medium, supplied as carbon-14 labelled sodium bicarbonate. Three groups of T. retortaeformis were compared, sheathed infective larvae, sodium hypochlorite treated, and carbon dioxide and hydrochloric acid stimulated exsheathed larvae. Samples of larvae and media were taken at series of intervals to examine for the incorporation of labelled carbon dioxide, as outlined in the Methods.

All radio-activity remaining after removal of the carbon dioxide is due to substances formed by metabolism of the larvae, so the treated medium was examined for non-volatile materials excreted as well as examining the sample of larvae.

Results

The residual incorporation of the radio-isotope into samples of $10^3$ larvae after ethanol extraction is given in Figure 37 for simple medium and complex medium over 4 hours incubation. Also shown on the graphs is the total non-volatile activity recovered from each sample of larvae plus medium. The difference, between the total and that retained by the larvae, is a measure of the substances excreted by, or extracted from, the larvae.

When the carbon dioxide retention level reached about 15,000 CPM per $10^3$ larvae, the activity incorporated showed a plateau. This occurred at about 1 to 2 hours in simple
medium, but was not reached by 4 hours in complex medium where the rate of labelled carbon dioxide incorporation was much slower.

Isotope labelled substances continued to accumulate in the medium throughout the course of the trial. This occurred at roughly the same rate in both the simple and complex media.

In the simple medium there was no obvious difference between the three groups of larvae. In the complex medium the untreated sheathed larvae appeared to incorporate the labelled carbon dioxide into non-volatile substances at a lower rate than the groups of treated larvae.

Control groups of cultures treated in the same way but without larvae did not give the carbon dioxide fixation as reported above.
Figure 37

TRIALS ON CARBON DIOXIDE UTILISATION

Incorporation of Carbon-14 from Labelled Sodium Bicarbonate by Third-stage T. retortaeformis in Culture, into Non-volatile Substances (within or released by the Larvae)

Exsheathment stimulus:

A - Nil
B - NaOCl
C - CO₂/HCl
**TABLE 1**

<table>
<thead>
<tr>
<th>Total Activity (medium and larvae)</th>
<th>Washed Larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>○○○○○○</td>
</tr>
<tr>
<td>B</td>
<td>△△△△△△</td>
</tr>
<tr>
<td>C</td>
<td>□□□□□□</td>
</tr>
</tbody>
</table>

**SIMPLE MEDIUM**

**COMPLEX MEDIUM**

**INTERVAL OF INCUBATION** (minutes)

Notes:
- Observations indicated that some third-stage larvae and fourth-stage larvae will develop to the fourth stage, and some will reach the fifth stage. Further work is needed to determine if this is due to age of the nematode or other factors.
- Development of the nematode was found to be affected by various conditions, including temperature and pH.
- The data was collected under standardized conditions for the development of T. articulatum.
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DISCUSSION

(i) Development and Growth of the Nematode

The observations (Section A) showed that some third-stage infective *T. retortaeformis* larvae will exsheath and develop in culture to the moult third-stage (i.e. the pre-fourth-stage) within 3 days. This is similar to the report (Silverman 1962; Silverman, Poynter, and Podger 1962) that *T. colubriformis* developed to the fourth-stage in culture, and some of these reached the sheathed fourth-stage as early as 72 hours (P.H. Silverman 1970 pers. comm.). J.T. McI. Neilson (1970 pers. comm.) also obtained development of *T. colubriformis* to the very early fourth-stage using Silverman's technique. F.W. Douvres (1971 pers. comm.) obtained development of the parasitic third-stage of *T. colubriformis* with a tissue culture system.

However, the casting of the old cuticle at the third ecdysis to free the pre-fourth-stage was not reported in vitro, and growth of developing *Trichostrongylus* (which commences with the liberated fourth-stage), has not been found in culture. A similar failure has been met in attempts to culture *T. axei* through developmental stages (S.E. Leland 1971 pers. comm.; F.W. Douvres 1971 pers. comm.).

Media at a pH around 6.5 seemed to provide optimum conditions for the development of *T. retortaeformis* observed in this study. This is within the normal pH range in the proximal duodenum (Robinson 1935; Hobson 1948;
Dukes 1955 p.413) which the adult nematodes usually inhabit.

The lack of growth of *T. retortaeformis* in culture is verified by the very low retention of radio-isotope labelled amino acids from the media (Section B). An uptake was found of the order of $10^1 - 10^3$ CPM per hour for 10,000 third-stage larvae (equivalent to ca. 1 mg dry weight, and ca. 0.5 mg Lowry protein). This compares with an uptake of the order of $10^4 - 10^6$ CPM per hour, calculated for a similar biomass of two free-living nematodes growing in culture, *Caenorhabditis* (W.L. Nicholas 1970 pers. comm.), and *Panagrellus* (Pasternak and Samoiloff 1970).

There appeared to be a plateau of retention of labelled amino acids that was the same for both live and killed *T. retortaeformis*, which suggests that the uptake may be inanimate absorption, and not incorporation. However, other workers have exposed parasitic nematodes (as follows) to carbon-14 labelled amino acids in culture, without reports of growth, and recorded uptake as incorporation (Hankes and Stoner 1956, *Trichinella* larvae; Stoner and Hankes 1958, *Trichinella* larvae; Weatherly, Hansen, and Moser 1963, *Ascaridia* adults; Jaffe and Doremus 1970, *Dirofilaria* larvae). When the reported uptakes of carbon-14 were estimated as approximate CPM uptake per hour for 1 mg dry weight, to make a crude comparison, it was found that they were much closer to the level for *Trichostrongylus* (passive absorption?) than to the level for *Caenorhabditis* (active incorporation). These workers did not use controls of killed nematodes, and thus it is not known whether *Trichostrongylus* differs from the other species. J.J. Jaffe (1970 pers. comm.) had reasoned that since amino acid uptake (incorporation?) is an energy requiring process, it would not occur with killed nematodes.

Fairbairn (review: 1957) considered that the notion of cuticular absorption of nutrients, instead of ingestion, should be abandoned, and Roberts and Fairbairn (1965)
stated that the level of absorption obtained in Weatherly's experiments was scarcely significant. It may be that certain nutrients must be ingested to be incorporated for growth. However, cuticular absorption alone could provide a sufficient turnover of nutrients for maintenance of dormant nematodes, for example arrested larvae in host tissues (McCoy, Downing, and van Voohis 1941), or T. retortaeformis arrested in culture.

It is surmised that particular substances that act as growth promoting factors (GPF) and ecdysis stimulating factors (ESF) were missing from the cultures in this study. The identity of whatever factors are required for Trichostrongylus is a matter for conjecture at this stage. They appear not to be known environmental factors, because when cultured together, Cooperia and Ostertagia develop while Trichostrongylus does not (Leland 1963). Similarly, although Nippostrongylus and Strongyloides occupy the same habitat and follow the same cycle in the rat, only Nippostrongylus develops, even when they are cultured together in the same tube (P.P. Weinstein 1971 pers. comm.).

Secondly, the unknown factors do not appear to be a once only trigger stimulus from the host, because nematodes recovered from the host after commencing development stop growing in culture.

It seems likely that an unknown ESF, and perhaps GPF, is a steroid, by analogy with arthropods (e.g. Karlson and Sekeris 1966). A steroid is an essential requirement for the growth of free-living nematodes (Hieb and Rothstein 1968), and also the effect of host steroids occurs during the pre-patent growth of parasitic nematodes (Ford 1968). None of the steroids examined in this study, or present in the tissue extracts, were able to fulfil the function for T. retortaeformis. Other essential requirements, such as haem (Hieb, Stokstad, and Rothstein 1970), for the growth of free-living nematodes (review: Rothstein and Nicholas 1969) were present in the tissue extracts in the media.
Lapage (1933b) suggested a close relationship between nematodes and intestinal bacteria, and found that microorganisms were carried in the gut of *T. retortaeformis*. Surface sterilisation does not disinfect larvae of microorganisms in the gut (Davey 1938a; Glaser and Stoll 1940; Jensen and Siemer 1969). It is possible that an unknown factor may normally be supplied to the nematode by a particular symbiotic micro-organism, as for *Neoaplectana* sp. (Poinar and Thomas 1966), and in a similar fashion to the requirement of certain insects (see Trager 1953; Gilmour 1961). Bacterial flora in the normal host exhibit a favourable influence on the development of intestinal nematodes (e.g. Wescott and Todd 1964; Wescott 1968), while the reverse effect was seen in an abnormal host (Newton, Weinstein, and Jones 1959). Stefanski and Przyjalkowski (1966, 1967) showed that the nematode responses varied with the species of bacteria present. As an alternative to the synthesis of essential nutrients, micro-organisms may inactivate toxic substances present in the environment of the nematode, as discussed by Weinstein, Newton, Sawyer, and Sommerville (1969), and could metabolise inhibitory products excreted by nematodes in culture.

(ii) Viability of Larvae and Catabolism

Most *T. retortaeformis* larvae remained alive in culture for 3 days, irrespective of the medium. In some trials, observed up to 15 days, 60% of third-stage larvae survived in balanced salt solution after physiological exsheathment.

Although the uptake of carbon-14 labelled amino acid appeared to be inanimate, possibly by cuticular absorption (Weatherly, Hansen, and Moser 1963), a turnover of the label occurred in viable larvae. The disappearance of carbon-14 in solution from labelled amino acid suggests deamination (Mahler and Cordes 1966 pp.686) and excretion.
by *T. retortaeformis* of volatile products such as carbon
dioxide or volatile fatty acids. The end products of
metabolism in nematode parasites have been discussed by

Discharge of amino acids in culture is common in other
nematodes (Balasubramanian and Meyers 1971), and
*Caenorhabditis* excretes more amino acids than are incorporated
(Rothstein 1963). The shrunken (live) *T. retortaeformis*
larvae observed in culture were suggested by Lapage (1935b)
to result from an osmotic effect, and may follow the
excretion of amino acids following from the metabolism of
protein and peptides.

Amino acids may be catabolised by nematode parasites
in culture as a source of energy, with the release of
ammonia and amines (Rogers 1952; Haskins and Weinstein 1957).
In culture it seems that any amino acid uptake by live
*T. retortaeformis* larvae, over the threshold seen in dead
nematodes, may have been catabolised for energy production.

It is suggested that the initiation of parasitic stages
by exsheathment (second ecdysis) may switch on the
turnover of amino acids, and thus the sheathed larvae when
cultured had a considerably higher retention of carbon-14
than the exsheathed larvae because they were less able to
catabolise the amino acids absorbed. Wilson (1965) found
that exsheathed third-stage *Nippostrongylus* larvae in
culture utilised endogenous protein more rapidly than any
other source of energy, but confused the issue by
continuing to refer to these larvae as infective larvae,
a term which is normally applied in strongylate nematodes
to the sheathed third-stage. All other studies on infective
larvae have shown that the metabolism of lipids is the
major source of energy (some references in Barrett 1969).
However, a change in lipid composition occurs with the
temperature elevation at the initiation of the parasitic
mode of life (Barrett 1968).

