SUSCEPTIBILITY OF DIFFERENT INSECT SPECIES TO INFECTION BY *HYMENOLEPIS DIMINUTA* AND *MONILIFORMIS MONILIFORMIS*

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You teach best

What you most need to learn

There is no such

Thing as a problem

Without a gift for you in its hands

You seek problems

Because you need their gifts...

Don't be dismayed at good-byes

A farewell is necessary before

You can meet again

And meeting again, after

Moments or lifetimes, is

Certain for those who are friends...

Here is a test

To find whether your

Mission on earth is finished

If you're alive,

It isn't

Extracts from "Illusions" by Robert Bach.

ACKNOWLEDGEMENTS

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CHAPTER SIX

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SUMMARY

This thesis focuses mainly on the interactions of the helminth parasites, *Hymenolepis diminuta* (Cestoda) and *Moniliformis moniliformis* (Acanthocephala), with the immune system of insect intermediate hosts. Chapter 1 (Introduction) gives details of the life cycles of each parasite species, their interactions with the insect host immune system and an introduction to the concepts of immune stimulation and immunosuppression. The following summary refers to the work presented in experimental Chapters 2 to 6. In these, particular emphasis has been placed on the simultaneous infection by the two species of parasite within one host, the cockroach *Periplaneta americana*, with interest concentrated on both ecological and immunological aspects of such infections.

Chapter 2

Natural concurrent infections of rats with *H.diminuta* and *M.moniliformis* may increase the chances of individual insects being infected by both parasites simultaneously. Although previous work has shown *H.diminuta* and *M.moniliformis* can co-exist in the small intestine of the rat, it was considered important to determine the length of time the two species would be simultaneously patent. This time period would occur during maximum rate of egg production by both parasite species, determined from numbers of eggs found the rat's faeces. It was concluded that although eggs of both species were found in the faeces for approximately 11 weeks post-infection, the chances of an insect acquiring an infection would be highest at the time of peak egg production. This was approximately 6 weeks in the mid-patent period of *M.moniliformis*.

Chapter 3

The initial processes leading to an infection of the intermediate host by *H.diminuta* and *M.moniliformis* were examined; ie. egg ingestion, hatching,

passage along the insect gut and penetration of the midgut wall.

Adaptations by the larvae of both species of parasite to infect the intermediate host were shown to influence host-specificity. *H.diminuta* and *M.moniliformis* hatched in the guts of a wide variety of insect species, but only *M.moniliformis* acanthors penetrated the gut wall of *P.americana*. *H.diminuta* oncospheres only penetrated the gut walls of the locust, *Schistocerca gregaria* and their natural hosts, the flour beetles *Tribolium confusum* and *Tenebrio molitor*.

Transit time for food material passing along the gut was found to be important in the synchronisation of parasite hatching with arrival at the site of gut penetration. *P.americana* holds food items and parasites in the foregut (crop) until partially digested before allowing them into the midgut. Thus any *H.diminuta* oncospheres stimulated to hatch by the insect's mouthparts only progressed as far as the crop until initial digestion was completed; the time taken for crop contents to be passed in to the midgut tended to exceed the time larvae remained active and capable of gut penetration. In contrast, *M.moniliformis* acanthors hatch over a longer time period and therefore enter the midgut in a state capable of gut penetration. The abilities of the two species of parasite to tunnel through the midguts of different insect species was compared *in vitro* using a qualitative assay technique. *H.diminuta* oncospheres were unable to penetrate the tissues of *P.americana* midgut.

Chapter 4

Eggs from both species of parasite were fed simultaneously to insects. It was initially proposed that oral infections of cockroaches with *M.moniliformis* might facilitate penetration of the cockroach gut by *H.diminuta* oncospheres, if the gut tissues were sufficiently disrupted by the former parasite. However, only locusts could be simultaneously

infected with both species orally, infected, and viable *H.diminuta* oncospheres fed to cockroaches were found to adversely affect the success of a simultaneously offered dose of *M.moniliformis* eggs (acanthors).

A hypothesis was put forward to explain this result; that *H.diminuta* oncospheres perturbed the midgut tissues in their unsuccessful attempt to burrow through the gut wall, thus initiating a wound-healing response by the host's immune system. This resulted in gut-penetrant *M.moniliformis* larvae being killed by a melanotic encapsulation reaction.

Unfortunately, light and electron microscopy has revealed little evidence of such a wounding in the gut which might have initiated such an immune response.

Chapter 5

H.diminuta oncospheres were injected directly into the cockroach haemocoele, as it has been previously shown that a small number of parasites survive. By repeatedly passaging the few surviving cysticercoids from each infection through the rat/cockroach system it was hoped to raise a cockroach-infective strain of *H.diminuta*. However, their infectivity to cockroaches did not increase in successive generations; several explanations for the possible failure of this selection programme have been put forward.

Intrahaemocoelic injections of pre-hatched *H.diminuta* oncospheres or *M.moniliformis* acanthellae into the host made it possible to by-pass the gut and thus investigate concurrent haemocoelic infections of cockroaches with both species of parasite. When *H.diminuta* was injected into *M.moniliformis*-infected cockroaches, prevalence and intensity of the former were significantly elevated compared to naive controls, indicating that a putative immunosuppressive action from *M.moniliformis* facilitated

H.diminuta development. In some instances, *H.diminuta* was found to have burrowed through the envelope surrounding *M.moniliformis* and continued normal development within, unmolested by the host's haemocytes. This was considered as further evidence for the protective nature of the acanthocephalan envelope.

Chapter 6

In Chapter 6, assays for aspects of haemocyte behaviour were performed on insects (in particular, *P.americana*) experimentally infected with either *M.moniliformis* or *H.diminuta*. It was found that the phenomenon seen in Chapter 4, whereby *H.diminuta* adversely affected the success of *M.moniliformis* when fed simultaneously to cockroaches, appears to be a direct consequence of the stimulatory effect of *H.diminuta* on the immune system. Conversely, the developing larvae of *M.moniliformis* were shown to depress haemocytic activity; possible explaining why elevated numbers of injected *H.diminuta* survive in *M.moniliformis*-infected cockroaches.

To investigate the affects of immune stimulation on the survival of parasites, locusts were injected with Zymosan, a derivative of yeast cell walls containing β1,3-glucans. *H.diminuta* oncospheres, injected into Zymosan-stimulated locusts appeared to be partially encapsulated, resulting in a temporary arrest in their development when compared to controls.

An assay was devised to observe the encapsulation of materials *in vitro* by the haemocytes of *P.americana*. This method was used to show differences in the haemocytic encapsulation reaction to the different larval stages of *M.moniliformis* and *H.diminuta* . Both the gut-penetrant stages of each parasite (ie. oncospheres and acanthors) were encapsulated, whereas the haemocoelic stages (ie. cysticercoids and acanthellae to cystacanths) remained free of haemocytes.

Chapter 7

Finally, in Chapter 7, a model for the alternative pathways leading to success or failure of parasitism by *H.diminuta* and *M.moniliformis* in the insect host has been discussed. The results presented here contribute to the fuller understanding of how immune stimulation and immunosuppression affect the survival of helminth parasites, particularly in the cockroach host. These two phenomena have also been shown to be effected by the parasites themselves.

Note

Some of the work described in this dissertation has been published in the following papers:

Holt, R.H.F. (1989). *Hymenolepis diminuta* utilises the envelope surrounding *Moniliformis moniliformis* in order to survive in the cockroach host. *J. Parasitol.*, 75, 160-162.

Lackie, A.M. and Holt, R.H.F. (1989). Immunosuppression by larvae of *Moniliformis moniliformis* (Acanthocephala) in their cockroach host (*Periplaneta americana*). *Parasitology*, 98, 307-314.

CHAPTER ONE

INTRODUCTION

THE LIFE CYCLES OF *HYMENOLEPIS DIMINUTA* AND *MONILIFORMIS MONILIFORMIS*

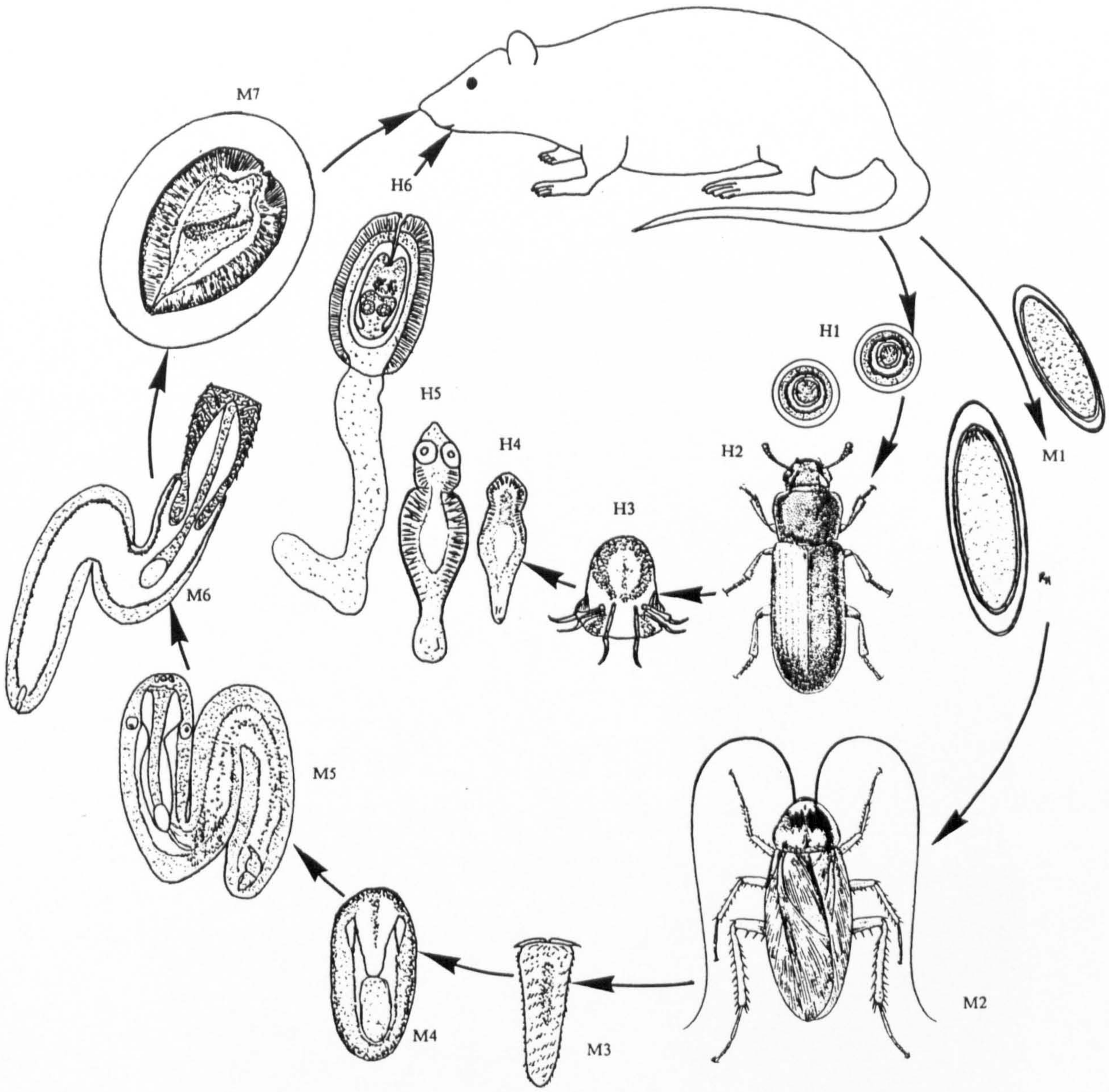


Figure 1.1.

Both *Hymenolepis diminuta* and *Moniliformis moniliformis* occur in the same definitive host, the rat, and infect beetles and cockroaches as their respective intermediate hosts. The adult stages of both parasite species produce eggs (H1 and M1) which pass out of the host with the faeces. Flour beetles, eg. *Tribolium confusum* (H2), ingest *Hymenolepis* eggs which then hatch to release oncospheres (H3). Oncospheres penetrate the gut wall of the beetle to gain access to the haemocoele in which the cysticercoid larvae develop (H4 = stage 2, H5=stage 3 and H6=stage 5 larvae; after Voge and Heyneman, 1957). *Moniliformis* infects cockroaches, eg. *Periplaneta americana* (M2). The eggs hatch to release gut-penetrant acanthors (M3) which then metamorphose once in the haemocoele. Development sequence shown: Stage I acanthella (M4), Stage III acanthella (M5), Stage IV acanthella (M6) and cystacanth (M7) (after King and Robinson, 1967).

FIGURE 1.2 SCANNING ELECTRON MICROGRAPH OF AN ONCOSPHERE LARVA OF *HYMENOLEPIS DIMINUTA*

MH = Median hooks

DLH = Dorso-lateral hooks

VLH = Ventral-lateral hooks

(After Ogren, 1957)

Scale bar = 10μm

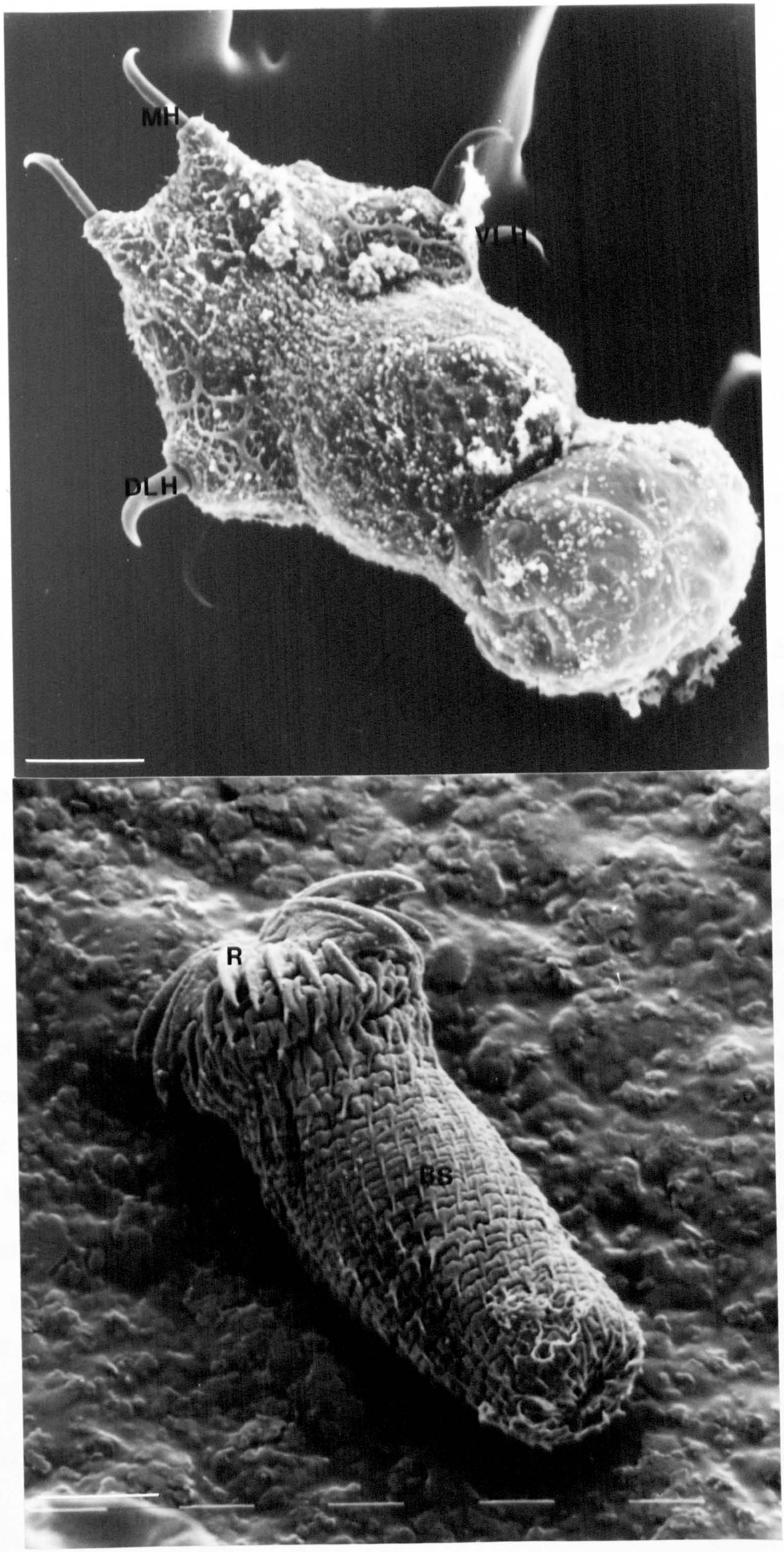
FIGURE 1.3 SCANNING ELECTRON MICROGRAPH OF AN ACANTHOR LARVA OF *MONILIFORMIS MONILIFORMIS*

BS = Helical rows of spines on hind body

R = Rostellar blades and hooks (rostellum)

(After Whitfield, 1971)

Scale bar = 10μm


MH
VCH
DLH
R
BS

1.1 GENERAL BACKGROUND

The fields of parasitology and invertebrate immunology have been integrated in this work, bridging some of the gaps in the knowledge on parasite development in the insect host and their interactions with the host immune system.

Some of the first observations of the invertebrate immune system were made by Metchnikoff in 1884, working with cladocerans. Even though over 100 years have lapsed since then some of the fundamental questions about the internal defence mechanisms of invertebrates remain unanswered. Much of the modern work on parasite interactions with the insect immune system stems from work by Salt (1956, 1957, 1960, 1961, 1963, 1965, 1968, 1970a, see later sections).

Like vertebrates, invertebrates (e.g. arthropods, molluscs and annelids) possess the ability to discriminate "self" from "non-self", but lack the immunoglobulins (antibodies) and immunological memory which enables vertebrates to respond to sequential doses of antigen. However there is some evidence of short term memory capabilities in the cockroach *Periplaneta americana* (Karp and Rheins, 1980; Rheins, Karp and Butz, 1980; Rheins and Karp, 1984) which, through studies in the future, may also be found to apply to other insect species.

The parasites *Hymenolepis diminuta* (Cestoda) and *Moniliformis moniliformis* (Acanthocephala) both develop in the same definitive host, the rat, and also have larval stages in a wide variety of species of insects (Burt, 1980; see also Figure 1.1). This thesis examines the relationship both species of parasite have with the immune system of the intermediate host and tries to derive some explanation for their mechanisms for survival.

This introduction is organised into sections dealing with the different aspects of infections of *M.moniliformis* and *H.diminuta* in both rat definitive hosts and the insect intermediate host. The initial section gives

an introduction to the life-cycles (Figure 1.1) of the parasites, beginning with their final host, the rat, and then continues to describe egg production and the processes involved in infecting the intermediate host. The last sections will deal mainly with parasite-parasite and parasite-host interactions.

1.2 *HYMENOLEPIS DIMINUTA* IN THE DEFINITIVE HOST

Development of *H.diminuta* in the final host begins once the body wall of the ingested intermediate insect host has been digested away in the rat host's stomach so that digestive enzymes act on the fibrous wall of the cysticercoids. Pepsin begins breaking down the cyst wall in the stomach and bile salts then activate the scolex within (Rothman, 1959). After excystment is completed in the duodenum, the small mobile scolex attaches itself to the lining of the gut using its four suckers (*Hymenolepis diminuta* is known as being "unarmed" as the scolex is not equipped with a circle of hooks, normally found in other species of *Hymenolepis*). Goodchild and Harrison (1961) reported the whole process as far as attachment takes approximately 4 hours, 3 for the outer wall of the cysticercoid to be broken down and then a further hour to reach the duodenum. By 24 hours post infection the scolex migrates into the second quarter of the small intestine and development begins. Growth begins slowly at first (Chandler, 1939) but then increases in rate exponentially between 1 and 7 days post infection (Roberts 1961, 1980; Goodchild and Harrison, 1961) The worm eventually becomes patent in approximately 17 days post infection.

The adult worm is usually approximately 45cm long, although this varies considerably according to age, size, sex and diet of the rat host as does egg output (Beck, 1952). The adult worm may produce large numbers of eggs per day; values ranging from as low as 1149 (Hager, 1941) to a vast 300,000 per worm per day (maximum output for one worm, Kino and Kennedy, 1987, see also

Chapter 2 for further details). The life span of *H.diminuta* seems to be limited only by the longevity of the host (Burt, 1980; Read, 1967) as worms which have been experimentally transferred from an ageing host to a young one continue to live indefinitely. However, rejection of the worms by what may be considered an unsuitable host does occur. In experimentally infected mice, worms are rejected from 8-10 days post infection (Hopkins, 1980) by action of the mouse immune system. The worms may destrobilate or be lost altogether.

Throughout its life span, the worm exhibits an age dependent forward migration (Tanaka and MacInnis, 1975) as well as a diurnal migration, travelling up and down the small intestine each day. Read and Kilejian (1969) demonstrated a shift in the amount of worm tissue in various parts of the intestine, with the most tissue in the anterior portion around 8.00am and the least at 4.00pm (see also Chandler, 1939; Chappell *et al.*, 1970; Hopkins, 1970a, b; Crompton, 1973; Holmes, 1973; see also Chapter 2).

1.3 *MONILIFORMIS MONILIFORMIS* IN THE DEFINITIVE HOST

As with *Hymenolepis diminuta* , the intermediate host of *M.moniliformis* has to be ingested by the final host in order for the life cycle to continue (Nickol, 1985), although in some instances some hosts such as amphibians acquire an infection but act only as paratenic host for the worm (Moore, 1946). *M.moniliformis* unlike *H.diminuta* requires only to be activated in the host intestine; excystment requiring the action of digestive enzymes is unnecessary (Stoddart and Crompton, 1988). Once the body of the host has been digested away, bile salts (sodium glycocholate) stimulate the cystacanth into activity (Graff and Kitzman, 1965) so that it can evert its hooked proboscis (rostellum) for attachment to the wall of the anterior portion of the duodenum, without the preliminary migratory phase as with *H.diminuta* (Moore, 1946). The hooks on this proboscis are arranged in distinct patterns and

therefore hook configuration is frequently used as a diagnostic feature to determine the species of the acanthocephalan in question (Van Cleave, 1953).

M.moniliformis is dioecious, the males being the smaller of the sexes (4-13 cm) and the larger female (10-30 cm) responsible for producing approximately 5,500 eggs ("shelled acanthors", Nicholas and Hynes, 1963; West, 1964) per day after a prepatent period of 38 days (Crompton and Nickol, 1985; see also Chapter 2).

1.4 *HYMENOLEPIS DIMINUTA* IN THE INTERMEDIATE HOST

Once gravid, the proglottids of *H.diminuta* detach from the posterior end of the worm and travel down the intestine of the rat. During transit the proglottid wall breaks down so that the eggs are released and mix with faecal material, eventually passing out of the rat with the faeces.

The structure of the egg, more correctly referred to as shelled oncosphere or hexacanth (although for convenience the term "egg" will be retained) has been described by many authors (see for example, Lethbridge, 1971b), although the description of the egg given here is by Holmes and Fairweather (1982).

There are 6 concentric layers or zones around the oncosphere contained in the egg as follows:-

outer layer = "shell", 0.7 to 1.2 μm thick, total diameter 70μm.

zone I = outer cytoplasmic layer, containing proteins, lipids and carbohydrates

zone II = gelatinous layer (protein based)

zone III = embryophore (sulphur-rich proteins)

zone IV = layer beneath the embryophore

zone V = oncospheral membrane

The processes involved in emergence of the oncosphere have been described both *in vitro* (Berntzen and Voge, 1965; Lethbridge, 1972; Holmes and Fairweather, 1982) and *in vivo* (Lethbridge, 1971a; Moczon, 1977) and in both cases the processes involved are essentially the same. Firstly the eggshell must be mechanically disrupted, either by the insect's mouthparts during ingestion, or by agitating the eggs in suspension with glass beads *in vitro* This process is necessary to stimulate the oncospheres into activity, for without mechanical damage the shell is impermeable to enzymes and the oncosphere within cannot be activated fully (Voge and Berntzen, 1961; Berntzen and Voge, 1965). Stimulation of oncosphere activity without mechanical disruption is possible, using distilled water or salt solutions, but the effects are short-lived and hatching does not occur (see Isobe, 1926 in Ubelaker, 1980).

Once the shell is cracked the inner envelope layers then swell as water is taken in by action of the colloidal gelatinous layer. Also the oncosphere becomes fully active and can be seen to moving its three pairs of hooks (see Figure 1.2) to facilitate escape from the shell and subshell layers. At this stage the oncosphere is capable of hatching *in vitro* without the aid of enzymic action, but this is a lengthy process, taking several hours, often resulting in the oncosphere's glycogen reserves being depleted (Lethbridge, 1971a). *In vivo* hatching is speeded up by the action of the host's digestive enzymes on the cytoplasmic and gelatinous layers. Lethbridge (1972) studied the effects of various extracts from the mid guts of beetles and cockroaches on the layers surrounding the oncospheres *in vitro* He concluded that amylases, although capable of digesting a major proportion of the layers, were not entirely responsible for this and that proteolytic enzymes were also required for the breakdown of the subshell layers. This agrees with other workers, for example Voge and Graiwer (1964) and Berntzen and Voge (1965) who used trypsin and amylase in a suitable saline to hatch the oncospheres

completely *in vitro* Holmes and Fairweather (1982) also investigated the extent to which the host facilitated oncosphere emergence by using eggs in which the oncospheres had been killed with liquid nitrogen. They then found that the oncospheres would still hatch even though dead, and thus concluded that the host gut enzymes play a major role in the hatching process.

Temperature also has a significant role in controlling the rate of hatching. Voge and Berntzen (1961) and Berntzen and Voge (1965) found that the optimum temperature for hatching was 25°C; higher temperatures in the region of 37-39°C either immobilised or decreased the mobility of the oncospheres within the membranes of the shell.

Normally *in vivo* the whole process of hatching takes approximately the same time as it does *in vitro* providing temperatures are similar. Lethbridge (1971a) reported that breakdown of the cytoplasmic layer was completed after 6 minutes and then penetration and escape from the embryophore by 12 to 15 minutes after activation.

Once free of the embryophore and oncospheral membrane, the oncosphere (Figure 1.2) travels across the gut lumen to the gut wall and then penetrates the midgut wall in order to reach the haemocoele (see later section on gut penetration). Once in the haemocoele the oncosphere undergoes metamorphosis to produce the early cysticercoid (Ogren, 1962).

Development of *H.diminuta in vivo* from oncosphere to the infective cysticercoid was first described by Grassi and Rovelli in 1892 (see Ubelaker, 1980), and later by Rothman (1957). Voge and Heyneman (1957) divided up the growth sequence into developmental stages, easily recognisable at the light microscope level and it is these which will be used throughout this work. Briefly, these stages are:-

stage 1 = 12-48 hours post-infection at 30°C, spherical appearance, 30-70μm diameter

stage 2 = 48-72 hours, oncospheral hooks migrate towards posterior, body cavity begins to form and overall shape becomes more ovate, size up to 100μm

stage 3 = 72 to 96 hours, scolex formation and body elongation takes place and the overall shape appears "tripartite" (350μm long).

stage 4 = 96 to 100 hours, the scolex retracts into the main body of the cysticercoid so that overall length is now 250-300μm. The vestigial oncospheral hooks are still present at the end of the tail of the cysticercoid.

stage 5 = 100-140 hours, the anterior wall closes around the scolex so that the cysticercoid is now infective to the final host, some increase in tail length occurs. See also Figure 1.1.

The whole process of development takes approximately 120 hours at 30°C, but the above authors found that this could occur in as little as 96 hours. The pattern of cysticercoid development has been described more recently by Richards and Arme (1984a,b) who studied development of the cysticercoid and the cyst wall at an ultrastructural level. They revealed a complex pattern of cellular organisation where the tegumentary, muscular, fibrous and inner cyst components of the cysticercoid wall develop at differing rates.

Voge and Turner (1956a,b) found that *H.diminuta* cysticercoids developed best at 30°C, although the rate of development increased steadily from 25-35°C. They attributed the increase in growth rate to the elevated metabolic activity of the host beetle which resulted in a higher intake of nutrients. These nutrients could then be used by the parasites themselves (Dunkley and Mettrick, 1971).

Very little work has been carried out to determine the optimal diet for an intermediate host to support maximum parasitic development although this

may have an application in keeping helminths in the laboratory. Voge (1959) found that when flour beetles were fed on a flour diet supplemented with sugars (such as sucrose, raffinose and maltose) they lived longer and would also support a greater number of viable *H.diminuta* cysticercoids than those fed on flour alone. On the latter "poor" diet, cysticercoid development remained incomplete, the scolices not withdrawing correctly thus rendering the larvae non-infective to the final host.

Cysticercoids of *H.diminuta* may also be cultured *in vitro* from the oncosphere stage (reviewed by Evans, 1980). Their development follows the same pattern as *in vivo* but tends to be somewhat slower, taking more than 18 days (Voge, 1975; Voge and Green, 1975) with reducing agents being essential additives to the culture media.

1.5 *MONILIFORMIS MONILIFORMIS* IN THE INTERMEDIATE HOST

Moniliformis moniliformis (also formerly known as *Moniliformis dubius*, see Amin, 1985) was first described by Grassi and Calandruccio in 1888 (quoted in Moore, 1946) as *Echinorhincus quercinus* occurring in *Mus decumanus* and *Myoxus quercinus* They also found its intermediate host to be the common beetle, *Blaps micronata*. Later in 1898, Margalhaes described *Moniliformis moniliformis* occurring in *Periplaneta americana* found in Rio de Janiero. Moore (1946) described the development of *M.moniliformis* in the cockroach, *P.americana* (see also Schmidt, 1985).

The fully developed shelled acanthors (referred to as "eggs" in this work) of *M.moniliformis* consist of an acanthor larva enclosed in a four-layered ovate shell. The acanthor larva itself is covered by a thin membrane (diameter of greatest and smallest dimensions are 86μm x 35μm) followed by a shell 4μm thick (diameters 95μm x 44μm). Unlike *H.diminuta* eggs, *M.moniliformis* eggs possess an outer membrane around the shell arranged in hoops and spirals (external diameter of membrane 120μm x 70μm, see Whitfield,

1971).

The stimuli for hatching of *M.moniliformis* acanthors are different to those of *H.diminuta* oncospheres. The egg shells do not seem to require the same physical disruption as with *H.diminuta* , and instead hatching is initiated by a solution of "electrolytes" (ie. a suitable ionic solution) at pH > 7.5 *in vitro* (Edmonds, 1966). It has been suggested that the rate and success of *M.moniliformis* hatching increases with a rise in the internal osmotic pressure which would occur, for example, in the crop of recently-fed *P.americana* (see section 1.7.2). A rise in internal osmotic pressure and thus hatching would be favoured in solutions (see Starling, 1985) which initiate a flux of permeant ions and water flow into the acanthor.

Using 0.3M $NaHCO_3$ at 26°C hatching can be induced *in vitro* in approximately 1 hour. Edmonds (1966) also reported that the hatching acanthor secretes chitinase, possibly to aid digestion of parts of the shell membrane and, along with hook action, facilitate hatching. *In vivo* hatching has been shown to be slower, taking from 2 to 6 hours (see below). Edmonds (1966) found that the viability of the acanthors could be increased if they were stored in distilled water for a period of time. Improvements were most apparent after storage for between 3 to 5 days after which viability was found to be near 50%. Later J. Lackie (1973) found that storage in 60% sucrose not only increased viability but also allowed the eggs to remain viable almost indefinitely.

Once hatched the acanthor larvae (Figure 1.3) penetrate the midgut wall of the cockroach in much the same way as *H.diminuta* does in the flour beetles. The reported timing of this event *in vivo* varies between authors. Moore (1946) states 24-48 hours are taken for the acanthors to hatch, then after "considerable wandering through the tissues of the midgut" they appear on the outer (ie. haemocoelic) surface of the gut 10 to 12 days later. Robinson and Strickland (1969) state that gut penetration may take an even

longer time, up to 14 days, but also show that penetrant acanthors are found on the outer surface of the gut after only 24 hours post infection.

Once in the haemocoele, development to the infective stage takes place. Moore (1946) describes larval development in a series of stages which take approximately 55 days to complete, although the development rate is very much dependent on temperature (J. Lackie, 1972). King and Robinson (1967) break down cystacanth development into 9 stages which will be referred to in this work as described below (see also Figure 1.1). In most cases the stages are recognisable by eye, using body form as an indicator.

Acanthor I	=	newly hatched, motile larvae (see Figure 1.3)
" II	=	larvae penetrating the tissues of the insect gut, and remaining attached to the gut wall
Acanthellae I	=	body spines are lost and the rostellar hooks are displaced
" II	=	nuclear rings of the lemnisci appear, body form recognisable by eye
" III	=	vermiform stage, the larvae undergo rapid growth and elongation
" IV	=	lemnisci form, body form recognisable by eye
" V	=	the larvae reduce in length and become rounder and flatter
" VI	=	proboscis invagination takes place
Cystacanth	=	after further growth the larvae are now infective to the final host (approximately 5 to 8 weeks at 27°C) and are surrounded by a fully developed envelope.

Temperature has a major role in the regulation of growth rate of *M.moniliformis*. Too high (38°C) and *M.moniliformis* is incapable of surviving in the host even though cockroaches can withstand higher temperatures (J. M. Lackie, 1972). At low temperatures development is slowed or completely arrested until warming occurs (King and Robinson, 1967).

Although much of the work regarding host nutrition and the development of *M.moniliformis* has concentrated on the situation in the rat (see Starling, 1985), the diet of the intermediate host may also affect *M.moniliformis* development as found by Cable and Dill (1967) working on another acanthocephalan *Paulisentus fractus*. They found that a poor diet of the host (a copepod, *Tropocyclops prasinus*) resulted in the retardation in parasite growth.

During development from acanthella I onwards a membranous envelope covering the whole surface of the parasite is formed. Gradually this envelope is raised from the parasite's surface, eventually resulting in the parasite "floating" free within its confines. The envelope seems to function as a protective barrier against the effectors of the insect immune system and therefore study of its role has important implications to this work (Mercer and Nicholas, 1967; Robinson and Strickland, 1969; J.M. Lackie and Rotheram, 1972; Rotheram and Crompton, 1972; Lackie and Lackie, 1979; O'Brien, 1988). Also this envelope forms a permeable membrane which allows for nutrient uptake and disposal of metabolic wastes by the parasite, although the mechanism by which the parasite achieves the above processes are as yet unknown.

