THE PHYSIOLOGICAL INTERACTIONS BETWEEN NEMATODE PARASITES AND THEIR INSECT HOSTS.

by

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ABSTRACT.

The hatching of Hammerschmidtiella diesingi in the midgut of the host Periplaneta americana has been investigated. The organisation of the egg layers has been examined and data on the composition and properties of the layers and membranes has been collected using a variety of techniques including histochemistry and chromatography. The fate of the egg layers during hatching has been followed and the possible effect of the host on the mechanism of hatching has been elucidated.

The interrelationships between host and parasite during migration and further development of the released larvae has been investigated, particularly during the processes of exsheathment and sex determination. The normal sequence of host development has been altered by surgically interfering with the normal functioning of the neuroendocrine system. Either cautery of the median neurosecretory cells or removal of the corpora allata, in addition to producing profound alterations in the physiology of the host, have markedly decreased the parasite burden. It seems likely that these operations are greatly interfering with the subsequent development of the late larval stages of the parasite. Both in vivo and in vitro effects of hormone extracts and juvenile hormone upon the nematode development have been analysed. The nematodes themselves have been examined for the presence of neurosecretory cells.

Studies were also carried out on the relationship between the nematode-bacterium complex DD-136 strain of Neoaplectana carpocapsae Weiser, and its several insect hosts. The effect of several factors: the nematode population size, the host species, host age, host weight, the nutritional status of the host, and the temperature of the host, on the development of the nematode in the host was investigated. Information

was also collected on the relationship between the bacteria, carried by the nematode, Achromobacter nematophilus, and the insect host. A field trial using N. carpocapsae for the control of Scolytus scolytus is described.

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GENERAL INTRODUCTION

The physiological interrelationships between insects and their parasites are imperfectly understood. This gap in knowledge is particularly striking considering the major importance of insects as both primary and intermediate hosts and vectors for a wide variety of parasites.

Several methods have been used for the control of insects including the application of potent chemical pesticides. However, these frequently have unpleasant and unwanted side effects. An alternative to such control measures lies in the use of nematodes in biological control.

In order to integrate nematodes into a control programme, it is imperative to understand effects other than those which result in mortality of the host. Information is required concerning effects on fecundity, vigour, increases or decreases in feeding by the host. Such debilitating effects of the nematodes of course makes them potentially invaluable as control agents since they can reduce the harmfulness of the host without removing it completely from the ecosystem, perhaps only to be replaced by another pest. This use of nematodes as biological control agents rests on the application of the nematodes in their most infective state to an insect in its most susceptible state under the most favourable external conditions. Such information can only be accrued by a study of the physiological states of both host and parasite at each stage in their life histories and under different imposed environmental conditions.

The physiological state of an insect is determined in part by its endocrine system which mediates between the environment and the animal itself. It is, therefore, of fundamental

importance to understand the physiological processes of both host and parasite which are controlled by the neuroendocrine system.

The endocrinology of insects has been reviewed comprehensively by Highnam and Hill (1969) and by Wigglesworth (1954, 1970) and is only outlined briefly here.

Development in insects is controlled by the endocrine system which permits the precise timing of developmental events which characterise insect life histories. The neurosecretory cells of the brain produce thoracotrophic hormone which is carried to the corpora cardiaca and then released into the blood. The site of action of this hormone is the thoracic glands which are stimulated to produce ecdysone which initiates deposition of the new cuticle and moulting. Secretion of juvenile hormone by the corpora allata ensures the development of another larval instar. Williams (1956) first isolated juvenile hormone which was identified some ten years later by Röller, Dahm, Sweely and Trost (1967). Substances with the activity of juvenile hormone have since been extracted from a wide variety of sources (Schneiderman and Gilbert, 1958; Williams, Moorehead and Pulis, 1959; Schneiderman, Gilbert and Weinstein, 1960).

The thoracic glands disappear during or soon after the last moult and the cerebral neurosecretory system and corpora allata are involved in the control of reproductive development. In many instances the secretions of these glands are controlled by stimuli such as feeding and mating. In the majority of insects, the corpora allata are essential for the full development of the eggs (Highnam, 1964). Juvenile hormone released from the corpora allata regulates the synthesis of protein in the fat

body (Engelmann, 1969; Engelmann, Hill and Wilkens, 1971) as well as facilitating uptake into the oocytes (Highnam, Lusis and Hill, 1963; Pratt and Davey, 1972). The median neurosecretory cells in some insects are also concerned with the control of oocyte growth and vitellogenesis (Hill, 1962).

Hormones may also be involved in the control of a range of metabolic processes including carbohydrate and lipid utilisation and other physiological processes such as water balance.

Nematodes are also dependent on environmental stimuli for the instigation of certain physiological events in their life cycle and these effects may be exerted via the endocrine system. Hitcho and Thorson (1971) discovered that some nematodes possess substances with juvenile hormone activity but as yet there is no proof that these substances play any definitive role in the control of physiological processes within the nematodes. However, nematodes have been shown to possess neurosecretory cells themselves (Gersch and Scheffel, 1958; Ishikawa, 1961; Davey, 1966; Rogers, 1968). Davey (1964) found that some of the primary sense cells on the lips of Ascaris stain with paraldehyde fuchsin and suggested (Davey, 1972) that stimulation of these cells may lead directly to release of hormones. In Phocanema sp. the neurosecretory cells appear to aid the process of moulting by producing a hormone resembling α ecdysone (Rajulu, Kulasekarapandian and Krishnan, 1972) which acts upon the excretory gland to bring about the secretion of a moulting fluid (Davey and Kan, 1968). It is possible, however, that the life cycles of some parasitic nematodes may have become so integrated with that of the host that they may have become dependent on them for the supply of an external stimulus for certain developmental

processes.

In vitro studies by Meerovitch (1965) first showed that the development of a nematode *Trichinella spiralis* was affected by application of farnesol, a chemical possessing insect juvenile hormone activity. Later *in vitro* studies (Johnson and Viglierchio, 1970; Shanta and Meerovitch, 1970; Webster and Craig, 1969; Dropkin, Lower and Acedo, 1971; Hitchot and Thorson, 1971; Davey, 1971; Thong and Webster, 1971; Hansen and Buecher, 1971) using a wide range of substances with juvenile hormone activity have all shown disruptive effects upon nematode development.

It is likely that some parasites which develop in the haemocoel of the host and thus in direct contact with the circulating hormones may be dependent upon or affected by them. Although there is no direct evidence for this there are examples where the nematode parasite and insect host life cycles seem to be synchronised to some degree (Götz, 1964).

Recently a great deal of research has been carried out on the physiological interactions between nematode parasites and the insect gut in which they dwell. Although one would assume no direct contact between the nematodes and the host hormones, substances with the activity of juvenile hormone have been found in insect faeces (Karlson and Schmialek, 1959). It seems possible that there may be some effect of the secretions of the host endocrine system either directly or indirectly on the presence of these nematodes in the host.

The present investigations were designed to elucidate various aspects of the host-parasite relationship and to suggest the possible utilisation of this knowledge in the application of

nematodes, either alone, or as part of an integrated system as biological control agents against insects.

To this end, two basic systems have been studied in detail. The first, that between the gut dwelling nematodes Hammerschmidtiella diesingi and Leidynema appendiculata and their host Periplaneta americana to elucidate and correlate the infestation and development of the parasites with the physiological and developmental events which occur within the host. Particular attention has been paid to the endocrine controlled phenomena such as growth, moulting and reproductive development of the insect and the effect of these on the developmental stages of the nematode.

The second system studied was that between the nematode-bacterium complex Neoaplectana carpocapsae Weiser, which has been found to inhabit the haemocoel of insects. An attempt has been made to discover the variation in susceptibility between different stages of the insect life cycles to parasitism. I have also endeavoured to show the separate and combined effects of bacteria and nematodes in debilitating the hosts and the concurrent physiological changes. The effect of the changing physiological state of the host due to such factors as hormonal manipulation, starvation and exposures to various temperatures on the nematode-bacterium complex life cycle have also been described. This has enabled possible control programmes under the most favourable conditions for both the host and the parasite to be suggested.

PART I

The physiological interactions between *Hammerschmidtiella diesingi* and *Leidynema appendiculata* and their host *Periplaneta americana*.

Section I

The structure of the egg of H. diesingi and the physiological role of P. americana in its hatching process

Introduction

Periplaneta americana, the American cockroach, can establish infestation wherever food is stored, manufactured or handled. It was chosen because stocks were readily available, there is a great deal of information on its biology and it is naturally infected with populations of both Hammerschmidtiella diesingi and Leidynema appendiculata.

Both of these nematodes were first discovered in Blatta orientalis by Hammerschmidt (1838; 1847) and were originally named by him Oxyuris diesingi and Oxyuris blattae respectively. Oxyuris diesingi was later placed in the genus Aorurus Leidy 1849 by Leidy (1850). After being renamed and redescribed, eventually, in 1932 Chitwood created a new genus Hammerschmidiella to contain O. diesingi stating that this genus differed from Aorurus in the position of the female vulva. Chitwood recorded the hosts of H. diesingi as B. orientalis and P. americana L.

Oxyuris blattae was similarly renamed and redescribed several times (Lee, 1959) before Chitwood (1932) in his revision of the Blattidae called it L. appendiculata Leidy 1850. He recorded the hosts as P. americana, B. orientalis and Blaberus atropos. A further description of L. appendiculata appeared in 1934 when Dobrovolny and Ackert dealt with the life history of the species.

The fourth larval and adult stages of both of these nematodes

are found in the hind gut of P. americana. Here the adult females lay their eggs which eventually pass out with the insect faeces. Lee (1959) demonstrated that H. diesingi was oviparous and that at the time of deposition the eggs are at the 2-4 cell stage and that development proceeded until a motile, elongated larva was formed within the egg shell. This first stage larva subsequently underwent a moult to the easily identifiable, non-motile, ovoid second stage larva, the infective "Resting Embryonated Stage" (R.E.S.) described by Dobrovolny and Ackert (1934). Lee (1959) also found that the developmental time to the R.E.S. varied with the temperature. Dobrovolny and Ackert (1934) working with L. appendiculata, believed that hatching took place in the midgut of the host but were unable to demonstrate it. Todd (1941) stated that two moults took place in the egg of L. appendiculata, the first outside of the cockroach and the second inside the new host after the eggs had been taken in during feeding. The only success in hatching the eggs so far has been obtained by Todd (1941) in unspecified protein solutions and Lee (1959) in the excised midguts of P. americana. According to Lee (1959) the difficulty in hatching these eggs lies in the inactive second stage larva (R.E.S.) which has to be induced to moult into the active third stage larva and in the release of the opercula cap of the egg to allow the enclosed larva to escape.

The first part of this work involved an investigation of the possible role of the host, if any, in the hatching of the nematode eggs. To this end the structure of the nematode eggs has been examined so that this information could be correlated with the results of both in vivo and in vitro studies on the hatching of the eggs. In an endeavour to fully understand the establishment

of nematode populations within the host, attempts were made to rear a colony of nematode free cockroaches. The rationale in producing such nematode free insects was that they could then be experimentally infected with a single species of nematode, so eliminating many of the parameters which could interfere with the host parasite relationship. An anthelmintic, Piperazine adipate, was used for this purpose.

1. CULTURE METHODS

(i) Maintenance of stocks of Periplaneta americana

Adult insects were obtained from a biological supplier (Gerard and Haigh, Sussex, England). They were kept in glass tanks with fine zinc mesh covers, in the dark at a constant temperature of 25°C. The insects were reared on equal quantities of Winalot dog biscuits and Spillers "Shapes" ground to a coarse powder with 5% by weight Yestamin (dried brewers yeast) added. Cultures were supplied with water.

(ii) Collection of nematodes and the culture of their eggs

The mature female worms of H. diesingi and L. appendiculata were usually extracted from naturally infected adult female P. americana, as insects of this sex carried a higher parasite burden (Table 1). The dissections were carried out in 0.15M sodium chloride which Lee (1960) had shown to be isosmotic with H. diesingi adults. It was noticed that gravid female nematodes released during the dissection, started to deposit their eggs within one minute in this medium. I decided to use this solution for the culture of the nematode eggs. The female nematodes were placed in a few drops of 0.15M sodium chloride on a cavity slide. Each cavity slide was placed in a petri dish lined with damp

filter paper and kept at 30°C in the dark. After 24 hours the adult nematodes were removed.

Results

From Table 1 it can be seen that there were more nematodes in adult female insects. H. diesingi was more abundant than L. appendiculata.

It was found that eggs contained third stage larvae 8 days after culture at 30°C.

Table 1 - Average parasite burden of adult P. americana

	H. diesingi		L. appendiculata	
	Males	Females	Males	Females
Female P. americana (20 insects)	1.14 ± 0.39	8.42 ± 1.2	0.75 ± 0.10	2.90 ± 0.60
Male P. americana (20 insects)	0.60 ± 0.02	2.95 ± 0.16	0.50 ± 0.05	1.65 ± 0.02

Discussion

Due to the abundance of H. diesingi in P. americana the eggs from these nematodes only were used in further experiments.

As described by Lee (1959) the first stage larva was formed in the egg after 24 hours and the second stage larva after 3 days at 30°C. The second stage larva was designated the infective stage as it was thought that a single moult occurred in the egg and that the second stage larva hatched in the host. However, after 8 days at 30°C it was found that eggs contained the motile third stage larva. This is the first time that a second moult inside the egg of these nematodes outside of the host has been recorded.

In this respect the eggs of H. diesingi and L. appendiculata resemble those of Enterobius vermicularis (Chandler, Alicata and Chitwood, 1941) in which the third stage larva is the infective stage for the definitive host, the first two stages, including the second moult to the third stage larva having taken place in the egg.

As the induction of this second moult was a handicap to previous attempts (Lee, 1959) to study both in vivo and in vitro hatching of the nematodes, these results appreciably enhance the prospect of further studies in this field.

2. NEMATODE FREE COCKROACHES AND THEIR EXPERIMENTAL REINFECTION

Introduction

Piperazine or diethylendiamine has been firmly established as the drug of choice for the treatment of a number of intestinal nematodes of vertebrates since Fayard (1949) demonstrated the anthelmintic properties of this molecule.

According to Del Castillo (1969) who classified anthelmintic drugs with regard to their selective action on parasitic nematodes, piperazine adipate exerts its effect on the neuromuscular system of nematodes. Norton and de Beer (1957) found that piperazine caused flaccid paralysis of the large, parasitic nematode Ascaris by blocking the neuromuscular junctions. Fiakpui (1967), using in vitro techniques, investigated the action of the drug on the smaller, free;living Caenorhabditis briggsae and he also found that piperazine exerted its effect by acting as a competitive blocking agent of the receptor site at the neuromuscular junctions but that this was reversible when the nematode was placed in a drug-free environment. He also suggested that development of

juvenile nematodes was retarded.

In this work both *in vivo* and *in vitro* experiments to show the effect of piperazine adipate on the oxyurid nematodes, from the hind gut of *Periplaneta americana* were carried out, and the infectivity of drug treated eggs in the host assessed.

Methods

(i) Effect of piperazine $NH.(CH_2)_2.NH.CH_2.CH_2.6H_2O$ on the worm burden of adult *P. americana*

Adult insects, which had received neither food or water for 72 hours, were placed singly in weighed petri dishes which were aerated and lined with foil. The petri dishes containing the insects were re-weighed and the weight of each cockroach determined by difference. From this the average weight of the cockroaches was determined (1.31 ± 0.77g). A dose range for administration to the insects was extrapolated from the data used for small animals (Merck Index). Batches of 5 insects, chosen at random, were each given a dose of 0.125, 0.25, 1.00 or 5.00mg of piperazine in 1ml distilled water (5mg/ml==20mM). In order to do this the cockroaches were anaesthetised and a length of catheter tubing, attached to a calibrated micrometer syringe, pushed into the crop. The control insects received an equal aliquot of distilled water. The insects were kept at 24°C and fed on 2g food after 12 hours. After intervals of 24, 48, 72 and 96 hours from the time the dose had been administered, the insects were killed with carbon dioxide and dissected. The presence of different developmental stages of the nematodes, from eggs to adults, in the host gut was noted. The number of nematodes was counted. The faeces were examined and any nematodes present were recorded.

(ii) The effect of piperazine on the adults, juveniles and eggs of H. diesingi and L. appendiculata in vitro

Adult and juvenile nematodes were removed from the host gut and placed in 0.15M sodium chloride where they could normally be expected to survive for up to one month. Eggs were collected from the adult female nematodes as previously described (Section 1, 1(ii)). In assay preparations, the adult and juvenile nematodes were used in batches of 50 and the eggs in batches of 100. The anthelmintic was dissolved in distilled water to give a solution of 50mg/ml. A replicate range of concentrations of 10, 25, 50, 100, 250 and 500 g nematicide/ml final medium was made up e.g. 0.2ml of nematicide solution was added to 19.8ml culture medium to give a concentration of 500 g/ml. The effect of each of these concentrations of piperazine on the nematodes was observed after 1 and 2 hour exposure and on the eggs at 24 hour intervals for 14 days. Control preparations contained in the test specimens in 0.15M sodium chloride only. The assay on the adult and juvenile nematodes was carried out at 24°C and on the eggs at 30°C.

(iii) The infection of cockroaches with piperazine treated eggs

Using both the control eggs and those exposed to the several concentrations of piperazine above, approximately 100 eggs from each batch were fed, on damp bread, to each of 6 cockroaches. The insects had previously been kept for 4 days in a cage with a fine wire mesh base, so that the risk of infection with nematode eggs from their own faeces was minimised. Half of the cockroaches were dissected after 24 hours and the rest after 48 hours. The percentage hatch of the eggs was calculated.

Results

None of the doses of piperazine administered to the cockroaches

in the first experiment was completely effective (Table 2). Doses of 1.00 and 5.00mg/ml piperazine drastically reduced the number of adult nematodes and the juveniles were removed by every dose above 0.125mg/ml but complete eradication of the host burden was not achieved.

The in vitro experiments demonstrated the susceptibility of adult, larval and egg stages of H. diesingi and L. appendiculata to piperazine. Each concentration of the drug induced irreversible paralysis in both the adults and juveniles but in no case was 100% of the nematodes affected. The numbers affected by the drug increased with time (Fig. 1). The juveniles were more susceptible than the adults to the higher concentration of the chemical, but less susceptible to the lower concentrations (Fig. 2).

None of the batches of eggs exposed to the several concentrations of piperazine for up to 12 days developed beyond the second larval stage (Fig. 3). The time taken for development of the exposed eggs to the first and second stage larvae, exceeded that of the control eggs.

None of the piperazine treated eggs which were administered to the cockroaches hatched in the host. In the control insects, hatch of 72% of the untreated eggs containing third stage larvae occurred.

Discussion

The fact that a flaccid paralysis was induced in both adult and juvenile nematodes after exposure to the drug showed that these oxyurid nematodes reacted in a similar way as Ascaris to the drug as described by Norton and de Beer (1957). However, the fact that this phenomenon appeared to be irreversible when the nematodes were returned to 0.15M sodium chloride, although throwing

Table 2 - The effect of piperazine on worm burden of P. americana in vivo

		Host gut				Faeces			
Dose	Time (hr.)	Females	Males	Juveniles	Eggs	Females	Males	Juveniles	Eggs
0.125g	24	9	-	4	+	-	-	-	+
(0.5mM)	48	6	-	5	+	-	-	-	+
	72	8	1	3	+	-	-	-	+
	96	6	-	2	+	-	-	-	+
	Control	8	2	6	+	-	-	-	+
0.25	24	6	-	3	+	-	-	-	+
(1mM)	48	7	-	4	+	2	-	3	+
	72	4	1	7	+	2	-	-	+
	96	1	-	3	+	5	-	-	+
	Control	6	-	-	+	-	-	-	+
1.00	24	1	-	-	+	6	-	-	+
(4mM)	48	1	-	-	+	7	1	4	+
	72	2	-	-	+	5	-	6	+
	96	1	-	-	+	6	1	4	+
	Control	7	-	6	+	-	-	-	+

151

5.00 (20mM)	24	1	-	-	+	9	-	6	+
	48	3	-	-	+	6	-	4	+
	72	2	-	-	+	8	-	5	+
	96	2	-	-	+	6	1	6	+
	Control	10	2	6	+	-	-	-	+

Fig. 1

The action of piperazine on adult nematodes after 1 and 2 hours.

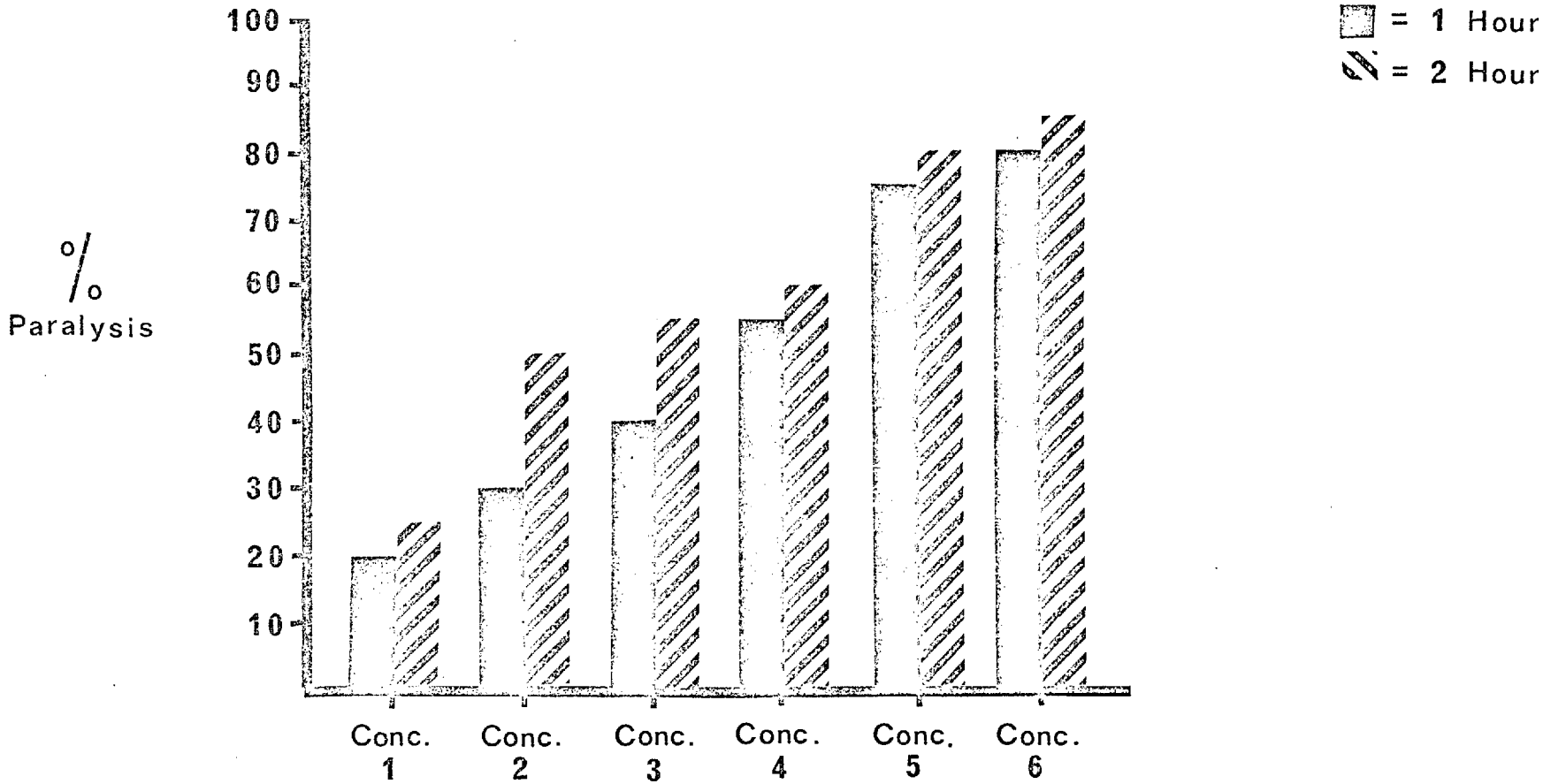

% Paralysis
100
90
80
70
60
50
40
30
20
10
Conc. 1
Conc. 2
Conc. 3
Conc. 4
Conc. 5
Conc. 6
= 1 Hour
= 2 Hour

Fig. 2

The action of piperazine on juvenile nematodes after 1 and 2 hours.

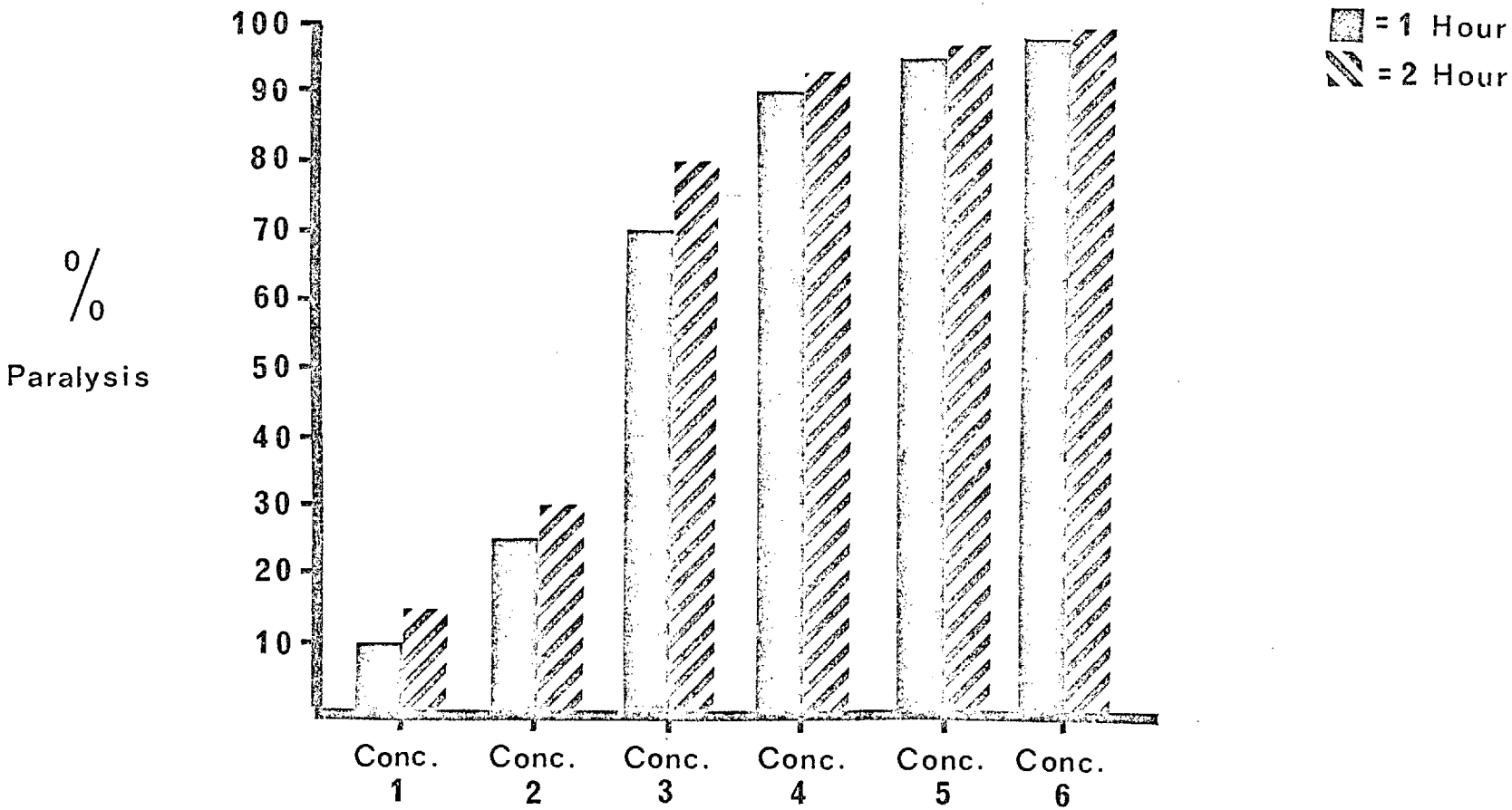
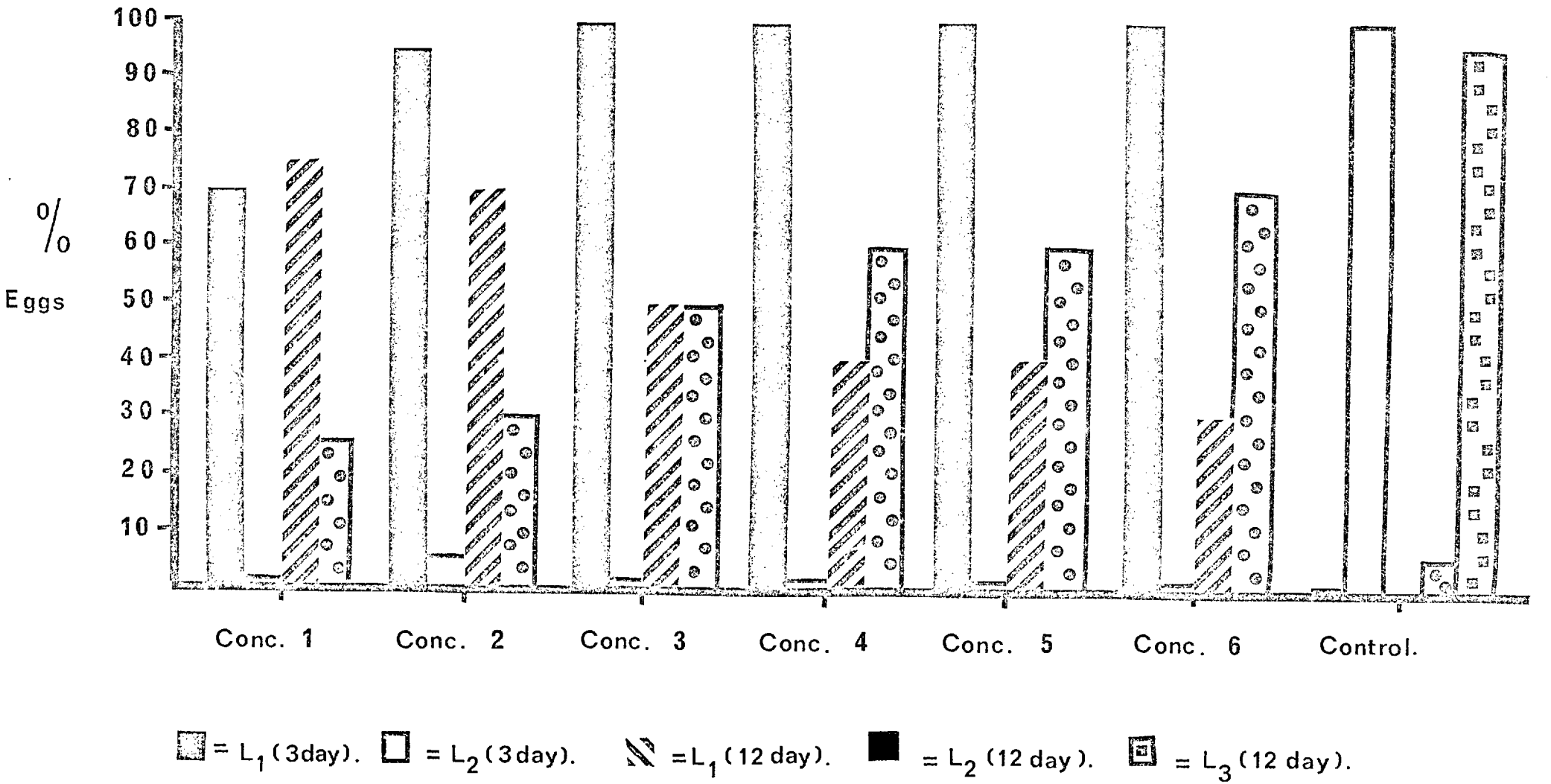

%
0
Paralysis
100
90
80
70
60
50
40
30
20
10
Conc. 1
Conc. 2
Conc. 3
Conc. 4
Conc. 5
Conc. 6
= 1 Hour
= 2 Hour

Fig. 3

The development of eggs after 3 day and 12 day exposure to piperazine at 30°C.


100
90
80
70
60
50
40
30
20
10
%
Eggs
Conc. 1
Conc. 2
Conc. 3
Conc. 4
Conc. 5
Conc. 6
Control.
= L1 (3day).
= L2 (3day).
= L1 (12 day).
= L2 (12 day).
= L3 (12 day).

little light on the reactions involved, suggests that all nematodes do not react in the same way to it. This is supported by the work of Vanfleteren and de Roets (1972), who found piperazine to exert only small to moderate toxic effect on the free living nematodes C. briggsae and Turbatrix aceti in vitro.

The in vitro experiments showed that the majority of juvenile and adult nematodes were affected by the anthelmintic after a relatively short exposure to it. Except for the greater effectiveness of the drug at the higher concentrations, these in vitro results showed little relationship to the results in vivo. Higher drug doses were used in the in vivo experiments in order to compensate for the fact that there was less chance of the drug coming into direct contact with the nematodes after its passage through the length of the gut, but it appeared that the doses decided upon were inadequate. If, however, larger doses still had been used, it is possible that the drug could have had pharmacological effects on the host.

It was concluded that this method of obtaining nematode free cockroaches was not feasible, and in the ensuing experiments adult female cockroaches with a natural infestation of mixed populations of both nematodes were used.

3. DEFINITIVE EXPERIMENTS ON THE STRUCTURE OF THE NEMATODE EGG AND ITS MODE OF HATCHING.

Introduction

In general, the fertilised nematode egg is enclosed within three distinct membranes. The outermost layer which contains both protein and carbohydrate is often referred to as the albuminous layer and is a secretion of the parental uterine wall (Chitwood,

1938). However, most of the Rhabditoidea do not form this layer at all (Chitwood and Chitwood, 1950). Meloidogyne eggs are deposited embedded in a gelatinous material secreted by the rectal glands, while Heterodera eggs are contained within a cyst formed by tanning of the cuticle of the female that produced them (Ellenby, 1946; Rogers, 1961). Both the complex middle coat and the innermost membrane are formed after fertilisation by the egg itself (Jacobs and Jones, 1939). The most obvious component of the middle coat is chitin which is not found in any other part of the nematode. The amount of chitin in the chitinous layer appears to vary greatly in different nematodes (Monné and Hönig, 1954). In the Ascaroidea and Oxyuroidea this thin layer is largely composed of chitin with a little protein. In others there is only a small amount of chitin and most of the layer consists of protein. The innermost or vitelline membrane is a lipoprotein. In the eggs of Ascaris suum nine amino acids have been identified in the vitelline membrane (Jaskoski, 1962) while the lipid constituent has been shown to be composed of ascaroside esters. The composition of this layer appears to differ in different nematodes.

In view of these differences in the egg shells of nematodes the mechanisms of hatching in different species will vary. Nematodes bearing stylets frequently utilise them for mechanical abrasion of the eggshell during hatch, while in other nematodes hatch occurs spontaneously when the larvae have reached the appropriate stage of development. The process of spontaneous hatch requires no alteration of the external environment, but a physiological stimulus provided by the embryo itself. Wilson (1958) found that the best hatch of Trichostrongylus retortae formis

was achieved in distilled water and that hatching was delayed by salts or sucrose. In the process of hatching the vitelline membrane became permeable to water and changes occurred in the hard outer shell. In contrast, eggs that are not self stimulating require a change in the external environment, generally provided by the host, to induce this process. Until this happens the eggs can survive and retain their infectivity. The hatch of eggs by this type of process has been investigated by Rogers (1958, 1960) and Fairbairn (1960, 1961) using Ascaris lumbricoides. At the onset of hatching the vitelline membrane becomes permeable (Fairbairn, 1960). Rogers (1958) suggested the secretion or activation of a chitinase which permeates the vitelline membrane and dissolves the hard shell. He also showed that the stimulus for hatching of A. lumbricoides eggs consisted of a combination of effects due to undissociated carbonic acid or dissolved gaseous carbon dioxide, a pH near 7.0 and a low oxidation-reduction (redox) potential. Temperature also affected hatch. Rogers (1960, 1961) showed that the process of hatching was similar in Toxocara mystax and Ascaridia galli. Anya (1966) in an experimental study of hatching in the oxyurid nematode parasite of the colon of mice, Aspiculuris tetraptera, indicated that the major factors in the initiation of hatching are the pH and temperature of the environment and that the process is aerobic. He concluded that neither carbon dioxide nor reducing agents were important factors in the hatching of eggs of this nematode as they are in the hatching of ascarids. He suggested that the physiology of hatching in ascarids is not necessarily the typical nematode pattern.

Definitions of hatching

It is necessary to point out here, for reasons that will be discussed later, that I have divided the process of hatching into two phases and have designated them "opercula release" and "total hatch".

"Opercula release" refers to the release of the opercula cap from the egg at a line of weakness at one end.

"Total hatch" is used to describe the situation where the opercula cap has been released and the larva inside the egg has actively escaped from the confines of the inner membrane.

A. The structure of the eggs of H. diesingi

Experimental procedures and results

(i) Examination by light microscope

Eggs containing first, second and third stage larvae were examined by transmitted light. The shape and size can be seen in Fig. 4, as can the fact that the operculum is only visible on the eggshells containing second and third stage larvae. Although difficult to define under transmitted light, disruption of eggs by ultra-sonication showed the presence of two layers, the outer eggshell and the inner vitelline membrane which surround the embryo which lies in a bathing fluid. The outer surface of the eggshell was further investigated.

(ii) The scanning electron microscope

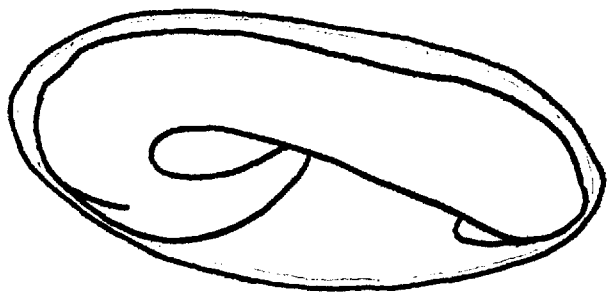
Adult female nematodes were extracted from cockroach hind guts in 0.15M sodium chloride and placed in this saline on cavity slides in damp filter paper lined petri dishes at 30°C. The eggs were collected at deposition and at various times up to eight

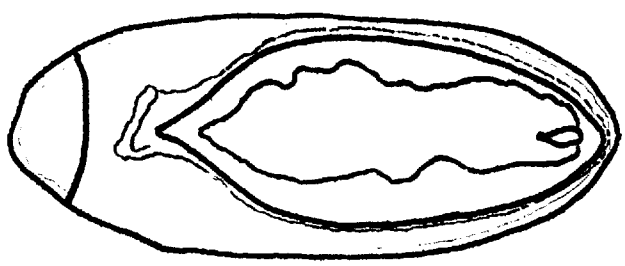
Fig. 4

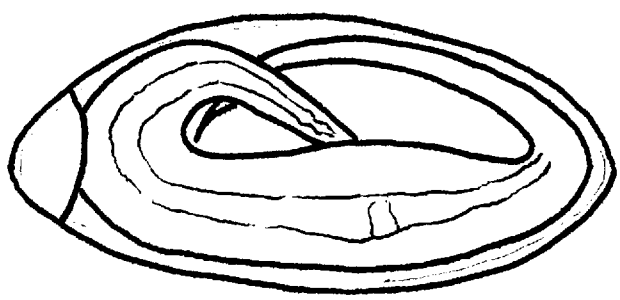
Top:
Egg containing first stage larva.

Middle:
Egg containing second stage larva.

Bottom:
Egg containing third stage larva.







50μ

days afterwards. In order to prevent the eggs exploding through excessively rapid evaporation of water at high vacuum in the electron microscope, they were dehydrated by storing on filter paper pads in a desiccator over calcium chloride pellets for 48 hours.

Dehydrated eggs were transferred to double sided sellotape mounted on a circular alloy specimen platform which fitted the goniometric chuck of the microscope. The projecting surfaces of the eggs were then coated with a thin layer of gold-palladium to reflect the incident electron beam. The coated specimens were then examined in a Cambridge Stereoscan Mark II scanning electron microscope (Cambridge Instrument Company Ltd.) operating at 20KV. Photographs of the surface of the eggshell were taken at magnifications between X1,200 and X12,100.

The photograph (Fig. 5) shows the clearly defined line of weakness which separates the operculum at the distal end of the egg from the rest of the eggshell. The surface of the eggshell is pitted and gives the appearance of canals which may connect with the inner layer of the shell.

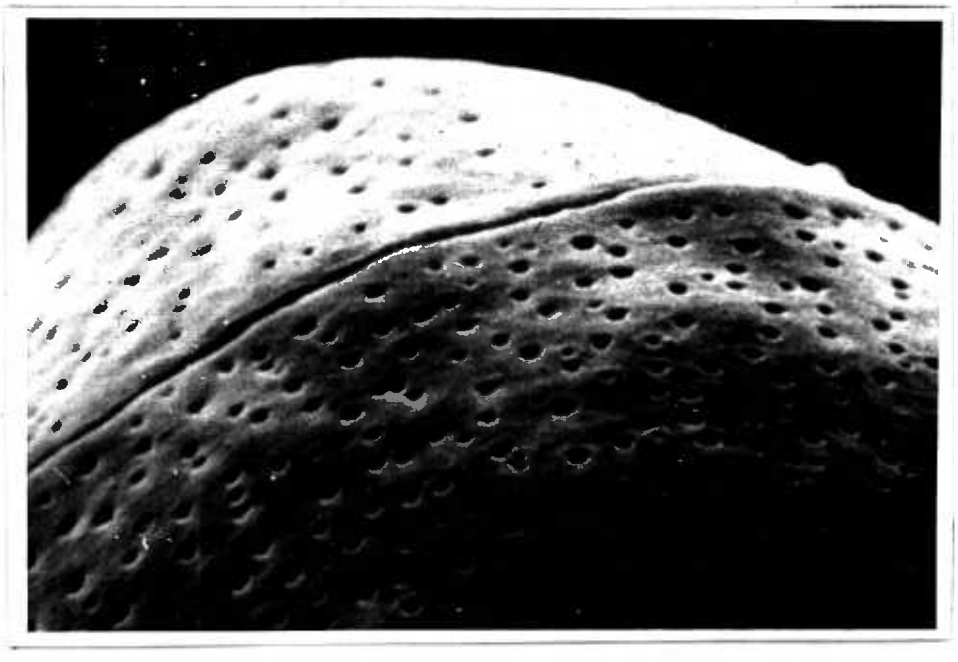
(iii) Histochemistry

It was found that intact eggs were impervious to the fixatives normally employed in histochemical methods (Section 1, 3.a.(vi)1.), so that all techniques were carried out on eggs that had first been mechanically damaged by sonication. The histochemical methods used are given by Pearse (1960).

Some paraffin sections were stained following the Himes-Moriber technique (Himes and Moriber, 1956) to aid the identification of the individual membranes. This technique also gave information concerning the distribution of protein since the napthol yellow

Fig. 5.

Stereoscan photograph of the surface of *H. diesingi* egg. X 10,500.



component of the stain gives a specific protein reaction. The periodic acid-Schiff (PAS) test was employed to demonstrate carbohydrate containing compounds, including glycogen. As a control, some sections were incubated with saliva at 37°C for 60 mins. Lipid was localised by incubating mechanically damaged eggs in saturated alcoholic sudan IV.

The Himes-Moriber technique showed that protein was distributed in the outer eggshell and that a separate inner vitelline membrane occurred which did not exhibit a positive reaction to this test. The distribution of carbohydrate containing compounds was investigated but negative results were obtained for both the outer and inner membranes. The presence of lipid in the subshell membrane was confirmed by the positive staining reaction after exposure to saturated alcoholic sudan IV. Lipid was not found in the outer egg shell.

(iv) Physico-chemical methods for determining linkages between proteins

The importance of protein structures and the linkages between the protein molecules in maintaining the integrity of the egg layers was examined as follows. To allow adequate penetration of the solutions, intact and mechanically damaged eggs were incubated in a range of solutions (as suggested by Brown, 1950) at room temperature for 24 hours. After incubation the eggs were examined microscopically for any structural defects.