Some further switch apparently needs to be activated
for *T. retortaeformis*, presumably following the next
(third) ecdysis, for the anabolic utilisation of amino acids and growth, which has not been observed for this nematode in culture.

Active metabolism by the third-stage *T. retortaeformis* larvae in culture was verified by the vital utilisation of radio-isotope labelled carbon dioxide. The possible pathways for carbon dioxide fixation known in parasitic nematodes have been reviewed by von Brand (1966 pp.79).

(iii) Arrested Development and the Accumulation of Larvae

The larvae in culture separate into those which do not appear to change after exsheathment (EL₃), and those which show some development. The discussion relates to the latter.

There was always an accumulation of *T. retortaeformis* larvae at the late third-stage (LL₃), and even in the larvae which developed to the pre-fourth-stage there was a failure of the third ecdysis. Silverman (1965b) reported that *Dictyocaulus* required an ecdysis stimulating factor (ESF) in the medium for the ecdysis of the third-stage to the early fourth-stage. This factor was present in some liver extracts (DIFCO peptone) and not in others. The requirement for ESF was reduced when the larvae had been held at the infective stage for some time before incubation.

It is suggested that a required ESF for *Trichostrongyulus* was absent from the cultures that have been examined. It was not supplied for *T. retortaeformis* by the addition of DIFCO peptone, host digestive enzymes, or reducing chemicals, and larvae that had been held for some time before culture did not progress beyond the late third-stage without it. Davey (1966; Davey and Kan 1967) put forward the view that ecdysis is under endocrine control (review: Rogers and Sommerville 1968), and a specific ESF may be required in the environment in excess of a threshold level, to trigger release of the substances
causing ecdysis. The threshold level may be modified by the prior history of the larvae.

The late third-stage at which the development of *T. retortaeformis* larvae in culture becomes arrested was found to be indistinguishable from the stage at which development in the host becomes inhibited (Frontispiece). In the host this is a histotrophic phase associated with the mucosa of the gut, and in culture the larvae in this phase become inactive and coil up.

Weinstein and Jones (1956) stated that *Nippostrongylus* larvae reach the late third-stage in all culture media (Thus in the absence of a ESF), and these larvae survive in an arrested state for fairly long periods (Weinstein 1958). *Nippostrongylus* larvae also become inhibited at the late third-stage in the skin (Taliaferro and Sarles 1939) or in the lungs (Haley 1958b). Further, Leland (1967a) reported a consistent accumulation of late fourth-stage *Cooperia* larvae in culture, and suggested that the fourth ecdysis was a critical step, presumably due to the requirement of a fourth-stage ESF (ESF₄). Sommerville (1960) showed that inhibition of *Cooperia* in the intestine also occurs prior to the fourth ecdysis. It is noted that *Nippostrongylus* larvae, and perhaps other species, which apparently require an ESF₃ to continue development (as above), may require an ESF₄ as well, because they can become inhibited at the fourth-stage in the intestine (Chandler 1932b).

By comparison, the gastric species of trichostrongylids, *Haemonchus* and *Ostertagia*, develop through the third ecdysis to the early fourth-stage but do not grow further, when cultured in salt solutions (review: Silverman 1965b). It is thus apparent that arrested development of these species in culture is not due to the absence of an ESF, and is probably due to the lack of a growth promoting factor (GPF). Inhibition of *Haemonchus* and *Ostertagia* also occurs in the stomach at the early fourth-stage (e.g. Roberts 1957; Sommerville 1954; respec.).
It is therefore suggested that *T. retortaeformis*, and probably other species, will develop in culture after exsheathment, until the same stage is reached at which inhibition is expressed in the host, irrespective of the medium in which they are incubated (providing it is not toxic).

It is surmised that development of larvae becomes arrested (inhibited) when the concentration of ESF (intestinal species) or GPF (gastric species) in the environment is below a critical threshold requirement. Thus inhibition tends to occur preceding (ESF requirement) or following (GPF requirement) an ecdysis, as pointed out by Sommerville (1960). Presumably inhibition could occur at other times if GPF subsequently became limiting. It follows from the Discussion of inhibition in Part II that the critical threshold level for individual larvae will vary, and may be altered within a population by selection. Some conditions under which inhibition is seen may be explained as a block of the response by larvae to ESF or GPF (e.g. resistant host, toxic medium), as an alternative to a low environmental level of specific ESF or GPF (e.g. abnormal host, inadequate medium), or to a raised threshold level (e.g. prolonged aging of infective larvae).
PART IV

GENERAL SUMMARY

*T. retortaeformis*, a streptolyrate nematode of the rabbit (*Oryctolagus cuniculus*), was considered to be representative of the species of *Trichostrongylus* inhabiting the small intestine of grazing mammals. The field occurrence of, and progress in experimental investigations with, *T. retortaeformis* were briefly reviewed.

The theme of *T. retortaeformis* infections in the characteristics of the host-parasite association, as outlined in a foreword. A comprehensive review was presented of variation in features influenced by physiological factors (i.e., excluding frank immunological or pathological reactions). Further, attempts to culture the parasites were considered (with a view to examining directly the effect of factors from the host on features of the nematode), and previous reports reviewed.

Characteristics of *T. retortaeformis* Infection in the Normal Host

Features of the Pre-patent Period

*T. retortaeformis* infective larvae within the sheath (uncast second-stage cuticle) were ca. 630 μ long. On ingestion by a susceptible host the larvae uncasted (second ecdysis), and within 2 days the third-stage larvae became slightly smaller (ca. 360 μ long). During this
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The theme of this study was variations in the characteristics of the host-parasite association, as outlined in a foreword. A comprehensive review was presented of variation in features influenced by physiological factors (i.e. excluding frank immunological or pathological reactions). Further, attempts to culture the parasites were considered (with a view to examining directly the effect of factors from the host on features of the nematode), and previous reports reviewed.

Characteristics of T. retortaeformis Infection in the Normal Host

Features of the Pre-patent Period

T. retortaeformis infective larvae within the sheath (uncast second-stage cuticle) were ca. 630 μ long. On ingestion by a susceptible host the larvae exsheathed (second ecdysis), and within 2 days the third-stage larvae became slightly smaller (ca. 560 μ long). During this
interval metamorphosis to the late third-stage occurred, the cuticle became thickened, and an obvious notch in the tail of larvae deepened.

Around 2 to 3 days after ingestion larvae began to grow, and the third ecdysis occurred. Prior to the ecdysis the tail notch deepened so that a terminal knob appeared, and the pre-fourth-stage cuticle formed anterior to it. Third-stage larvae were not recovered greater than 700 µ long.

The larvae grew very rapidly for a week, doubling their length every two days.

The sexes of fourth-stage larvae were readily distinguished after 4 days. The fourth ecdysis occurred around day 5 to day 7 when the males were ca. 2.7 mm long, and the females ca. 3.2 mm long. Adult nematodes measured at the end of the pre-patent period were up to 9.5 mm (females) and 7.5 mm (males).

The pre-patent period was found to be 10 to 13 days. With low levels of infection (less than 500 nematodes), it was sometimes longer than 2 weeks (possibly because the numbers of eggs passed earlier, if any, were less than the minimum detectable level).

In a proportion of *T. retortaeformis* (roughly of the order of 2% of the larval dose) development was arrested at the late third-stage prior to the third ecdysis, when the larvae were ca. 560 µ long. These larvae are very difficult to detect in preparations from the host gut.

No evidence was found of a vasculo-pulmonary migration of larvae. Except for inhibited larvae found in the stomach of adult rabbits, no *T. retortaeformis* were recovered outside the small intestine. Nearly all were found in the proximal region of the intestine.

However, larvae were missing from some post mortem preparations within a few days of infection. It was suggested that immediately after an ecdysis larvae were destroyed in the pepsin digestion of the gut wall, probably before their freshly exposed cuticle had
Features of the Patent Period

With weekly sampling of egg production, the initial patent period commenced at 2 weeks after infection. With moderate dose levels of infective larvae, it terminated with negative counts at week 7, or at week 4 when a more rapid initial rise in egg count was found. However, with lower levels of infections (around 1,000 nematodes established), negative egg counts were not found until week 10, or in some cases did not occur in observations up to 12 weeks. With low levels of infection (less than 500 adult nematodes) the egg counts fluctuated about the minimum detectable level.

The mean egg production per adult female *T. retortaeformis* in moderate infections (around 2,000 to 5,000 nematodes) was usually around 100 to 200 eggs over 24 hours, but varied. Zero egg output was obtained even from populations of adult females with eggs in the uterus. Mean production up to 400 eggs per female per day was found at low levels of initial infection. After a loss of part of the infection, the mean egg production per female in the residual population was lower than before.

A rhythm of egg production was detected with a cycle length of around 22 days between peaks. It was suggested that egg production may be stimulated by host-hormones, and the rhythm was integrated with a hormonal cycle in the host. The more precipitous falls in egg output were associated with a loss of the nematodes present. This loss usually occurred after the first or second peak egg count, and thus troughs in egg output followed the first two peaks more closely (i.e. sharper fall) than later. It was suggested that rising egg output was suppressed (hence a peak) by an immunological response, when egg production exceeded a threshold (the threshold level and response being influenced by cycling hormonal levels).
Variations of egg size were found to relate to cycles of egg production. Small eggs were passed at the beginning of a patent period, and the incidence of eggs larger than the median volume increased towards the end of each cycle. A heterogeneous population of adult females was suggested, with instances of overlapping of egg production so that a rise commenced during the decline in egg output of the previous cycle. The cycles of egg output occurred in the established T. retortaeformis adult population, because inhibited larvae were not present to replace nematodes lost after each peak egg count.

Mature nematodes persisted for over a year in rabbits without reinfection. Adult rabbits could be infected, even after receiving regular small doses for over a year.

With small doses (a few hundred infective larvae at a time), up to 100% were recovered as adults at 3 to 4 weeks after the beginning of infection.

Characteristics of T. retortaeformis in Culture

T. retortaeformis infective larvae were exsheathed by treatment with 0.05% sodium hypochlorite, or by a pseudo-gastric stimulus with carbon dioxide and hydrochloric acid (maximum exsheathing with $10^{-3} \text{N HCl}$ - ca.$\text{pH}$ 3, plus 100% CO$_2$, at 38°C, for 1 - 2 hours, on a roller/rocking).

Many third-stage larvae underwent metamorphosis in culture, irrespective of the medium used. Around 2 - 3 days of incubation, larvae reached the late third-stage, where their development was arrested. The developed larvae became slightly smaller (ca. 560 µ long), and tended to coil. They were morphologically indistinguishable from larvae whose development was inhibited in the host.