The origin of the envelope, either parasite- or host-derived, has been disputed for some time. Crompton (1964) proposed that the envelope surrounding the acanthocephalan *Polymorphus minutus* in its host *Gammarus pulex* was derived from the serosal membrane covering the host gut wall. It was

proposed that as the penetrant parasites approached the haemocoelic interface with the gut they remained just under the serosal membrane where they used host tissue to create the envelope. Mercer and Nicholas (1967) observed a similar process whereby it was proposed that *M.moniliformis* formed its envelope, not from the gut serosa, but from encapsulating *P.americana* haemocytes. They described how the newly penetrant larvae were encapsulated and that "vesicular elements of the capsule originate as extensions of the plasma membrane of the haemocytes". They also concluded, on the basis of histological evidence, that an amorphous component of the envelope was parasite-derived. J. Lackie and Rotheram (1972) disagreed with the host derivation of the envelope on the grounds that:-

a, they could culture the acanthors *in vitro* and a membranous coat would form (unfortunately complete development of the parasite was never achieved).

b, after calculating tissue volume occupied by the envelope, they found that it was fully developed by the stage where stretching and elevation from the parasite surface occurred.

c, No haemocytes were found to be present on the envelope surface once elevation of the envelope had commenced, and therefore any growth could not have been formed from haemocytic material.

J. M. Lackie (1972) and Lackie and Lackie (1979) showed conclusively that the gut serosa and the action of the penetration itself was not necessary for the production of the envelope. Acanthors injected directly into the cockroach haemocoele developed normally as did those injected into *Schistocerca gregaria*.

1.6 TRANSMISSION OF PARASITES TO A SUITABLE HOST

Viability of a parasite within a population of hosts depends on the parasite itself utilising some suitable mechanism for transport from one host to another, whether it is a passive or an active mechanism. For the parasites

in this work this is a relevant consideration at 2 stages, dispersal of the eggs from final host to intermediate host and then when the infective stage is eaten by the final host. Both *H.diminuta* and *M.moniliformis* produce large numbers of eggs therefore increasing the chances of infecting the intermediate host, although losses at this stage are very high. Keymer (1980, 1982) and Keymer and Anderson (1979) examined the effects of administering doses of *H.diminuta* eggs in differing spatial distribution patterns and observed the infection of *Tribolium confusum* with time. They found that with exposure to increased doses of eggs the level of infection rose linearly for a small increase in egg density then levelled off as physical and behavioural constraints prevented the hosts from ingesting any more eggs. This distribution is typical of a predator/prey relationship when the parasite eggs in this case are considered as prey items.

Other factors affecting the ingestion of parasite eggs include the availability of alternative food substances. A wide range and large quantities of readily available food will effectively reduce the proportion of parasite eggs in the host's diet and will result in lowered parasite prevalence (ie. the proportion of hosts infected in a population) and intensity of infection (ie. mean number of parasites per host). Keymer and Anderson (1979) also found that spatial distribution of eggs in the host's environment is important in affecting the resultant distribution of the parasite population within the host population. Normally this is found to be overdispersed or aggregated (Anderson, 1982; Crompton *et al.*, 1984) where many hosts have few or no parasites and a few hosts in the population have many parasites. This is due to several factors working together which, when superimposed, tend to increase the degree of overdispersion. For example, out of a population of intermediate hosts only a small proportion of individuals might be in the vicinity of eggs deposited by the final host and an even smaller proportion of these intermediate hosts will find and ingest the eggs; this again being dependent on the nutritional status of the host. Not all the

hosts will ingest the same number of eggs and of those eggs which successfully hatch within the host, the host immune system and physical barriers to infection will selectively reduce the numbers of parasites developing to only the "fittest parasite" or those in the most susceptible hosts.

Keymer and Anderson (1979) set up an experiment to reproduce the factors leading to overdispersion of parasites within a population. Parasite eggs were arranged in various patterns within an arena, then presented to *T.confusum* They found that the most aggregated patterns of eggs in the arena led to the increased overdispersion of parasites within the hosts while at the same time the overall infection intensity remained the same.

It is possible for a host population to become overburdened with parasites so that the adversely affected individuals within the population experience a drop in growth rate and fecundity, as was found in an experimental infection of *H.diminuta* (Keymer, 1980; Maema, 1986) and *Hymenolepis microstoma* (Tan and Jones, 1969) in flour beetles. Similarly Granath (1980) found that both the parasites (*H.diminuta*) and host (*T.molitor*) suffered ill effects at high infection densities and low temperatures. In the wild such a heavy infection would result in the reduction of the pool of available hosts for the parasites, eventually leading to the decline of the parasites themselves. Therefore it is evident that an optimal distribution pattern exists so that the population of parasites is at equilibrium within its population of hosts. Above a critical threshold of parasite density within a population the parasites may cause an increase in host mortality which will eventually have a regulatory effect on the size of the parasite population itself. Conversely if the parasite levels are too low there will be too few infected hosts to maintain the population, leading to the eventual demise of the parasites altogether. This level, which combines mortality and reproductive rates with parasite transmission is defined as the transmission threshold (see Anderson, 1982). The determination of this

threshold level is very important for the planning of vector and disease control programme (Anderson, 1982; Macdonald, 1965).

Rau (1979) examined a natural population of *H.diminuta* found at a riding stable in Quebec. Here there were several species of insect intermediate host available to *H.diminuta* plus an abundance of rat final hosts. Frequency distribution of this natural population in the insect hosts fitted the negative binomial, with *Tribolium obscurus* being the most heavily infected host. Rau concluded that this species of beetle was twice as likely to transmit *H.diminuta* to rats than the other insects present as it was found to support a higher prevalence and intensity of infection. Also *T.obscurus* lived longer, and had a shorter larval period than the other species of beetle. Rau suggested that some of the insects such as the larvae of *T.molitor* ingested large numbers of parasite eggs, but because the parasites do not develop successfully in the larvae (as also shown by Lethbridge, 1971a; Voge and Graiwer, 1964) they were effectively being removed from the parasite population.

1.7 BARRIERS TO INFECTION

The parasites investigated in this study require entry into the haemocoele in order to continue their life cycles. To do so they have to cross, by-pass or evade the physical, biochemical, physiological and immunological barriers presented by the host insect.

This section reviews the literature concerning how parasites in general, and more specifically *H.diminuta* and *M.moniliformis*, overcome these obstacles to successful infection.

1.7.1 THE INSECT GUT

The cuticle covering an insect is extremely tough, water-proof and in itself an effective barrier to most micro-organisms. A more obvious path into

the internal environment of an insect is therefore via the alimentary canal.

Bacteria, protozoa and nematodes are all known to occur in the insect gut as parasites or commensals (see Noble and Noble, 1982 for general text) and may even be pathogenic to the insects themselves as well as to the victims of vector-borne diseases (Wedberg *et al.*, 1949). In the case of *H.diminuta* and *M.moniliformis* the conditions within the guts of most insects are known to provide the appropriate stimuli or enzymes to initiate hatching of their eggs (Chapman, 1985a, b) and cockroach gut enzymes in particular are known to facilitate *H.diminuta* egg-hatching (Lethbridge, 1972, see also Chapter 3 for further details).

Many haemocoelic parasites infect insects via the gut, and therefore must reach a particular region, normally the midgut, before penetration to the haemocoele can take place (see below). Having successfully hatched, the main barrier to infection at this stage is the gut wall itself. Neither the foregut nor hindgut of insects such as the cockroach is easily penetrable due to the cuticular lining (intima) of both (see Chapman, 1985). However the midgut is not protected in this way and is penetrable to parasites suitably equipped to cut through the peritrophic membrane, epithelial cells and musculature (see section 1.7.3).

1.7.2 PASSAGE OF FOOD AND PARASITES ALONG THE INSECT GUT

Once a parasite is ingested by the host insect, it is subject to the same conditions as other ingested food items. This includes the mass movement of ingested matter through, and eventually out of the gut. For a parasite which can only penetrate a particular part of the gut, for example *H.diminuta* in the midgut of *T.molitor*, egg hatching and oncospheral penetration has to be co-ordinated so that the distance travelled down the gut with the ingested meal coincides with the hatching of the larvae near a suitable site of penetration.

Gut transit times for food vary considerably between insect species and also on the size and constituents of the meal which has been taken. In reviews by Chapman (1985b) and Dow (1986) flour beetles are shown to have a generally faster (5 hours in adults, 2 hours in larvae) gut transit time than cockroaches (20-100 hours) which may be important in comparing hatching rates of parasites *in vivo* Also enzyme secretion (which is particularly relevant to the hatching of *H.diminuta*) is dependent on the size of the meal and tends to increase with food intake.

In *T.molitor*, protease and amylase secretion increases with intake of food (Dadd, 1956), possibly controlled by mechanisms resulting from mechanical stimulation of the gut (Chapman, 1985b). Similarly, protease activity increases 1 hour after *P.americana* feeds (Agrawal, 1981), possibly under hormonal control from the salivary glands and secretagogue mechanisms (whereby food chemicals directly stimulate the secretory epithelium). *P.americana*, in particular, is capable of storing large amounts of food in the crop, therefore effectively increasing the gut transit time. The degree of extension of the crop regulates the rate of crop emptying into the midgut, mediated by stretch receptors in the crop wall (Engelmann, 1968). Also the rate of crop emptying is proportional to the concentration of sugars in the crop (Treherne, 1957) and is controlled by a second regulatory factor, osmotic feedback from the haemolymph. This feedback mechanism acts on the valve between the proventriculus and the crop, so that rate of crop emptying depends on the duration and frequency of the opening of this valve. Thus if a cockroach takes a large meal, food may not be released from the crop via the proventriculus into the midgut until the osmotic pressure rises, possibly delaying access any parasite may have to the midgut wall. This delay may be result in the oncospheres running out of glycogen reserves before they reach the midgut or even suffering ill effects from prolonged exposure to enzymes in the gut.

1.7.3 PENETRATION OF THE INSECT GUT BY *HYMENOLEPIS DIMINUTA* AND *MONILIFORMIS MONILIFORMIS*

Hymenolepis diminuta oncospheres utilise 2 mechanisms in order to penetrate the gut wall. Firstly, their primary mode of penetration is by the use of 3 pairs of curved hooks (see figure. 1.2), 1 slender median pair with curved tips and 2 pairs with shorter thicker blades arranged laterally towards the anterior of the oncosphere (Holmes and Fairweather, 1982). The oncosphere uses these hooks in what has been called a "breast stroke" swimming action through the tissues (Lethbridge, 1971a). Each cycle of muscular contraction forces the hooks out into the gut tissue then backwards, pushing the oncosphere forwards in the process. In a heavy infection, ie. 80-100 burrowing larvae, Moczon (1977) described this tunnelling action as causing extensive damage to the epithelium of the midgut wall. This may affect survival of the host and certainly causes pathological changes. However in normal levels of infection, the rapid turnover of cells in the midgut (4 days for *T.molitor* and 40-120 hours for *P.americana*) is usually enough to repair damage so that the host suffers no lasting ill effects.

The second mode by which *H.diminuta* oncospheres may facilitate gut penetration is by the use of penetration gland secretions. Lethbridge and Gijsbers (1974) stained these glands with neutral red and observed their decrease in size as the oncospheres passed through the gut wall, concluding that the oncospheres were using an enzyme(s) to breakdown the cells. Ubelaker (1980) reviews the role of the penetration glands and concludes that even if they have a role in digesting the gut-muscle tissues (Moczon, 1977) the biochemical properties of the secretions are not fully understood.

Penetration of the midguts of beetles by *H.diminuta* oncospheres has been

shown to occur in the anterior third of the midgut taking 45-75 minutes (Lethbridge, 1971a) with some degree of specificity to muscle type and morphology. In cases of beetles with midgut papillae (Snodgrass, 1935; Chapman, 1982, 1985a), some authors (Voge and Graiwer, 1964) have speculated that penetrant oncospheres use these groups of regenerative epithelial cells as a means of avoiding the need to penetrate the tougher gut musculature. Evidence to support this comes from work by Lethbridge (1971a) who found that adult *T.molitor* which have such papillae are susceptible to *H.diminuta* infection whereas the larvae of the beetle which have no papillae and a thicker gut wall are less susceptible. Moczon (1977) disagrees with this, stating that the musculature itself is not an effective barrier to penetration.

Lethbridge (1971a) also considered that the peritrophic membrane, being thicker in larval *T.molitor* than in adults, may also prevent oncospheral penetration. This membrane, formed from a lattice-work of chitin fibrils in a protein - carbohydrate matrix, encloses the food as it passes down the midgut (Chapman, 1985a). Its functions have been described as mainly protective, for example, preventing the abrasive food of phytophagous insects damaging internal gut cells, or as a barrier to micro-organisms. The peritrophic membrane also serves as a selectively permeable membrane controlling enzymatic action on food substances in the gut and also conserves and compartmentalises the activation of enzymes so that some enzymes are inactive until they have passed through the membrane (Terra and Ferriera, 1981). Evidently the larvae of *H.diminuta* and *M.moniliformis* are capable of penetrating the peritrophic membranes of their natural hosts (Lethbridge, 1971a) but it may present an effective barrier in other insects.

The process of penetration by the acanthors of *M.moniliformis* has been described by Whitfield (1971). The body of the acanthor is covered in 20 rows of spines, helically arranged, and pointing posteriorly. Also the rostellum is equipped with two pairs of cutting blades approximately 20μm long (Figure

1.3). Burrowing involves the larvae cutting through the gut tissues, using the rostellar blades everted by a "piston-like" action caused by contraction of muscles in the hind body, to make a pathway while the other body spines prevent any backward slipping. The acanthors also secrete chitinase, which may weaken the peritrophic membrane. Migration across the gut wall of *P.americana* has been reported to take as long as 10-12 days which seems inordinately long when considering that the larvae can move at 2cm per hour for up to 4 hours *in vitro* (Whitfield, 1971). However this only reflects the ability of the acanthor to move through a fluid media, and the rate of movement through gut-tissue is probably much slower.

1.7.4 IMMUNE MECHANISMS IN THE GUT

So far the insect gut has been considered as a physical barrier to parasitic infection. The gut might also play an important role in the immune defence mechanisms of insects, as has already been shown in mammals, such as in the guts of rats and mice responding to cestodes (Hopkins, 1980).

Early work by Wedberg *et al* (1949) showed that various species of micro-organisms would pass through the gut of *Blaberus craniifer* without affecting the insect and there were also some ingested bacteria which did not appear in the faeces. The authors did not express an opinion as to the fate of these micro-organisms; it is possible that they were simply digested and killed but later work has shown that the immune system of the insect may react to organisms within the gut lumen.

A reaction against *Bacillus popilliae* was reported to be mounted by the midgut epithelial cells of the European chafer, *Amphimallon majalis* (Kawanishi *et al.*, 1978). The bacteria normally replicate in the gut lumen, then infect the nidi of the regenerative epithelium, but in this case an inflammatory response by the gut cells and haemocytes subjacent to the epithelial lesions

prevented the infection from spreading. Nematodes are also known to survive in the gut cells of insects (Poinar and Hess, 1974). Cawthorn and Anderson (1977) observed the reaction of *Blatella germanica* and *Acheta pennsylvanicus* hindgut cells to the nematode *Physaloptera maxillaris*. They found that, the cockroaches formed melanised capsules around the nematodes and killed them while in the colon, whereas the crickets had little or no defence against this infection.

Dow's (1986) review of the insect midgut describes recent work on the properties of endocrine cells found in the cockroach midgut (Endo and Nishiitsutsuji-Uwo, 1980, 1982; Andries and Tramu, 1985). The precise function of these cells has yet to be determined, but Dow has suggested that they may be involved in sending chemical signals to various functional systems in the insect including the immune response. It is possible that they can detect harmful chemicals or damage in the gut and alert the immune system to react accordingly (see also Chapters 4 and 6).

From the evidence above the immune response may take effect when a parasite comes into contact with the midgut wall. Once the gut is penetrated and the parasite survives to enter the haemocoele, it is then intimately in contact with the effectors of the immune system and must prevent the host from immunorecognition. The insect's immune system, although very different from the vertebrate system, is a very effective barrier to parasite survival.

1.7.5 THE INSECT IMMUNE SYSTEM

The basic principle behind the evolution of an immune system is to maintain the integrity of a colony of cells and at the same time prevent invasion by non-self organisms or materials. Even the most simple multicellular animals, the Porifera and Coelenterata, have cells involved in recognising and rejecting foreign or non-self material. This action can be considered as an immune response and may protect, for example, one species of coral from overgrowth by another. A general review of invertebrate immunity

is provided by Ratcliffe *et al.*, (1985).

The components of the insect immune system, both cellular and humoral, are situated mainly in the haemocoele. The cellular component consists of circulating haemocytes, classified throughout the literature in a confusing and large number of ways, some systems relying on morphological and granular content of the cells and others on the function of the cells. For examples of classification see Gupta (1979), Rowley and Ratcliffe (1981), Brehelin and Zachary (1986). Lackie (1988a) describes the various classes of haemocytes, the ones referred to in this work are as follows:-

Prohaemocytes - Small, round cells, normally associated with insect larvae and considered as the stem cells from which other cell types develop.

Plasmatocytes - Normally the most abundant cells in the haemolymph, which contain a variable number of granular inclusions, especially in cockroaches. *In vivo* the cells are phagocytic and involved in defence reactions, such as capsule and nodule formation against invasive particles (see below). The cells appear rounded or spindle-shaped and may also come out of suspension, flatten, but remain motile *in vivo* and *in vitro* Some workers refer to another cell type, the granulocytes, as a separate cell type from plasmatocytes, but in the insects used in this work they probably constitute a behavioural subclass of plasmatocyte (Lackie, 1988).

Coagulocytes - These are granular cells which lyse readily on contact with foreign surfaces, possibly coating them with a gel or granular coagulum.

The cellular components of the immune system act against non-self materials and molecules in several ways, determined mainly by the size of the particle being dealt with by the haemocytes as follows:-

1.7.5.1 PHAGOCYTOSIS

Phagocytosis involves the internalisation of small particles such as yeasts and bacteria, and also haemocytes have been shown to phagocytose injected latex beads and colloidal carbon under experimental conditions. Both coagulocytes and plasmatocytes (Brehelin and Hoffmann, 1980; Guzo and Stoltz, 1987; M. Carr and A. Lackie, unpublished) can phagocytose *in vivo* and *in vitro* though the plasmatocytes form the largest proportion of the phagocytosing cells. The proportions of these cells may vary within a species, depending on (a) the material the cells are phagocytosing (Ratcliffe and Walters, 1983), (b) when the cells are examined after phagocytosis commences and (c) if the cells are stimulated prior to the introduction of particles into the haemolymph. Gunnarsson (1988a) has shown that phagocytic activity of *Schistocerca gregaria* haemocytes can be non-specifically stimulated *in vivo* by an injection of saline. Dularay and Lackie (1987) found a similar non-specific stimulation after an injection of Sepharose beads into *P.americana.* In these cases it seems that the insect's immune system is responding to a wound or the presence of a foreign surface. The proportion of phagocytic cells may also be increased chemically by injected β1,3-glucans *in vivo* (Gunnarsson, 1988a) and *in vitro* (Ratcliffe *et al.*, 1984; Leonard *et al.*, 1985a; Huxham and Lackie, 1988). Phagocytosis can clear low doses of bacteria from the haemolymph, but if the dose was increased to over $10^3 \mu l^{-1}$ haemolymph, Ratcliffe and Walters (1983) found that nodule formation was a more efficient clearance mechanism in *Galleria mellonella* larvae.

1.7.5.2 NODULE FORMATION

Nodule formation is a process by which small haemocytic aggregates form around foreign particles, normally bacteria, and remove them from circulation

in the haemolymph (Salt, 1970a). In *Galleria mellonella*, once bacteria are trapped by haemocytes, the resulting nodule becomes melanised (Ratcliffe and Gagen, 1976, 1977) and adheres to the lining of the haemocoele. However, melanisation of nodules does not occur in all insect species and also depends on the nature of the stimulus (Gunnarsson and Lackie, 1985; Guzo and Stoltz, 1987). In her review of haemocyte behaviour, Lackie (1988a) summarises the information on stimulators of nodule formation. Briefly these include bacteria, viruses, vertebrate erythrocytes, yeasts and fungal spores, protozoan parasites and xenogeneic haemocytes and a wide variety of abiotic materials. Also nodules form in a response to soluble molecules such as bacterial endotoxin, Laminarin and Zymosan supernatant (see Chapter 6), galactose-rich glycoproteins (eg. porcine stomach mucin) and in addition there are examples of non-stimulatory soluble chemicals, such as dextran (an α1,6-glucan) and bovine serum albumin (nonglycosylated protein).

Locusts which had received a prior injection of Zymosan supernatant (containing β1,6- and β1,3-glucans in solution) or cockroaches injected with pieces of *Blaberus craniifer* tissue were found to be highly sensitive to an injection of saline 24 hours afterwards which stimulated the production of large numbers of haemocytic nodules (Gunnarsson, 1988a; Lackie and Dularay, unpublished). It has been reasoned that this was probably a response from the pre-activated haemocytes which had subsequently become more sensitive to a reaction initiated by puncturing the cuticle and epidermis during the process of injecting saline. Any injection probably initiates a small wounding response which involves the release of wound factors into the haemolymph (Lackie, 1988a).

1.7.5.3 ENCAPSULATION

Any objects within the haemocoele which are too big to be phagocytosed and require a greater number of cells than usually involved in nodulation are

surrounded by concentric layers of haemocytes; ie encapsulated. As the processes involved in encapsulation are also described in Lackie (1988a) a brief summary will be provided here.

There are 3 steps in the process, initial contact and cell adhesion, haemocyte recruitment to form the capsule and finally cessation of the reaction. In *Clitumnus extradentatus* (Schmit and Ratcliffe, 1978), *Locusta migratoria* and *Melolontha melolontha* (Brehelin *et al.*, 1975) and *Bombyx mori* (Sato *et al.*, 1976) it has been shown that initial contact in the process of encapsulation is made by coagulocytes and granular cells which rapidly adhere and degranulate onto a foreign surface, although it appears that this early degranulation of coagulocytes is absent in *P.americana.* Ratcliffe (1986) proposed that this initial degranulation coated the target with components of the phenoloxidase system which then allowed plasmatocytes to adhere to form the main body of the capsule. The thickness of the capsule varies depending on the number of haemocytes available, for example *P.americana* has a higher concentration, and larger number of haemocytes in its haemolymph than *S.gregaria*, and thereby produces thicker capsules (Lackie *et al.*, 1985). Capsule thickness also depends on the relative electrostatic charges or hydrophobicity of the haemocytes and their targets (Lackie, 1983, 1986a; Takle, 1986; Vinson, 1974) and also the surface carbohydrate composition (Lackie and Vasta, 1988; see also discussion of recognition, section 1.7.5.5).

Cessation of the reaction occurs after the layers of haemocytes forming the capsule become flattened, interdigitate and the surface becomes smoothed with a layer of extracellular material (Takle, 1986). Any other haemocytes in the vicinity of the capsule then "lose interest" in the capsule.

The killing mechanism involved in encapsulation is not fully understood. The melanin produced in the centre of a capsule as an end product of the phenoloxidase cascade (Soderhall, 1982) has been thought to be associated with the production of a variety of toxic phenolic compounds, although in several species which exhibit melanising ability, melanin did not appear to have a

bactericidal effect (Walters and Ratcliffe, 1983; White *et al.*, 1985). Some parasitic species are able to survive total encapsulation, for example some helminth larvae (Lackie, unpublished) and also the hyphae of *Metarhizium anisopliae* (Gunnarsson, 1987) remain viable within haemocytic capsules. Melanotic encapsulation can occur with few or no haemocytes (ie. humoral encapsulation) as in the case of many of the Diptera (Gotz, 1986) and it seems that phenoloxidase is not just compartmentalised in the haemocytic portion of the haemolymph as a whole. In the literature, humoral defence and cellular defence mechanisms are often regarded separately, although this is rather an artificial division, as many of the humoral factors are derived from haemocytes.

1.7.5.4 HUMORAL DEFENCE

In cellular encapsulation, the amount of prophenoloxidase released by attaching granular cells is relatively small, and the bulk of the capsule is formed by the haemocytes. The main difference between cellular and humoral encapsulation is that in the latter case the resultant capsule is cell-free. The material forming the capsule is not from attaching cells but from the haemolymph (Gotz, 1986). In some dipteran species (eg. *Chironomus*), with few circulating haemocytes, humoral encapsulation predominates and has been shown to be effective against nematodes and bacteria (Gotz, 1973).

Humoral defence relies mainly on antibacterial substances which are produced in the fat body. There have been many antibacterial proteins characterised, the production of which can be induced in *Hyalophora cecropia* pupae by injection of non-pathogenic bacteria. Antibacterial proteins are classified in 2 main groups, attacins (active against gram positive and negative bacteria) and cecropins (active against *Escherichia coli*) (Boman, 1986). Some of the phagocytes in *H.cecropia* have been shown to associate

closely with the fat body and thereby induce the fat body to produce these proteins (Faye, 1978). Trenczek (1986) has also shown that *H.cecropia* haemocytes can themselves synthesise cecropins and attacins. Similar proteins to attacins and cecropins have been found in other holometabolous insects such as *Galleria mellonella* (Hoffman *et al.*, 1981) and *Manduca sexta* (Spies *et al.*, 1986) but have not been found in hemimetabolous insects such as the cockroach (Takle, personal communication) or the cricket. However *Locusta migratoria* has been shown to produce a different antibacterial protein in response to infections of bacteria but not to eukaryotes (Lambert and Hoffman, 1985; see also Boman and Hultmark, 1987 for a review of antibacterial molecules).

Lysozyme is also produced in response to infection (Chadwick, 1970; Spies *et al.*, 1986). Chadwick (1970) found that increased levels of lysozyme did not necessarily increase the antibacterial capabilities of the insect and therefore it is not known whether lysozyme itself is an effective bactericide. Boman and Hultmark (1987) considered the role of lysozyme and suggested that it digested the peptidoglycan layer of bacterial cell walls which are exposed after attacins and cecropins have attacked the outer membranes.

1.7.5.5 IMMUNE RECOGNITION

It has been argued that haemocytes either directly recognise foreign bodies and wounds during random contact or are responding to soluble factors released from wounds (Salt, 1970; Lackie, 1988a). To be effective these soluble factors must cause the behaviour of the haemocytes to change so that they come out of circulation and adhere only to the appropriate (ie. damaged or "non-self") surface. However, any soluble molecule present in the haemolymph will be rapidly dispersed from a point source which would seem to make the targeting of a precise area impossible. Therefore it would be reasonable to suggest that a general attachment of haemocytes occurs onto many

surfaces, but only those attaching to and recognising a wound or foreign body actively recruit further haemocytes to form a permanent capsule.

Recognition of a wound deep in the tissues, for example within the gut, as mentioned earlier in this chapter, requires that haemocytes respond to a soluble molecular signal either by chemokinesis (a general change in rate of movement or frequency of turning) or chemotaxis (a change in direction and rate of movement associated with following a concentration gradient, J. M. Lackie, 1986). A concentration gradient can only be maintained where there is little or no circulation and therefore this system could only work within the tissues.

Cherbas (1973) identified a wound factor, "haemokinin", which when released from various damaged tissues taken from moths (*H.cecropia*, *Philosamia cynthia* and *Antheraea polyphemus*) altered the behaviour *in vitro* of haemocytes. Similarly other workers (Harvey and Williams, 1961) have shown that damaged tissue causes an increased demand for oxygen and have related this with possible factors released from the wounded area.

Phenoloxidases are found both within the cuticle (see Brunet, 1980; Andersen, 1985) and haemolymph of insects. In the haemolymph, activation of the prophenoloxidase system may cause the release of "wound factors" which alter the behaviour of the haemocytes. Haemocyte adhesion (Johansson and Soderhall, 1988), locomotion (Takle and Lackie, 1986; Huxham and Lackie, 1988) and exocytosis (Ratcliffe *et al.*, 1984) are all affected *in vitro* by these factors, which might also be produced *in vivo* when the cuticle is damaged. The prophenoloxidase system can also be activated *in vitro* in the presence of calcium ions by injected β1,3-glucans (eg Laminarin and Zymosan supernatants) (Pye, 1974; Söderhäll, 1981; Ashida *et al.*, 1983; Dularay and Lackie, 1985; Leonard *et al.*, 1985b).

Arthropod lectins have been discussed with regard to their role in the recognition arm of the immune system (Renwrantz, 1986) although their precise function still remains unknown. Evidence suggests that they occur on

haemocyte membranes and in serum, where they may bind specifically to foreign molecules either produced by or found on the surface of invasive foreign bodies. However, much of the early work on opsonisation of bacteria or erythrocytes for phagocytic uptake was inconclusive or negative (Scott, 1971; Rowley and Ratcliffe, 1980). One disadvantage with research into lectin production is that the major lectins found in the haemocytes or haemolymph possibly mask the properties of the minor lectins of other specificity (Lackie, 1988a; Renwrantz, 1986); for example, most lectins purified from insects are specific for galactose as found in *P.americana* (Lackie and Vasta, 1986, 1988; Kubo and Natori, 1987), grasshoppers (Stebbins and Hapner, 1985, 1986), *Sarcophaga* (Komano *et al.*, 1980) and *H.cecropia* (Castro *et al.*, 1987). When injected into *P.americana*, a galactose-rich glycoprotein (porcine stomach mucin) stimulated large numbers of nodules *in vivo* (Lackie and Vasta, 1988). It has also been found that *Sarcophaga* galactose-specific lectin is released as a response to wounding and to an injection of sheep erythrocytes, which are subsequently lysed (Komano and Natori, 1985); these authors concluded that the lectin probably acts to recognise and remove foreign or damaged tissues.

1.7.5.6 BASEMENT MEMBRANE

Recognition and recruitment of haemocytes onto abiotic surfaces may work by another system independent of wound factor control, as the targets themselves might be inert but are still subject to immediate encapsulation. Thus the haemocytes must have some ability to to target inert non-self materials which do not produce "haemokinin"-like substances.

In insects the lining of the haemocoele is coated with what appears (under the electron microscope) to be a type of basement membrane ie. it is thicker than subepithelial basement membrane (see Ashhurst, 1979), and contains a non-fibrillar type IV collagen to which proteoglyacans and glycoproteins are attached (Timpl *et al.*, 1981). Haemocytes are sensitive to

the integrity of this membrane, which if damaged or changed in some way is readily encapsulated (Salt, 1961, 1970a; Scott, 1971; Rizki and Rizki, 1980; Lackie, 1986b, c). Thus any foreign surface implanted in the haemocoele may be recognised by its lack of components characteristic of "self" basement membrane. Also haemocyte recognition may be facilitated by the adsorption of plasma factors onto the foreign surface.

The carbohydrate molecules associated with basement membrane appear to be associated with different tissues within the insect, as shown by using exogenous lectins. For example, galactose-specific peanut agglutinin binds to *P.americana* midgut basement membrane but not to that of Malpighian tubules or ovaries (R. Martin and A. Lackie, unpublished). A similar study of the salivary glands of *Aedes aegypti* has shown differences in the lectin-binding characteristics over their surface. Perrone *et al.* (1986) have suggested that malaria sporozoites may have specific carbohydrate receptors which enable them to find and penetrate the appropriate region of the mosquito salivary gland.

Transplantation experiments in insects have demonstrated the remarkable lack of recognition of allogenic and some xenogeneic implants and cuticular grafts (Lackie, 1986b). It appears that variability in basement membrane composition between closely related species is not high enough to induce an immune response, although if the basement membrane is damaged the transplanted tissues are encapsulated.

Disturbance of the molecular integrity of the membrane may also account for the recruitment of haemocytes to the lining of the haemocoele of *Schistocerca gregaria* during the initial stages of an infection of the fungus *Metarhizium anisopliae* (Gunnarsson, 1988b). The fungal hyphae invade the insect by penetrating the cuticle which itself is closely associated with the epidermal layer beneath. Before penetration was completed, haemocytes were seen adhering to the haemocoelic surface below the infected area of cuticle and were thought to be reacting to the damaged basement membrane of the

epidermal layer.

1.7.5.7 PHYSICOCHEMICAL FACTORS IN RECOGNITION

Lackie (1988a) stated that surface charge and carbohydrate composition are two of the parameters which influence haemocytic response. The electrostatic charge on the surface of a foreign body has been known for some time to affect haemocyte adhesion and encapsulation (see Vinson, 1974 for charges on *Heliothis virescens* haemocytes; Dunphy and Nolan, 1982 for *Choristoneura fumiferana*; Lackie, 1983, 1986a, for *P.americana* and *S.gregaria*).

A highly negative charge on polystyrene substrata was found to provoke the thickest encapsulation reaction *in vitro* using isolated *P.americana* haemocytes (Lackie, 1986a) and similarly *in vivo* negatively charged CM-Sepharose beads provoked a greater response than neutral Sepharose. However, locust haemocytes encapsulated neutral but not negative beads (Lackie, 1983). By using both cell electrophoresis and measurement of cationised ferritin binding, Takle and Lackie (1985) found that locust cells carried a higher negative charge than those of *P.americana.* This might in part explain why locusts are tolerant of a relatively wide range of parasites and abiotic implants. On the basis of electrostatic repulsion and attraction in cell-substratum adhesion (Curtis, 1972), it would be harder for locust haemocytes to make contact with parasites which are predominantly negatively charged.

Lackie (1988a) has also suggested that the haemocytic encapsulation response *in vivo* is influenced by the carbohydrate composition of the surface of foreign bodies. Lackie and Vasta (1988) found that Sepharose beads conjugated to galactose-rich glycoproteins provoked significantly thicker capsules compared to those beads which were conjugated to sialic acid-rich glycoproteins, a result in direct contrast to that predicted on the basis of surface charge.

1.7.5.8 ALTERATION OF THE HOST IMMUNE RESPONSE

The ability of the immune system to recognise and respond to foreign material depends on the past history of the host, and may be influenced by the presence of parasites in the haemocoele. Parasite survival in the host depends on the avoidance or suppression of the immune system; this ability depends on past experience of the parasite in evolutionary terms. Some parasites may immunosuppress the host so that other parasites, perhaps unrelated to the primary infection, survive in the haemocoele where they would normally be recognised and encapsulated. This effect is very well documented in the case of parasitoid wasps, which during their life cycle parasitise and kill lepidopteran larvae. Although this mode of parasitism is unlike that of *H.diminuta* and *M.moniliformis*, which both form chronic rather than acute infections, the mode of immunosuppression is of relevance to these helminths and therefore a few examples are given below.