Both 98% formamide and 6M urea were used to disrupt any electrovalent linkages present in the eggshell proteins. Any disulphide (s-s groups) were reduced with 0.5% alkaline thioglycollic acid (Goddard and Michaelis, 1934) or treatment with 5% sodium sulphide. Covalent linkages other than those

due to s-s bonds were disrupted with sodium hypochlorite.

Covalent linkages: Intact eggs were unaffected by either formamide or 6M urea. When mechanically damaged eggs were exposed to these solutions the embryos swelled markedly. The vitelline membrane was unaffected.

Disulphide bonds: Neither 5% sodium sulphide or 0.5% thioglycollic acid had any effect on intact eggs. In mechanically damaged eggs the embryos disintegrated. No change was apparent in the vitelline membrane.

Other linkages: After 20 mins. exposure to 1% sodium hypochlorite, the outer eggshell of the intact eggs was removed, while the contents of the eggshell and the vitelline membrane were unaffected after 24 hours. The vitelline membrane and embryo disintegrated after 24 hours in the case of mechanically damaged eggs.

(v) Investigation into the role of the eggshell and the vitelline membrane

Studies with intact eggs showed that 1% sodium hypochlorite dissolved the eggshells within 20 mins. but that it had no effect on the subshell membrane or the enclosed embryo. Hypochlorite treatment, therefore, exposed the subshell membrane and enabled experiments to be designed to investigate its permeability and its role in protecting the embryo from its external environment during its development to the R.E.S. and beyond. Information was also obtained on the function of the eggshell and subshell membrane in H. diesingi by comparing the performance of intact, mechanically damaged and hypochlorite treated eggs in each of the experiments.

(vi) Permeability of the vitelline membrane to dyes, fixatives and enzymes

1. Dyes and Fixatives

Embryonated eggs and those containing first, second and third stage larvae were pipetted into separate sets of tubes containing one of the following dyes in 0.9% saline; neutral red (1:10,000 w/v), methylene blue, toluidine blue, trypan blue and Evan's blue (all 0.01% w/v). The eggs were recovered after 60min. The ability of the dyes to pass through the shell and vitelline membrane was determined by comparing them with mechanically damaged eggs which had been immersed in the same dye solutions for the same period.

Similarly, all the types of eggs were incubated in saline solutions containing 5-30% formalin, 10-50% ethanol and 1% osmium tetroxide. After 60 min., intact, mechanically damaged and hypochlorite treated eggs were removed from each fixative solution, washed repeatedly in 0.9% saline and transferred to 0.15M sodium chloride. Efficiency of the vitelline membrane in intact and hypochlorite treated eggs was determined for the embryonated and first and second larval stages by their ability to develop further and the third stage larvae by their reaction when exposed to the hatching stimuli (Section 1, 3.B).

After 60 min. in solutions of toluidine blue, methylene blue, trypan blue and Evan's blue, none of the embryos in intact and hypochlorite treated eggs were stained. This indicated that the vitelline membrane was impermeable to these dyes in eggs of all ages, since mechanically damaged eggs, in which the shell and vitelline membrane had been ruptured, showed an almost uniform staining.

After 60 minutes in solutions of toluidine blue, methylene blue, trypan blue and Evan's blue, none of the embryos were intact and hypochlorite treated eggs were stained. This indicated that the vitelline membrane was impermeable to these dyes in eggs of all ages, since mechanically damaged eggs, in which the shell and vitelline membrane had been ruptured, showed an almost uniform staining.

When intact and hypochlorite treated eggs were kept in neutral red for the same period, the stain passed through the vitelline membrane and stained the embryo. In mechanically damaged eggs, the embryos became stained after 10 minutes.

The activity of first and third stage larvae in mechanically damaged eggs ceased abruptly as soon as they were exposed to 5 - 30% formalin, 10 - 50% ethanol and 1% osmium tetroxide. Similar stage larvae in eggs with the vitelline membrane intact were still mobile 60 minutes after exposure.

2. Enzymes

Intact, mechanically damaged and hypochlorite treated eggs were washed and incubated in enzymes. All of the enzymes, with the exception of pepsin, were dissolved in M/15 phosphate buffer at the optimum pH of each enzyme. Pepsin (British Drug Houses, Poole, England) was prepared in 015M sodium chloride and adjusted to pH 2 by the dropwise addition of 0.1N hydrochloric acid. The eggs were incubated in 2ml of each enzyme solution at room temperature for varying periods of time up to 60 minutes.

Neuraminidase (0.1% pH 5.2), B-galactosidase (0.5% pH 7.2), B-glucuronidase (10mg/ml pH 6.8) (Sigma Chemical Company, USA and London) and Hyaluronidase (1mg/ml pH 7.0) (Hoch-Light Labs., Staines, Bucks) were used to determine distribution of the sugar

units.

Pancreatin (0.5% pH 7.8), papain (0.5% pH 6.2), ficin (0.5% pH 6.2), pepsin 0.5% pH 2.0), crude trypsin (0.5% pH 7.8) (British Drug Houses), crystalline trypsin (1mg/ml pH 7.8) (Koch-Light Laba) were employed to indicate the presence of proteins in the structure of eggshell and vitelline membrane.

The effect of lipase (0.5% pH 7.8) (Koch-Light Labs) on the vitelline membrane was observed.

The four enzymes, hyaluronidase, neuraminidase, B-glucuronidase and B-galactosidase used to determine the presence of sugar derivatives were without effect on the eggshell and vitelline membrane.

Although the proteolytic enzymes utilised digested the embryo in damaged eggs, they brought about no change in the egg shell or vitelline membrane.

However, lipase dissolved the vitelline membrane of hypochlorite heated eggs after only 15 minutes exposure to it. This allowed the second or third stage larvae to escape from the confines of the eggshell. The enzyme had no effect on the intact egg.

(vii) Analysis of the eggshell

1. Treatment of the eggshells.

A sample of approximately 15,000 eggs was washed in 0.1% sodium hydroxide to separate them and then rinsed in distilled water several times. The eggs were disrupted after two presses with Biotec X-press equipment (Biotec Ltd. South Croydon). The eggshell fragments were transferred to centrifuge tubes and washed six times with distilled water. The sample was dried at 40°C.

2. Extraction of the lipids

The egg shell fragments were placed in 15ml diethyl ether and left for two days. The sample was centrifuged and the lipid fraction decanted. This process was repeated twice. The protein fraction was hydrolysed at 110°C in 4ml of 5N hydrochloric acid in a sealed 5ml ampoule for 24 hours. The acid hydrolysate was evaporated 8 times on a watchglass over steam to remove excess hydrochloric acid. 5ml of distilled water was used for each evaporation. The residue was dissolved in 2ml 0.025N. hydrochloric acid. This procedure was a modification of that used by Jaskoski and Butler (1971) in the preparation of tick eggs for amino acid analysis.

3. Automatic amino acid analysis

An amino acid analysis of a sample of eggs prepared as described above was carried out in the Department of Biochemistry at Imperial College.

4. Chromatography

The egg residue containing the amino acids as prepared above was dissolved in 3ml of 10% isopropanol and the pH brought to neutrality by the addition of a small amount of Gomori's 0.2 phosphate buffer as described by Jaskoski (1962).

Ascending two dimensional thin layer chromatography was carried out using glass plates coated with cellulose. A solution of n-butanol: acetic acid: water (8:2:2) was used as the irrigant in single dimension chromatograms. For two dimensional chromatograms the solvents used were n-butanol: acetic acid: water (8:2:2) for the first dimension and n-propanol: water 7:3) for the second dimension. Single dimension chromatograms were developed for a distance of 16cm in 6-7 hours. Two dimensional chromatograms were developed for 13cms in each

direction in 9-10 hours.

Amino acid standards plus standards of glucosamine, ornithine and citrulline were prepared as 0.1% solutions in 10% isopropanol. Amino acids were visualised with a 0.3% solution of ninhydrin in n-butanol to which 3ml of glacial acetic acid had been added (Stahl, 1965).

Ascending chromatograms of the eggshell hydrolysate were developed in n-butanol, acetic acid and water (4:1:1) and visualised with 0.25% ninhydrin in acetone.

Thirteen amino acids were separated in the eggshell hydrolysate by thin layer chromatography and identified by reference to their position on a standard map and their Rf values. In addition, methionine, aspartic acid, glutamic acid and ammonia were located by the automatic amino acid analyser which also separated leucine and isoleucine into separate peaks. This machine also supplied quantitative data on the amino acid composition of the eggshells. From this information each substance is presented as percentage total in Table 3. Tryptophan was not found by either chromatography or automatic amino acid analysis but as it is unstable during acid hydrolysis it is possible that it is responsible for some of the ammonia determined by the analyser (Table 3).

Table 3. Substances from the eggshell located by automatic amino acid analysis

Substance	% Total
Threonine	5.21
Serine	9.45
Glycine	14.69
Alanine	10.36
Valine	0.20
Isoleucine	3.15
Leucine	6.65
Tyrosine	1.64
Phenylalanine	2.79
Histidine	2.18
Lysine	4.43
Arginine	6.26
Omithine	Trace
Methionine Sulphoxide	0.60
Proline	1.74
Aspartic acid	9.43
Glutamic acid	11.76
Ammonia	10.09

5. Chitin determination

The Chitosan test of Campbell (1929) was applied on a small fraction of intact eggshells that were not subjected to extraction of lipids or hydrolysed.

The chitosan test was positive.

B. Hatching of the oxyurid eggs

Estimation of hatching.

Previous workers investigating the hatching of nematode eggs (Rogers, 1960: Fairbairn, 1961) found that some eggs started to hatch in the culture medium, before they had been exposed to any hatching stimulus. They called this phenomenon "background hatching" and Fairbairn gave this as high as 10% in his experiments. In order to avoid any possible variation introduced into experimental results due to this "background hatch", before use all eggs were washed by centrifugation in the buffer to be used in the subsequent incubation as recommended by Anya (1966). Eggs which showed no evidence of "opercula release" after this process were used in subsequent experiments.

Experimental procedures and results

(i) The role of pH

The effect of pH on hatching of eggs was investigated within the pH range 1-10. Preliminary experiments had indicated that at an equivalent pH, there was no significant difference between the proportion of eggs which will release their operculum in any of the following buffers; phosphate, tris-(hydroxymethy) - amino-methane, veronal acetate (Pearse, 1960), citrate and borate (Clark, 1928). In all of the experiments reported here veronal acetate has been used except in the pH range 2 and below, where dilutions of 1% hydrochloric acid with distilled water were used. The pH was checked with a glass electrode.

Approximately 100 eggs containing second or third stage larvae were placed in each of five solid watch glasses containing 2ml of the buffer adjusted to the relevant pH.

A glass cover was secured to each watch glass with vaseline. The watch glasses were incubated for 24 hours at either 24, 29 or 35°C. The experiment was carried out in the pH range 1-10.

The percentage opercula release obtained at each pH and at each temperature for eggs containing either second or third stage larvae is shown in Figure 6. The most suitable pH range for opercula opening for both stages appears to be in the range pH 4.1 - 7.2.

(ii) The role of temperature

The results shown above indicate that temperature was also an important factor in the preliminary stages of egg hatch. This factor was further investigated.

Batches of eggs were put into incubation dishes containing buffer at the pH optimum for opercula release and sealed, as described previously. The buffer had previously been heated for 1 hour at the temperature of subsequent incubation. At the end of 24 hours, the number of eggs in all dishes which showed opercula release was noted and the percentage hatch calculated. A separate series of experiments was conducted for each of the following temperatures: 10, 20, 24, 29, 32, 35, 37, 40, 45, 50 and 60°C.

The relationship of temperature to the percentage opercula release is shown in Figure 7.

(iii) The role of oxygen

Anya (1965) found that the presence of oxygen was essential for the development of oxyurid nematode eggs to the infective stage outside of the host. Rogers (1949) and later Crompton,

Fig. 6

The relationship between pH of the hatching medium and the percentage of eggs of H. diesingi which release their opercula at 24, 29 and 35°C.

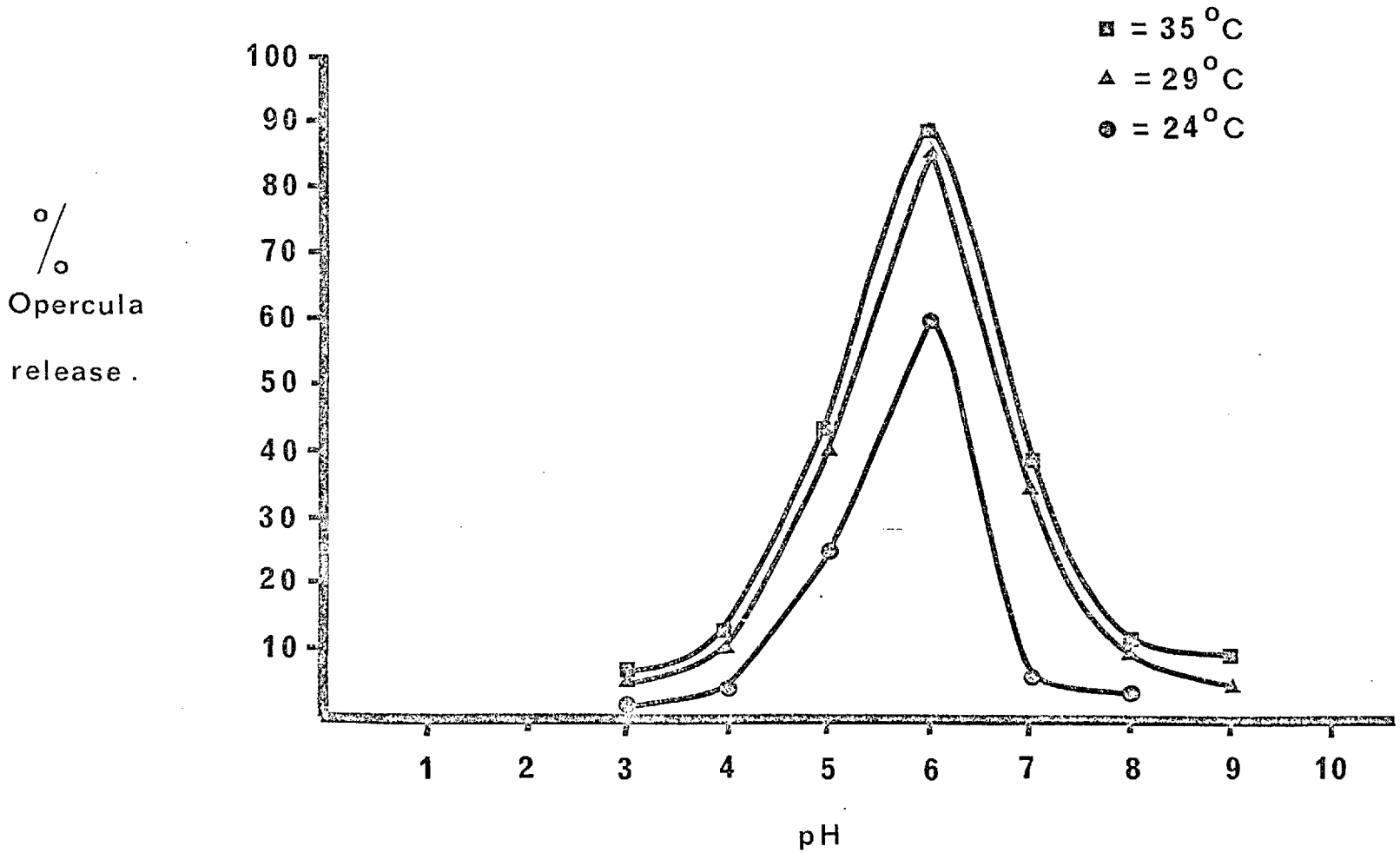
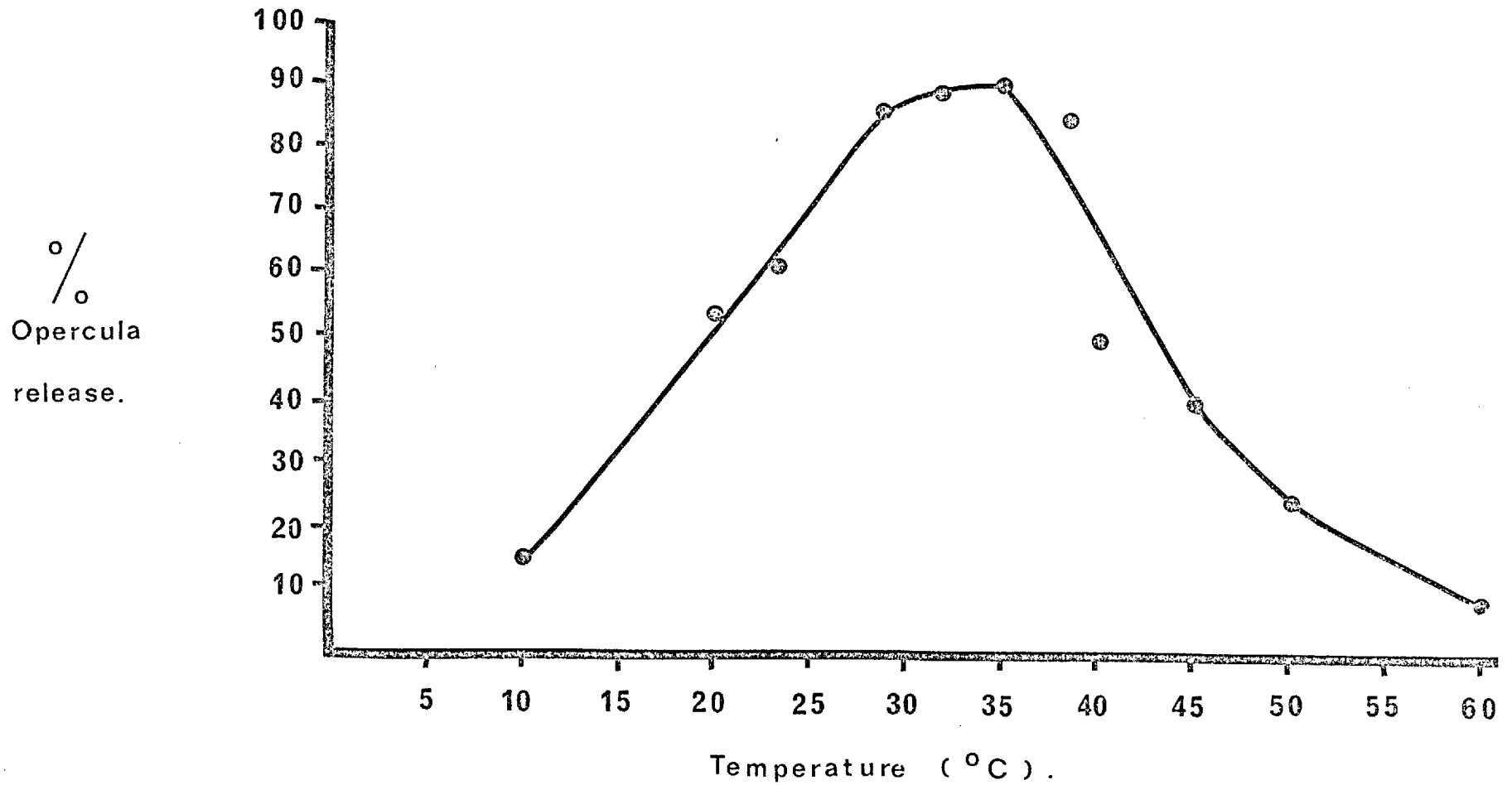

% Opercula release .
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
pH
= 35 °C
= 29 °C
= 24 °C

Fig. 7

The relationship between temperature and the percentage opercula release by eggs containing second or third stage larvae of H. diesingi at pH 6.2.


% Opercula release.
100
90
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
50
55
60
Temperature (°C).

Shrimpton and Silver (1965) showed that near anaerobic conditions exist in the centre of the lumen of the vertebrate alimentary canal system. This led Anya (1966) to suggest that the absence of oxygen or its presence in very low concentrations may provide part of the stimulus for the hatching process. The role of oxygen in the hatching process was investigated.

Approximately 1,500 eggs were placed in each of two flasks, which were filled with veronal acetate buffer (pH 6,2) standing in a water bath at 30°C for a period of 5 hours. Nitrogen was bubbled into one jar and air drawn through the other. At the end of the experimental period, 4 samples of at least 100 eggs were withdrawn from each flast and the percentage opercula opening was estimated. The experiment was repeated several times.

The average results obtained can be seen in Table 4. The low percentage opercular release in near anaerobic conditions contrasted markedly with those in air. Eggs containing either second or third stage larvae showed similar reactions to the conditions with the highest opercula release in 100% nitrogen of 14.1 and 15.8 respectively, compared with 81.4 and 80.4% in air.

Table 4. The percentage hatch in vitro of eggs of H. diesingi in the presence and absence of oxygen after 5 hours incubation at 30°C and pH 6.2.

	100% Nitrogen	Air
Eggs containing	12.6	70.2
second stage larvae	14.1	79.8
	12.2	81.4
Eggs containing	15.8	78.6
third stage larvae	14.6	80.4
	14.2	79.4

(iv) The role of carbon dioxide

In 1963, Rogers suggested that carbon dioxide was the major factor which initiated the hatching of eggs of ascarids and perhaps of other nematodes but that he had also found (1960) that large concentrations of carbon dioxide can inhibit hatching. Wallace (1963), showed that carbon dioxide inhibits the hatch of H. rostochiensis eggs. However, the results of preliminary experiments suggested that carbon dioxide does not play a primary role in the initiation of hatch of H. diesingi eggs. The following experiment was carried out to determine the role, if any, of carbon dioxide in the hatching process.

Two flasks set up as described previously with the exception that the air passed into one of the flasks was bubbled first through a container of 1N sodium hydroxide to remove carbon dioxide from it. The experiment was carried out at 30°C, for 5 hours and the percentage hatch determined as before.

The percentage hatch of eggs kept in each of the gas phases are recorded in Table 5. It can be seen that carbon dioxide does not appear to paly any role whatsoever in the initiation of hatching of the eggs.

Table 5. The percentage hatch of eggs of H. diesingi in air and air minus carbon dioxide after incubation for 5 hours at 30°C and pH 6.2.

	Air minus carbon dioxide	Air
Eggs containing	81.6	80.3
second stage larvae	79.4	80.6
	81.2	79.3
Eggs containing	80.4	81.4
third stage larvae	79.2	80.6
	79.8	81.2

(v) The role of reducing agents.

Rogers (1960) has shown that the presence of reducing agents, together with the addition of salts, variation in pH and temperature, affect the rate of hatch of Ascaris eggs in the presence of dissolved gaseous carbon dioxide and unidssociated carbonic acid.

The effects of two reducing agents, sodium dithionite and cysteine on hatching were investigated in the presence of carbon dioxide.

About one hundred eggs, containing either second or third stage larvae, were added to solid watch glasses containing veronal acetate buffer and either sodium dithionite or cysteine to give a final concentration of 1mg/ml. The pH of each solution was adjusted to 6.2. Each watch glass was covered and incubated for 24 hours at 30°C.

Table 6 shows the results of these experiments. Neither sodium dithionite nor cysteine has any effect on the initiation of hatch of eggs containing either second or third stage larvae under the conditions specified.

Table 6 The percentage hatch of H. diesingi eggs in the presence or absence of reducing agents.

	Control	Cysteine present	Sodium dithionite present
L_2	79.8±2.4	78.2±1.6	79.3±2.0
L_3	79.6±1.8	79.1±2.1	80.8±2.3

L_2 = Eggs containing second stage larvae

L_3 = Eggs containing third stage larvae

(vi) The role of salt solutions

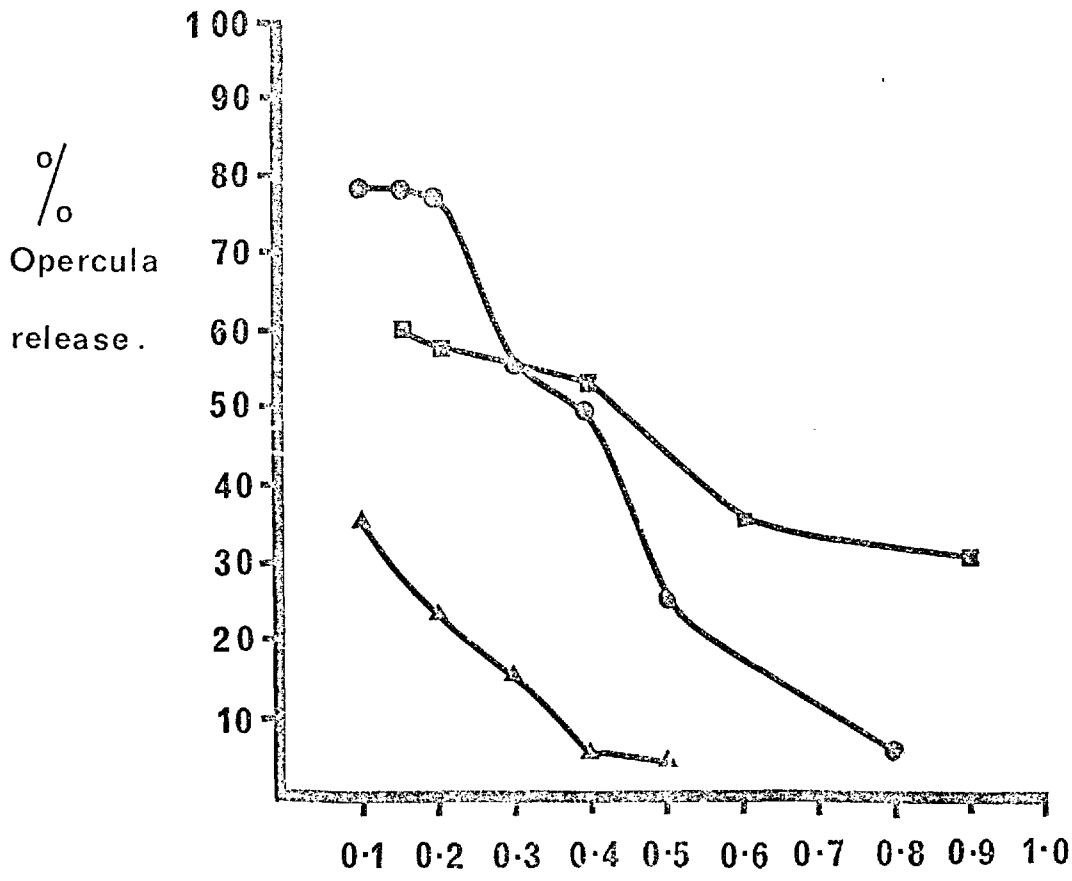
Most of the previous work, that by Wilson (1958), Dropkin, Martin and Johnson (1958), Rogers (1960) and Anya (1966) have shown that certain concentrations of salts have an inhibitory effect on the hatching of nematode eggs. To be more specific Anya (1966) found that the hatching of eggs of A. tetraptera was inhibited by sodium chloride and sucrose in concentrations of 0.1-1.0 M. Nevertheless, Rogers (1960) showed that the presence of sodium chloride at concentrations below 0.1M increased the hatch of A. lumbricoides eggs. It was decided to follow the lines of investigation taken by Anya (1966) and examine the effect of sodium chloride, sucrose and a physiological saline on the hatching of these oxyurid eggs.

Veronal acetate buffer was used to make up all solutions which were subsequently adjusted to pH 6.2. The following concentrations of sodium chloride were used: 0.05, 0.08, 0.15, 0.2, 0.3, 0.5, 0.8, 1.0M. To 2ml of the buffer each containing these concentrations were added approximately 100 eggs containing second or third stage larvae. The sealed assay specimens were incubated at 30°C for 12 hours. The percentage hatch was determined at the end of this period. Sucrose was made up in the following concentrations: 0.1, 0.2, 0,3, 0.4, 0,5M and the percentage hatch determined. A balanced salt solution was used. This was made up by dissolving the salts in Fenwicks (1939) saline solution in veronal acetate buffer. From this a series of different concentrations of the solution were made up: 0.15, 0.2, 0.4, 0.6, 0.9 and 1.0M. The percentage hatch was again determined.

The results of these experiments can be seen in Figure 8. Opercula release was depressed by sodium chloride above the concentration of 0.15M and by sucrose and the saline at

Fig. 8

Opercula release by eggs containing second or third stage larvae of *H. diesingi* in sucrose, sodium chloride and saline.



Key

△ = Sucrose

▨ = Saline

⊙ = Sodium chloride

concentrations greater than 0.1M. As Anya states (1966), at high concentrations the sucrose is very viscous and any indications of hatch are hard to discern, this may lead to underestimation of the true hatch.

(vii) Hatch of H. diesingi eggs in different parts of excised guts of P. americana

Lee(1959) showed that the eggs containing second stage larvae could hatch completely, to release third stage larvae, in the extirpated midgut of P. americana after 24 hours at 30°C. Experiments based on his method were carried out using eggs containing second and third larvae stages and the crop, midgut and hindgut of the host.

The crop, midgut and hindgut of 10 insects were dissected out and ligatured at one end with fine thread. Approximately 100 eggs containing either second or third stage larvae were introduced into the open end of the gut in 0.15M sodium chloride through a silicone repellent coated pipette. The open end was then secured and each piece of gut was placed in a test tube containing insect saline. The top of each tube was covered with parafilm and the tube supported in a water bath at 24°C and incubated for 24 hours. After this period, the parts of the guts were dissected and the percentage opercula release and complete hatch estimated.

From the results in Table 7 it can be seen that the crop was the site of opercula release by eggs containing both second and third stage larvae. This process recurred in the midguts but the percentage release of the operculum by eggs containing third stage larvae was six times the percentage of eggs containing second stage larvae. Although 8% total hatch is recorded in the midgut

for eggs containing second stage larvae, the ovoid larvae released remained immobile and did not moult to release the third stage larva. In contrast, 69% of the third stage larvae were released from the confines of the eggshell and its membrane in the excised midgut. The third stage larvae were actively swimming after 24 hours. There was no evidence for complete hatch in the crop, nor for any initiation of hatch in the hindguts. The empty eggcases of the free larvae were found.

Table 7 The hatch of H. diesingi eggs in excised parts of the gut of P. americana.

	% Opercula release		% Total hatch	
	L_2	L_3	L_2	L_3
Crop	26	63	0	0
Midgut	12	71	8	69
Hindgut	0	0	0	0

L_2 = Eggs containing second stage larvae

L_3 = Eggs containing third stage larvae

(viii) The hatch of eggs in midgut homogenates.

The midguts of several insects were dissected out and homogenised in saline. During this process they were kept as cool as possible by repeated immersion in ice. A series of homogenates were made up to give a range of concentrations of 1, 2, 4 and 5 midguts/ml. One hundred eggs containing second or third stage larvae were added to test tubes containing each concentration of these homogenates. The tubes were sealed with parafilm and incubated in a water both at 24 C for 12 hours. At the end of this period the percentage of the eggs which had hatched was determined.

Homogenised hindguts were used as controls.

From Table 8 it can be seen that the eggs containing second stage larvae released their opercula and complete hatch occurred in each concentration of midgut homogenates but as soon previously, the released second stage larvae did not undergo a further moult and remained immobile. In contrast the eggs containing third stage larvae hatched completely and were motile. The percentage hatch gradually increased with increased concentration but was steadily maintained at the highest concentrations. No hatch occurred in the controls.

Table 8 The percentage total hatch in midgut homogenates

	Concentration of homogenate (Midguts/ml)			
	1	2	4	5
Eggs containing second stage larvae	3	6	5	2
Eggs containing third stage larvae	49	55	67	68

(ix) In vitro hatch in midgut extracts.

Whole insect midguts were excised, blotted on filter paper to remove any adherent haemolymph, and homogenised in ice cold saline pH6.2, to give an initial concentration of 2 midguts/ml. The homogenate was centrifuged at 170g for 10 minutes and the pale yellow liquid lying between the sedimented gut debris and the surface lipids was withdrawn and used as a sourse of crude enzymes. The experimental design was identical with that for the effect of midgut homogenates.

The results were exactly comparable with those obtained in the previous experiment.

1. The effect of heating the midgut extract.

A freshly prepared saline extract (conc. 1 midgut/ml) was maintained in a water bath at 80°C for 0.5, 10 and 15 minutes. At the end of each time period, 1ml of the extract was transferred to a clean test tube, cooled at 24°C and mixed with 100 eggs containing either second or third stage larvae. Eggs were withdrawn from each of the extracts at 5 minute intervals for up to 1 hour and examined for hatching.

None of the eggs exposed to the heated extract exhibited total hatch.

2. The effect of varying the pH of the extract.

Variations in the pH of the extract were examined by pipetting into each of 11 tubes 100 eggs (which had previously been exposed to a pH of 6.2 and had released their opercula caps), 0.5ml of saline extract (2 midguts/ml) and 0.5ml of M/15 phosphate buffer (pH range 5-8). At one minute intervals some of the eggs were withdrawn and examined for release of larvae.

From Table 9 it can be seen that maximum hatch occurred over the pH range 6.8 - 7.8.

Table 9. The effect of varying the pH of the extract on the percentage hatch of H. diesingi eggs.

pH	5.0	5.5	6.0	6.5	6.8	7.0	7.2	7.4	7.6	7.8	8.0
% Hatch	0	0	9	22	40	45	55	60	52	48	25

(x) Hatch in salivary gland homogenates.

Pairs of salivary glands were dissected out of the cockroaches and homogenised in ice cold saline to give an initial concentration of 2 pr salivary glands/ml. Batches of 100 eggs containing second or third stage larvae were added to replicates of the homogenates at 24°C. The pH was adjusted to 5.2. The experiment was carried out for 12 hours.

Exposure to these extracts did not instigate hatching in any of the eggs.

(xi) Hatch in purified enzymes.

The oxyurid eggs were incubated in solutions of each of the enzymes found in the midgut of P. americana. One millilitre aliquots of the following solutions were prepared in M/15 phosphate buffer and incubated with 100 eggs (which had already initiated hatch under stimulation) for 60 minutes at 24°C. The following enzymes were used: Bacterial α-amylase (300mg/ml pH 6.8), α-chymotrypsin (30mg/ml), trypsin plus α-chymotrypsin 20 + 30mg/ml) both at pH 7.8, lipase (20mg/ml PH 7.8) (Koch-Light Labs., Staines, Bucks); twice crystalline hog pancreatic α-amylase (100 mg/ml pH 7.0), trypsin (20mg/ml) and trypsin (20mg/ml), at pH 7.8 (British Drug Houses, Poole, England).

Intact eggs exposed to these enzymes for the experimental period were unaffected. However, complete hatch occurred in 61% of the eggs which had been immersed in lipase after hatch had been initiated in them. When the opercula cap had been released in these eggs, the vitelline membrane disappeared after 15 minutes exposure to lipase.

(xii) The in vivo hatching of eggs

Nymphs 2, 4 and 6 months old together with adult P. americana were starved for two days and then given pieces of moistened bread infected with eggs containing either second or third larval stage H. diesingi. The cockroaches were killed after 30 minutes, 1, 3, 5, 12 and 24 hours and their crops, midguts and hindguts examined for stages of the nematode.

After 30 minutes and 1 hour eggs were found only in the crop. The opercula cap of the eggs at the R.E.S. or later stage had been released at the line of weakness. The eggs in the second larval stage remained enclosed by the inner membrane in the open ended eggshells, they were non-motile. The third stage larvae were in the same position but were motile. At 3 and 5 hours complete hatch of both second and third stage larvae had occurred and non-motile second stage larvae and actively swimming third stage larvae were found in the midgut. After 12 and 24 hours, the insects dosed with second stage larvae had less than 2% of the original egg dose in the form of active third stage larvae in the hindgut. Non-motile second stage larvae were still found in the midgut. In contrast 62% of the eggs containing third stage larvae used in the original dose were found as active third stage larvae in the hindgut, 7% were found in the midgut. The different ages of P. americana were all susceptible to H. diesingi.

(xiii) The effect of starving P. americana on the in vivo hatch of H. diesingi.

Twenty adult female P. americana were starved for 5 days. At the end of this period 50 infective eggs of H. diesingi were introduced into the crop of each insect using a catheter. The cockroaches were anaesthetised with carbon dioxide and a catheter

which had been rinsed with silicone repellent, introduced into the insects mouth. The catheter was attached to a calibrated micrometer syringe, containing the eggs, so that the dose introduced into the insect could be controlled. The cockroaches were killed after 1,6, 12 or 24 hours, their guts excised and examined for stages of the nematodes. The results are averages of 5 insects in each case.

After 1 hour, 65% of the eggs had released their opercula caps in the crop. Both the crops and midgut contained eggs after 6 and 12 hours. Although hatch had been initiated in 72% of the eggs found none of the larvae had escaped from the confines of the vitelline membrane. After 24 hours 60% of the eggs were found in the hindgut and of these 2% were empty, total hatch having occurred.

(xiv) The effect of starving P. americana on the midgut lipase and esterase activities.

Twenty adult female P. americana were starved for 5 days. At the end of this period the midguts were excised from the insects and the total midgut lipase and esterase activities measured.

The colorimetric method of Selligman and Nachlas (1963) was used. The principle of this method is that lipase and esterase hydrolyse 2-naphtyl laurate to 2-naphthol and lauric acid. In the absence of taurocholate, most of the hydrolysis is due to esterase, while in the presence of taurocholate, the hydrolysis is due to esterase and lipase. The difference corresponds to the lipase activity. Two molecules of 2-napthol are coupled with tetrazotized o-dianisidine to give a purple azo dye which can be extracted with ethyl acetate and determined colorimetrically.

The method was carried out according to the authors with the modifications by Gordon (1968).

The mean values for the enzyme activities in fed and starved cockroaches are shown in Table 10.

Table 10. The effect of starvation on the esterase and lipase activities in P. americana.

	Fed	Starved
E_2 Esterase activity	0.1305 ± 0.070	0.1054 ± 0.079
E_1-E_2 Lipase activity	0.0991 ± 0.082	0.0839 ± 0.0167

Those results showed that the midgut esterase and lipase activities were unaffected by starvation of the insect.

Discussion.

The reproductive tract of female nematodes is responsible for the secretion of all of the substances found in their eggs with the exception of chitin which is secreted by the egg itself (Lee, 1965). It also seems probable that the female is responsible for the gelatinous coat surrounding the eggs as was found by Lee (1961) who investigated the origin of this substance around the eggs of another cockroach inhabiting oxyurid, Thelastoma bulhöesi.

Although the presence of chitin has been determined as a constituent of the eggshells of H. diesingi, it was not possible to determine its location in relation to the structure of the eggshell as after treatment of the eggshells it appeared as small scattered areas in the eggshell material. The chitin is synthesised and extruded from yolk material. According to Fairbairn (1960), it is probable that the yolk provides the

glycogen and trehalose to form the glucose from which the N-acetyl glucosamine units of chitin are formed. Pollak and Fairbairn (1955) have shown that Ascaris oocytes contain glutamine or related substances. According to Pogell (1956), the conversion of glucose-6-phosphate to glucosamine occurs in the presence of glutamine, which serves as a source of amino groups. Fairbairn (1955) has shown that Ascaris oocytes also contains a source of acetate which is needed for the acylation of glucosamine to N-acetyl glucosamine. Glucosamine was not identified on the chromatograms, nor was it found during the automatic amino acid analysis but this does not preclude the presence of chitin. McFarlane (1962) working on the house cricket eggshells, failed to detect glucosamine even though the eggshell is composed of cuticles which are chitinous.

Proteins are commonly stabilised by electrovalent linkages involving polar groups present on the side chains of some of their amino acid constituents and attractive forces of this type can normally be diminished by solvents of high dielectric constant, such as formamide and 6M urea (Brown, 1950). The failure of eggshells to change after prolonged immersionin both of these solvents, may indicate that electrovalent linkages are not important to the eggshell structure. Neither were eggshells disrupted by alkaline thioglycollic acid or sodium sulphide, both of which destroy the disulphide bridges which are often responsible for stabilising many structural proteins. The insignificance of the s-s bonds was further indicated by the absence of cystine from the chromatographic and automatic analyser results and the fact that sulphur bearing amino acids account for a very small fraction of the eggshell proteins.

Sodium hypochlorite, however, rapidly dissolved the shells. The precise action of sodium hypochlorite is not understood but it is thought to cleave the peptide bonds in the protein chain as well as some of the bonds in aromatic residues (Brown 1950). A Characteristic of tanned protein shells of digenetic fluke and pseudophyllidean cestode eggs is their insolubility in solvents other than sodium hypochlorite (Smyth and Clegg 1959). It has been found that tanned shells of other helminths are resistant to papain, as are the shells of the oxyurid eggs under discussion. Evidence for tanning in the cysts of the plant parasitic nematode Heterodera rostochiensis was presented by Ellenby (1946), while Clarke, Cox and Shepherd (1967) found that the eggshell hydrolysates contained only very small amounts of the aromatic and more particularly the hydroxy aromatic amino acids. Only a small amount of these amino acids are found in the oxyurid eggs. However, the most plentiful amino acids in Heterodera rostochiensis cysts were proline, aspartic acid, glycine, serine and hydroxyproline. Clarke, Cox and Shepherd (1967) suggested that the eggshell contains a collagen-like protein. Similar results have been obtained for Ascaris eggs by Kreutzer (1953) and Jaskoski (1962). Collagen like proteins occur in the cuticle of other nematode species e.g. Toxocara mystax, Strongylus equinus (Bird, 1956) and an oxyurid nematode Aspiculuris tetraptera (Anya, 1966a).

It seems unlikely that conventional tanning is responsible for the hard, protective coat provided by the eggshells of H. diesingi or that structural support is provided by a collagen-like protein. It is possible that an alternative route for tanning may exist when suitable protein substrates are available. Smyth and Clegg (1959) stated that histidine, tyrosine and phenylalanine, all present in the oxyurid eggshells, could act

as phenols and be modified to quinnones which are then autotanned. This is supported by later work which showed that tyrosine is directly involved in tanning in Fasciola hepatica, while histidine is employed in Diphyllobothrium latum and Schistocephalus solidus (Bogomolova and Pavlova, 1961). Further evidence can be gathered from the insects. In the Blowfly puparium, the tanning is derived principally from tyrosine. It seems likely, therefore, that the resistance of the outer protein and chitin egg layer is due to a form of tanning, as indicated by their properties, colour and amino acid composition. However, the mechanism by which such a process could be brought about is not understood.

The failure of sodium hypochlorite to disrupt the vitelline membrane, although dissolving the outer coat, was particularly useful for the study of the composition and the role of the vitelline membrane.

The most striking property of the vitelline membrane was its permeability. The fact that neutral red penetrated the outer coat of the egg but not the membrane showed that the shell material was freely permeable to compounds of quite high molecular weight and provides further evidence that it is the sub-shell membrane and not the eggshell that blocks the entry of substances into the egg. This is supported by the work of Fairbairn and Passey (1955) and Wottage (1937), who showed that in A. lumbricoides eggs, the scleroprotein layer is dyed by basic vital stains and is permeable to water, but these substances do not penetrate into the perivitelline space once the vitelline membrane has been formed.

The vitelline membrane, therefore, plays an important role in the protection of the embryo from unfavourable conditions that may exist outside of the host. A possible role of this egg shell

layer in the hatching of the nematode larvae was indicated by the digestion of the membrane by lipase. The vitelline membrane has variously been referred to as being composed of a preponderance of lipid soluble substances (Fairbairn, 1960), esterified glycosides with the solubility characteristics of lipids (Lee, 1965) and waxy (Monné, 1955), but has been shown in Ascaris and Parascaris by Fairbairn (1960) to be a homogeneous layer composed of a hexose called ascarylose. This occurs in the glycosides which are the main components of the yolk of these nematodes. The positive staining of the vitelline membrane and its digestion by lipase, indicate that in the oxyurid nematode eggs, the vitelline membrane is composed of a lipid substance. Some further information on the structure of the vitelline membrane comes from Sarda and Desnuelles (1958) work using pancreatic lipase. They found that this crude preparation, as used in the preliminary experiments, presented here, does not act on dissolved substrate, but is only active when absorbed at an ester-water interface, apparently because the active centre of the enzyme is only then exposed.

However, the effect of lipase on the vitelline membrane was only achieved in the hatching experiments after the release of the opercula cap. This release was dependent on several stimuli provided by the host; pH, temperature, oxygen and electrolyte concentration.