Under certain conditions larvae developed to the moult third-stage (pre-fourth-stage), but the third ecdysis did not occur. It was surmised that an ecdysis stimulating
factor (ESF) requirement was not provided in the cultures. Separation of the old and new cuticles occurred at low pH levels (less than 6), but appeared to be an artifact.

The addition of carbon dioxide or of bicarbonate, irrespective of the medium, appeared to promote development. A pH around 6.5 gave optimal results for development and survival.

Maximum development of larvae was associated with lower survival, but both features appeared enhanced by the presence of Caenorhabditis briggsae Maintenance (CbM) Medium, and tissue extracts.

Growth of exsheathed infective larvae, or of larvae taken from the gut of the rabbit, was not observed. Mating and egg production in adults was not observed. It was postulated that a chemical or physical growth promoting factor (GPF) requirement was not met in the cultures.

A feature of exsheathed T. retortaeformis larvae in culture was a failure to retain radio-isotope from carbon-14 labelled amino acids at a higher level than that adsorbed by killed control larvae. This is consistent with the failure to observe growth.

However, the live larvae showed an active turnover of the labelled substrate. It was suggested that physiological exsheathment triggers the capacity of third-stage larvae to catabolise amino acids taken up, and to excrete metabolites. A retention of higher levels of radio-isotope in sheathed infective larvae suggested that they lack this capacity. The viability of larvae in culture was confirmed by the fixation of carbon-14 labelled carbon dioxide into non-volatile metabolites.
Sources of Variation of *T. retortaeformis* Characteristics

Factors Derived from the Treatment and Status of the Infective Larvae

The exsheathment of infective larvae (in response to a pseudo-gastric stimulus) was facilitated in relation to the period that larvae were held before infection. This was paralleled by increasing infectivity, referred to as maturation, for a period of about 4 weeks at 20°C. Those larvae first reaching the infective stage (3 to 4 days after the start of incubation of eggs in faeces) were scarcely infective. Infectivity increased by 1% every 3 to 4 hours until maximal infectivity was approached, which was about the time of optimal yields from faecal culture. The initial rate of egg count rise, and the egg production per female, also increased following a maturation period.

The difference in nematode recovery, due to variations in infectivity between a 1 week and a 4 week incubation, exceeded the experimental variation between groups of hosts in other observations.

The surviving larvae held at ambient temperature (20°C) for longer than 4 weeks before infection, referred to as aging, showed decreasing infectivity, although the potential for exsheathment continued to increase. The same result was obtained with larvae held in the cold (4°C), referred to as storage, for more prolonged periods. Parasitic development appeared delayed, with a slower rate of initial egg count rise and initially a depressed egg output per female. It was suggested that the more slowly developing nematodes, which persist longer in the host, have a useful function when the free-living environment is unfavourable for transmission of the parasite. Infective larvae with the potential for fastest development may be those which use up their energy reserves and become uninfected first, and are thus selected against during aging.

A greater inhibition of development was observed from stored larvae than from fresh larvae (as noted below).
Factors Derived from the Administration of Infective Larvae

It was found by comparison between experiments that a greater proportion of *T. retortaeforis* were established from low or moderate dose levels (up to ca. 5,000 infective larvae) than from high dose levels. The egg production per female was greater at lower levels of infection.

Inhibition, or arrest, of larval development occurred with high larval doses, and was not detectable at significant levels in moderate levels of infection. Inhibition was suggested to occur when (i) a proportion of the larvae in a batch had a higher threshold requirement for an ESF or GPF than the level available in the environment. The threshold level of individual larvae appears to vary, and a higher level was selected for (or modified) by aging of larvae before infection of rabbits. (ii) A sufficient quantity of ESF or GPF for normal development may be lacking in the environment (as shown by arrest of larvae in culture, and the greatest proportion of inhibited nematodes at highest levels of infection). (iii) The availability of ESF or GPF to the larvae may be depressed by a resistant state of the host (thus a greater degree of inhibition was found in rabbits that had a previous infection, and in older rabbits).

The splitting of a given level of infective larvae into multiple doses over 18 days lead to a higher number of nematodes established in the rabbit. The rate of development was not delayed. There was a much greater loss of infection after the establishment phase than in a single dose infection. Although the egg production per female was initially greater, the egg output overall was depressed.

In rabbits that had experienced a previous infection, the number of *T. retortaeforis* becoming established, and the egg production per female, were depressed, although
there was no delay in the initial rate of egg count rise. However, more nematodes remained at the residual phase than in a primary infection.

A greater inhibition of larval development was observed when rabbits had had a previous infection (as noted above).

It was concluded that experience of the parasite, firstly, from a previous infection lead to suppression of subsequent establishment of the nematode, and secondly, during a primary infection lead to suppression after the nematodes were established. The mechanisms are presumably immunological.

Factors Derived from the Treatment and Status of the Host

When rabbits were fed on laboratory pellets (which included meals from cereal and animal sources) the egg production per female nematode was considerably greater than from rabbits fed lucerne. The number of *T. retortaeformis* and the initial rate of egg count rise were similar on the two diets (except in the bigger rabbits which had more nematodes when fed laboratory pellets). However, the greatest expulsion of nematodes after the establishment phase occurred in rabbits fed laboratory pellets, while there was no loss apparent in the rabbits fed lucerne.

More nematodes were recovered during the first 11 days of infection from juvenile female rabbits than from males, possibly related to more rapid sexual maturation of the females. On the other hand, the egg production per female nematode was much greater in the male rabbits.

A difference in photoperiod had no detectable effect on establishment of infection in young male rabbits.

During the first 15 days of infection, early pregnancy had no effect (except in the bigger rabbits which had more nematodes when pregnant). Lactation caused a faster initial
rise in egg count, but did not alter the number of nematodes established. Weaning does of their litter at the beginning of infection caused a marked reduction in the number of nematodes recovered, and an even more marked suppression of egg production by the remaining female nematodes.

Lactation, following parturition 30 days after infection, prevented the loss of infection which occurred in the control does, and resulted in persistent egg production by the adult nematodes already present (including a rise in egg output from those does in which the egg count had already fallen to zero). Between days 30 and 45 after infection with 5,000 larvae, the lactating rabbits passed a geometric mean of 94,800 eggs each day, compared to only 300 per day from the control does.

The elevated egg production seen in lactation, and the higher egg production early in the infection of rabbits fed laboratory pellets (cf. lucerne) and of male rabbits (cf. female), were suggested to be due to anabolic effects of factors in the diet, and of reproductive or growth hormones.

In general, it was found that the greater the number of *T. retortaeformis* established, or the greater the initial egg production, then the greater the probability of (and the more severe) the expulsion of nematodes about 4 weeks after infection. This loss of infection was associated with reduced egg laying in the residual female nematodes. The mechanisms are presumably immunological.

It was suggested that more slowly developing nematodes (e.g. from aged infective larvae), and lower levels of established nematodes (e.g. in secondary infection or on green feed) - each of which is respectively less likely to precipitate loss of infection - would provide a major contribution for the post-parturient rise in *T. retortaeformis* egg counts from lactating does.
Variation in Host Features

Effects on host body weight gain were noted in some experiments. A positive regression of faecal output ("appetite") and body weight gain was found.

At a high level of infection, body weight was lost, but the effect was delayed early in infections with larvae that had been stored in the cold (and whose parasitic development was delayed). The stored larvae appeared more pathogenic later in the infection than the larvae not stored. At a moderate level of infection, a greater depression of body weight gain was caused by infections with larvae that had been stored in the cold for only 2 days, than by infections with larvae not exposed to cold. Further experimental observations with non-infected control rabbits are required.

In the variations summarised above under factors derived from the administration of infective larvae, a direct effect was seen during establishment of infection - the greater the number of nematodes, the greater the depression of body weight in the rabbits. The body weight gain, therefore, was greatest in those rabbits which were resistant at the beginning of an infection. This effect was taken to be normal.

Anomalous effects occurred during establishment of *T. retortaeformis* infection - there was greater parasite production (eggs) and greater host production (body weight) together, in rabbits fed laboratory pellets (cf. lucerne). It was suggested that this is due to anabolic factors favouring both the host and parasite, i.e. the thriving host theory. Two alternative theories discussed do not appear applicable to this study.

A major paradox occurred with the loss of infection, because it was invariably associated with a loss of body weight (including the rabbits which were least affected up to this time). The severity of the effect was directly related to the level of infection initially established, and thus to the expression of immunity during an infection.
irrespective of experimental factors. It was suggested that the mechanism of expulsion itself, interferes with the well being of the host.

Thus the body weight gain is not necessarily related to the number of nematodes present. These observations, at moderate levels of infection, support the dictum quoted by H. McI. Gordon:

"Happy Hosts have Happy Parasites".
REFERENCES

Manuals


Reports


Authors and Editors


Anon. see Manuals and Reports at front of references.


Dunsmore J.D., (1966a), See Anon. (1966r).


Filmer J.F., (1948), see Anon. (1948r).


Leland S.E., (1967a). The In-vitro Development of Cooperia pectinata, a Nematode Parasite of Cattle, from Third-Stage Larvae to Adults, including Egg Production. J. Parasit. 53: 630-633.


Mönig H.O., (1934). Veterinary Helminthology and Entomology (1st Edn.). Bailliére Tindall & Cox, London; Williams and Wilkins, Baltimore, 402 pp. (see also Lapage G. (1962), and Soulsby E.J.L. (1968)).


Piatkowska W., (1966). Sposoby Rozpoznawania grubosciennych
jaj Nicieni P a sozytujacych u Czlowieka i Zwierzat Domowych,
Najczesciej Spotykanych w Probach Ziemi z otoczenia

on the Peripheral Lymphatic Tissue of Neonatally

Hormones and Immunological Maturation, in Wolstenholme

obacter nematophilus (Achromobacteraceae: Eubacteriales)
in the Development of the Nematode, DD-136 (Neoaplectana

Poole J.B., (1954). Studies on the Micro-environment of the
Free-living Stages of Nematodes. Thesis, McGill University,
Montreal 115 pp. cited after Andersen, Wang, and Levine
(1966) and Andersen and Levine (1968), which see.

Disease Produced in Guinea Pigs by Trichostrongylus
colubriformis, with observations on Natural and Artificially
Acquired Immunity. J. Parasit. 48 (Proc.): 52 (abst. 130).

Prasad D., (1959). The Effects of Temperature and Humidity on
the Free-Living Stages of Trichostrongylus retortaeformis.

Linstow) et du Strongylus strigosus (Dujardin) Bull. Soc.