Walker (1959) first described how the cynipid wasp *Pseudeucoila bochei* parasitised the larvae of *Drosophila melanogaster*, where the parasites were in some way prevented from haemocytic encapsulation. She suggested that some form of immunosuppressive agent from the female wasp was responsible. Nappi and Streams (1969) followed up this work, finding a change in number and also in type of cells associated with the encapsulation of the susceptible eggs of another cynipid wasp *P.mellipes*. However with *P.bochei* no such change was observed, this parasite had survived by actively suppressing the host, preventing any change in the haemocytes. Thus *P.mellipes* can only parasitise wasps already infected with *P.bochei*, a type of association regularly encountered in parasitic wasps. This example of obligatory multiparasitism was also observed by Nappi (1975), who noted that the last eggs oviposited by the female during laying were more prone to encapsulation than the first. Guzo

and Stoltz (1985, 1987) found a similar situation in the tussock moth *Orgyia leucostigma*, parasitised simultaneously by *Cotesia melanoscela* and *Hyposter exiguae*; in this case the authors found a virus associated with the venom and calyx fluids of *C.melanoscela* which seemed to be responsible for the immunosuppression of the host. A similar virus was also found in the calyx and venom fluids of *Hyposter fugitivus*, which after purification, also acted as an immune suppressant (Guzo and Stoltz, 1987). More recent studies by Davies and Vinson (1986) have shown that the molecular components of the fibrous layer surrounding the eggs of *Cardiochiles nigriceps* seem to delay encapsulation, rather than utilising viruses and the effects of venom. The eggs of *Nemeritis canescens* (=*Venturia*) have a similar fibrous layer, in which "particles entangled in long projections from the chorion are the effective agent in preventing encapsulation" while the effectiveness of the host immune system against other invasive agents remains unimpaired (Rotheram, 1967; Salt, 1970b; Bedwin, 1979).

Lackie (1986d) and Yoshino and Boswell (1986) point out the danger of immunosuppression as a strategy for parasite survival. They reasoned that if the immune system of an infected host was totally suppressed it would be unable to respond to other pathogens. This might lead to an increased mortality in the host population resulting in fewer available hosts. However, it seems that those parasites which use immunosuppression in this way only infect the living host for a short period of time, after which the parasite kills the host. Evidence presented below appears to contradict this reasoning as *M.moniliformis* was found to immunosuppress the cockroach host.

Other parasites have immunosuppressive capabilities; the nematode *Mermis nigrescens* infects *Schistocerca gregaria* and as a consequence the locusts are made susceptible to experimental infection (by intrahaemocoelic injection) of two species of trypanosomatid protozoans (Ibrahim *et al.*, 1986). Similarly destruxins, produced as secondary metabolites from the fungus *Metarhizium anisopliae*, reduce the ability of the locust to form nodules in response to

particles, or chemicals of microbial origin (Vey *et al.*, 1985; Huxham *et al.*, 1988). Also *M.anisopliae* suppresses the effect that β1,3-glucans have on the activation of prophenoloxidase in *P.americana* and *S.gregaria* (Huxham and Lackie, 1986; Huxham *et al.*, 1988).

The opposite effect of immunosuppression occurs when the immune system of an insect has been recently stimulated. Immunocompetence at the cellular level in *Galleria mellonella* larvae increases after latex beads are injected into the haemocoele so that the larvae acquire the ability to phagocytose a normally non-phagocytosable strain of *Bacillus thuringiensis* (Mohrig *et al.*, 1979). Similarly, by stimulating the immune system of *P.americana* with an injection of Sepharose beads or implants of *Blaberus craniifer* tissue, cuticular grafts of normally acceptable *Blatta orientalis* are rejected (Dularay and Lackie, 1987) and at the same time nodule-forming and phagocytic ability are enhanced.

Thus it seems that the immune system can be manipulated in two directions. First so that a broader range of non-self material is accepted or ignored by the haemocytes in the haemolymph (ie. immunosuppression) which may or may not reduce the general immune capabilities of the insect. Secondly, non-specific stimulation of the insect immune system may result in an increase in recognition of all but a narrow range of self-like materials and also a general increase in responsiveness.

1.7.6 INTERACTIONS OF *HYMENOLEPIS DIMINUTA* WITH THE IMMUNE RESPONSE OF THE INTERMEDIATE HOST

For a parasite to survive in the host haemocoele there are 2 possible mechanisms, either to avoid or to suppress immunorecognition and/or effector mechanisms. Avoidance of recognition could be achieved in a similar way as the successfully xenografted tissues survives in its "host" insect (Lackie, 1986c) - if a parasite surface appears to be enough like host self tissue it

will escape recognition (see section 1.7.5.6 on basement membrane).

Lackie (1976) suggested that *H.diminuta* avoided encapsulation at least in part by appearing as host tissue to the immune system of the host. However *H.diminuta* does not only develop in the flour beetle but also in *S.gregaria* (Lethbridge, 1971c; Lackie, 1981 and see also Chapter 5) and occasionally in *P.americana* at low densities when pre-hatched larvae are injected directly into the haemocoele. As it is already known that *P.americana* will reject *S.gregaria* tissue implants, it seems unlikely that *H.diminuta* is inherently "tissue typed" to only one of its hosts. Instead it could utilise some active mechanism of adsorption of host-like molecules onto its surface thereby creating a disguise while in the haemolymph.

The mechanism for disguise could well be mediated by the microvilli which cover the whole surface of the cysticercoids from the early larval stages onwards (Ubelaker *et al.*, 1970; Heyneman and Voge, 1971; Lackie, 1976; Richards and Arme, 1984a). Several workers have found beetle haemocytes in close association with the microvillar layer, and have suggested that this layer acts in defence of the cysticercoid by lysing attacking haemocytes. Lackie (1976) also found cell lysis to occur at the cyst surface *in vitro* but concluded that this was not part of the protective function of the cysticercoid surface. Moreover to actively lyse large numbers of haemocytes would seem a dangerous method of preventing encapsulation, as this would more likely initiate a wounding response resulting in wholescale attack by the immune system. Richards and Arme (1985a), examining the haemocyte debris on the surface of the cysticercoid, suggested that normally the negative electrostatic charge on the surface of the cysticercoid may prevent encapsulation, but when small areas of the surface are disrupted during morphological changes at the time of scolex retraction they are open to temporary haemocytic attack.

The host response to a parasite may be mediated by changes in the

endocrine system (Nappi, 1973a,b). Hurd and her co-workers have shown that *H.diminuta* affects the endocrine system of *T.molitor*, causing alterations in vitellogenins and egg production. Hurd and Arme (1984a,b; 1986a,b; 1987a,b) Hurd *et al.*, (1982) Hurd *et al.*, (1987) and Hurd and Weaver (1987) have also shown that *H.diminuta* alters the amino acid composition of the beetle's haemolymph; it was originally thought that this might have been due to parasite inhibition of juvenile hormone production by the *corpora allata*. However, recent evidence (Hurd and Weaver, 1987) has disproved this and as yet no explanation to why haemolymph proteins change during infection has been offered.

Although the mechanism whereby *H.diminuta* avoids/evades the immune system has still not been ascertained, from evidence presented below it seems unlikely to have the same type of mechanism as *M.moniliformis* .

1.7.7 INTERACTIONS OF *MONILIFORMIS MONILIFORMIS* WITH THE IMMUNE RESPONSE OF THE INTERMEDIATE HOST

Periplaneta americana haemocytes are known to react to the early larvae of *M.moniliformis* . Even as the acanthor stage is burrowing through the gut wall of the host, haemocytes begin to aggregate around the haemocoelic side of the gut adjacent to the site of entry (Robinson and Strickland, 1969), and once in the haemocoele the larvae are weakly encapsulated. However the capsule only lasts for a few days, the haemocytes disappear leaving the larvae to continue development.

Rotheram and Crompton (1972) proposed that the microvilli covering the surface of the parasite were in some way responsible for the disappearance of the haemocytes, as the microvilli were involved in the raising of the envelope, possibly incorporating host molecules in the process.

If *M.moniliformis* appears similar to host "self", then it would be expected to provoke a response similar to that of its own host's tissue if

transplanted to another insect. Lackie and Lackie (1979) tested this hypothesis by transferring cystacanths raised in locusts to *P.americana* and found that as with transplanted tissue there was no reaction to the parasites. Either the parasites did not use the above "disguise" technique or surface molecular turnover was very rapid allowing the molecules of the new host to replace the old before immunorecognition took place.

Brennan and Cheng (1975) stated that *M.moniliformis* actively suppresses the host's immune system, thereby preventing the parasites from being encapsulated and melanised. It was proposed that the material forming the amorphous coat on the acanthor protected the parasite by inhibiting enzymes of the phenoloxidase pathway. By blocking tyrosinase action the parasite prevented the production of toxic quinones and hence the melanisation process. On this basis, the authors argued that the temporary encapsulation of the acanthors, as they entered the haemocoele after penetrating the gut, was because the amorphous coat had been damaged during penetration; this then allowed immunorecognition to take place until the amorphous layer had been restored. However, the above authors did not use whole living material; instead they used sonicated acanthors, so that not only were parts of the amorphous layer present in the preparations injected into cockroaches but also damaged tissues of an indefinable nature. If immunosuppression does take place it cannot therefore be said to be conclusively due to action of the amorphous layer of the envelope alone.

1.8 THE AIMS OF THE WORK DESCRIBED IN THIS THESIS

Given that a low proportion of the cockroaches *Periplaneta americana* and *Blatta orientalis* can become infected after intrahaemocoelic injections of *Hymenolepis diminuta* oncospheres; and that preliminary experiments had suggested that the infectivity of the parasite for *P.americana* could be increased by selection, the original aim of this project was to select

"cockroach-adapted" strains of *H.diminuta*. It was intended that this strain was to be used to study (a) the nature of the parasite's adaptation to its new host, by means of comparing the host's immune response to adapted and unadapted strains, and (b) the interaction of the parasite with inbred laboratory stock and local "wild-caught" *B.orientalis*, in order to provide insight into the evolution of parasite adaptation to new hosts. Attempts to select a cockroach-adapted strain by intrahaemocoelic injection and by oral infection were unsuccessful, as described in Chapter 5, but the results provided some very interesting information, on the interactions of *H.diminuta* with *P.americana*, which were exploited in the subsequent work.

Since wild-caught rats have been found to be naturally and simultaneously infected with the acanthocephalan *Moniliformis moniliformis* and the cestode *Hymenolepis diminuta*, it was interesting to determine whether a single species of insect host could become simultaneously infected with both parasites after eating faeces from those rats harbouring concomitant infections; in other words would it be possible for rats to pick up their double infection by eating single individual insects?

The aims of the project were thus extended:-

1) To determine which range of insect species could become infected after ingesting eggs of *H.diminuta* and/or *M.moniliformis*, by examining the ability of larvae to hatch in the insect gut and penetrate to the haemocoele.

2) To determine the effect of pre-existent haemocoelic infections of *M.moniliformis* on the establishment of *H.diminuta* in the cockroach *P.americana*.

3) To manipulate (stimulate or suppress) the immune response of *P.americana* and examine its effect in the intrahaemocoelic establishment

of *H.diminuta* .

4) In the course of the work, it became apparent that the 2 species of parasite themselves caused dramatic changes in immune responsiveness, and it became necessary to devise a variety of assays for haemocytic behaviour ("cellular immunity") in order to measure and compare the parasites' effects.

CHAPTER TWO

CONCURRENT INFECTIONS OF RATS WITH *HYMENOLEPIS DIMINUTA* AND *MONILIFORMIS MONILIFORMIS*

2.1 INTRODUCTION

In the wild, rats have been reported to carry infections of both *Moniliformis moniliformis* and *Hymenolepis diminuta* simultaneously (Holmes, 1973 and Holland, 1987). Holland not only describes instances where *M.moniliformis* and *H.diminuta* occur in the same host, but also a third parasite, *Nippostrongylus braziliensis*, forms a multi-species infection with the other two.

The importance of rats as carriers of both *M.moniliformis* and *H.diminuta* is twofold. Firstly it will mean both parasites can complete their life cycles in the same localised environment if intermediate hosts are available. Secondly, as both parasites have intermediate hosts which are found in stored food and refuse, these hosts may become infected from rat droppings left in domestic situations and pass on the infections to man.

Co-parasitism in the final host could also lead to a similar situation in an insect, providing this intermediate host's feeding habits, morphology and physiology were suitable. The occurrence of simultaneous infections in the intermediate host will form the basis of Chapter 4 and Chapter 5. In this chapter the work will concentrate on determining whether or not laboratory strains of *H.diminuta* and *M.moniliformis* can co-parasitise the rat final host and for approximately how long.

If the parasites used in this study can co-exist in the rat, there will therefore be a period of time in which the eggs from both species will be simultaneously expelled in the rat's faeces.

H.diminuta is well known for its longevity, and can out-live its mammalian host if experimentally transferred to a new one before the original host dies of old age (see Arai, 1980). During this time the worm produces a large number of oncospheres per day after a prepatent period of 17 days. The number of eggs produced per worm per day varies considerably, values ranging from 1149 (Hager, 1941), 70-80,000 (Hesselberg and Andreasson, 1975), 125,000

(Boddington and Mettrick, 1981) to as many as 282,000 ± 85,700 (Kino and Kennedy, 1987). This vast range of reproductive output, as reported by the above authors, is most likely the result of the significant control host diet has on parasite development (Beck, 1952). This, along with other factors such as different strains of parasite used in experiments and crowding effects within the host intestine (Boddington and Mettrick, 1981; Hager, 1941; Hesselberg and Andreasson, 1975) compounds the difficulties experienced when attempting to make comparisons of parasite fecundity.

Female *M.moniliformis* will only live for an average of 144 days, although longevity is influenced by sex and diet of the host. After a prepatent period of 38 days a single female may release as many as 5,500 eggs (shelled acanthors) per day (Crompton, 1985).

From this it is evident that the time in which both species' eggs will be found together in the rat faeces is primarily dependent on the prepatent period and longevity of *M.moniliformis* in a joint infection. Theoretically, in such an infection, the eggs from both species should be present from about 38-144 days, providing that possible interspecific competition and physiological alterations of the rat intestine do not effect the growth rate of the worms.

The following experiments test whether the laboratory strains of both parasite can coexist and for approximately how long their eggs are present in the faeces together. In the second experiment, the abundance of parasite eggs in the rat faeces was estimated from collections at 5 day intervals. Counting absolute numbers of parasite eggs produced by the worms was difficult to standardise and unnecessarily time-consuming for the level of information required in this study. By collecting eggs every 5 days some accuracy in predicting the precise times of egg production is lost, and therefore this should be taken into account when considering the results. However this does have the advantage of allowing faecal material to accumulate so that average

abundance of eggs produced by the worms with time can be assessed.

In the laboratory, concurrent infections have been set up to explore cases in which *H.diminuta* and *M.moniliformis* jointly infect rats, in order to concentrate mainly on the interactions between the two parasites and their host (Holmes, 1961, 1962, 1973, and Holland, 1987). In all the cases studied *M.moniliformis* successfully out-competes *H.diminuta* for prime position in "the zone of viability" in the gut where optimal levels of nutrients occur. As a result, *H.diminuta* is excluded from the anterior quarter of the gut and both species of parasite suffer some reduction in body weight (see Roberts and Mong, 1968; Crompton *et al.*, 1984).

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS

2.2.1.1 RATS

Adult male Wistar rats, bred in the animal suite in the Department of Zoology, were used throughout this series of experiments. The rats were maintained at 21±2°C in a 12 hour light : dark regime and fed on CRM rat diet and water *ad libitum*

2.2.1.2 BEETLES

Tribolium confusum were raised and maintained in the insectary in the Department of Zoology. They were housed in plastic tanks partly filled with wholemeal flour covered in a layer of filter paper and kept at 28±2°C at 12 hours light : dark regime. Their diet was supplemented with lettuce and apple.

2.2.1.3 COCKROACHES

Periplaneta americana were raised and maintained in the insectary as above, housed in glass tanks with dry peat and cardboard egg cartons as

bedding. They were fed on rat diet and water *ad libitum* supplemented with fruit and Beemax.

2.2.1.4 PARASITES

Hymenolepis diminuta

H.diminuta was maintained in Wistar rats and the flour beetle, *Tribolium confusum* Lightly anaesthetised rats were infected by intubation with 10 mature cysticercoids each. The worms were then allowed to develop for at least 3 weeks before use. The strain of *H.diminuta* used throughout most of this work is a wild type strain obtained from a rat caught on a farm near Exeter in 1986 and will be referred to as Exeter strain (C. Kennedy, personal communication). Anaesthesia and intubation were carried out under home office licence number PIL. 60/00895 and project licence PPL. 60/00370.

To infect beetles, mature *H.diminuta* were first dissected from the small intestine of a freshly killed rat. Mature proglottids were then teased away from the posterior end of the worms in Hepes buffered saline (HBS, pH 7.2, Huxham and Lackie, 1986) using fine forceps and a dissecting microscope (Wild) at 25x magnification; mature eggs were circular in profile with fully formed oncospheres within the shell layers. Small portions of proglottid containing mature eggs were distributed evenly on a piece of filter paper and presented to approximately 50-100 beetles (fasted for 48 hours previously) in a 120mm diameter crystallising dish. Mature cysticercoids were dissected from the beetles after 8-10 days at $28\pm2^{o}C$ or approximately 3 weeks at room temperature. For this beetles were CO_2 anaesthetised and teased apart using fine forceps in a small petri dish of HBS, recovering the cysticercoids with a Pasteur pipette.

Moniliformis moniliformis

M.moniliformis was maintained in Wistar rats and the cockroach

P.americana. Lightly anaesthetised rats were infected by intubating 10 cystacanths in HBS into each. The worms were then left to develop for at least 7 weeks before use. The *M.moniliformis* used throughout these experiments was originally obtained from the Rice Institute, Texas and will be referred to as Texas strain.

To obtain cystacanths, mature female *M.moniliformis* were dissected from the small intestine of a rat. Eggs were "stripped" from the female worm into HBS by holding the anterior end with forceps, cutting the posterior 2-3mm away and then pulling the whole worm through partially closed forceps. The eggs were centrifuged out of suspension at 250g for 3 minutes and were added to a 60% sucrose solution for storage. The eggs can be stored in this state at $4^{o}C$ for at least 4 months (J.M. Lackie 1973) without any loss in viability.

Cockroaches, fasted for two days, were fed on this egg-in-sucrose suspension by pipetting small drops onto the egg boxes in their enclosure. Cystacanths could be dissected from the cockroaches after 6 weeks at $28\pm2^{o}C$. For this, CO_2 anaesthetised cockroaches were decapitated, legs removed and then the abdomen cut open with a circumventral incision in a petri dish of HBS. Cystacanths were flushed out of the body cavity with jets of HBS from a pasteur pipette and collected in a suitable container of fresh HBS.

2.2.2 HEPES-BUFFERED BALANCED SALT SOLUTION

Hepes-buffered balanced salt solution, pH 7.2 (HBS) was that developed for use with cockroach haemocytes (Huxham and Lackie, 1986).

2.2.3 EXPERIMENTAL PROCEDURES

2.2.3.1 COMPATIBILITY OF LABORATORY STRAINS OF BOTH PARASITE SPECIES IN THE RAT HOST

Batches of *M.moniliformis* cystacanths and *H.diminuta* cysticercoids dissected from stock infections were collected in plastic tissue culture wells

in HBS in groups of 5 or 10 of each parasite as follows.

Experimentals

Group A 3 batches of 5 *M.moniliformis* + 5 *H.diminuta*

" B " " " 10 *M.moniliformis* + 10 *H.diminuta*

Controls

" C " " 5 *M.moniliformis* only

" D " " 5 *H.diminuta* only

" E " " 10 *M.moniliformis* only

" F " " 10 *H.diminuta* only

Each batch was then administered to individual rats by intubation (as above) and the rats maintained at 21°C ±2°C for 1 month. After this period the rats were killed by cervical dislocation and the small intestine removed and carefully dissected with a single longitudinal cut. Counts were then made of numbers of each species of worm, their sex if applicable and their relative position in the gut (ie. 1st, 2nd, 3rd or 4th quarter, measured from the anterior end of the small intestine).

2.2.3.2 PATENCY AND FECUNDITY OF THE PARASITES IN A CONCURRENT INFECTION

Batches of *M.moniliformis* and *H.diminuta* larvae were prepared as in section 2.2.3.1 in groups of 5 of each parasite as follows.

Experimentals

Group A 3 batches of 5 *M.moniliformis* + 5 *H.diminuta*

Controls

" " B 3 " 5 *M.moniliformis*

" " C 3 " 5 *H.diminuta*

Each batch was then administered by intubation (as above) to each of a total of 9 rats, which were then housed in 9 separate cages and maintained as above.

Faecal samples were removed from each of the cages at 5 day intervals and either examined immediately for the presence of parasite eggs or stored at 4°C in Sterilin Universal containers for later processing.

Estimates of abundance of parasite eggs were made by taking approximately 5g (wet weight) of faeces from each cage sample and mixing thoroughly with 5ml of HBS (ie. 50% weight/volume mixture). A 1g subsample of this homogenate was filtered through a coarse mesh (0.5mm) to remove large solid particles, then 10ml of HBS added to the finer graded fraction. This suspension was then gently centrifuged for 3 minutes at 250g, the supernatant removed and the pellet placed in a 4cm petri dish with approximately 10ml of HBS for examination. Egg abundance was assessed at four levels using a Wild dissecting microscope at 25x magnification, ie. no eggs present (-), few eggs present (+), eggs seen frequently (++) and abundant (+++). After 140 days the rats were killed, their small intestines removed and dissected, and any parasites in the small intestine were counted as in section 2.2.3.1.

2.3 RESULTS

2.3.1 COMPATIBILITY OF LABORATORY STRAINS OF BOTH PARASITE SPECIES IN THE RAT

Both species of parasite survive in the rat host together when introduced in groups of either 5 or 10, although the average proportion of parasites surviving in the lower density double infections seem to be slightly higher than those in the higher density infections (Table 2.1).

In the 10+10 mixed infection, *H.diminuta* suffers a reduction in percentage survival compared to controls and the 5+5 infection. In the majority of cases *M.moniliformis* was found to be in a more anterior position than *H.diminuta* in the small intestine of the rat.

2.3.2 PATENCY AND FECUNDITY OF THE PARASITES IN A CONCURRENT INFECTION

From the results on Table 2.2, estimates (to the nearest 5 days) of the prepatent period and patent period were calculated (summarised on Table 2.3). This assumes that there is only a short time delay between eggs being released from the parasites and their appearance in the faeces.

In the concurrent infections, *H.diminuta* was found to develop slightly more slowly than in controls (ie. it had a longer prepatent period), but usually outlived *M.moniliformis*.

Eggs of both species were found together in the faeces from 35-40 days post infection, to 75-85 days post infection, this range being limited only by the length of the patency period of *M.moniliformis*. *M.moniliformis* ceased producing eggs between 115 and 120 days post-infection, but a reduction in number was noted at around 95-105 days post-infection.

On dissection of the rat after 140 days, the majority of *M.moniliformis* were absent from the gut, *H.diminuta* was found in its normal anteriad position (Table 2.4) in the first and second quarters of the small intestine.

TABLE 2.1 NUMBER AND POSITION OF WORMS IN CONCURRENT INFECTIONS OF *MONILIFORMIS MONILIFORMIS* (TEXAS) AND *HYMENOLEPIS DIMINUTA* (EXETER)

Rat N°	Group	Dose	N° *Moniliformis* Females	Males	Small int'n position (quarter)	N° *Hymenolepis*	Small int'n position (quarter)
1	A	5+5	2	2	1st	5	2nd
2	A	5+5	3	2	1st	5	2nd-3rd
3	A	5+5	3	2	2nd	2	2nd-3rd
4	B	10+10	2	5	2nd	6	2nd-3rd
5	B	10+10	3	7	2nd	7	2nd-3rd
6	B	10+10	7	2	2nd	9	2nd-3rd
7	C	*H.diminuta*				5	1st
8	C	control				4	2nd
9	C	5 per rat				4	1st
10	D	*Moniliformis*	3	2	1st		
11	D	control	2	2	1st		
12	D	5 per rat	3	2	1st		
13	E	*H.diminuta*				7	1st-2nd
14	E	control				8	1st-2nd
15	E	10 per rat				10	1st-2nd
16	F	*Moniliformis*	2	3	1st		
17	F	control	4	5	1st-2nd		
18	F	10 per rat	5	2	2nd		

TABLE 2.2 EGG OUTPUT BY *MONILIFORMIS MONILIFORMIS* AND *HYMENOLEPIS DIMINUTA* IN SINGLE AND CONCURRENT INFECTIONS

Day pi	Joint infections						*H.diminuta* control			*M.moniliformis* control		
	Rat 1		Rat 2 #		Rat 3		Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
	* mm	hd	mm	hd	mm	hd						
5	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	+	-	-	-	-	-
20	-	+	-	+	-	-	++	++	+	-	-	-
25	-	++	-	+	-	+	++	+++	+	-	-	-
30	-	+++	-	+++	-	+	+++	+++	++	-	-	-
35	-	+++	-	+++	+	++	+++	+++	++	+	-	-
40	+	+++	+	+++	+	+++	+++	+++	++	++	++	+
45	++	+++	+	+++	++	+++	+++	+++	++	+++	+++	++
50	++	++	+++	+++	+++	+++	+++	+++	++	+++	+++	++
57	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++	++
64	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++	++
71	+++	++	++	+++	+++	+++	+++	+++	++	+++	+++	++
78	+++	++	++	+++	+++	+++	+++	+++	++	+++	+++	++
85	+++	+++	++	+++	++	+++	+++	+++	++	+++	+++	++
90	+++	+++	++	+++	++	+++	+++	+++	++	+++	+++	++
95	+++	+++	+	+++	++	+++	+++	+++	++	++	+++	++
100	++	++	+	+++	++	++	+++	+++	++	++	+	++
105	++	++	+	+++	+	+++	+++	+++	++	++	+	++
110	+	++	+	+++	+	+++	+++	++	++	++	+	++
115	+	++	-	+++	+	+++	+++	++	++	+	+	++
120	+	++	-	+++	-	+++	+++	++	++	+	-	+
125	-	++	-	+++	-	+++	+++	++	++	-	-	+
130-40	-	++	no data		-	+++	+++	++	++	-	-	+

Relative abundance of eggs in faeces given by:-
- = no eggs
+ = few
++ = frequent
+++ = abundant

* mm = *M.moniliformis* and hd = *H.diminuta*
\# Rat 2 was killed on day 125 for examination

TABLE 2.3 SUMMARY OF RESULTS FROM TABLE 2.2

Conditions		*H.diminuta* infections		*M.moniliformis* infections	
		Prepatent period (days)*	Patent period (days)*	Prepatent period (days)*	Patent period (days)*
Controls	1	15	>140	35	85
	2	20	>140	40	75
	3	20	>140	40	100
mean values		18.3	>140	38.3	86.7
Expt.	1	20	>140	40	80
	2	20	no data after 125	40	70
	3	25	>140	35	80
mean values		21.6	>140	38.3	76.7

* = To the nearest 5 days.

TABLE 2.4 NUMBER OF PARASITES RECOVERED FROM RATS AFTER 140 DAYS POST INFECTION

Conditions	Rat N^{O}	Group	Number *H.diminuta*	Position in Gut (quarter)	Number *M.moniliformis*	Position in Gut (quarter)
Experimental	1	A	5	1st-2nd	1(female)	1st
double	2*	A	5	1st-2nd	0	-
infection	3	A	4	1st-2nd	1 (male?)	1st-2nd
H.diminuta	1	C	5	1st-2nd	-	-
control	2	C	5	1st-2nd	-	-
	3	C	2	1st	-	-
M.moniliformis	1	B	-	-	0	-
control	2	B	-	-	0	-
	3	B	-	-	1	1st

* = After 125 days pi.

2.4 DISCUSSION

The data presented in this section clearly demonstrate that a concurrent infection of the Exeter strain of *H.diminuta* and Texas strains of *M.moniliformis* is possible in rats. More importantly from the point of view of the acquisition of infection by the insect intermediate host, eggs of both species occur simultaneously in the rat faeces for a period of approximately 6-11 weeks.

Work in the past has concentrated on the interactions of the two species of worm in the small intestine of the rat, where through processes of interference competition (Holland, 1987; Holmes, 1961, 1962, 1973) *M.moniliformis* displaces *H.diminuta* from its prime site of nutrient absorption in the small intestine (Crompton and Whitfield, 1968; Crompton *et al.*, 1984) so that *H.diminuta* lies in a more posterior position. The relevant findings of the work described here were similar; *H.diminuta* was always found in a more posterior position in the gut than *M.moniliformis* when in concurrent infections. The mechanism involved in this displacement remains the subject of much speculation. Suggestions by many of the above authors include that, the excretion of toxic substances by *M.moniliformis* effects *H.diminuta*, the rat host's physiology is modified thereby affecting the position of the parasites or interspecific competition between *M.moniliformis* and *H.diminuta* for space and nutrients results in the observed changes in position.

Infective stage density, ie. numbers of viable parasite eggs per unit area, has to be above a minimum threshold for the parasite to effectively transmit to and survive in the host population. If the concentration of eggs per unit area available for ingestion by the intermediate host was below this threshold subsequent prevalence of the parasite in the host population would also drop so that after several generations the parasite population would "crash".

For an intermediate host to ingest the eggs of two parasite species, the

eggs of both must be at high concentration to increase the chances of being eaten together in the same meal. In practice this will depend largely on how fecund the two species of parasite are, and for how long the two parasites remain simultaneously patent.

There is some discrepancy in the literature concerning patterns of egg production by *M.moniliformis* and *H.diminuta* in concurrent infections (see section 2.1). Results in section 2.3.1 showed a slight reduction in the relative abundance of *H.diminuta* eggs produced in concurrent infections; Holland (1987), also found this phenomenon in concurrent infections, especially at high infection densities. Hager (1941), Boddington and Mettrick (1981) and Roberts and Mong (1968) studied the relationship between numbers of eggs produced per worm and the density of worms in the rat small intestine; they also found that net egg production remained nearly constant even though individual worm size and productivity was inversely proportional to number of eggs. This contrasted with Hesselberg and Andreasson's (1975) finding, which showed that maximum numbers of eggs were obtained from single worm infections and that net production dropped with increasing numbers of worms in the intestine. Therefore any reduction on fecundity cannot be conclusively attributed to intraspecific or interspecific competition alone in the concurrent infections.

Duration of patency was particularly important when considering that *M.moniliformis* will only produce eggs for approximately 106 ± 16 days (Crompton *et al.*, 1972). This is therefore the limiting factor in the length of time the eggs of *M.moniliformis* and the much longer lived *H.diminuta* (Arai, 1980) could be found in the faeces together. Information in the literature is particularly sparse when considering the period of patency of both parasite species in concurrent infections, but is of particular relevance to this study when considering the simultaneous passage of two parasites to one intermediate host. The prepatent period of *H.diminuta* in the two-species infections (in

this work, measured from the time of infection to the appearance of eggs in the faeces) was longer than in controls. *M.moniliformis* eggs appeared in the faeces at the same time in concurrent and single species infections but disappeared slightly earlier at the end of the parasite's patent period.

Even though the prepatent period for *H.diminuta* in the two-species infection seems to be longer, consequent loss in fecundity during the initial stages of the infection will not affect the chances of a simultaneous infection of the intermediate host occurring. This is because *M.moniliformis* is not producing eggs during this period, and it is only when both infections are patent that an insect might be simultaneously infected.

Thus the rat faeces contaminated with eggs from both species serve to ensure that the mixed eggs remain at a locally high density when presented as a meal to an insect host. However the rat will only produce a limited amount of such faeces during the patent period of *M.moniliformis* (approximately 77 days) and of these only the faeces produced at the time of *M.moniliformis* peak egg production will favour one insect ingesting both species, ie. for approximately 44 days in the middle of the infection.

A non-specific inflammatory response can lead to the expulsion of parasites from the host intestine. Such a response was initiated by the presence of *Trichinella spiralis* during its intestinal phase in mice and during this time challenge infections of *Hymenolepis microstoma* were unsuccessful (Howard *et al.*, 1978). *H.microstoma* developed normally when *T.spiralis* was present in the bile duct or if *H.microstoma* was established before *T.spiralis*. *Hymenolepis citelli* is normally rejected from the mouse intestine after approximately 20 days. Its survival may be prolonged if in a concurrent infection with a second species of parasite, *Nematospiroides dubius* (= *Heligmosomoides*), which appears to have an immunosuppressive affect on the host and delays rejection (Alghali *et al.*, 1985). Evidently, from the two examples above, it is of prime consideration to which parasite establishes itself in the host first if one of the parasites alters the state of the hosts

immune system. However both *H.diminuta* and *M.moniliformis* seem to have little or no affect on the host in low-density infections and it has therefore been assumed that at the level of investigation required in this chapter the sequence of infection is unimportant for the survival of either species.

2.5 SUMMARY

1. Both Exeter strain of *H.diminuta* and Texas strain of *M.moniliformis* will infect rats concurrently in the small intestine of the host.

2. Interactions between the two species of parasite and the rat host results in *M.moniliformis* occupying the prime position for nutrient absorption in the small intestine causing *H.diminuta* to occupy a more posterior position. This result agrees with observations made by previous authors.

3. There was a slight reduction in relative fecundity of *H.diminuta* during the concurrent infections with *M.moniliformis*, whereas *M.moniliformis* showed no change. Further work would be required to verify this effect more accurately, possibly by incorporating some method of counting absolute numbers of eggs produced by the parasite.

4. It was concluded that although eggs of both species of parasite were present in the faeces for approximately 77 days, the chances of passing on both species simultaneously to an intermediate host would be highest at the time of peak egg production. This was for approximately 44 days in the middle of the patent period of *M.moniliformis*.

5. It has been assumed that the sequence in which *H.diminuta* and *M.moniliformis* infect the rat host has little affect on the establishment

and fecundity of either species in concurrent infections; only the patent period of *M.moniliformis* dictates the time interval during which the eggs of both parasite species will be present in the faeces.

CHAPTER THREE

MECHANISMS OF PARASITE HATCHING AND GUT PENETRATION

IN VIVO AND *IN VITRO*

3.1 INTRODUCTION

In Chapter 1, the insect gut was discussed in its role as a barrier to parasitic infection. This chapter will deal with the passage of the parasites along the gut, the hatching of *H.diminuta* and *M.moniliformis* larvae *in vivo* and finally the penetration of the gut wall *in vitro* and *in vivo* by the larvae of both species.

3.1.1 PASSAGE OF INGESTED MATERIAL ALONG THE INSECT GUT

To study the gut as a barrier to parasitic infection it is necessary to know when, after ingestion, the eggs and larvae arrive at a suitable site of penetration. The aim of this section is to ascertain where the larvae of *H.diminuta* and *M.moniliformis* hatch and when they arrive in the midgut of their host. The timing could be critical for survival of the parasite, especially if it can only remain mobile for a limited time after activation, as is the case with *H.diminuta* (Ogren, 1969; Ogren *et al.*, 1969) or is adversely affected by prolonged exposure to enzymes. This timing is dependent on several factors, related to feeding behaviour and co-ordination of digestion in the insect.