In vitro, the most suitable pH range for opercular release was in the range pH 4.1 - 7.2. Day and Powning (1949) found that the pH of the midgut of P. americana depended on the nature of the food ingested. They stated that the pH of the crop was 4.5 - 6.5 and the midgut 5.2 - 6.5. So that the range necessary to facilitate hatch could normally be expected in the host.

This was found to be true in the in vivo experiments, where opercular release occurred in the crop. As found by Anya (1966) and Rogers (1958), temperature also affected the process of hatching as shown by the fact that the optimum release of the opercular cap occurred at an effective pH at 30°C. The temperature of cockroaches is, of course, a reflection of its surroundings. P. americana is normally found in abundance in areas with a relatively high mean temperature and during the course of the experiments were kept at 25°C. The experiments showed that carbon dioxide was not necessary for stimulation of the hatching process, however, the role of oxygen was clear. It is unlikely that the lumen of the gut at the site of opercula release is anaerobic, for this region of the gut is served by visceral trachea which are in intimate association with the gut wall.

Although Rogers (1960), found that reducing agents played an important part in the hatching of eggs of Ascaris lumbricoides and T. mystax and a lesser role in the case of Ascaridia galli (Rogers, 1961) in this series of experiments such compounds had no effect on the percentage of opercula release in the oxyurid nematodes studied. These results agree with the work by Anya (1966) and to some extent with Edmonds (1966) who worked on the hatch of an acanthocephalan Moniliformis dubius in the midgut of the cockroach.

Hatching requires a change in the permeability of the vitelline membrane to permit osmotic uptake of water as well as movement of inorganic and organic substances into or out of the perivitelline space. The alteration in the osmoregulatory capacity of the membrane may be influenced by surface-active agents or the presence of various ions in the external medium. The initiation of hatch of the nematodes studied was suppressed

by NaCl above the concentration of 0.15M and by sucrose and physiological saline at concentrations greater than 0.1M. Wilson (1958), found that the normal change in permeability of the vitelline membrane was delayed in the presence of electrolytes. This delay could not be ascribed to the colligative properties of the salt used but was controlled by the mobility of its slower ion, regardless of whether this is the anion or the cation. The effect of the salt on hatching is a consequence of the penetration of the egg membranes by both components of the salt, with specific effects different ions being relatively unimportant. Apparently the salt stabilises the vitelline membrane in some manner so that it retains its ability to act as a water resistant barrier for an extended period of time, delaying the osmotic uptake of water which initiates the hatching process. Ellenby and Gilbert (1958) showed that monovalent ion chlorides reduce hatching, while divalent ion chlorides stimulate it. Effects due to osmotic differences between salines were ruled out by adjusting all salines to be osmotically equivalent to 2mM potassium chloride. Further studies indicated that hatch is affected not by the increased cation concentration but by the nature of the cation with potassium always decreasing hatch and calcium always increasing it.

Once the osmotic uptake of water occurs, the hydrostatic pressure within the eggshell increases with the result that the enclosed larva loses mobility just before hatching. The actual rupture of the vitelline membrane, releasing the larva, may be completely due to increased hydrostatic pressure or to an attack on the outer layers from within by a hatching fluid secreted by the embryo. In the case of H. diesingi it would appear that the

ability of the nematode to secrete such a hatching fluid has been lost and the parasite now relies on the secretion of a hatching fluid e.g. lipase, by the host.

It is suggested that the very low percentage hatch obtained by Lee (1959) in his *in vivo* experiments were due to the fact that the eggs administered to the insects were not in the infective stage. Any "hatch" which he obtained resulted in the release of immobile second stage larvae in the host which did not develop further. The percentage hatch obtained was equivalent to the background hatch found by other workers.

From the experiments carried out *in vivo*, the area in the host gut where each stage in the hatching process occurred could clearly be seen. The crop was the site of opercula release. It was possible that the mechanical action of the insect mouthparts during ingestion could have aided this process. However, none of the eggs found in the crop showed any sign of mutilation, the opercula cpa always having sheared clearly at the line of weakness. This showed that the biting mouthparts of the host played no part in the hatching process.

Total hatch was completed in the midgut. All of the typical conditions found in both crop and midgut of the living host have been reproduced *in vitro* and the results obtained *in vitro* closely correlate with those found *in vivo*.

P. americana is susceptible at each stage in its life history to infection with this nematode, provided that it is stimulated to produce midgut enzymes. This would normally be the case as infective eggs are ingested by the host during feeding on oothecae, food or faeces. The availability of food and the feeding cycles of the host will play an important part in the establishment of the parasite burden carried by the insect.

Once inside the insect the hatching of the egg is dependent purely on physiological stimuli provided by the host.

Section II

The physiological interactions between the larval and adult stages of H. diesingi and L. appendiculata and their host P. americana.

1. The effect of median neurosecretory cell cautery, allatectomy and removal of the corpora allata-corpora cardiaca complex from P. americana on the nematode burden and the physiological state of the host.

Introduction

A physiological relationship between oxyurid nematodes and P. americana has been demonstrated. The nematodes hatch in the fore and midgut of the insect; further development of these larvae into fourth stage juveniles and finally to mature adults takes place in the hind gut. All of these stages, are, therefore, exposed to the effects of pH, ionic concentration, compounds with possible juvenile hormone activity, as well as to various excretory products and to a flow of undigested and unabsorbed food from the foregut.

It was essential to discover which, of any, of these factors affected the development of the juveniles and the status of the adults. It was decided to attempt to solve this problem by in vivo and in vitro methods. First, it was decided to vary the physiological state of the host so upsetting the normal environment of the parasites and then look at the effects on the parasites in vivo.

It was mentioned in the general introduction that insects control many of their physiological processes by hormones. Interference with the normal functioning of the endocrine system

was carried out by a series of neurosurgical experiments during which the median neurosecretory cells (m.n.c.) of P. americana were cauterised, the corpora allata removed or the corpora allata and corpora cardiaca extirpated. The effect of these operations on the nematode burden of the host have been investigated, together with the effects of cautery and allatectomy on several of the physiological processes within the host which may directly or indirectly, influence the development of the juvenile nematodes or control the size of the parasite population.

Previous work in this field has been carried out by Gordon (1968) who described how removal of the m.n.c. of adult female Blatta orientalis resulted in a significant reduction in the number of adult male and female H. diesingi in the gut of the cockroach. He also found that removal of the cardiacum - allatum complex had the same effect, while reimplantation of the corpora allata failed to prevent this reduction in numbers (Gordon, 1968; 1970). From these observations Gordon (1970) suggested that the m.n.c.-corpus cardiacum complex of the host was essential to the well being of the parasites. However, Davey and Hominick (1973) working on H. diesingi and L. appendiculata in P. americana having obtained a similar reduction in the parasite burden after cardiatoctomy-allatectomy, suggested that the effect on the parasite burden was an indirect one, probably due to the reduction of food intake by the host following the extirpation resulting in a change in the nutritional status of the parasites.

Methods

(i) Cautery of the median neurosecretory cells of P. americana.

The insect was anaesthetised with carbon dioxide and it's

head inserted into a triangular hole cut in the base of the inner section of a match box. This was then slotted into the outer cardboard frame. The specimen was, therefore, secured inside the match box, on it's back with only it's head protruding.

A piece of cuticle was cut away from the frons and epicranium on three sides only and bent backwards. This exposed thebrain and overlying fat body. After removing the fat body, the surplus haemolymph was absorbed with tissue and the median neurosecretory cells of the brain, identified from work by Gorbman and Bern (1962) and from stained whole brain mounts prepared previously following the method of Dogra and Tandan (1964), were destroyed using a microcautery needle. The cuticle was returned to its original position and the wound sealed with Newskin (Veno Drug Company, St Helens, Lancashire). The application of a drop of hard wax had previously been used for this purpose but this would not adhere adequately to the waxy cuticle of P. americana.

This procedure was repeated for the operated controls but the mnc. were not cauterised. Control insects were anaesthetised only.

(ii) To test the effectiveness of the operation.

The morphology of the brain and the size and appearance of the ovaries were used as the main criteria for evaluation.

After autopsy, the brains were fixed in Bouin, embedded in paraffin wax and transverse sections cut serially at 7µ, then stained with P.F. (Ewen, 1962). The sections were examined for the presence or absence of neurosecretory cells.

The length of the longest terminal egg follicle was measured and every terminal oocyte was graded according to its state of resorption. When insects produced one or more oothecae during the

experiment, allowance was made in the measurement of the maximum oocyte lengths by adding onto the existing maximum oocyte length, the length of the ripe oocytes (4mm) less the mean maximum oocyte length of the penultimate oocytes, (0.93±0.01mm) multiplied by the number of oothecae produced. This gave a measurement of the maximum growth of the terminal oocyte over the two week experimental period. The percentage of ovary affected by resorption bodies was also determined for each insect.

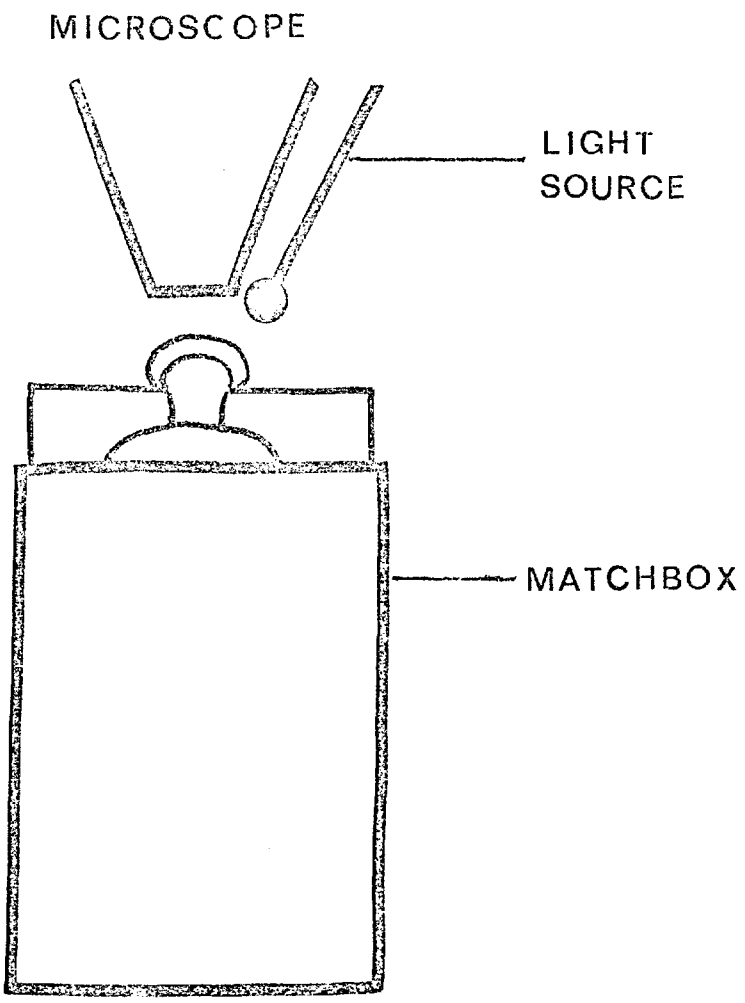
After two weeks m.n.c. cautery, P. americana appeared bloated due to an increase in the volume of the haemolymph and this factor could also be taken as a criterion for effective m.n.c. cautery. In this respect P. americana seemed to resemble Blatta orientalis, Anisotarsus, Iphita and Schistocerca in that the m.n.c. appears to produce a diuretic factor.

(iii) The method used for removal of the corpora allata and the retrocerebral complex

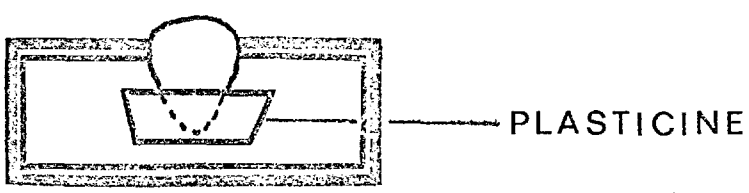
Either operation was carried out in a steady stream of carbon dioxide so that the insect was completely anaesthetised throughout the process. The insect was placed in a matchbox (Figure 9). The cervical membrane was cut dorsally between the cervical sclerites and folded back over the head. This exposed two longitudinally directed tracheal trunks which lead to the head region. These were pushed to each side so that the foregut could be traced forwards to the back of the brain. At this stage superfluous haemolymph was mopped up and the fat body from the anterior part of the neck was removed. After doing so the corpora cardiaca were visible as paired bodies lying dorsal to the foregut. From the corpora cardiaca, the nervi allati could be traced on

Fig. 9

Operational set-up for removal of corpora allata or the retrocerebral complex.



END VIEW.



either side of the foregut to the corpora allata which were small round bodies. The corpora cardiaca could be traced forwards to the nervi corporis externi (N.C.C.E.) and the nervi corporis cardiaci interni (N.C.C.I.). These nerves respectively provide a connection between the lateral and median neurosecretory cells of the pars-intercerebralis and the corpora cardiaca. Either the corpora allata alone could be exterpated, or the whole retrocerebral complex could be lifted out by the severance of both pairs of N.C.C.E. and N.C.C.I. After the operation, the cervical membrane was replaced and the wound coated with Newskin.

The operated controls were treated in exactly the same way except that the corpora allata or the retrocerebral complex was left intact.

Confirmation that the operations had been successfully performed again came from a close examination of the terminal oocytes, in particular the growth and percentage resorption of the terminal, oocytes and also the production of oothecae.

(iv) Measurement of food intake by P. americana.

Before and after the operations, control and experimental insects were kept individually in petri dishes. The insects were fed and watered daily. The faeces were collected every 48 hours and weighed. The faecal weight was taken as a measurement of food intake.

(v) Measurement of parasite burden in P. americana.

At the end of the experimental period the hind gut was removed from the insect by severing the gut at the junction of the midgut with the hindgut and at the rectum. It was placed

in 0.15M sodium chloride in a petri dish and slit longitudinally. The gut contents were shaken into the saline and any nematodes attached by their tails to the gut wall teased away from it with a mounted hair. The hind gut wall was removed and the nematodes in the petri dish counted.

(vi) Determination of haemolymph volume.

The red dye amaranth or azorubin S, the trisodium salt of 3-hydroxy-4-(4-sulpho-1-naphthylazo)2,7, naphthalene disulphonic acid (MW 604) was used.

Animals were weighed and then injected with 5µl of 0.2% amaranth using an Agla micrometer syringe and a 30G hypodermic needle inserted through an intersegmental membrane. After allowing 5 minutes for dispersal and uniform distribution, a 5µl sample was withdrawn through the neck membrane. The dyed blood was diluted with 0.5ml water and the optical density read at 520 nm using a Pye SP 600 spectrophotometer against a blank using normal undyed blood from another animal. The blood volume was calculated using the following equation:

$$V = (dg_1/g_2) - a$$

g_1 = wt. dye injected

g_2 = wt. dye recovered

d = vol. of sample

a = vol. saline injected with dye

(vii) Analysis of insect haemolymph for protein, lipid and carbohydrate content.

Ten microlitres of haemolymph were obtained by puncturing the membrane at the base of the right hind leg of the insect. This was transferred by micropipette to a tube containing 0.5ml

5% trichloroacetic acid. Another 0.5ml trichloroacetic acid was added to the tube which was centrifuged for 5 minutes. The supernatant was removed and placed in another tube. Ether: ethanol 0.5ml (6:1) was added to the residual solution which was sonicated for 2 minutes. This solution was centrifuged for 5 minutes. The supernatant was removed and added to the supernatant collected previously. The lipid extraction with 0.5ml ether: ethanol was repeated.

(a) The protein test.

The test for protein was carried out on the residual material after addition of 0.5ml 1N sodium hydroxide. The Hartree (1972) modification of the method of Lowry, Rosenbrough, Farrand Randall (1951) was used.

Ten microlitres of the test solution were made up to 1ml in distilled water, 0.9ml of solution A (see Table 11) was added and the whole was placed ina water bath at 50°C for 10 minutes then cooled to room temperature, 0.1ml of solution B (Table 11) was added and the solution left for at least 10 minutes. To this was added 3ml of solution C (Table 11) and the whole was mixed rapidly, then heated again for 10 minutes at 50°C. The tube was allowed to stand for 30 minutes and the colour read on a Unicam SP 600 spectrophotometer against a reagent blank (1ml water + 0.9ml A + 0.1ml B + 3.0ml C) at 650 nm. A series of bovine serum albumen standards were treated in the same way. A linear relationship exists within the range 0-120mg(Figure 10). Solutions were, adjusted, if necessary, to give optical densities within the linear range.

Fig. 10

Calibration curve for protein analysis. Hartree (1972) modification of the method of Lowry et al. (1951).

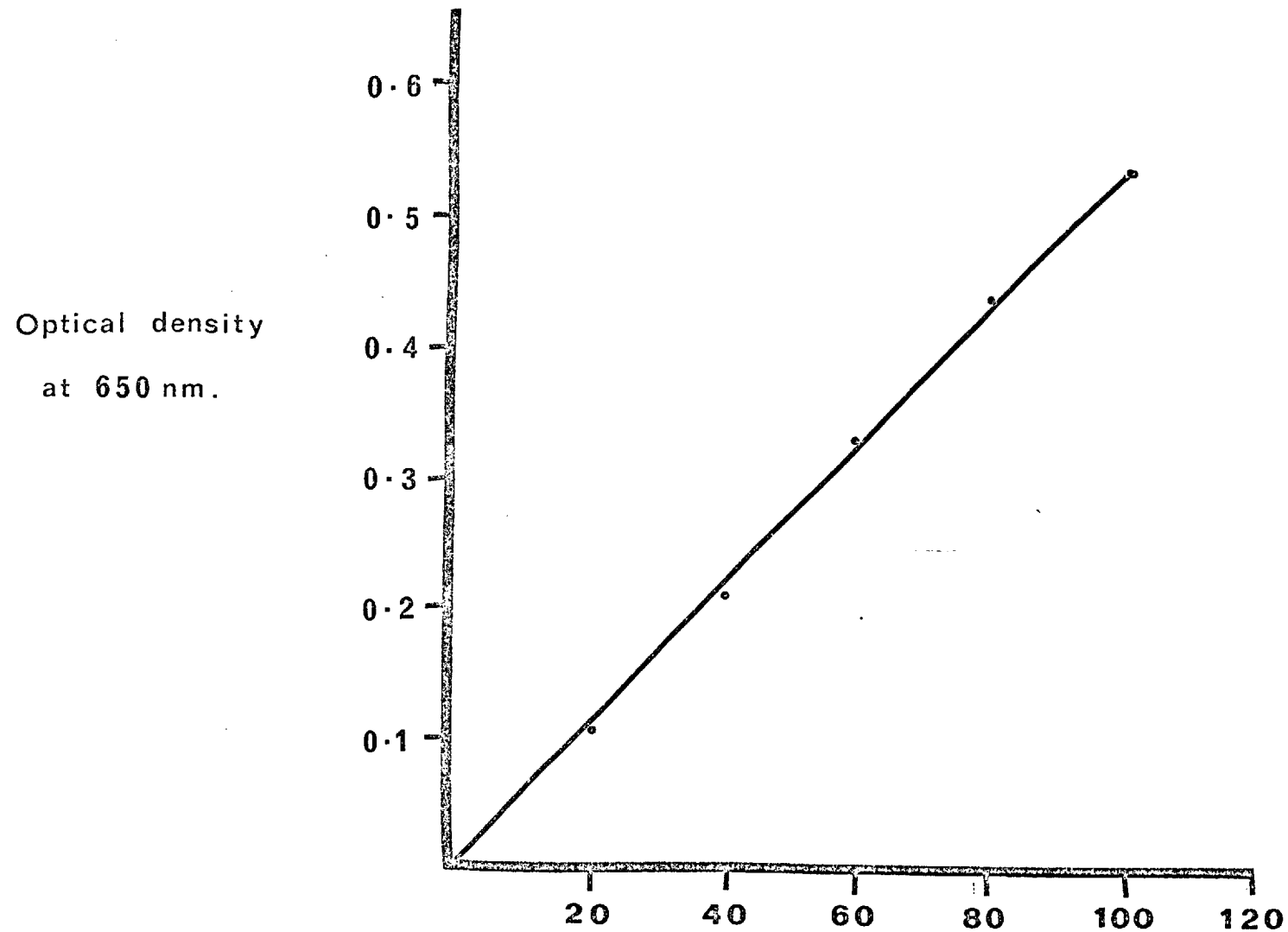

Optical density
at 650 nm.
0·6
0·5
0·4
0·3
0·2
0·1
20
40
60
80
100
120
ug Bovine serum albumin.

Table 11

Solutions used in Hartree modification

Solution A. Potassium sodium tartrate 2gm and 100gm sodium carbonate are dissolved in 500ml in sodium hydroxide and diluted with water to 1 litre.

Solution B Potassium sodium tartrate 2gm and 1gm hydrated copper sulphate are dissolved in 90ml water and 10ml IN sodium hydroxide is added.

Solution C One volume Folin-Ciocalteu reagent is diluted with 15 vol water.

(b) The carbohydrate test.

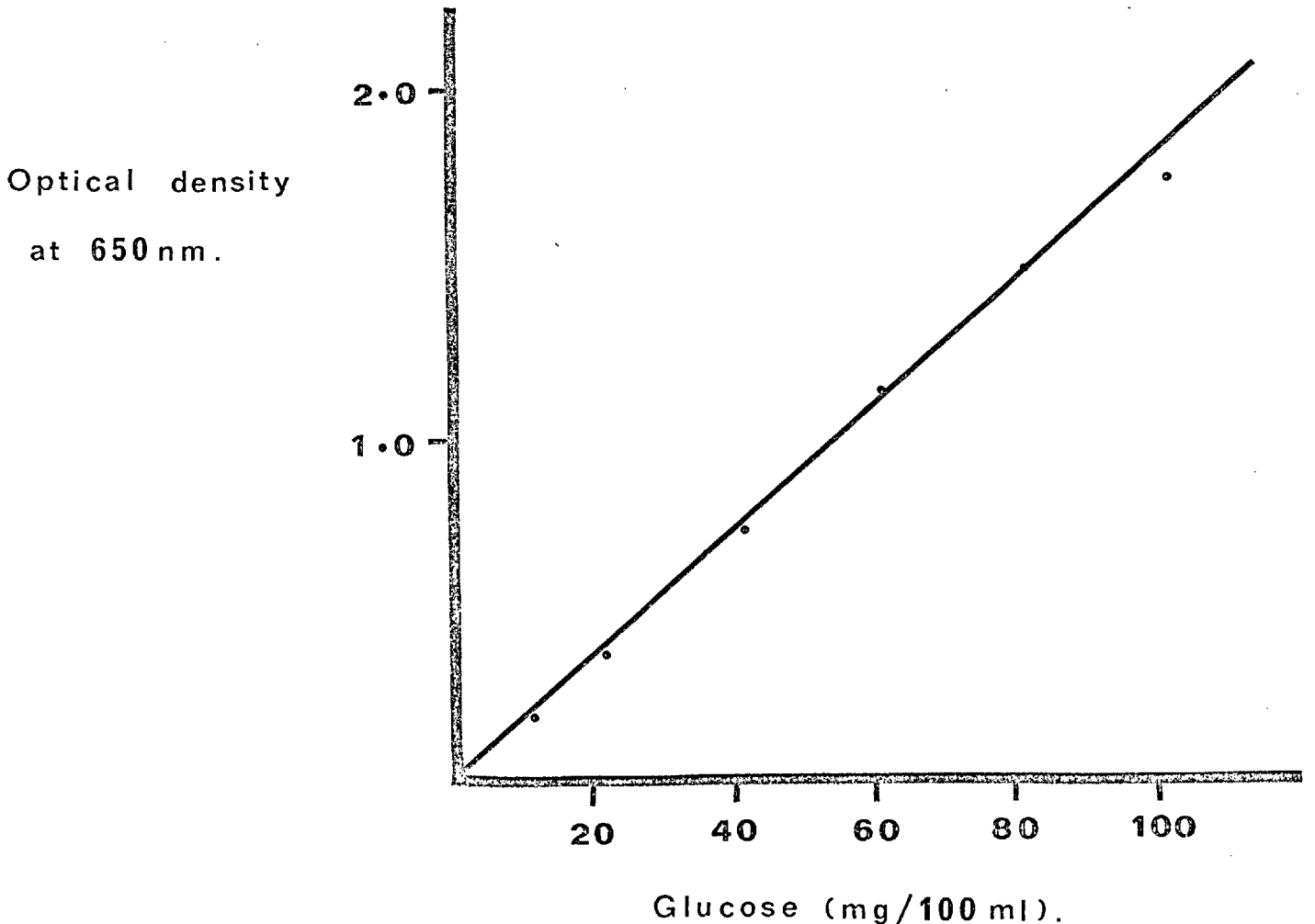
To samples from the lower phase were added 5ml of anthrone reagent (0.05% anthrone, 1% thiurea in 72% sulphuric acid). The whole was heated at 100°C for 12 minutes, then cooled in iced water and the colour read against a suitable reagent blank using a Unicam SP600 spectrophotometer at 650nm. The optical densities of a series of standard soloutions of glucose were used to quantify the anthrone positive material in the haemolymph (figure 11).

(c) The lipid test.

Ten microlitre samples were taken and put into 0.5ml concentrated sulphuric acid and heated at 100°C for 12 minutes. After cooling in iced water 50 ml were added to 0.5ml of vanillin reagent (Boehringer Biochemica Total Lipid Test). The colour developed was read after 30 minutes and before 1 hour at 546nm. A 5ml Boehringer test standard lipid (1gm% cholesterol) was included in each batch.

Fig. 11

Calibration curve for anthrone test.


Optical density
at 650 nm.
2·0
1·0
20
40
60
80
100
Glucose (mg/100 ml).

(viii) Method of measuring the total midgut protease activity.

The method used was that described by Charney and Tomarelli (1947) which was employed successfully by Day and Powning (1949), Thomsen and Möller (1959: 1959a: 1963) and Gordon (1968). This method essentially depends upon the breakdown of a protein derivative (sulphamilamide azocasein) into coloured components, which are soluble in trichloroacetic acid and which can be determined spectrophotometrically.

The azocasein substrate was prepared according to the method of Charney and Tomarelli (1947) with the adaptation by Gordon (1968) in that the solid azocasein was dissolved in 0.05N Caustic soda instead of 1% sodium bicarbonate. In this way a 1% solution of azocasein was prepared. Each determination was carried out as follows:-

0.5ml of 0.2M phosphate buffer pH 7.5 was added to 0.5ml of 1% azocasein. The pH was chosen as it was shown by Wigglesworth (1928; 1961) to be the optimum for midgut proteinases in P. americana. The azocasein/phosphate buffer solution was warmed to 37°C in a water bath. The complete midgut and contents were homogenised in a glass homogeniser with 1ml of phosphate buffer and the homogenate was added to the substrate. The midgut homogenate/azocasein suspension was then incubated for 1 hour at 37°C. For each set of determinations, a blank was prepared by adding 1.5ml of phosphate buffer to 0.5ml of azocasein substrate solution. The blank was incubated. After 1 hour the reaction was stopped by the addition of 2ml of 5% trichloroacetic acid to each sample and blank. Ten minutes later, the protein precipitate of each solution was filtered off. To 2ml of the filtrate, 2ml of 0.5N caustic soda were added, the blank was treated in the same way. Each sample was then read at 440nm on a spectrophotometer

against the blank. The spectrophotometer readings of the samples were used to compare the protease activities, since these values were directly proportional to the enzyme activities.

(ix) Method of measuring the Intracellular and Extracellular midgut protease activities.

This was essentially the same method as used for the total midgut protease activity, with the following modification:-

Each midgut was slit open by a longitudinal incision and the contents washed into a glass homogenised with 1ml phosphate buffer. This suspension of midgut contents was then assayed for its proteolytic activity by the colourimetric method Charney and Tomarelli (1947) and this represented the extracellular fraction of the total midgut protease activity. The remainder of the midgut was sonicated with 1ml phosphate buffer and treated in exactly the same way. This represented the intracellular fraction of the total midgut protease activity.

(x) Method of measuring the total midgut amylase activity.

The midgut was homogenised mechnaically in a glass homogeniser with 4ml of phosphate buffer pH 5.8, Wigglesworth (1927; 1961) has demonstrated that the optimum pH for all carbohydrases of P. americana is 5.9. When totally homogenised, the homogenate was centrifuged for 10 minutes and the supernatant was used as the enzyme solution. One ml. of 1% soluble starch plus 0.5ml of phosphate buffer were warmed for 10 minutes at 37°C. This constituted the substrate and to this 0.5ml of enzyme solution were added. After 10 minutes incubation, the reaction was terminated by the addition of 0.5ml of digest to 9.5ml of "Colour reagent". The "C.R" was previously prepared by the the addition

of 9.5ml of distilled water to 0.5ml of stock solution (0.2% iodine in 2% potassium iodide. Then 2ml of coloured sample were diluted to 8ml with 6ml distilled water and the resulting solution read on a spectrophotometer at 660 nm against a distilled water blank. This constituted the sample reading. For each determination, a blank was prepared as follows:-

To 9.5ml of "C.R." were added 1ml of 1% soluble starch and 0.5ml of enzyme solution. From the resulting coloured solution, 2ml were extracted and diluted to 8ml with 6ml distilled water. This constituted the BLANK reading and was read at 660 nm on a spectrophotometer. The BLANK reading gave the reading before incubation and the SAMPLE reading gave the spectrophotometer reading after incubation. The activity of the enzyme was then expressed in arbitrary units from the formula:-

$$\frac{\text{"BLANK" reading - "SAMPLE" reading}}{\text{"BLANK" reading}}$$

(xi) Method of measuring the total midgut lipase and esterase activities.

See Section 1. 3B(xiv)

(xii) The treatment of insect haemolymph and faeces for the detection of substances with the activity of juvenile hormone.

(a) The bioassay.

A wax test bioassay using Galleria mellonella as a test animal (de Wilde, Staal, de Kort, de Loof and Baard, 1968) with the modifications of Johnson (1972) was used to measure the substances with the activity of juvenile hormone in the haemolymph and faeces of the insects.

(b) Preparation of the extract.

Ten haemolymph samples of 10µl were obtained from each experimental group and each transferred to a glass homogeniser containing 1ml ether: ethanol (6:1, v/v), then homogenised for 2 minutes. A further 2ml ether: ethanol was added to each tube, the contents of which were then poured into centrifuge tubes containing 1-2ml ether: ethanol and 2ml water. Each tube was mised thoroughly then centrifuged at 2,000 r.p.m. for 5 minutes. The upper layer was removed from each tube and the extraction repeated with the lower layer. At the end of this process the ether extracts were evaporated under nitrogen, in a water bath at room temperature, this procedure took about 10 minutes in each case. The smears remaining at the bottom of the tubes were washed out using a small amount of ether: ethanol into smaller tubes for immediate assay or to be stored at -20°C.

(c) Preparation of the extract for assay.

A clean glass slide was smeared with lanolin. A drop of ethanol was spotted onto the centre of the slide where it dried almost immediately. The extract was added dropwise into the confines of this area. When this had been completed a known weight of olive oil was mixed into the extract, together with a known weight of wax (M.pt. 56°C). The head of a pin was heated in a flame and the wax mixed into the extract on the slide.

(d) Galleria mellonella assay

Only pupae with clear brown eyespots were used i.e. those under 24 hours old. The pupae were anaesthetised in carbon dioxide for 3-5 minutes. For the assay, one mm of cuticle and its underlying epidermis was excised from the median crest on

the mesonotum of Galleria. Using a slightly warmed mounted needle, the wound was sealed with a 1mg quantity of the mixture to be assayed. Six days after the operation the cuticle of the newly moulted adults was inspected.

(xiii) Method of measuring the concentration of sodium and and potassium ions in the insect haemolymph.

A 5µl haemolymph sample was taken from the insect and mixed with 2ml concentrated sulphuric acid. The concentrations of sodium and potassium ions in each sample were determined by comparison with standard solutions of sodium chloride and potassium iodide respectively in a Perkin Elmer Atomic Absorption Spectrophotometer Model 290B using an air-acetylene flame.

(xiv) Design of the Experiment

Group 1 comprised 55 adult female insects; these were the unoperated controls.

Group 2 comprised 14 adult female insects; these were operated controls for Group 3, the m.n.c. cauterised insects.

Group 3 comprised 14 adult female insects; these were m.n.c. cauterised.

Group 4 comprised 38 adult female insects; these were operated controls for Groups 5 and 6 the allatectomised insects and those with both corpora allata and corpora cardiaca removed respectively.

Group 5 comprised 18 adult female insects; these were allatectomised.

Group 6 comprised 20 adult female insects; both the corpora allata and corpora cardiaca were removed from these insects.

All insects were isolated in separate dishes and fed daily. The cauterised insects together with their controls and operated controls were sampled after 2 weeks. The insects which were allatectomised or had both corpora allata and corpora cardiaca removed were sampled after 4 weeks.

RESULTS

(i) Evidence that the operations were carried out successfully

Examination of the sections of the pars-intercerebralis revealed that the removal of the m.n.c. was successful. The deep purple staining cells were absent from the brains of all of the m.n.c. cauterised insects but present in both control groups.

Examination of the terminal oocytes and estimations of the fecundity of the insects afforded further confirmation that the operations were successfully performed. Of the 14 operated insects, 3 each produced an ootheca. However, a total of 12 oothecae were produced by the 14 operated controls and 13 oothecae by the 14 unoperated controls.

Microscopic examinations of the terminal oocytes showed resorption bodies to be present in all stages from R.B. 1-6 (see Lusis, 1963) in the ovaries of all three groups of insects but were more commonly resorbed at the R.B. 4-6 level. Measurements of the maximum length of the terminal oocytes and the percentage of ovary bearing resorption bodies are summarised in Table 12. An analysis of the results shows that the mean maximum oocyte length of the m.n.c. cauterised insects was significantly less than that of the operated controls and the unoperated controls, the two latter groups did not vary significantly from each other. However, it is apparent that removal of the m.n.c. did not completely suppress oocyte development. Also, the percentage of the resorbed ovary of the m.n.c. cauterised insects was significantly greater than the corresponding value for the operated controls and the unoperated controls. There was no significant difference between the two control groups.

All of the allatectomised insects survived the 4 week experimental period, of these 2 insects produced oothecae. The 18 unoperated control insects produced 16 oothecae and the same number of surviving operated control insects produced 15 oothecae. Table 13 shows the mean results for the percentage of oocytes affected by the resorption bodies and the maximum length of the terminal oocyte. As with the cauterised insects, removal of the

corpora allata significantly increased the percentage of oocyte resorption compared with the control groups, but there was no significant difference between the latter. Similarly, the mean maximum terminal oocyte length in allatectomised insects was significantly less than either of the control groups, which again did not vary significantly from each other.

No oothecae were produced by the 20 insects which had their corpora allata and corpora cardiaca removed and which survived the 4 week experimental period. However, 15 and 17 oothecae were produced by the operated and unoperated control insects respectively. All of the control insects survived the experimental period. Table 14 shows the mean results for the percentage of oocyte affected by resorption bodies and the maximum length of the terminal oocytes. There was a significant increase in the percentage of oocyte resorption in the experimental insects, compared with the operated controls and unoperated controls. There was no significant difference between the control groups. The mean maximum terminal oocyte length of the insects which had had their corpora allata and corpora cardiaca removed was significantly less than either the operated or unoperated controls.

Table 12. Oocyte measurements for control and 2 week m.n.c. cauterised cockroaches.

	No of insects	% Resorption	lmax.(mm)
Controls	14	26.47±5.8	5.14±0.34
Operated controls	14	26.40±5.6	5.01±0.31
Operated	14	84.29±6.3	2.32±0.07

Comparison of means

% Resorption

Operated - Operated controls	t = 6.810	P>0.001
Operated - Controls	t = 6.707	P>0.001
Operated Controls - Controls	Not significant	

lmax (mm)

Operated - Operated Controls	t = 8.251	P>0.001
Operated - controls	t = 8.022	P>0.001
Operated Controls - Controls	Not significant.	

Table 13. Oocyte measurements for control and 4 week allatectromised insects.

	No of insects	% Resorption	lmax.(mm)
Controls	18	24.78±5.3	4.91±0.29
Operated controls	18	24.70±5.3	5.02±0.28
Operated	18	84.01±5.4	2.46±0.16

Comparison of means

% Resorption

Operated - Operated controls	t = 7.771	P>0.001
Operated - Controls	t = 7.750	P>0.001
Operated controls - controls	Not significant	

lmax (mm)

Operated - Operated controls	t = 7.855	P>0.001
Operated - Controls	t = 3.207	P>0.01<0.001
Operated controls - Controls	Not significant	

Table 14. Oocyte measurements for control and operated insects 4 weeksafter corpora allata and corpora cardiaca removed from cockroaches.

	No. of insects	% Resorption	lMax.(mm)
Controls	20	25.99±4.54	4.73±0.22
Operated Controls	20	25.87±4.52	4.91±012.
Operated	20	84.31±5.63	2.12±0.09

Comparison of means

% Resorption

Operated - Operated Coltrols	t = 8.096	P>0.001
Operated - Controls	t = 8.066	P>0.001
Operated Controls- Controls	Not significant	

lmax.(mm)

Operated - Operated Controls	t = 12.4	P>0.001
Operated - Controls	t = 11.347	P>0.001
Operated Controls - Controls	Not significant	

(ii) The effect of removing various endocrine glands on the parasite burden of the insects.

All of the operated insects, unoperated and operated controls survived the experimental periods.

The effects of m.n.c. cautery on the adult parasite burden of the cockroaches can be seen in Tables 15 and 16. It is evident that cautery of the m.n.c. resulted in a significant decrease in the total numbers of both H. diesingi and L. appendiculata. The mean total burden of adults of each parasite in the cauterised insects was significantly less than in the operated controls and in the unoperated controls. The two control groups did not vary significantly from each other with regard to their respective levels of infestation of total adult H. diesingi and L. appendiculata. When the numbers of adult male and female nematodes in the operated insects are compared separately with the numbers in the control groups, it is found that although the numbers of adult female nematodes of both species were significantly reduced, the total adult male populations were lost completely from the operated insects.

The effects of m.n.c. cautery on the levels of third and fourth stage larvae in the insects can be seen in Tables 17 and 18. The cauteries resulted in the loss of all third and fourth stage larval L. appendiculata and fourth stage H. diesingi from the operated insects, while a number of third stage larvae of H. diesingi remained. There was no significant difference in the numbers of third and fourth stage larvae of either species between the control groups.

The effects of allatectomy on the total adult parasite burden are shown in Tables 19 and 20. It is obvious that this operation has similar effects on the total adult parasite burden

of both species as those caused by m.n.c. cautery. As a result of this operation there is a significant decrease in the total number of adult H. diesingi and L. appendiculata, as compared with either operated or unoperated control groups, while the control groups did not vary significantly from each other. However, when the numbers of adult males and females of both species are considered separately, it can be seen in Table 20 that although allatectomy significantly reduced the numbers of both male and female H. diesingi and female L. appendiculata, the numbers of adult male L. appendiculata bordered on the significance level. The numbers of third and fourth stage larvae of each species was reduced significantly as a result of this operation (Tables 21 and 22).

Extirpation of both corpora allata and corpora cardiaca again resulted in a significant decrease in the total adult populations of both nematodes as shown in Tables 23 and 24. Although the female parasite burden in each case was markedly decreased, the number of adult male H. diesingi in the operated group compared with the operated controls bordered on the significance level. The adult male population of L. appendiculata was significantly reduced after this operation. A further result was a decrease in the numbers of third and fourth stage larvae of both H. diesingi and L. appendiculata shown in Tables 25 and 26. The number of larvae in the control groups did not vary significantly from each other.

Table 15. Numbers of adult H. diesingi in the hind gut of control and 2 week m.n.c. cauterised cockroaches.

	No. of insects	No. adult female parasites	No. adult male parasites	Total no. of adult parasites
Controls	14	8.42±1.2	1.14±0.39	9.57±1.9
Operated controls	14	7.21±1.1	0.85±0.23	8.07±0.98
Operated	14	1.07±0.39	0.0	1.07±0.39

Comparison of means

Number of adult female parasites

Operated - operated controls	t = 4.834	P>0.001
Operated - controls	t = 4.581	P>0.001
Operated controls - coltrols	Not significant	

Number of adult male parasites

Operated - operated controls	t = 3.632	P>0.01<0.001
Operated - controls	t = 3.153	P>0.01<0.001
Operated controls - controls	Not significant	

Total number of adult parasites

Operated - operated controls	t = 4.993	P>0.001
Operated - controls	t = 4.375	P>0.001
Operated controls - controls	Not significant	

Table 16. Numbers of adult L. appendiculata in the hind gut of control and 2 week m.n.c. cauterised cockroaches.

	No. of insects	No. adult female parasites	No. adult male parasites	Total No. of adult parasites
Controls	14	2.90±0.60	0.75±0.10	3.65±0.80
Operated controls	14	2.66±0.30	0.91±0.09	3.55±0.70
Operated	14	0.80±0.10	0.0	0.80±0.10

Comparison of means

Number of adult female parasites

Operated - operated controls	t = 5.942	P>0.001
Operated - controls	t = 3.494	P>0.01<0.001
Operated controls - controls	Not significant	

Number of adult male parasites

Operated - operated controls	t = 10.224	P>0.001
Operated - controls	t = 7.575	P>0.001
Operated controls - controls	Not significant	

Total number of adult parasites

Operated - operated controls	t = 3.90	P>0.001
Operated - controls	t = 3.54	P>0.01<0.001
Operated controls - controls	Not significant	

Table 17. Number of third and fourth stage larval H. diesingi in the hindgut of control and 2 week m.n.c. cauterised insects.

	No. of insects	No. of third stage larvae	No. of fourth stage larvae
Controls	14	4.85 ± 0.73	7.0 ± 1.28
Operated controls	14	5.5 ± 0.84	7.42 ± 0.84
Operated	14	7.44 ± 0.14	0.0

Comparison of means

Number of third stage larvae

Operated - operated controls	$t = 3.309$ $P>0.05<0.02$
Operated - controls	$t = 3.290$ $P>0.01<0.001$
Operated controls - controls	Not significant

Number of fourth stage larvae

Operated - operated controls	$t = 8.939$ $P>0.001$
Operated - controls	$t = 5.520$ $P>0.001$
Operated controls - controls	Not significant

Table 18. Number of third and fourth stage larval L. appendiculata in the hindgut of control and 2 week m.n.c. cauterised insects.

	No. of insects	No. of third stage larvae	No. of fourth stage larvae.
Controls	14	$2.16^{+}0.63$	$2.85^{+}0.40$
Operated controls	14	$1.87^{+}0.56$	$2.89^{+}0.30$
Operated	14	0.0	0.0

Comparison of means

Number of third stage larvae

Operated - operated controls	t = 3.4	P>0.01<0.001
Operated - controls	t = 3.434	P>0.01<0.001
Operated controls - controls	Not significant	

Number of fourth stage larvae

Operated - operated controls	t = 9.697	P>0.001
Operated - controls	t = 7.1428	P>0.001
Operated controls - controls	Not significant	

Table 19. Numbers of adult H. diesingi in the hind gut of control and 4 week allatectomised cockroaches.

	No. of insects	No. adult female parasites	No. adult male parasites	Total No. of adult parasites
Controls	18	7.11±0.91	1.11±0.25	8.21±1.10
Operated controls	18	6.77±0.88	1.07±0.21	7.61±1.05
Operated	18	0.83±0.23	0.55-0.05	0.88±0.22

Comparison of means

Number of adult female parasites

Operated - operated controls	t = 6.726	P>0.001
Operated - controls	t = 6.678	P>0.001
Operated controls- controls	Not significant	

Number of adult male parasites

Operated - operated controls	t = 4.0636	P>0.001
Operated - controls	t = 4.222	P>0.001
Operated - controls - controls	Not significant	

Total number of adult parasites

Operated - operated controls	t = 6.2892	P>0.001
Operated - controls	t = 6.544	P>0.001
Operated controls- controls	Not significant	

Table 20. Numbers of adult L. appendiculata in the ~~third~~ hind gut of control and 4 week allatectomised cockroaches.