Railliet A., (1889). Developpement Experimental du Strongylus
strigosus (Dujardin) et du Strongylus retortaeformis


Ransom B.H., (1920) cited in Ransom B.H., and Foster W.D.,
(1920). Observations on the Life History of Ascaris

Ransom B.H., (1923). Use of the Guinea Pig as an Artificial


Rohrbacher G.H., (1957). The Effect of Green Feed upon the Development of Trichostrongylus axei in the Laboratory Rabbit. J. Paras. 43 (Proc.): 11-12 (abst. 4).


Sarles M.P., (1929a). The Effect of Age and Size of Infestation on the Egg Production of the Dog Hookworm, 


Sarles M.P., (1930). The Occurrence of Self-Cure and Protection in Rabbits Receiving Repeated Infections of 
Trichostrongylus calcaratus. J. Parasit. 17: 114 (abst.).

Sarles M.P., (1932a). Development of an Acquired Resistance in Rabbits by Repeated Infection with an Intestinal Nematode, 


APPENDIX I

A List of References to STRONGYLATA IN LAGOMORPHS
LIST OF REFERENCES
TO STUDIES OF
STRONGYLATA IN LAGOMORPHS

(Oryctolagus, Sylvilagus, Lepus)

FAMILY ANCYLOSTOMIDAE
GENUS ANCYLOSTOMA:
GENUS NECATOR:

FAMILY DICTYOCALVIDAE
GENUS DICTYOCAULUS:

FAMILY HELIGMOSOMIDAE
GENUS NIPPOSTRONGYLUS:

FAMILY PROTOSTRONGYLIDAE
GENUS MUELLERIUS:

Noda 1963
Yamanaka 1960
Stiles and Goldberger 1906
Yoshida and Fukutome 1967
Shetty, Himes, and Edds 1970
Soliman 1953
Wade, Fox, and Swanson 1960
Wade, Swanson and Fox 1961
Harley and Gallicchio 1970
Rendtorff 1961
Sulzer 1963+
Sulzer and Goodchild 1963
Thorson 1953a, 1953b
Beresford-Jones 1966

The full reference is given in the main body of the thesis.

Herman (1942 pp.171) listed 374 papers, on the parasites of rabbits, published since 1780.

Reports are omitted which only show the occurrence of nematodes, and incidence of infected animals, in field samples.

+ Denotes thesis.
FAMILY STEPHANURIDAE
GENUS STEPHANURUS: Alicata 1971

FAMILY TRICHONEMATIDAE
GENUS OESOPHAGOSTOMUM: Alicata 1933
Berger and Ribelin 1969
Grigorev 1967
Kotlan 1948
Wood 1958+

FAMILY TRICHOSTRONGYLIDAE
GENUS COOPERIA: Alicata 1958
Beach 1963+, 1964, 1965
Leland and Wallace 1966
Wood 1958+
Wood and Hansen 1960

GENUS GRAPHIDIUM: Bull 1953a, 1964
Cameron 1923
Dudzinski and Mykytowycz 1963
Dujardin 1845
Dunsmore 1966a, 1966c, 1968
Dunsmore and Dudzinski 1968
Enigk 1938a, 1938b
Gilruth 1906
Goodey 1922
Hall 1916
Lapage 1933a, 1933b
Mykytowycz 1959
Mykytowycz and Hesterman 1958
Railliet 1888, 1889
Railliet and Henry 1909
Thomas and Urquhart 1956
Wetzel and Enigk 1937

GENUS HAEMONCHUS: Daskalov 1966
Wood 1958+
Wood and Hansen 1960

GENUS HYOSTRONGYLUS: Kotlan 1948, 1949, 1952
Tromba and Douvres 1958

GENUS NEMATODIRUS ETC: Arnold 1941
Bernard 1965a
Chandler 1924
Dikmans 1937a
Sommerville 1963
GENUS OBELISCOIDES:

Alicata 1932, 1965
Chandler 1924
Dixon 1965a
Drum 1965
Fernando 1968, 1969
Frandsen 1965, 1966
Glasser and Stoll 1940
Graybill 1923
Russell, Baker, and Raizes 1966
Russell, Ward, and Baker 1970
Sollod, Hayes, and Soulsby 1968
Sollod and Allen 1971
Stockdale, Fernando, and Lee 1970
Worley 1963a, 1963b
Worley and Thompson 1963

GENUS OSTERTAGIA:

Daskalov 1966
Wood 1958+
Wood and Hansen 1960

GENUS TRICHOSTRONGYLUS...

T. affinis:

Dikmans 1937b
Dixon 1965a, 1965b
Graybill 1924
Cauthen 1958
Ciordia and Bizzell 1960
Ciordia, Bizzell, and Porter 1965
Ciordia, Bizzell, Porter, and Dixon 1966
Dixon 1965a
Drudge, Leland, Wyant, and Elam 1955
El-Rawi 1960+
Herlich and Ryan 1970
Kates and Thompson 1965
Leland and Drudge 1957
Rohrbacher 1957, 1958, 1960a, 1960b
Rohrbacher, Porter and Herlich, 1958
Ross 1970
Sinha 1967
Wood 1958+
Dikmans 1937b
Dixon 1963, 1965a
Hall 1916
Ransom 1912
Sarles 1930, 1932a, 1932b, 1934
Stoll 1932

T. axeii:

T. calcaratus:
T. colubriformis:
Bernard 1965a
Brackett and Bliznick 1949
Ciordia, Bizzell, and Porter 1965
Ciordia, Bizzell, Porter, and Dixon 1966
Dikmans 1937b
Dixon 1965a
Drudge, Leland, Wyant, and Elam 1955
El-Rawi 1960+
Gawad 1964+
Gazzigava 1929
Kates and Thompson 1965
Roberts 1935
Rohrbacker 1958, 1960a
Rohrbacker, Porter, and Herlick 1958
Samson 1970
Sommerville 1963
Williams and Palmer 1964
Wood 1958+
Wood and Hansen 1960

T. retortaeformis:
Bailey 1968
Bondy 1965
Brumpt 1921
Bull 1953a, 1953b, 1964
Clapham 1947
Crofton 1947, 1948a, 1948b, 1954
Davey 1938a
Dudzinski and Mykytowycz 1963
Dujardin 1845
Dunsmore 1965b, 1966a, 1966c, 1968
Dunsmore and Dudzinski 1968
Goodey 1922
Gupta 1961, 1962
Hall 1916
Lapage 1933a, 1933b
Michel 1952a, 1952b, 1952c, 1963a, 1968
Mykytowycz 1959
Mykytowycz and Hesterman 1958
Nnochiri 1950+
Poole 1954+
Prasad 1959
Railliet 1889
Sommerville 1963
Whitten 1948
Wilson 1954, 1958
Zeder 1800

T. sigmodontis:
T. vitrinus:
Thatcher and Scott 1962a, 1962b
Bernard 1965a
Roberts 1935
APPENDIX II

(Draft paper for publication)

EFFECTS OF ADMINISTERING TO DOMESTIC RABBITS LARVAE OF TRICHOSTRONGYLUS RETORTAEFORMIS THAT HAD BEEN SUBJECT TO COLD STORAGE FOR VARYING LENGTHS OF TIME

A collaborative experiment, with

DR. J.D. DUNSMORE
DIVISION OF WILDLIFE RESEARCH
CSIRO
CANBERRA
INTRODUCTION

In recent years it has been realised that the pattern of development of particular nematode parasites within their hosts is a function of a wide range of variables. Host immunity, although its mechanism of action is still not clear, is an important regulator of parasite numbers in many host-parasite systems. Host age, sex, and physiological and social status all play a part in influencing the numbers of parasites naturally found in wild animals.

It is now also realised that the physiological status of a nematode, influenced by the conditions under which infective forms have been reared and stored, is important in determining the developmental potential of these parasites in experimental infections. By implication it must be assumed that these factors are important in natural infections.

The present experiment was designed to further analyse the role of larval age and storage time, using as a laboratory system the natural host parasite association of *Trichostrongylus retortaeformis* and the rabbit, *Oryctolagus cuniculus*. Additionally, because seasonal cycles of parasitism in the field are common, an attempt was made to determine if the development of *T. retortaeformis* could be affected by subjecting the host to different photoperiods.

Quite unexpectedly in the experiment we found that similar doses of infective larvae, that had been exposed to cold for different lengths of time, but that had indistinguishable patterns of development, affected their hosts to different degrees as judged by a variety of relevant parameters.
MATERIALS AND METHODS

i. Experimental Design

The experiment was of factorial design testing the development of four batches of *T. retortaeformis* larvae in rabbits, of which half were exposed to an artificially increasing day length.

ii. Rabbits

Sixteen 5-week-old male N.Z. White rabbits were purchased as a single uniform lot from a commercial rabbit breeding establishment known to be free from stongylate nematodes. Using a random number system they were immediately divided into four experimental groups and each allotted a permanent cage. Through the course of the experiment they were maintained on a diet of lucerne pellets.

iii. Larvae

The strain of *T. retortaeformis* had originally been isolated from wild rabbits collected from the south coastal region of N.S.W. The strain had since been maintained in laboratory rabbits. Some care was taken to ensure that the larval batches differed in as few respects as possible. Faeces were collected from the donor rabbit and incubated for 8 or 12 days at 20°C. The infective larvae were isolated by a Baerman technique and if not used immediately were stored in about 1 cm depth of water at 4°C. Appendix II Table 1 includes all the relevant features of each of the four batches of larvae; the reference number for each batch of larvae corresponds to that for a group of rabbits.

When preparing the batches of larvae for administration to the rabbits, counts were made of the numbers of live larvae in each of four aliquots of 0.1 ml. The distribution of these counts in each case did not significantly differ from Poisson. The volume containing 2500 larvae was calculated and dispensed; the appropriate 95% Poisson confidence limits have been included in Appendix II Table 1.
The batches of *T. retortaeformis* larvae used to infect the corresponding groups of rabbits

<table>
<thead>
<tr>
<th>Group &amp; Batch</th>
<th>Source of Eggs for Culture of Larvae</th>
<th>Duration of Incubation and Storage</th>
<th>Number of Live Larvae per Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lab Stage of Passage Infection Number (days)</td>
<td>Incubation at 20°C* (days)</td>
<td>Storage at 4°C (days)</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>12</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>8</td>
<td>378</td>
</tr>
</tbody>
</table>

* from the time of collection of the donor faeces

@ 95% confidence limits of Poisson distribution calculated from the means of the actual numbers counted.

(See also Table 2)
On day 0 the larvae were given to the rabbits by stomach tube, with adequate water to ensure that all reached the stomach.

iv. Caging and Lighting

All rabbits were individually caged and the two light-treatment groups that were to be exposed - one to normal (midwinter) day length and the other to a regime of increasing day length - were in separate racks of cages in the same room. The normal light group was exposed to the normal daylight entering from windows. A set of fluorescent lights with a time-switch control was placed close against the other rack of cages. This provided almost the entire source of light to these rabbits and was so arranged that very little light escaped from the vicinity of the cages; additional blinds above and below the racks of cages ensured that none of the artificial light reached the other rabbits.