In omnivorous insects such as the cockroach *P.americana*, salivation is stimulated by food intake and the presence of food-chemicals, detected by sensilla on the mouthparts and antennae (Chapman, 1985b). After being chewed, food items and saliva pass back into the crop where the bulk of digestion takes place. The cells lining the crop of the cockroach do not secrete digestive enzymes, these are produced anteriorly in the salivary glands and posteriorly in the midgut. This results in a mixture of amylase, invertase, maltase, lipase and proteases (Wigglesworth, 1927) normally at pH 4.6-6.8 due to microbial action on carbohydrates. Just after feeding there is an increase in protease activity, which Day and Powning (1949) found was stimulated not

only by protein (gelatin in this case) but also by starch.

After experimental starvation for 3 days, *P.americana* exhibits a delay of 1 hour after feeding in initiation of protease activity, and in some species, for example *Leucophaea maderae*, this delay can last as long as 12-14 hours, even if the food has already reached the midgut (Agrawal, 1981). This could be due to a delay in the neurosecretory mechanisms controlling secretion of enzymes as suggested by Rounds (1968). After injecting a homogenate of midgut from a recently fed to a starved insect the latter exhibited elevated protease activity possibly attributable to a transfer of hormonal signals. This effect was most notable at sunset which coincides with the time at which cockroaches normally begin feeding. However, due to the imprecise nature of the injected homogenate the nature of these hormonal signals was not elucidated. Chapman (1985b) summarises that enzyme secretion in the cockroach is more likely to be stimulated by a secretagogue mechanism, whereby food chemicals directly stimulate the secretory epithelium of the gut.

As the cuticular lining of the crop of most insect species prevents absorption of nutrients (Madrell and Gardiner, 1980), ingested material is then passed down into the midgut where, in the midgut caecae, absorption takes place. Passage of food from the crop into the midgut of the cockroach is regulated by stretch receptors and osmotic feedback mechanisms. Treherne (1957, 1958a,b) found that rate of crop emptying in locusts and cockroaches was proportional to the luminal osmotic pressure in the crop. Above a certain value, the valve between the proventriculus and midgut opens, the extent and duration of opening being dependent on osmotic pressure of the crop. This mechanism prevents nutrient uptake in the caecae from saturating. Also if the crop is too full, the cockroach will delay further feeding until some of the crop contents have passed into the midgut (see also, Davey and Treherne, 1963a, b, 1964).

Flour beetles feed on stored foodstuffs, living their whole life cycle in its food source, flour. Even though their relatively short gut is normally

kept full, feeding tends to be intermittent. Protease activity, as in the cockroach, increases in response to ingestion of food, including flour as well as cellulose powder and water (Chapman, 1985b). Dadd (1956) suggested that protease activity was under humoral control, triggered by mechanical stimulation of the gut. This was concluded after haemolymph was transferred from a recently fed to a starved beetle, the effect being an increase in the proteolytic activity of the starved beetle.

Thus it seems that many factors contribute to rate of passage of ingested material passing along the gut. These include amount of food ingested, temperature, carbohydrate and sugar contents, moisture content and even time of day.

3.1.2 HATCHING OF *HYMENOLEPIS DIMINUTA*

Voge and Graiwer (1964) and Lethbridge (1971a) described the processes involved in the hatching of *H.diminuta* oncospheres from their eggs (correctly termed shelled embryophores or shelled oncospheres) followed by their penetration of the midgut wall of the flour beetle *Tenebrio molitor.* The hatching process involves four stages (1) mechanical rupturing of the egg shell which leads to (2) activation of the oncosphere within. Then (3) enzymatic action in the midgut digests away the gelatinous coat surrounding the oncosphere to (4) allow the larva's escape into the gut lumen (Holmes and Fairweather, 1982)

Mechanical rupturing of the shell of *H.diminuta* by the insect's mouth parts normally occurs as the parasite is ingested along with food. Lethbridge (1971a) reported *T.molitor* could ingest approximately 200 eggs per minute and of these around 50% would remain undamaged. These undamaged eggs were therefore not activated, and would pass out of the gut with the faeces and still remain viable to infect other hosts. However a small percentage of the

eggs ingested were totally destroyed by the beetle's mouthparts. Lethbridge (1971b) further demonstrated the imperviousness of the egg shell by exposing the eggs to hypochlorite, enzymes, fixatives and dyes and found that the larvae remained viable.

H.diminuta oncospheres can escape the confines of the egg and subshell layers, providing the shell has been ruptured, without the aid of enzymatic action, using only their own mechanical means and secretions from penetration glands. This process is relatively lengthy, taking up to 2 hours, and only a small proportion of oncospheres are capable of completing hatching this way (Holmes and Fairweather, 1982). This may be due to stored glycogen, normally reserved for gut penetration, being used up prematurely in burrowing through the subshell layers (Lethbridge, 1971a).

Hatching is normally facilitated by the action of host gut enzymes on the subshell layers, once the shell has been broken by the insect's mouthparts, as demonstrated by Voge and Berntzen (1961) and Berntzen and Voge (1965) for *H.diminuta* and other *Hymenolepis* species. Lethbridge (1972) also investigated the effects of enzyme extracts from *T.molitor* midgut on the hatching of *H.diminuta in vitro*. He found that at a "concentration" of 1 midgut per ml in saline, at pH 6.6-6.8, the cytoplasmic layer and embryophore would dissolve at slightly different rates in approximately 5-12 minutes, though the rates of digestion of each layer could be manipulated by altering the pH. Using electrophoresis, Lethbridge isolated 4 bands from the midgut extract, 2 of which exhibited proteolytic activity, 1 which showed amylase activity and the fourth had no enzyme activity at all. The two proteases together were very effective in inducing hatching, one was found to destroy the embryophore and weaken the cytoplasmic layer and the second, conversely, was found to destroy the cytoplasmic layer and weaken the embryophore. Similarly, it was found that enzymes extracted from cockroach midguts were also very effective in digesting the subshell layers; however, these enzyme preparations would have contained a multitude of undefined enzymes thus limiting the significance of

the results.

Several workers have investigated the effects of other enzyme preparations on the hatching of *H.diminuta* larvae *in vitro* Lethbridge (1972), using preparations of α-amylase (at 300µg ml^{-1}, pH 6.8), found 10% of the larvae hatched within 60 minutes after the embryophore but not the cytoplasmic layers had weakened, and in a defined medium of trypsin, chymotrypsin and peptidase, hatching was achieved in a similar time as *in vivo* Lackie (1976, 1981) adapted an *in vitro* method for hatching *H.diminuta* oncospheres originally developed by Bernzen and Voge (1965) which involved digestion by α-amylase at 0.75 % in HBS to remove the subshell layers, resulting in complete hatching in approximately 15 minutes.

The enzymes involved in hatching *in vivo* are still undefined, many workers reporting different findings possibly due to some commercial enzyme preparations, for instance bacterial amylase, being contaminated with proteases. However it does seem that amylases weaken the carbohydrate matrix of the gelatinous layer allowing the oncospheres to tear through the rest of the subshell layers using their hooks (Holmes and Fairweather, 1982).

Once free of its shell, the oncosphere is still surrounded by the oncospheral membrane, the precise function of which is unknown. Initially this membrane may protect the oncosphere from damage by enzymes in the host gut, but it is lost at a later stage.

3.1.3 PENETRATION OF THE HOST GUT BY— *HYMENOLEPIS DIMINUTA*

H.diminuta oncospheres penetrate the tissues of the gut using their 3 pairs of recurved hooks in a clawing fashion, possibly facilitated by proteolytic secretions from penetration glands. The biochemical action of penetration gland secretions is not fully understood. Lethbridge and Gijsbers (1974) showed how the contents of the glands, when stained with neutral red,

diminished as the oncosphere progressed through the gut. This action was described by Moczon (1977) as causing the muscle cells in the gut to swell and soften, possibly through osmotic effects, although this was based on histological evidence alone.

The hooks, 1 slender median pair and 2 shorter thicker lateral pairs (Holmes and Fairweather, 1982) are extended into the gut tissues, then retracted in a sweeping motion in cycles lasting approximately 3 seconds (Ogren, 1969). These movements propel the oncosphere forwards, destroying epithelial cells in its path as it progresses towards the haemocoele (Moczon, 1977). The rate of burrowing is largely dependent on temperature, and continued burrowing is dependent on the glycogen reservoirs carried by the oncosphere. Ogren (1969) and Ogren *et al.* (1969) demonstrated by staining techniques that glycogen levels in the oncosphere were barely detectable after approximately 3.5 hours of activity.

Penetration of the gut of adult *T.molitor* by *H.diminuta* oncospheres is known to occur in the anterior third of the midgut, taking 45-75 minutes to accomplish (Lethbridge, 1971a). However, the oncospheres cannot penetrate the gut of larval *T.molitor*; this inability is attributed to differences in the gut morphology. The larvae not only have a thicker peritrophic membrane, but also they lack the midgut papillae present in the adult beetles (Snodgrass, 1935; Chapman, 1982, 1985a)

Voge and Graiwer (1964) and Lethbridge (1971a) found that the penetrant oncospheres use the papillae of regenerative epithelial cells as a means of by-passing the tougher gut muscles as they burrow through the adult midgut. Oncospheres which burrowed through the gut between the papillae only got as far as the circular muscle layer unless they diverted along the basement membrane into an adjacent papilla; only very few oncospheres successfully penetrated the musculature. In *T.molitor* larvae, Lethbridge (1971a) reported that the oncospheres took approximately 45 minutes to cross the peritrophic

membrane and then only very few larvae were able to penetrate the gut over 2 hours later.

Moczon (1977) in contrast, reported that *H.diminuta* oncospheres were capable of penetrating the musculature of adult *T.molitor* guts. He proposed that their inability to penetrate larval gut was due to biochemical differences in muscle tissue which made it an unsuitable substrate for penetration gland secretions to act on. However it would seem unlikely that the penetration gland secretions would be so specific in their action, as *H.diminuta* has been reported to successfully infect 63 species of insect from 7 different classes (Arai, 1980), although the modes of gut penetration have not been described in these cases.

Voge and Graiwer (1964) also suggested that the rapid gut emptying time of the larval beetle compared to the adult (ie. 2 and 5 hours respectively, Chapman, 1985b; Dow, 1986) may sweep the oncospheres along the gut too rapidly for them to hatch and grasp the gut epithelium with their hooks. However, Lethbridge (1971a) also considered this and found it not to have any significant effect.

3.1.4 HATCHING OF *MONILIFORMIS MONILIFORMIS*

The processes involved in the hatching of *M.moniliformis* shelled acanthors (termed "eggs" throughout this work) show several distinct differences to those of *H.diminuta*

The stimuli required for inducing *M.moniliformis* to hatch were investigated by Edmonds (1966). He found that solutions of various electrolytes (see Chapter 1) would initiate complete hatching without the need for mechanical rupturing of the shell or enzymatic action. In 0.25M sodium bicarbonate solution at pH greater than 7.5 (or pH 6 in the presence of CO_2), Edmonds found that acanthors would hatch *in vitro* in 20 to 60 minutes at a range of temperatures between 10 and 37^{o}C. However, *in vivo* this was found to take much longer, not starting until 2 hours after ingestion. Once stimulated

the acanthor becomes active within the shell, making a regular series of movements as described by Whitfield (1971). Here the two pairs of rostellar blades are pushed forwards then swept laterally backwards, describing an arc which effectively cuts through the subshell layers and later the gut tissue. Chitinase is also secreted by the acanthor to breakdown the chitin in the shell membranes and aid hatching (Edmonds, 1966).

Suitable physiological stimuli from the host and active participation by the acanthor therefore combine to result in hatching. However, Nickol (1985) emphasises that hatching alone does not necessarily result in a successful infection. Other factors including gut penetration and survival in the haemocoele are important considerations in the success of *M.moniliformis* and similar parasites in their intermediate host. An average of approximately 25% of mature acanthors fed to *P.americana* will reach cystacanth stage when administered in low doses, but as the dose is increased, proportionally more acanthors are lost. As yet a saturation level for numbers of parasites per host has not been found (see Crompton and Nickol, 1985 for general text on the Acanthocephala).

3.1.5 PENETRATION OF THE HOST GUT BY *MONILIFORMIS MONILIFORMIS*

Once hatched in the insect's gut the acanthors have to reach the haemocoele in order to metamorphose and continue development to the cystacanth stage. To do so they have to penetrate the gut wall of the cockroach.

Using their array of cutting blades and posteriorly directed spines in the cycle of movements as described above (Whitfield, 1971), the acanthor burrows "ratchet-like" through the tissues of the lower midgut wall in about 1 to 14 days (Moore, 1946; Robinson and Stickland, 1969) although this time is now considered to be shorter (J.M. Lackie, 1973). Whitfield (1971) suggested that chitinase, secreted by the acanthor during hatching, is also used to weaken

the peritrophic membrane in the initial stages of gut penetration.

The route taken by the acanthor through the gut wall has been described as involving "considerable wandering" (Moore, 1946) possibly causing extensive cell damage. However as the gut epithelial cells of the cockroach are replaced every 40-120 hours or more slowly if the insects are starved (House, 1974) the effects are probably not long lasting.

Eventually the acanthor reaches the haemocoelic side of the gut wall where the first stage of development will take place.

The aim of the first experiment in this section is to determine approximate transit times for a set dose of ingested parasite eggs to reach the midgut. By counting the numbers of eggs in various stages of hatching in different sections of the gut, estimates of transit times and success in hatching can be made.

3.2 MATERIALS AND METHODS

3.2.1 INSECTS

Cockroaches

Adult male *P.americana*, *P.australasiae*, *Blatta orientalis*, and *Blatella germanica* were raised in the departmental insectary as described for *P.americana* in section 2.2.1.3.

Beetles

T.confusum were raised as described in section 2.2.1.4.

Locusts

Schistocerca gregaria were obtained from Bioserv, and housed in purpose-built vivaria kept at 32±^{0}C to 28^{0}C (day : night temperatures) at 12 hours day : night (artificial lighting) regime. They were fed on dried bran and water

ad libitum with an occasional supplement of wheat shoots.

All insects were starved for two days prior to the start of each experiment to ensure that they would feed readily on the parasite eggs.

3.2.2 PARASITES

Hymenolepis diminuta

Eggs of *H.diminuta* were obtained from approximately 25 worms (from 3 rats, at approximately 3 weeks post infection), as in section 2.2.1.4. They were washed twice in HBS by centrifugation at 250g for 3 minutes, then replacing the supernatant with fresh HBS and repeating. The final suspension was adjusted to a concentration of $5x10^3$ eggs per ml in 60% sucrose solution by estimating concentration of a subsample on a Fuchs Rosentahl haemocytometer where :-

$$\frac{N^o \text{ Eggs mm}^2 \times 5000 \times 40}{9} = N^o \text{ Eggs per ml of stock suspension}$$

Moniliformis moniliformis

Eggs of *M.moniliformis* were collected from approximately 15 worms (from 3 rats at approximately 7 weeks post infection), washed twice and stored in 60% sucrose overnight at 4^oC as in section 2.2.1.4. This solution was then filtered using a 25µm pore Nitex mesh (Plastoc Associates) to retain the mature shelled acanthors which were then concentrated to give $5x10^5$ oncospheres per ml as above.

3.2.3 DETERMINATION OF EGG VIABILITY

Natural variation in egg viability tends to occur between the eggs produced in different *H.diminuta* and *M.moniliformis* infections. Therefore an estimate of the average viability of the pooled egg suspensions obtained from the 3 rats was made for each species:

Hymenolepis diminuta

A small subsample of *H.diminuta* eggs taken either from 1 infected rat or a pooled suspension were hatched *in vitro* by an adaptation of the method developed by Lackie (1976). Eggs were mechanically cracked by agitating them with 3mm diameter glass beads in HBS and then transferred to a separate vessel in clean HBS. The oncospheres were induced to hatch in a solution of 0.75% α-amylase (bacteria-derived, Sigma) in HBS at 28°C in approximately 10-15 minutes. Viability was estimated by observing a drop of egg suspension on a slide at 250 x magnification where moving oncospheres, both free in the enzyme preparation and those inside unhatched eggs were counted as viable, and non-motile or malformed oncospheres as non-viable. Viability was calculated as the percentage of motile oncospheres in the total number counted.

Moniliformis moniliformis

The eggs suspension collected from female worms using the above method contained small numbers of immature eggs and ovarian balls (J.M. Lackie, 1973) as well as the mature shelled acanthors. Therefore the numbers of fully developed eggs in suspension are diluted by the immature eggs.

Estimation of percentage of eggs capable of hatching was made by adapting a method developed by J.M. Lackie (1973). Here an aliquot of suspension was placed in 0.3M $NaHCO_3$ (Edmonds, 1966) and left for 1 hour at 26°C. Without disturbing the eggs, the majority of the supernatant was then drawn off, leaving a small volume of concentrated egg suspension. After agitating gently, a drop was placed on a slide and as above, numbers of hatched or activated acanthors counted and compared to total numbers of eggs.

3.2.4 QUALITATIVE AND QUANTITATIVE ANALYSIS OF PARASITE EGG-HATCHING AND PASSAGE ALONG THE INSECT GUT

3.2.4.1 FEEDING METHOD

Insects were housed in individual plastic containers with a small hole cut in the lid. They were allowed to adapt to room temperature for approximately 30 minutes until they appeared quiescent.

Eggs in the 30% sucrose solution were administered to the larger species of cockroaches by Oxford pipette, ensuring the eggs were well mixed beforehand. The pipette containing the aliquot of eggs was carefully inserted through the hole in the lid of the container so that the tip was held near the head of the cockroach. Then a small amount of egg suspension was expelled to form a hanging drop. Once the cockroaches had started to feed from the pipette tip further egg suspension was slowly expelled from the pipette until the whole dose was taken. Any insects refusing to feed or only taking part of the dose were rejected at this stage. A clean pipette tip was used for feeding each individual cockroach and upon examining the tips, a small amount of suspension was found to adhere to the inner surfaces. The residue of egg suspension on each pipette tip appeared to be a nearly constant amount and was therefore assumed to be of negligible significance.

The locusts were fed in a similar manner, although they were found to take the eggs more readily if they were positioned on the rim of the container so that they could grasp the pipette tip with their forelegs.

T.molitor and *B.germanica* would not feed by the pipetting method and so were fed by presenting them with a small drop of egg suspension on Nescofilm. They were observed and allowed to feed on the egg suspension for 15 minutes. Again any insect refusing or taking only a small part of a dose was rejected at this stage.

M.moniliformis was fed only to *P.americana*, *S.gregaria* and *T.molitor* whereas *H.diminuta* was fed to all insect species present.

In summary the dosing regimes for both species of parasite egg suspension

were as follows:-

Periplaneta americana 50 1$\mu$of eggs suspended in sucrose containing approximately 250 eggs.

Periplaneta australasiae 40μl of eggs suspended in sucrose containing approximately 200 eggs.

Blatta orientalis 40μl of eggs suspended in sucrose containing approximately 200 eggs.

Schistocerca gregaria 100μl of eggs suspended in sucrose containing approximately 500 eggs.

Blatella germanica fed for 5-15 minutes on egg suspension.

Tenebrio molitor fed for 5-15 minutes on egg suspension.

Feeding times were staggered as the dissection techniques used below were relatively time consuming. After feeding all insects were housed in an incubator at 28^{o}C and supplied with water *ad libitum*

3.2.4.2 DISSECTION AND EXAMINATION OF THE INSECT GUTS

Timing

Each insect was timed from the start of feeding then dissected at set intervals later. At approximately hourly intervals, up to 24 hours, insects were dissected to assess the position of eggs in the gut. This information was used to gauge when to dissect the main groups for quantitative analysis. The main groups were dissected at 30-45 minutes, 4 hours, 8 hours and 48 hours post-feeding and their guts examined in detail for eggs and parasite larvae.

Dissection

Insects were anaesthetised with CO_2 at the appropriate time post-feeding and the legs, wings and elytra removed as appropriate and were then immersed in a small petri dish of HBS. The whole gut was then dissected out from the body leaving the head still attached to the foregut, taking care to reduce stretching and bending to a minimum to prevent the gut contents from being disturbed.

A qualitative assessment of the position of the eggs in the gut was made by cutting the gut at the junction between the fore- and midgut, then squeezing the contents out of each section. This simply revealed whether or not the eggs had been passed from the crop into the midgut.

For the quantitative analysis, the guts were removed as above and ligated with silk thread immediately posterior to the proventriculus or crop and at the junction of midgut and hindgut.

The guts were then transferred into clean HBS in a small petri dish with grid squares drawn on the underside for reference during counting. The crop region was opened by making a longitudinal cut from the head to the first ligature, and the contents flushed out with a gentle jet of HBS from a Pasteur pipette. The gut was transferred to a second similar dish of HBS where the process was repeated for the midgut.

Counts of the whole eggs, oncospheres in gelatinous coats, free oncospheres and empty shells constituting more than half the original shell were made at 50x magnification in both sections of the opened gut.

3.2.5 *IN VITRO* PENETRATION OF THE INSECT GUT BY *MONILIFORMIS MONILIFORMIS* AND *HYMENOLEPIS DIMINUTA*

Feeding

The insects were fed with parasite eggs as in section 3.2.4.1 and maintained at 28°C until the time of dissection.

Beetles

Fifteen minutes after feeding the beetle guts were dissected from the body of the insects as in experiment 3.2.4.2 and silk thread was attached to both ends of the gut. The guts were then transferred to clean HBS and the outer surface cleaned of extraneous fat and connective tissue with fine forceps and HBS jetted from a Pasteur pipette.

The guts were suspended by the silk threads in individual conical tubes filled with D73 cockroach medium (Lackie and Huxham's medium, in Crompton and Lassiere 1987). A small piece of wire bent into a "U" shape was placed over the mid point of the gut in order to prevent the gut floating and keep it reasonably taut (Figure 3.1). All the tubes were then maintained at 28°C in a waterbath.

After 2 hours the tubes were gently agitated to remove any loosely adherent larvae from the outer surface of the guts, which were themselves then removed from the tubes for examination. Any larvae on or near the surface of the gut could be seen at 50x magnification under a dissecting microscope (Wild) using high intensity back lighting. Some of the guts were fixed in 2.5% glutaraldehyde in HBS for 1 hour, mounted on slides in glycerine jelly and used for microphotography.

The remaining medium in the tubes was centrifuged at 250g for 5 minutes and the supernatant drawn off. A small quantity of medium plus the pelleted fraction was examined at 100 x magnification for the presence of parasite larvae.

Cockroaches and Locusts

From the qualitative experiment, transit time for food and eggs to reach the midgut was found to be approximately 7-8 hours for cockroaches and 3-4 hours for locusts. Thus in later experiments both cockroaches and locusts were left for 7 and 3 hours respectively after feeding to ensure that the eggs

had reached the midgut. The guts were then dissected and suspended in medium (Figure 3.2), and after 2-6 hours inspected for presence of larvae as above.

3.2.6 *IN VITRO* PENETRATION OF GUT SACS BY *IN VITRO*-HATCHED LARVAE

The method used above tested whether or not parasite larvae from ingested eggs, were capable of penetrating the insect gut. In this experiment, larvae hatched *in vitro* were introduced directly into a "sac" of gut, from which the peritrophic membrane had been removed *in vitro*

For technical reasons the only insects suitable were those with large and relatively easily manipulated guts such as *P.americana*, *T.molitor* and *S.gregaria*.

Guts were removed from uninfected insects as in section 3.2.4.2 and the head and hindgut cut away. The guts were then placed in a large petri dish of HBS and viewed under a dissecting microscope at 12-25x magnification.

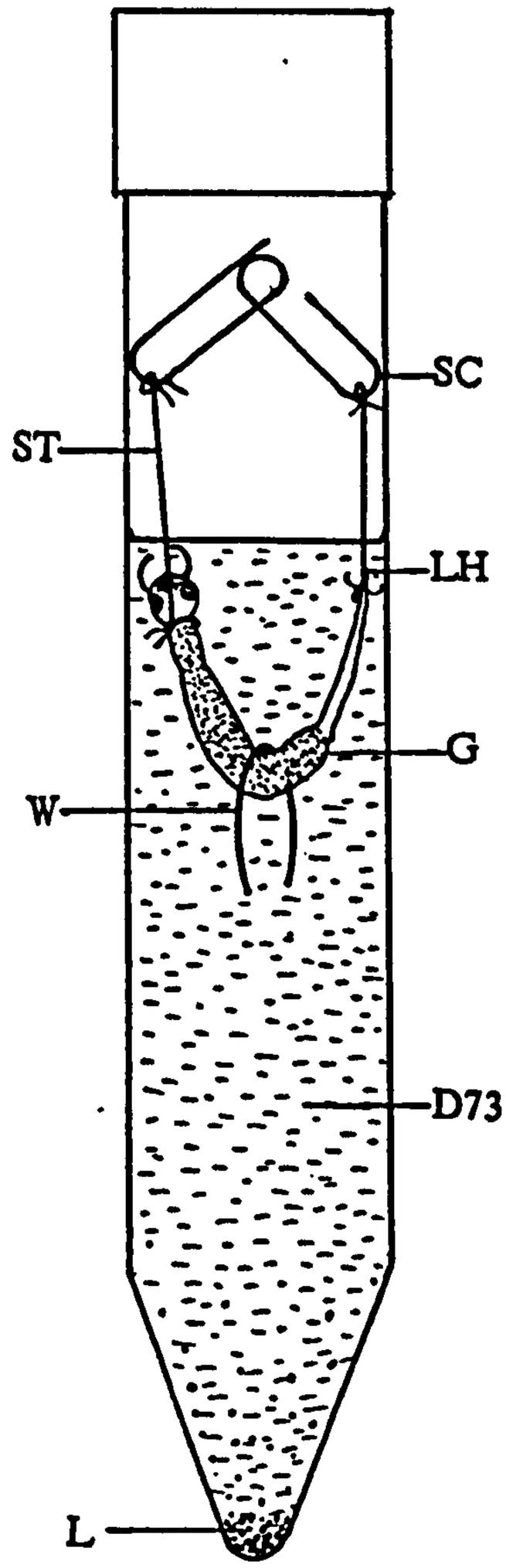
A flexible plastic capillary tube, approximately 30cm long, 0.5mm external diameter and 0.25mm bore, was connected to a 26g 3/8 needle and 1ml syringe filled with HBS (Figure 3.2). A small amount of HBS was flushed through the tube to remove air bubbles and then it was inserted into the anterior 3mm portion of the gut and held in position with silk thread tied around the outside of the gut using a slip knot. Once secure, a small amount of HBS was injected into the gut to create a small positive pressure. This pushed the gut contents enclosed in the peritrophic membrane towards the posterior open end of the gut where it could be extracted with the aid of fine forceps. Once cleared of debris the posterior end of the gut was partially closed with a slip knot of silk.

Suspensions of pre-hatched *H.diminuta* and *M.moniliformis* larvae, prepared as in part 3.2.3, were washed twice by centrifugation at 250g for 3 minutes and resuspended in HBS. A small aliquot of either suspension was placed on a

slide and examined at 100x magnification to ensure hatching had taken place and that the majority of the parasite larvae were viable.

A 1ml syringe (without a needle) was filled with a suspension of larvae and connected to the capillary tube tied to the gut. After allowing a few minutes for the larvae to settle to the bottom of the syringe, a small volume of suspension was injected into the gut via the capillary tubing (Figure 3.2). By observing the gut and tube through the microscope, the amount of suspension flowing into the gut could be regulated so as to maximise the number of larvae introduced. The slip knot around the posterior end of the gut was tightened to seal the gut, then the anterior knot was tightened as the tubing was removed from the anterior end. The sealed guts containing hatched parasites were transferred into 3cm plastic tissue culture dishes (Nunc) partially filled with D73 cockroach medium, which were then floated in a water bath at 28^{o}C. After approximately 2 hours the outer surface of the gut and the contents of the dish were microscopically examined for the presence of parasite larvae as in section 3.2.5.

FIGURE 3.1 APPARATUS USED IN PENETRATION OF THE GUT *IN VITRO*



L Parasite larvae

SC Spring clip

ST Silk thread

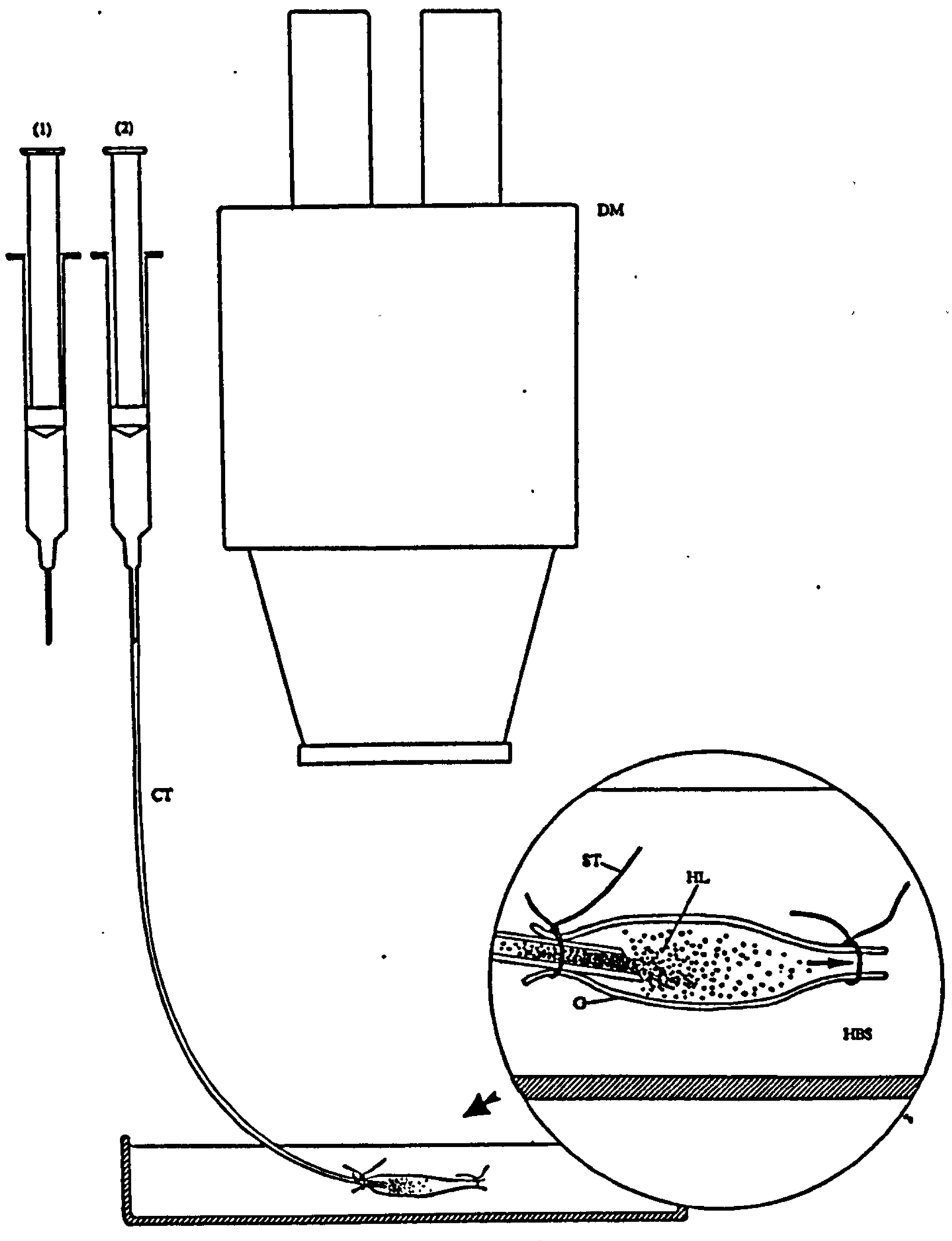
LH Ligated hindgut

G Beetle or cockroach gut with head attached

D73 D73 cockroach medium

W Wire weight

FIGURE 3.2 APPARATUS USED IN PENETRATION OF GUT SACS *IN VITRO*



(1) 1ml syringe containing HBS

(2) 1ml syringe containing pre-hatched oncospheres/acanthors

CT Plastic capillary tubing (0.5mm diameter, 0.25mm bore)

DM Dissection microscope

ST Silk thread

HL Hatched larvae

G Gut sac

3.3 RESULTS

3.3.1 VIABILITY OF PARASITE EGGS

Hymenolepis diminuta

From 3 counts each of 500 eggs from the pooled suspension hatched *in vitro* as above, the percentage found to be viable was 90%. In some cases, passive hatching of non-motile oncospheres occurred; these were counted as non-viable.

Moniliformis moniliformis

From 3 counts of 500 eggs from the pooled suspension hatched *in vitro* as above, the percentage found to be viable was 76%.

3.3.2 DISSECTION OF THE INSECT GUT AT HOURLY INTERVALS FOR QUALITATIVE ANALYSIS OF PARASITE EGG POSITION

A qualitative estimate of the positions of parasite eggs (whole or otherwise) was made in conjunction with the quantitative study below. The hourly observations showed that eggs were present in the crops of the insects soon after feeding (Table 3.1). This was followed by a movement of eggs from the crop into the midguts within 2 hours in *T.molitor*, more gradually over 2-8 hours in locusts and 5-8 hours in the case of cockroaches. However it was not until approximately ,8 hours later that large numbers of eggs were found in the midguts of the majority of insects, apart from *T.molitor* where a large number were seen only 2 hours after feeding.

By 24 hours the crop contents of all the insects were much depleted, the eggs having passed into the midgut or out of the gut altogether.

Due to a shortage of locusts, only a limited number were used for observing the hatching of *M.moniliformis* in the gut.

3.3.3 QUANTITATIVE ASSESSMENT OF PARASITE EGG HATCHING AND MOVEMENT ALONG THE GUT

From the dissections of the insect guts at times determined from the above observations, the progress of *H.diminuta* and *M.moniliformis* eggs with time can be monitored as they pass along the insect gut (Tables 3.2 and 3.3).

Counting the numbers of free larvae of *H.diminuta* and *M.moniliformis* was difficult because of their small size and mixing with the debris from the gut lumen. It was also considered that oncospheres and acanthors would be missing from the gut lumen if they had already begun to penetrate the tissues of the gut wall. Therefore counts of nearly whole or empty eggshells were made as they were more readily detectable in the gut and could be taken as indicators of hatched larvae. For these counts, assumptions were made to correlate eggshell numbers with numbers of hatched larvae. Presumably there was a small number of non-viable eggs hatched in the gut, their shells were included in the total numbers of viable eggs hatched. Therefore it was assumed that the shells from non-viable eggs occurred at low and constant levels along the whole length of the guts of all the insects examined. It was also assumed that larvae and eggshells travelled down the insect gut at similar rates.