	No. of insects	No. adult female parasites	No. adult male parasites	Total No. of adult parasites
Controls	18	3.51±0.50	0.82±0.20	4.23±0.70
Operated controls	18	2.86±0.40	0.78±0.41	3.89±0.60
Operated	18	1.10±0.20	0.0	1.10±0.20

Comparison of means

Number of adult female parasites

Operated - operated controls	t = 4.0487	P>0.001
Operated - controls	t = 4.496	P>0.001
Operated controls - controls	Not significant	

Number of adult male parasites

Operated - operated controls	t = 1.930	P≯0.1<0.05
Operated - controls	t = 4.1414	P>0.001
Operated controls - controls	Not significant	

Total number of adult parasites

Operated - operated controls	t = 4.456	P>0.001
Operated - controls	t = 4.3532	P>0.001
Operated controls - controls	Not significant	

Table 21. Number of third and fourth stage larval H. diesingi in the hindgut of control and 4 week allatectomised insects.

	No. of insects	No. of third stage larvae	No. of fourth stage larvae
Controls	18	$1.11^{+}0.33$	$8.38^{+}0.91$
Operated controls	18	$1.16^{+}0.31$	$6.66^{+}0.78$
Operated	18	0.0	0.0

Comparison of means

Number of third stage larvae		
Operated - operated controls	t = 3.754	P>0.001
Operated - controls	t = 3.373	P>0.01<0.001
Operated controls - controls	Not significant	
Number of fourth stage larvae		
Operated - operated controls	t = 8.560	P>0.001
Operated - controls	t = 9.218	P>0.001
Operated controls - controls	Not significant	

Table 22. Number of third and fourth stage larval L. appendiculata in the hindgut of control and 4 week allatectomised insects.

	No. of insects	No. of third stage larvae	No. of fourth stage larvae
Controls	18	0.88 ± 0.20	3.11 ± 0.13
Operated controls	18	1.01 ± 0.30	2.89 ± 0.11
Operated	18	0.0	0.0

Comparison of means

Number of third stage larvae

Operated - operated controls	t = 3.377	$P > 0.01 < 0.001$
Operated - controls	t = 4.422	$P > 0.001$
Operated controls - controls	Not significant	

Number of fourth stage larvae

Operated - operated controls		$P > 0.001$
Operated - controls		$P > 0.001$
Operated controls - controls	Not significant	

Table 23. Numbers of adult H. diesingi in the hindgut of control insects and those 4 weeks after removal of both corpora allata and corpora cardiaca.

	No. of insects	No. adult female parasites	No. adult male parasites	Total no. of adult parasites
Controls	20	6.2±0.68	0.85±0.23	7.83±0.80
Operated controls	20	6.0±0.73	0.70±0.22	6.72±0.86
Operated	20	1.0±0.22	0.20±0.08	1.2±0.77

Comparison of means

Number of adult female parasites

Operated - operated controls	t = 3.946	P>0.001
Operated - controls	t = 4.1969	P>0.001
Operated controls- controls	Not significant	

Number of adult male parasites

Operated - operated controls	t = 2.1459	P>0.1<0.05
Operated - controls	t = 6.9	P>0.001
Operated controls - controls	Not significant	

Total number of adult parasites

Operated - operated controls	t = 4.8167	P>0.001
Operated - controls	t = 6.027	P>0.001
Operated controls - controls	Not significant	

Table 24. Numbers of adult *P. appendiculata* in the hindgut of control insects and those 4 weeks after removal of both corpora allata and corpora cardiaca.

	No. of insects	No. female parasites	No. male parasites	Total No. of adult parasites
Controls	20	3.10±0.60	0.89±0.20	3.76±0.04
Operated controls	20	2.93±0.30	0.91±0.08	3.80±0.04
Operated	20	0.81±0.10	0.0	0.81±0.10

Comparison of means

Number of adult female parasites

Operated - operated controls	t = 7.090	P>0.001
Operated - controls	t = 3.829	P>0.01<0.001
Operated controls - controls	Not significant	

Number of adult male parasites

Operated - operated controls	t = 11.518	P>0.001
Operated - controls	t = 4.472	P>0.001
Operated controls - controls	Not significant	

Total number of adult parasites

Operated - operated controls	t = 27.943	P>0.001
Operated - controls	t = 27.570	P>0.001
Operated controls - controls	Not significant	

Table 25. Number of third and fourth stage larval H. diesingi in the hindgut of control insects and those 4 weeks after removal of both corpora allata and corpora cardiaca.

	No. of insects	No. of third stage larvae	No. of fourth stage larvae
Controls	20	0.80 ± 0.24	7.40 ± 0.80
Operated controls	20	0.85 ± 0.24	6.00 ± 0.83
Operated	20	0.0	0.0

Comparison of means

Number of third stage larvae

Operated - operated controls	$t = 3.5564$	$P > 0.001$
Operated - controls	$t = 3.347$	$P > 0.01 < 0.001$
Operated controls - controls	Not significant	

Number of fourth stage larvae

Operated - operated controls	$t = 7.2315$	$P > 0.001$
Operated - controls	$t = 9.251$	$P > 0.001$
Operated controls - controls	Not significant	

Table 26. Number of third and fourth stage larval L. appendiculata in the hindgut of control insects and those 4 weeks after removal of both corpora allata and corpora cardiaca.

	No. of insects	No. of third stage larvae	No. of fourth stage larvae
Controls	20	0.86±0.20	2.98±0.21
Operated controls	20	0.88±0.30	2.66±0.1
Operated	20	0.0	0.0

Comparison of means

Number of third stage larvae		
Operated - operated controls	t = 2.9431	P>0.02<0.01
Operated - controls	t = 4.5263	P>0.001
Operated controls - controls	Not significant	
Number of fourth stage larvae		
Operated - operated controls	t = 26.86	P>0.001
Operated - controls	t = 14.197	P>0.001
Operated controls - controls	Not significant	

(iii) The effects of 2 week m.n.c. cautery and 4 week allatectomy on the enzyme activity in P. americana.

Total midgut protease activity.

It can be seen from Table 27 that there was no effect of m.n.c. cautery on the total midgut protease activity. Table 28 shows that the midgut protease activity is also unchanged 4 weeks after allactectomy. Comparison between the values for operated insects and either of the control groups showed no significant difference, neither was there a significant difference between control groups.

Intracellular and extracellular midgut protease activity.

An analysis of the data (see Table 29 and 30) showed that there was no effect of either cautery or allatectomy on the intracellular or the extracellular protease activities.

Total midgut amylase activities.

The results set out in Tables 31 and 32 demonstrate that cautery of the m.n.c. had no effect on the activity of amylase in the insects' midgut. However, although there was no difference between the activity of this enzyme in either control groups 4 weeks after allatectomy, the level of activity in the operated insects compared with that in the either control group only borders on the level of significance.

Total midgut lipase and esterase activities.

The summary of the results in Tables 33 and 34 show that neither cautery or allatectomy interfere in any way with the activities of lipase or esterase.

Table 27. The effect of m.n.c. cautery on the total midgut protease activity.

	No. of Insects	Spectrophotometer reading.
Controls	14	0.101±0.019
Operated controls	14	0.102±0.022
Operated	14	0.110±0.021

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Table 28. The effect of allatectomy on the total midgut protease activity.

	No. of insects	Spectrophotometer reading
Controls	18	0.103±0.010
Operated controls	18	0.111±0.013
Operated	18	0.099±0.003

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Table 29. The effect of m.n.c. cautery on the intracellular and extracellular midgut protease activities.

Intracellular midgut protease activities.

	No. of insects	Spectrophotometer reading.
Controls	14	0.133±0.009
Operated controls	14	0.133±0.014
Operated	14	0.123±0.012

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Extracellular midgut protease activities.

	No. of insects	Spectrophotometer reading
Controls	14	0.073±0.005
Operated controls	14	0.073±0.008
Operated	14	0.074±0.016

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Table 30. The effect of allatectomy on the intracellular and extracellular midgut protease activities.

Intracellular protease activities.

	No. of insects	Spectrophotometer reading
Controls	18	0.126±0.012
Operated controls	18	0.127±0.012
Operated	18	0.113±0.003

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Extracellular protease activities.

	No. of insects	Spectrophotometer reading
Controls	18	0.060±0.007
Operated controls	18	0.062±0.007
Operated	18	0.067±0.004

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Table 31. The effect of m.n.c. cautery on the midgut amylase activity.

	Number of insects	"Activity" values
Controls	14	0.736±0.008
Operated controls	14	0.737±0.005
Operated	14	0.742±0.022

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Table 32. The effect of allatectomy on the midgut amylase activity.

	Number of insects	"Activity" values
Controls	18	0.728±0.020
Operated controls	18	0.728±0.017
Operated	18	0.680±0.010

Comparison of means

Operated - operated controls	$t = 2.264$ $P>0.05<0.02$
Operated - controls	$t = 2.0$ $P>0.1<0.05$
Operated controls - controls	Not significant

Table 33. The effect of m.n.c. cautery on the total midgut lipase and esterase activities.

	No. of insects	E_2 (esterase activity)	$E_2 - E_1$ (lipase activity)
Controls	14	0.163±0.074	0.101±0.015
Operated controls	14	0.155±0.037	0.096±0.008
Operated	14	0.155±0.026	0.097±0.012

Comparison of means

Esterase activity

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Lipase activity

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Table 34. The effect of allatectomy on the total midgut lipase and esterase activities.

	No. of insects	E_2 (esterase activity)	$E_2 - E_1$ (lipase activity)
Controls	18	0.099 ± 0.014	0.060 ± 0.010
Operated controls	18	0.098 ± 0.011	0.058 ± 0.010
Operated	18	0.103 ± 0.012	0.059 ± 0.020

Comparison of means

Esterase activity

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Lipase activity

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

(iv) The effect of m.n.c. cautery and allatectomy on the faecal output of the insects.

The faeces produced by each insect were removed from the containers every 48 hours, dried and weighed. The weighings were taken before and after the operations.

The results are tabulated in Tables 35 and 36. It can be seen that there is no difference between the weight of faeces produced by m.n.c. operated insects and those in either of the control groups. There was no difference in the weight of faeces produced by either of the control groups over the 2 week experimental period. However, it was noted that the faeces produced by the operated insects were more dry and granular than those produced by the control insects 2 days after the operation had been performed until the end of the experiment.

The removal of the corpora allata from insects had no effect on the faecal output and presumably on the food intake by the insects compared with either control group.

Table 35. The effect of m.n.c. cautery on the food intake (faecal output) of the cockroaches.

	Operated controls		Controls		Operated	
		Faecal output/insect/ 48 hours (mg)		Faecal output/insect/ 48 hours (mg)		Faecal output/insect 48 hours (mg)
Day of Experiment	No. of Insects		No. of insects		No. of insects	
2	14	11.0	14	10.2	14	10.8
[4]		10.6		10.6		9.2
6		10.8		9.7		11.9
8		12.2		7.2		6.3
10		11.7		11.6		8.8
12		10.5		12.1		10.3
14		11.6		11.9		11.4
16		14.8		13.2		10.2
18		15.1		10.6		9.9

[ ] = day of operation.

Table 36. The effect of allatectomy on the food intake (faecal output) of the cockroaches.

Day of experiment	No. of insects	Operated controls: Faecal output/insect/48 hours (mg)	Controls: Faecal output/insect/48 hours (mg)	Operated: Faecal output/insect 48 hours (mg)
2	20	9.8	10.1	11.7
4		6.6	9.7	12.4
6		7.3	10.4	13.5
8		8.9	10.7	11.2
10		10.7	10.6	9.7
12		11.2	11.7	8.9
14		10.9	12.6	6.8
16		11.7	13.5	9.8
18		12.4	14.1	8.9
20		13.1	11.2	11.7
22		10.3	9.7	10.3

Table 36 (continued)

Day of experiment	Operated controls Faecal output/insect/ 48 hours (mg)	Controls Faecal output/insect/ 48 hours (mg)	Operated Faecal output/insect 48 hours (mg)
24	10.1	6.8	10.9
26	10.9	8.9	11.7
28	9.6	9.5	10.8
30	9.7	11.1	7.3

☐ = day of operation

(v) The effects of m.n.c. cautery and allatectomy on the haemolymph volume of the insects.

The m.n.c. operated insects were obviously "bloated" in appearance 2 days after cautery had been performed. At the end of the 2 week experimental period these insects were so swollen that their abdomens touched the base of their containers and they had difficulty in walking.

The effect of m.n.c. cautery on the haemolymph volume of P. americana is summarised in Table 37. It can be seen that there is a significant difference in the haemolymph volume of the operated insects compared with that of unoperated control insects. The haemolymph volumes of the control insects were not significantly different from each other.

In contrast, the removal of the corpora allata from the experimental insects did not produce the same effect as cautery of the m.n.c. The haemolymph volume of allatectomised insects did not vary significantly from insects which had been "sham" operated or the unoperated controls (Table 38), nor did the mean value for the operated group differ significantly from those obtained for operated or unoperated control insects for the m.n.c. cauterised insects.

Table 37. Effect of 2 week m.n.c. cautery on haemolymph volume (µl)

	No. of insects	Haemolymph volume (µl)
Controls	14	160.28±3.46
Operated controls	14	163.50±3.00
Operated	12	429.83±6.98

Comparison of means

Operated - operated controls	t = 35.020	P>0.001
Operated - controls	t = 34.570	P>0.001
Operated controls - controls	Not significant	

Table 38. Effect of 4 week allatectomy on haemolymph volume (µl)

	No. of insects	Haemolymph volume (µl)
Controls	16	160.06±3.70
Operated controls	18	160.11±2.85
Operated	16	155.05±3.00

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

(vi) The effects of m.n.c. cautery and allatectomy on the concentrations of sodium and potassium in the haemolymph of P. americana.

Concomitant with the increase in the haemolymph volume of cauterised insects there is a large increase in the concentration of sodium ions in the haemolymph. This is significantly different from the concentration found in both control groups (Table 39). However, the level of sodium ions in the blood of allatectomised groups is unaffected by the operation as shown in Table 40.

Cautery also causes a significant rise in the level of potassium ions in the haemolymph of operated insects, but this is not of the same order as the increase in sodium ions (Table 39). Allatectomy did not significantly change the level of potassium ions in the haemolymph of the operated insects, as compared with the level in either of the control groups (Table 40.)

Table 39. Effect of 2 week m.n.c. cautery on the concentration of sodium and potassium ions in the haemolymph of P. americana.

		Concentration of ions (mM/litre)	
	No. of insects	Sodium	Potassium
Controls	10 (12)	2.25 ± 0.40	0.73 ± 0.09
Operated controls	10 (12)	2.24 ± 0.47	0.69 ± 0.10
Operated	10 (12)	19.23 ± 0.62	1.37 ± 0.14
	Comparison of means		
Operated - operated controls		$t = 21.533$ $P > 0.001$	$t = 3803$ $P > 0.001$
Operated - controls		$t = 22.688$ $P > 0.001$	$t = 3.659$ $P > 0.01 < 0.001$
Operated controls - controls		Not significant	Not significant

Table 40 Effect of 4 week allatectomy on the concentration of sodium and potassium ions in the haemolymph of P. americana.

		Concentration of ions (mM/litre)	
	No. of insects	Sodium	Potassium
Controls	14 (14)	1.60±0.27	0.73±0.08
Operated controls	14 (14)	1.58±0.31	0.77±0.10
Operated	14 (14)	1.74±0.28	0.83±0.12
Comparison of means			
Operated - operated controls		Not significant	Not significant
Operated - controls		Not significant	Not significant
Operated controls - controls		Not significant	Not significant

(Brackets denote number of insects used for potassium ion determinations).

(vii) The effects of m.n.c. cautery and allatectomy on the carbohydrate, protein and lipid levels in the haemolymph of P. americana.

After 2 week m.n.c. cautery, there was a significant change in the concentration of blood carbohydrates. Table 41 shows that there is a significantly greater concentration of carbohydrate in the haemolymph of 2 week m.n.c. cauterised insects than in either of the control groups. The control and operated control groups did not vary significantly from each other with regard to their respective concentrations of haemolymph carbohydrates. In contrast, cautery caused a decrease in the protein concentration in the haemolymph of operated insects as compared with either of the control groups (Table 42), while there was no significant change in the concentration of lipids in the haemolymph of operated insects (Table 43).

Both the carbohydrate and protein levels in the haemolymph of allatectomised insects were unchanged as shown in Tables 44 and 45 respectively. However, there was a significant increase in the level of lipid in the haemolymph of allatectomised insects as compared with either of the control groups as can be seen in Table 46.

Table 41. Effect of 2 week m.n.c. cautery on the concentration of haemolymph carbohydrates.

	No. of insects	Concentration of carbohydrates (gm.glucose equiv/100ml blood)
Controls	14	1.27±0.16
Operated controls	14	1.08±0.26
Operated	14	2.13±0.14

Comparison of means

Operated - operated controls	t = 3.025	P>0.01<0.001
Operated - controls	t = 3.675	P>0.01<0.001
Operated controls - controls	Not significant	

Table 42. Effect of m.n.c. cautery on the concentration of haemolymph protein.

	No. of Insects	Concentration of protein (gm.equiv. bovine serumalbumen/ 100ml blood)
Controls	14	0.25±0.03
Operated controls	14	0.25±0.03
Operated	14	0.13±0.03

Comparison of means

Operated - operated controls	t = 2.371	P>0.05<0.02
Operated - controls	t = 2.558	P>0.02<0.01
Operated controls - controls	Not significant	

Table 43. Effect of 2 week m.n.c. cautery on the concentration of haemolymph lipids.

	No. of insects	Concentration of lipids (gm % cholesterol)
Controls	14	3.32±0.12
Operated controls	14	3.10±0.10
Operated	14	3.11±0.13

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Table 44. Effect of 4 week allatectomy on the concentration of haemolymph carbohydrates.

	No. of insects	Concentration of carbohydrates (gm glucose equiv/100ml blood)
Controls	16	1.14±0.12
Operated controls	16	1.16±0.15
Operated	16	1.15±0.14

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Opeated controls - controls	Not significant

Table 45. Effect of 4 week allatectomy on the haemolymph protein levels.

	No. of insects	Concentration of protein (gm equiv bovine serum albumen/100ml blood).
Controls	18	0.26±0.04
Operated controls	18	0.27±0.03
Operated	18	0.26±0.03

Comparison of means

Operated - operated controls	Not significant
Operated - controls	Not significant
Operated controls - controls	Not significant

Table 46. Effect of 4 week allatectomy on the concentration of haemolymph lipids.

	No. of insects	Concentration of lipids (gm. % cholesterol)
Controls	16	2.9±0.14
Operated controls	16	3.0±0.06
Operated	16	5.2±0.02

Comparison of means

Operated - operated controls	t = 38.128 P>0.001
Operated - controls	t = 17.424 P>0.001
Operated controls - controls	Not significant

(viii) The effects of m.n.c. cautery and allatectomy on the presence or absence of substances with the activity of Juvenile hormone in the haemolymph and faeces of P. americana.

The results from the bioassays were divided into three categories and given appropriate scores according to Johnson (1972).

A positive result, given the score of 2 was judged by the dark brown colour and characteristic surface structure of the patch of pupal cuticle in the wounded area. A negative result, scoring zero, occurred when there was a patch of normal adult cuticle in the wounded area. Alternatively, if there was a response but the area of cuticle involved was small, this result was given a score of one.

For each experimental group the results were expressed as follows. Ten extracts had been made and ten pupae bioassayed. As each extract had been diluted with 10mg of olive oil and wax, then the maximum score possible would be 20 (i.e. 10 positive results). This would be epxressed as a 100% response. The minimum score would be expressed as a 0% response. Scores between these two extremes would be expressed as a percentage of the maximum.

The effect of m.n.c. cautery on the presence or absence of a substance with juvenile hormone activity in the haemolymph and faeces of P. americana can be seen in Figure 12. Low levels of the substance could be detected in both the haemolymph and faeces of operated insects until day 10, a comparatively higher percentage response was found in both control groups which continued throughout the experimental period. The percentage

response of the control groups varied widely. This was also true of the control groups for the allatectomised insects (Figure 13). The response in the control groups on the days that were sampled showed a wide range of variation. No substance with juvenile hormone activity was detected in the haemolymph of the allatectomised insects but there was a low percentage response in the faeces on the first day of sampling after the operation had been performed.

Fig. 12

The effect of n.n.c. cautery on the presence or absence of substances with the activity of juvenile hormone in the haemolymph and faeces of *P. americana*.

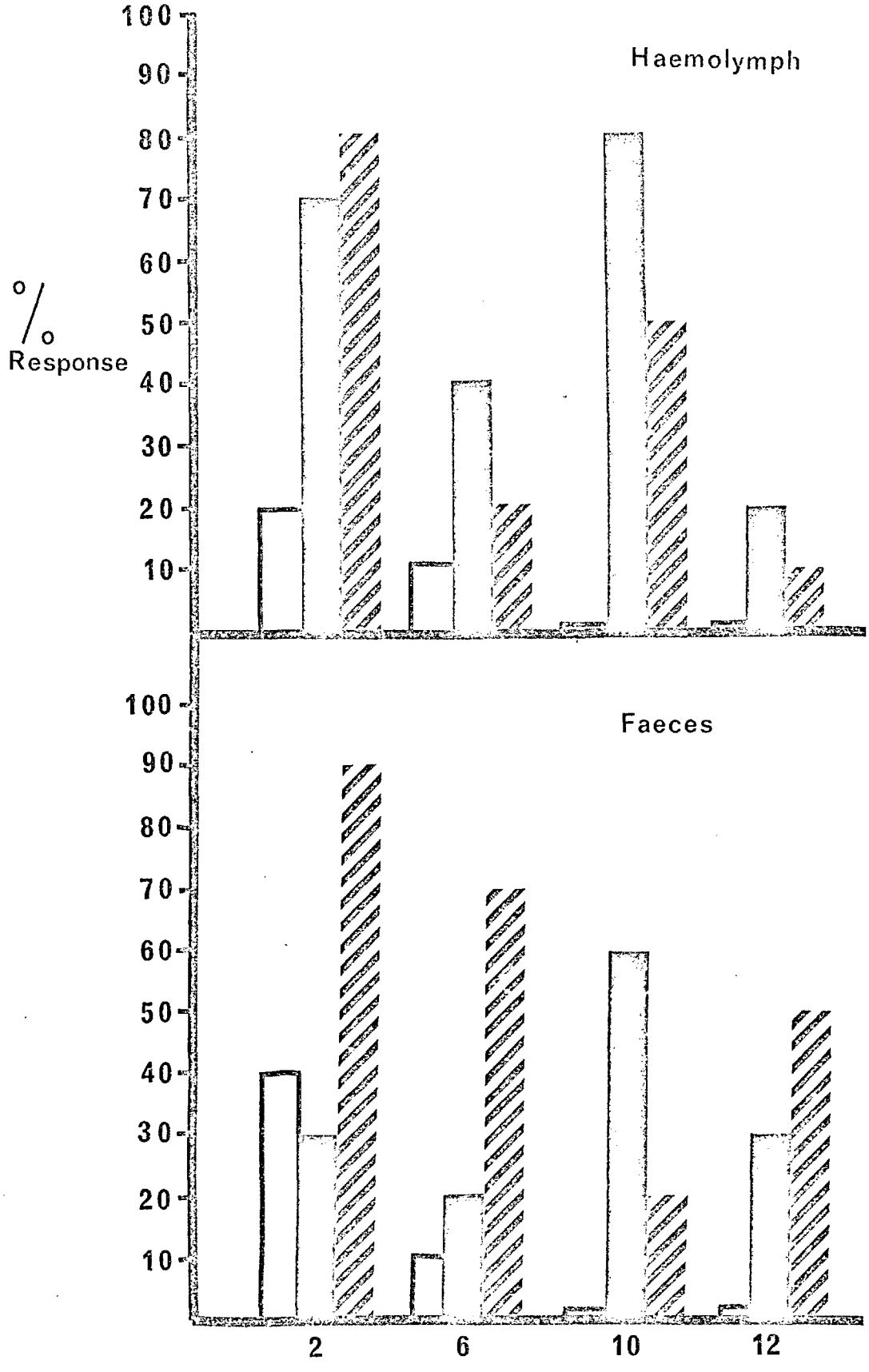
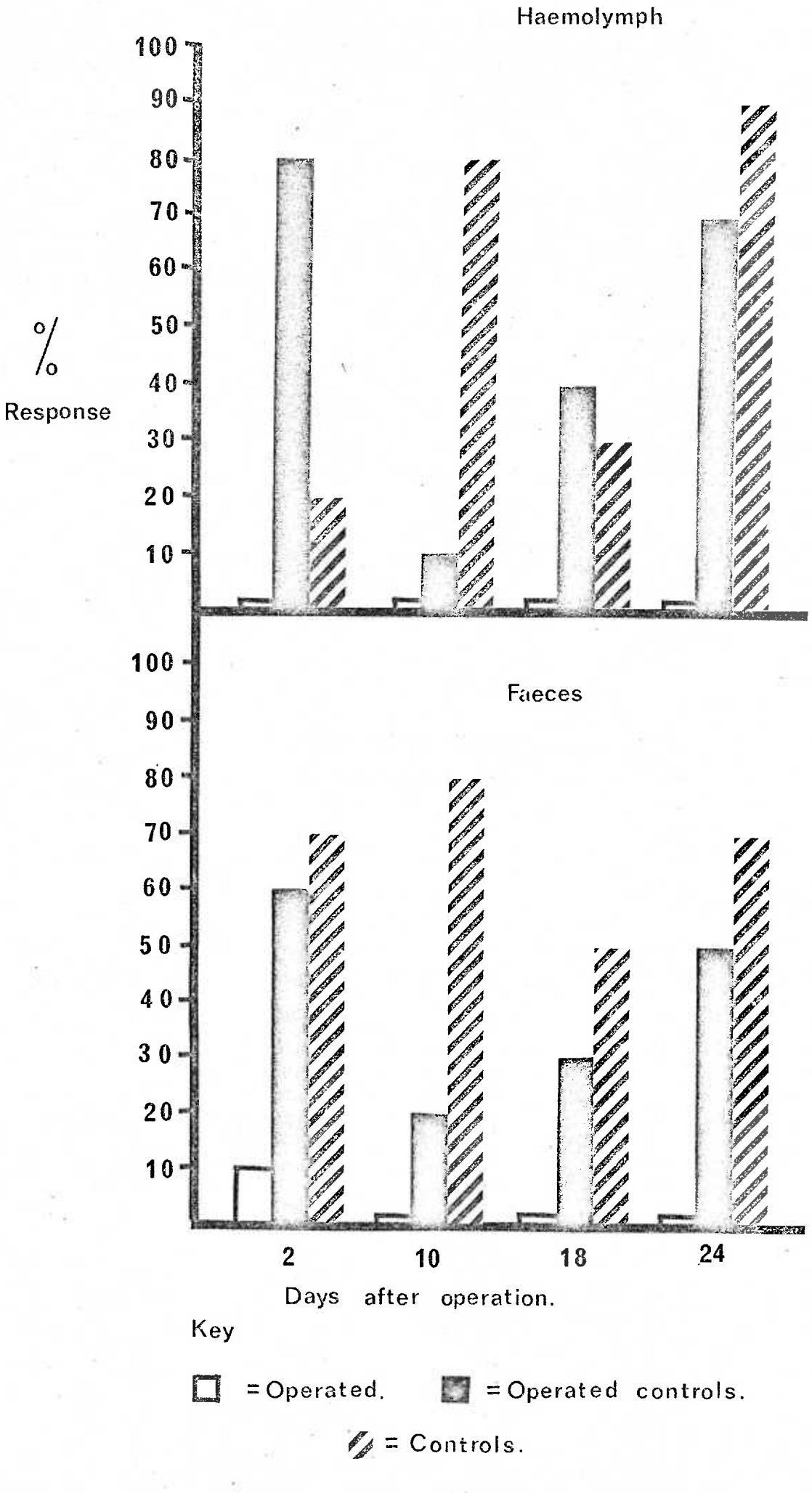

Haemolymph
Faeces
% Response
100
90
80
70
60
50
40
30
20
10
2
6
10
12
Days after operation.
Key
= Operated.
= Operated controls.
= Controls.

Fig. 13

The effect of allatectomy on the presence or absence of substances with the activity of juvenile hormone in the haemolymph and faeces of *P. americana*.


Haemolymph
100
90
80
70
60
50
40
30
20
10
%
Response
Faeces
100
90
80
70
60
50
40
30
20
10
2
10
18
24
Days after operation.
Key
= Operated.
= Operated controls.
= Controls.

Discussion

It was evident from the histological examination of the pars-intercerebralis and from the effects produced on the fecundity of the m.nc. cauterised insects that cautery of the m.n.c. was successfully carried out. Both allatectomy and removal of both corpora allata and corpora cardiaca were also successfully performed as indicated by the percentage oocyte resorption and reduction in the terminal oocyte length observed.

The results of each of these experiments showed that total numbers of adult H. diesingi and L. appendiculata were reduced in operated insects compared with the operated and unoperated control groups. However, when the numbers of adult male and female H. diesingi are considered separately it was found that after both allatectomy and removal of both corpora allata and corpora cardiaca, although there was a significant decrease in the numbers of adult female nematodes in the operated groups compared with the controls, the decrease in the adult male population in some cases bordered on the significance level. An explanation of these results may lie in the suggestion by Lee (1959) that sex in this nematode is not genetically predetermined but that the adult male parasite is actually a neotenic fourth stage larva. If this is the case then it is the overall effect of the operation on the total numbers of adult parasites which is the important factor to consider. A possible explanation for the decrease in the total number of adult parasites is that the adults must have been destroyed and/or eliminated from the host, in which case it could be concluded, as did Gordon (1968) who looked at the effect of 2 week cautery on the levels of infestation of adult H. diesingi and Blatta orientalis, that the adult nematodes are dependent upon a supply of

neurosecretory material from the insect host for their existence.

Surgical manipulation of the insect endocrine system by any of the operations resulted in the loss of third and fourth stage larvae from the hind gut of operated insects, except in the case of 2 week m.n.c. cautery. Here, a number of third stage larvae of H. diesingi resided in the gut of the operated insects. As development from third stage larvae to fourth stage larvae normally takes place within 19-21 days, one would expect to find some third stage larvae in the hindguts of insects from each of the control groups together with a number of fourth stage larvae. The fact that a larger percentage of third stage larvae were found in the operated insects than in the control groups and that there was an absence of fourth stage larvae indicates that the third stage larvae were unable to resume growth or moult to the fourth stage larvae in the environment resulting from cautery of the m.n.c. The fact that nematodes stop growing when they moult and that the requirements for growth to resume might be limiting or critical. The possibility that nematodes might be more vulnerable to adverse conditions when moulting than at other times needs careful consideration.

The absence of third stage larvae in 4 week allatectomised and cardiaca-allata operated insects and the very low numbers in the control groups agrees with the developmental time of the larval third to fourth stage. However, the total absence of fourth stage larvae in both of the operated groups compared with the numbers found in the control groups, signifies that any third stage larvae that did develop to the fourth stage or any fourth stage larvae present in the hind gut could not exist in the hind gut of these operated insects.

From the results outlined above it is obvious that the overall effect on the operations performed on the insects was a loss of larval and adult stages from the hind gut of the host. This decrease in parasite burden may have been due directly to the absence of a hormone produced by the neuroendocrine system of the host, or may have been induced indirectly through a change in the physiological state of the host and consequently inducing a change in the physiological environment of the parasite.

There are many factors which will influence and change the environment of the gut living parasites. A particularly important factor is the amount and quality of food ingested. Concomitant with this factor is the activity of the enzymes secreted by the insect to digest the food.

Considering the effect of this factor upon the third stage larvae, feeding would not seem to be important since the onset ofmoulting is commonly associated with a period of inactivity or lethargy which may last from an hour to several days (Veglai, 1915; Hansen and Buecher, 1964; Hechler and Taylor, 1966). By the time the nematode regains its activity a new cuticle has been formed. The significance of the lethargy is probably due to the fact that at this time the lining of the nematode mouth and oesophagus is being removed. Larvae in the fourth stage undergo rapid developmental changes as they mature to adults and for this they require energy derived from constant feeding. Thus changes in the supply of food would greatly affect this stage of the nematode.

According to Hominick and Davey (1973) at normal to moderately reduced levels of nutrition in the host the adult population of L. appendiculata dominates in the host gut, but

at reduced levels of nutrition it is H. diesingi which predominates. According to the literature both H. diesingi and L. appendiculata are bacteria feeders although no specific work has been carried out to verify this. However, it seems more likely in fact that these nematodes are not specifically bacterial feeders but will ingest any available particulate matter at random in the host gut. From measurements of the guts of these nematodes Hominick and Davey (1973) have shown that L. appendiculata is capable of ingesting large particulate matter, while H. diesingi takes in only fine particulate matter. Evidence for the support of this random particulate feeding of these nematodes comes from a simple experiment where the adult nematodes have been allowed to browse on damp black filter paper, after a few minutes black filter paper fibres are evident in their alimentary tracts.

If H. diesingi and L. appendiculata were specifically bacterial feeders then nutritional changes in the host would undoubtedly affect the status of the bacteria in the gut and in turn the nematodes would be unable to feed, die and subsequently be lost from the host. It was for this reason that the food intake of the host, activity of the enzymes secreted by the insect were investigated together with the levels of carbohydrates, lipids and protein in the haemolymph which would reflect, to some extent, any changes in the environment of the parasites.

Engelmann (1965) has shown that removal of the m.n.c. in the cockroach Leucophaea maderae results in a decrease in food intake by the insect. The present study has shown that the food intake of P. americana is unaffected by either m.n.c. cautory or allatectomy. It can be inferred from the results that the

activity of the midgut proteases is not under the control of neuroendocrine factors. It seems more likely that enzyme secretion follows stimulation by food. No variations were found in the activity of intracellular or extracellular protease activities, or those of lipase or esterase following the operations. Although m.n.c. cautery induced no variation in the activity of amylase, allatectomy exerted a subtle effect. Amylase is not usually produced by the midgutcells themselves, but is passed back from the salivary glands. So it seems that food has been ingested normally by the operated insects and that digestion of it has not been affected. However, changes in the physiological state of the host due to interference with the assimilation of this food would be indicated by changes in the levels of carbohydrates, lipids and proteins in the haemolymph.

Removal of the m.n.c., but not the corpora allata, caused an increase in the blood carbohydrate level. This could have been due to increased absorption of carbohydrates by the midgut which would have a direct bearing upon the parasites, since an increase in uptake of food materials by the midgut would automatically result in lowering the food materials available in the parasites environment. However, the fact that the parasite population was lost from insects after allatectomy, which left both m.n.c. and corpora cardiaca intact, even though there was no effect on the carbohydrate metabolism after this operation, leads one to disregard this idea. Another possible reason why this elevation of carbohydrate levels should occur after cautery has been suggested by Goldsworthy (1967). He has found that there is a hyperglycaemia factor in Locusta migratoria, similar in effect to that found by Steele (1961) in P. americana and he suggested that this increase in carbohydrate level may be a

result of a reduced protein metabolism induced by cautery of the m.n.c. Therefore, the amount of energy required from the carbohydrates would be reduced. As a result, carbohydrate levels would tend to build up. The results obtained in these experiments on P. americana in fact showed that m.n.c. cautery caused a decrease in the concentration of protein in the haemolymph of operated insects. The result could be interpreted as similar to those obtained by Hill (1962, 1963) from Schistocerca gregaria, who found that after m.n.c. cautery the haemolymph proteins remained at a fairly stable low level, while in the control insects the haemolymph protein level rose steadily as oogenesis proceeded. If this is also occurring in P. americana, then in fact there will be no change in the environment of the parasites. Changes are more likely to be occurring in the protein metabolism of the normally developing female controls. It should also be noted that there was a change in the lipid metabolism of allatectomised insects. Therefore, it seems that decrease in parasite burden cannot be attributed to any effect mediated via the total midgut enzyme activities, nor can it be attributed to any effect on the feeding of the insect. Neither does it seem possible that the changes in the metabolism of the host investigated could greatly affect the environment of the parasites.

It has been mentioned that the adoption of a parasitic mode of existence presupposes dependence on the host to supply many factors essential to the life and well being of the parasite. This dependence induces adaptation of the parasite to an osmotic and ionic environment regulated to be favourable to the cells and tissues of the host. The need for active osmoregulation depends on the environment normally inhabited by the nematode.

Species such as H. diesingi and L. appendiculata, which are parasites of the gut, inhabit a medium in which the ion content fluctuates but normally remains hypertonic to nematode body fluid. Most nematodes can tolerate a considerable increase in the osmotic pressure of their body fluid, so that hypertonic external conditions present no great regulatory problem. Such conditions were induced in the gut of P. americana after cautery of the m.n.c., the massive increase in haemolymph volume resulting in dry, hyperosmotic conditions in the parasites environment. This phenomenon together with fluctuations in the ionic content of the haemolymph and so probably in the parasites environment are possible reasons for the decrease in parasite burden.

The fact that a large number of third stage H. diesingi survived the two week cautery period suggests that they were able to adapt to such stringent conditions. The best documented case for regulation of water balance in larval nematodes is reported by Weinstein (1952) who worked on third stage larval Nippostrongylus muris and Ancylostoma caninum. These adjust to variations in osmotic pressure of the external medium by a change in the rate of excretion of fluid by the excretary system. While these two can regulate water balance actively when they encounter hypo or hyper conditions in this way, other larvae absorb or lose water through the cuticle in response to environmental conditions.

However, while no clear cut relationships have been observed, it is possible that the abundance or deficiency of certain ions in the environment may effect larval development. Data obtained by Oteifa (1953), Curtis (1964) and Marks and Sayre (1964) indicates that inorganic substances offset

parasitic nematode populations through their effect on the metabolism of the host organism. Nevertheless, a direct effect of the ions on larval metabolic processes cannot be ruled out.

The methods of osmoregulation in adult nematodes vary. In addition to the elimination of water from the body across the cuticle, by the alimentary tract and the excretory system, internal osmotic pressure can be reduced by excretion of metabolic waste products or of electrolytes from the body fluid, while adjustments to hypotonic conditions can be made by uptake of ions from the bathing fluid. H. diesingi is capable of osmoregulation in hyperosmotic salines, while it lacks the ability to adjust to hypoosmotic conditions. The fact that the nematode returns to its original length in hyperosmotic salines and fails to do so in hyperosmotic sucrose indicates that the regulatory mechanism involves the uptake of ions from the environment. Adult H. diesingi have been shown by Lee (1960) to be able to withstand the osmotic stress induced in the gut of cockroaches during their moult. Experiments have shown, however, that the adults of L. appendiculata are not so efficient at osmoregulation in hyperosmotic media as are H. diesingi and so it is possible that L. appendiculata could be lost from the host in cases where H. diesingi could survive.

Even though it has been shown that adult H. diesingi can survive in hyperosmotic conditions it was suggested that it could only do so if there was adequate provision for the uptake of ions. The results of the experiments carried out indicated that cautery of the m.n.c. interfered with the normal ionic balance of the parasites environment. Selective ion movement has been

suggested as an osmoregulatory mechanism by several workers. In hypertonic saline, either a water efflux, an ion influx or both could occur. An efflux of water must cause shrinking. This shrinking would be less extensive if ions were able to enter the body to effect more rapid osmotic equilibrium. Stephenson's (1944) data showed that in Rhabditis terrestris the rates of penetration of the cuticle of the four cations tested were $Na^{+} > Ca^{++} > Mg^{++} > K^{+}$.

The small size of H. diesingi precluded an analysis of the body fluid to determine quantitative changes in composition resulting from ion movement. Work on Ascaris (Hobson, 1952) has shown that the nematode body fluid is hyposmotic to the intestinal contents of the pig. The increase in body fluid osmolarity when placed in saline is accompanied by changes in ionic composition of the body fluid. Ion movement occurs primarily ocross the body wall of Ascaris. It was found that the Ca^{++} and Mg^{++} concentration of the body fluid remained relatively constant irrespective of the concentrations of these ions in the external medium, and while the levels of Na^{+} and K^{+} in the body fluid were found to be variable, the variations did not appear to depend directly on the concentration of these ions in the bathing solution. Therefore, it would not appear that variation in the osmotic pressure or availability of ions in the cockroach gut would be directly responsible for the loss of nematodes from the host.

However, if the concentration of potassium (mM) is in the region of 2 - 8, Sanborn and Arthur (1965) have shown that there is a low frequency of spiking resulting in single slow waves or abortive spikes and this leads to maintained relaxation of the nematode muscles. If this situation arose, then it is possible

that the adult nematode population would be pushed out with the faeces during peristaltic movements of the hind gut as they would be unable to maintain their position. However, allatectomy produced no such effects upon the water or ionic balance in the host, even so the nematode population was lost. It now becomes necessary to investigate the role of the insect neuroendocrine system in ensuring the survival of the nematode parasites. This has been carried out by *in vivo* and *in vitro* experiments recorded in the next section.

2. THE EFFECTS OF NEUROSURGERY, REIMPLANTATION OF NEURO-ENDOCRINE GLANDS AND TOPICAL APPLICATION OF JUVENILE HORMONE TO THE HOST ON THE DEVELOPMENT AND ESTABLISHMENT OF H. DIESINGI WITHIN IT AND THE EFFECT OF GLAND EXTRACTS AND JUVENILE HORMONE ON H.DIESINGI AND L. APPENDICULATA IN VITRO.

Introduction

In the previous chapter it has been shown that m.n.c. cautery, allatectomy and removal of both corpora allata and corporata cardiaca all produce a decrease in the parasite burden of the host. Some of these operations changed the immediate environment of the parasites, but decreases in the nematode population could not be directly attributed to any or all of these physiological changes. A more direct study of hormonal effects was required with particular attention being paid to the effects of the corpora allata and juvenile hormone upon larval and adult nematodes. This was carried out in the next series of experiments.

An attempt was made to establish a population of H. diesingi in cockroaches which had been cauterised, allatectomised or had both corpora allata and corpora cardiaca removed. This was carried out by dosing the operated insects with infective eggs of the nematode. The results of the previous experiments have shown that neither m.n.c. cautery or allatectomy has any significant effect on the activity of midgut esterase and lipase. So that if operated insects were given a dose of infective eggs, the eggs should be able to hatch normally. Using this method, the development and establishment of a nematode population could be followed. The role, if any, of the corpora cardiaca and that of the corpora

allata in this process was examined by injecting extracts from either of these glands into operated insects. The role of juvenile hormone was investigated by the topical application of the hormone to operated insects dosed with nematode eggs.

The *in vivo* studies of the effects of the host neurosecretion upon the thelastomatid parasites suggested that a study of the direct effect of neurosecretory materials on the nematodes *in vitro* would be a rewarding line of research. The effects of gland extracts and juvenile hormone on *H. diesingi* and *L. appendiculata* *in vitro* have been carried out here for the first time.

A. THE EFFECTS OF NEUROSURGERY AND REIMPLANTATION OF EXTRACTS OF NEUROENDOCRINE GLANDS ON THE DEVELOPMENT AND ESTABLISHMENT OF H. DIESINGI IN P. AMERICANA.

Materials and methods.

In the experimental group each insect was dosed with 50 eggs on the day snown in the experimental design shown below. The experimental period was measured from the day of dosing. Insects were dissected on days 1, 5, 10, 15 and 25 of the experimental period. Insects which were injected with gland extracts were first injected 2 days before they were dosed and thereafter every 5th day until the end of the experimental period.

(i) METHOD OF REIMPLANTING CORPORA ALLATA OR CORPORA CARDIACA INTO OPERATED P. AMERICANA.

Homogenates of known numbers of corpora allata or corpora cardiaca from adult female *P. americana* were made up in glass homogenisers in 0·15 M sodium chloride and a dose range

equivalent to a single gland to 4 pairs of either gland made by dilution. The dose in 10µl saline were injected into the haemolymph of the insects between the third and fourth sclerites of the insect abdomen using an Agla syringe.

(ii) METHOD OF DOSING P. AMERICANA WITH INFECTIVE STAGE EGGS OF H. DIESINGI.

The eggs were cultured to the third larval stage as described in Section 1.1.ii.