During the experiment the normal day length was about 10½ hours. Commencing ten days before the rabbits were infected, the daily photoperiod was increased by ½ hour each day till 2 days after the administration of the larvae, at which time the artificial day length for their treatment group was 16 hours. The day length was maintained at this length for the remainder of the experiment.

v. Faecal Collections and Egg Counts

Twice weekly, commencing 8 days after infection, 24-hour collections of faeces were made from each rabbit. The faeces were weighed, and two separate trichostrongylid egg counts performed for each rabbit by a modified McMaster egg counting technique; each count used 2 grams of faeces. For statistical analyses, the estimated number of eggs passed per day was calculated and transformed to log (number of eggs plus one).
vi. Necropsy

On day 29 all rabbits were killed by cervical dislocation and dissected immediately. The adrenal glands, spleen and appendix of each rabbit were collected into 10% formalin.

The entire small intestine was slit open and the contents collected. The mucosa was scraped away and digested for 3 hours in 1% pepsin and 1.5% HCl at 38°C. The intestinal contents and the digested mucosa were fixed by pouring into sufficient boiling absolute ethanol to give a final alcohol concentration of about 70%.

To count the nematodes the volume of each suspension was adjusted to 1 litre and all the worms counted in ten aliquots each of 10 ml. For statistical analyses the number of worms counted in the total 10% sample was transformed to log (number of worms plus one).

From each rabbit ten male and ten female T. retortaeformis were mounted in water and their images projected onto photographic paper and recorded. The images were measured and the lengths of the worms calculated by comparison with a calibrated scale.

vi. Statistical Methods

All analyses were by analysis of variance. Initially two-way analyses were performed but after realising that the light treatment had not altered any of the measured variables only one-way analyses were done, sorting each of the variates according to the batch of T. retortaeformis larvae that had been given. Final tests to detect specific between-group differences were by Duncan’s new multiple range test as described by Steel and Torrie (1960 pp.107).

RESULTS

There was no suggestion in any of the data that varying exposure to light had any influence on any of the factors being studied and accordingly this will be omitted.
in all the mentioned results; complete analyses were, however, carried out to detect any such influence.

i. Body Weight Changes

At the commencement of the experiment there was no significant between-group difference in their body weights. Although only a few body weight measurements were made during the course of this experiment a significant and unexpected factor became apparent quite early (see Appendix II Figure 1a and Table 2). Groups 1, 3 and 4 gained weight steadily and at a similar rate. But during days 0-19 the Group 2 rabbits gained only a mean of 112 grams while Groups 1, 3 and 4 gained 500, 544 and 438 gm respectively. This difference was significant (p < 0.05) and the other three groups did not significantly differ from each other (Duncan's new multiple range test, see Appendix II Table 3).

ii. Faecal Output

Faeces were collected and weighed twice weekly so that any mention of the total amount of faeces passed up to a particular day actually refers only to the amount collected at these times.

Up to day 19 Groups 1 and 2 rabbits passed significantly less faeces than did Groups 3 and 4 (Appendix II Table 3). The Group 2 rabbits passed less faeces at all seven of the 24 hour faecal collections than did any of the other groups (see Appendix II Figure 1b); although this was significantly less than Groups 3 and 4 it did not significantly differ from Group 1. The between-group differences in total (of the 7 x 24-hour collections made) faecal output followed a similar pattern to their differences in weight gain. Group 3 rabbits produced most faeces and gained the most weight; Group 2 produced least faeces and gained least weight and Groups 1 and 4 were similar and intermediate in both respects. In addition,
over all 16 animals a highly significant linear regression related body weight gain to total faecal output.

iii. Trichostrongylus Egg Production

All rabbits in Groups 1, 2 and 3 passed Trichostrongylus eggs on day 12 after having been negative on day 8 (Appendix II Figure 1c). The egg production from each rabbit in these groups (1, 2 and 3) remained fairly static until days 22 - 26 which were followed by a declining productivity. The mean total daily egg production of these three groups were not significantly different at any point. Despite the appearance of remaining high and steady during the first 10 - 15 days of egg production the productivity curves could all be adequately described by the same simple negative linear regression relating log total daily egg production to time. This suggests that following the initial peak, egg production declined exponentially and at a similar rate in each of these three groups. However a simple linear regression fitted non-transformed data equally well suggesting that over the time period of our observations the decline in egg production may have been arithmetic.

Trichostrongylus eggs were not detected in the Group 4 rabbits until day 15 when all became positive. The egg production of the members of this group remained significantly less than that of the other groups right through the experiment, and showed no tendency to the decline that was apparent in the others.

iv. Trichostrongylus Recovered at Necropsy

All nematodes recovered were adult. Groups 1, 2 and 3 had similar numbers of T. retortaeformis at necropsy. They had a geometric mean of 1,940 parasites, significantly more than the mean of only 340 recovered from the Group 4 rabbits (Appendix II Figure 2). There were no significant between-group differences in the lengths of either the male
### APPENDIX II

#### TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Rabbit Number</th>
<th>Weight Gain to day 19 (gms)</th>
<th>Faecal Output to day 19 (gms)</th>
<th>Total Egg Output ($10^{-3}$)</th>
<th>Nematodes Recovered</th>
<th>Egg Output per female in last 24 hours*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>88</td>
<td>775</td>
<td>419</td>
<td>717.8</td>
<td>900</td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>89</td>
<td>350</td>
<td>242</td>
<td>1,112.9</td>
<td>1,460</td>
<td>40.6</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>500</td>
<td>250</td>
<td>407.5</td>
<td>750</td>
<td>46.2</td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>375</td>
<td>291</td>
<td>1,043.0</td>
<td>1,080</td>
<td>161.0</td>
</tr>
<tr>
<td></td>
<td>Mean #</td>
<td>500</td>
<td>300</td>
<td>763.3</td>
<td>940</td>
<td>53.3</td>
</tr>
<tr>
<td>2.</td>
<td>96</td>
<td>50</td>
<td>121</td>
<td>547.4</td>
<td>790</td>
<td>70.0</td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>-75</td>
<td>129</td>
<td>627.2</td>
<td>960</td>
<td>52.7</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>350</td>
<td>303</td>
<td>1,151.5</td>
<td>1,450</td>
<td>97.6</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>125</td>
<td>140</td>
<td>1,266.6</td>
<td>890</td>
<td>63.5</td>
</tr>
<tr>
<td></td>
<td>Mean #</td>
<td>112</td>
<td>173</td>
<td>841.2</td>
<td>882</td>
<td>69.2</td>
</tr>
<tr>
<td>3.</td>
<td>93</td>
<td>550</td>
<td>475</td>
<td>996.4</td>
<td>640</td>
<td>179.1</td>
</tr>
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<td></td>
<td>84</td>
<td>600</td>
<td>413</td>
<td>1,088.0</td>
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<td>46.6</td>
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<td>90</td>
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<td>360</td>
<td>584.4</td>
<td>740</td>
<td>112.1</td>
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<tr>
<td></td>
<td>87</td>
<td>525</td>
<td>575</td>
<td>1,148.3</td>
<td>750</td>
<td>108.0</td>
</tr>
<tr>
<td></td>
<td>Mean #</td>
<td>544</td>
<td>456</td>
<td>923.5</td>
<td>764</td>
<td>100.3</td>
</tr>
<tr>
<td>4.</td>
<td>95</td>
<td>250</td>
<td>411</td>
<td>79.0</td>
<td>140</td>
<td>119.0</td>
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<td>414</td>
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<td>350</td>
<td>196</td>
<td>144.8</td>
<td>150</td>
<td>117.3</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>550</td>
<td>331</td>
<td>189.6</td>
<td>120</td>
<td>137.5</td>
</tr>
<tr>
<td></td>
<td>Mean #</td>
<td>438</td>
<td>338</td>
<td>145.5</td>
<td>122</td>
<td>139.0</td>
</tr>
</tbody>
</table>

* Nematodes recovered at day 29.

a Total of the first four 24-hour faecal collections.

* Total egg output in the last 24 hours before autopsy divided by the number of female *T. retortaeformis* recovered.

# For columns 3 and 4 these are arithmetic means; for the last three columns geometric means are presented.
The major significant between-treatment differences as revealed by Duncan's multiple range test. There is a significant difference between any two means not underscored by the same line (p < .05).

<table>
<thead>
<tr>
<th>Weight Gain to Day 19 (gms)</th>
<th>Group</th>
<th>2</th>
<th>4</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.E. = 79</td>
<td>Mean</td>
<td>112</td>
<td>438</td>
<td>500</td>
<td>544</td>
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</table>

<table>
<thead>
<tr>
<th>Faecal Output to Day 19 (gms)</th>
<th>Group</th>
<th>2</th>
<th>1</th>
<th>4</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.E. = 46</td>
<td>Mean</td>
<td>173</td>
<td>300</td>
<td>338</td>
<td>456</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Log$_e$ Total Egg Production $^Z$</th>
<th>Group</th>
<th>4</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.E. = 0.2076</td>
<td>Mean</td>
<td>11.9328</td>
<td>13.5455</td>
<td>13.6426</td>
<td>13.8589</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Log$_e$ Total T. retortaeformis</th>
<th>Group</th>
<th>4</th>
<th>3</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.E. = 0.0903</td>
<td>Mean</td>
<td>3.5543</td>
<td>5.1263</td>
<td>5.3204</td>
<td>5.3316</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spleen Weight (mg)</th>
<th>Group</th>
<th>4</th>
<th>3</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.E. = 65</td>
<td>Mean</td>
<td>697</td>
<td>771</td>
<td>826</td>
<td>1128</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spleen Wt.(mg)/Kg. Body Weight</th>
<th>Group</th>
<th>3</th>
<th>4</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.E. = 41</td>
<td>Mean</td>
<td>346</td>
<td>361</td>
<td>414</td>
<td>661</td>
</tr>
</tbody>
</table>

$^Z$ The total of the egg production during the seven 24-hour faecal collection periods.
Appendix II Figure 2

The sex/fertility ratio of the nematodes from the rabbits of Groups 1, 2, and 3 was almost 1:3. In the Group 4 rabbits there were relatively many more females so that the ratio was about 3:1. This was not significantly less than that for Groups 1, 2, and 3, but did not significantly differ from that for Group 2. It appears from the last column of Appendix II Table 2 that the egg productivity of the female worms recovered from the Group 4 rabbits was considerably higher than that from the nematodes in the Group 3 rabbits, with Group 2 and 4 ranked between. A significant linear regression was computed according that there was a significant positive correlation between the egg output per female nematode and the number of time that the worm was observed to be found in the stool. The figure shows the number of nematodes of different species per 100 g of wet weight and the number of eggs produced per 100 g of wet weight and the number of eggs produced per 100 g of wet weight and the number of eggs produced per 100 g of wet weight.