Details of hatching and transit times along the guts can be seen on Tables 3.2 and 3.3 and from Figures 3.3-3.11, where the species of insect studied are shown to have subtle differences in their modes of gut action on the ingested parasites as follows.

Periplaneta americana (see Figures 3.3 and 3.9)

Oncospheres of *H.diminuta* began to hatch in the crop from 30-45 minutes, and a small number of free but non-motile larvae were found here 48 hours later. The peak numbers of eggshells of both *H.diminuta* and *M.moniliformis* in the midgut occurred at about 8 hours after feeding. A small number of motile oncospheres and oncospheres with their subshell layers still intact were also

present at this time. *M.moniliformis* were found in the process of hatching from 4 hours onwards, both in the crop and midgut, but the numbers of empty shells in the crop soon after feeding were found to be lower than those of *H.diminuta*

After 24 hours, the crop had released most of its content and by 48 hours the midgut had also emptied.

Periplaneta australasiae (see Figure 3.4)

Hatching of *H.diminuta* larvae occurred in the first 4 hours after ingestion. However release of crop contents was slow so that relatively large numbers of parasites were still present at 48 hours post-feeding.

Similarly to *P.americana*, a peak in the number of eggshells present in the midgut was found at the 8 hour dissection, although in this case the release of eggs from the crop of the insects seem to be somewhat slower. A few mobile oncospheres plus oncospheres with their subshell layers intact were found in the midgut at 8 hours after feeding.

Blatta orientalis (see Figure 3.5)

Similar to the above species of insect, oncospheres were found to hatch in the crop in relatively large numbers 4 hours after ingestion.

The timing of oncospheres and eggshells passing out of the crop into the midgut seemed to be later than in *P.americana*, without the peak seen at 8 hours as in the other cockroach species. This may represent a slower and more gradual release of the crop content by the cockroaches, or simply that the peak did not occur around the time of the 8 hour dissection.

At 48 hours the numbers of oncospheres found in the midgut rose noticeably although they were found to be non-motile.

Blatella germanica (see Figure 3.6)

Motile oncospheres were found free in the crop and midgut 4 hours after

ingestion. A peak in the midgut content was again found in the 8 hour sample corresponding with a release of crop content around 6 hours post feeding. Here the crop seemed to empty relatively quickly, whereas in the midgut, numbers of shells declined gradually.

The large standard deviation around the mean number of eggshells in the gut presumably stems from the method of feeding the parasites to the insects in this experiment, where instead of dosing by pipette the insects were allowed to feed for a set time.

Schistocerca gregaria (see Figures 3.7 and 3.11)

The passage of eggs along the locust gut appeared to be rather different from that in cockroaches.

The large numbers of eggs of both *M.monilifomis* and *H.diminuta* found in the crop of the locust soon after feeding corresponds well with numbers of eggs administered in the dose. Crop emptying occurred between 4-8 hours after feeding, but great difficulty was found in tracing eggs once they were in the midgut. However, most eggs found were unhatched.

Tenebrio molitor (see Figures 3.7 and 3.10)

The standard deviation around the mean number of *H.diminuta* eggshells was large, as with *B.germanica*, and reflects the method of feeding in this experiment. The ratio of whole to broken shells was found to be approximately 1:1 agreeing with Lethbridge's (1971a) results.

Passage of food down the gut of *T.molitor* was quicker than in the cockroaches, broken eggs being present in the midgut within one hour. A peak in midgut egg content was found at the 4 hour dissection and after this there is no evidence of further eggs hatching (ie. there were no oncospheres with intact subshell layers but no shells). By 48 hours only a few broken shells were left in the midgut, the rest having passed into the hindgut and eventually out of the beetle.

The above situation is similar for the time taken for *M.moniliformis* larvae to travel down the gut although the patterns of hatching are somewhat different. There is some evidence for the beetle's mouthparts causing some *M.moniliformis* eggs to be broken during ingestion, hence the presence of empty shells in the crop region, but the main increase in numbers of empty shells was found 4-8 hours after ingestion.

3.3.4 *IN VITRO* PENETRATION OF THE INSECT GUT WALL BY *MONILIFORMIS MONILIFORMIS* AND *HYMENOLEPIS DIMINUTA*

The larvae of *H.diminuta* and *M.moniliformis* successfully penetrated the gut tissues of their natural hosts. However only the acanthors of *M.moniliformis* were found to penetrate the guts of all the species of insects examined where they could be seen near or on the surface of the gut when examined under a dissecting microscope. *H.diminuta* oncospheres were found to penetrate only the guts of *T.molitor*, they could be seen, using bright back lighting, in the midgut papillae (Figure 3.12.1) or free in the medium. The qualitative results are summarised below in Table 3.4.

3.3.5 *IN VITRO* PENETRATION OF GUT SACS BY *IN VITRO*-HATCHED LARVAE

Similar results were obtained by using pre-hatched larvae injected directly into the gut *in vitro* from which the peritrophic membrane had been removed, although numbers of penetrant larvae were greater and penetration was achieved sooner. In this and the previous experiment the results are given qualitatively. Due to the small size of the larvae of both parasite species and the difficulty in detecting larvae hidden within the tissues of the gut, the results in this and in section 3.3.5 have been given qualitatively as estimates of abundance (see Table 3.5).

TABLE 3.1 PRELIMINARYTEST FOR PRESENCE OF PARASITIC EGGS ALONG THE GUT OF THE INSECT HOST

		H. diminuta										*M.moniliformis*			
Time *	P.ame		P.aus		B.ori		B.ger		S.gre		T.mol		P.ame		T.mol	
#	c	mg	c	mg	c	mg	c	mg	c	mg	c	mg	c	mg	c	mg
1/2	+	-	++	-	+	-	++	-	++	-	++	+	+	-	++	-
1	++	-	++	-	±	-	++	-	++	-	++	++	++	-	++	++
2	++	-	++	-	+	-	++	-	++	+	++	++	++	-	++	++
3	++	-	++	-	++	-	++	+	++	+	++	++	++	-	++	++
4	++	-	++	-	++	-	++	+	++	+	+	++	+	-	+	++
5	++	-	++	+	++	-	++	+	++	+	+	++	++	-	+	++
6	++	-	+	+	++	+	+	+	++	+	+	++	++	-	+	++
7	+	+	+	+	++	+	+	++	++	++	+	++	+	++	+	++
8	+	++	+	++	+	++	+	++	++	++	+	++	+	++	+	++
9	+	++	+	++	+	++	+	++	++	++	+	++	+	++	+	+
10	+	++	+	++	+	++	+	++	++	++	+	++	+	++	+	+
11	+	++	+	++	+	++	+	++	++	++	+	++	++	++	+	++
12	+	++	+	++	+	+	+	+	++	++	+	++	+	++	+	+
13	+	++	+	++	+	+	+	+	++	++	+	++	+	++	+	+
14	+	++	+	+	+	+	+	+	++	++	+	++	+	++	-	+
15	+	++	+	++	+	+	+	+	+	+	+	++	+	++	+	+
16	+	++	+	++	+	+	+	+	++	++	+	++	+	++	-	+
17	+	++	+	++	+	+	+	+	++	++	+	++	+	++	+	+
18	+	++	+	++	+	+	+	+	++	++	+	+	+	++	+	++
19	+	++	+	++	+	+	+	+	++	++	+	+	+	++	+	+
20	+	++	+	++	+	+	+	+	+	+	+	+	+	+	+	++
21	+	++	+	++	+	+	+	+	++	+	-	+	+	++	-	+
22	+	++	+	++	+	+	+	+	+	+	+	++	-	+	+	+
23	+	++	+	+	+	+	+	+	+	+	+	+	+	++	+	+
24	+	++	+	+	+	+	+	+	+	+	+	+	+	++	+	+

* P.ame = *P.americana*
P.aus = *P.australasiae*
B.ori = *B.orientalis*
B.ger = *B.germanica*
S.gre = *S.gregaria*
T.mol = *T.molitor*

\# c = crop
mg = midgut
- = no eggs present
+ = eggs present
++ = eggs abundant

TABLE 3.2 SUMMARY OF DATA FROM *IN VIVO* HATCHING OF *HYMENOLEPIS* EGGS AND TRANSIT TIMES ALONG THE GUT (MEAN VALUES ± S.D)

Insects/time	CROP								MIDGUT							
	WE		GC		FO		BS		WE		GC		FO		BS	
	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d
P.americana																
30-45 mins	91.00	26.50	54.00	20.74	7.14	11.05	52.00	28.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4 hours	77.14	11.04	33.00	12.32	33.71	17.26	82.57	14.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8 hours	48.71	12.88	0.00	0.00	10.86	7.56	25.71	8.79	52.71	31.87	6.29	5.91	5.29	4.07	102.86	21.20
24 hours	13.00	8.91	0.00	0.00	3.29	3.73	9.86	5.70	36.71	17.38	0.00	0.00	5.71	6.50	47.86	20.00
48 hours	1.43	2.15	0.00	0.00	1.14	3.02	2.14	3.34	0.86	1.21	0.00	0.00	0.00	0.00	0.71	1.89
P.australasiae																
30-45 mins	73.14	28.59	27.29	11.54	5.00	4.69	40.43	15.54	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4 hours	82.50	13.05	30.50	13.26	36.00	34.40	35.33	9.79	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8 hours	33.86	11.35	8.00	4.62	13.14	7.52	25.14	10.38	44.57	22.81	8.86	8.19	16.00	11.11	54.71	15.40
24 hours	5.57	3.74	0.00	0.00	1.29	2.63	24.14	11.60	27.57	17.49	0.00	0.00	4.71	6.55	17.86	11.80
48 hours	6.70	9.43	0.00	0.00	13.00	22.05	28.00	14.40	49.86	40.05	0.57	1.51	40.57	43.77	19.00	14.70
B.orientalis																
30-45 mins	63.43	24.26	37.31	20.58	4.00	8.47	39.43	23.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4 hours	71.43	19.92	23.14	13.72	44.57	25.42	70.43	22.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8 hours	36.71	12.07	6.29	3.68	6.29	5.50	44.57	18.13	58.57	27.59	3.71	4.89	7.29	6.90	20.57	7.30
24 hours	19.71	16.57	0.00	0.00	4.71	8.46	21.00	12.40	20.29	12.16	0.00	0.00	9.86	10.98	21.86	12.86
48 hours	33.00	40.87	0.00	0.00	20.14	26.99	15.00	28.02	9.57	9.61	0.00	0.00	4.71	6.21	15.43	16.20
S.gregaria																
30-45 mins	384.30	61.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4 hours	357.90	31.60	0.00	0.00	0.00	0.00	3.57	4.76	32.14	27.36	0.00	0.00	5.00	11.18	17.00	32.00
8 hours	141.43	54.29	0.00	0.00	0.00	0.00	0.00	0.00	210.00	46.55	6.14	7.20	1.71	3.73	10.43	14.13
24 hours	38.86	15.09	0.00	0.00	0.00	0.00	0.71	1.25	92.86	21.19	0.00	0.00	0.43	0.79	7.00	8.50
48 hours	1.71	2.63	0.00	0.00	0.00	0.00	2.86	6.34	2.29	5.22	0.00	0.00	0.00	0.00	4.00	6.42
T.molitor																
30-45 mins	42.43	29.25	24.43	17.91	13.43	8.52	36.71	19.27	33.37	12.91	6.71	6.37	11.86	7.73	23.86	17.60
4 hours	6.67	4.18	0.33	0.82	5.00	7.67	14.00	3.52	31.50	22.45	0.67	1.63	6.83	6.62	36.67	10.50
8 hours	0.57	1.13	0.00	0.00	0.00	0.00	2.88	3.67	44.71	24.54	0.00	0.00	0.71	1.25	34.29	11.00
24 hours	1.00	1.29	0.00	0.00	0.00	0.00	1.86	2.54	21.86	12.19	0.00	0.00	0.86	1.21	12.86	7.20
48 hours	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.57	5.33	0.00	0.00	0.00	0.00	8.00	9.26
B.germanica																
30-45 mins	33.00	35.03	20.71	21.94	7.57	7.46	30.43	23.70	1.29	2.21	0.00	0.00	0.00	0.00	0.00	0.00
4 hours	17.29	16.00	0.71	1.25	23.86	12.55	31.29	21.25	19.43	12.31	1.00	1.53	14.29	10.19	18.57	12.00
8 hours	10.14	9.08	0.00	0.00	0.00	0.00	6.29	5.47	20.14	18.85	0.00	0.00	8.86	9.58	33.29	15.00
24 hours	1.71	2.21	0.00	0.00	0.43	0.79	1.57	1.81	17.57	13.26	0.00	0.00	0.00	0.00	17.00	10.00
48 hours	1.14	2.27	0.00	0.00	0.00	0.00	2.00	2.38	8.43	16.37	0.00	0.00	2.57	5.16	7.29	7.80

WE = Whole eggs GC = Larvae with gelatinous coat FO = Free oncospheres BS = Broken shells

TABLE 3.3 SUMMARY OF DATA FROM *IN VIVO* HATCHING OF *MONILIFORMIS* EGGS AND TRANSIT TIMES ALONG THE GUT (MEAN VALUES ± S.D)

Insects/time	CROP						MIDGUT					
	SA		FA		ES		SA		FA		ES	
	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d	$\bar{x}$	s.d
P.americana												
30-45 mins	103.71	30.91	0.00	0.00	6.57	5.80	0.00	0.00	0.00	0.00	0.00	0.00
4 hours	26.71	18.38	32.57	25.90	62.90	25.60	0.60	1.50	1.60	4.20	4.10	11.00
8 hours	19.29	10.56	23.70	8.10	32.60	20.70	7.00	5.20	50.60	11.90	55.70	10.60
24 hours	7.57	5.22	2.00	2.10	7.40	6.50	14.70	8.80	44.10	20.00	117.10	47.90
48 hours	2.14	2.27	0.00	0.00	1.60	2.90	7.70	8.30	10.30	7.20	89.90	53.30
T.molitor												
30-45 mins	49.30	20.10	0.00	0.00	6.30	6.70	9.90	7.70	0.00	0.00	1.40	3.00
4 hours	12.00	9.20	5.30	5.70	13.40	15.70	35.10	19.30	7.70	5.51	36.70	23.90
8 hours	3.70	4.30	0.00	0.00	3.10	5.90	48.70	17.30	9.40	4.90	31.91	18.65
24 hours	0.00	0.00	0.00	0.00	0.00	0.00	11.90	6.10	3.60	2.84	7.40	7.00
48 hours	0.34	0.80	0.00	0.00	0.73	1.90	5.30	7.30	4.10	3.60	3.00	5.90
S.gregaria												
30-45 mins	351.80	68.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4 hours	302.20	60.45	7.20	7.90	14.00	7.80	25.80	11.52	2.00	2.55	70.40	39.53
8 hours	77.00	51.90	49.80	42.66	105.40	73.56	57.00	23.90	14.80	11.95	266.00	89.96
24 hours	37.00	29.30	13.60	13.70	48.00	22.50	46.00	33.60	8.00	8.92	104.00	18.38
48 hours	1.40	2.19	0.00	0.00	3.00	4.47	6.40	6.30	0.00	0.00	17.00	12.55

S A = Shelled acanthors F A = Free acanthors E S = Empty shells

TABLE 3.4 PENETRATION OF THE MIDGUT BY LARVAE OF *MONILIFORMIS MONILIFMORMIS* AND *HYMENOLEPIS DIMINUTA IN VITRO*

Insects	Presence of larvae on gut surface		Presence of larvae in medium	
	oncospheres	acanthors	oncospheres	acanthors
P.americana	-	++	-	-
P.australasiae	-	++	-	++
B.orientalis	-	++	-	+
S.gregaria	*+	*+	*+	-
T.molitor	++	+	++	++
B.germanica	-	++	-	++

- = none present
+ = few present
++ = several present
* = average of less than 1 larva per gut

TABLE 3.5 *IN VITRO* PENETRATION OF GUT SACS BY *IN VITRO*-HATCHED PARASITE LARVAE

Insects	Presence of larvae on gut surface		Presence of larvae in medium	
	oncospheres	acanthors	oncospheres	acanthors
P.americana	-	++	-	++
T.molitor	+++	+++	++	++
S.gregaria	++	++	+	+

- = none present
+ = few present
++ = several present
+++ = many present

Figure 3.3 **Passage of *Hymenolepis diminuta* egg shells along the gut of *Periplaneta americana*.**

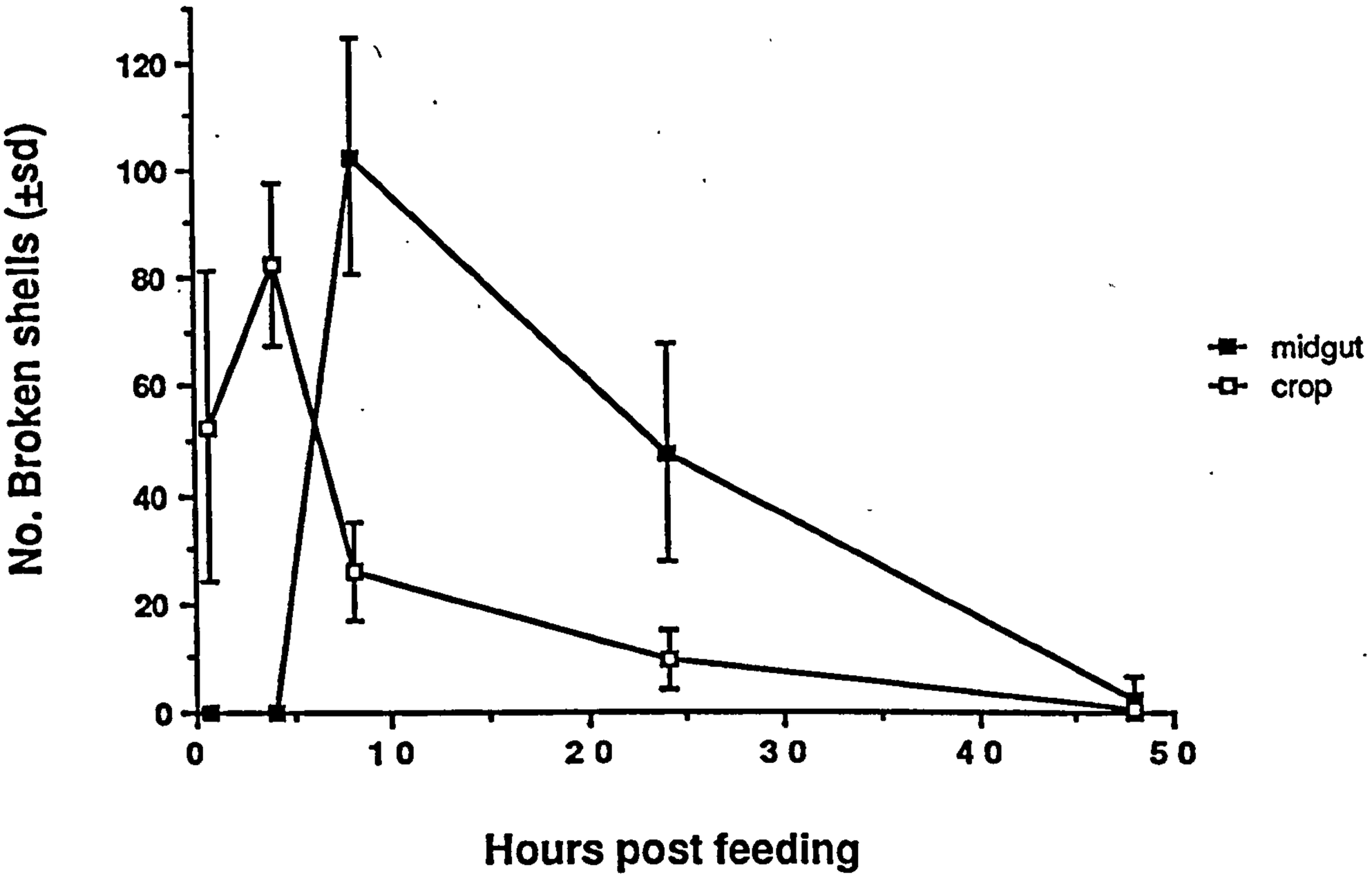


Figure 3.4 **Passage of *Hymenolepis diminuta* egg shells along the gut of *Periplaneta australasiae*.**

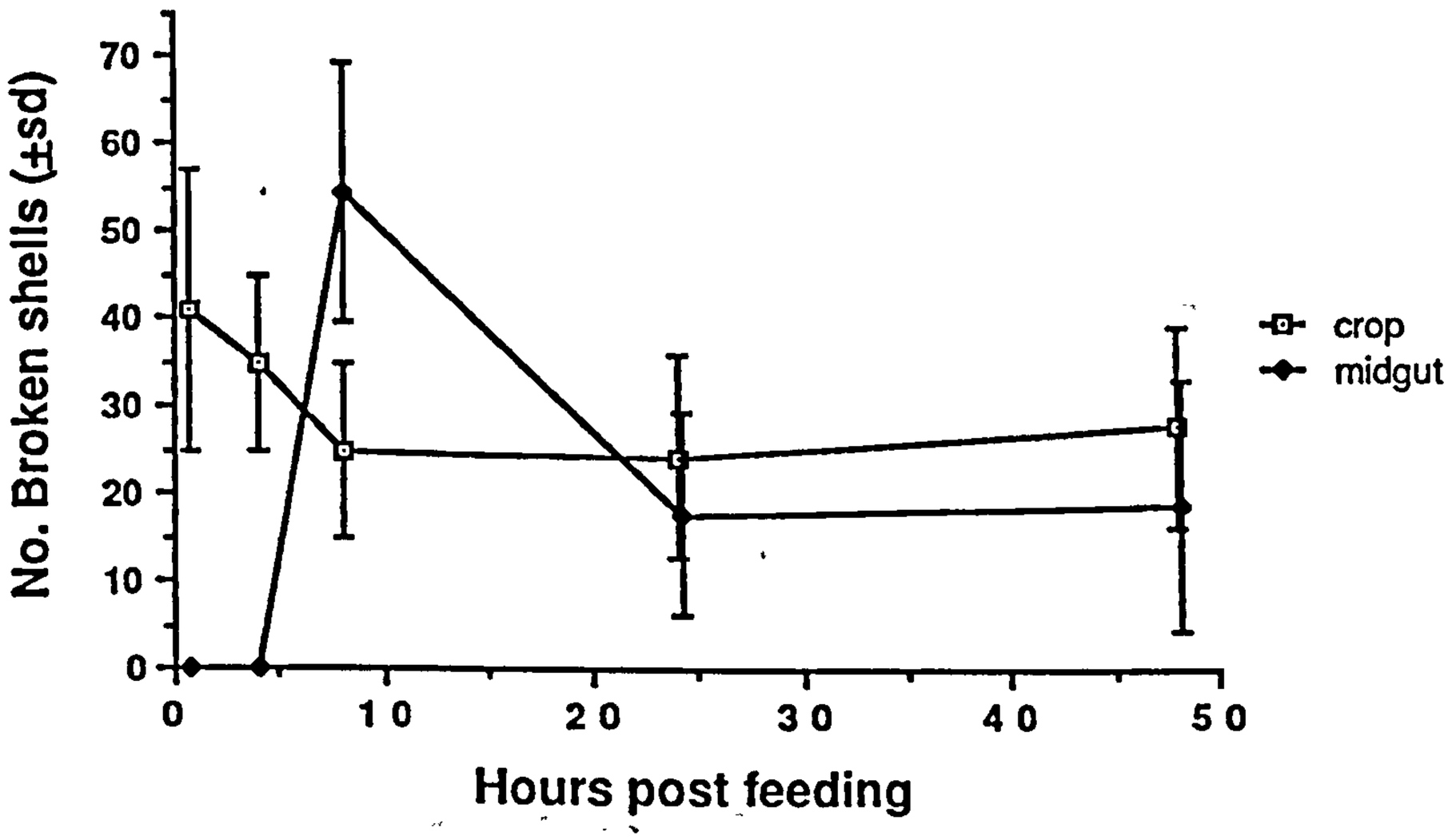


Figure 3.5 **Passage of *Hymenolepis diminuta* egg shells along the gut of *Blatta orientalis*.**

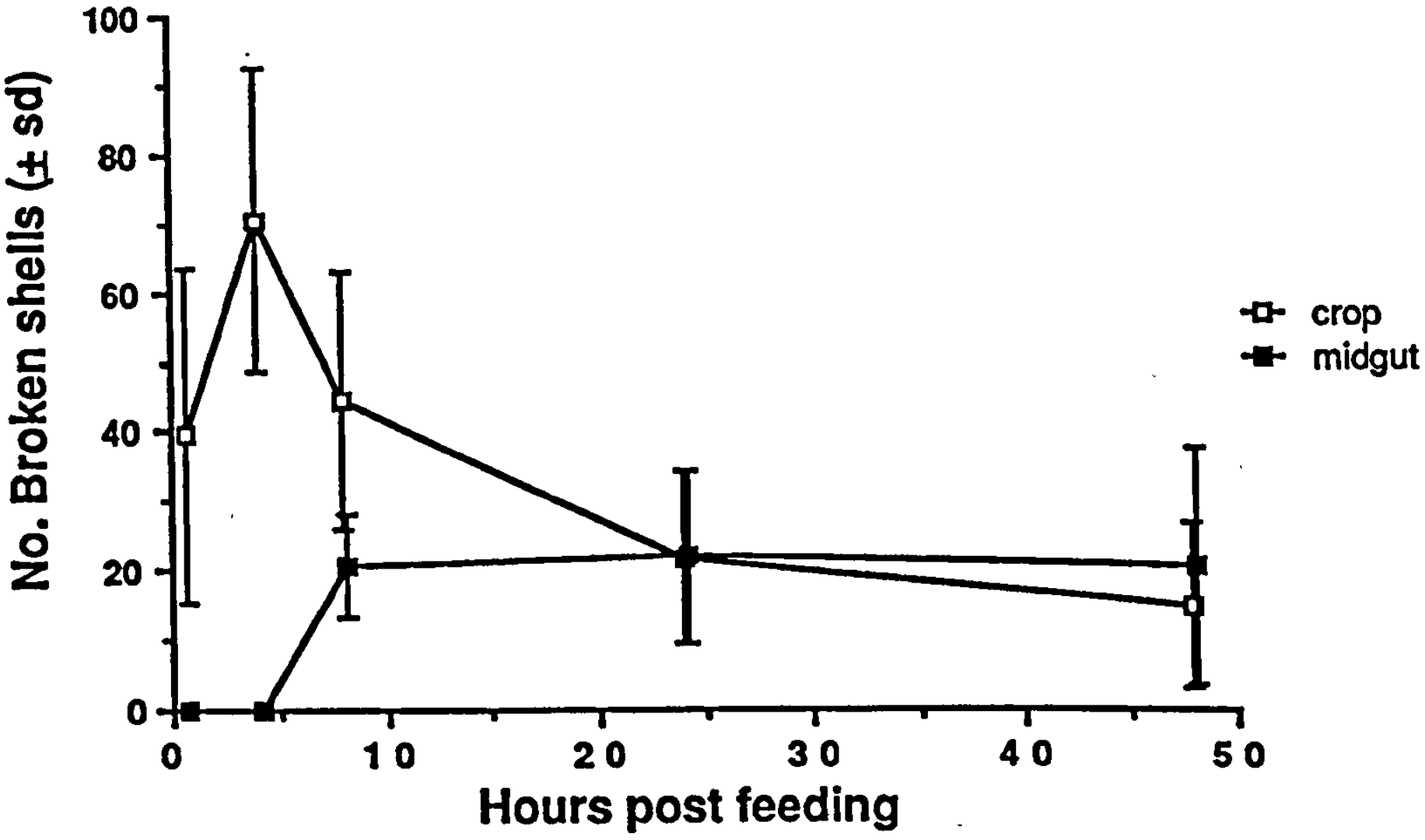


Figure 3.6 **Passage of *Hymenolepis diminuta* egg shells along the gut of *Blatella germanica*.**

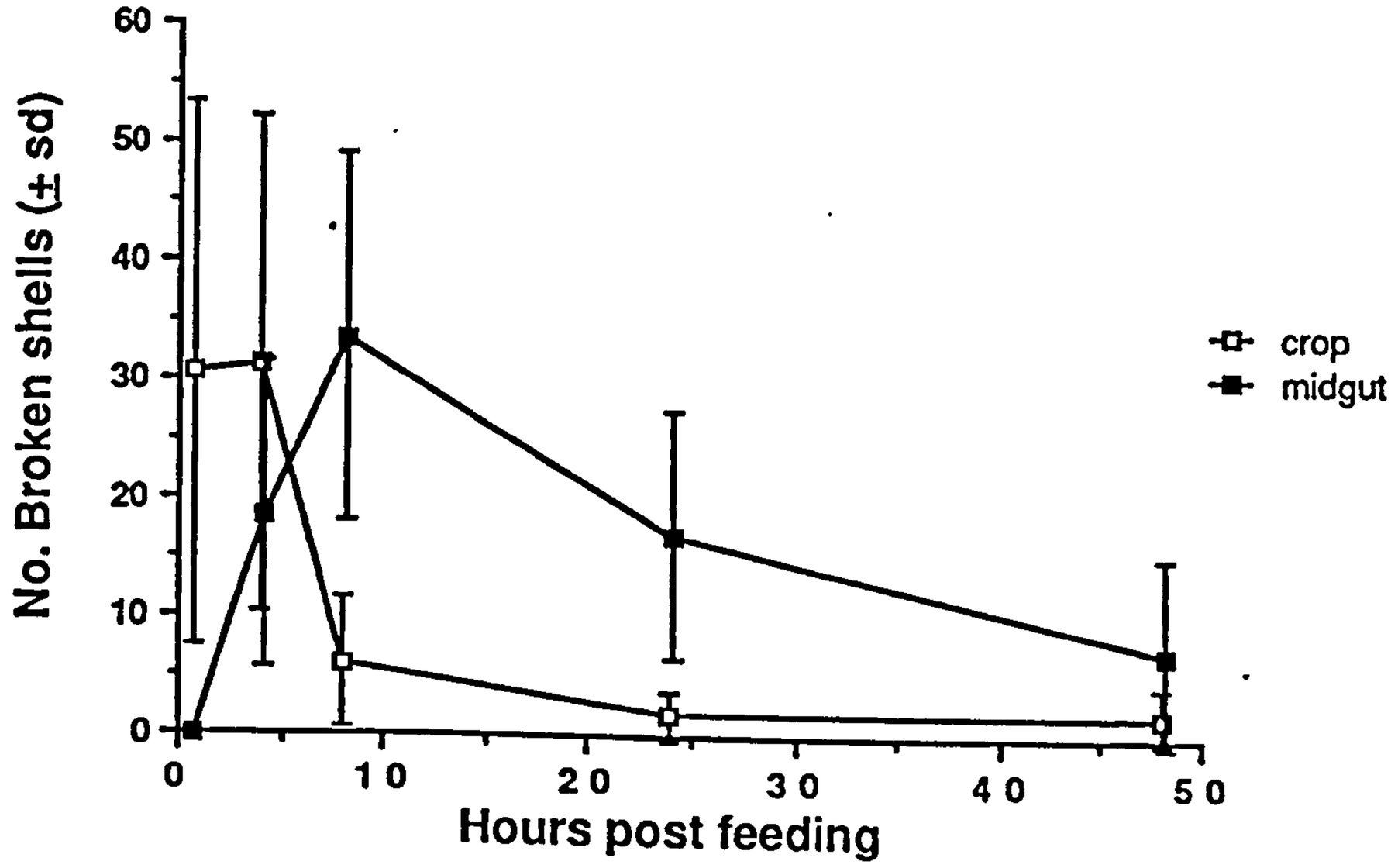


Figure 3.7 **Passage of *Hymenolepis diminuta* egg shells along the gut of *Schistocerca gregaria*.**

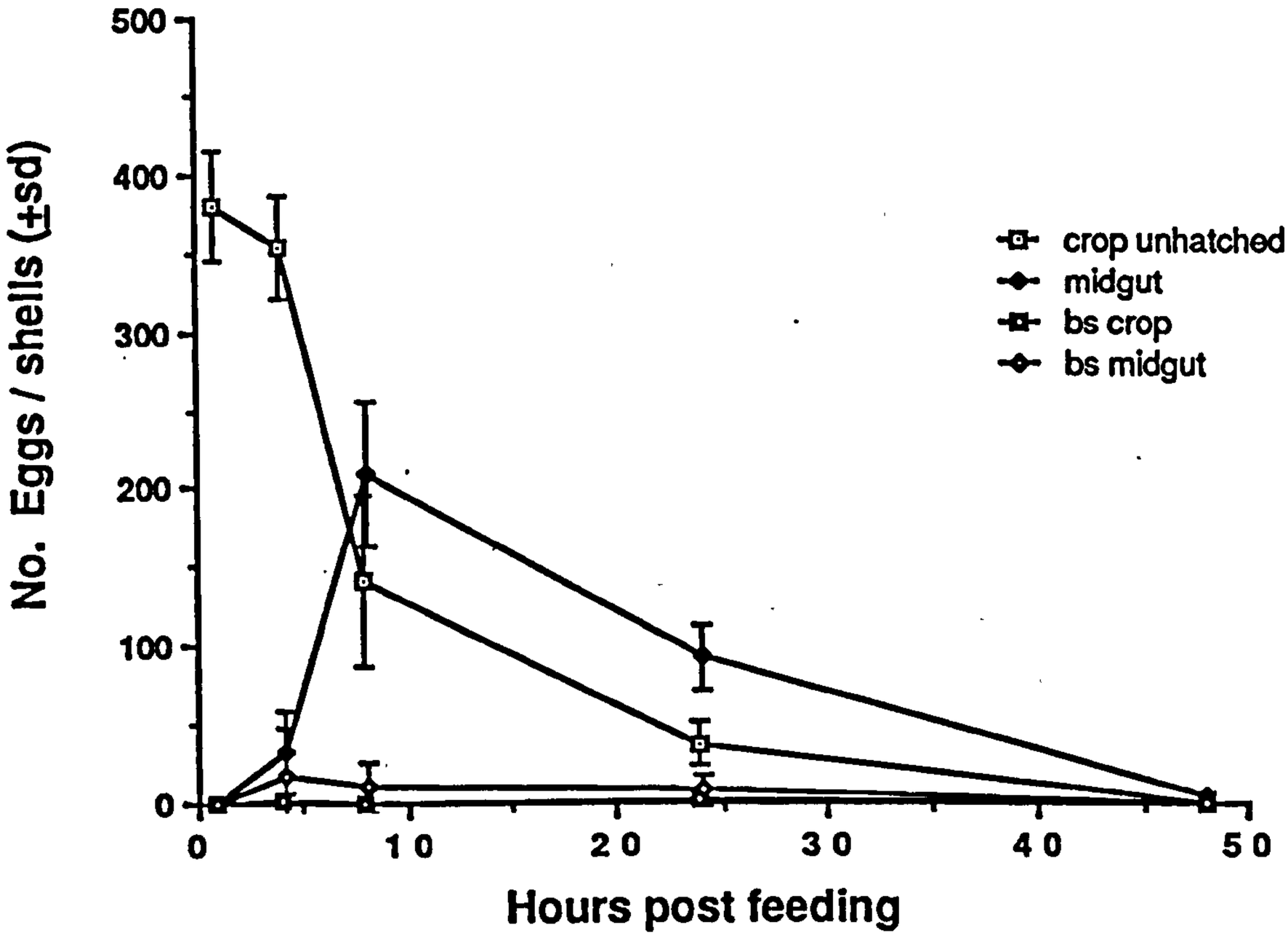


Figure 3.8 **Passage of *Hymenolepis diminuta* egg shells along the gut of *Tenebrio molitor*.**