The cockroaches were anaesthetised with carbon dioxide and a catheter which had been rinsed with silicone repellent, introduced into the insects mouth. The catheter was attached to a calibrated micrometer syringe, containing the eggs, so that the dose introduced into the insect could be controlled. The standard dose administered was 50 eggs. Control insects were anaesthetised only.

(iii) EXPERIMENTAL DESIGN

As shown previously, it was not possible to rear nematode free cockroaches, so that all cockroaches used in these experiments carried natural parasite burdens. This necessitated the inclusion of Groups 1-6 in the experimental design. These groups indicated the standard of infection present in the control experimental insects before the introduction of infective eggs into them. Deduction of the nematode levels found in Groups 1-6, on days 1, 5, 10, 15, 20 and 25 of the experiment, from those found in the experimental animals enabled the numbers of nematodes developing in the dosed experimental groups to be measured and then compared with the number developing in the operated dosed insects.

A. SYSTEM CONTROL GROUP

Group	No. of insects	
1	20	Unoperated controls
2	20	M.n.c. operated controls
3	20	Allatectomy operated controls
4	20	M.n.c. cauterised
5	20	Allatectomised
6	20	Corpora allata/cardiaca removed.

B. EXPERIMENTAL GROUPS

Group	No. of insects	
7	20	Unoperated controls. Dosed day 16.
8	20	M.n.c. operated controls. "
9	70	M.n.c. operated "
10	20	" +1g.p.e.ca "
11	20	" 2 " "
12	100	" 3 " "
13	100	" 4 " "
14		" 1 g.p.e.cc "
15		" 2 " "
16		" 3 " "
17		" 4 " "

Groups 7 - 17 repeated for allatectomised and ca/cc removed. All dosed 30 days after operation.

Results.

Comparison of the unoperated and operated controls in Group A with unoperated and operated controls in Group B showed that it was possible to establish a population of H. diesingi in the gut of P. americana. Infective eggs were able to hatch normally and after 1 day some third stage larvae had migrated to the hind gut. After 5 days, a high population of third stage larvae was established in the hind gut. This

remained steady until day 20 when some of the nematodes were found to have exsheathed to the fourth larval stage. By day 25 some of the fourth stage larvae had begun to mature to the adult and both male and female gonads could be observed. The number of nematodes which had initiated male adult development outnumbered those that had initiated female development by a ratio of 2:1.

Examination of the faeces of m.n.c. cauterised insects that had been dosed with infective eggs of H. diesingi showed the presence of third stage larvae after 10 days. Although at this time the majority of the larvae still resided in the hind gut of the host, the number of larvae found in the faeces gradually increased towards the end of the experimental period. Of the larvae found in the hind gut on day 15, over half of them were non-motile and none had exsheathed. On day 20, no larvae were found in the hindguts of cauterised dosed insects.

The number of third stage larvae found in the guts of dosed allatectomised insects or those dosed after removal of both corpora allata and corpora cardiaca at day 10 did not vary significantly from the number found at this time in dosed cauterised insects and the same course of events seems to have taken place in the host gut from then on. The nematodes, in the third stage, had become establisned in the hosts over the first 10 days and this was followed by their gradual expulsion over the next 10 days until none remained.

The reimplantation of extracts of concentrations of corpora allata into any of the operated insects gave seemingly anomalous results. After cautery, allatectomy, or when corpora

allata and cardiaca had been romoved, repeated injection of a single pair of corpora allata to the dosed insects had various effects. In the minority of cases the third stage larval population was maintained throughout the experimental period and the majority of the nematodes exsheathed. When this occurred, the results were comparable with those found in the unoperated control insects. However, in others although the hatch of eggs was normal the third stage larvae were expelled from the host or the population of third stage larvae decreased after the second injection or even third injection but with a subsequent injection the population stabilised and the remaining nematodes continued to develop. Similar results were obtained when higher concentrations of corpora allata gland extracts were used. These results indicated that of the gland extracts used, only a certain percentage of them were viable.

In contrast, the injection of any concentration of corpora cardiaca gland extracts into any of the operated insects failed to prevent the loss of all third stage larvae, from any of the hosts, within 15 days.

B. THE EFFECTS ON NEMATODE DEVELOPMENT OF TOPICAL APPLICATION OF JUVENILE HORMONE TO INSECTS WHICH HAVE BEEN M.N.C. CAUTERISED, ALLATECTOMISED OR CORPORA ALLATA AND CARDIACA REMOVED.

This experiment ran concurrently with the one previously described, so that population results from control insects from these groups were used for comparative means in assessing the effects on the nematode populations of repeated topical application of juvenile hormone to operated insects. Cauterised

insects and their controls were dosed with nematode eggs 16 days after the operation. Allatectomised, cardiaca-allata operated and their controls were dosed with eggs 30 days after the operations had been performed. Topical applications were first made ? days before the insects were dosed with eggs and subsequently every 5th day for 25 days.

(i) METHOD OF TOPICAL APPLICATION OF JUVENILE HORMONE TO P. AMERICANA.

The juvenile hormone (Synthetic - JA, Calbiochem) was dissolved in acetone. By dilution a range of concentrations from 25 - 250 mg/ml of juvenile hormone was made up. The cockroaches were anaesthetised and their elytra were cut off to expose the thorax dorsally. Aliquots of 10µl of a known dose of juvenile hormone (250ng, 500ng, 1·0mg or 2·5mg) in acetone was applied. using a microcap, to the insect. Acetone only was applied to control insects.

(ii) EXPERIMENTAL DESIGN

Group	No. of insects	
50	M.n.c. cauterised+acetone.	
51	"	+ 250ng J.H.
52	"	+ 500ng J.H.
53	"	+ 1·0mg J.H.
54	"	+ 2·5mg J.H.

Groups 50 - 54 repeated for allatectomised insects and those wnich had cardiaca and allata removed.

RESULTS

The topical application of acetone had no effect on the development of the nematodes within the insects. Results

obtained were comparable with those for dosed unoperated insects in the previous experiments.

The repeated application of either 100 or 200ng juvenile hormone to dosed cauterised and allatectomised insects or to those which had had their corpora allata and cardiaca removed resulted in the loss of the parasites from each host within the first 15 days of the experiment. The dead third stage larvae were found in the faeces. However, the application of either of the two higher doses of juvenile hormone to any of the operated insects resulted in the maintenance of the larval population and the initiation of adult development within individual nematodes. No immature female nematodes were found at the end of the experimental period but the initiation of male gonad development was observed.

C. EFFECTS OF APPLICATION OF JUVENILE HORMONE AND EXTRACTS FROM THE BRAINS OF P. AMERICANA AND LOCUSTA MIGRATORIA TO H. DIESINGI AND L. APPENDICULATA IN VITRO.

Materials and methods.

Fourth stage larvae, adult male and female H. diesingi and L. appendiculata were collected from the gut of P. americana. Third stage larvae of both species were hatched in vitro as described in Part 1. A series of incubation dishes were set up containing a known volume of 0·15M sodium chloride. At least ten specimens of each stage from each species of nematode were placed in the incubation dishes to which a known dose of juvenile hormone or homogenised brain extract was added.

The juvenile hormone in acetone and the insect brain

extracts were prepared as described previously. Doses of 10µl of juvenile hormone and gland extracts in the range equivalent to a single gland to 4 pairs were added to the incubation dishes with constant stirring.

(i) EXPERIMENTAL DESIGN FOR EACH NEMATODE AGE GROUP AND SPECIES.

Group	Treatment
1	Control : saline
2	Control : saline + 10µl acetone
3	Saline + 250 ng J.H.
4	Saline + 500ng "
5	" 1·0mg "
6	" 2·5mg "
7	" + single gland equivalent c.a. P. americana
8	" + 1 g.p.e. c.a. "
9	" + 2 " " "
10	" + 3 " " "
11	" + 4 " " "
12	" + single gland equivalent c.c. "
13	" + 1 g.p.e. " "
14	" + 2 " " "
15	" + 3 " " "
16	" + 4 " " "
17	" + single g.e.ca + single g.e.cc "
18	" + 1 g.p.e. c.a. + 1 g.p.e. cc. "
19	" + 2 " " + 2 " " "
20	" + 3 " " + 3 " " "
21	" + 4 " " + 4 " " "

Treatments 7 - 21 inclusive repeated with extracts from L. migratoria

Results.

All of the stages which were kept in incubation dishes in saline could survive in this medium for up to one month if the containers were sealed. All of the nematodes used in these experiments were examined at daily intervals for up to one week. Nematodes from the same age group of different species are considered together except where specified.

In saline the third stage larvae formed a fourth stage larval cuticle on the third day but would not exsheath. The adult female nematodes released their eggs normally during the first 2 days of the experiment but after this the deposition of them gradually declined. The cultures were unaffected by the addition of acetone to the saline.

When 250 or 500ng juvenile hormone were added to the culture medium containing the third stage larvae during the first 48 hours of the experimental period, no effects on the nematodes were observed. If, however, these doses were applied to the third stage larvae which had already produced their fourth stage larval cuticle on the third day then they would exsheath within 24 hours. Of the third stage larvae of H. diesingi treated with 250ng juvenile hormone on the third day, 16 survived, 14 of these exsheathed, while of the 15 surviving larvae treated with 500ng juvenile hormone, at the same time, 12 exsheathed. When 1·00mg (15 worms, 11 survivors) or 2·5mg juvenile hormone (16 worms, 10 survivors) was administered to the third stage larvae before or after the third day, although the fourth stage larval cuticle had been formed as in the controls, no exsheathment occurred. Similar results were obtained for the third stage larvae of L. appendiculata.

None of the fourth stage larvae of either species used showed any sign of sexual maturation at the beginning of the experiment. After 1 week in saline, although active, the control larvae showed no sign of maturation although all survived. However, when 250ng juvenile hormone were administered to fourth stage larvae (H. diesingi; 10 worms, 9 survivors; L. appendiculata: 10 worms, 8 survivors) four of each species showed initiation of the male sex organs on the fifth day. No females were observed. Few of the fourth stage larvae which were exposed to higher doses of juvenile hormone survived the experimental period, of those that did, none showed any sign of sexual maturation.

No observable effects were produced by the exposure of adult male nematodes to this dose range of juvenile hormone. However, all survived the experimental period.

Adult female nematodes, however, in contrast to the controls continued to produce eggs for the duration of the experimental period when exposed to 250 and 500ng or to 1·00mg juvenile hormone. Those exposed to greater concentrations of the hormone survived the experimental period but behaved as the controls.

The application of a single gland pair equivalent (g.p.e.) of corpora allata from P. americana to the third stage larval nematodes was ineffective. However, the exposure of these nematodes to a single gland equivalent of corpora allata from L. migratoria or to 1 g.p.e. of corpora allata from P. americana induced exsheathment, in a few cases, when applied after the 4th stage larval cuticle had been produced. In the first group, of the 17 H.diesingi which survived the

experimental period 7 exsheathed normally, in the latter group, 18 nematodes survived and 4 exsheathed. Comparable results occurred after exposure of the third stage larvae of L. appendiculata to these concentrations. of corpora allata. Applications of higher concentrations of corpora allata from either insect produced no changes in the third stage larvae of H. diesingi or L. appendiculata compared to the controls. No overt changes were produced in the fourth stage larvae or adult nematodes exposed to the dose range of corpora allata from either insect.

The addition of any dose of corpora cardiaca from either P. americana or L. migratoria to the incubation dishes containing third stage larvae, fourth stage larvae or adult nematodes produced no effect. The numbers of experimental nematodes surviving for one week were comparable with those in the control dishes.

However, the application of a single gland equivalent of both corpora allata and corpora cardiaca from either P. americana or L. migratoria resulted in exsheathment of the third stage larvae of H. diesingi. When administered after 2 days; 9 of the larvae exposed to the glands from P. americana and 11 of those exposed to extracts from L. migratoria exsheathed. Similarly, 4 and 6 of the third stage larvae of L. appendiculata exposed as above to gland extracts of P. americana and L. migratoria respectively exsheathed. None of the other stages of the nematodes were affected.

Exposure to 1 g.p.e. of corpora allata and cardiaca from either insect produced the same effect on the third stage larvae as described above, while application of higher doses

of both glands to third and fourth stage larvae or adult nematodes produced no effect on them compared with the controls.

D. THE STAINING OF LARVAL AND ADULT H. DIESINGI AND L. APPENDICULATA FOR NEUROSECRETION.

Method

The whole mount staining technique of Dogra and Tandan (1964) was used after the nematodes had been killed by heat.

Results.

The results in all cases were negative.

DISCUSSION

The experimental results have shown that third stage larvae of either *H. diesingi* or *L. appendiculata* can only survive for up to two weeks in the gut of insects which have undergone neurosurgery. However, it was shown that exsheathment could be initiated in them both *in vivo* and *in vitro* by exposure to homogenised extracts of corpora allata or to doses of juvenile hormone. This poses the question as to the role of these substances in the exsheathment process. It has been demonstrated by Rogers (1963) that exsheathment in *Trichostrongylus* larvae is due to the activity of an exsheathing fluid which is secreted by cells at the base of the oesophagus and released through the excretory pore of the larva. This fluid acts on the sheath so that the inner cuticle layer is eventually dissolved and the outer layer splits open by movement of the larva (Rogers and Sommerville, 1960).

It has been suggested that the release of an exsheathment fluid is the end result of a neurosecretory process (Sommerville, 1957; Rogers, 1965; Davey and Kan, 1967). With an appropriate stimulus, neurosecretory cells release hormones that activate receptor tissues, stimulating the production or release of an exsheathment fluid. Davey and Kan (1968) working with *Phocanema decipiens*, a nematode parasite of vertebrates, provided experimental evidence indicating the production and release of an unidentified neurosecretory hormone involved in ecdysis. The fact that some parasitic nematodes require a stimulus from the host before these processes can begin indicates that in some

species, part of the hormonal pathway has been lost. The host stimulus acts indirectly and the links coupling it with the release of fluids by the nematode larvae are so far unknown.

Although there is little direct experimental evidence to support such conjectures, cells near the nerve ring of Ascaris show histochemical evidence of neurosecretion. In view of the cell constancy in nematodes, these cells could have neurosecretory functions in the larval stages as well. Staining for such cells in H. diesingi and L. appendiculata have proved unsuccessful, so that it appears possible that these nematodes do not have funtional neurosecretory cells, in which case, instead of relying on the host for a physiological stimulus for the activity of their own neurosecretory cells to produce an exsheathment fluid, they may indeed rely on the host neurosecretion for the initiation of this process itself. Alternatively if the nematode does possess neurosecretory cells but they have not been apparent due to the staining method used, it is possible that host endocrines provide the stimulus to the nematode neurosecretory cells for activation.

It is thus necessary to examine the reason why nematodes which had formed a new cuticle would ecdyse when extracts of corpora allata or juvenile hormone were added but those that had not fully formed their new cuticle when these extracts were added would not. Davey (1971), working on Phocanema decipiens, found that when juvenile hormone was present from the beginning of ecdysis, it stimulated the neurosecretory cells to secrete, this lead to premature activation of the

excretory cells which passed out moulting fluid before the new cuticle was fully formed. Application of juvenile hormone after deposition of a new cuticle stimulated the neurosecretory cells which resulted in the release of exsheathment fluid into the space between the cuticles and exsheathment proceeded normally. It can be construed that the same course of events took place in this series of experiments.

Although Davey (1971) found that application of juvenile hormone to nematode larvae evoked a positive response, other workers have not found this so. Dropkin, Lower and Acedo (1971) tested the effects of juvenile hormone and its analogues on nematode growth and found that if concentrations of 25 or 50mg/ml were administered seven out of ten of the insect juvenile hormone treatments inhibited growth of the nematodes. It is possible that unless a large range of different doses is tried then the critical concentrations which evoke response may be missed. This brings into consideration the range of concentrations of corpora allata and juvenile hormone which initiated results in this series of experiments. Anomalous results were obtained using homogenised extracts of corpora allata. There is very little information on the secretory activity or the quantity of active constituents in the corpora allata of adult female P. americana or L. migratoria at any one time. It has been indicated by certain workers that secretion by the allata of both of these insects is cyclic. It is, therefore, reasonable to presume that if the corpora allata are extracted from adult insects at random as the juvenile hormone content of them

could not be quantitatively determined, amounts of gland extract applied need not have reflected a true range of hormone. It is also possible that after injection into the haemocoel of an insect the homogenised extracts were dispersed amongst the tissues, so that little penetrated to the gut via the malpighian tubules. However, the malpighian tubules probably allow the excretion of compounds such as juvenile hormone or its breakdown products (Maddrell, 1973) into the gut lumen but, as we have seen, although the presence of substances with the activity of juvenile hormone can be shown in the faeces of P. americana, there is, as yet, no means of quantifying them.

The in vitro application of known doses of juvenile hormone gives more indication of the levels which will affect the nematodes. The absence of abnormalities produced in the morphology of treated nematodes indicates that any effects produced were not purely pharmocological.

The results discussed so far indicate that there is a dependence by the third stage larvae on a neuroendocrine stimulus from the host for their development. The fourth stage larvae and adults will now be considered.

Although extracts of corpora cardiaca had no effect on the fourth stage larvae, exposure of them to homogenised extracts of corpora allata and to doses of juvenile hormone resulted in the initiation of development of sex organs. In the cases observed the general trend was for development of male sex organs. When the development of both male and female sex organs was initiated, the number of male out-numbered the females by ?:1. Sexuality in nematodes is not,

of course, always genetically predetermined. As mentioned previously, Lee (1959) suggested that sex is not predetermined in H. diesingi and the adult male nematode is a neotenic fourth stage larva. Christie (1929) suggested that it is the environment of the parasite in the host which was the sex determining factor as exemplified by Agamermis decaudata and Mermis subnigrescens in Melanoplus sp. Christie observed that if this insect was infected with a small number of infective ova of M. subnigrescens, then the nematodes which developed from them were all female. However, if high infections were administered then all male parasites developed. In the in vivo experiments performed here, doses of 50 infective eggs were administered which, if considered as a large dose agrees with Christie's theory. However, sexual development of any kind only occurred in vitro after exposure of relatively low numbers of fourth stage larvae to either juvenile hormone or extracts of corpora allata, so that although this does not negate Christie's theory, it introduces the necessity of another factor in the parasites' environment in the host, not investigated by Christie, for development of the parasite.

In fact, Johnson and Viglierchio (1970) found that the activity of two closely related farnesyl derivatives was specific for males in vitro, while Shanta and Meerovitch (1970) found that low concentrations of farnesyl methyl ether inhibited development of male copulatory appendages. This reintroduces the factor, previously discussed, that any results observed depend on the exact concentration of hormone extract applied.

The results obtained in this series of experiments demonstrated the effects of corpora allata and juvenile hormone on the fecundity of both H. diesingi and L. appendiculata; while the corpora cardiaca exerted no such effect. However, Nadakal and Nayar (1968) published results on the effect of implantation of corpora cardiaca into P. americana on the fecundity of three unidentified species of thelastomatids within them. These workers used only five experimental insects and implantation only affected the nematode egg counts in the faeces of two of them.

PART II

The physiological interactions between the nematode - bacterium complex DD-136 strain of *Neoaplectana carpocapsae* and it's hosts.

Introduction

The nematode used in this series of experiments was the DD-136 strain of Neoaplectana carpocapsae Weiser. Recently there has been a great deal of discussion concerning the taxonomy of the 19 species of the genus Neoaplectana, Steiner (1929) (Rhabditoidea: Steinernematidae). The nematode in question has, in the past, been referred to as N. carpocapsae, DD-136 and N.dutkyi. Poinar, Hansen, Yarwood and Weiser (1972) clarified the status of this particular nematode and only the main points of the controversy are reported here.

In 1955 Weiser published his description of N. carpocapsae from diseased codling moth larvae in 1953 in Czechoslovakia. In the same year Dutky and Hough (1955) found a nematode resembling N. chresima, Steiner from dead codling moth larvae in Virginia, U.S.A., this was never described but became widely known as DD-136. In 1964, Schmiege compared the morphological features of Weiser's nematode and DD-136 and decided that they were probably the same. This was followed by work by Jackson (1965) who reported morphological and physiological differences between the nematodes, he first introduced the taxon N. dutkyi. In an attempt to solve this problem crosses of the American and Czechoslovakian strains were made by Poiar (1967), Hansen, Yarwood Jackson and Poinar (1968) and Stanuszek (1972). Allof these workers showed the nematodes to be conspecific. However, Turco, Thames and Hopkins (1971) claimed N. carpocapsae, Weiser and DD-136 were separate species on the basis of morphological variation. Poinar, Hansen, Yarwood and Weiser (1972) after taking into account all of the available information dismissed the claims of Turco et al and concluded that the DD-136 nematode should remain a strain of N. carpocapsae Weiser.

The DD-136 strain of *N. carpocapsae* first gained popularity as an instrument for the biological control of insects in 1959 (Dutky 1959). It has given promising results against agricultural pests both above and below ground and its use in biological control of medically important insects in aquatic habitats has been investigated (Briand and Welch, 1963). It has been possible to consider *N. carpocapsae* in the field of biological control because of its wide host range (Niklas, 1967) and the ease with which it lends itself to mass propagation.

The nematode is cultivated in laboratories mainly *in vivo* by passage through the larvae of *Galleria mellonella*, although stock cultures have been successfully kept for several years *in vitro*.

The larvae of *G. mellonella* are very susceptible to infection by these nematodes. Poinar and Himsworth (1967) have shown that the insect haemolymph shows almost no defensive reactions against the third stage infective larvae. The juvenile nematodes usually enter the host haemocoel by burrowing directly through the gut wall. Once in the haemocoel the nematodes develop to the adult state and mate. The mature females produce masses of eggs which usually hatch inside her body. The juveniles produced may reach the adult stage, mate and produce a new generation in the same host. The juveniles which arise from the second generation adult females together with some of the first generation juveniles may not complete their development but, as third stage infective larvae, leave the host cadaver.

An unusual aspect of these nematodes is that the infective larvae carry a bacterium, *Achromobacter nematophilus* in the ventricular portion of the intestine. As soon as the larvae reach the host haemocoel, the bacteria are released through

the anus of the nematode. In the haemocoel the bacteria rapidly multiply setting up a fatal septicaemia in the host.

Attempts at practical application of nematodes must be preceeded by laboratory tests which provide information as to whether the nematodes obtained from G. mellonella are capable of adapting themselves to new hosts. To test their ability a series of experiments has been carried out using a range of host species. Several facets of the invasive process have been studied; the influence of the number of infective larvae on development of the nemas in the host and on the mortality of the hosts; the influence of the size of the initial dose on the number of developing giant females; the influence of the hosts body weight and stage of development on the density of giant females produced and on the production of larvae by them. The physiological state of the host was also varied by neurosurgery and the subsequent effect on the nematode development investigated. The interrelationship between the nematodes and the bacteria was also studied. The other phenomena which were observed included the effect of temperature on the duration of the life cycle of the nematodes together with the number of nematode generations which a particular host could sustain before migration of invasive larvae into the environment.

After carrying out these experiments it was possible to design field experiments in which the invasive larvae of the nematode were used to their best advantage against the susceptible stages of Scolytus scolytus in Elm trees.

A. Comparison of techniques for the mass propogation of the DD-136 strain of Neoaplectana carpocapsae.

Materials and Methods.

A suspension of infective stage larvae was obtained from D.P. Harlam, U.S Delta States Agricultural Research Centre, Stoneville, Mississippi, U.S.A. Both in vivo and in vitro methods were used for the cultivation of the nematode.

1. In vivo method.

Inocculation chambers (Dutky, Thompson and Cantwell, 1964) were set up. These consisted of disposable petri dishes lined with damp filter paper. Twenty Galleria mellonella larvae (av. wt 90mg) were added to each dish which contained 0.5ml nematode suspension (20,000 infective stage larvae/ml). The dishes were sealed and held at 32°C overnight then placed in an incubator at 24°C for 5 days. At the end of this period the insect larvae were removed and placed in nematode traps. The traps used were modified versions of those devised by White (1929) and Dutky et al (1964). They consisted of autoclaved enamel trays in which were laid filter paper covered half petri dishes open side down. The infected G. mellonella larvae were placed on the filter paper. Dilute formaldehyde (0.001%) was added to each tray to half the height of the petri dish halves. The trays were covered with foil and placed in an incubator at 24°C. The nematodes were harvested from the trays twice a week. After each harvest, fresh formaldehyde was added to the trays. The harvested nematodes were allowed to settle in a flask and the solution decanted. A known volume of formaldehyde solution could be added and the number of nematodes in the suspension

counted. Table 47 shows the yield of N. carpocapsae infective stage larvae from G. mellonella under these conditions.

2. In vitro methods.

(a) Test tube slants were made according to the method of Dutky et al (1964) but to each slant was added ¾cm cube of pigs' liver prepared by dicing the cooked liver and autoclaving (Glaser, McCoy and Girth, 1942). Each tube was then inoculated with 0.2 - 0.25ml nematode suspension (40,000 nemas/ml) which had previously been surface sterilised as recommended by Dutky et al (1964). These cultures were kept at 24°C. A climax population of infective stage larvae was obtained every 3 weeks. Table 47 shows the average yield of N. carpocapsae cultured in this way.

(b) The second in vitro method was identical with that described above with the modification that sterilised petri dishes were used to hold the culture medium containing the nematodes. The average nematode harvest per dish can be seen in Table 47.

3. Storage in infective stage larvae of N. carpocapsae.

The infective stage nematodes were stored in shallow dishes containing 0.001% formaldehyde in tap water and covered with parafilm. The nematodes were kept at 4°C and oxygenated every month by bubbling compressed oxygen into the trays for 1 minute. According to Dutky et al (1964), kept in this state, the larvae have a shelf life of 5 years. In order to check this phenomenon during the course of the experiments every 6 months for 2 years approximately 5,000 ensheathed third stage larvae were harvested from liver cultures and washed several times in distilled water.

Then they were stored in the usual way with not more than 1,000 larvae in each tray. The viability of the nematodes which had been stored for 6 months, 1 year, 1½ years and 2 years was checked in two ways. First, the number of active larvae per tray was used as an estimate of survival. Secondly, the capacity of the stored nematodes for maturation and reproduction was tested by adding samples of 100 of the active nematodes to liver slants and following their development.

Results.

The cultive method results can be seen in Table 47. The in vivo method gave a higher hield of nematodes than either of the in vitro methods.

Table 47. Yield of infective stage larvae of N. carpocapsae in vivo and in vitro

In vivo	3.5×10^6
In vitro method 1.	2.5×10^5
In vitro method 2.	2.5×10^5

The survival experiments showed that after 1 year 90% of larvae were active in water at room temperature, while only 80% of the nematodes which had been kept for more than one year were active under the same conditions.

When nematodes of one year old and less were placed in the cultive medium first stage larvae appeared after 7 days and the critical population level after 2 weeks. However, with increasing age some populations failed to reproduce and if they did so the number of offspring was reduced.

Discussion.

The quickest method of producing the largest number of nematodes was by passage through G. mellonella larvae. The disadvantages of this method lay in the fact that it was time consuming, as a culture of G. mellonella larvae had also to be reared, and that stacking of the trays took up a great deal of space. The in vitro methods circumvented these obstacles. Both in vitro methods yielded the same number of larvae. The petri dish method had, however, the advantage that all stages of the nematode could easily be picked out of the culture for use in experiments.

The effects of storage on N. carpocapsea agree with those of Jackson (1973) who looked at N. glaseri. He too found that reproductive capacity decreased when the nematodes were more than one year old. As he suggested it is necessary to keep a careful record of the nematodes to be used in experiments.

B. The susceptibility of insect species to N. carpocapsae

Materials and methods.

The nematodes were mass reared by passage through G. mellonella as described. Before use in the tests the larvae were washed twice in distilled water. Specimens of Scolytus scolytus were provided by C. Walker, Forestry Commission, Alice Holt, Wrecclesham, Surrey. The other insects used, Periplaneta americana, Tenebrio molitor and Ephestia Kühniella were taken from stock cultures at Imperial College. For each test, at least ten insects were placed into each of six petri dishes containing a small amount of suitable food. The six dishes were paired at random. Three dishes were test inoculated with approximately 500 infective stage nematode larvae in 2ml of

distilled water. The three other dishes received 2ml of distilled water. The experiments were carried out in triplicate in a constant temperature room at 24°C for 1 week.

At the end of the experimental period the number of dead insects was noted and all of the insects were dissected. The percentage of the insects from the test dishes that carried haemocoelic infections was counted. The mean values of numbers of dead insects in the control and test dishes were compared. As the samples used were small, it was necessary to test that the variance of each sample was the same, this was done by a variance ratio test. When the variances were assumed equal a t test was carried out. When it was found that the variances could not be assumed to be equal a modified t test was carried out (Davies, 1971).

Results

With the exception of adult female P. americana, all of the insects tested were highly susceptible to infection by N. carposapsae. A significant number of the insects died as a result of septicaemia induced by the presence of the nematode borne bacteria in the haemocoel.

The percentage of T. molitor larvae parasitised in the three trials were 63, 50 and 60% (Table 48). The parasitised insect cadavers retained their colour but became flaccid. Parasitism of 83, 70 and 73% was found in T. molitor adults (Table 48), while the external appearance of the insect cadavers remained unchanged. High percentages of parasitism were recorded by both larvae and adults of S. scolytus· 97, 80 and 80% and 93, 77 and 80% respectively (Table 48). Parasitised larvae became flaccid and turned brown, while the adult insects

which had been killed by the parasite showed no outward sign of decomposition. A high incidence of parasitism, 93, 80 and 93% was recorded for E. kühniella larvae (Table 48). A greenish discolouration and flaccid appearance characterised the nematode infected larval cadavers. In E. kühniella, the developing nematodes were concentrated in the head capsule and anterior portion of the larvae. This contrasted with the distribution present in the other insects where the nematodes were found throughout the entire body cavity.

The haemolymph of infected T. molitor, S. scolytus and E. kühniella contained nematodes at all developmental stages. However, adult female P. americana showed no Neoaplectanid infection in the haemocoel, the nematodes were found only in the gut and faeces. In both instances the nematodes were still in the ensheathed infective stage. No parasitism was recorded for P. americana (Table 48).

Table 48. Number of dead larval and adult insects after exposure to N. carpocapsae for 1 week.

Species tested		Number dead Mean ± S.E		Variance ratio	% Parasitised
Tenebrio molitor larvae	Test	5.444	0.915	67.750	63,750, 60
	Control	0.111	0.111		
Variances not assumed equal		t = 5.789 (with 8 D.F) P<0.001			
Tenebrio molotor Adults	Test	7.667	0.624	9.692	83, 70, 73
	Control	0.889	0.200		
Variances not assumed equal		t = 10.348 (with 10 D.F) P<0.001			
Scolytus scolytus larvae	Test	6.778	1.077	20.889	97, 80, 80
	Control	0.333	0.236		
Variances not assumed equal		t = 5.844 (with 9 D.F.) P<0.001			
Scolytus scolytus adults	Test	7.667	0.707	18.000	93, 77, 80
	Control	0.333	0.167		
Variances not assumed equal		t = 10. 094 (with 9 D.F) P<0.001			
Ephestia Kühniella larvae	Test	16.667	0.687	17.000	93, 80, 93
	Control	0.333	0.167		
Variances not assumed equal		t = 23,099 (with 9 D.F) P<0.001			

Table 48 (continued).

		Number dead		Variance	%
		Mean	± SE	ratio	Parasitised
Periplaneta americana adult	Test	0.333	0.167	2,250	0, 0, 0
	Control	0.111	0.111		

Variances assumed equal t = 1.109 (with 16 D.F) P ?0.1

Discussion

The results showed that with a single exception all of the insects tested were susceptible to N. carpocapsae. Using this method of infection it is necessary for the host to be at a feeding stage so that infective larvae can be taken up during feeding. It is then necessary for the nematodes to penetrate the host gut to gain access to the haemocoel. The success of the nematode depends, therefore, on the feeding mechanism of the insect, the rate of passage of food through the gut and the kind and structure of the insect gut lining. As intact infective stage nematodes were found in the gut and faeces of P. americana but not in the haemocoel indicated that the nematodes had gained access to the host gut without interference from the insect mouthparts but had been unable to penetrate to the haemocoel. The process of penetration may depend on stimuli from the host or it may be blocked by the structure of the host gut. In the next section this process is investigated further.

The method of infection used in these experiments was useful for a quick assessment of susceptibility but gave no indication of the number of nematodes gaining access to the haemocoel or developing there. In the next section a more specific method of infection of hosts with known doses of nematodes is examined in order that the host-parasite relationship can be further investigated.

C. Introduction of the parasite into hosts.

Introduction.

The method used previously for the infection of the hosts relied on the uptake of the infective larvae by the insect during feeding. This method gave an indication of susceptible hosts but precluded tests on non-feeding stages such as pupae or larvae in pre-moult state. Neither was it possible, using this method, to determine the number of invasive larvae taken up by any particular insect.

In the ensuing experiments two methods of giving predetermined doses of larvae to any one host were tested, both were independent of the feeding of the host. Introduction of the nematodes per os and per anus has also been carried out. These experiments were designed to compare the efficiency of the different methods of inoculation. It was also hoped that they would give some indication as to the site of penetration in the host gut by the nematodes into the haemocoel together with the role of the host in and site of exsheathment of the larvae.

P. americana has been included in the experiments in order to gain information on the lack of susceptibility of this insect to N. carpocapsae.

Materials and methods.

1. Microinjection technique.

Each insect was immobilised by ether in an anaesthesia chamber. Each stage and species of insect was injected per os, per anus or in the case of intrahaemocoelic injections a place was chosen to prevent any loss of haemolymph from the insect

during the process. The equipment consisted of a supported microinjector conbining a sterile, calibrated syringe and a sterile hyperchrome stainless steel hypodermic needle. The syringe was filled by wide bore hypodermic needles. This equipment allowed a known dose of infective larvae to be accurately dispensed into each insect.

2. Inoculation cocoon method.

The test insect was wrapped in filter paper which had been impregnated with a known dose of infective stage larvae in suspension. The insect was inserted into a plastic microcentrifuge tube and the cap secured.

3. Experimental design.

Group I. Twenty G. mellonella and T. molitor larvae, T. molitor and P. americana adults were each injected per os with 100 infective stage larvae. Each insect was dissected after 4 days and the numbers of dead and developing nematodes distributed throughout the gut, haemocoel and faeces counted.

Group II. This group of insects was of the same size and consisted of the same species as Group I. These insects were each injected per anus with 100 infective stage larvae and dissected 4 days later as above.

Group III. Consisted of larvae of T. molitor and G. mellonella. Samples of five insects were ligatured at segmental intervals down their bodies. In all, 8 groups were ligatured at 8 different positions. Each ligatured insect was injected per os with 100 infective larvae in carmine suspension, left for 30 minutes,

1 hour, 3 hours or 6 hours and the progress of the nematodes from gut to haemocoel evaluated.

Group IV. Twenty of each G. mellonella, T. molitor and S. scolytus and P. americana adults were injected intra-haemocoelically with 100 infective larvae. After 4 days the insects were dissected and the number of developing nematodes counted.

Group V. Twenty of each G. mellonella and T. Molitor pupae were infected by the cocoon method with 100 infective stage larvae. After 4 days the insects were dissected and the number of developing nematodes in the haemocoel of each species counted.

Results.

Group I. Ninety six percent of the nematodes injected per os were found to have become established in the haemocoels of G. mellonella larvae and T. molitor adults; 84% in T. molitor larvae but none were found in the haemocoel of P. americana. When the insect guts were examined discarded third stage larvae sheaths were found in G. mellonella and T. molitor larvae and T. molitor and P. americana adults. Sixty percent of the nematodes found in the gut of P. americana retained their sheaths. Inactive nematodes were found in the faeces of T. molitor larvae and P. americana adults. Cast sheaths were also found in the faeces of T. molitor and G. mellonella larvae and T. molitor adults.

Group II. After 4 days no nematodes were found in the haemocoel of any of the injected insects except in one G. mellonella larva in which 3 nematodes were found. In the other insects, nematodes which had been injected were found in the hind gut

and faeces but non had exsheathed. Some of the nematodes present in the hind gut were still active.

Group III. When ligatured G. mellonella were injected per os, penetration of the majority of the nematodes occurred in the first 30 minutes of the experiment via the gut wall in the anterior third of the alimentary tract. Penetration occurred in the same region of the tract of T.molitor but the process was slower. It was only after 1 hour that the majority of the nematodes had penetrated into the haemocoel. After 30 minutes ensheathed nematodes were found in the haemocoel.

Group IV. After intrahaemocoelic injections between 90-94% of all nematodes injected began to develop except in the case of P. americana where only 15% of the nematodes became established. The other nematodes found in the haemocoel were totally immobilised.

Group V. Variable results were obtained in this experiment. When G. mellonella was exposed to the nematodes, the number found in the haemocoel of individual insects varied between 0 and 12. For comparison, the numbers found inside of T. molitor pupae fell in the range 0 - 33.

Discussion.

Penetration by the nematodes into the haemocoel of susceptible insects occurs in the arterior part of the host gut. It is equally obvious that the host gut can represent an insurmountable barrier to this process. The guts of G. mellonella larvae and T. molitor adults seem to be easily penetrated. The

decreased penetration of the foregut of T. molitor larvae indicated that this process is hindered somewhat by the gut wall itself and the process may be affected by the rapid passage of food through the tract of these larvae, so that although capable, the nematodes do not have the opportunity to penetrate.

From the first experiment it could be seen that the majority of nematodes exsheathed in the gut of the host. This indicated that this process might be a necessary preliminary or necessary part of penetration. Injections directly into the haemocoel, however, showed that exsheathment and subsequent development could also occur.here. The most pertinent fact to emerge from this experiment was that infective larvae of N. carpocapsae could survive and exsheath in the haemocoel of P. americana, so indicating that the structure of the gut precludes the initial essential penetration. This is probably due to the thick periotrophic membrane which lines the tract for the main part. It could have been interesting to have tried to infect these insects per os during a period of moulting to see if this indeed was the case.

Introduction of the nematodes per anus showed that the nematodes could not penetrate this region of the digestive tract. Although some were found to survive there, the majority were pushed out of the host body with the faeces. The fact that some nematodes were found in the haemocoel of a single G. mellonella larva does not invalidate this idea. In the final experiment it was shown that direct penetration could occur and it possible that nematodes had migrated from the anus of this insect and penetrated from the outside.

The method of this active penetration from the host environment through the exoskeleton into the host haemocoel has

been debated. The pupae of G. mellonella are covered with a hard chitinous integument and the mouth and anus are closed, they are, however, immobile. Both Sandner and Stanuszek (1971) rule out the possibility of the nematodes entering through the tracheal system, however, Weiser (1966) accepts this system as one of the routes of penetration of N. carpocapsae into the larvae of G. mellonella. Access via the tracheal system seems to be the most likely method. However, as the exoskeleton of T. molitor pupae forms a less impregnable integument, it seems likely that active penetration into this insect could occur. This could be the reason why a greater number of nematodes were found in pupae of T. molitor than in G. mellonella. This experiment did show that direct contact is not a very efficient method of infecting these insects.

D. Investigation into the relationship between N. carpocapsae and to hosts.

Materials and methods.

The nematodes were reared in vivo as described previously. Specimens of T. molitor, G. mellonella and E. kühniella were taken from stock cultures at Imperial College, S. scolytus were provided by the Forestry Commission. Known numbers of nematodes were introduced into the insects using the intra-haemocoelic injection technique.

Experiment 1: Influence of the size of the initial dose of the infective larvae on the mortality of the hosts.

Method: At least thirty specimens of each insect were injected with a dose of 10, 50, 100, 500 or 1,000 invasive larvae. The

insects were kept at 24°C. The number of dead insects was counted at intervals of 12 hours. The dead insects were dissected to establish that the nematode - bacterium complex was the cause of death.

Results: The results showed that the greater the initial dose, the greater the rapidity of killing the larvae, pupae and/or adults of the insects tested. The relationships have been shown by means of mortality curves for each of the doses used (Figures 14 - 19).

All doses were lethal for 100% of the larvae and pupae of G. mellonella. Compared with the larvae, the pupae of this insect died three times more quickly with doses of 10 and 50 invasive larvae and four times more quickly with doses of 100, 500 or 1,000 invasive larvae.

T. molitor proved to be far more resistant to the effect of the nematodes. Although all doses proved to be lethal for 100% of the larvae, pupae and adults used in the experiment, the survival times of the insects varied considerably. Doses of 1000 and 500 larvae were twice as effective in killing the adults as the larvae, while the most susceptible stage of this insect was the pupa.

The order of susceptibility for S. scolytus was larvae > pupae > adults. The larvae of E. kühniella were particularly susceptible to doses of 500 and 1,000 larvae. When lower doses were administered the mortality of the insects was comparable to G. mellonella larvae.

Fig. 14

Mortality curve for *G. mellonella* larvae and pupae.

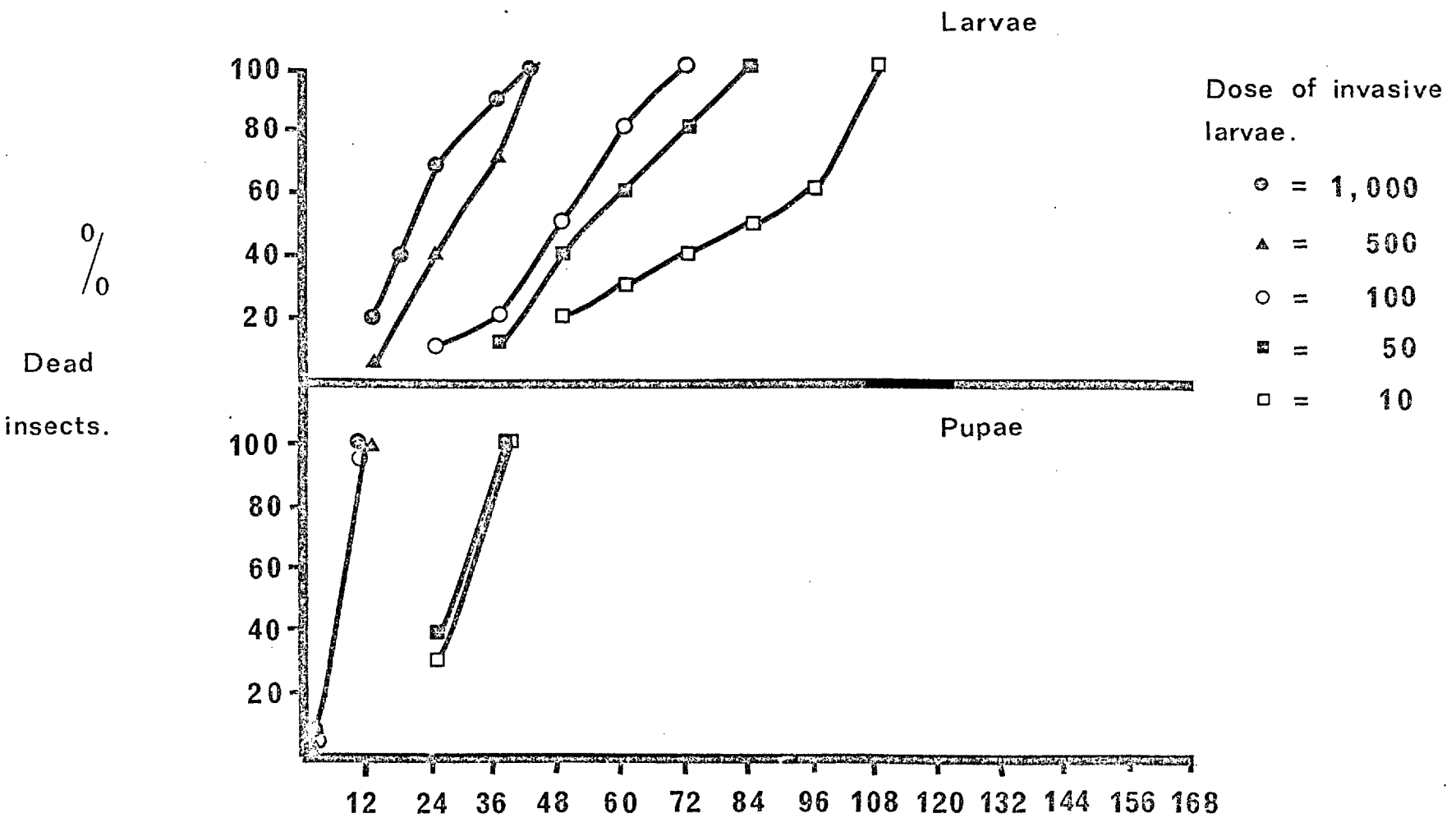

Larvae
Pupae
100
80
60
40
20
12
24
36
48
60
72
84
96
108
120
132
144
156
168
HOURS
% Dead insects.
Dose of invasive larvae.
= 1,000
= 500
= 100
= 50
= 10

Fig. 15

Mortality curve for T. molitor larvae and adults.