(Individual rabbits are in the same order as in Table 2)
or female nematodes although there were quite marked differences between the lengths of the nematodes recovered from different individual rabbits.

The male/female ratio of the *T. retortaeformis* from the rabbits of Groups 1, 2 and 3 was similar, averaging 0.83. In the Group 4 rabbits there were relatively many more females so that the ratio was 0.59. This was significantly less than that for Groups 1 and 3 but did not significantly differ from that for Group 2.

It appears from the last column of Appendix II Table 2 that the egg production of the female worms recovered from the Group 4 rabbits was considerably higher than that from the nematodes in the Group 1 rabbits with groups 3 and 4 ranked between. A significant linear regression was computed suggesting that there was a significant positive relationship between the egg output per female nematode on day 29 and the length of time that the infective larvae had been stored at 4°C prior to being used in this experiment.

v. Organ Weights

There were no significant differences between the groups in the weights of their adrenal glands or appendices. However the spleens of the Group 2 rabbits were significantly (*p < 0.01*) heavier (mean of 1198 mg, or 661 mg/Kg body weight) than those of the other three groups (mean of 765 mg, or 374 mg/Kg body weight). There were no significant differences between Groups 1, 3 and 4 in this respect.

Considering all rabbits there were significant negative linear regressions relating spleen weight to gain in body weight and to total faecal output.
DISCUSSION

This experiment was designed to study the effect of larval storage time on development of *T. retortaeformis* in its natural host, *O. cuniculus*. Consequently the varying pathogenicity was not anticipated, and the experimental design was not adequate to lead to a complete analysis of this interesting effect on the rabbit. However, the results obtained are both striking and significant although difficult to interpret at this stage.

The three young batches of *T. retortaeformis* larvae, stored for 0, 2, and 57 days after harvest from faecal culture, had all been produced by the same populations of parasites within a single uniform lot of donor rabbits; their preparation differed only in the time they had been exposed to the storage temperature of 4°C. For none of the parasite parameters that were studied did we detect any significant between-batch differences. These included the length of the prepatent period, the magnitude of the peak egg count, the rate of decline of the egg count, the numbers, sex-ratio and lengths of the recovered nematode and the egg production per female nematode at necropsy. It can reasonably be inferred from these data that there was no difference in the infectivity of each of the batches of larvae. (However, Ford suggests that a decline in infectivity due to storage was already biologically evident in Group 3 (mean 1,692), although not statistically less than in Group 1 (mean 2,045) or in Group 2 (mean 2,065) – see Figure 29).

There is little experimental data published on the *T. retortaeformis* in *O. cuniculus* system, with which a comparison can be made. Michel (1952b) described the "normal" pre-patent period, presumably in domestic rabbits, as "about a fortnight". In our experiment all rabbits infected with larvae of Batches 1, 2 and 3 had a high egg count on day 12 after having passed no eggs on day 8.
Patency is reached with these strains of host and parasite at 10 or 11 days after infection (Ford, this thesis), somewhat earlier than reported by Michel.

Additionally Michel (1952c, 1963a) observed an exponential decrease in the egg count occurring "over a period of many months". In our experiment the decline occurred at the same rate in all the groups and could equally well be described as arithmetic or exponential; a negative linear regression could be fitted to the egg production data in either the raw or the log-transformed form. If the trend is regarded as exponential, the decline, if it continued to zero would take many months to reach it; if arithmetic, zero would be reached in little over a month after infection. An exponential decline would be in agreement with other observations of Michel (1963b) on Ostertagia ostertagi in calves, at which time he made the interpretation that the form of the curve for egg count decline is due to a succession of adult populations, all decreasing exponentially and each producing fewer eggs per female than the last, apparently under increasing influence of host immunity. However, the succession of adult populations postulated by Michel for O. ostertagi and for T. retortaeformis using doses of 100,000 larvae, is precluded from experiments with moderate numbers of infective larvae, because as in our experiment, there is no reservoir of inhibited larvae (see below).

In this experiment, the mean recovery rate for Groups 1 to 3 on day 29, was as high as 77% of the administered dose of larvae. Without inhibited larvae present, it is very likely that the parasites recovered represented at least the bulk of the originally established population. If this is so, the observed decline in egg output is in fact a true reflection of a decline in egg productivity of each individual female parasite.

This interpretation of the data is additional evidence that both the infectivity of the larvae and the rate of decline in egg production of the adult females did
not significantly differ between the batches of T. retortaeformis larvae that had been stored for 0, 2 and 57 days.

On the other hand, the parasite populations developing from the larvae stored for 378 days were quite different. It is known that a high proportion of T. retortaeformis infective larvae will survive in the cold more than 12 months (Gupta 1961; Ford 1970 unpublished). However, infectivity of these surviving larvae was greatly reduced, as the mean percentage establishment in Group 4, measured at day 29, was only 14%, compared to the combined mean of 77% for the younger larvae. The nematodes had grown to the same length in all groups by day 29.

The very much lower peak egg production reached in the Group 4 rabbits was not followed by the decline that was apparent in the rabbits that had received the younger larvae. It has been observed earlier (Ford, this thesis) that low T. retortaeformis egg counts, from burdens of less than 500 nematodes, may persist for many months at a level of the order of 10,000 eggs per day or 100 eggs per gram of faeces. It is also possible that the rabbits of Groups 1, 2 and 3 would have shortly produced low egg counts of this magnitude and continued at this level for a prolonged period. In that event the egg count pattern of the Group 4 rabbits could be considered as the equivalent of the later stages of the picture in Groups 1, 2 and 3.

Additionally it is clear that patency was delayed in the Group 4 rabbits and this is presumably an effect of the prolonged cold storage of the larvae. Ford (this thesis) has also observed delayed maturation with T. retortaeformis larvae that had been stored for 14 weeks.

Stockdale, Fernando, and Lee (1970) and Armour, Jennings, and Urquhart (1969b) have shown that environmental factors may affect the percentage of an infecting dose of larvae that become inhibited in the early fourth stage of development. Accordingly it had been thought likely that we would find a percentage of the recovered T. retortaeformis
still in either the late third or the early fourth stage of development when the hosts were necropsied on day 29 and a very careful examination was made to detect these small parasites if present.

Although Michel (1952c) has established that arrested development at the late third-stage can occur with *T. retortaeformis* in experimental infections, parasites in an inhibited state of development were not recovered in any of the rabbits used in this experiment, including those that received the larvae stored 378 days, or in a similar experiment by Ford (this thesis) investigating aging of the same strain of larvae at 20°C. Further, despite extensive search using digestion techniques, arrested stages of *T. retortaeformis* have never been detected in wild rabbits in Australia (Dunsmore, unpublished). On the other hand, Bull (1953a) reported finding larvae, apparently arrested, in wild rabbits in New Zealand. Earlier reports from other countries do not appear to have considered the possibility of inhibited forms in field surveys.

There are at least three possible reasons why we failed to detect arrested development of *T. retortaeformis* in this experiment although it has been reported by others.

1. The characteristic may be genetically controlled, and the wild strain present in Australia may have a very low potential for the character. It has been found that several laboratory passages of species of *Ostertagia* drastically reduce the ability of the resultant laboratory strain to display the characteristic of inhibition, compared with a freshly isolated field strain (Armour, Jennings, and Urquhart 1969b; Connan 1969). The characteristic might be expected to be selected against in conditions in which it confers no particular advantage to the parasite. Such conditions will be very apparent in the usual laboratory situation in which parasite strains are being selected for their ability to maintain high rates of egg output while the free-living stages are provided with optimum conditions for larval development.
ii. At the moderate levels of infection used in this experiment numbers of parasites may be below a level at which arrested development becomes apparent. Michel used doses of 100,000 larvae and reported up to 30% arrested development; we used doses of only 2,500 larvae. It has been shown for a number of trichostrongylid species that a smaller proportion of a small dose of larvae will become arrested than if a large dose is used (Martin, Thomas, and Urquhart 1957; Dunsmore 1960; Russell, Baker, and Raizes 1966).

iii. Arrested development may only occur, or may occur more often in older rabbits. Ford (this thesis) has evidence suggesting this. It should be noted that Michel (1952b, 1952c) apparently used adult rabbits; those used in this experiment were only about 8 weeks old when infected.

Additionally there are other reasons for the expression of arrested development that have been incriminated in particular cases but seem less likely to apply in this experiment. Nor do we have data to suggest which of the above is more likely to apply in this case. But we see no reason to suggest that our failure to detect arrested *T. retortaeformis* in this experiment is any significant departure from the previous results of other workers especially Michel (1952c) who worked with at least ostensibly the same host-parasite system.

The observation that the productivity of eggs per female was highest in the Group 4 rabbits, those that had fewest parasites, may be at least partly explained by Michel's (1969c) suggestion that "the capacity of a worm growing in a ---- lightly infected host is greater than that of a worm developing in a ---- heavily infected host".

Although it had been thought possible that varying the photoperiod may have influenced the pattern of development of the parasite this did not prove to be the case. James and Johnston (1967) presented evidence that suggested that altering the photoperiod may have influenced parasite burdens...
in sheep but an experiment by Ford (1967 unpublished) with Haemonchus contortus in sheep did not support the suggestion. It can only be said that there is no sound evidence suggesting that daylight length affects the course of infections with nematode parasites.

It is clear that the rabbits were more severely affected by the parasites, when infected with larvae briefly exposed to the cold (Group 2), than when infected with larvae not exposed to cold (Group 1) or stored in the cold for 8 weeks (Group 3). This occurred despite the fact that all gross parasitological measurements did not detect significant differences between the populations of T. retortaeformis derived from these three batches of larvae.

Group 2 rabbits passed less faeces than Groups 1 and 3, suggesting a more depressed appetite, and their weight gains were reduced relative to the others, especially up to 19 days after infection. Additional confirmation of the more marked pathogenicity of Batch 2 larvae was obtained at necropsy when the rabbits that had received these larvae were found to have much larger spleens.

In the absence of an uninfected group of control rabbits, it is not possible to confidently assess the pathogenicity of each of the above three indistinguishable groups of established parasite populations. However, the Group 4 rabbits had only low infections and could be regarded as "almost normal" controls. On the basis of weight gains and splenic weight those rabbits that received larval Batches 1 and 3 were not affected by their parasite burden as judged by a comparison with Group 4 rabbits.