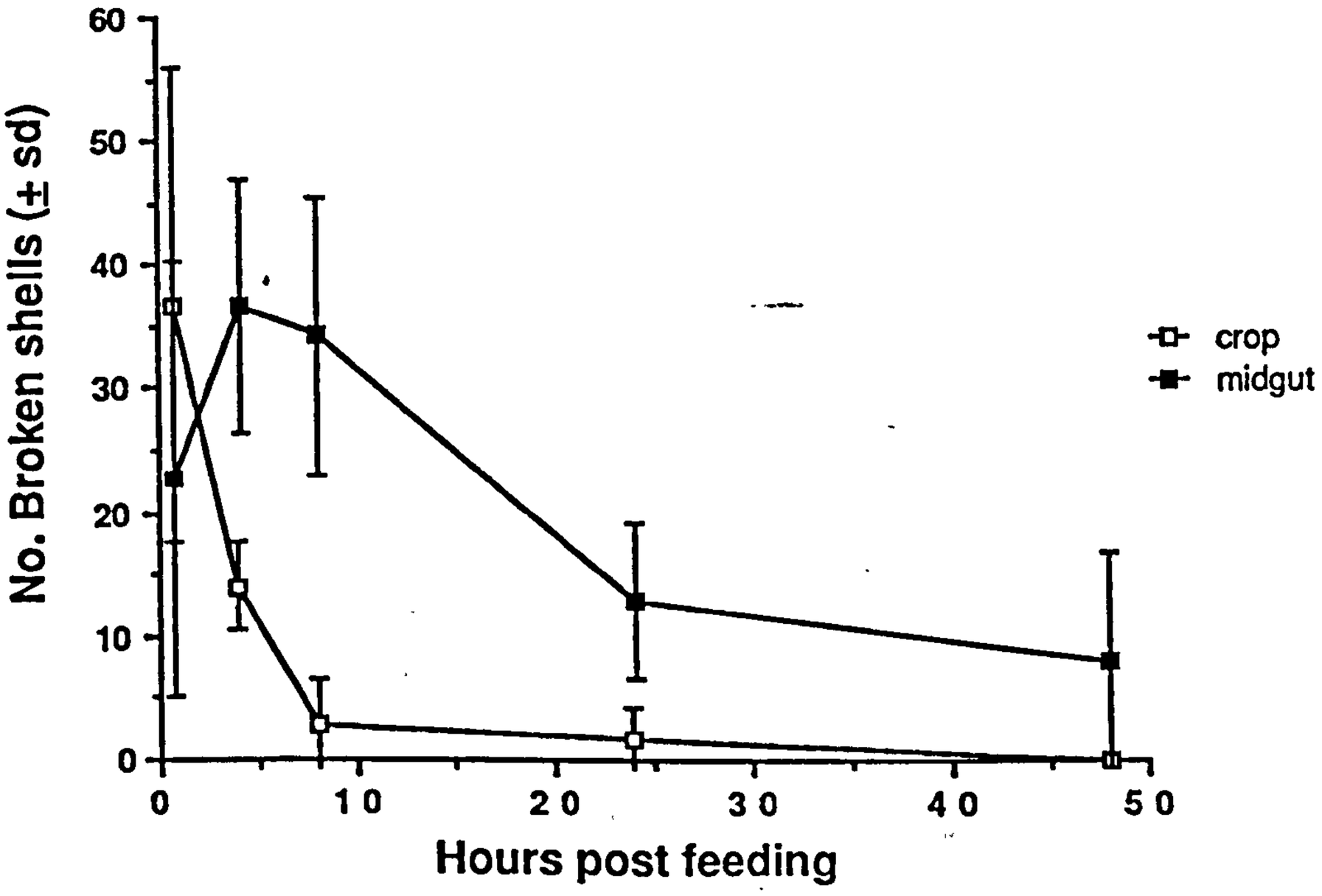


Figure 3.9 **Passage of *Moniliformis moniliformis* egg shells along the gut of *Periplaneta americana*.**

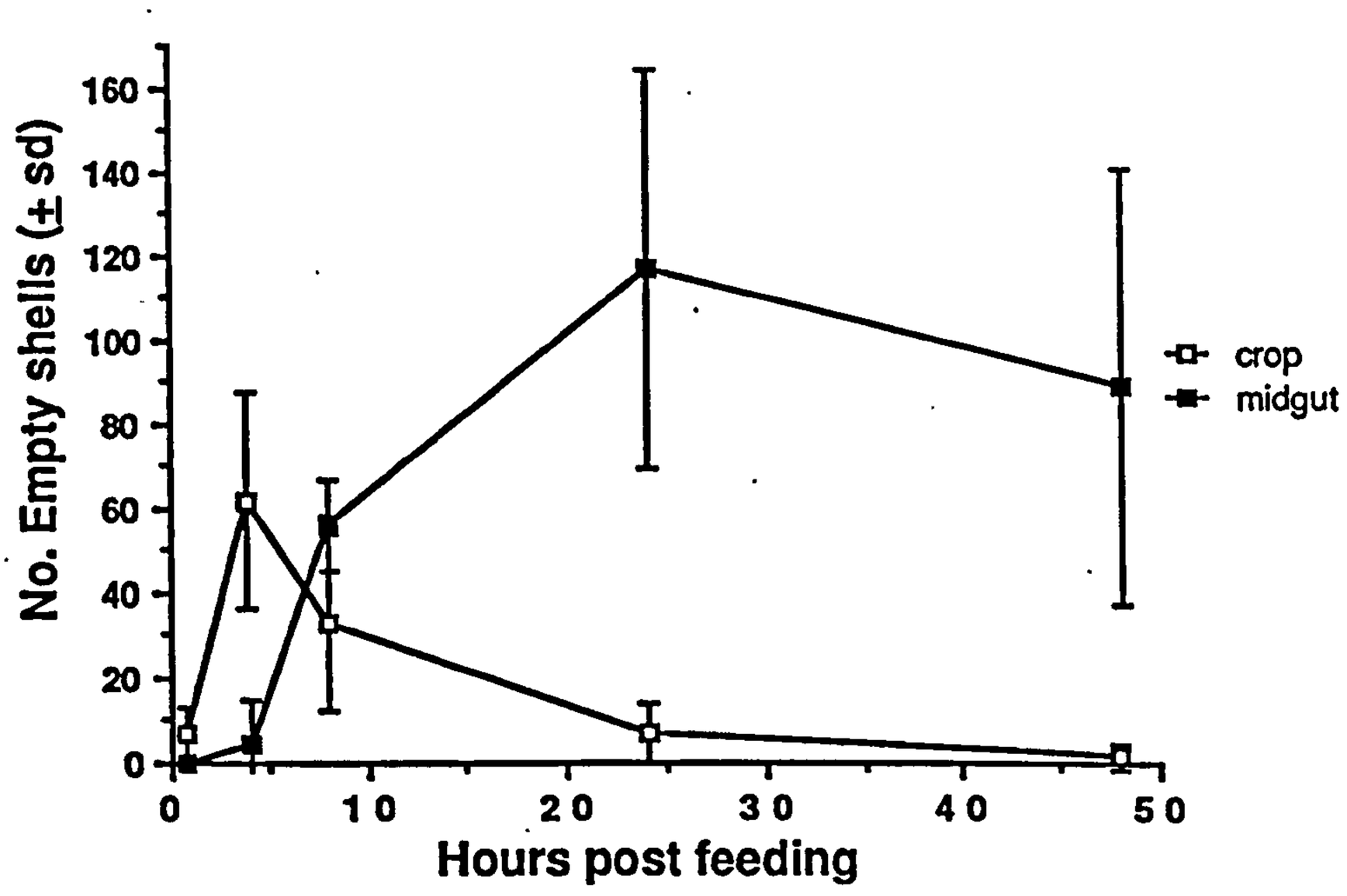


Figure 3.10 **Passage of *Moniliformis moniliformis* egg shells along the gut of *Tenebrio molitor*.**

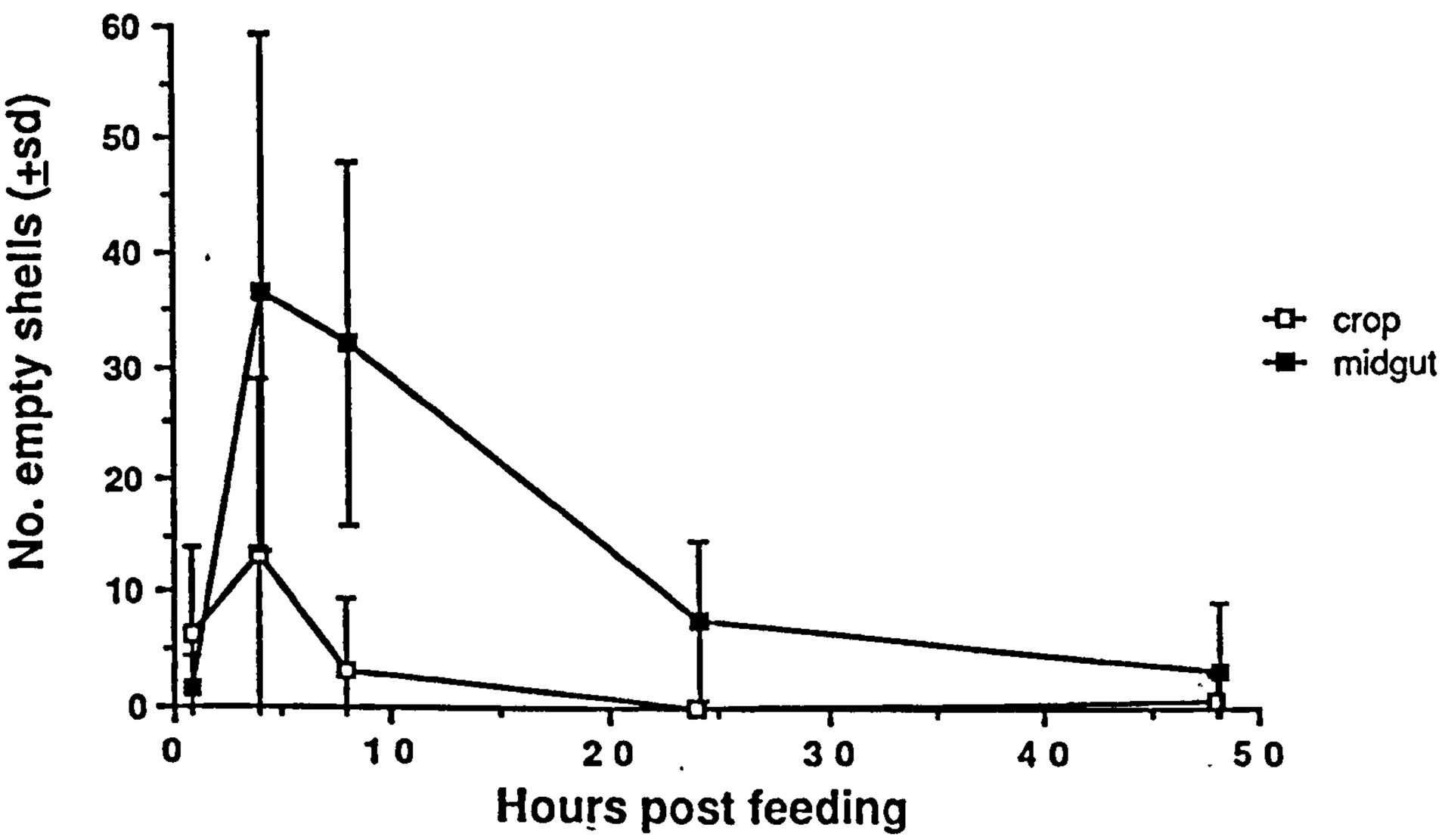


Figure 3.11 Passage of *Moniliformis moniliformis* egg shells along the gut of *Schistocerca gregaria*.

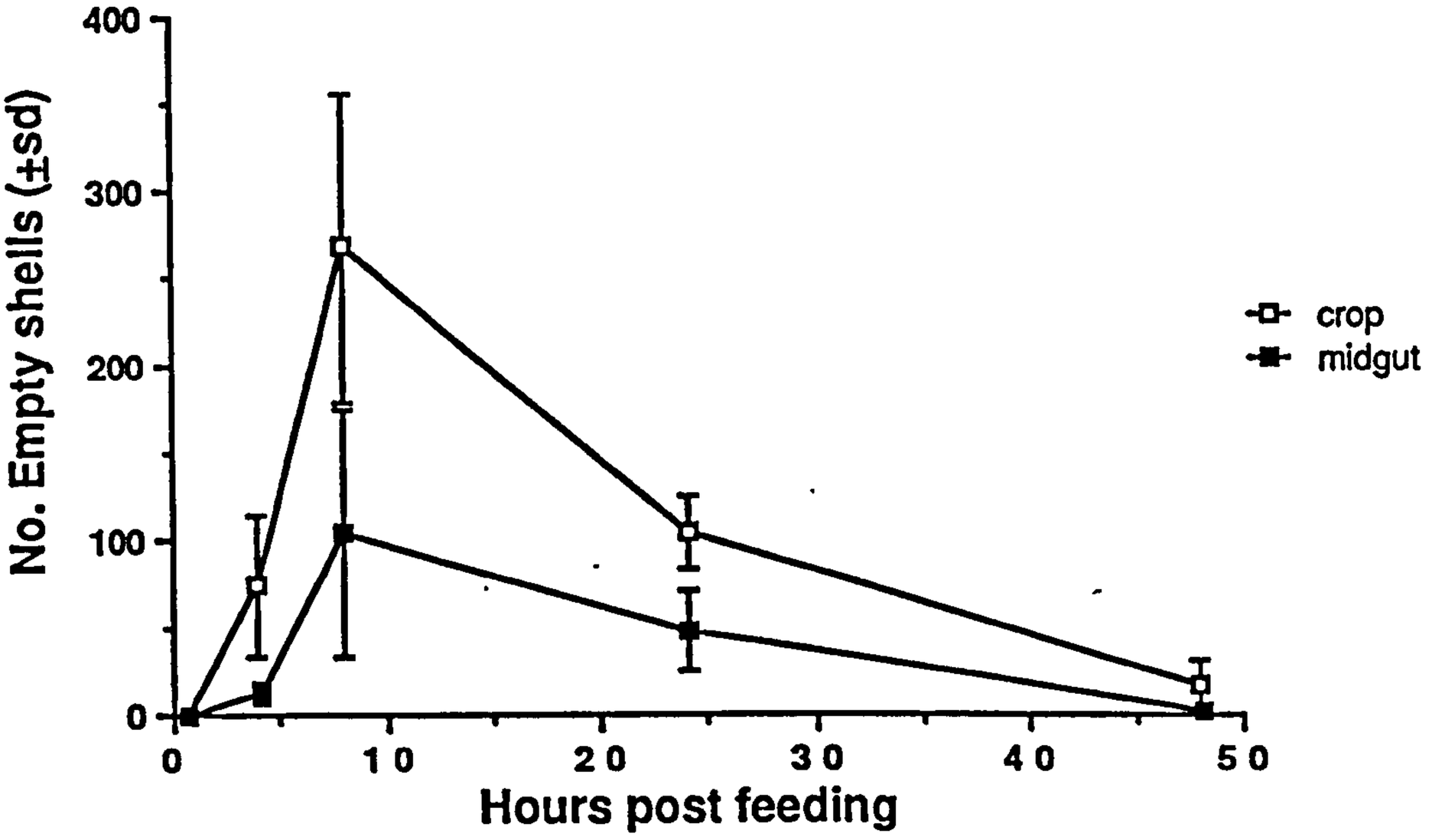


FIGURE 3.12.1 SCANNING ELECTRON MICROGRAPH OF THE PAPILLAE ON THE HAEMOCOELIC SURFACE OF THE MIDGUT OF *TENEBRIO MOLITOR*

P = Midgut papillae

CM = Circular muscles

LM = Longitudinal muscles

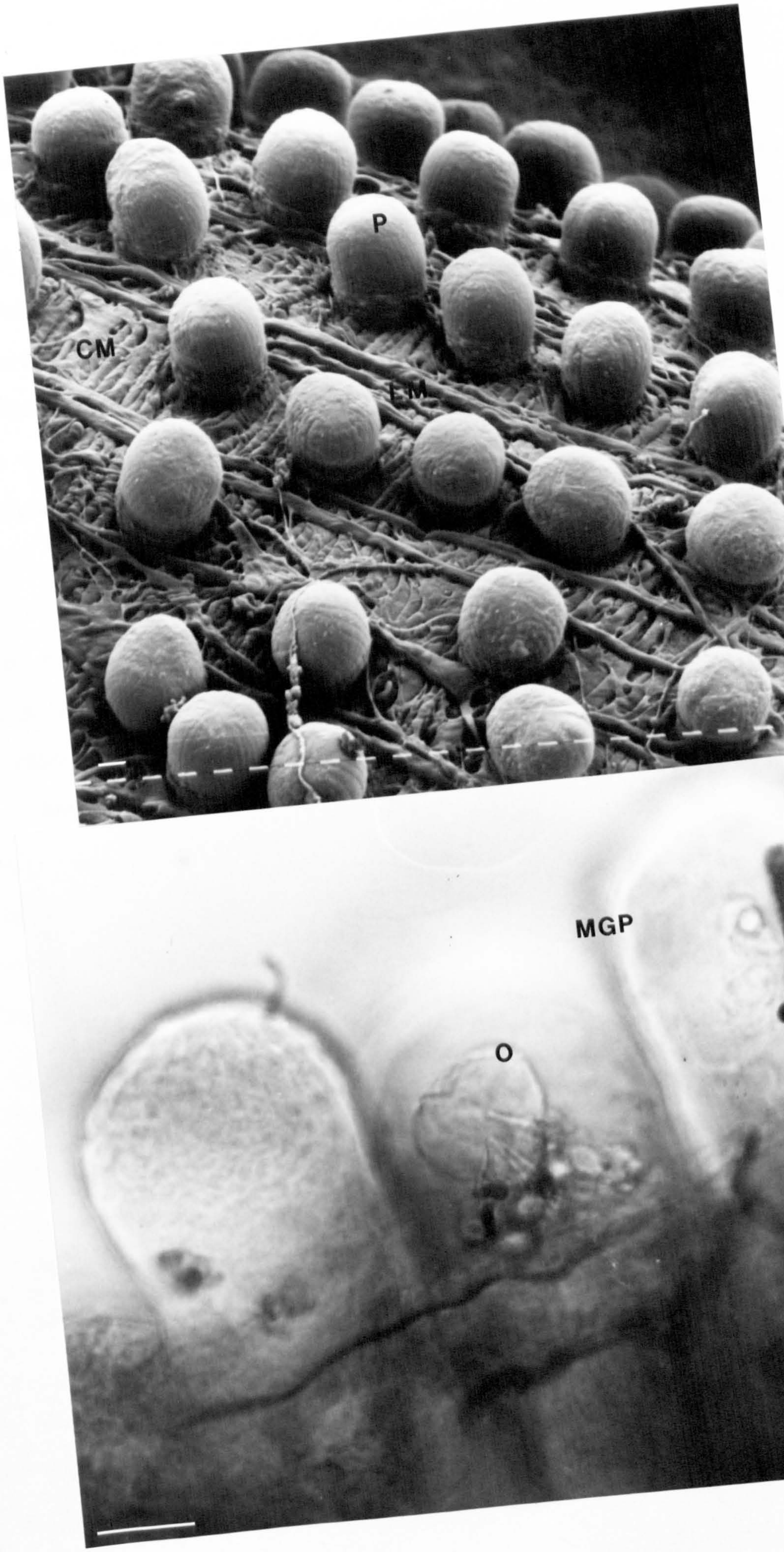
Scale bar = 50μm

FIGURE 3.12 PHOTOMICROGRAPH OF A *HYMENOLEPIS* ONCOSPHERE PENETRATING THE MIDGUT PAPILLAE OF *TENEBRIO MOLITOR IN VIVO*

O = Penetrant oncosphere

MGP = Midgut papillae

Scale bar = 50μm


P
CM
EM
MGP
O

3.4 DISCUSSION

Successful hatching of either *H.diminuta* or *M.moniliformis* is dependent on different factors for each parasite. *In vitro* the values for egg viability of each species of parasite were found to be reasonably close, approximately 90% for *H.diminuta* and 76% for *M.moniliformis*. However when *H.diminuta* is ingested, the percentage viability of the dose of eggs is of little significance compared to whether or not the insects mouthparts break open the shells to initiate hatching. In the first experiment only approximately 50 % of *H.diminuta* eggs were cracked during the process of ingestion by most of the insects studied, a value agreeing with that of Lethbridge (1971a), and in locusts virtually all the eggs remained whole. This was probably a direct result of the mechanics of the locust mouthparts being suited for chewing and biting large fragments of plant material therefore allowing smaller items such as *H.diminuta* eggs to pass into the crop undamaged.

Mechanical cracking is not essential for the stimulation of hatching of *M.moniliformis*, and only seems to effect a small proportion of the ingested acanthors in this experiment. Instead hatching is a gradual process, requiring exposure to certain electrolytes of at least 0.2M and pH greater than 7.5 as described by Edmonds (1966). By 8 hours only approximately 30 % of the mature acanthors remained unhatched in the guts examined, which correlates well with the 76 % viability estimate at the start of the experiment.

Once ingested, eggs are held in the crop where the majority of the digestive processes take place before the ingested material is allowed to pass into the midgut.

It is possible that the length of time recorded for eggs remaining in the crop is influenced considerably by elements within the design of the experiment itself. The following examples show how different factors may alter transit times of food along the gut.

Osmotic pressure and concentration of sugars in the crop as well as degree of fullness dictate the rate of crop emptying (Treherne, 1957; Davey and Treherne, 1963a, b, 1964). Consequently the sucrose solution used as an incentive for the insects to feed may effectively speed up the rate of crop emptying and reduce the time taken for food substances to travel down to the midgut.

Conversely, because the insects were starved prior to ingesting the parasite eggs and sucrose, there may be a delay until the digestive enzymes are produced (Day and Powning, 1949, Dow, 1986) thus resulting in a delay in the digestion of the subshell layers of *H.diminuta* Similarly as the insects were not fed after receiving the doses of eggs, this may again effect the progression of the food along the gut and production of enzymes.

Cockroaches are known to swallow air to inflate the crop if starved for prolonged periods (Chapman, 1985b) which is very likely to effect the way in which subsequently ingested material passes down the gut. The effects of starving cannot be totally avoided in this experiment, as a fast was required prior to dosing to ensure the insects would readily take the egg suspension. In preliminary experiments it seemed that a fasting period of 2 days was the best compromise before the cockroaches began to fill their crops with air.

Lethbridge (1972) reported that enzyme extracts of cockroach gut facilitated oncosphere hatching *in vitro* and from the results obtained here it is evident that hatching also occurs quickly *in vivo* As previously mentioned (see Chapter 1), *M.moniliformis* hatches in the presence of a suitable electrolyte and although it is not fully understood which chemical components within the crop are responsible for acanthor activation, this occurs in a wide range of insect species as demonstrated in this work. For *M.moniliformis*, unlike *H.diminuta* which is activated as soon as the shell is cracked, hatching is a more gradual process, where not until 4-8 hours after ingestion are large numbers of acanthors found in the crop and midgut. This could be regarded as an adaptation by *M.moniliformis* to parasitise insects, such as cockroaches,

which have a slow rate of crop emptying so that the acanthors are not hatched prematurely in the crop.

In comparison, *H.diminuta* appears to hatch too early in cockroaches, so that large numbers of oncospheres are hatched in the crop and will not reach the midgut until their ability to burrow has possibly ceased.

Several workers in the past have studied the passage of food materials along the gut and suggested that if the rate of movement is too high, larvae would be swept along the gut and would not have a chance to grasp the gut wall to begin burrowing (Voge and Graiwer, 1964). In the case of *H.diminuta* in the gut of cockroaches it would seem the converse may be true, and the rate at which food items pass along the gut may be too slow (total transit time of 20-100 hours in *P.americana*, Bignell, 1980) for *H.diminuta* to reach a suitable site of penetration before running short of energy reserves. This is supported by the fact that the transit time for food materials passing down the gut of *T.molitor* is shorter than in the cockroach and corresponds well with the time taken for *H.diminuta* to hatch and reach the midgut (ie. approximately 1/2 to 4 hours).

In some of the insects studied eggs were released from the crop steadily, so that it was virtually empty after 24-48 hours. This corresponded with the gradual flow of these eggs through the midgut. However some insects, *P.australasiae* in particular, only allowed a small flow of eggs from the crop via the proventriculus into the midgut, leaving a large number of eggs and non-motile oncospheres in the crop for as long as 48 hours (Table 3.2 and Figure 3.4). This slowing of crop emptying may be a consequence of the starving/feeding regimes used in the experiment, but it is effective in preventing infective stages reaching the midgut. *H.diminuta* larvae in this case were eventually released from the crop, the majority being non-motile and presumably rendered incapable of penetrating the gut through energy depletion or prolonged exposure to enzymes.

It seems that *H.diminuta* oncospheres have more than one chance of being mechanically activated when ingested by a cockroach. A few partially hatched, active larvae were observed with their subshell layers still intact in the midgut, corresponding with the general release of crop contents at around 8 hours post feeding. It seems unlikely that they remained in this state since passing through the mouthparts of the insect, and it is therefore possible that they were "shelled" by the grinding action of the chitinous plates in the proventriculus (Chapman, 1982, 1985a). This resulted in a small proportion of the oncospheres hatching directly into the midgut where they potentially could begin to penetrate the gut tissues, but unfortunately, due to their small size and low numbers they could not be traced in the *in vitro* gut-penetration experiment.

Leger and Cavier (1970) investigated a similar system to that discussed here. They found that *in vivo*-hatched larvae of *Hymenolepis nana* survived when injected into the haemocoele of the cockroach *Leucophaea maderae*, but when the insects were fed with the eggs, the larvae failed to infect the insects. On careful examination of the histology of the gut wall they concluded that the peritrophic membrane played a significant role in preventing penetration and infection by the oncospheres.

However results of the *in vitro* penetration experiments clearly show the difference in ability of *M.moniliformis* and *H.diminuta* to burrow through the gut tissues, and it appears that the peritrophic membrane has little influence on this difference *per se*. Even when the peritrophic membrane is removed in the *in vitro* assay, oncospheres are still unable to penetrate the midgut wall. *Hymenolepis citelli* was also used in preliminary experiments to determine whether the oncospheres, which closely resemble those of *H.diminuta*, were capable of penetrating the midguts of a similar range of hosts. Results were the same; the oncospheres were only able to penetrate the midguts of the flour beetles.

H.diminuta oncospheres followed the path across the gut wall of *T.molitor*

that was described by Lethbridge (1971a). Penetrant oncospheres were able to cross the midgut wall via the tissues of the midgut papillae, thus avoiding the midgut musculature. Figure 3.12 shows oncospheres as they appeared in the papillae in section 3.3.4 of the *in vitro* penetration assay. Therefore it does seem that they follow a pathway of least resistance or that only those which, by chance, enter papillae are able to get through.

M.moniliformis were also shown to penetrate beetle midgut tissue, although their route of penetration seemed to be more variable as they appeared both on the papillae and on the muscular surface of the gut.

Both parasites are able to penetrate the gut of *S.gregaria*, but only in very low numbers. The large cross-sectional area of the locust gut could prevent all the larvae apart from those nearest the periphery of the gut from penetrating, especially if the larvae had to burrow through the contents of a full gut.

M.moniliformis is capable of penetrating the gut wall of *P.americana*, whereas *H.diminuta* does not even penetrate the guts of the smaller cockroaches. In an attempt to infect cockroaches via the oral route with *H.diminuta*, preliminary experiments were carried out using recently ecdysed insects. In these it was assumed that the cuticular-lined regions of their guts would be comparatively soft therefore allowing the larvae to penetrate. However results were always negative.

M.moniliformis acanthors burrow by the use of their rostellar hooks in an action which seems to form a hole not much larger than the diameter of their body, anchoring themselves during each stroke with backward pointing spines. *H.diminuta* oncospheres on the other hand use relatively large sweeping strokes with their 3 pairs of hooks which normally propel the larvae through the loosely packed regenerative cells of the midgut papillae. It is therefore possible that in the cockroach midgut, oncospheres are not properly equipped to deal with the more closely packed cells or simply cannot begin to burrow

and soon run out of glycogen stores.

It seems that there are a combination of factors taking effect within the gut which make the cockroach an unsuitable host for *H.diminuta*, whereas these factors have been overcome by *M.moniliformis*. Either *H.diminuta* is withheld too long in the crop where penetration is made impossible by the intima and then runs short of energy reserves, or the larvae cannot penetrate the midgut wall.

3.5 SUMMARY

1. Both *H.diminuta* and *M.moniliformis* larvae hatched in the guts of a wide variety of insect species, indicating that the appropriate stimuli were present. *M.moniliformis* hatched in and penetrated the guts of all the insect species investigated (eg. cockroaches, locusts and beetles)but did not develop fully in beetles. *H.diminuta* oncospheres penetrated beetle guts and developed as expected, and also penetrated and developed to a lesser extent in locusts, confirming observations made by Lethbridge (1971a); the oncospheres were unable to penetrate cockroach guts.

2. Lethbridge's (1971a) observation that *H.diminuta* oncospheres enter the midgut papillae in order to penetrate the midguts of flour beetles, was also confirmed here.

3. Transit time for food materials along the gut is important in the synchronisation of parasite hatching with arrival at the site of gut penetration. *P.americana* holds food items and parasites in the crop

until digested before allowing them into the midgut. Thus any oncospheres stimulated to hatch by the insect's mouthparts can only progress as far as the crop until initial digestive processes are completed and here they appear to run short of glycogen reserves. As *M.moniliformis* acanthors hatch relatively slowly compared to *H.diminuta* oncospheres, they are active and capable of gut penetration when they leave the crop and enter the midgut.

4. Approximately 50% of *H.diminuta* eggs eaten by cockroaches (approximately 50%) remain whole and viable within the crop. Some of these eggs are cracked causing subsequent hatching as they pass through the proventriculus so that a small number of active *H.diminuta* oncospheres reach the midgut. However, no evidence of midgut penetration by these few larvae was found.

5. The results of the *in vitro* gut-penetration assays confirmed the observations made *in vivo* *M.moniliformis* is capable of penetrating the midgut tissues of a wide variety of insects, whereas *H.diminuta* is limited to beetles and (at low success rates) locusts. The peritrophic membrane appeared to play little role in preventing penetration of *M.moniliformis* in cockroaches. Reasons for the differing ability of *H.diminuta* and *M.moniliformis* larvae to penetrate cockroach gut have been suggested to be related to the differing burrowing methods used by the two species.

CHAPTER FOUR

SIMULTANEOUS ORAL INFECTION OF INSECTS WITH *HYMENOLEPIS DIMINUTA* AND *MONILIFORMIS MONILIFORMIS*

4.1 INTRODUCTION

In Chapter 3 it was established that the oncosphere larvae of *H.diminuta* are incapable of penetrating the midgut wall of cockroaches. However this alone cannot be considered as conclusive evidence for the inability of *H.diminuta* to infect them. Lackie (1976) infected cockroaches with *H.diminuta* by injecting pre-hatched larvae directly into the abdominal haemocoele, demonstrating that the physiological and nutritional requirements of the larvae had been met in this host.

Crompton (personal communication) suggested that the massed burrowing action of large numbers of *M.moniliformis* on the midgut wall of the cockroach might facilitate simultaneous penetration by *H.diminuta* if the two parasites were administered in a single dose. Moreover if a parasite's mechanism for survival involved immunosuppression of the host it might permit a secondary infection by another parasite species not normally capable of surviving in this host. Thus it is possible that *M.moniliformis* might immunosuppress *P.americana* thereby conferring protection on *H.diminuta* from the cockroach immune system (see Chapter 6, also Brennan and Cheng, 1975).

There are numerous examples of one parasite assisting the establishment of a second species, many of which involve parasitoid wasps (for example see Guzo and Stoltz, 1985); interspecific reactions of species which develop intrahaemocoelically have been studied. This type of reaction in the haemocoele will be studied in greater detail in Chapter 6 whereas in this chapter work will concentrate on the interactions of parasites with the insect midgut.

Almost nothing is known about the effect a parasite or pathogen may have on the insect when burrowing through the gut wall. Glinski and Jarosz (1986) attempted to induce a defence response in *Galleria mellonella* larvae by feeding with pathogenic bacteria (*Pseudomonas aeruginosa, Serratia marcescens* and *Xenorhabdus nematophilus*). Apart from a non-significant increase in blood

lysozyme activity they found no reaction unless the bacteria were injected into the haemocoele. Chippendale and Kilby (1969) suggested that the midgut pericardial cells, amongst other tissues in *Pieris brassicae*, may be involved in haemolymph protein biosynthesis and that proteins may cross the intestinal epithelium to reach the midgut lumen or haemolymph.

Any two parasite species in a single host may also interact to the detriment of one or both parasites. Gordon and Whitfield (1985) described the occurrence of *H.diminuta* and *Raillietina cesticillus* together in the flour beetle *T.confusum* and found that in cases where *H.diminuta* were allowed to develop first, a subsequent challenge infection of *R.cesticillus* was able to develop as in homologous infections. But if the primary infection was of *R.cesticillus*, fewer of a subsequently-administered dose of *H.diminuta* developed than normal. The authors suggested that the reason for this difference might be because *R.cesticillus* activated the beetle's immune system which prevented normal growth of *H.diminuta* while at the same time having no effect on the development of *R.cesticillus*.

It has been previously established (Chapter 2) that faeces from rats with concomitant infections of *M.moniliformis* and *H.diminuta* could contain eggs of both parasite species. Thus, the aim of this chapter is to determine whether certain insect species, *P.americana* in particular, can become infected by feeding on a mixed dose of eggs and whether the parasite species interact to the benefit or detriment of one another.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS

4.2.1.1 PARASITES

The first set of experiments was carried out with the eggs of *H.diminuta* and *M.moniliformis* in the faeces collected from rats used in heterologous and homologous infections in Chapter 2.