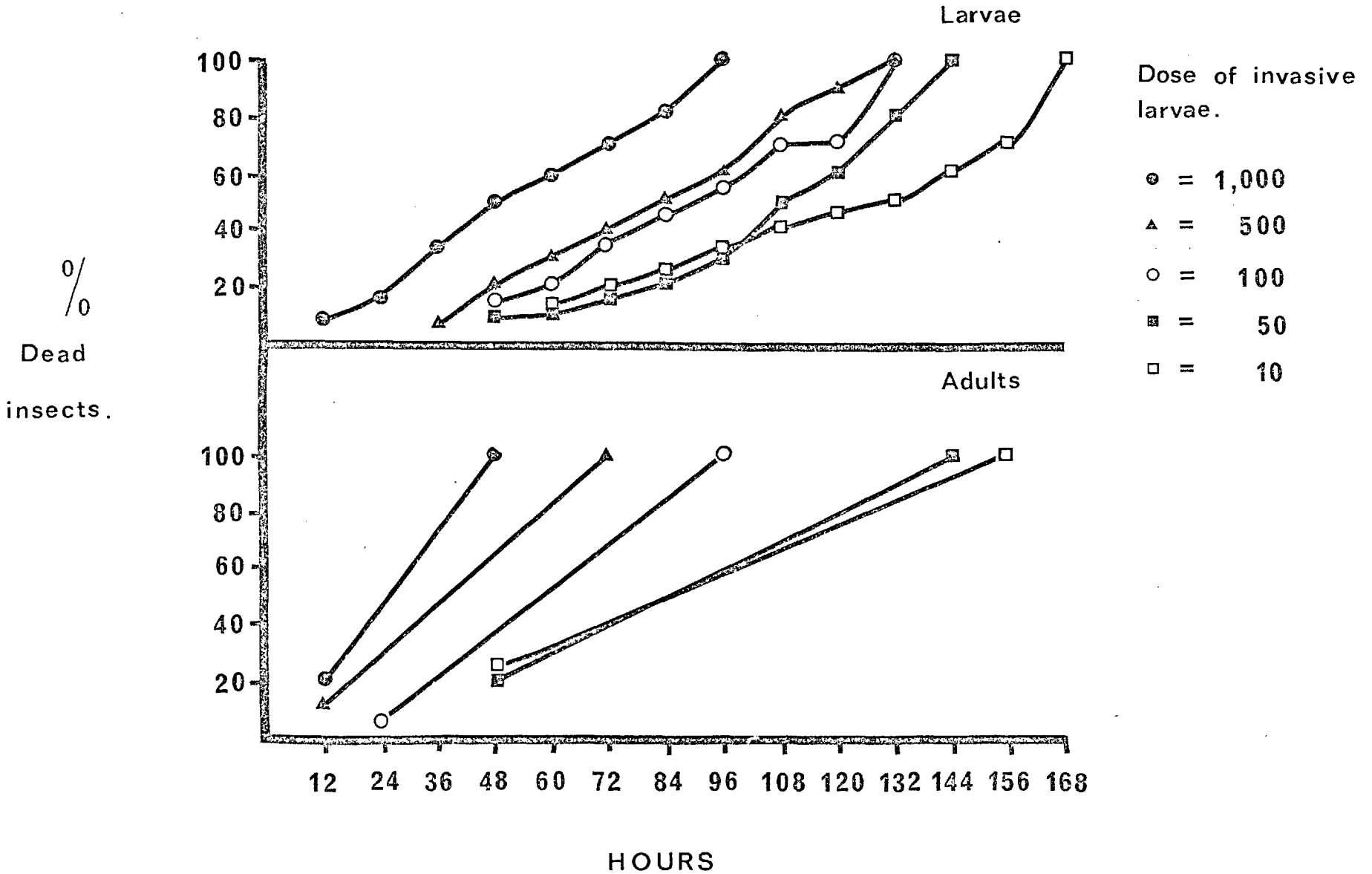

Larvae
100
80
60
40
20
Adults
100
80
60
40
20
12 24 36 48 60 72 84 96 108 120 132 144 156 168
HOURS
% Dead insects.
Dose of invasive larvae.
= 1,000
= 500
= 100
= 50
= 10

Fig. 16

Mortality curve for *T. molitor* pupae.

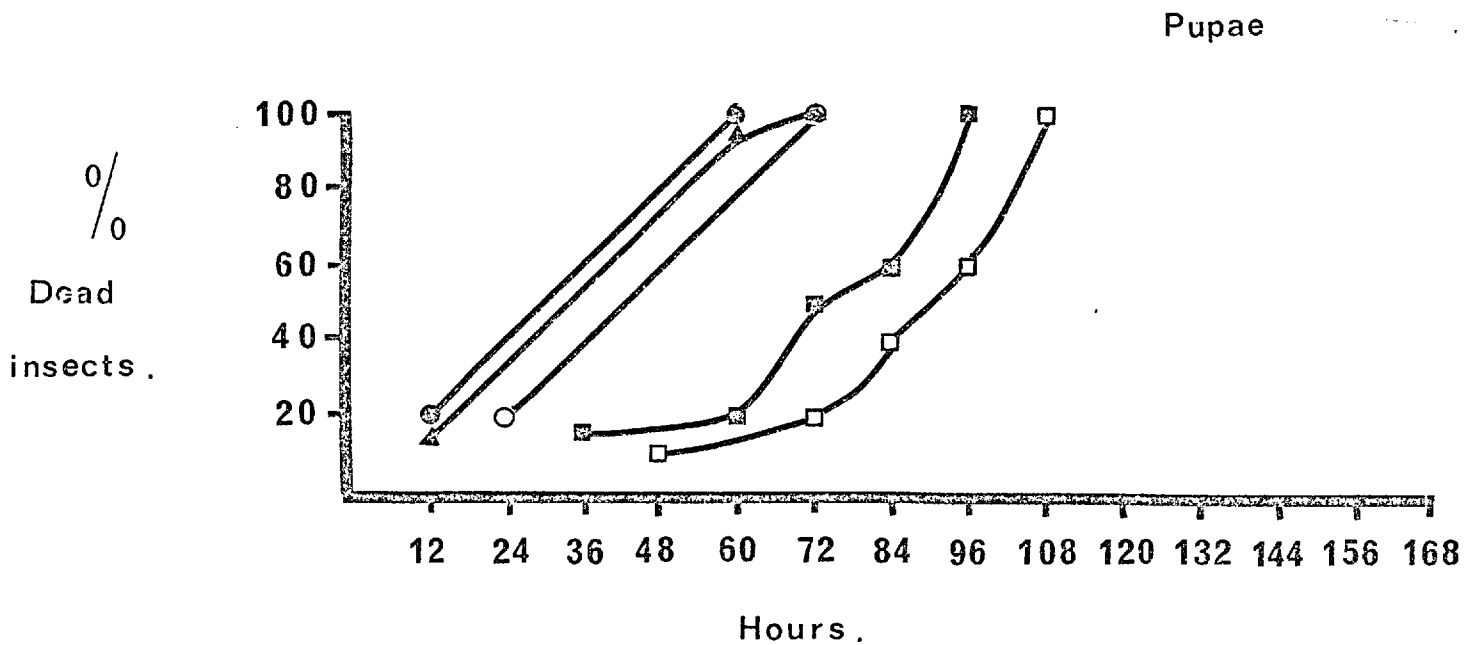

Pupae
%
Dead
insects.
100
80
60
40
20
12 24 36 48 60 72 84 96 108 120 132 144 156 168
Hours.
Dose of invasive larvae.
● = 1,000 ▲ = 500 ○ = 100 ■ = 50 □ = 10

Fig. 17

Mortality curve for S. scolytus larvae and adults.

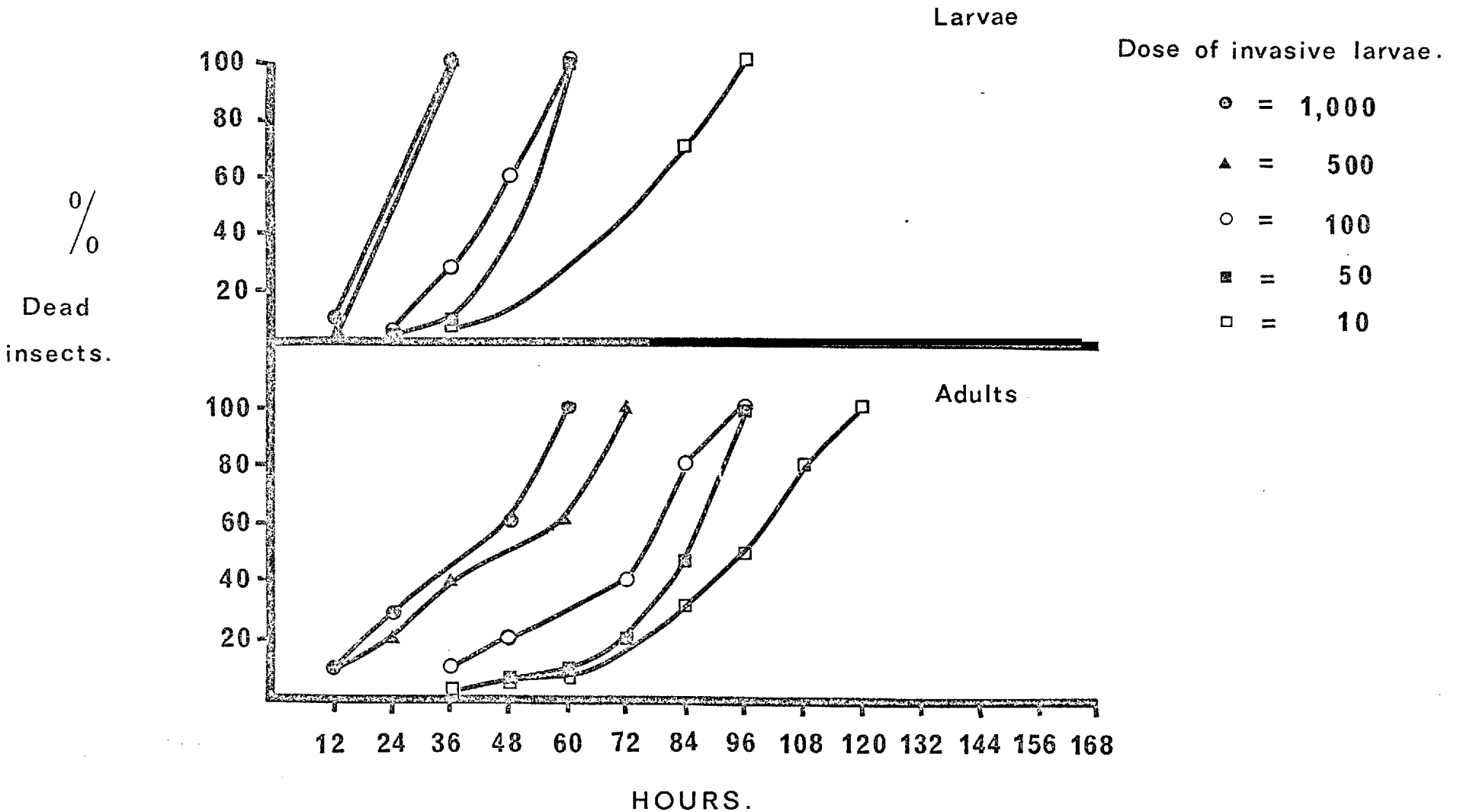

Larvae
Adults
Dead insects.
%
100
80
60
40
20
12 24 36 48 60 72 84 96 108 120 132 144 156 168
HOURS.
Dose of invasive larvae.
⊙ = 1,000
▲ = 500
○ = 100
■ = 50
□ = 10

Fig. 18

Mortality curve for S. scolytus pupae.

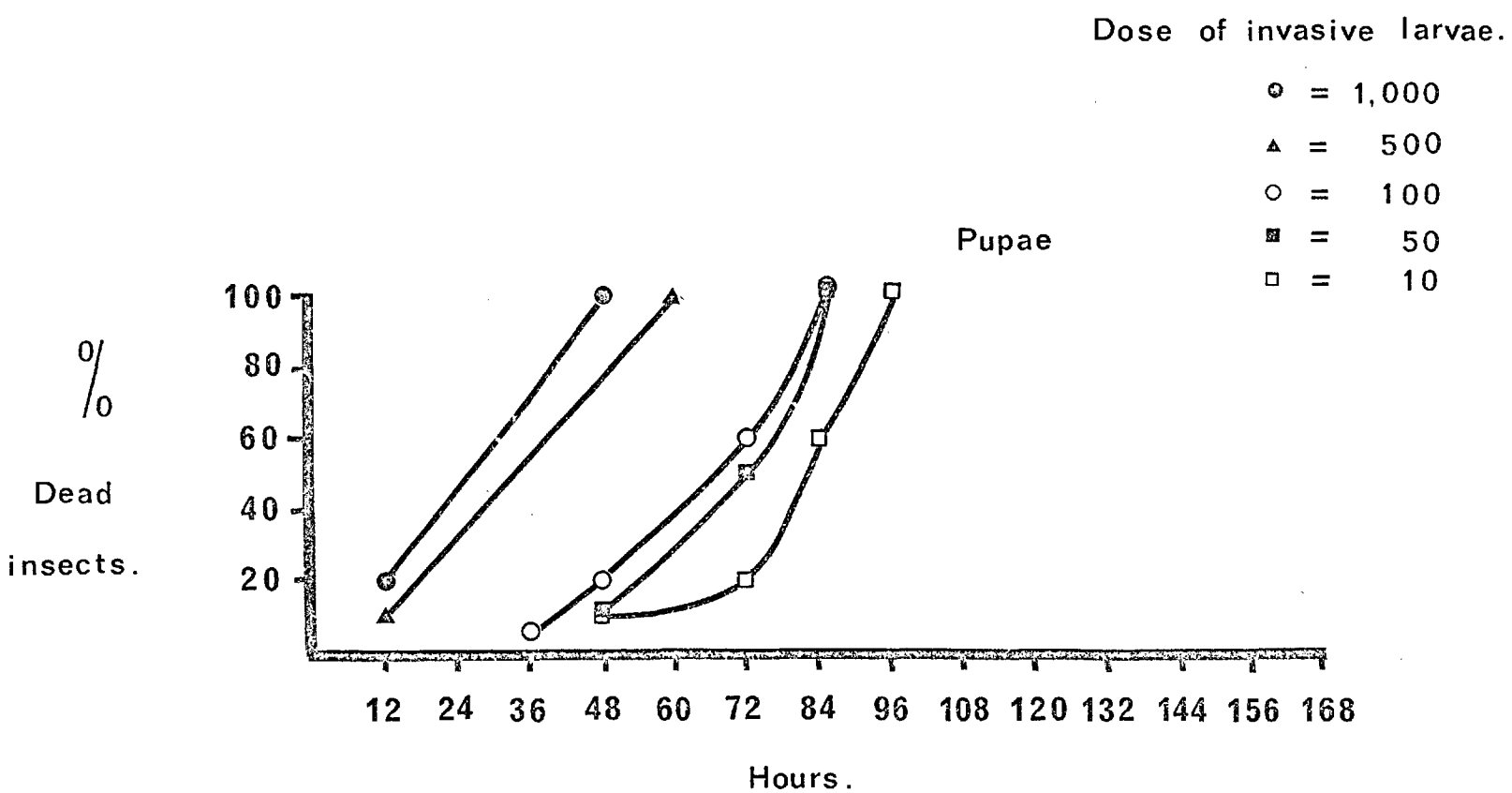
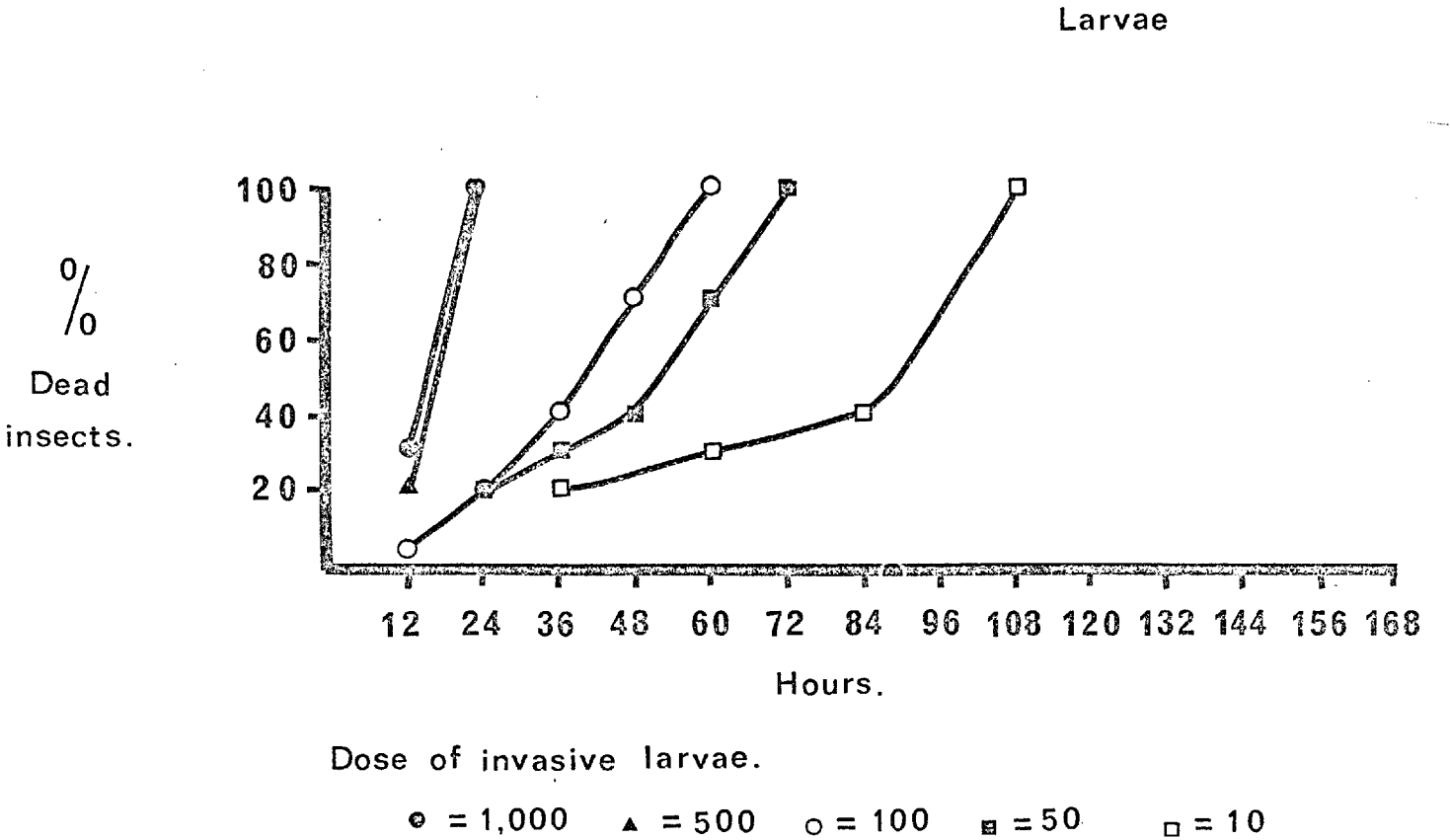

Dose of invasive larvae.
= 1,000
= 500
= 100
= 50
= 10
Pupae
%
Dead
insects.
100
80
60
40
20
12 24 36 48 60 72 84 96 108 120 132 144 156 168
Hours.

Fig. 19

Mortality curve for *E. kühniella* larvae.


Larvae
%
Dead
insects.
100
80
60
40
20
12 24 36 48 60 72 84 96 108 120 132 144 156 168
Hours.
Dose of invasive larvae.
● = 1,000 ▲ = 500 ○ = 100 ■ = 50 □ = 10

Experiment 2. Influence of size of initial dose of invasive larvae on the numberof giant females developing.

Method. Doses of 10, 100 or 1,000 invasive larvae were administered to at least 30 specimens of each insect species and stage. The insects were kept for 6 days at 24°C and at the end of this period were dissected and the size and number of giant females, were measured.

Results. The relationship between the size of the initial dose and the number of giant females developing are given in Table 49.

The number of giant females which develop in hosts under constant conditions varied within wide limits and never attained a value equal to the initial dose. When the larvae of G.mellonella and S. scolytus, the pupae of T. molitor and adults of T. molitor and S. scolytus were given an initial dose of 10 invasive larvae, the maximum percentage of these insects had low classes of numbers of giant females. In contrast, the maximum percentage of S. scolytus pupae had a slightly higher number of females, while the maximum percentage of G. mellonella pupae, T. molitor and E. kühniella larvae had even larger numbers of giant females.

The injection of an initial dose of 100 invasive larvae per host resulted in a far smaller percentage of low classes, the peak of production of giant females lay between 10 - 70 giant females for all insects.

After exceeding the dose of 100 invasive larvae per host, this peak shifted. The maximum percentage of G. mellonella larvae had high numbers of giant females while the maximum percentage of the pupae of this species had lower numbers of giant females. The percentage of T. molitor larvae was evenly distributed over the giant female class range 80 - 600. In T. molitor pupae of the

Table 49 - The influence of size of initial dose on the number of giant females developing.

Host		No. of giant females																				
		1	2	5	8	10	20	50	60	70	80	90	100	200	300	400	500	600	700	800	900	1000
Dose 10																						
G. mellonella	larvae	72*	14	10	4	-																
"	pupae	8	41	49	2	-																
T. molitor	larvae	11	27	55	7	-																
"	pupae	61	35	3	1	-																
"	adults	88	6	4	2	-																
S. scolytus	larvae	86	7	4	3	-																
"	pupae	18	53	27	2	-																
"	adults	73	15	10	2	-																
E. kuhniella	larvae	3	28	62	7	-																
Dose 100																						
G. mellonella	larvae	1	1	5	6	8	42	20	12	2	2	1	-									
"	pupae	1	1	4	6	10	39	24	10	3	1	1	-									

Table 49 (Contd).

Host		1	2	5	8	10	20	50	60	70	80	90	100	200	300	400	500	600	700	800	900	1000
T molitor	larvae	3	5	10	10	12	16	22	12	8	1	1	-									
"	pupae	1	1	4	6	10	39	24	10	3	1	1	-									
"	adults	2	5	5	6	17	25	30	6	2	1	1	-									
S. scolytus	larvae	1	1	3	7	10	31	25	10	8	2	2	-									
"	pupae	1	1	2	8	8	10	12	12	15	28	3	-									
"	adults	1	1	1	6	8	12	42	21	6	1	1	-									
E. kühniella	larvae	1	1	2	2	14	36	20	12	7	4	1	-									
Dose 1.000																						
G. mellonella	larvae	1	1	1	1	1	1	1	1	2	2	2	2	2	2	10	18	22	26	3	1	-
"	pupae	1	1	1	1	1	1	1	1	1	1	1	2	2	16	24	30	10	2	2	1	-
T. molitor	larvae	1	1	1	1	1	1	3	5	5	7	8	8	8	8	8	10	10	2	1	1	-
"	pupae	1	1	1	1	1	1	1	1	1	1	2	12	16	18	18	8	6	6	2	2	-
"	adults	1	1	1	1	1	1	1	1	1	1	1	3	7	10	16	26	14	11	1	1	1

Table 49 (Contd).

Host		1	2	5	8	10	20	50	60	70	80	90	100	200	300	400	500	600	700	800	900	1000
S. scolytus	larvae	1	1	1	1	1	1	2	4	4	4	4	8	10	10	10	10	10	8	6	4	-
"	pupae	1	1	1	1	1	1	1	1	1	1	2	2	6	10	12	12	12	14	12	8	-
"	adults	1	1	1	1	1	1	1	1	1	1	1	1	8	20	20	20	10	8	1	1	-
E. kühniella	larvae	1	1	1	1	1	1	1	1	1	1	1	2	5	8	20	24	15	10	3	2	-

%Percentage of test insects which contained one giant female.

peak was distributed in the 200 - 400 giant female range. In contrast 26% of T. molitor adults had 400 giant females. Again in S. scolytus larvae, pupae and adults, there was no well defined peak of product ion. However, a maximum 24% of E. kühniella larvae produced 500 giant females

Experiment 3. The influence of host body weight and density of giant females on the production of larvae.

Method: All of the insects used were weighed and arranged in classes differing by 20mg. The insects were dosed with invasive larvae and kept at 24°C for 8 days. At the end of this period the insects were dissected and the number of giant females and first and second stage larvae counted.

Results: Figures 20 - 23 show the mean production of larvae. It can be seen that in all cases the maximum yield of larvae was obtained from 50 giant females and the maximum from 200 giant females. However, there were vast differences between the number of larvae yielded by 50 giant females in different insects and insects of different ages. The maximum yields from 50 giant females was always higher in the pupae, compared with larvae of the same species and even higher in the adults as demonistrated by S. scolytus and T. molitor. The yield of larvae from 50 giant females in E. kühniella was significantly lower than any of the other insects.

The number of larvae produced was not directly proportioned to the weight of the insect. Maximum yields occurred in G. mellonella larvae over the weight range 120 - 180 mg, in pupae of the same species over the range 100 - 140mg. In T. molitor and E. Kühniella larvae the yield was fairly standard over the entire weight range.

Fig. 20

The influence of G. mellonella body weight and density of giant females on the production of larvae.

Key

△ = 200 adult females.

◎ = 100

◭ = 50

▣ = 10

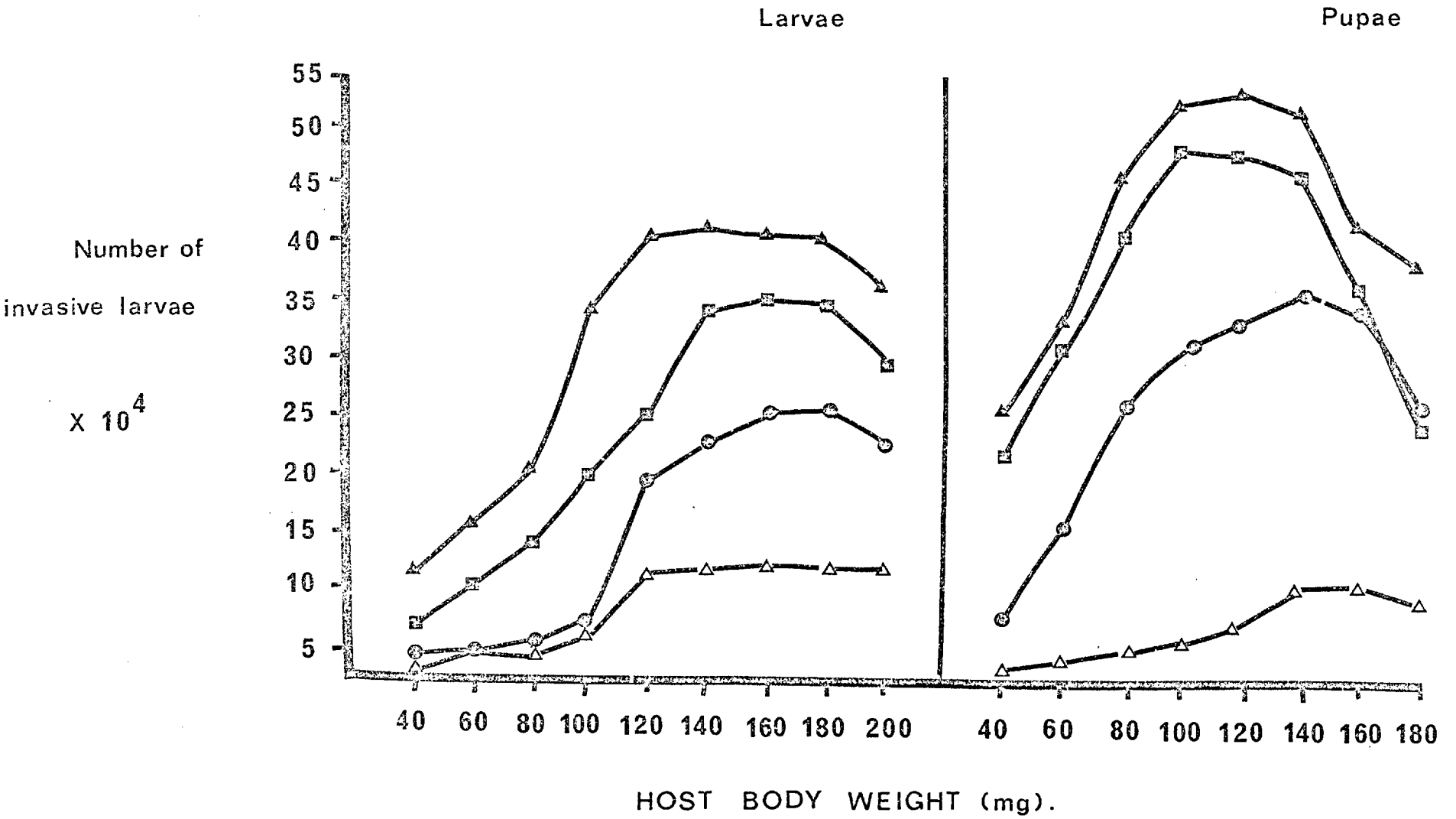

Larvae
Pupae
Number of
invasive larvae
X 10^4
55
50
45
40
35
30
25
20
15
10
5
40 60 80 100 120 140 160 180 200
40 60 80 100 120 140 160 180
HOST BODY WEIGHT (mg).

Fig. 21

The influence of S. scolytus body weight and density of giant females on the production of larvae.

Key

 = 200 adult females.

 = 100

 = 50

 = 10

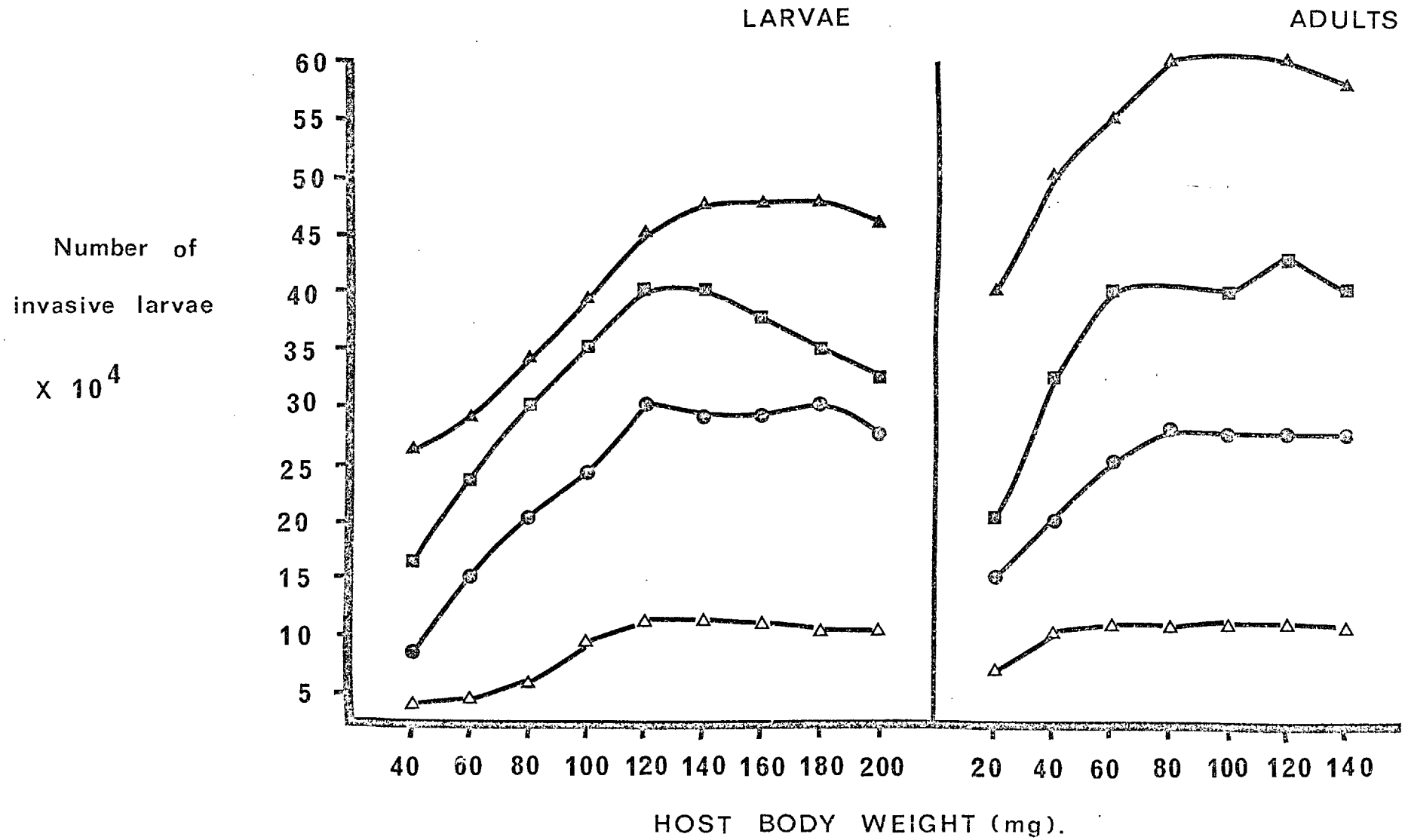

LARVAE
ADULTS
Number of
invasive larvae
X 10^4
60
55
50
45
40
35
30
25
20
15
10
5
40 60 80 100 120 140 160 180 200
20 40 60 80 100 120 140
HOST BODY WEIGHT (mg).

Fig. 22

The influence of T. molitor body weight and density of giant females on the production of larvae.

Key

△ = 200 adult females.
◎ = 100
▲ = 50
▣ = 10

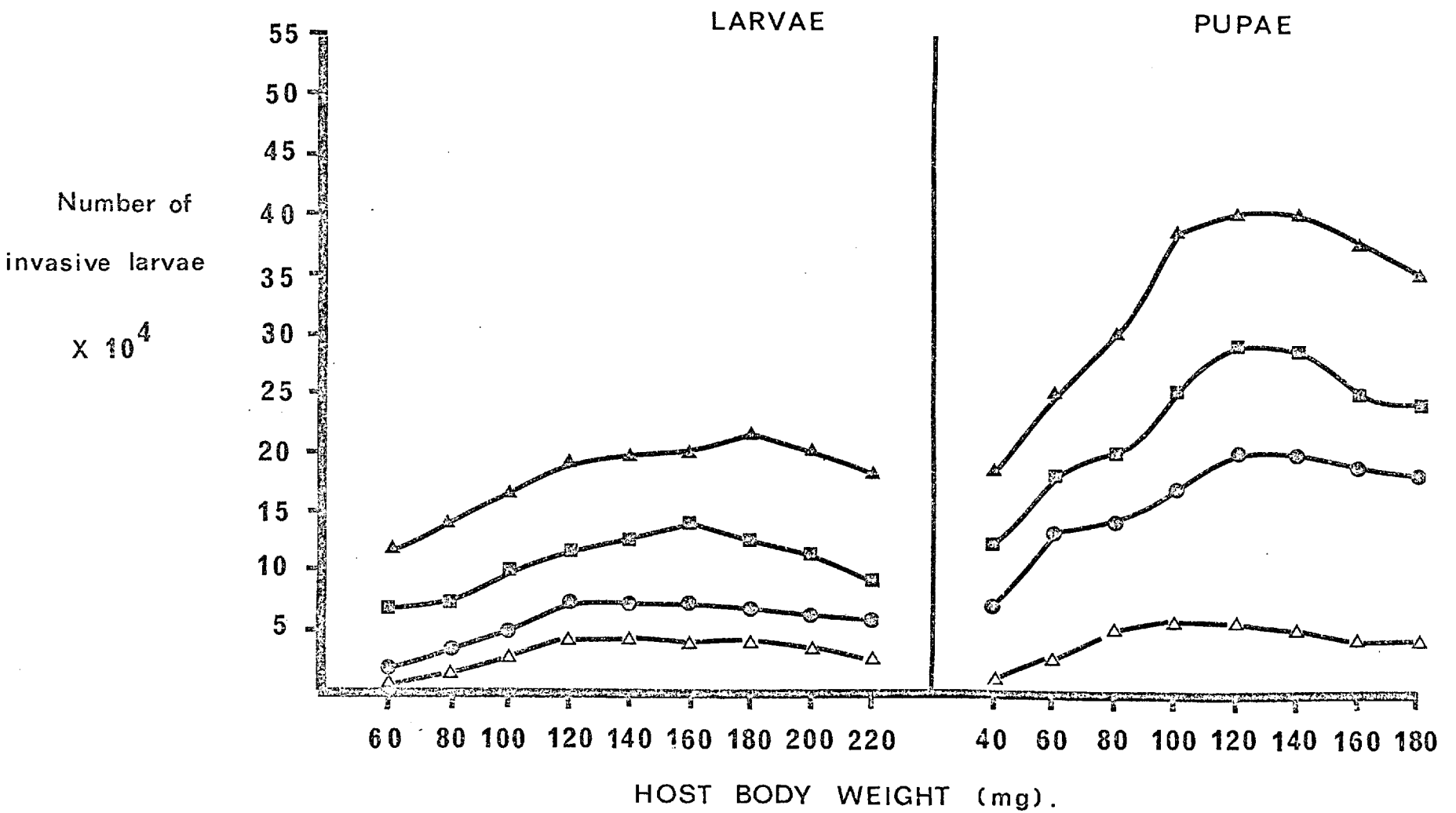
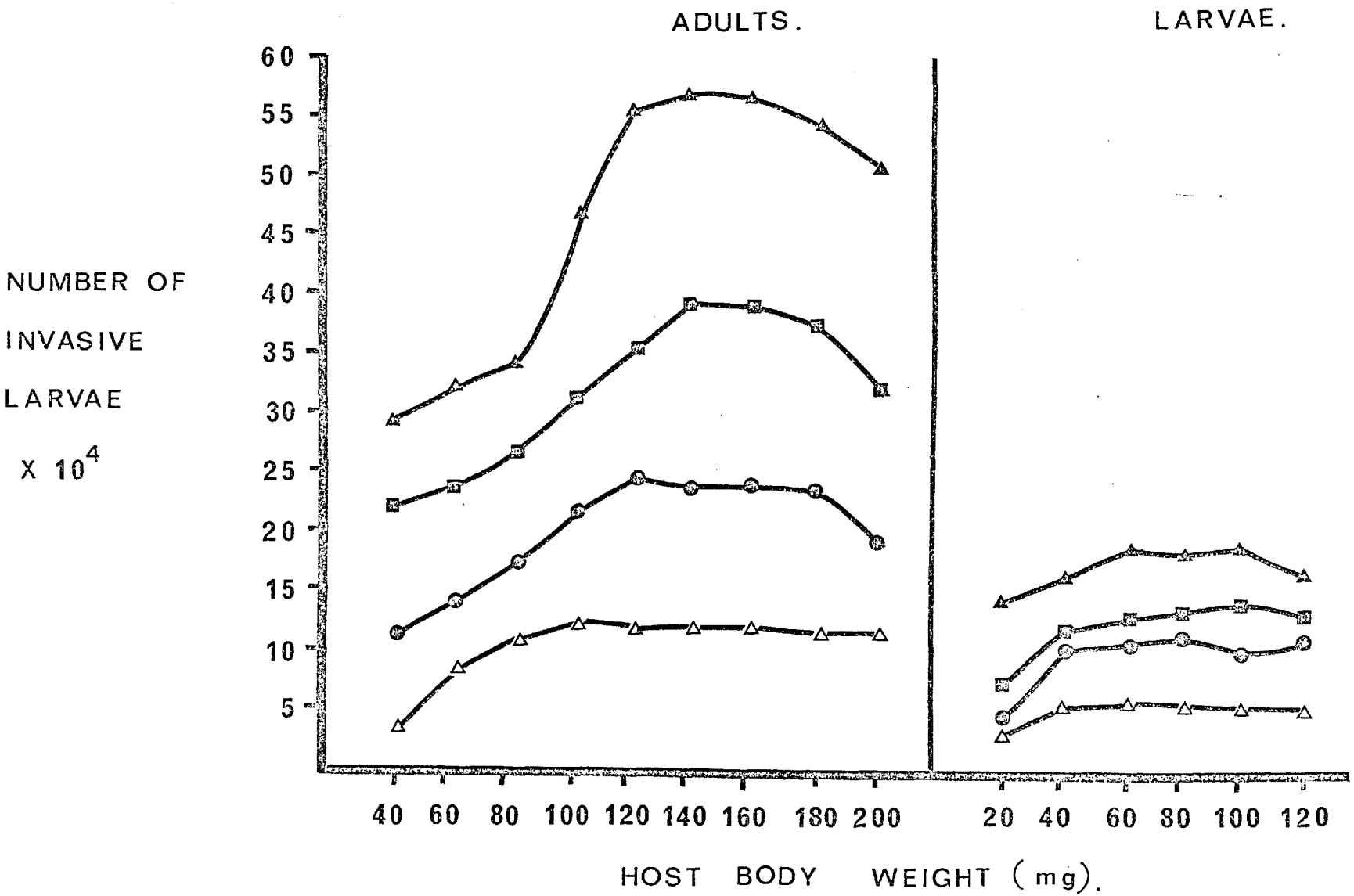

LARVAE
PUPAE
Number of
invasive larvae
X 10^4
55
50
45
40
35
30
25
20
15
10
5
60 80 100 120 140 160 180 200 220
40 60 80 100 120 140 160 180
HOST BODY WEIGHT (mg).

Fig. 23

The influence of *T. molitor* and *E. kühniella* body weight and density of giant females on the production of larvae.

Key

Δ = 200 adult females.
● = 100
▲ = 50
■ = 10


ADULTS.
LARVAE.
NUMBER OF
INVASIVE
LARVAE
X 10^4
60
55
50
45
40
35
30
25
20
15
10
5
40 60 80 100 120 140 160 180 200
20 40 60 80 100 120
HOST BODY WEIGHT (mg).

Experiment 4. The influence of the stage of hosts' development and density of giant females on the degree of mortality of nematode larvae produced.

Method: The insects were dosed and kept as described previously but dissected after 14 days. The number of dead nematode larvae of the first generation progeny was counted.

Results: The degree of mortality is expressed (Figures 24 - 27) in percentage of dead nematodes from the maximum production obtained at a given stage of the hosts development, with a known density of giant females. It can be seen that an increase in the density of giant females results in an increase in the numbers of dead nematode larvae. There is, however, an obvious difference in the levels of mortality in the larval insects compared with the pupal stages. The nematode larval mortality in adult insects, although higher than in the larvae was, in the case of S. scolytus lower than in pupae, while in T. molitor, was of the same order as that in the pupae.

Fig. 24.

Influence of the stage of hosts' development and density of giant females on the degree of mortality of nematode larvae produced.