The primary pathogenic effect of parasites of the genus Trichostrongylus is believed to be a depression of appetite (Gordon 1964; Symons 1969b). The rabbits that received Batch 2 larvae, and to a lesser extent those that received Batch 1 larvae, passed less faeces than those that received larvae of Batch 3 or 4, and it seems therefore that the appetites of these rabbits must have been depressed.
The rabbits that received the Batch 2 larvae, and which were apparently subject to the greater depression of appetite, were unable to maintain their rate of weight gain; while those that received Batch 1 larvae and had a detectable but less marked apparent appetite depression, were still able to maintain a rate of weight gain parallel to that of the other groups of rabbits.

In the absence of more data it is not possible to intelligently comment on the possible causes of the enhanced pathogenicity of fresh *T. retortaeformis* larvae that had been exposed to cold for two days following isolation from culture.

Ford (this thesis, 1971a) showed that the larvae of *T. retortaeformis* need a period of maturation, after reaching the apparently infective stage, before they are of optimum infectivity. Thus it is clear that to eliminate undesirable sources of variability, larvae of identical age and reared in a precisely repeatable manner should be used in experimental work. It has been shown, also for *T. retortaeformis*, that the size of resulting infective larvae is affected by relatively minor variations in the culture temperature (Gupta 1961), or in the nature of the incubation medium (Wilson 1954). To our knowledge, no tests have been made of the differential infectivity of larvae reared in media of slightly differing composition, but this would be a logical extension of those observations. Ciordia, Bizzell, Porter, and Dixon (1966) showed for *T. colubriformis* in rabbits that infectivity did vary with the culture temperature.

It seems that care in the selection of the strain of parasite being used, and precise control of the conditions of incubation of the pre-infective stages and of aging of the infective stage, are desirable features of any experiment of a quantitative nature.

Some results reported here further complicate this with the suggestion that even if different batches of infective larvae present indistinguishable patterns of
development within their host, they may still vary in their pathogenicity for that host.

SUMMARY

Groups of 4 rabbits were all given 2,500 *Trichostrongylus retortaeformis* larvae that had all been incubated in similar fashion but stored for 0, 2, 57 or 378 days at 4°C before being used. The rabbits were killed 29 days post-infection. There were no significant differences between the parasite burdens of the first three groups in either egg count trends or in the parasites recovered at necropsy. In the rabbits that received larvae that had been stored for 378 days the pre-patent period was prolonged, the peak egg count reached was significantly lower and described a different curve to that of the other groups, and fewer *T. retortaeformis* were recovered.

The rabbits that received larvae which had been stored at 4°C for 2 days before use were more severely affected by the parasites than the other groups of rabbits. They gained less weight and passed less faeces, suggesting their appetites were depressed; and at autopsy they had significantly larger spleens than the other rabbits. It seems that the larvae stored for 2 days, and to a lesser extent those used immediately after culture, had a more severe depressing effect on the appetite of their hosts than did the larvae stored for longer periods. This was reflected in their relatively reduced rates of weight gain.

The results emphasise the need to standardise experimental methods of culture and storage in any experiments designed to look at either the patterns of development, or the pathogenicity, of parasitic nematodes.
APPENDIX III

SUMMARIES OF STATISTICAL
ANALYSES OF VARIANCE

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>(\bar{F})</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effect</td>
<td>1</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>Replicates</td>
<td>3</td>
<td>4.539 **</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>3</td>
<td>0.006</td>
<td></td>
</tr>
</tbody>
</table>

The mean results and significant differences are illustrated in Figure 10.

** \(p \leq 0.02\)

(Analyses from results in tables for Part II, Sections B and C, Experiments 7 to 12)
TABLE 11 - A

EXPERIMENT 7

Adult Nematodes

SUMMARY OF ANALYSIS OF VARIANCE

TRANSFORMED VALUES: $\log (n^2/10 + 1)$

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>$s^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Storage of Larvae</td>
<td>1</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.539 **</td>
</tr>
<tr>
<td>Replicates</td>
<td>3</td>
<td>0.006</td>
</tr>
<tr>
<td>Residual</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

** t TEST

The mean results and significant differences are illustrated in Figure 10.

** $p \leq 0.01$
### Inhibited Larvae

**SUMMARY OF ANALYSIS OF VARIANCE**

TRANSFORMED VALUES: $\log \left( \frac{n^2}{10} + 1 \right)$

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>$s^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Storage of Larvae</td>
<td>1</td>
<td>2.228 (*)</td>
</tr>
<tr>
<td>Replicates</td>
<td>3</td>
<td>1.541</td>
</tr>
<tr>
<td>Residual</td>
<td>3</td>
<td>0.357</td>
</tr>
</tbody>
</table>

*There were no significant interactions between the main effects.*

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>$s^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residual</td>
<td>3</td>
<td>0.357</td>
</tr>
</tbody>
</table>

**t TEST**

The mean results and significant differences are illustrated in Figure 11.

(*) $p < 0.10$
TABLE 13 - A

EXPERIMENT 8

Total Nematodes
(Group 1 - 5)

SUMMARY OF ANALYSIS OF VARIANCE

TRANSFORMED VALUES: log (n²/10 + 1)

F TEST

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>S²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Age of Larvae</td>
<td>4</td>
<td>2.323 **</td>
</tr>
<tr>
<td>Day of P.M.</td>
<td>1</td>
<td>0.665 **</td>
</tr>
<tr>
<td>Sex of Host</td>
<td>1</td>
<td>0.046</td>
</tr>
<tr>
<td>Residual</td>
<td>13</td>
<td>0.034</td>
</tr>
</tbody>
</table>

(There were no significant interactions between the main effects)

T TEST

The mean results and significant differences for age of larvae are illustrated in Figure 13.

** p ≤ 0.01
TABLE 13 - B

EXPERIMENT 8

**Total Nematodes**
(Groups 2 - 5)

**SUMMARY OF ANALYSIS OF VARIANCE**

**TRANSFORMED VALUES:** $\log (n^2/10 + 1)$

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>$s^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main Effects:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of Larvae (A)</td>
<td>3</td>
<td>0.085 *</td>
</tr>
<tr>
<td>Day of P.M. (B)</td>
<td>1</td>
<td>0.699 **</td>
</tr>
<tr>
<td>Sex of Host (C)</td>
<td>1</td>
<td>0.100 *</td>
</tr>
<tr>
<td><strong>Interactions:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B x A</td>
<td>3</td>
<td>0.025</td>
</tr>
<tr>
<td>B x C</td>
<td>1</td>
<td>0.057 (*)</td>
</tr>
<tr>
<td><strong>Residual</strong></td>
<td>6</td>
<td>0.012</td>
</tr>
</tbody>
</table>

**t TEST**

**AGE OF LARVAE MEANS**
- 8 days: 1.886
- 15 days: 2.080
- 30 days: 2.156
- 60 days: 1.856

Significant differences:
- $\geq 0.134$ *
- $\geq 0.203$ **

**DAY OF P.M. MEANS**
- day 5: 1.785
- day 11: 2.203

Significant differences:
- $\geq 0.094$ *
- $\geq 0.143$ **

The mean results and significant differences for sex of host are illustrated in Figure 15.

(*) $p \leq 0.10$
* $p \leq 0.05$
** $p \leq 0.01$
TABLE 13 - C

EXPERIMENT 8

Egg Production per Day
(Groups 2 - 4)

SUMMARY OF ANALYSIS OF VARIANCE
TRANSFORMED VALUES: $\log (n^2 + 1)$

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>$s^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Age of Larvae</td>
<td>2</td>
<td>0.070 (*)</td>
</tr>
<tr>
<td>Sex of Host</td>
<td>1</td>
<td>0.241 **</td>
</tr>
<tr>
<td>Residual</td>
<td>2</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**F TEST**

**AGE OF LARVAE MEANS**

- 8 days: 4.485
- 15 days: 4.739
- 30 days: 4.851

Significant differences:
- $\geq 0.012$ *
- $\geq 0.028$ **

**SEX OF HOST MEANS**

- male: 4.892
- female: 4.491

Significant differences:
- $\geq 0.008$ *
- $\geq 0.019$ **

The mean results and significant differences for age of host are shown in Figure 15.

(*) $p \leq 0.10$

* $p \leq 0.05$

** $p \leq 0.01$
TABLE 13 - D

EXPERIMENT 8

Egg Production per Female
(Groups 2 - 4)

SUMMARY OF ANALYSIS OF VARIANCE

TRANSFORMED VALUES: log (n² + 1)

F TEST

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>S²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Age of Larvae</td>
<td>2</td>
<td>0.029 *</td>
</tr>
<tr>
<td>Sex of Host</td>
<td>1</td>
<td>0.314 **</td>
</tr>
<tr>
<td>Residual</td>
<td>2</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The mean results and significant differences for sex of host are illustrated in Figure 15.

*  p ≤ 0.05
** p ≤ 0.01
### EXPERIMENT 9

**TABLE 14 - A**

**Adult Nematodes**  
(Groups 2 - 5)

**SUMMARY OF ANALYSIS OF VARIANCE**

**TRANSFORMED VALUES: log (n^2/10 + 1)**

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>( s^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Week of P.M.</td>
<td>3</td>
<td>0.355</td>
</tr>
<tr>
<td>Previous Infection</td>
<td>1</td>
<td>1.087</td>
</tr>
<tr>
<td>Diet</td>
<td>1</td>
<td>0.005</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>0.319</td>
</tr>
</tbody>
</table>

**F TEST**

**t TEST**

The mean results and significant differences are illustrated in Figure 16.
### TABLE 14 - B

EXPERIMENT 9

**Egg Production per Female**
(Groups 2 - 5)

#### SUMMARY OF ANALYSIS OF VARIANCE

**TRANSFORMED VALUES**: $\log(n^2 + 1)$

#### F TEST

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>$s^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Week of P.M.</td>
<td>3</td>
<td>0.911</td>
</tr>
<tr>
<td>Previous Infection</td>
<td>1</td>
<td>0.422</td>
</tr>
<tr>
<td>Diet</td>
<td>1</td>
<td>1.006</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>0.537</td>
</tr>
</tbody>
</table>

#### t TEST

The mean results and significant differences are illustrated in Figure 17.
# TABLE 15 - A

## EXPERIMENT 10

### Adult Nematodes

(Weeks 4 and 6)

### SUMMARY OF ANALYSIS OF VARIANCE

TRANSFORMED VALUES: $\log (n^2/10 + 1)$

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>$s^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks of P.M. (A)</td>
<td>1</td>
<td>2.960 **</td>
</tr>
<tr>
<td>Previous Infection (B)</td>
<td>1</td>
<td>0.081</td>
</tr>
<tr>
<td>Dosing (C)</td>
<td>1</td>
<td>0.011</td>
</tr>
<tr>
<td>Diet (D)</td>
<td>1</td>
<td>0.513</td>
</tr>
<tr>
<td>Initial Body Weight (E)</td>
<td>1</td>
<td>0.027</td>
</tr>
<tr>
<td>Interactions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A x B</td>
<td>1</td>
<td>0.898</td>
</tr>
<tr>
<td>A x C</td>
<td>1</td>
<td>2.234 *</td>
</tr>
<tr>
<td>A x D</td>
<td>1</td>
<td>2.381 *</td>
</tr>
<tr>
<td>A x E</td>
<td>1</td>
<td>1.199 (*)</td>
</tr>
<tr>
<td>B x C</td>
<td>1</td>
<td>0.677</td>
</tr>
<tr>
<td>B x D</td>
<td>1</td>
<td>0.120</td>
</tr>
<tr>
<td>B x E</td>
<td>1</td>
<td>0.810</td>
</tr>
<tr>
<td>C x D</td>
<td>1</td>
<td>0.193</td>
</tr>
<tr>
<td>C x E</td>
<td>1</td>
<td>0.332</td>
</tr>
<tr>
<td>D x E</td>
<td>1</td>
<td>0.800</td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>0.345</td>
</tr>
</tbody>
</table>

### F TEST

The mean results and significant differences are illustrated in Figure 19.