The second set of experiments was carried out using suspensions of eggs of both species in sucrose solution as in Chapter 3, section 3.2.4.1.

4.2.1.2 INSECTS

Adult male *P.americana* (see section 2.2.1.3), adult *Tenebrio molitor* (see section 2.2.1.2) and adult *Schistocerca gregaria* (see section 3.2.1) were maintained as described in previous sections.

All insects were starved for 48 hours prior to the start of the experiments.

4.2.2 EXPERIMENTAL PROCEDURES

4.2.2.1 ADMINISTRATION OF MIXED DOSES OF EGGS IN RAT FAECES

Faecal material from rats infected with *H.diminuta* and *M.moniliformis* was collected and made into a paste by adding HBS (approximately 50% weight/volume) and mixing thoroughly. A 1g sub-sample of this homogenate was then filtered through a coarse mesh (0.5mm) to remove the larger solid particles and processed to estimate egg abundance as in section 2.2.3.2.

Samples of faecal material classified as containing "abundant" numbers of eggs were then formed into 0.2ml pellets, each containing approximately 800-1000 parasite eggs. The pellets were formed by extruding the faecal material

from a 1ml plastic syringe with the lower end cut away. In this manner, pellets of faecal material were formed containing approximately :-

a, 800-1000 *H.diminuta* eggs

b, 800-1000 *M.moniliformis* eggs

c, a 1:1 mixture of 400-500 *H.diminuta* eggs plus 400-500 *M.moniliformis* eggs

The faecal pellets were presented to batches of insects that were housed in plastic boxes (approximately 280mm x 160mm x 90mm) and maintained at $28\pm1^{o}C$. The insects were allowed to feed on the faecal pellets *ad libitum*, replacing pellets as necessary, for 3 days. The insects were then transferred to clean enclosures and fed on rat diet and water *ad libitum*, and maintained at $28\pm1^{o}C$.

After 21 days post-feeding, the insects were anaesthetised with CO_2, legs, wings, head and elytra removed and then dissected in a small petri dish containing HBS. Parasites were gently flushed out of the body cavity with HBS jetted from a Pasteur pipette and were counted at 12-25x magnification using a Wild dissecting microscope.

This experiment was repeated 3 times using the eggs in faeces recovered from different rats in each case. In each repeat approximately 5 insects from each batch were retained for dissection at a later date to determine if normal parasitic development had taken place.

4.2.2.2 ORAL ADMINISTRATION OF PARASITE EGGS IN SUSPENSION

Three suspensions of parasite eggs in 60% sucrose were prepared using eggs obtained by the method described in section 3.2.4.1. Two consisted of *M.moniliformis* and *H.diminuta* at concentrations of $5x10^3$ ml^{-1} and one contained a 1:1 mixture of the two. Groups of *P.americana* and *S.gregaria* were fed by Oxford pipette (see Chapter 3 section 3.2.4.1) with doses of each

suspension as follows.

P.americana group 1 : 50μl of egg suspension containing approximately 250 *H.diminuta* eggs.

group 2 : 50μl of egg suspension containing approximately 250 *M.moniliformis* eggs.

group 3 : 50μl of egg suspension containing approximately 125 *H.diminuta* and 125 *M.moniliformis* eggs.

S.gregaria group 1 : 100μl of egg suspension containing approximately 500 *H.diminuta* eggs

group 2 : 100μl of egg suspension containing approximately 500 *M.moniliformis* eggs

group 3 : 100μl of egg suspension containing approximately 250 *H.diminuta* and 250 *M.moniliformis* eggs.

T.molitor was fed for 5 minutes on a drop of each suspension presented on Nescofilm.

In all the above groups, any insect refusing, or only taking a part of the dose was rejected at this stage. All the insects were then maintained at 28$\pm$1^{o}C for 21 days post-feeding. After this time the insects were CO_2 anaesthetised, dissected as described above, and counts made of any developing *H.diminuta* and *M.moniliformis* larvae found in the haemocoele.

This experiment was repeated 3 times using different batches of eggs in each case, and for each replicate approximately 5 insects were retained for dissection at a later date.

4.2.2.3 ORAL ADMINISTRATION OF PARASITE EGGS INCLUDING NON-VIABLE *HYMENOLEPIS DIMINUTA* EGGS

This experiment was designed to ascertain whether the decrease in

M.moniliformis development (as found in sections 4.3.1 and 4.3.2) was either a consequence of dilution with the solution of *H.diminuta* eggs or of action by the live parasites.

Three suspensions of parasite eggs were prepared in 60% sucrose as follows, one containing only *M.moniliformis* eggs at $5x10^3$ ml^{-1}, the second with a 1:1 mixture of *H.diminuta* and *M.moniliformis* as in section 4.2.2.2 and the third a 1:1 mixture of *M.moniliformis* and non-viable *H.diminuta*

In the first two experimental repeats, *H.diminuta* eggs were rendered non-viable by heating them to 80°C for 10 minutes before mixing them with the *M.moniliformis* eggs. Non-viability was confirmed by attempting to hatch the eggs *in vitro* where although mechanical disruption plus the action of amylase caused passive hatching of many of the eggs, the larvae remained immobile within the embryophore. In later experiments, immature eggs of *H.diminuta* were used as a non-viable control in place of the heat killed eggs. These immature eggs were teased out of proglottids near the middle of the strobila of adult worms. They appeared more ovate than the mature eggs and the oncospheres were not fully developed. On attempting to hatch them *in vitro* immature oncospheres were released passively from the eggs but remained non-motile.

Each group of *P.americana* was fed using the same pipetting method described in Chapter 3,(section 3.2.4.1) and was then housed in plastic boxes and maintained at 28±1°C for 21 days by which time *M.moniliformis* had developed to stage I acanthellae. Rat diet cake and water were provided *ad libitum*

After this time the insects were CO_2 anaesthetised and examined for the presence of parasites as above.

3 insects from each group were dissected earlier, at 8 and 12 hours after infection, for histological examination of their midguts, see below.

4.2.2.4 HISTOLOGICAL AND ELECTRON MICROSCOPICAL EXAMINATION OF THE INSECT GUT

Histology

Guts removed from insects in section 4.2.2.3 were attached to pieces of card 25mm x 15mm so that they would remain straight during fixing. They were then placed in a small dish of Bouin's fixative and left overnight. After fixing, the guts were removed from the cards and dehydrated in a series of alcohols up to 100% (using a Shandon 2L Processor Mk. II).

The guts were then embedded in paraffin wax and both longitudinal and transverse 8μm sections cut using a Leitz 1512 microtome.

Sections were stained with Mallory's triple stain and mounted on glass slides using Histomount (National Diagnostics).

Scanning electron microscopy

Insect gut with head and hindgut still attached were removed from insects treated as in section 4.2.2.3 and attached to pieces of card as above. They were then fixed in 2.5% glutaraldehyde in HBS (pH. 7.2) at 4^{o}C overnight.

After glutaraldehyde fixation, the guts were rinsed with HBS 3 times for 5 minute intervals, and post fixed in 2% osmium tetroxide in distilled water for 1 hour. They were rinsed twice in distilled water for 5 minutes, and dehydrated in a series of acetone rinses (30%, 50%, 70%, 90%) for 10 minutes at each concentration followed by 3 rinses in 100% acetone (molecular-seive dried). Final dehydration was by critical point drying (CPD, Polaron Equipment Ltd.) in liquid CO_2 for 1 hour. The temperature was raised to approximately 45^{o}C to evaporate the pressurised CO_2 and the CPD then decompressed slowly over 15 minutes. Specimens were mounted on aluminium stubs using double sided adhesive tape then gold coated using a Polaron gold coater. The specimens were viewed using a Phillips 500 SEM.

Hatched oncospheres and acanthor larvae (see section 3.2.3 for hatching

proceedure) were also processed for SEM using the above protocol. They were enclosed in perforated cylinders throughout the fixing and dehydration processes to prevent loss (Figures 1.2 and 1.3).

4.3 RESULTS

4.3.1 ADMINISTRATION OF MIXED DOSES OF EGGS IN RAT FAECES

Table 4.1 and Figures 4.1-4.3 show the mean number of parasites recovered from each group of insects. There were no *H.diminuta* cysticercoids recovered from cockroaches.

M.moniliformis (stage I acanthellae) were found in the haemocoele of *P.americana*, with no sign of gut penetration by *H.diminuta* when the external surface of the midgut was examined. There is a significant difference (Student's t-test, $p < 0.05$) between the mean number of *M.moniliformis* larvae recovered from the homologous infection and the heterologous infection with *H.diminuta* in replicate 2; in all cases mean numbers of *M.moniliformis* are lower in the heterologous infections. The standard deviations from the means are high in all cases, reflecting the wide range in numbers of parasites recovered in each group (Figure 4.1)

H.diminuta developed, as expected, in the flour beetle *T.molitor* (Figure 4.2), again there was a wide range in the numbers of parasites recovered. There was no statistical difference between the numbers of *H.diminuta* in the homologous and heterologous infections.

The infections of flour beetles with *M.moniliformis* were of limited success. In most cases the developing larvae appeared malformed or partially covered in haemocytes and not as advanced as the *M.moniliformis* of the same age found in *P.americana* (ie. they appeared as malformed stage I acanthellae).

No *M.moniliformis* were found to develop beyond stage II acanthella when left to develop for a further period of time.

Successful infections of locusts with *H.diminuta* and *M.moniliformis* (Figure 4.3) reflect the results of the *in vitro* gut penetration experiments in the previous chapter. Both species of parasite infect their natural hosts as expected, but only the locust, *S.gregaria*, seems to be able to act as host to both simultaneously. In *S.gregaria*, both species of parasite were found in the haemocoele, although only at very low prevalence and intensity of infection. The larvae of both parasite species were less developmentally advanced than those of the same age found in their natural hosts. The cysticercoids of *H.diminuta* were mainly at stage 5 in all the locusts but several were only found to have developed as far as stage 3. Similarly many of the *M.moniliformis* larvae had only reached the first acanthella stage compared to stage II larvae in controls.

4.3.2 ORAL ADMINISTRATION OF PARASITE EGGS IN SUSPENSION

The results are given in Table 4.2 and Figures 4.4-4.6.

As in the previous experiment, only *M.moniliformis* (stage I acanthellae) were found in the haemocoele of *P.americana*, with no signs of *H.diminuta* larvae having penetrated the midgut wall.. In this experiment the difference between numbers of acanthellae in the homologous and heterologous infections of cockroaches was even greater than with eggs administered in faeces (Figure 4.4). Notably fewer larvae were present in the heterologous infection than in controls (Student's t-test, significant at $p<0.01$ for replicate 3, although the other replicates were not statistically significantly different, $p>0.05$). There was also a visible increase in the numbers of encapsulated and melanised *M.moniliformis* larvae found on the haemocoelic surface of the gut in the cockroaches fed with both species of parasite.

H.diminuta and *M.moniliformis* were found in the flour beetles (Figure

4.5), although *M.moniliformis* was again malformed and less mature (less than stage 1 acanthella) than larvae present in *P.americana*. Some of the beetles were retained until 4 weeks post feeding, and when dissected the *M.moniliformis* larvae present in the haemocoele appeared to be partially melanised and had not developed much further than those found in 21 days post infection.

In *S.gregaria* (Figure 4.6) both species of parasite were found in the haemocoele but only at relatively low prevalence and intensity of infection in relation to the size of the dose given. Numbers of *M.moniliformis* in the heterologous infection were slightly higher although not statistically different ($p>0.05$) from those found in the homologous infections.

4.3.3 FURTHER INVESTIGATIONS INTO THE EFFECT ON INTENSITY OF *MONILIFORMIS MONILIFORMIS* INFECTIONS BY *HYMENOLEPIS DIMINUTA*

The results are presented in Table 4.3 and Figure 4.7.

The results clearly show the surprising effect that *H.diminuta* has on the numbers of *M.moniliformis* developing in concurrent oral infections. Only a small fraction of the expected numbers of *M.moniliformis* developed when administered along with *H.diminuta* (Student's t-test $p<0.001$ level in all cases), and microscopic examination of the haemocoelic surface of the cockroach midgut revealed elevated numbers of encapsulated and melanised acanthors compared to those in control insects. However, the numbers of *M.moniliformis* which developed in the homologous infections were not statistically different from the heterologous control infections where non-viable *H.diminuta* were included in the dose. The numbers of *M.moniliformis* eggs in the heterologous infection were diluted to half the original concentration when mixed with the suspension of *H.diminuta* eggs. The results show that this does not appear to affect the numbers of *M.moniliformis*

developing in the control insects (with the non-viable *H.diminuta*) and therefore the reduction found in the heterologous infection is probably caused by the live oncospheres.

Throughout this chapter the mean numbers of larvae developing between individual batches of eggs vary . This is assumed to be due to natural variation in the batches of parasite eggs obtained from different rats and the variable susceptibility of the insect hosts.

4.3.4 HISTOLOGICAL AND SEM EXAMINATION OF THE INSECT GUT FOR EVIDENCE OF PARASITIC WOUNDING

See Figure 4.8.

Eggs and some hatched oncospheres were found in the guts at 8 and 12 hours post-feeding. Figure 4.8 shows a transverse section of the midgut of *P.americana* with *H.diminuta* eggs and hatched oncospheres at 8 hours post-feeding. However, the only oncospheres found were those in the gut lumen surrounded by the peritrophic membrane. There was no evidence from the histological sections that oncospheres had made contact with or penetrated the midgut wall.

SEM studies of the haemocoelic surface of the gut also failed to reveal signs of damage on the cockroach gut possibly caused by oncospheres (See Figure 3.12.1 for electron micrograph of the haemocoelic surface of *T.molitor* midgut).

TABLE 4.1 ORAL ADMINISTRATION OF EGGS IN FAECAL PELLETS

Insect species	Exp'tl repeat	Mean number of Parasites (± sd) Homologous infections *Hymenolepis* $\bar{x}$	*Hymenolepis* sd	*Moniliformis* $\bar{x}$	*Moniliformis* sd	Heterologous infections *Hymenolepis* $\bar{x}$	*Hymenolepis* sd	*Moniliformis* $\bar{x}$	*Moniliformis* sd	N°. insects n
P.americana										
	1	0.00	0.00	10.00	8.17	0.00	0.00	3.50	6.23	6
	2	0.00	0.00	7.00	6.71	0.00	0.00	1.71	1.81	7
	3	0.00	0.00	11.57	15.12	0.00	0.00	5.00	6.95	7
T.molitor *										
	1	2.14	2.34	0.43	0.79	2.00	2.83	0.14	0.38	7
	2	2.43	2.88	0.00	0.00	2.71	3.68	0.14	0.38	7
	3	3.86	4.38	2.14	5.24	4.29	6.47	0.00	0.00	7
S.gregaria										
	1	1.00	1.53	0.57	1.13	1.86	2.54	2.14	2.85	7
	2	1.43	2.30	1.57	1.51	2.29	3.68	2.86	3.08	7

* = *M.moniliformis* were found to develop as far as stage II acanthellae in *T.molitor*

TABLE 4.2 ORALLY ADMINISTERED EGGS IN SUSPENSION

Insect species	Exp'tl - repeat	Mean number of Parasites (± sd) Homologous infections Hymenolepis $\bar{x}$	Hymenolepis sd	Homologous infections Moniliformis $\bar{x}$	Moniliformis sd	Heterologous infections Hymenolepis $\bar{x}$	Hymenolepis sd	Heterologous infections Moniliformis $\bar{x}$	Moniliformis sd	N°. insects n
P.americana										
	1	0.00	0.00	12.63	12.24	0.00	0.00	3.00	5.81	8
	2	0.00	0.00	17.71	23.14	0.00	0.00	5.00	6.45	7
	3	0.00	0.00	22.14	18.40	0.00	0.00	1.29	1.38	7
T.molitor *										
	1	4.29	4.68	0.00	0.00	6.43	7.07	0.29	0.76	7
	2	8.71	7.74	0.14	0.38	5.86	6.09	0.00	0.00	7
	3	4.29	4.15	0.29	0.49	7.14	6.49	0.29	0.76	7
S.gregaria										
	1	2.63	2.50	10.00	18.43	2.50	4.38	4.25	5.39	8
	2	5.57	5.97	1.57	2.82	0.43	0.79	3.29	4.57	7
	3	1.86	3.34	1.86	3.18	2.00	2.65	1.29	1.70	7

* = *M.moniliformis* were found to develop as far as stage II acanthella in *T.molitor*.

TABLE 4.3 EFFECTS OF *HYMENOLEPIS DIMINUTA* ON THE DEVELOPMENT OF *MONILIFORMIS MONILIFORMIS* IN CONCURRENT ORAL INFECTIONS OF *PERIPLANETA AMERICANA*

Experimental repeats		Mean number of *M.moniliformis* larvae in :- *Moniliformis* only	*Moniliformis* + *Hymenolepis*	*Moniliformis* + NV *Hymenolepis*
1	mean	44.29	5.75	*37.00
	sd	24.64	5.99	22.00
2	mean	29.70	3.60	*19.09
	sd	34.25	9.19	20.59
3	mean	12.78	2.40	#12.63
	sd	7.19	4.01	7.96
4	mean	39.75	6.70	#55.80
	sd	28.73	6.48	27.35
5	mean	33.56	3.40	#34.88
	sd	23.61	4.27	28.49

* = Heat killed *Hymenolepis*
# = Immature *Hymenolepis*

NV = Non-viable

Figure 4.1 **Simultaneous infections of *Periplaneta americana* with *Hymenolepis diminuta* and *Moniliformis moniliformis* in rat faecal material.**

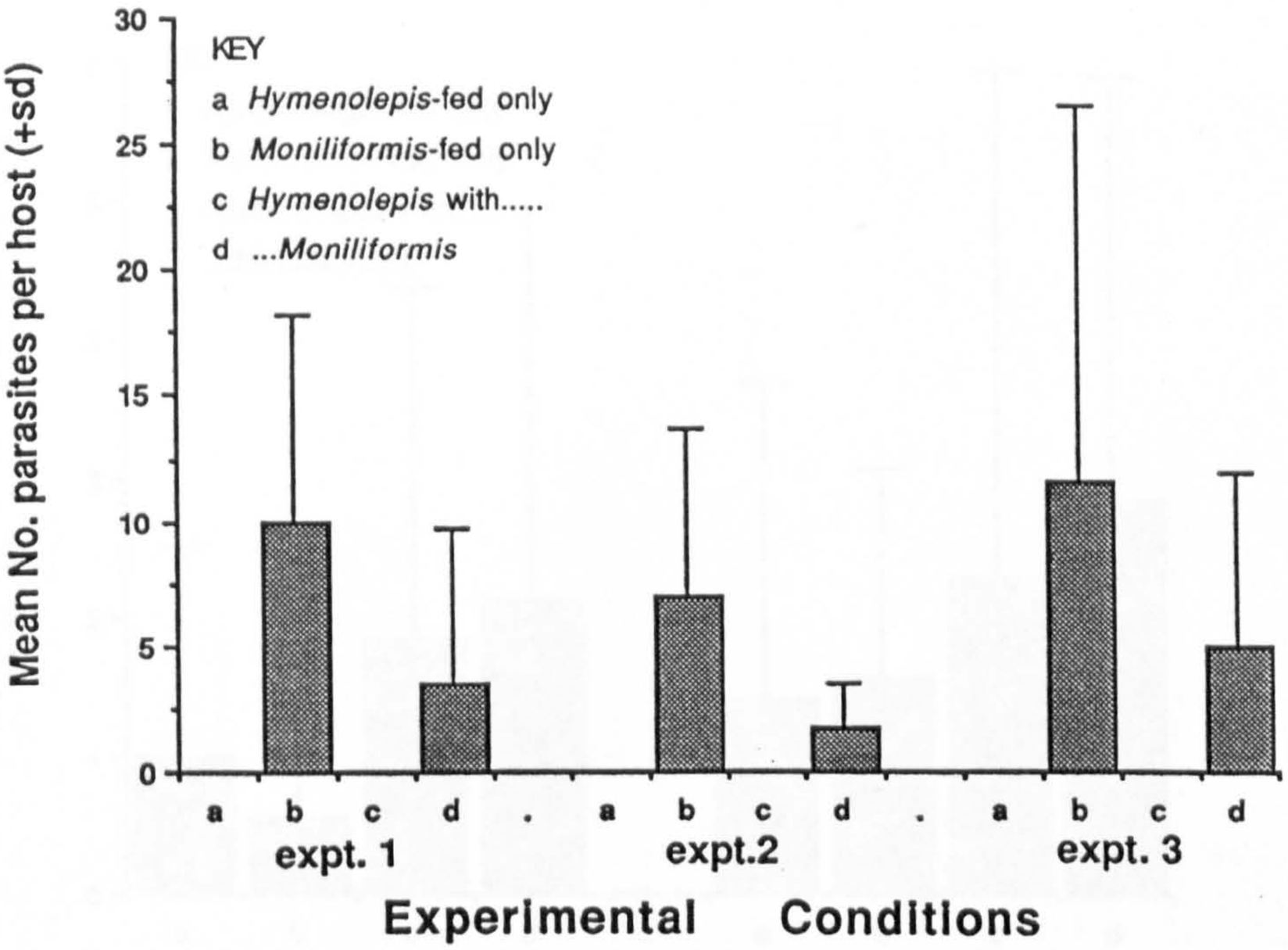


Figure 4.2 **Simultaneous infections of *Tenebrio molitor* with *Hymenolepis diminuta* and *Moniliformis moniliformis* in rat faecal material**

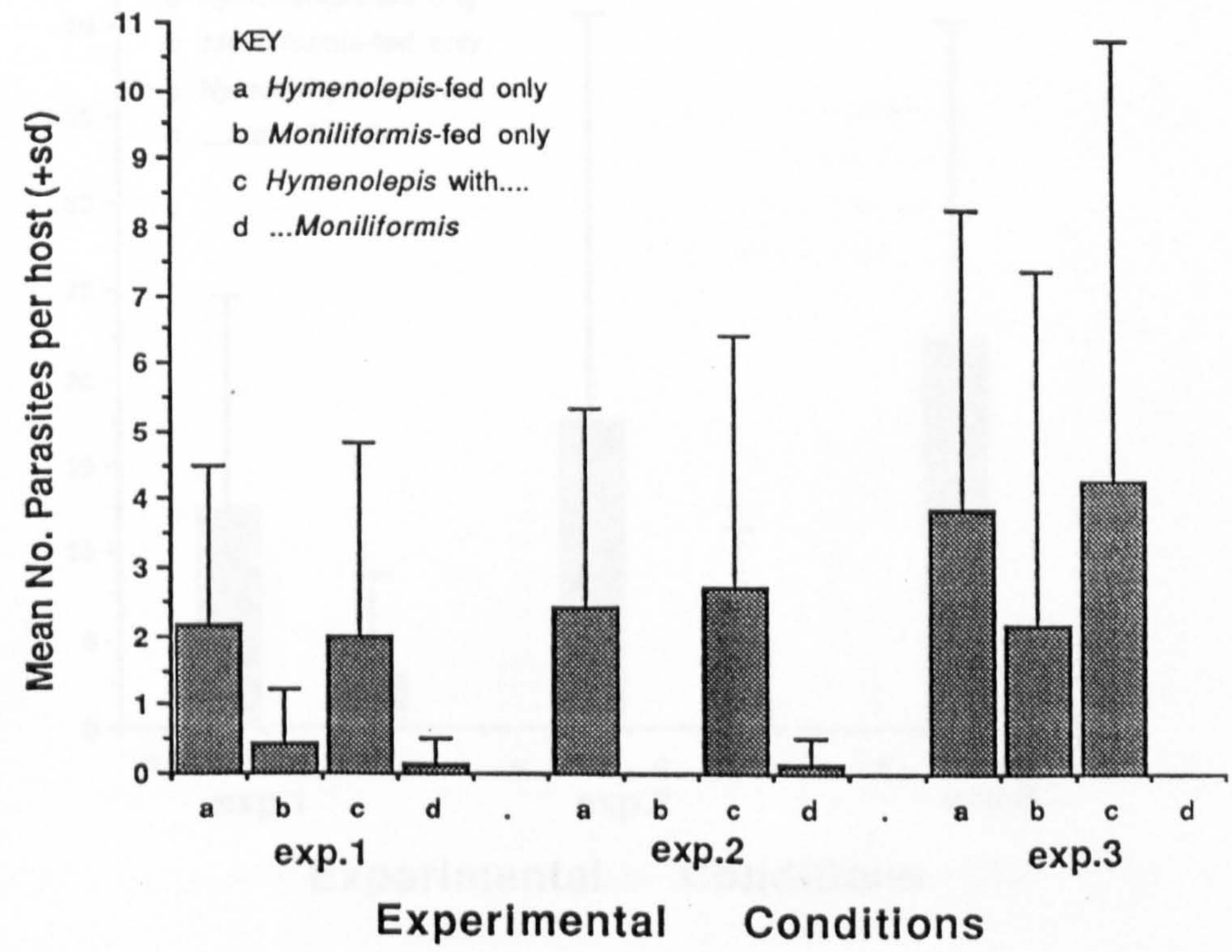


Figure 4.3 Simultaneous infections of *Schistocerca gregaria* with *Hymenolepis diminuta* and *Moniliformis moniliformis* in rat faecal material.

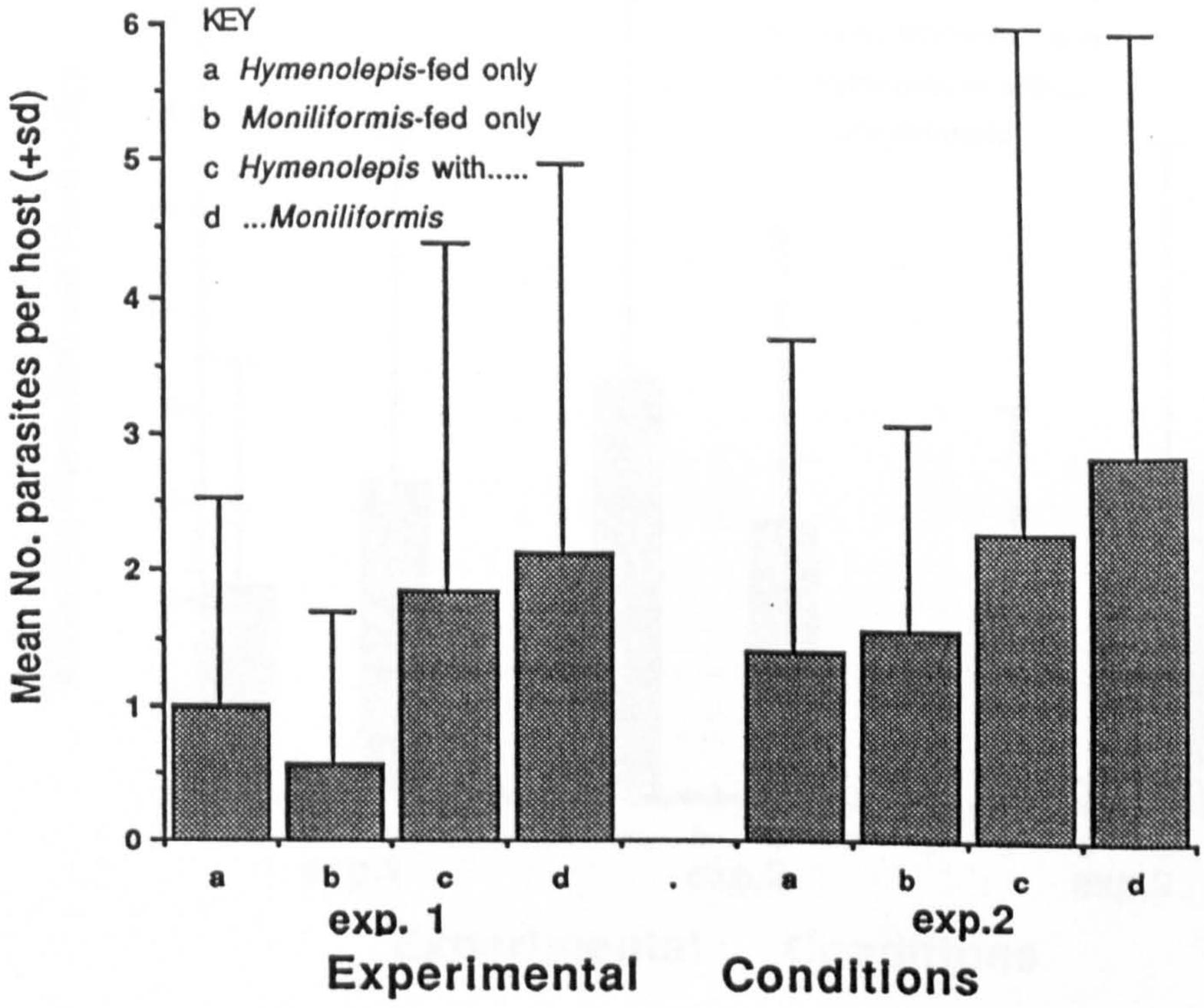


Figure 4.4 Simultaneous infections of *Periplaneta americana* with *Hymenolepis diminuta* and *Moniliformis moniliformis* in sucrose solution.

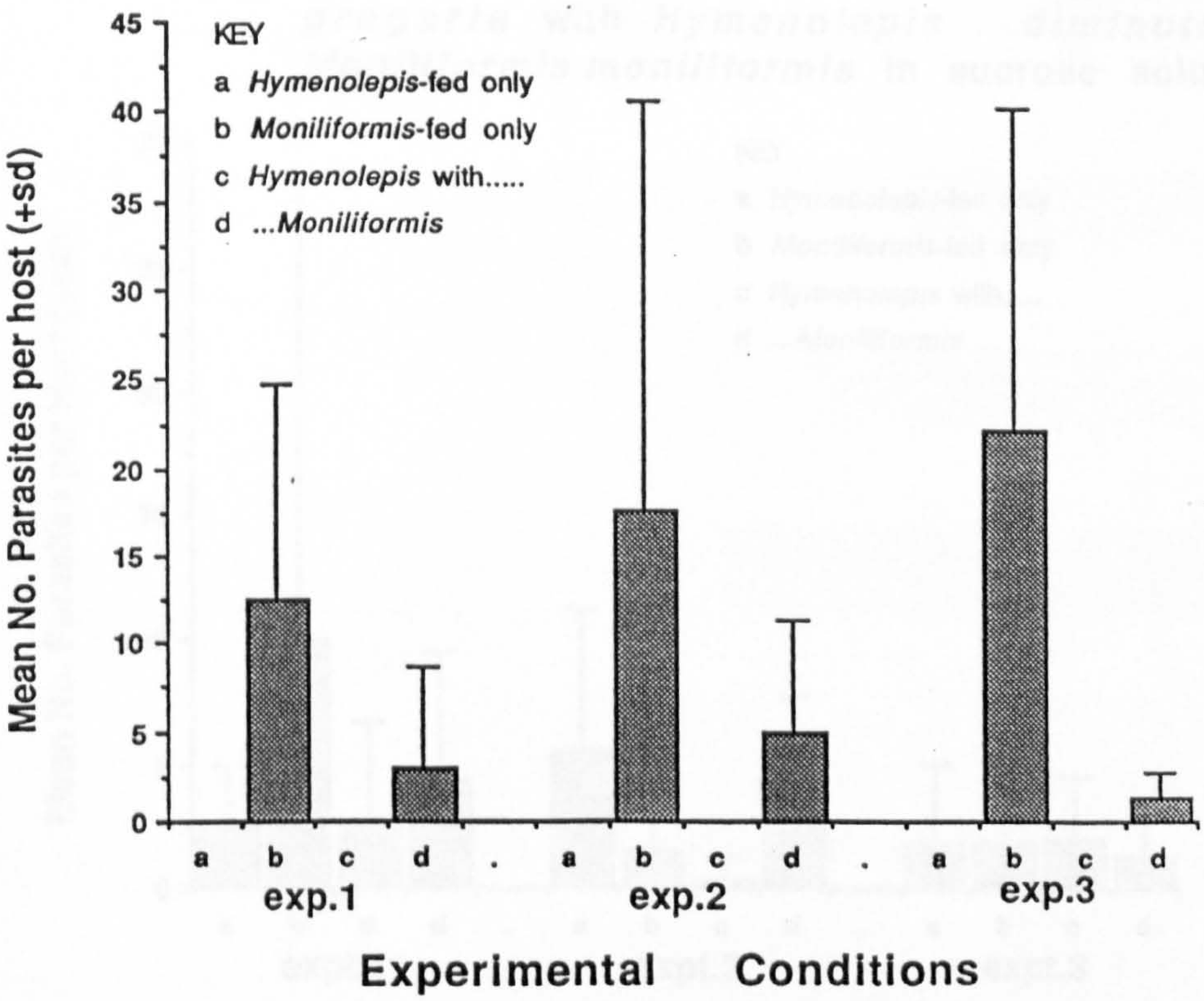


Figure 4.5 **Simultaneous infections of *Tenebrio molitor* with *Hymenolepis diminuta* and *Moniliformis moniliformis* in sucrose solution.**