G. mellonella larvae and pupae.

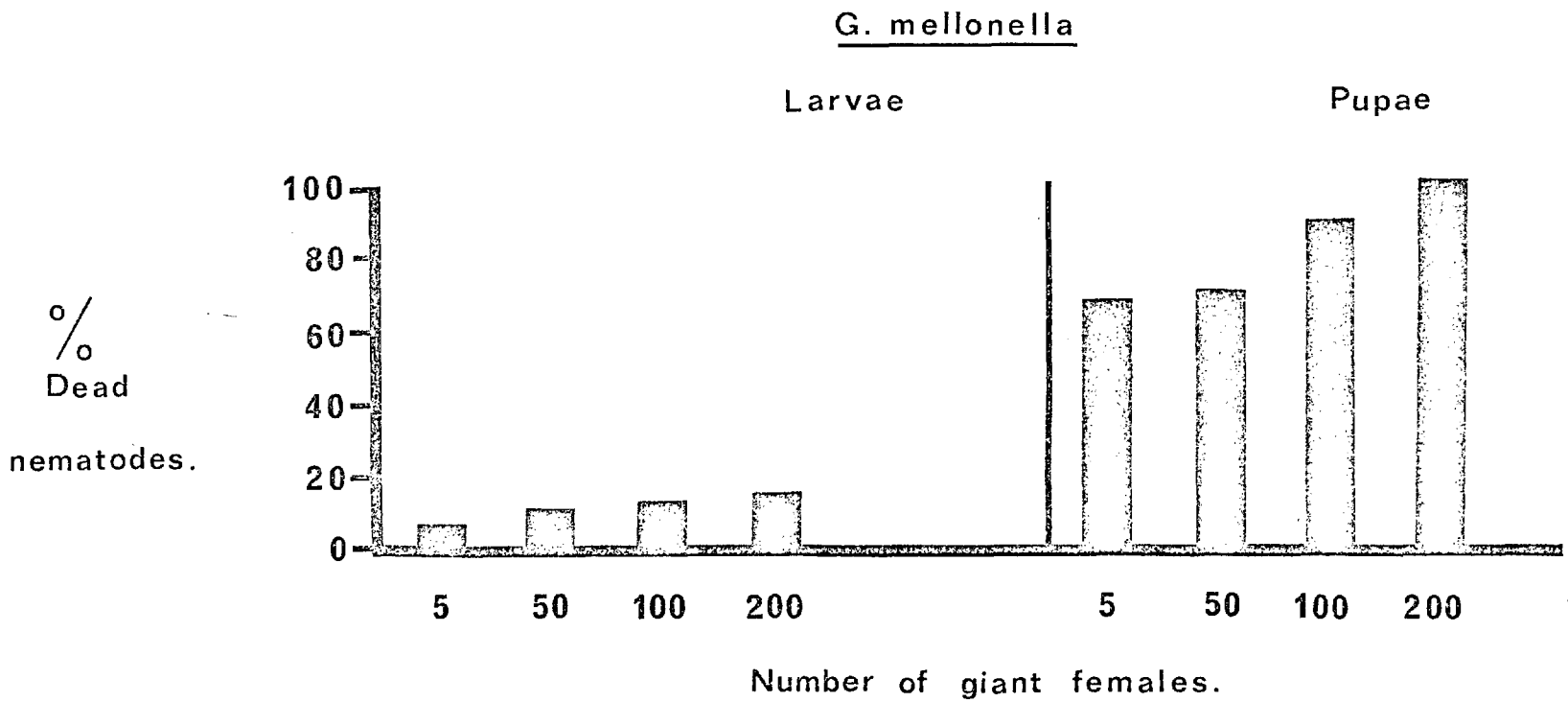

G. mellonella
Larvae
Pupae
100
80
60
40
20
0
% Dead nematodes.
5
50
100
200
5
50
100
200
Number of giant females.

Fig. 25.

Influence of the stage of hosts' development and density of giant females on the degree of mortality of nematode larvae produced.

S. scolytus larvae and adults.

S. scolytus

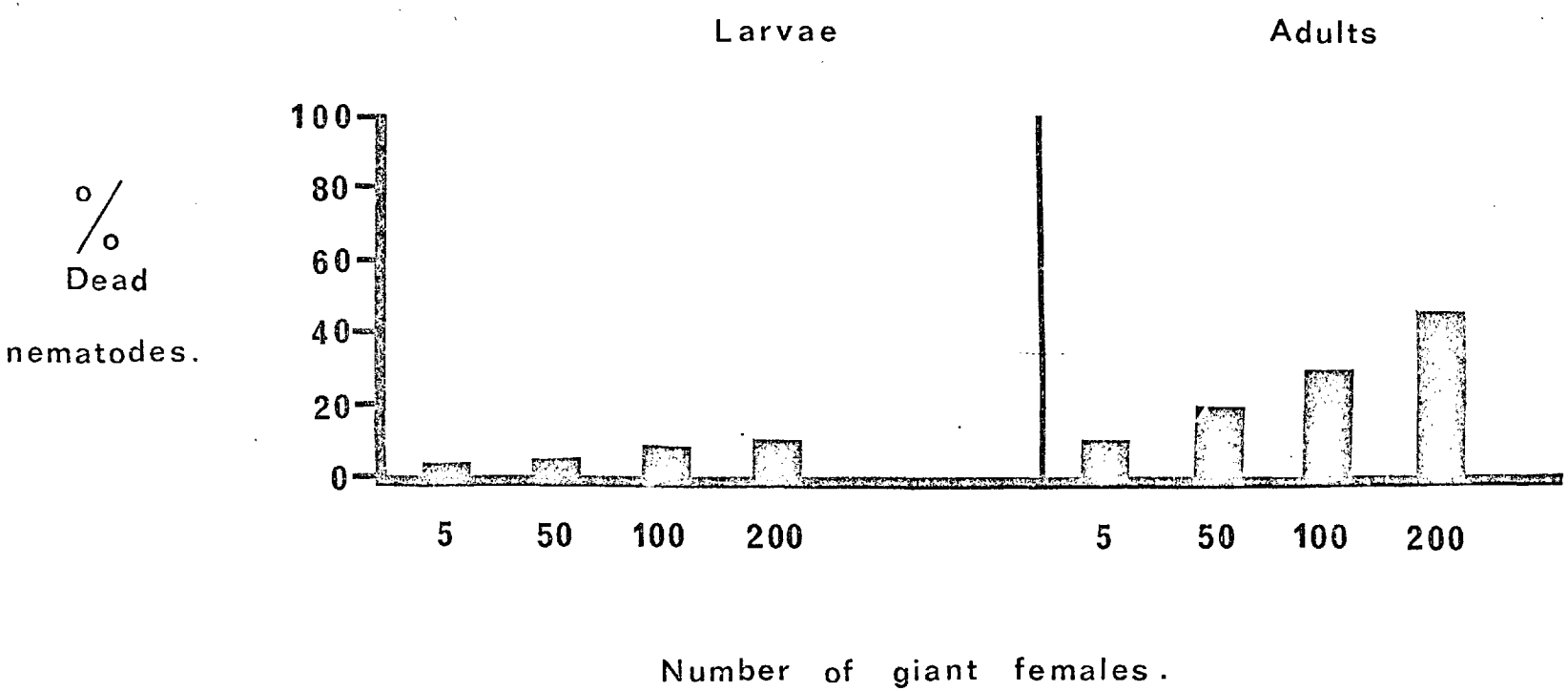

Larvae
Adults
% Dead nematodes.
100
80
60
40
20
0
5
50
100
200
5
50
100
200
Number of giant females.

Fig. 26.

Influence of the stage of hosts' development and density of giant females on the degree of mortality of nematode larvae produced.

T. molitor larvae and pupae.

T. molitor

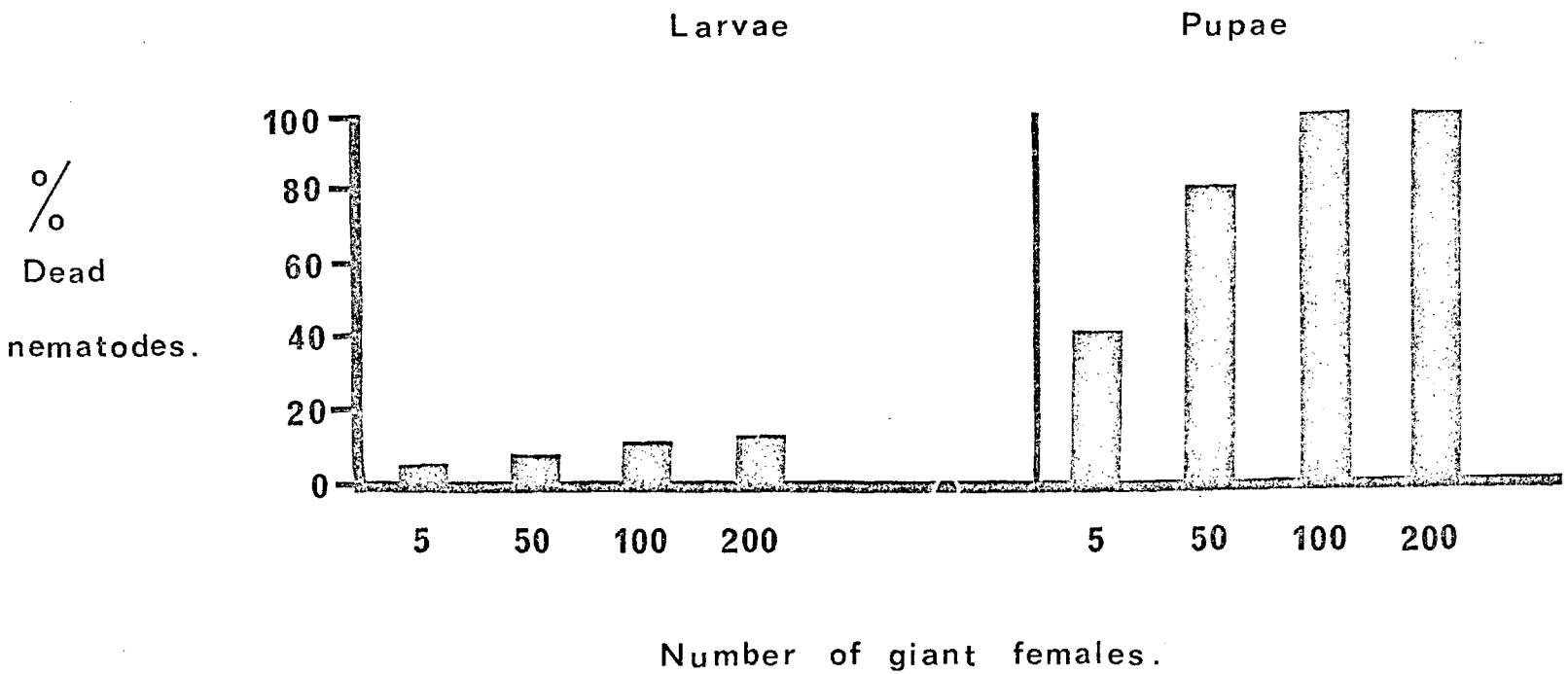
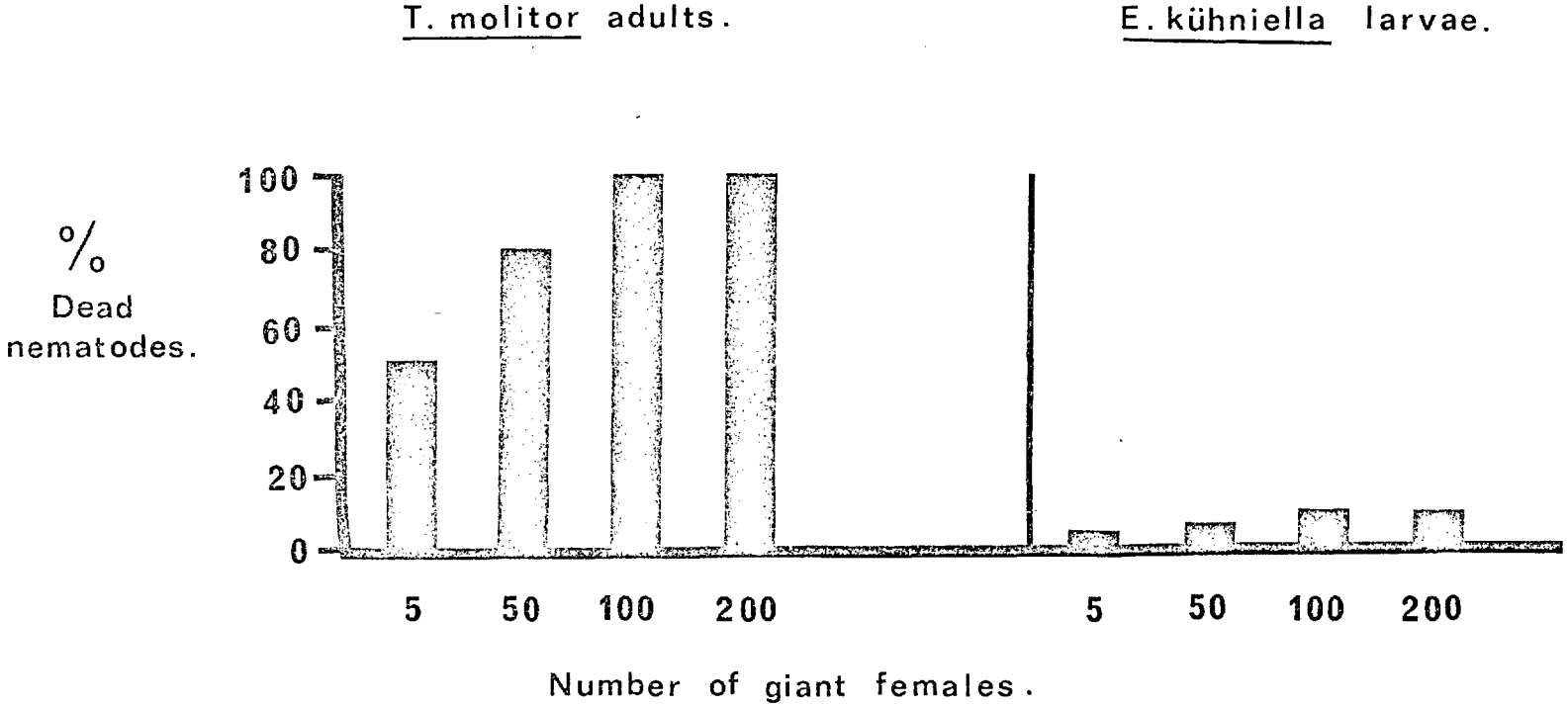

Larvae
Pupae
%
Dead
nematodes.
100
80
60
40
20
0
5
50
100
200
5
50
100
200
Number of giant females.

Fig. 27.

Influence of the stage of hosts' development and density of giant females on the degree of mortality of nematode larvae produced.

T. molitor adults and E. kühniella larvae.


T. molitor adults.
E. kühniella larvae.
%
Dead
nematodes.
100
80
60
40
20
0
5
50
100
200
5
50
100
200
Number of giant females.

Experiment 5. The effect of temperature on the number and duration of generations per host.

Method: Larvae of G. mellonella, of standard weight, were injected with 50 invasive larvae and their divided into three groups. Group 1 was kept at 24°C, Group 2 at 18°C and Group 3 at 4°C. Specimens from each group were dissected at daily intervals and the course of development of the nematodes followed.

Results: The development of N. carpocapsae at 24°C can be seen in Figure 28. Over the 15 day experimental period two generations occurred and a third was initiated. The second generation differed from the first in the duration of the larval fourth stage. However, when the insects were kept at 18°C (Figure 29) only two generations were produced. In the first generation the third and fourth larval stages were prolonged, however, this was not repeated in the second generation. When exposed to 4°C, each stage of the nematode development was prolonged, the progeny of the first generation only being released at 13 days (Figure 30).

Fig. 28.

Development of *N. carpocapsae* at 24° C.

24°C

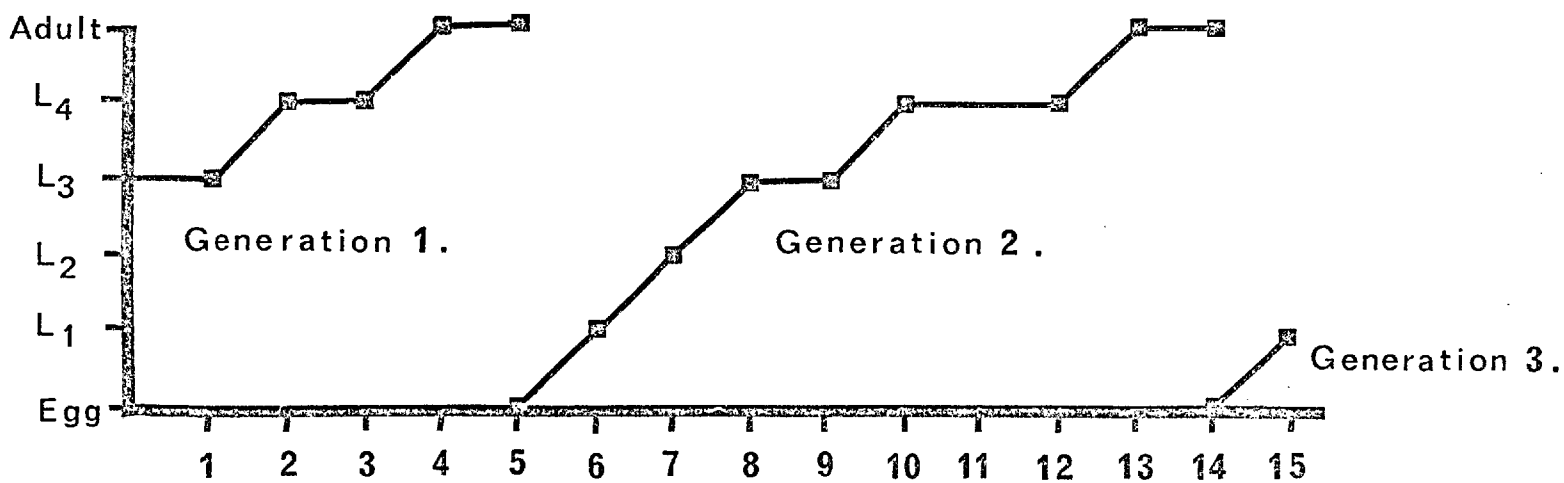

Adult
L4
L3
L2
L1
Egg
Generation 1.
Generation 2.
Generation 3.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days.

Fig. 29.

Development of *N. carpocapsae* at 18°C.

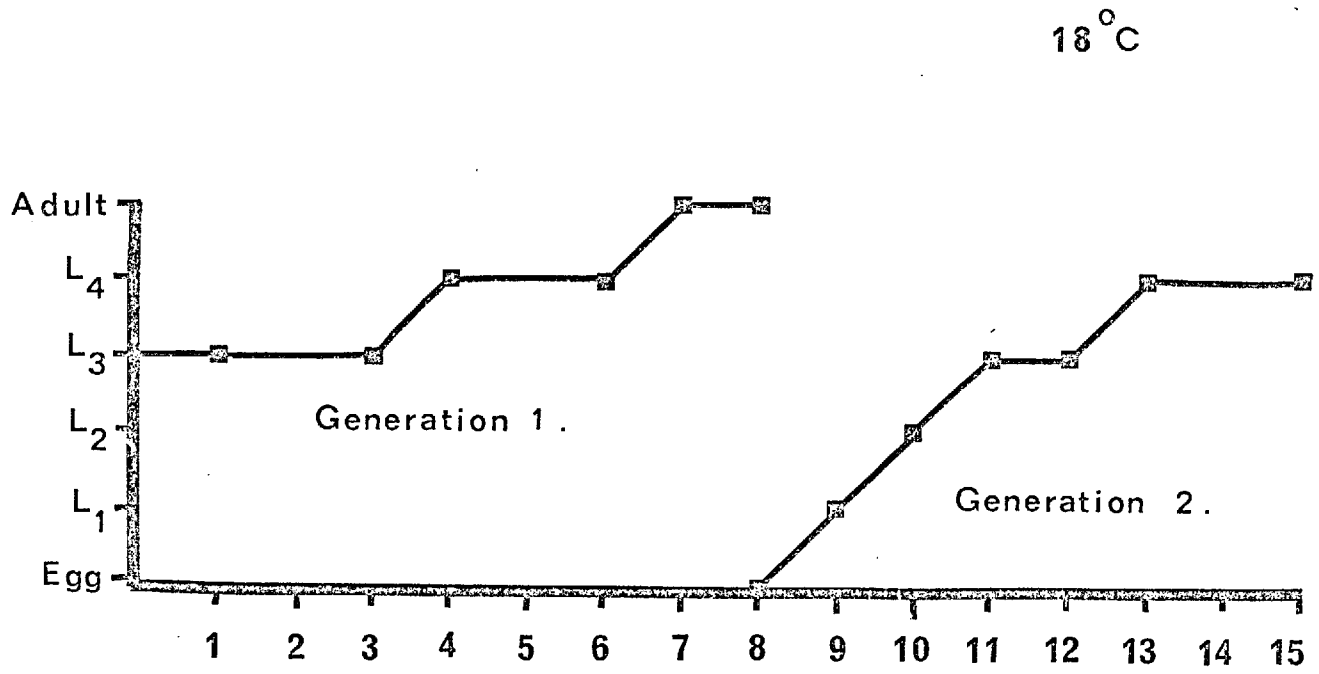
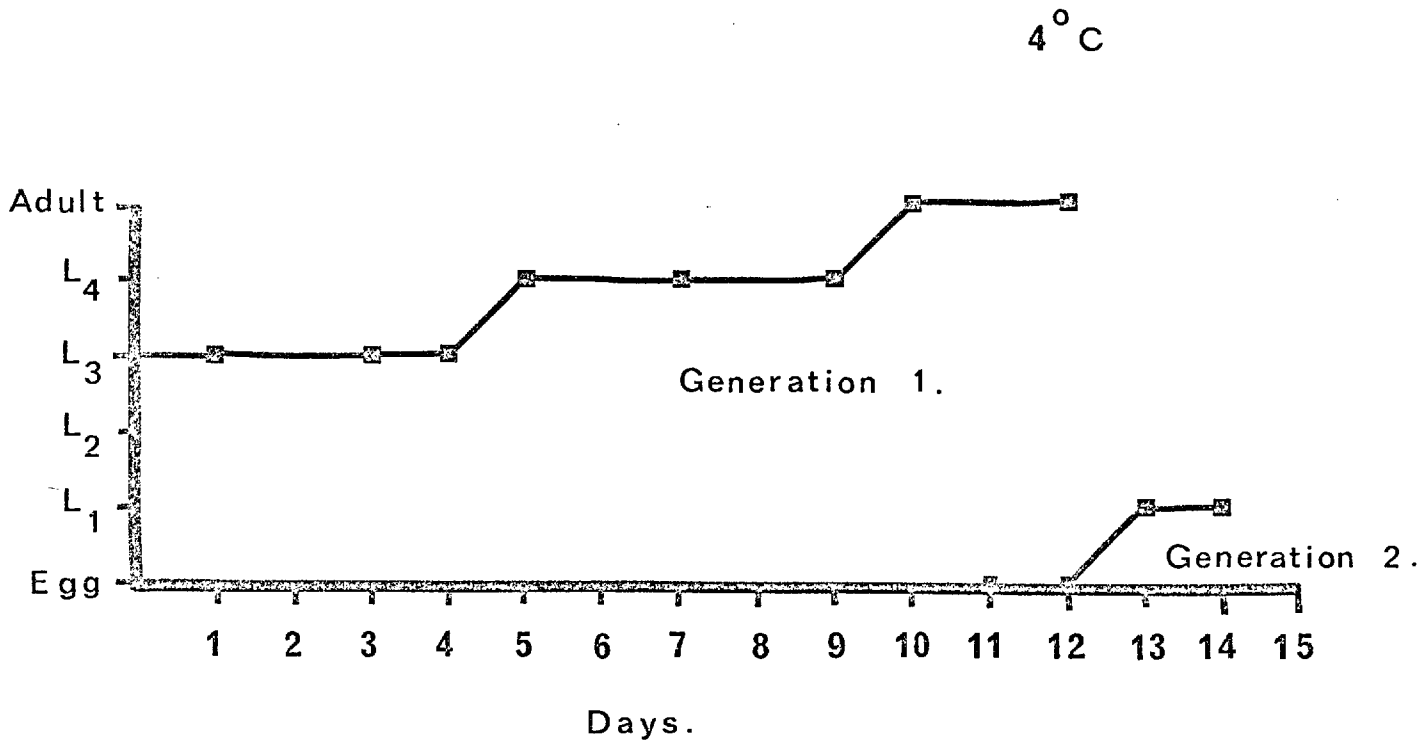

18 °C
Adult
L4
L3
L2
L1
Egg
Generation 1.
Generation 2.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days.

Fig. 30.

Development of *N. carpocapsae* at 4°C.


4°C
Adult
L4
L3
L2
L1
Egg
Generation 1.
Generation 2.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days.

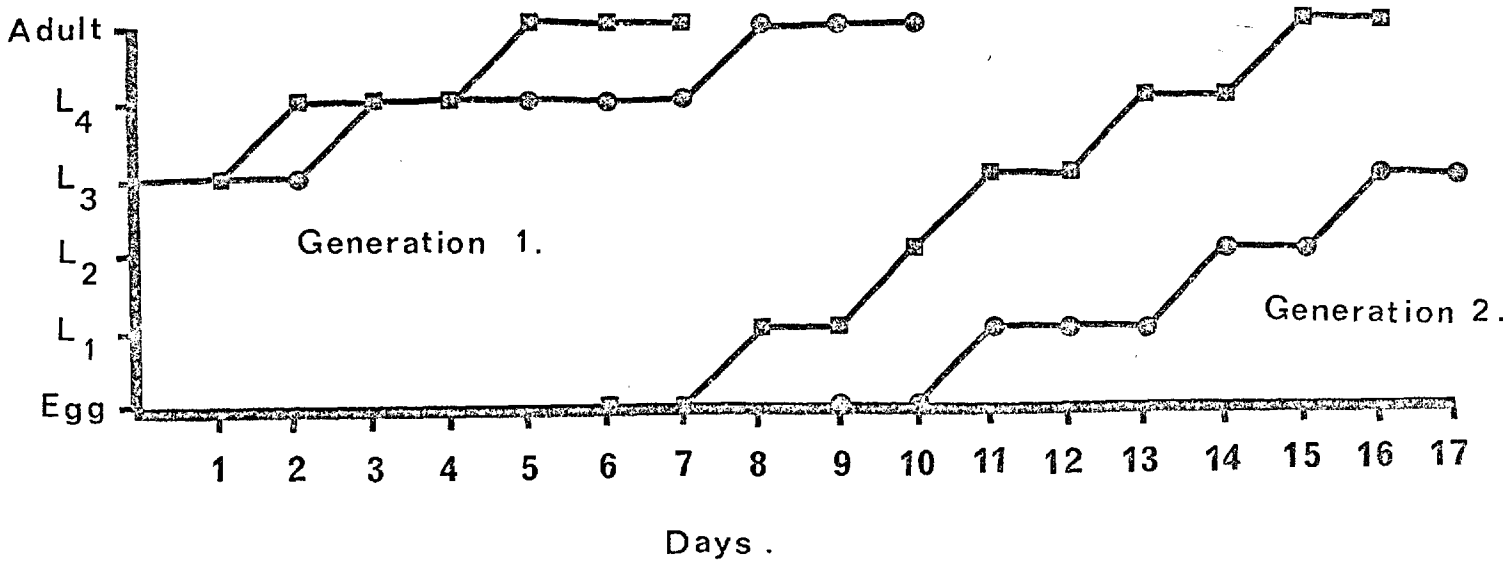
Experiment 6: The effect of the nutritional state of the host on the development of nematodes within them.

Method: Adult insects which had been starved for one week were used in this experiment, fed insects were used as controls. All insects were dosed with 50 invasive larvae. They were kept at 24°C and specimens from starved and control groups were dissected at daily intervals.

Results: Figures 31 and 32 show the effect of starvation of the host on the development of nematodes in adult T. molitor and S. scolytus. The overall effect in both insects was that the life cycle of the nematode was extended in duration. The fourth stage, the feeding stage, of the nematode was particularly prolonged.

Fig. 31.

Effect of starvation of host on the development of nematodes in adult *T. molitor*.



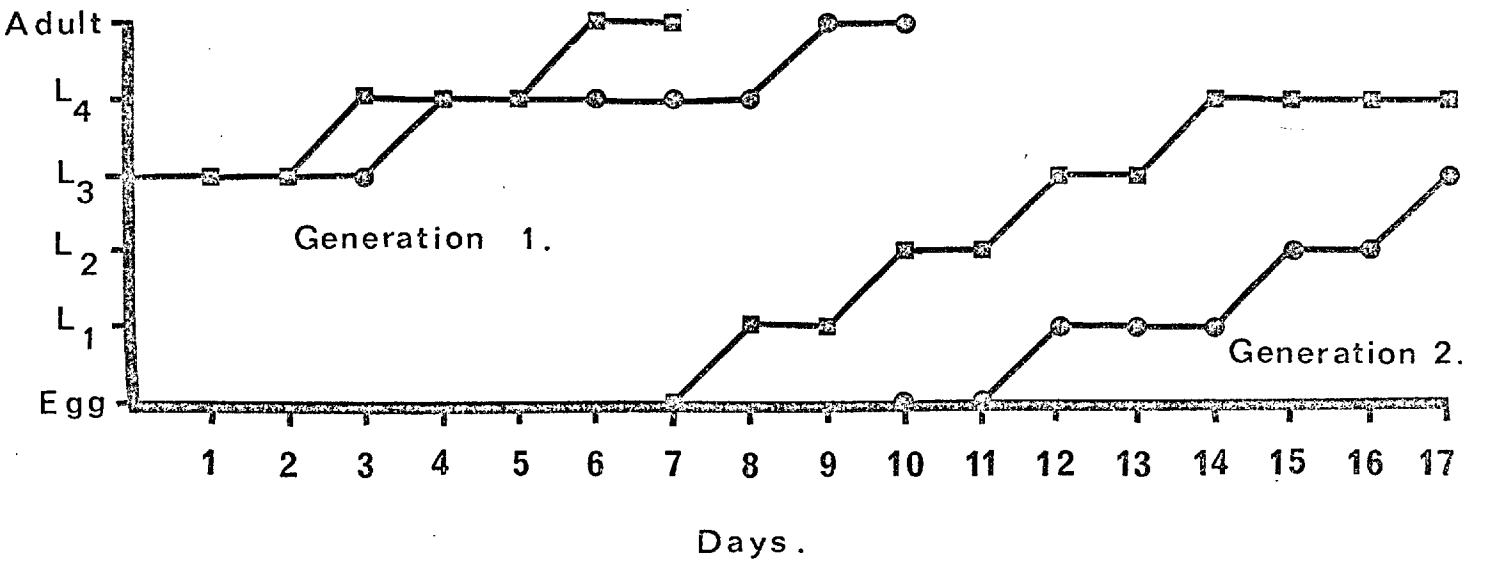
Key

■ = Fed

⊙ = Starved

Fig. 32.

Effect of starvation of host on the development of nematodes in adult *S. scolytus*.



Key

■ = Fed

⊗ = Starved

Discussion

From the result presented it can be concluded that the species of host, its stage of development, its nutritional status are important in influencing the establishment and development of N. carpocapsae infections.

It was found that the rate of mortality of the insects increased with increase in the dose of invasive nematode larvae. This can be explained from the current ideas on the life cycle of this nematode. The mortality of a host is due to the bacteria carried by the nematode into the host haemocoel. The rate of mortality must depend on the multiplication of these pathogens in the insects' body cavity. The rate will, therefore, depend on the size of the inoculum of bacteria carried into the insect and so ultimately depends on the number of larvae gaining access to the haemocoel. It has been shown using the intra-haemocoelic injection method that 98% of the dose of nematodes injected can develop in the host. So that one can assume that increase of the dose of invasive larvae will increase the simultaneous injection of bacteria into the host if every infective larva in fact carries an inoculum of bacteria. This will be investigated further in a subsequent section.

The premise, however, does not explain why there is variation in the mortality rate of different hosts inoculated with the same number of nematode larvae. As mortality is due to a septicaemia set up by the presence of the bacteria, it would seem that variations in mortality are due to variation of different hosts as food sources for bacterial populations

A second factor which could affect the extension of the hosts mortality, is the defense system of the host haemolymph. According to Poinar and Himsworth (1967) this factor plays only

a small part in the case of G. mellonella. Encapsulation of large numbers of infective larvae in Leptinotarsa decemlineata was reported by Sandner and Stanuszek (1971) but the rapid death rate of insects observed in the experiments described here indicated that if any haemolytic defence reactions did occur they were very weak. No evidence of such reactions was collected during this investigation.

A third factor worthy of consideration is the possible effect of the nematodes on the host. This will be investigated further in the next section.

Investigations into the effect of initial dose on the number of giant females developing gave information on the threshhold populations of nematodes which would develop before overcrowding effects became evident. This information has practical application in estimation of optimum initial dose.

The optimum giant female population for G. mellonella, T. molitor, S. scolytus and E. kühniella larvae, T. molitor pupae and adults dosed with 100 infective larvae was in the range 20 - 50; while G. mellonella pupae and S. scolytus adults it was 50 - 60 and in S. scolytus pupae 70 - 80. It has been mentioned previously that with relatively high doses of infective stages, the majority often differentiate to form males. This offers an explanation of some of the results but the opposite effect occurred in G. mellonella and S. scolytus pupae and in S. scolytus adults. It would appear that these insects offered a more suitable environment for both nematode and bacteria for survival. An increase in the initial dose tenfold resulted in an increase in the optimum number of giant females, that an insect could support, of the same order. This would indicate that the number of giant females which develop is directly related to the

number of bacteria present.

A factor not taken into consideration in earlier experiments was the effect of host weight on the density of giant females. It is from these results that species, age, size and the number of giant females on the production of larvae which is of prime importance to the parasite for propogation of its species.

The optimum number of giant females which were produced after a dose of 100 invasive larvae was approximately 50. From these further experiments we can see that this number of giant females per host is most likely to produce the maximum number of invasive larvae. Further experiments showed that the minimum number of these larvae were likely to die in the host and the maximum number survive. However, when larger numbers of giant females developed, although they produced larvae, the yield gradually declined with increased numbers of giant females and a similar decline was evident in the numbers of surviving larvae.

Both temperature and host nutritional status have a part to play in the development of the nematode-bacterium complex by affecting the number of generations and the generation time.

If we consider the effect of temperature on G.mellonella larvae. The usual culture temperature is 24°C and it is obvious why. Over a 2 week period there can be 2 complete generations and the initiation of a third. It was clear, however, that with each successive generation, certain stages were prolonged. It was also observed that with successive generations the number of progery declined. Both of these factors can be attributed to the food supply which must also be declining.

When kept at 18°C only 2 generations occurred and feeding stages in particular were prolonged. This can be attributed to the lower metabolism of both bacterium and nematode at this temperature but not to lack of available food for the host continued to feed. This temperature effect was more clearly defined when the nematode-bacterium complex was exposed to 4°C. The life cycle was prolonged to 12 days and this indicated that each stage of the nematode was taking a longer time to reach the threshold for further development. As the host was not feeding at this temperature, it was possible that the availability of food for the parasite must also be taken into consideration. It was found during the investigation that the nutritional state of the host did directly affect the state of N. carpocapsae. Induced starvation depleted the available food in the haemocoel of hosts for both bacteria and nematodes. This resulted in the feeding stages of the nematode being of particularly long duration, however, the stimuli for development were still present, so that the life cycle could be completed. The second generation of nematodes in both T. molitor and S. scolytus, as one would expect, were subjected to greater stress than the first and as a result were much prolonged.

E. Investigations into the relationship between Achromobacter nematophilus, Neoaplectana carpocapsae and an insect host.

We have seen in the previous experiments how the bacterium-nematode complex of N. carpocapsae succeeds in causing mortality of insect hosts. It is now necessary to investigate the individual roles of bacteria and nematodes in this process.

Methods:

1. Isolation of bacterium.

Infective stage larvae were collected from petri dish cultures and placed individually in hanging drops of insect haemolymph taken from a proleg of G. mellonella larvae. The cultures were kept overnight at 28°C then the bacteria were transferred to agar plates for culture.

2. Bacterial counts.

(a) From nematodes.

An infective stage nematode was placed in distilled water on a microscope slide. The anterior portion of the cuticle was cut away and the foregut exuded into the distilled water. The sample was air dried and treated with the Gram stain.

(b) From insect haemolymph.

A smear of insect haemolymph was made on a slide, air dried and treated with the Gram stain.

3. Isolation of nematodes.

This was carried out using the method of Dougherty and Calhoun (1948) with the modifications of Poinar and Thomas (1966).

4. Culture of G. mellonella

The surface sterilised insect eggs were placed on an autoclaved medium of honey, water and wax in plastic rearing boxes.

5. EXPERIMENTAL DESIGN.

a) Bacterial counts from infective stage nematode larvae.

Bacterial counts from 200 infective stage nematode larvae were made.

b) Insects infected with nematodes minus bacteria.

G. mellonella, T. molitor larvae and T. molitor adults were dosed with 10, 50, 100 or 1,000 infective stage nematodes, injected into the host haemocoel. At least 10 insects from each group were dissected at daily intervals and observations made on the development of the nematodes and mortality of the host. Haemolymph samples were taken from the same insects and a check made for the presence of A. nematophilus. Haemolymph samples taken from G. mellonella larvae were examined at daily intervals to determine the levels of carbohydrate, lipid and protein levels in the insects dosed with 10 nematodes compared with uninfected controls.

c) Insects infected with bacteria minus nematodes.

The haemocoels of at least ten adult T. molitor, larval T. molitor and G. mellonella were infected with a range of doses of A. nematophilus from 2,000 - 200,000 cells. The mortality rate of the hosts was measured.

Results.

a) Bacterial counts from infective stage nematode larvae.

The number of bacterial cells ranged from 40 - 300 per nematode. Only 2% of the nematodes examined did not contain A. nematophilus.

b) Insects infected with nematodes minus bacteria.

When G. mellonella larvae were injected with 10, 50, 100 or 1,000 nematodes the mortality rates of the insects tested were 6, 5, 4 and 2 days respectively; in T. molitor larvae 6, 5, 4, 2 days and in T. molitor adults 8, 7, 5 and 3 days. Haemolymph samples showed

that A. nematophilus was not present. The life cycle of the nematodes in each case was extended, particularly the feeding and adult female stages were prolonged. The adult females containing eggs, on average, survived for 9 days before the eggs within them hatched. The effects of the presence of 10 nematodes on the carbohydrate, protein and lipid content of the haemolymph of G. mellonella over the 6 day period, compared with controls can be seen in Table 50.

Table 50 The effect of infection with 10 bacteria free N. carpocapsae on the carbohydrate, protein and lipid haemolymph levels of G. mellonella larvae.

	Carbohydrate levels (gms. glucose equiv/ 100ml blood)		Protein levels (gm equiv. bovine serum alb/100ml blood)		Lipid levels (gm % cholesterol)	
Day	Dosed	Control	Dosed	Control	Dosed	Control
1	0.62*	0.68	0.061	0.076	1.16	1.17
2	0.58	0.69	0.025	0.081	1.02	1.19
3	0.56	0.72	0.025	0.071	1.00	1.12
4	0.56	0.61	0.023	0.060	0.95	1.06
5	0.52	0.76	0.018	0.082	0.95	1.10
6	0.50	0.65	0.017	0.073	0.90	1.12

* Average of 10 insects

c) Insects infected with bacteria minus nematodes.

When G. mellonella larvae were injected with 200,000 bacterial cells, all of the insects were dead after 1 day, the mortality rate was the same for both T. molitor larvae and adults. When 100,000 cells were introduced, G. mellonella and T. molitor larvae survived for 2 days but T. molitor adults for only one. The infection of the insects with 10,000 cells resulted in the survival of

G. mellonella for 2 days, T. molitor larvae for 3 days and T. molitor again for one. When the lowest dose, 2,000 cells, was administered, G. mellonella larvae survived 3 days, T. molitor larvae 4 days and T. molitor adults 2 days.

Discussion.

The bacterial counts showed that although the actual number of bacterial cells carried by an individual nematode varied over a fairly large range, the majority of nematodes did in fact carry an inoculum of bacteria. When Poinar (1966) investigated the association between A. nematophilus and N. carpocapsae, he did not specify the percentage of bacterium-free nematodes that he found. He did, however, specify that cells in the range of 10-200 per nematode were present in those he looked at. The difference in the number of bacterial cells found in this work and that of Poinar, may be due to the number of bacterial cells that the nematodes had been exposed to during development. An alternative lies in the fact that Poinar (1966) found bacterial cells packed inside intestinal cells of some nematodes, where he suggested they might multiply. Depending on the method used for collection of bacteria from infective stage larvae for counts, these cells may or may not be broken open and it is debateable whether these bacterial cells are always released.

These results indicate that even knowing the dose of the bacterium-nematode complex to which a particular insect has been exposed it is still difficult to establish the level of A. nematophilus which has entered the haemocoel or to what extent it has contributed to the death of the host. The injection of doses of A. nematophilus into a range of insects gave an indication of the effect of the bacteria on the host.

If one compares the mortality rates of the insects which have

been injected with 200,000, 100,000, 10,000 or 2,000 bacterial cells, with the mortality curves (Section D, Expt. 1) for insects injected with 1,000, 500, 50 or 10 nematodes containing bacteria, to which these doses approximate, then one finds that the mortality rates after injection of bacteria alone are much higher than after injection of nematodes and bacteria. There is a single exception to this and that is the death of G. mellonella larvae takes place 2 days after inoculation with 100,000 bacteria but after 1½ days after injection with 500 infective nematodes. With this exception it would appear, therefore, that the bacteria can exploit the host to the full in the absence of the nematodes. Perhaps this is due to the utilisation of the bacteria themselves as a food source by the nematodes when they are present. The nematodes may also provide competition for the bacteria in the utilisation of the available food.

The mortality rates did vary between insects and this gives some indication of the degree of exploitation of different host species and ages by the bacterium. The mortality rate of T. molitor adults was especially significant in that each dose of bacteria, with the exception of the smallest, proved lethal after 1 day. Comparing the two larval species used, G. mellonella proved more susceptible to the bacterium than T. molitor. The actual mechanism by which the bacterium degrades the host system during infection is little understood. However, Samdner and Seryczynska (1971) reported degenerative morphological changes in the fat body of infected insects.

The nematodes alone contribute to the death of the host. The presence of nematodes in the haemocoel of G. mellonella caused a decrease in the levels of carbohydrate, lipids and proteins in the haemolymph over a short period of time. Similar effects were found when the effect of another haemocoel dwelling nematode, Mermis nigrescens,

on its host Schistocerca gregaria was investigated (Gordon, Webster and Mead, 1971). The mortality rate of the larvae exposed to the nematodes was greater than the adult insect. This indicated that although the nematode development was slowed down, the nematodes were able to exploit larval hosts to a greater degree than adult hosts. The developmental time of the nematodes was greater in larval than in adult insects and this may indicate that a substance present in greater quantities in larval than in adult haemolymph is responsible in some part for the development of the nematodes. This is investigated further in the next section.

F. ROLE OF HOST ENDOCRINE SYSTEM IN THE DEVELOPMENT OF N. CARPOCAPSAE.

Methods and Experimental Design.

1. In vivo.

(a) Larvae of G. mellonella were divided into 3 groups each consisting of 20 insects. One hundred invasive larvae were injected into the haemocoel of the insects in Group I which had been ligatured behind the head. Group II insects were ligatured but not dosed. Group III insects were not ligatured but were dosed. All doses were administered a day after ligatures had been attached.

The experiment was repeated with T. molitor larvae.

(b) Larvae of G. mellonella were divided into 4 groups. Insects in Group I were ligatured half way down their body and 50 invasive larvae injected into the haemocoel at the anterior end and 50 into the haemocoel at the posterior end. Group II insects were ligatured in the same way but 50 nematodes minus bacteria were injected both anteriorly and posteriorly.. Group III insects were treated in a similar way but bacteria only (2,000) were injected into the two regions of the haemocoel. Group IV insects were ligatured but not dosed. Groups I, II and III were repeated twice. The first time the nematodes and or bacteria were injected anterior to the ligature and in the second, posterior to the ligature. Again all doses were administered 1 day after ligatures had been applied. Each group consisted of 30 insects. The entire experiment was repeated with T. molitor larvae.

(c) Ligatured and dosed Group II insects in (b) above were dosed with 2,000 A. nematophilus cells 4 days after the onset of the experiment.

The experiment period for a, b and c was 7 days after which

time all insects were dissected.

(d) Twenty one adult Locusta migratoria were allatectomised, left for 1 day and then divided into 3 groups. One hundred infective larvae were injected into the haemocoel of insects in Group I, 50 nematodes minus bacteria into Group II and a dose of 2,000 bacterial cells into the insects in Group III. Treatments I - III were repeated with insects (groups of 5) which had not been allatectomised and 2 further groups were controls, the first had been allatectomised the second had not. All insects were kept individually for 1 week, after which time they were dissected.