(*) $p \leq 0.10$

* $p \leq 0.05$

** $p \leq 0.01$
TABLE 15 - B

EXPERIMENT 10

Egg Production per Day
(Weeks 4 and 8)

SUMMARY OF ANALYSIS OF VARIANCE

TRANSFORMED VALUES: log (n^2 + 1)

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>s^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week of P.M. (A)</td>
<td>1</td>
<td>29.143 **</td>
</tr>
<tr>
<td>Previous Infection (B)</td>
<td>1</td>
<td>3.685</td>
</tr>
<tr>
<td>Dosing (C)</td>
<td>1</td>
<td>1.474</td>
</tr>
<tr>
<td>Diet (D)</td>
<td>1</td>
<td>0.701</td>
</tr>
<tr>
<td>Initial Body Weight(E)</td>
<td>1</td>
<td>0.032</td>
</tr>
<tr>
<td>Interactions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A x B</td>
<td>1</td>
<td>11.057 (*)</td>
</tr>
<tr>
<td>A x C</td>
<td>1</td>
<td>12.434 *</td>
</tr>
<tr>
<td>A x D</td>
<td>1</td>
<td>16.064 *</td>
</tr>
<tr>
<td>A x E</td>
<td>1</td>
<td>1.767</td>
</tr>
<tr>
<td>B x C</td>
<td>1</td>
<td>10.144 (*)</td>
</tr>
<tr>
<td>B x D</td>
<td>1</td>
<td>0.056</td>
</tr>
<tr>
<td>B x E</td>
<td>1</td>
<td>2.287</td>
</tr>
<tr>
<td>C x D</td>
<td>1</td>
<td>0.748</td>
</tr>
<tr>
<td>C x E</td>
<td>1</td>
<td>3.882</td>
</tr>
<tr>
<td>D x E</td>
<td>1</td>
<td>2.698</td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>2.702</td>
</tr>
</tbody>
</table>

(*) p ≤ 0.10
* p ≤ 0.05
** p ≤ 0.01

The results are plotted in Figure 18.
**TABLE 15 - C**

**EXPERIMENT 10**

**Egg Production per Female**

**SUMMARY OF ANALYSIS OF VARIANCE**

TRANSFORMED VALUES: $\log (n^2 + 1)$

### F TEST

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>d.f.</th>
<th>$s^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main Effects:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week of P.M. (A)</td>
<td>1</td>
<td>4.451 *</td>
</tr>
<tr>
<td>Previous Infection (B)</td>
<td>1</td>
<td>0.998</td>
</tr>
<tr>
<td>Dosing (C)</td>
<td>1</td>
<td>0.237</td>
</tr>
<tr>
<td>Diet (D)</td>
<td>1</td>
<td>0.161</td>
</tr>
<tr>
<td>Initial Body Weight(E)</td>
<td>1</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Interactions:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A x B</td>
<td>1</td>
<td>2.462 *</td>
</tr>
<tr>
<td>A x C</td>
<td>1</td>
<td>1.313</td>
</tr>
<tr>
<td>A x D</td>
<td>1</td>
<td>3.009 *</td>
</tr>
<tr>
<td>A x E</td>
<td>1</td>
<td>0.164</td>
</tr>
<tr>
<td>B x C</td>
<td>1</td>
<td>1.244</td>
</tr>
<tr>
<td>B x D</td>
<td>1</td>
<td>0.019</td>
</tr>
<tr>
<td>B x E</td>
<td>1</td>
<td>0.124</td>
</tr>
<tr>
<td>C x D</td>
<td>1</td>
<td>0.016</td>
</tr>
<tr>
<td>C x E</td>
<td>1</td>
<td>1.156</td>
</tr>
<tr>
<td>D x E</td>
<td>1</td>
<td>0.424</td>
</tr>
<tr>
<td><strong>Residual</strong></td>
<td>16</td>
<td>0.525</td>
</tr>
</tbody>
</table>

### T TEST

The mean results and significant differences are illustrated in Figure 20.

* $p \leq 0.05$
TABLE 15 - D

EXPERIMENT 10

Body Weight Gain per Week

SUMMARY OF ANALYSIS OF VARIANCE

VALUES: gms

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>( s^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Week of P.M. (A)</td>
<td>1</td>
<td>1,510 (*)</td>
</tr>
<tr>
<td>Previous Infection (B)</td>
<td>1</td>
<td>769</td>
</tr>
<tr>
<td>Dosing (C)</td>
<td>1</td>
<td>10,636 **</td>
</tr>
<tr>
<td>Diet (D)</td>
<td>1</td>
<td>22,215 **</td>
</tr>
<tr>
<td>Initial Body Weight (E)</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>Interactions: A x B</td>
<td>1</td>
<td>493</td>
</tr>
<tr>
<td>A x C</td>
<td>1</td>
<td>594</td>
</tr>
<tr>
<td>A x D</td>
<td>1</td>
<td>10,652 **</td>
</tr>
<tr>
<td>A x E</td>
<td>1</td>
<td>245</td>
</tr>
<tr>
<td>B x C</td>
<td>1</td>
<td>412</td>
</tr>
<tr>
<td>B x D</td>
<td>1</td>
<td>951</td>
</tr>
<tr>
<td>B x E</td>
<td>1</td>
<td>207</td>
</tr>
<tr>
<td>C x D</td>
<td>1</td>
<td>1,370 (*)</td>
</tr>
<tr>
<td>C x E</td>
<td>1</td>
<td>255</td>
</tr>
<tr>
<td>D x E</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>403</td>
</tr>
</tbody>
</table>

**T TEST**

The mean results and significant differences are illustrated in Figure 21.

(*) \( p \leq 0.10 \)

** \( p \leq 0.01 \)
**TABLE 16 - A**

**EXPERIMENT 11**

**Adult Nematodes**

**SUMMARY OF ANALYSIS OF VARIANCE**

TRANSFORMED VALUES: \( \log (n^2/10 + 1) \)

**F TEST**

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>( s^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Day of F.M. (A)</td>
<td>2</td>
<td>1.066 *</td>
</tr>
<tr>
<td>Reproductive Status (B)</td>
<td>1</td>
<td>1.749 *</td>
</tr>
<tr>
<td>Interaction: A x B</td>
<td>2</td>
<td>0.606</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>0.223</td>
</tr>
</tbody>
</table>

(Prior Body Weight Gain, and other interactions, did not contribute to the source of variation.)

**t TEST**

The mean results and significant differences are illustrated in Figure 22.

* \( p \leq 0.05 \)


TABLE 16 - B

EXPERIMENT 11

**Egg Production per Female**

**SUMMARY OF ANALYSIS OF VARIANCE**

TRANSFORMED VALUES: log \((n^2 + 1)\)

**F TEST**

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>(s^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects: Day of P.M. (A)</td>
<td>2</td>
<td>0.438</td>
</tr>
<tr>
<td>Reproductive Status (B)</td>
<td>1</td>
<td>0.461</td>
</tr>
<tr>
<td>Prior Body Weight Gain (C)</td>
<td>2</td>
<td>0.529</td>
</tr>
<tr>
<td>Interactions: A x B</td>
<td>2</td>
<td>0.509</td>
</tr>
<tr>
<td>B x C</td>
<td>2</td>
<td>2.618 *</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>0.314</td>
</tr>
</tbody>
</table>

(Other interactions did not contribute to the source of variation.)

**t TEST**

The mean results and significant differences are illustrated in Figure 23.

\[ * \ p \leq 0.05 \]
TABLE 16 - C

EXPERIMENT 11

Total Egg Production for 15 Day Period

SUMMARY OF ANALYSIS OF VARIANCE

TRANSFORMED VALUES: \( \log(\text{total } n^0 + 1) \)

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>( s^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effects:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period (A)</td>
<td>2</td>
<td>3.277</td>
</tr>
<tr>
<td>Reproductive Status (B)</td>
<td>1</td>
<td>3.892 (*)</td>
</tr>
<tr>
<td>Prior Body Weight Gain (C)</td>
<td>2</td>
<td>2.531</td>
</tr>
<tr>
<td>Interactions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A x B</td>
<td>2</td>
<td>2.633</td>
</tr>
<tr>
<td>A x C</td>
<td>4</td>
<td>2.857</td>
</tr>
<tr>
<td>B x C</td>
<td>2</td>
<td>1.992</td>
</tr>
<tr>
<td>Residual</td>
<td>4</td>
<td>0.813</td>
</tr>
</tbody>
</table>

**F TEST**

The mean results and significant differences are illustrated in Figure 25.

**t TEST**

The mean results and significant differences are illustrated in Figure 25.

\( (*) \) \( p \leq 0.10 \)
TABLE 17 - A

EXPERIMENT 12

SUMMARY OF ANALYSIS OF VARIANCE

TRANSFORMED VALUES: \( \log \left( \frac{n^2}{10} + 1 \right) \)

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>( s^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effect: Lactation Status</td>
<td>2</td>
<td>0.229</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>0.100</td>
</tr>
</tbody>
</table>

The mean results and significant differences are illustrated in Figure 27.
TABLE 17 - B

EXPERIMENT 12

Egg Production per Female

SUMMARY OF ANALYSIS OF VARIANCE

TRANSFORMED VALUES: \( \log (n^2 + 1) \)

**F** TEST

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>d.f.</th>
<th>( s^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Effect: Lactation Status</td>
<td>2</td>
<td>0.400 **</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>0.046</td>
</tr>
</tbody>
</table>

**t** TEST

The mean results and significant differences are illustrated in Figure 28.

\(* * p \leq 0.01\)
Thanks for this thesis are expressed to

Anne Clugston who typed all the printing masters for Lettercopy

My wife Leonie and friends who helped check the manuscript

Sylvia Ozolins who typed the tables for photographic reduction

Canberra, September 1971