(e) Five allatectomised insects which had been dosed with 50 nematodes minus bacteria were dosed after 3 days with 2,000 bacterial cells. Similarly dosed insects which had not been allatectomised were also given the same dose of bacteria. All insects were dissected 7 days after the beginning of the experiment.

(f) Ten, 1, 2 and 3 day 4th instar L. migratoria and 10, 1, 3 and 6 day 5th instar nymphs were injected in each case with either 100 infective larvae, 50 nematodes only or an inoculum of 2,000 bacteria as before. Control insects from 4th and 5th instars were not dosed. All insects were kept separately as before and dissected after 7 days.

In vitro.

(a) Homogenates of corpora allata, corporacardiaca or both, from P. americana and L. migratoria, were prepared in saline and juvenile hormone in acetone (see Section 2.2.C). Doses were applied to N. carpocapsae in petri dish culture in the presence or absence of A. nematophilus and the results observed at daily intervals. Control nematodes were not dosed. In preliminary experiments the nematodes were shown to be unaffected by acetone.

Results.

In vivo.

(a) All of the ligatured, dosed insects survived for 5 days. On dissection the nematodes had not progressed beyond the exsheathed third stage. All of the insects which had been dosed but not ligatured were dead within 3 days. Only two of the control ligatured G. mellonella died within the experimental period as did three T. molitor larvae.

(b) When G. mellonella larvae were ligatured halfway down their body, nematodes with bacteria, nematodes or bacteria only injected anterior to the ligature developed normally. However, when these same treatments were applied posterior to the ligature, except in one or two cases, the nematodes either with or without bacteria did not develop normally, but the bacteria, when present, did. The same results occurred when treatments were given either arteriorly or posteriorly. Of the insects that were ligatured only, 23 survived the experimental period. The majority of T. molitor that were ligatured in this way, including the controls, died within two days.

(c) The addition of bacteria to the nematodes in the haemocoel of the ligatured G. mellonella, either anterior or posterior to the ligature or both, had no effect on the development of the nematodes. At the end of the experiment the bacteria, which had been introduced, had become established in the host. No success was obtained using T. molitor larvae as the ligatured insects died.

(d) The injection of the nematode-bacterium complex into L. migratoria resulted in the death of all of the insects within three days. On dissection, it was found that the nematodes had not developed beyond the exsheathed third stage, however, the haemocoels were filled with A. nematophilus. When nematodes alone were

injected into allatectomised insects they did not develop, 18 of the insects survived. The deaths of the other two insects could not be directly attributed to the presence of the nematodes. All of the insects in Group III died as a result of being injected with an inoculum of bacteria. When insects which had not been allatectomised were dosed, all of the insects died within the experimental period. Dissection of the insects showed that the nematodes had developed normally in the presence of bacteria, normally, but at a slower rate in their absence. Of the insects which had been allatectomised but not dosed 4 out of 5 survived. The same survival figure was obtained for unoperated controls that had not been dosed.

(e) The addition of bacteria to nematodes in the haemocoel of allatectomised insects did not promote the development of the nematodes, but did increase the mortality rate of the hosts. All insects which had not been allatectomised but contained nematodes died one day after the addition of the bacteria.

(f) The nematodes in the presence or absence of bacteria developed in all 4th instar nymphs which resulted in death of the hosts. However, no nematode development occurred in 5th instar L. migratoria. All of the control insects survived.

In vitro.

(a) When a gland equivalent range of corpora allata or both corpora allata and corpora cardiaca from P. americana was added to nematodes in culture in the presence or absence of bacteria, a range of nematode development occurred from no development to the production of giant females containing eggs. There was no correlation between nematode development and the dose of gland extracts administered. The addition of corpora cardiaca was without effect. Similar results were obtained after addition of gland extracts from

L. migratoria. When doses of juvenile hormone were added to either culture, all of the nematodes developed to the adult stage.

Discussion.

We have seen that there is variation in the development of N. carpocapsae when placed in the haemocoel of different stages and species of insects in the absence of bacteria. The rate of development was unchanged when bacteria were added. As we have seen this depression in nematode development is not due to the lack of availability of food for the nematode in the form of bacteria, as the nematodes can utilise the host as a food source. Neither are the bacteria providing stimuli for development. In fact it follows that stimuli for nematode development must be found in the nematodes' environment, the haemocoel itself.

It has been shown that stimuli for development are present however low the host food reserves are but the nematodes could not develop when the flow of substances from the head of the insect was prevented by a ligature. The experimental results showed that as long as the nematodes were present in a part of the host haemocoel connected to the brain then they could develop normally. T. molitor larvae did not prove to be good experimental animals due to the form of the integument and the intersegmental folds. When ligatures were applied they tended to sever the body and this resulted in the death of the insects. In only two cases, nematodes posterior to a ligature in G. mellonella developed normally, this was probably due to inadequate ligatures so that the posterior end of the host was in fact in contact with the anterior end.

When considering the factor or factors which could stimulate development in the nematodes it is necessary to take into consideration the secretions from the brain itself. After allatectomy

nematode development was inhibited. However, the application of corpora allata to the nematodes did not convincingly affect the development of the nematodes but, as mentioned previously, it is difficult using extracts to determine the exact dose applied. The application of juvenile hormone directly to the nematodes was effective, so that it appeared that a secretion from the corpora allata may be important for the development of the nematodes, extracts of corpora cardiaca being ineffective. This hypothesis was substantiated when fourth or fifth instar nymphs were injected with nematodes. It was found that although the nematodes would develop in fourth instars, they would not develop in fifth instars. Johnson (1972) found that a substance with the activity of juvenile hormone could be detected in the haemolymph of fourth instars of L. migratoria but not in fifth. These results indicate that the substance promoting development of the nematodes is of similar configuration to juvenile hormone. Dutky, Kaplanis, Thompson and Robbins (1966) found that N. carpocapsae derives its sterol requirement from the insect host and that the nematodes convert a part of the cholesterol from the host to cholestenol. Hansen and Buecher (1971) found that compounds with juvenile hormone activity affected the viability of first stage larvae, but not egg laying or exsheathment of DD-136, nor exsheathment of N. glaseri.

G. THE CONTROL OF SCOLYTUS SCOLYTUS IN THE FIELD USING N. CARPOCAPSAE.

Introduction.

The part played by the elmbark beetle, S. scolytus, in transmission of the Dutch Elm Disease fungus, Ceratocystis ulmi, is well-known (Gibbs, 1971) and although root transmission and disease recurrence are not thought to be more important than in the past, it is clear that if the beetles could be controlled, the incidence of new disease may, as a consequence be much reduced.

The abandonment of a sanitation felling policy, coupled with the enormous numbers of trees killed by the disease has lead to a great deal of elm cordwood and timber left in the open for long periods. It has become impracticable to burn all of the cordwood or debark the timber, so a source of breeding material and hence a pool of infection has been created. Spraying of logs with an oil-based insecticide has a fairly good controlling effect but this is most effective for prevention of invasion into fresh material (Scott, King and Walker, 1973). Where the beetles have already bored in, the only 100% effective control, to date, is the destruction of the bark by burning.

However, these experiments have shown that the larval, pupal and adult stages of S. scolytus are susceptible to N. carpocapsae. The only difficulty in the biological control of S. scolytus using this nematode, lies in its application. The only access for the nematodes to the insects which live in galleries beneath the bark, is via the entrance holes made by the adult beetles. It is, therefore, necessary to apply sufficient nematodes in a medium which will adequately cover the bark surface and prevent desiccation of the nematodes before they have gained access to such holes and subsequently, the galleries.

Method.

Field trials were carried out at Madresfield, Glos. The experiment was timed as late as was practically possible, i.e. before the majority of insects had stopped feeding but at the estimated cessation of migratory invasions which would confound the controls.

Eighty eight logs (1 metre) were placed in a randomised block consisting of 22 replicates of 4 treatments. The logs were treated with invasive larvae in distilled water, in 10% glycerol or in 0.05% P.B.I.* wetting agent. The fourth group were controls which were left untreated. Each replicate of 4 logs was placed clear of the ground on bearers but removed from stacks for spraying. Each log was sprayed to run off with the treatment medium. The dose applied was approximately 5,000 nematodes per square metre. The experiment was left for 40 days after which a sample (5,000 mm^2) was taken from each log using a bark punch. The punch was heat sterilised between samples. Adult and larval stages of Scolytus scolytus were removed from the galleries in the bark. All insect stages collected were washed in alcohol then dissected in saline and the presence of any stages of N. carpocapsae recorded. The frass was extracted in distilled water and the presence of the invasive larvae of the nematode noted.

Results.

The results of the frass examination can be seen in Table 51. Using Fishers Exact Test, it was found that the presence of nematodes in the treated logs was significantly different than in the control logs at the 0.001 level. There was no significant difference between the treatments.

When larval and adult S. scolytus were found in the samples; infected and uninfected larvae were present in both the treated and

untreated logs, while, all adult beetles found in both groups of logs were infected.

Table 51 Nematodes in the frass of treated and untreated logs.

Treatment	Nematodes present	Nematodes absent
Glycerol 10%	18	4
Untreated	5	17
Wetting agent	18	4
Water	15	7

Discussion.

The most important fact to emerge from the results was that the nematodes, applied by spraying, could penetrate under the bark and so gain access to the insects. As treatments were equally efficient for dispersal of the nematodes, water need only be used in future spraying procedures.

One can assume that infected larvae in the treated logs had ingested the nematodes during feeding. The uninfected larvae found in treated logs were, from their size, at the non-feeding stage. It has been shown previously, that large numbers of infective larvae have to be in close proximity to an insect to ensure penetration. As relatively low numbers of invasive larvae occurred in the frass from the galleries, it is not surprising that non-feeding insect stages were not infected. It would, therefore, be necessary to apply greater quantities of nematodes in order to secure the infection of non-feeding stages. Another solution lies in the self-propagation of the nematodes inside infected insects. The release and migration of infective larvae from successive host cadavers would furnish vast

numbers of infective larvae in the galleries equivalent to repeated spraying of the logs.

The presence of infected larvae in the untreated logs was explained on the discovery of infected adult beetles in these logs. This indicated that the mortality time for adult beetles, after ingestion of nematode larvae, was of sufficient duration to allow migration to another log, and, of great significance for practical application, allowed spread of the lethal nematode infections.

GENERAL DISCUSSION.

The main points which became apparent during the course of this research have already been discussed. Under this heading are those points emerging from these separate discussions.

It has been demonstrated that physiological interactions occur between the host and parasite from the moment a parasite enters a host. The major part of this work has shown that a neuroendocrine relationship exists between the host and the parasite and it has been investigated here, both *in vitro* and *in vivo* for the first time.

Several lines of research by other workers have suggested that the existence of such a relationship would be likely. The work of Cleveland and Burke (1960) and Cleveland, Burke and Karlson in the same year indicated that the sexual cycles of the gut flagellates in the wood-feeding cockroach, *Cryptocercus punctulatus*, are closely attuned to the moulting cycle of the host and that gametogenesis in these flagellates depends on the ecdysone titre of the insect. Although it was not possible to correlate hormone titre in *Periplaneta americana* with infestation of *Hammerschmidtiella diesingi* and *Leidynema appendiculata*, the research indicated that secretions from the host do play a role in development and establishment of these nematode parasites.

Work on the same cockroach/nematode system has been carried out *in vivo* only by Hominick and Davey (1972,1973). They concluded that any effect of the host neuro-endocrines on the parasites was indirect and due to a change in feeding of the host. Similar deductions were made by Corbet (1968), who investigated

the relationship between two insects, Nemeritis canescens, a parasitoid of the caterpillar of Ephestia kuhniella. However, as Gordon (1968) pointed out, a study of the direct effects of host neurosecretory material upon the parasites could only be carried out in vitro and in this work an attempt has been made to do this, even though it has been limited by the lack of information, both qualitative and quantitative, on neuroendocrine secretions.

As it was impossible to obtain nematode-free cockroaches, I was unable to assess any effect of the parasites on the host. From observations of infected insects they seem, however, to be slight, although the insects were susceptible to infection throughout their lives.

This is in marked contrast to the effects shown of the DD-136 strain of Neoaplectana carpocapsae Weiser in several insect hosts. The significance of this work lay in the determination of the factors which affect the susceptibility of insects of different species and age to this nematode- bacterium complex. As a result of this research it was possible to carry out a field experiment using this nematode-bacterium complex as a control agent against Scolytus scolytus. This is the first time that a field trial of this nature has been carried out in this country against a bark boring beetle. Previously, laboratory spraying experiments have been carried out, using the same nematode in similar concentrations, with some degree of success (Moore, 1970). Final results of the experiment recorded here will be assessed after emergence, if any, of the beetles from the nematode sprayed logs.

In conclusion, this research has shown that laboratory work, in vivo and in vitro, into the

physiological interactions between an insect host and its nematode parasites produces a great deal of information on the biology of both host and parasite, and that this information can be applied in the field of biological control.

SUMMARY.

1. Different aspects of host-parasite interrelationships were made utilising *Hammerschmidtiella diesingi* and *Leidynema appendiculata* which inhabit the hind gut of *Periplaneta americana* and the DD-136 strain of *Neoaplectana carpocapsae* Weiser which parasitises the haemocoel of several hosts.

2. Attempts to obtain nematode-free cockroaches using piperazine adipate and to reinfect the insects with a single species of nematode were fruitless.

3. When investigating the nematode infections of *P. americana* a detailed study was made of the infective egg and its hatching process.

4. After interference with the host endocrine system the adult parasite burden was drastically reduced and juvenile nematodes were absent.

5. The median neurosecretory cells of the host govern its parasite population.

6. Reimplantation of extracts of the host endocrine system can result in the re-establishment of infection.

7. *In vitro* studies have shown that the host endocrine system is probably responsible for the induction of development of the nematode juvenile stages.

8. Penetration of the infective stage of *Neoaplectana* sp. occurs via the midgut or through the soft parts of the host exoskeleton.

9. Exsheathment of infective larvae may occur in either the host foregut or haemocoel.

10. The development of the nematodes in the host depends on several factors: nematode population size, the host species, host age, host weight, the nutritional status of the host and the temperature of the host.

11. The bacteria alone can kill the host. The development of the bacteria can be related to the physiological state of the host.

12. The nematodes are not dependent on the bacteria for development.

13. The development of the nematodes gradually debilitates the host and finally kills it.

14. In vitro and in vivo studies have indicated that the nematodes are dependent on secretions from the neuroendocrine system of the host for development.

15. H. diesingi and L. appendiculata could not be used successfully in a biological control programme.

16. Neoaplectana sp. are ideally suited for use as biological control agents.

17. Field trials using the DD-136 strain of N. carpocapsae Weiser against S. scolytus indicate that this nematode may be useful as a control agent for this bark beetle.

REFERENCES.

Anya, A. O. (1965). Studies on the biology, morphology and physiology of nematodes with special reference to Aspiculuris tetraptera. Ph.d. Thesis, University of Cambridge.

Anya, A. O. (1966). Experimental studies on the physiology of hatching of eggs of Aspiculuris tetraptera Schultz (Oxyuridae:Nematoda). Parasitology. 56: 733-744.

Anya, A. O. (1966a). The structure and chemical composition of the nematode cuticle. Observations on some oxyurids and Ascaris. Parasitology. 56: 179-198.

Bird, A. F. (1956). Chemical constitution of the nematode cuticle. Observations on the whole cuticle. Expl. Parasit. 5: 350-358.

Bogomolova, H. A., and Pavlova, L. J. (1961). Zheltonochnye kletki Fasciola hepatica i Diphyllobothrium latum i ich rol v obrazovanii obolochki idica; pitanii zarodysha. Helminthologia. 3: 47-59.

Briand, L. J., and Welch, H.E. (1963). Use of entomophilic nematodes for insect pest control. Phytoprotection. 44: 37-41

Brown, C. H. (1950). A review of the methods available for the determination of the types of forces stabilising structural proteins in animals. Q. Jl. microsc. Sci. 91: 331-339.

Campbell, F. L. (1929). The detection and estimation of insect chitin; and the irrelation of 'chitinisation' to hardness and pigmentation of the cuticula of the American cockroach, Periplaneta americana L.
Annals of the Entom. Soc. of America. 22: 401-426.

Castillo, J. del. (1969). Pharmacology of Nematoda.
In: Chemical Zoology. Vol. 3. 521-554.
Eds. M. Florkin and B.T. Scheer.
Academic Press, New York and London.

Chandler, A.C., Alicata, J.E., and Chitwood, M.B. (1941). Life History (Zooparasitica) 11. Parasites of Vertebrates.
In: An Introduction to Nematology.
Ed. J.R. Christie. Chapter 6. 267-301.
Babylon, New York.

Charney, J., and Tomarelli, R. M. (1947).
A colorimetric method for the determination of the proteolytic activity of duodenal juice. J. biol. Chem. 171: 501-505.

Chitwood, B. G. (1932).
A synopsis of the nematodes parasitic in insects of the family Blattidae.
Zeitschrift fur Parasitenkunde. 5: 14-50.

Chitwood, B. G. (1938).
Further studies on nemic skeletoids and their significance in the chemical control of nemic pests.
Proc. helminth. Soc. Wash. 6: 68-76.

Chitwood, B. G., and Chitwood, M.B. (1950).
'An Introduction to Nematology.'
Monumental Printing Co., Baltimore.

Christie, J. R. (1929). Some observations on sex in the Mermithidae. J. Exp. Zool. 53: 59-76.

Clarke, W. M. (1928). The Determination of Hydrogen Ions. 3rd Edt. London: Bailliere, Tindall and Cox.

Clarke, A.J., Cox, P.M., and Shepherd, A.M. (1967). The chemical composition of the egg shells of the potato cyst-nematode, Heterodera rostochiensis Woll. Biochem. J. 104: 1056-1060.

Cleveland, L.R., and Burke, A.W., Jr. (1960). Modifications induced in the sexual cycles of the protozoa of Cryptocercus by change of host. J. of Protozool. 7: 240-245.

Cleveland, L.R., Burke, A.W., Jr., and Karlson, P. (1960). Ecdysone induced modifications in the sexual cycles of the protozoa of Cryptocercus. J. of Protozool. 7: 229-239.

Corbet, S. A. (1968). The influence of Ephestia kühniella on the development of its parasite Nemeritis canescens. J. Exp. Biol. 48: 291-304.

Crompton, D.W.T., Shrimpton, F.H., and Silver, I.A. (1965). Measurement of the oxygen tension in the lumen of the small intestine of the domestic duck. J. Exp. Biol. 43: 473-478.

Curtis, G. J. (1964). The effect of potassium chloride on the infestation of sugar beet by beet eelworm Heterodera schachtii Schmidt. Ann. Appl. Biol. 54: 269-280.

Davey, K. G. (1964). Neurosecretory cells in a nematode, Ascaris lumbricoides. Canad. J. Zool. 42: 731-734.

Davey, K. G. (1966). Neurosecretion and molting in some parasitic nematodes. American Zoologist. 6: 243-249.

Davey, K. G. (1971). Molting in a parasitic nematode, Phocanema decipiens. Vl. The mode of action of insect juvenile hormone and farnesyl methyl ether. Int. J. for Parasitol. 1: 61-66.

Davey, K. G. (1972). In: Comparative Biochemistry of Parasites. Ed. H. Van den Bossche. Academic Press, London.

Davey, K.G. and Hominick, W. (1973). Endocrine relationships between nematodes and their insect hosts-A review. Expl. Parasit. 33: No. 2 212-225.

Davey, K. G., and Kan, S. P. (1967). Endocrine basis for ecdysis in a parasitic nematode. Nature, London. 214: 737-738.

Davey, K. G., and Kan, S. P. (1968). Molting in a parasitic nematode, Phocanema decipiens. lV. Ecdysis and its control. Can. J. Zool. 46: 893-898.

Davies, G. J. (1971). Computer programming in quantative biology. Academic Press, London.

Day, M. F., and Powning, R. F. (1949). Processes of digestion in certain insects. Aus. J. Sci. Res. SER. B. 2: 175-215.

Dobrovolny, C. G., and Ackert, J. E. (1934). The life history of Leidynema appendiculata (Leidy), a nematode of cockroaches. Parasitology. 26: 468-480.

Dogra, G. S., and Tandan, B. K. (1964). Adaptations of certain histological techniques for in situ demonstration of the neuroendocrine system of insects and other animals. Quart. J. micr. Sci. 105: 455-466.

Dougherty, E. C., and Calhoun, H. G. (1948). Techniques for temporarily freeing soil nematodes from bacteria by the use of antibiotics and methiolate. Anat. Rec. 100: 395.

Dropkin, V. H., Lower, W. R., and Acedo, J. (1971). Growth inhibition of Caenorhabditis elegans and Panagrellus redivivus by selected mammalian and insect hormones. J. of Nematology. 3: No. 4 349-355.

Dropkin, V. H., Martin, G. C., and Johnson, R. W. (1958). Effect of osmotic concentration on hatching of some plant parasitic nematodes. Nematologica. 3: 115-126.

Dutky, S. R. (1959). Insect Microbiology. Adv. Appl. Microbiol. 1: 175-200.

Dutky, S. R., and Hough, W. S. (1955). Note on a parasitic nematode from codling moth larvae Carpocapsa pomonella (Lepidoptera: Olethreutidae). Proc. Entomol. Soc. Wash. 57: 244.

Dutky, S. R., Kaplanis, J. N., Thompson, M. J., and Robbins, W.E. (1966). The isolation and identification of the sterols of the DD-136 insect parasitic nematode and their derivation from its insect hosts. Annual Meeting of the Society of Nematologists. Abstract. Nematologica. 13: 139-140.

Dutky, S. R., Thompson, J. V., and Cantwell, G. E. (1964). A technique for the mass propagation of the DD-136 nematode. J. Insect Path. 6: No. 4. 417-422.

Edmonds, S. J. (1966). Hatching of the eggs of Moniliformis dubius. Expl. Parasit. 19: No. 2. 216-227.

Ellenby, C. (1946). Nature of the cyst wall of the potato root eelworm, Heterodera rostochiensis, Wollenweber and its permeability to water. Nature, London. 157: 302-303.

Ellenby, C., and Gilbert, A. B. (1958). Influence of certain inorganic ions on the hatching of the potato root eelworm Heterodera rostochiensis Wollenweber. Nature, London. 182: 925-926.

Engelmann, F. (1965). The mode of regulation of the corpus allatum in adult insects. Arch. Anat. micros. Morph. exp. 54: 387-404.

Engelmann, F. (1969). Female specific protein: biosynthesis controlled by corpus allatum in Leucophaea maderae. Science. 165: 407-409.

Engelmann, F., Hill, L., and Wilkens, J. L. (1971). Juvenile hormone control of female specific protein synthesis in Leucophaea maderae, Schistocerca vaga, and Sarcophaga bullata. J. Insect Physiol. 17: 2179-2191.

Ewen, A. B. (1962). An improved aldehyde fuchsin staining technique for neurosecretory products in insects. Trans. Am. microsc. Soc. 81: 94-96.

Fairbairn, D. (1955). Lipids of the female reproductive organs in Ascaris lumbricoides. Can. J. Biochem. Physiol. 33: 31-37.

Fairbairn, D. (1960). The physiology and biochemistry of nematodes. In: Nematology. Fundamentals and Recent Advances with Emphasis on Plant Parasitic and Soil Forms. (Eds. J.N. Sasser and W.R. Jenkins) pp.267-296. University of North Carolina Press, Chapel Hill, North Carolina.

Fairbairn, D. (1961). The *in vitro* hatching of *Ascaris lumbricoides* eggs. Can. J. Zool. 39: 153-162.

Fairbairn, D., and Passey, B. I. (1955). Lipid components in the vitelline membrane of *Ascaris lumbricoides* eggs. Can. J. Biochem. Physiol. 33: 130-134.

Fayard, C. (1949). These pour le Doctorat de Medicine. 60pp. Thesis, Paris. (quoted from Semaine Hop. 35: 1778).

Fenwick, D. (1939). Studies on the saline requirement of the larvae of *Ascaris suum*. J. Helminth. 17: 211-228.

Fiakpui, E. Z. (1967). Some effects of piperazine and methyridine on the free-living nematode *Caenorhabditis briggsae* (Rhabditidae). Nematologica. 13: 241-255.

Gersch, M., and Scheffel, H. (1958). Sekretorisch tatige zellen im Nervensystem von *Ascaris*. Naturwissenschaften. 45: 345-346.

Gibbs, J. N. (1971). Dutch Elm Disease (*Ceratocystis ulmi*). Forestry Commission Leaflet. No. 19. 11pp.

Glaser, R. W., McCoy, E.E., and Girth, H.B. (1942). The biology and culture of *Neoaplectana chresima*, a new nematode parasitic in insects. J. Parasitol. 29: 123-126.

Goddard, D.R., and Michaelis, L. (1934). A study on keratin. J. Biol. Chem. 106: 605-614.

Goldsworthy, G. J. (1967). In: Neurosecretion and Parasitism in *Blatta orientalis*. R. Gordon. Ph.D. Thesis, Univ. Sheffield, 1968.

Gorbman, A., and Bern, H. A. (1962). A Textbook of Comparative Endocrinology. Wiley, New York.

Gordon, R. (1968). Neurosecretion and parasitism in Blatta orientalis L. Ph.D. Thesis, University of Sheffield.

Gordon, R. (1970). A neuroendocrine relationship between the nematode Hammerschmidtiella diesingi and its insect host Blatta orientalis. Parasitology. 61: 101-110.

Gordon, R., Webster, J.M., and Mead, D.E. (1971). Some effects of the nematode Mermis nigrescens upon carbohydrate metabolism in the fat body of its host, the desert locust Schistocerca gregaria. Can. J. Zool. 49: No. 4 431-434.

Götz, P. (1964). Der Einfluss unterschiedlicher Befallsbedingungen auf die mermithogene Intersexulität von Chironomus. (Dipt.) Zeitschrift fur Parasitenkunde. 24: 481-545.

Hammerschmidt, K. E. (1838). Helminthologische Beiträge. Isis (Oken) Leipzig. 5:351-358.

Hammerschmidt, K. E. (1847). Helminthologische Beiträge. Beschreibung einiger Oxyuris-Arten. Naturwiss. Abh. Wien. 1: 379-388.

Hansen, E. L., and Buecher, E. J. (1971). Effect of insect hormones on nematodes in axenic culture. Experientia. 27: 859-860.

Hansen, E., Buecher, E.J., and Yarwood, E.A. (1964). Development and maturation in Caenorhabditis briggsae in response to growth factor. Nematologica. 10: 623-630.

Hansen, E.L., Yarwood, E.A., Jackson, G.J., and Poinar, Jr., G.O. (1968).
Axenic culture of Neoaplectana carpocapsae in liquid media. J. Parasitol. 54: 1236-1237.

Hartree, E. F. (1972).
Determination of protein: A modification of the Lowry method that gives a linear photometric response.
Analytical Biochemistry. 48: 422-427.

Hechler, H. C., and Taylor, D. P. (1966).
In: Growth and Development. Chapter 9.
Chemical Zoology. Vol. 3.
Eds. M. Florkin and B.T. Scheer.
Academic Press, New York and London.

Highnam, K.C. (1964).
Hormones and behaviour in insects.
Viewpoints in Biology. 3: 219-260.

Highnam, K. C., and Hill, L. (1969).
'The Comparative Endocrinology of the Invertebrates.' 270pp.
Edward Arnold Ltd., London.

Highnam, K.C., Lusis, O., and Hill, L. (1963).
The role of the corpora allata during oocyte growth in the desert locust Schistocerca gregaria Forsk.
J. Insect Physiol. 9: 587-596.

Hill, L. (1962).
Neurosecretory control of haemolymph protein concentration during ovarian development in the desert locust.
J. Insect Physiol. 8: 609-619.

Hill, L. (1963).
The endocrine control of oocyte development in the desert locust Schistocerca gregaria Forskal.
Ph.D. Thesis, University of Sheffield.

Himes, M., and Moriber, L. (1956).
A triple stain for deoxyribonucleic acid, polysaccharides and protein.
Stain Technol. 31.

Hitcho, P. J., and Thorson, R. E. (1971).
Possible molting and maturation controls in Trichinella spiralis.
J. of Parasit. 57: No. 4, 787-793.

Hobson, A.D., Stephenson, W., and Beadle, L.C. (1952).
Studies on the physiology of Ascaris lumbricoides.
1. The relation of the total osmotic pressure, conductivity and chloride content of the body fluid to that of the external environment.
J. Exptl. Biol. 29: 1-21.

Hominick, W. M., and Davey, K. G. (1972).
Reduced nutrition as the factor controlling the population of pinworms following endocrine gland removal in Periplaneta americana L.
Can. J. Zool. 50: 1421-1432.

Hominick, W. M., and Davey, K. G. (1973).
Food and the spatial distribution of adult female pinworms parasitic in the hind gut of Periplaneta americana.
Int. J. for Parasit. 3: 759- 771.

Ishikawa, M. (1961). The secretory phenomenon of the nerve cell of Ascaris. Kiseichagaku Zasshi. 10: 1-5.

Jackson, G. J. (1965). Differentiation of three species of Neoaplectana (Nematoda: Rhabditida), grown axenically. Parasitology. 55: 571-578.

Jackson, G. J. (1973). The aging of Neoaplectana glaseri. Proc. helminth. Soc. Wash. 40: No. 1 74-76.

Jacobs, L., and Jones, M. F. (1939). Studies on oxyuriasis XXI. The chemistry of the membranes of the pinworm egg. Proc. helminth. Soc Wash. 6: 57-61.

Jaskoski, B. J. (1962). Paper chromatography of some fractions of Ascaris suum eggs. Expl. Parasit. 12: 19-24.

Jaskoski, B. J., and Butler, V. L. (1971). Rhipicephalus sanguineus: Amino acid composition of eggshell. Expl. Parasit. 30: 400-406.

Johnson, R. A. (1972). The activity of the corpora allata in the migratory locust, Locusta migratoria migratoides, R. and F. Ph.D. Thesis, University of Sheffield.

Johnson, R. N., and Viglierchio, D. R. (1970). Heterodera schachtii responses to exogous hormones. Expl. Parasit. 27: 301-309.

Karlson, P., and Schmialek, H. (1959). Nachreis der Exkretion von Juvenilhormon. Zeitschrift fur Naturforschung. 146: 821.

Kreuzer, L. (1953). Zur kenntnis des chemischen Aufbaus der Einhülle von Ascaris lumbricoides. 1. Mitteilung. Zeitschr. vergl. Physiol. 35: 13-26.

Lee, D. L. (1959). The biology, morphology and physiology of nematodes parasitic in cockroaches. Ph.D. Thesis, University of Cambridge.

Lee, D. L. (1960). The effect of changes in the osmotic pressure upon Hammerschmidtiella diesingi (Hammerschmidt, 1838) with reference to the survival of the nematode during moulting of the cockroach. Parasitology. 50: 241-246.

Lee, D. L. (1961). Studies on the origin of the sticky coat on the eggs of the nematode Thelastoma bulhoesi (Magalhaes, 1900) Parasitology. 51: 379-384.

Lee, D. L. (1965). The Physiology of Nematodes. Oliver and Boyd, Edinburgh and London. 154 pp.

Leidy, J. (1850). Description of nematoid entozoa infesting insects. Proc. natn. Acad. Sci. U.S.A. 5: 284-290.

Lowry, O. H., Rosenburg, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lusis, O. (1963). The histology and histochemistry of development and resorption in the terminal oocytes of the desert locust, Schistocerca gregaria. Q. Jl. microsc. Sci. 104:57-58.

Mc Farlane, J. E. (1962). The cuticles of the egg of the house cricket. Can. J. Zool. 40: (1) 13-21.

Maddrell, S. H. P. (1973). Personal communication.

Marks, C. F., and Sayre, R. M. (1964). The effect of potassium on the rate of development of the root-knot nematodes, Meloidogyne incognita, M. javanica and M. hapla. Nematologica. 10: 323-327.

Meerovitch, E. (1965). Studies on the in vitro axenic development of Trichinella spiralis. 11. Preliminary experiments on the effects of farnesol, cholesterol, and an insect extract. Can. J. Zool. 43: 81-85.

Monné, L. (1955). On the histochemical properties of the egg envelopes and external cuticles of some parasitic nematodes. Ark. Zool. 9: 93-113.

Monné, L., and Hönig, G. (1954). On the properties of the egg envelopes of various parasitic nematodes. Ark. Zool. 7: 261-272.

Moore, G. E. (1970). Dendroctonus frontalis infection by the DD-136 strain of Neoaplectana carpocapsae and its bacterium complex. J. of Nematology. 2: 341-344.

Nadakal, A. M., and Nayar, K. K. (1968). Neural and hormonal influence on the fecundity and egg-laying of certain oxyurid nematodes inhabiting the hind gut of the cockroach, Periplaneta americana, L. Indian Journal of Experimental Biology. 6: 29-32.

Niklas, O. F. (1967). Die Nematoden DD-136 (Neoaplectana sp.) und Neoaplectana carpocapsae Weiser, 1955 (Rhabditoidea) als Insektenparasiten. Eine Literatur-übersicht. Mitt. Biol. Bundesanst. Land Forstwirt. Berlin-Dahlem. 124: 40pp.

Norton, S., and de Beer, E. J. (1957). Investigations on the action of piperazine on Ascaris lumbricoides. Am. J. Trop. Med. Hyg. 6: 898-905.

Oteifa, B. A. (1953). Development of the root-knot nematode, Meloidogyne incognita, as affected by potassium nutrition of the host. Phytopathology. 43: 171-174.

Pearse, A. G. E. (1960). Histochemistry, Theoretical and Applied. J. and A. Churchill Ltd., London.

Pogell, B. M. (1956). Hexosamine formation in rat liver. Biochem. Biophys. Acta. 21: 205-206.

Poinar, Jr., G. O. (1966). The presence of Achromobacter nematophilus, Poinar and Thomas in the infective stage of a Neoaplectana sp. (Steinernematidae Nematoda). Nematologica. 12: 105-108.

Poinar, Jr., G. O. (1967). Description and taxonomic position of the DD-136 nematode (Steinernematidae, Rhabditoidea) and its relationship to Neoaplectana carpocapsae Weiser. Proc. helminth. Soc. Wash. 34: 199-209.

Poinar, Jr., G. O., and Himsworth, P. T. (1967). Neoaplectana parasitism of larvae of the greater wax moth Galleria mellonella. J. Invert. Pathol. 9: 241-246.

Poinar, Jr., G. O., and Thomas, G. M. (1966). Significance of Achromobacter nematophilus Poinar and Thomas (Achroobacteraceae:Eubacteriales) in the development of the nematode DD-136 (Neoaplectana sp. Steinernematidae). Parasitology. 56: 385-390.

Poinar, Jr., G.O., Hansen, E.L., Yarwood, E.A. and Weiser, J. (1972). Clarification of the status of the DD-136 strain of Neoaplectana carpocapsae Weiser. Nematologica. 18: 288-290.

Pollack, J. K., and Fairbairn, D. (1955). The metabolism of Ascaris lumbricoides ovaries. 2. Amino acid metabolism. Canad. J. Biochem. Physiol. 33: 307-316.

Pratt, G. E., and Davey, K. G. (1972). The corpus allatum and oogenesis in Rhodnius prolixus (Stal). 1. The effects of allatectomy. J. Exp. Biol. 56: 201-214.

Rajulu, G.S., Kulasekarapandian, S., and Krishnan, N. (1972). Nature of the hormone from a nematode Phocanema depressum Bayliss. Current Science. 41: 67-68.

Rogers, W. P. (1949). On the relative importance of aerobic metabolism in small nematode parasites of the intestinal tract. 1. Oxygen tensions in the normal environment of the parasite. Aust. J. Scient. Res. B. 2: 157-165.

Rogers, W. P. (1958). Physiology of the hatching of eggs of Ascaris lumbricoides. Nature, London. 181: 1410-1411.

Rogers, W. P. (1960). The physiology of infective processes of nematode parasites: the stimulus from the animal host. Proc. Roy. Soc. B. 152: 367-368.

Rogers, W. P. (1961). The Nature of Parasitism. The relationship of some metazoan parasites to their hosts. Academic Press, New York. 287pp.

Rogers, W. P. (1963).
Physiology of infection with nematodes: Some effects of the host stimulus on infective stages.
Ann. N. Y. Acad. Sci. 113: 208-216.

Rogers, W. P. (1965).
The role of leucine aminopeptidase in the moulting of nematode parasites.
Comp. Biochem. Physiol. 14: 311-321.

Rogers, W. P. (1968).
Neurosecretory granules in the infective stage of Haemonchus contortus.
Parasitology. 58: 657-662.

Rogers, W. P., and Sommerville, R. I. (1960).
The physiology of the second ecdysis of parasitic nematodes.
Parasitology. 50: 329-348.

Roller, H., Dahm, K.H., Sweely, C.C., and Trost,B.M. (1967). The structure of the Juvenile hormone. Angewandte Chemie. 6: 179-180.

Sanborn, R. C., and Arthur, E. J. (1965).
Osmotic and Ionic Regulation in Nematodes. Chapter 8. In: Chemical Zoology. Vol. 3. Eds. M. Florkin and B. T. Scheer. Academic Press, New york and London.

Sandner, H., and Stanuszek, S. (1971).
The role of nematodes as factors reducing populations of insect pests.
Report. Inst. of Ecol., Warsaw, Poland.

Sandner, H., and Seryczynska, H. (1971).
Changes in the ultrastructure of the fat body of G. mellonella L. under the influence of invasion by Neoaplectana carpocapsae Weiser (Nematoda, Steinernematidae)
Bulletin de l'Academie Polonaise des Sciences.
19: No.1 67-69.

Sarda, L., and Desnuelle, P. (1958).
Action de la lipase pancreatique sur les esters en emulsion.
Biochem. biophys. Acta. 30: 513-521.

Schmiege, D. C. (1964).
A note on the taxonomy of an undescribed insect parasitic nematode in the genus Neoaplectana.
Parasitology. 54: 233-236.

Schneiderman, H. A., and Gilbert, L. I. (1958).
Substances with juvenile hormone activity in Crustacea and other Invertebrates.
Biological Bulletin. 115: 530- 535.

Schneiderman, H. A., Gilbert, L.I., and Weinstein, M. J. (1960).
Juvenile hormone activity in micro-organisms and plants.
Nature, London. 188: 1041-1042.

Scott, T.M., King, C.J., and Walker, C. (1974).
Trials with insecticide sprays to control elm bark beetles in logs.
Q. Jl. For. LXVlll 2: 167-170.

Seligman, A.M., and Nachlas, M.M. (1963).
'Lipase.' In: Methods of Enzymatic Analysis. Ed. H.U. Bergmeyer.
Verlag Chemie. 776-778.
Academic Press.

Shanta, C. S., and Meerovitch, E. (1970).
Specific inhibition of morphogenesis in Trichinella spiralis by insect juvenile hormone mimics.
Can. J. Zool. 48: 617-620.

Smyth, J.D., and Clegg, J.A. (1959).
Egg-shell formation in trematodes in cestodes.
Expl. Parasit. 8: 286-323.

Sommerville, R. I. (1957). The exsheathing mechanism of nematode infective larvae. Exp. Parasit. 6: 18-30.

Stahl, E. (1965). Thin Layer Chromatography. Academic Press, New York.

Stanuszek, S. (1972). Revision of the genus Neoaplectana Steiner, 1929 (Rhabditoidea: Steinernematidae). Abstract XIth Int. Symp. Nemat. Reading.

Steele, J. E. (1961). Occurrence of a hyperglycaemic factor in the corpus cardiacum of an insect. Nature, London. 192: 680-681.

Steiner, G. (1929). Neoaplectana glaseri n. sp.: new genus parasite of the Japanese beetle. J. Wash. Acad. Sci. 19: 436-439.

Stephenson, W. (1944). The effect of certain inorganic chloride solutions upon the movement of a soil nematode (Rhabditis terrestris Stephenson) and its bodily size. Parasitology. 35: 167-172.

Thomsen, E., and Möller, I. (1959). Further studies on the function of the neurosecretory cells of the brain of the adult Calliphora erythrocephala female. In: The Ontogeny of Insects. Acta. Symp. Evol. Ins., Praha. 121-125.

Thomsen, E., and Möller, I. (1959a). Neurosecretion and intestinal proteinase activity in an insect, Calliphora erythrocephala. Nature, London. 183: 1401-1402.

Thomsen, E., and Möller, I. (1963). Influence of the neurosecretory cells and corpus allatum on intestinal proteinase activity in the adult blowfly Calliphora erythrocephala Meig. J. Exp. Biol. 40: 301-321.

Thong, C.H.S., and Webster, J.M. (1971). The effect of gonadotrophins on the *in vitro* growth of the free-living nematode *Cephalobus* sp. Canad. J. Zool. 49: 1059-1061.

Todd, A. C. (1941). An addition to the life history of *Leidynema appendiculata* (Leidy, 1850; Chitwood, 1932) a nematode in cockroaches. J. Parasit. 27: 34-35.

Turco, C.P., Thames, Jr., W.H., and Hopkins, S.H. (1971). On the taxonomic status and comparative morphology of species of the genus *Neoaplectana* Steiner (Neoaplectanidae: Nematoda). Proc. helminth. Soc. Wash. 38: 68-79.

Vanfleteren, J. R., and Roets, D. E. (1972). The influence of some anthelmintic drugs on the population growth of the free-living nematodes *Caenorhabditis briggsae* and *Turbatrix aceti* (Nematoda: Rhabditida.) Nematologica. 18: 325-338.

Veglia, F. (1915). Third and Fourth Reports pp.374-500. Director of Vet. Res., Union of South Africa.

Wallace, H. R. (1963). The Biology of Plant Parasitic Nematodes. Edward Arnold Ltd., London.

Webster, J. M., and Craig, S. (1969). The direct effect of plant and insect hormones on *Cephalobus* sp. J. of Nematol. 1: 308.

Weinstein, P. P. (1952). Regulation of water balance as a function of the excretory system of the filariform larvae of *Nippostrongylus muris* and *Ancylostoma caninum*. Expt. Parasit. 1: 363-376.

Weiser, J. (1955). Neoaplectana carpocapsae n. sp. (Anguillilata, Steinernematinae) nový cizopasnik housenek obaleče jabloňového Carpocapsa pomonella L. Acta. Soc. Zool. 19: 44-52.

Weiser, J. (1966). Nemoci hmyzu. Praha. pp.554.

White, G. F. (1929). A method for obtaining infective nematode larvae from cultures. Science. 66: 302-303.

Wigglesworth, V. B. (1927). Digestion in the cockroach. 11. The digestion of carbohydrates. Biochem. J. 21: 797-811.

Wigglesworth, V. B. (1928). Digestion in the cockroach. 111. The digestion of proteins and fats. Biochem. J. 22: 150-161.

Wigglesworth, V. B. (1961). The Principles of Insect Physiology. 5th Edition. pp. 309-360. Methuen and Co., Ltd., London.

Wigglesworth, V. B. (1964). The hormonal regulation of growth and reproduction in insects. Advances in Insect Physiology. 2: 247-336.

Wigglesworth, V. B. (1970). Insect Hormones. 159 pp. Oliver and Boyd, Ebinburgh.

Wilde, J. De., Staal, G.B., der Cort, C.A.D., de Loof, A., and Baard, G. (1968). Juvenile hormone titer in the haemolymph as a function of photoperiodic treatment in the adult colorado beetle (Leptinotarsa decimlineata Say). Proc. K. ned. Akad. Wet. (C) 71: 321-326.

Williams, C. M. (1956). The juvenile hormone of insects. Nature, London. 178: 212-213.

Williams, C.M., Moorehead, L.V., and Pulis,J.F. (1959). Juvenile hormone in the thymus, human placenta and other mammalian organs. Nature, London. 183: 405.

Wilson, P. A. G. (1958). The effect of weak electrolyte solutions on the hatching rate of the eggs of Trichostrongylus retortaeformis (Zeder). and its interpretation in terms of a proposed hatching mechanism of strongyloid eggs. J. exp. Biol. 35: 584-602.

Wottage, K. (1937). Die stofflichen veränderungen u der eizelle von Ascaris megalocephala nach der befruchtung. Protoplasma. 29: 31-59.