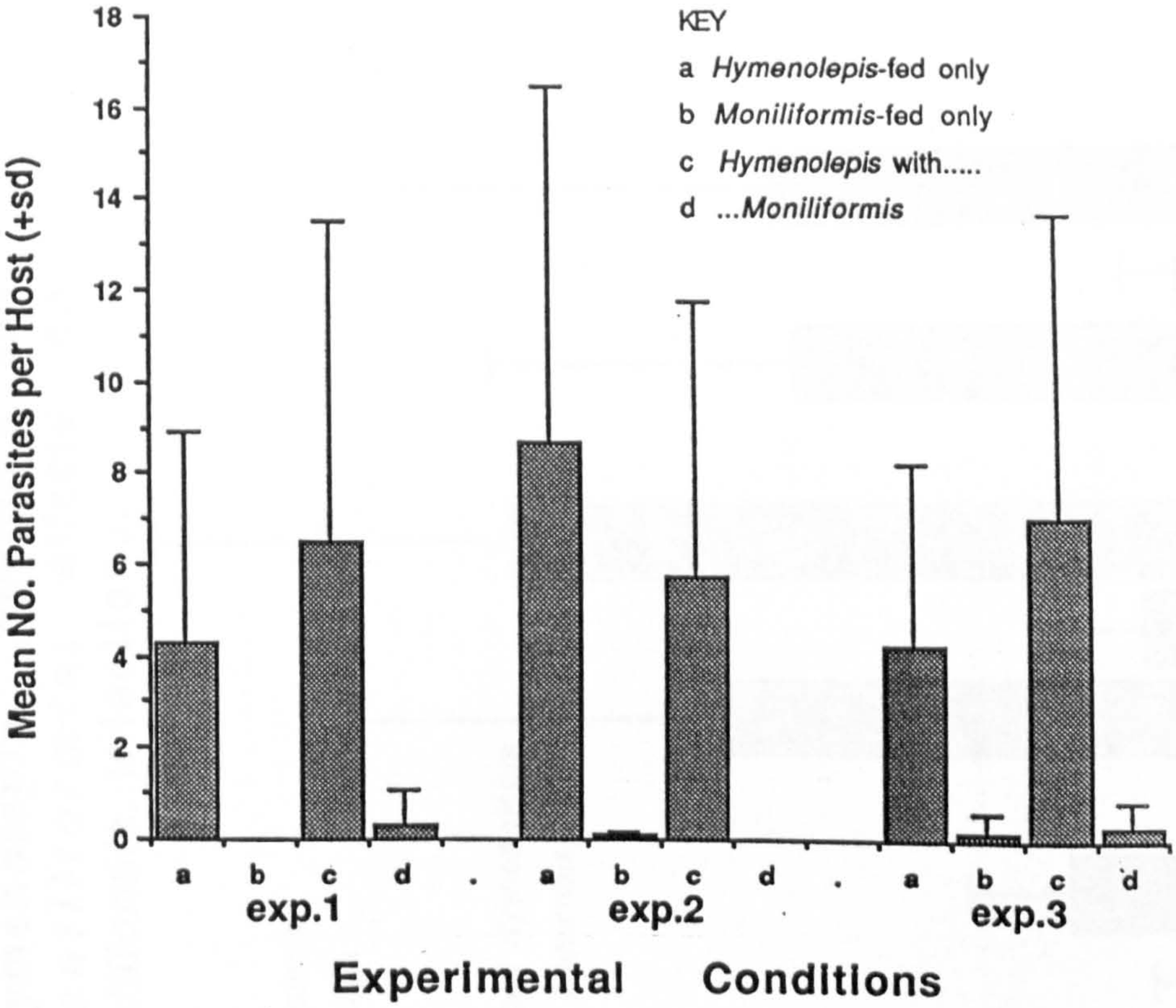


Figure 4.6 **Simultaneous infections of *Schistocerca gregaria* with *Hymenolepis diminuta* and *Moniliformis moniliformis* in sucrose solution.**

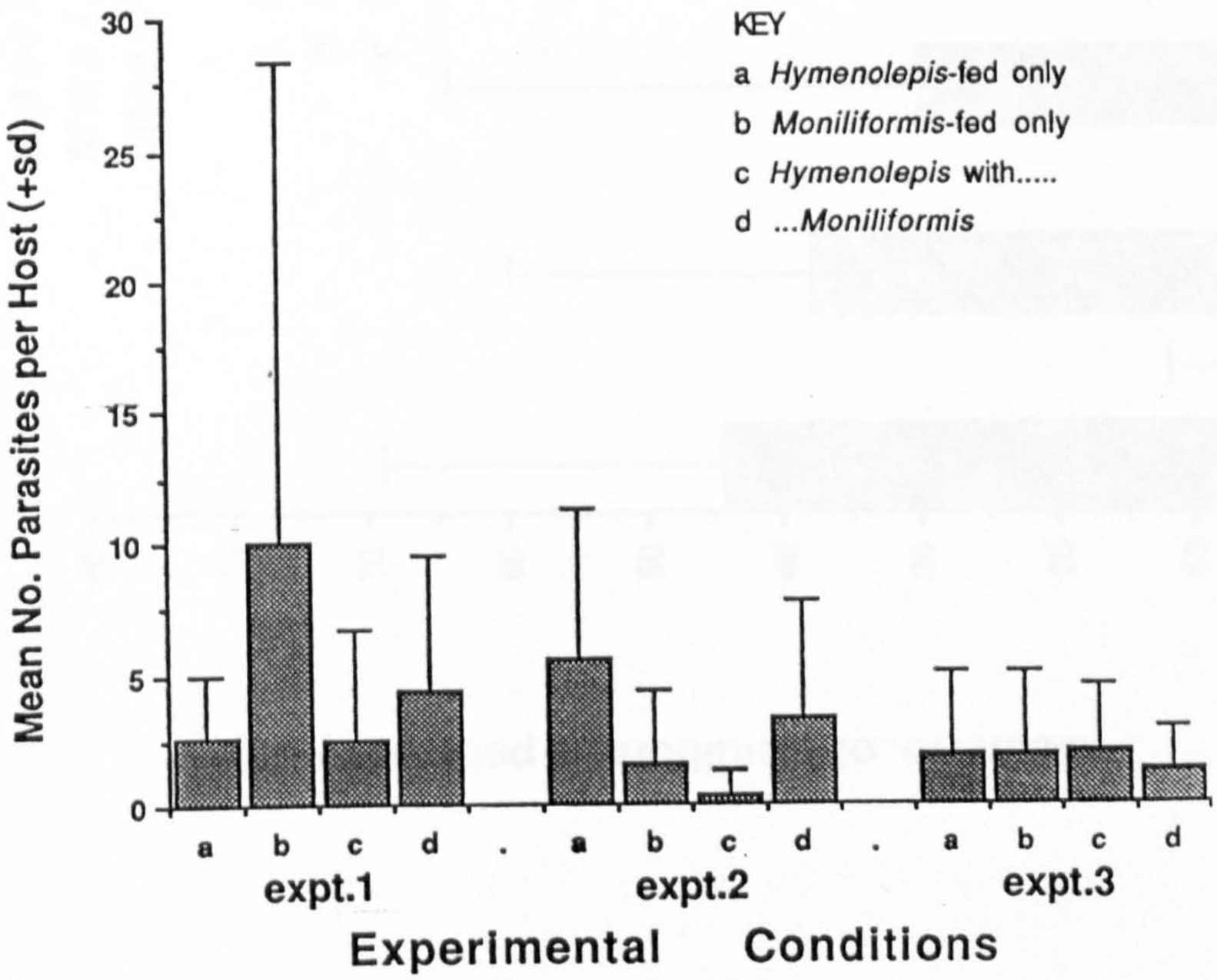


Figure 4.7 **Simultaneous infection of *Periplaneta americana* with *Hymenolepis diminuta* and *Moniliformis moniliformis*: effects on intensity of *M. moniliformis* infections.**

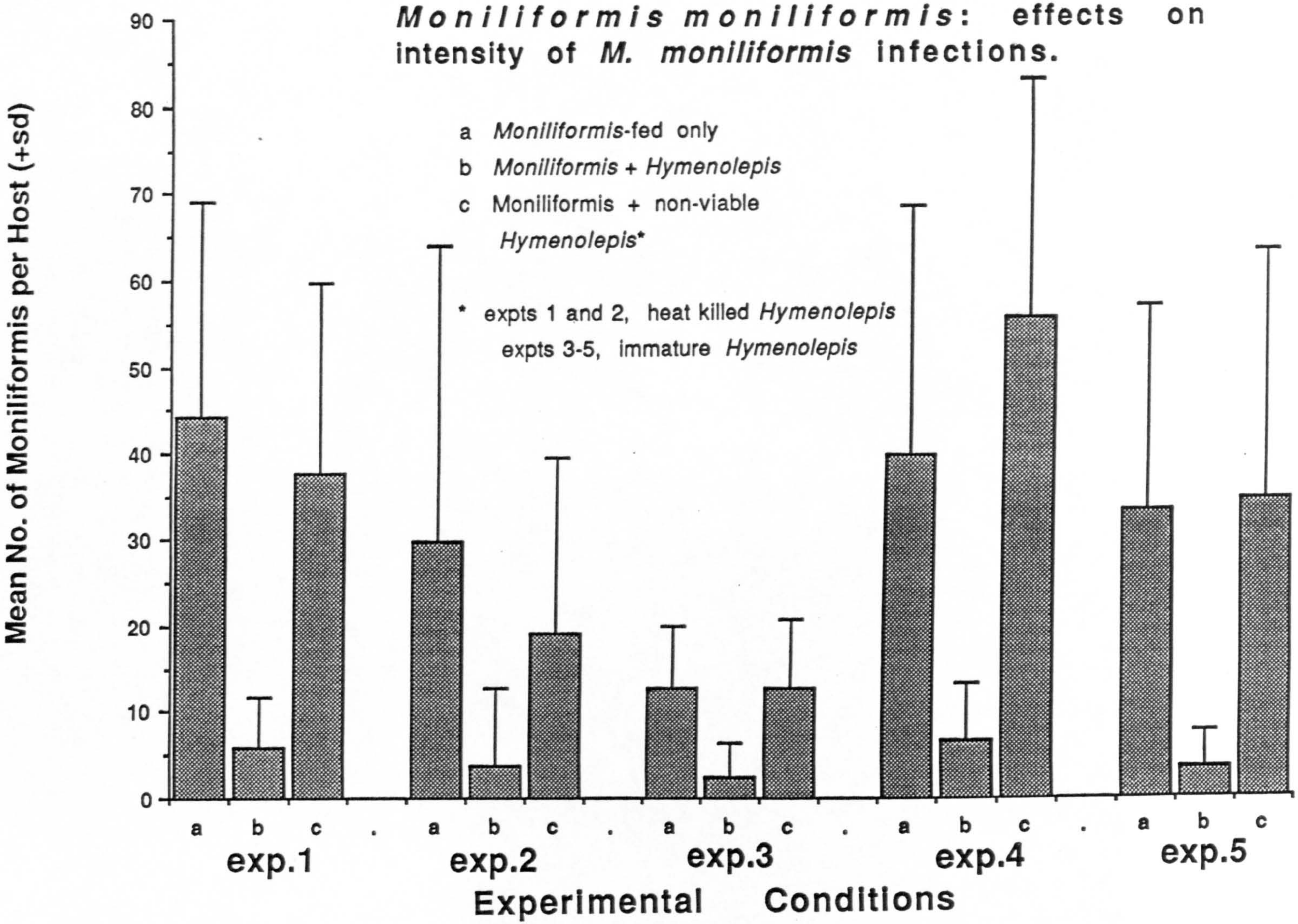
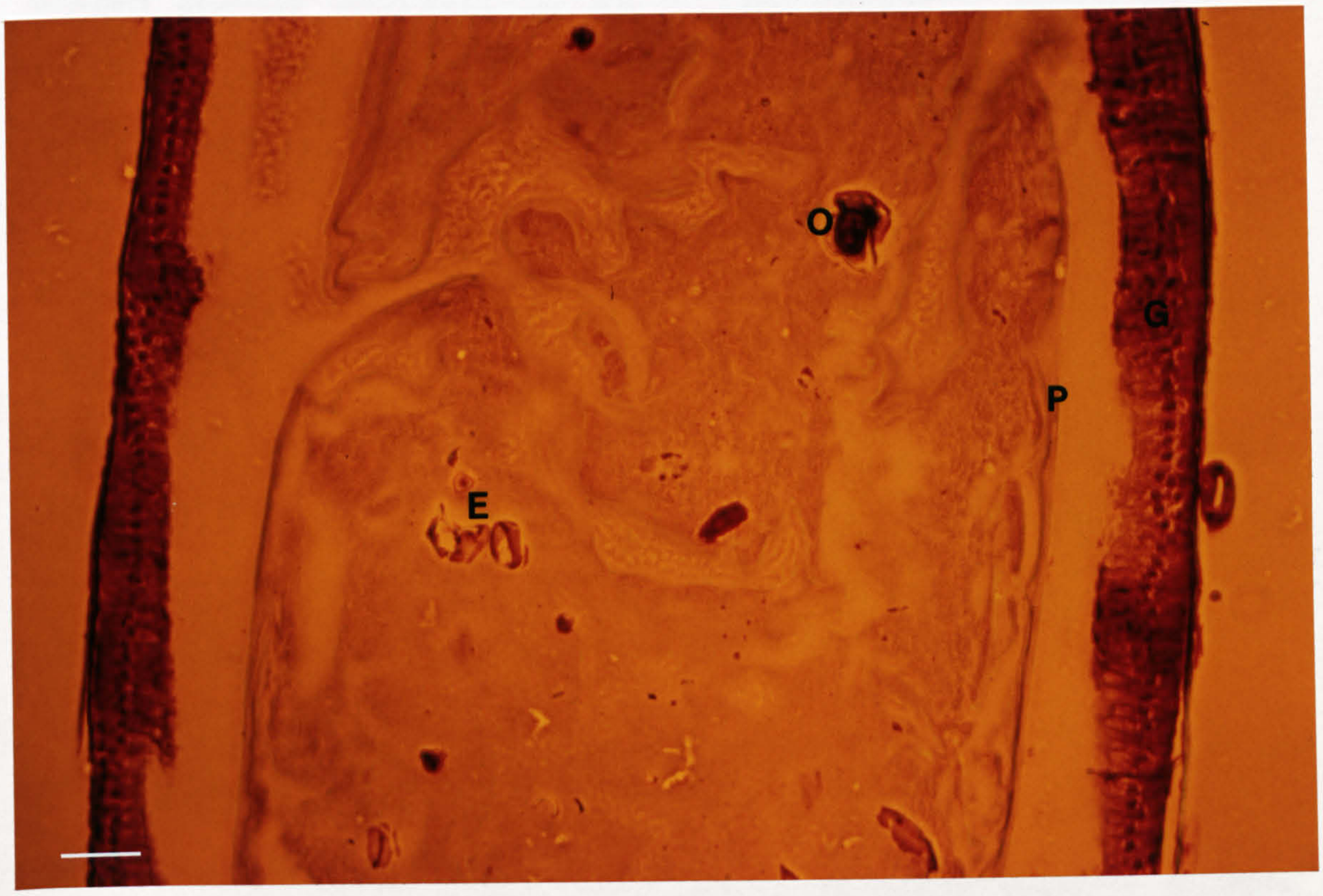


FIGURE 4.8 TRANSVERSE SECTION OF *PERIPLANETA AMERICANA* GUT CONTAINING EGGS AND ONCOSPHERE LARVAE OF *HYMENOLEPIS DIMINUTA* (8 HOURS POST-FEEDING)



E = *Hymenolepis* egg shells

O = Hatched oncospheres

G = gut wall

P = peritrophic membrane

Scale bar = 0.25mm

4.4 DISCUSSION

An attempt was made to find a species of insect which could be orally infected with *H.diminuta* and *M.moniliformis* simultaneously. Locusts were shown to become infected with both parasites after ingesting the eggs of both parasites, but only a small proportion of the large doses of eggs administered (see sections 4.3.1 and 4.3.2) was found to develop, reflecting the low numbers observed penetrating the gut *in vitro* (Chapter 3, sections 3.3.4 and 3.3.5). However once in the haemocoele, both parasites species develop normally, although a little slower than in their normal hosts.

Lethbridge (1971c), found similar results where oral infections of locusts with *H.diminuta* resulted in only very few of the larvae developing, but when prehatched oncospheres were injected directly into the haemocoele, large numbers developed. Lethbridge did not ascertain why orally administered *H.diminuta* failed to infect locusts in large numbers, but it seems likely that the gut presents a major barrier to oral infection which was by-passed by the intrahaemocoelic injection.

In the literature there is little information presented on locusts acting as intermediate hosts for acanthocephalans. Moore (1962) described the life cycle of *Mediorhyncus grandis*, of which the larvae infected crickets, grasshoppers and locusts including *Schistocerca americana* under experimental conditions, but were melanised after a short period in beetles. The development of *M.grandis* in the haemocoele of its host was found to be similar to that of *M.moniliformis*, where the mature cystacanth forms a membranous envelope totally enclosing the parasite.

The development of *M.moniliformis* in the locust *S.gregaria* appears to follow the same pattern as in *P.americana*, although in this study it seems to be a little slower at the same temperature. At 27°C cystacanth development is completed in approximately 5-8 weeks in *P.americana* (King, 1955; King and Robinson, 1967). In initial experiments, the remaining life span of the adult

locusts infected with *M.moniliformis* was too short for the parasite to develop to the cystacanth stage. However, when newly-emerged final instar locusts were infected they survived long enough for cystacanths to develop fully. Preliminary experiments using third instar to pre-adult instar locusts showed that they could be orally infected and the parasites would survive through the processes of successive moults.

Lackie (1976, 1979, 1981, 1986b, c, e), working on a range of insects including locusts and cockroaches has shown that the immune system of *S.gregaria* will accept as "self" a wide range of xenografted tissues and parasites and will only weakly encapsulate certain abiotic implants. Therefore it is not surprising that once *M.moniliformis* acanthors reach the haemocoele, there appears to be little or no immune response mounted by the locust to prevent the parasites from developing normally.

Due to the very low prevalence and intensity of *M.moniliformis* and *H.diminuta* in oral infections of *S.gregaria*, even when the dose is very concentrated, the locust would probably be an unsuitable host in a natural situation. This alone, apart from the differences in geographical distribution and availability of final hosts would probably result in the parasites being below threshold levels to survive within the locust population (Anderson, 1986; Anderson and May, 1978).

In the initial stages of planning the experiments in this chapter, it was thought that a concurrent infection of *H.diminuta* and *M.moniliformis* might lead to the success of both parasites in the cockroach (see section 4.1). However, a different and more interesting result with respect to the immune system of the host has been found. When the two species of parasite are administered together, not only are the *H.diminuta* unsuccessful but there is also a significant reduction in the numbers of *M.moniliformis*.

Initially this was thought to be a consequence of diluting the *M.moniliformis* eggs by half when mixing them with the suspension of *H.diminuta* eggs, resulting in a smaller proportion of *M.moniliformis* reaching the

haemocoele. However, when diluted with non-viable *H.diminuta* eggs the reduction in *M.moniliformis* numbers was not seen and numbers of *M.moniliformis* comparable to homologous infections were recovered. It is worth noting that J.M. Lackie (1973) found that numbers of *M.moniliformis* developing after a single dose of eggs did not always correlate with numbers of eggs administered, and an increased dose did not necessarily result in an increase in the numbers of cystacanths recovered. Therefore it seems that the presence of viable, motile *H.diminuta* larvae in the gut of *P.americana* is in some way affecting the survival of *M.moniliformis*.

Gordon and Whitfield (1985) found that the presence of *R.cesticillus* in *Tribolium confusum* reduced the number of *H.diminuta* developing in challenge infections. The above authors could not explain these results in the terms of competition for space and nutrients and suggested that the immune system was possibly being stimulated by one of the parasites which led to the reduction in numbers of the other. Although in this case both parasites developed in the intermediate host, it is possible that a similar stimulation of the immune system by one parasite species is responsible for the reduction in numbers of *M.moniliformis* in the heterologous infections in this work.

The hypothesis put forward to explain the observed reduction in prevelence and intensity of *M.moniliformis* in heterologous "infections" is that *H.diminuta* oncospheres damage the midgut tissues of *P.americana* in their unsuccessful attempt to burrow through the gut wall, and therefore initiate a wound-healing response by the host's immune system. Once stimulated in this way the immune system becomes more sensitive to non-self (Lackie, 1986a) lowering the threshold at which immune recognition occurs so that penetrant acanthors are recognised as foreign and encapsulated. Normally acanthors are weakly encapsulated by the host's haemocytes when they reach the haemocoele, but lose this capsule after a short period of development (Rotheram and Crompton, 1972). However if the capsule was considerably thicker, which may

have been the case if the immune system was stimulated, the acanthors may not all be able to escape the covering of cells, resulting in high levels of mortality. Some of the penetrant acanthors are always encapsulated and melanised in this way, but in the two-species infections the number of melanised acanthors on the haemocoelic surface of the midgut appeared much higher. This supports the hypothesis that the immune system had been stimulated. Further investigations on the effect of parasitic infectin on haemocyte behaviour are discussed in Chapter 6.

From histological examination at the light microscope level of the epithelium of *P.americana* midgut, there is no evidence of tissue damage which could have been caused by oncospheres, although this is very difficult to assess at a cellular level using light microscopy; therefore this phenomenon cannot be explained at this stage. Examination of the internal surface of the gut by SEM may reveal more detail about the state of the cells in the lining of the midgut. Similar studies have been used to show that *Bacillus thuringiensis* Kurstaki HD-1 crystal endotoxin causes extensive, but temporary damage to the internal surfaces of the midgut lumen of *Manduca sexta* (Spies and Spence, 1985) but this has not been linked with the state of the immune system.

It has also been considered that unknown substances produced by the oncospheres of *H.diminuta* could possibly effect the immune system of the host. Penetration gland secretions, normally produced by the oncosphere during gut penetration (Lethbridge, 1971a; Lethbridge and Gijsbers, 1974), may damage the gut wall of the cockroach and stimulate the immune system without the oncospheres themselves making contact with the tissues. The nature of this stimulus, whether physical or chemical, would be an interesting topic for future studies.

4.5 SUMMARY

1. From the range of insects fed with eggs of both parasite species, only locusts became infected simultaneously with *H.diminuta* and *M.moniliformis*; only a very small proportion of those larvae fed to the locusts developed. As expected, both species of parasites were shown to penetrate the gut of and develop in their usual species of intermediate host.

2. Viable oncospheres fed to cockroaches, adversely affected the success of concurrently-fed *M.moniliformis* larvae. Large numbers of melanised and encapsulated objects were found which appeared to be the remains of penetrant acanthors adhering to the haemocoelic surface of the gut.

3. The hypothesis put forward to explain the observed reduction in prevelence and intensity of *M.moniliformis* in heterologous infections is that *H.diminuta* oncospheres damage the midgut tissues of *P.americana* in their unsuccessful attempt to burrow through the gut wall, and therefore initiate a wound-healing response by the host's immune system. This results in gut-penetrant larvae, *M.monilifomis* in this case, being encapsulated and melanised

4. Light and electron microscopy has shown little evidence of wounding in the gut, although penetrant oncospheres were not seen. Further studies of the internal surfaces of the midguts may reveal the source of the immune stimulation.

CHAPTER FIVE

HYMENOLEPIS DIMINUTA IN THE COCKROACH HOST AND THE EFFECT OF *MONILIFORMIS MONILIFORMIS* ON PARASITE SURVIVAL

5.1 INTRODUCTION

The gut barrier prevents *Hymenolepis diminuta* from infecting cockroaches by their normal route (Chapters 3 and 5), but if oncosphere larvae, hatched *in vitro*, are injected directly into the haemocoele of *Periplaneta americana* a very small proportion of the larvae survive and develop to the cysticercoid stage (Lackie, 1976). Similarly, Leger and Cavier (1970) experimentally infected the cockroach *Leucophaea maderae* with *Hymenolepis nana*. Evidently the physiological environment of the cockroach haemocoele is adequate for *Hymenolepis* to survive, but the immune system is capable of recognising and destroying the majority of injected parasites, which appear as small encapsulated and melanised objects adhering to the lining of the haemocoele.

Preliminary experiments (see Lackie, 1986d) demonstrated an increase in survival of *H.diminuta* after successive passages using cockroaches as intermediate hosts, and it was suggested that it might be possible to select for a strain of *H.diminuta* capable of infecting *P.americana*. However this would depend on whether this enhancement in survivorship was an inheritable factor and not an artefact of the procedures used. Also, to adapt easily to the new host, the parasite should not have to undergo extensive physiological and morphological changes. Schom *et al.* (1981) demonstrated changes in the adaptability of *Hymenolepis citelli* to its usual intermediate host *Tribolium confusum* after passaging through this system 16 times. The highly infective parasites were selected against in this case, as they tended to cause high mortality of the hosts, eventually leading to the general reduction of highly infective lines.

There are already several laboratory strains of *H.diminuta*, each originating from single worms. The Texas strain, derived from parasites maintained at the Rice Institute, Texas, U.S.A., has been maintained for more than 22 years in laboratory rats (*Rattus norvegicus*) and *T.confusum* and *Tenebrio molitor*, whereas the Japanese strain is more recent, having been

obtained from *Rattus rattus* in 1985 from Tokyo, Japan (Kino and Kennedy, 1987). Pappas and Leiby (1986) reviewed the origins and morphologies of these strains and found that each has distinct characteristics in regards to egg size, numbers of eggs produced and numbers and distribution of testes in the proglottids, but do not appear to differ in their infectivity to rats. Kino and Kennedy (1987) compared Japanese and Texas strains of *H.diminuta*, and concluded that Texas strain was better adapted to *Rattus norvegicus* in which the Japanese strain only produced small worms and fewer eggs. Infectivity to the intermediate host was also investigated, where each parasite seemed to be better adapted to living in a particular species of beetle, ie. Japanese strain were more successful in *Tribolium confusum* than Texas strain, but were less so in *Tenebrio molitor.*

The first section of this chapter deals with attempts to select a strain of *H.diminuta* infective to cockroaches for use in the study of host-parasite immune interactions. It was intended that, if this programme were successful the survivorship of the resulting strain could be compared with that of normal strains. Also if this cockroach-adapted strain changed the host environment in some way, perhaps by immunosuppression, it may confer some protection on normal-strain *H.diminuta* injected into the same cockroach haemocoele.

Two ways of attempting to increase the ability of *H.diminuta* to survive in *P.americana* (which are the main aims of this chapter) are:-

1. Increase the infectivity of the parasite by selective breeding, or

2, reduce the immunological capability of the host by immunosuppression. This may be possible through the use of a second parasite species capable of surviving as a concomitant infection in this particular host.

It has also been suggested that a *H.diminuta*-susceptible strain of cockroach could be raised to fulfill the same purpose in this work as that of an infective strain of parasite. However this would seem impractical

because of the time scale involved for selecting for infectable cockroaches.

Preliminary studies by Lackie (Lackie and Holt, 1988) have shown that the haemocytes of *M.moniliformis* parasitised *P.americana* were less able to phagocytose latex beads (0.2μm diameter, Polyscience, U.K.) than non-parasitised animals which may be regarded as evidence of immunosuppression. Because rats can be infected with both *M.moniliformis* and *H.diminuta* (Chapter 2), it would be interesting to determine if pre-existent or concomitant infections of *M.moniliformis* would lead to an increase in *H.diminuta* establishment.

5.2 MATERIALS AND METHODS

5.2.1 INSECTS

5.2.1.1 COCKROACHES

Periplaneta americana, *Periplaneta australasiae*, *Blatta orientalis* and *Blatella germanica* were bred and maintained in the department's insectary at 28$\pm$1^{o}C, and fed on rat diet and water *ad libitum* with an occasional supplement of fruit and wheatgerm as described in section 2.2.1.3.

5.2.1.2 LOCUSTS

Schistocerca gregaria were obtained from Bioserv and maintained as described in section 3.2.1.

5.2.2 PARASITES

H.diminuta was maintained in Wistar rats and *T.confusum* as described in section 2.2.1.4. In the first series of experiments Japanese, Texas and Exeter strains of *H.diminuta* were used. (See sections 2.2.1.4 and 5.1 for origin of parasites).

M.moniliformis (Texas strain) was maintained in Wistar strain rats and cockroaches as described in section 2.2.1.4.

5.2.3 MEDIA AND SALINES

Cockroach cell culture medium (CM) was that developed and described as Huxham and Lackie's medium in Crompton and Lassiere (1987). Medium was supplemented with heat inactivated foetal calf serum (FCS) (Flow) at 10% (vol/vol).

HEPES-buffered balanced salt solution, pH 7.2 (HBS) was that developed for use with cockroach haemocytes (Huxham and Lackie, 1986).

5.2.4 EXPERIMENTAL PROCEDURES

5.2.4.1 SELECTION OF A STRAIN OF *HYMENOLEPIS DIMINUTA* INFECTIVE TO *PERIPLANETA AMERICANA*

A preliminary experiment was carried out to determine which of the 3 laboratory strain of *H.diminuta* were most infective to cockroaches. Infections were made by injecting hatched larvae directly into the haemocoele as follows:-

In vitro hatching

Eggs were mechanically cracked by agitating them with glass beads for 30 seconds, and the oncospheres were hatched out in 0.75% α-amylase in HBS at 28°C in approximately 15 minutes (Lackie, 1976). Hatched larvae were washed in HBS, concentrated by centrifugation at 250g for 3 minutes, filtered through 50μmnylon mesh (Nitex, Plastoc Associates U.K.) to remove unhatched eggs and broken egg-shells, then washed twice more before being resuspended at $5x10^4$ ml^{-1}. 10μl of this suspension containing approximately 500 oncospheres was injected, using a microsyringe, ventrally into the abdominal haemocoele of CO_2-anaesthetised cockroaches. Each insect was swabbed around the site of

injection beforehand with 70% ethanol.

To monitor the viability of each batch of *H.diminuta* eggs used, oncospheres were also injected at a lower dose (10μl HBS containing approximately 250 oncospheres) into *S.gregaria* (Lethbridge, 1971c).

All insects were housed in plastic boxes and kept at 28±1°C with food and water provided *ad libitum*

Dissection of insects and recovery of parasites

At about 5 to 8 days post infection, locusts were dissected to assess the development of the parasites, and when the majority were found to have reached stage 5 (Voge and Heyneman, 1957) the batches of cockroaches were dissected. Insects were anaesthetised with CO_2, their heads, legs and wings removed and the body cavity opened by a circumlateral incision. In a small petri dish of HBS the gut was carefully removed from the body and discarded, ensuring any parasites adhering to the surface were dislodged beforehand. The thoracic musculature, in particular that of the coxae, was teased apart with fine forceps to remove any parasites.

In the first experiment a comparison of the infectivity of the different strains of *H.diminuta* to cockroaches was made by injecting a known dose of each into batches of *P.americana*. After 8 days the insects were dissected as above and counts made of the parasites recovered. This was repeated three times using different batches of eggs in each case.

Once it had been determined which of the three strains of *H.diminuta* was most infective by intrahaemocoelic injection to cockroaches, it was passaged through the cockroach / rat cycle in an attempt to enhance the infectivity of the strain. Numbers of parasites recovered from each cockroach were recorded, and stage 5 cysticercoids intubated in groups of 5-10 into rats. After approximately 3 weeks, rats were killed by cervical dislocation and the small intestine dissected to remove the tapeworms. Eggs from these worms were then hatched and injected into cockroaches as described above to continue the

passaging process. Similarly a few cysticercoids were passaged from locust to rat to compare the progress of the infection.

5.2.4.2 EFFECT OF *MONILIFORMIS MONILIFORMIS* ON THE SURVIVAL OF *HYMENOLEPIS DIMINUTA* WITHIN THE COCKROACH HAEMOCOELE

In order to obtain hosts containing a standard number of *M.moniliformis* larvae, donor cockroaches that had been infected orally were dissected at approximately 2 weeks post-infection and stage I-II acanthellae (King and Robinson, 1967) were collected.

These larvae were washed twice in HBS, then washed in CM + 10% FCS. Naive recipient *P.americana* (adult females), anaesthetised with CO_2, were each given 40 larvae in 10μl CM + 10% FCS by injection using a sharpened glass micropipette attached via a flexible tube to a 1ml syringe which was used as a pump.

Control insects received either 10μl CM + 10% FCS in which larvae had been temporarily stored or pieces of ovariole dissected from naive female *P.americana*. The total size of the pieces of ovariole was comparable to that of the parasites injected into the experimental hosts. Ovariole was used as a control because transplanted allogeneic tissues, in common with acanthellae and cystacanths, are not encapsulated by haemocytes (Lackie, 1979).

After 7 days, pre-hatched *H.diminuta* oncospheres (approximately 500 in 10μl HBS) were then injected into all 3 groups of cockroaches. Insects were maintained at 28$\pm$1^{o}C and provided with food and water *ad libitum* for 7-10 days, after which they were dissected and the numbers of unencapsulated *H.diminuta* larvae counted.

5.3 RESULTS

5.3.1 SELECTION OF A STRAIN OF *HYMENOLEPIS DIMINUTA* INFECTIVE TO *PERIPLANETA AMERICANA*

From the 3 replicate infections, each with different batches of eggs, the data were pooled to assess differences in the ability of the 3 laboratory strains of *H.diminuta* to infect cockroaches (Table 5.1).

The geometric mean of the number of parasites of each strain per host was calculated after $\log_{10}$ transforming the data (x+1) then performing unpaired t-tests. The null hypothesis was rejected at $p > 0.05$ level for Exeter strain versus Japanese (ie. a significant difference exists in the ability of the two strains to infect cockroaches), but retained for Exeter versus Texas, and Texas versus Japanese.

As infections by Exeter strain *H.diminuta* were most prevalent, this strain was chosen for further passaging through the cockroach / rat system.

In all the infections including those below, large numbers of encapsulated and melanised particles were found adhering to the inner lining of the haemocoele, in particular the dorsal diaphragm. On examining them at x250-500 magnification (Leitz-Ortholux) they appeared to contain oncospheral hooks, and were therefore assumed to be the melanised remains of oncospheres which failed to develop to the cysticercoid stage.

Table 5.2 summarises the results of passaging Exeter strain *H.diminuta* through groups of *P.americana*, *P.australasiae* and *S.gregaria*. In total, 72 separate batches of eggs were used to infect *P.americana*, *P.australasiae* and *S.gregaria* and the results combined for each passage number. *Blatta orientalis* and *Blatella germanica* were also injected by this method, but none were found to be infected in 3 groups of 12 of each species.

After each successive passage in both *P.americana* and *P.australasiae*, mean numbers of *H.diminuta* larvae recovered decreased rather than increased,

and by the fourth passage no larvae were recovered from the insects. There was a significant decrease (t-test of $\log_{10}$ transformed data x+1 $p<0.05$) between the geometric mean number of parasites in passages 2 and 3 in *P.americana*. Prevalence and intensity of infection were found to be slightly higher in *P.australasiae*, but again there was no evidence of any increase in survival of the parasite in successive passages.

The values for highest percentage prevalence and intensity of infection are also given in Table 5.2. These data are taken from the most successful infections made during each passage using separate batches of eggs, from which the resulting cysticercoids were used in subsequent passages. However these data also show the general trend leading towards the eventual failure of the parasites, particularly in *P.americana*.

As expected, large numbers of *H.diminuta* cysticercoids with a prevalence of 100% were recovered from locusts, indicating that viability of the oncospheres in all preparations was high. However there is no evidence to suggest that there was any enhancement in survival of the cysticercoids after each successive passage in locusts although this was unlikely to be found after only 3 passages.

5.3.2 EFFECT OF *MONILIFORMIS MONILIFORMIS* ON THE SURVIVAL OF *HYMENOLEPIS DIMINUTA* LARVAE WITHIN THE COCKROACH HAEMOCOELE

Both the prevalence and intensity of infection by injected *H.diminuta* increased when *M.moniliformis* was already present within the haemocoele (Table 5.3). *H.diminuta* larvae that developed to the cysticercoid stage were subsequently shown to be infective to rats. When the data for the 3 experimental replicates are pooled and plotted as a frequency distribution (Figure 5.1), it can be seen that the effect of the pre-existent *M.moniliformis* infection is to shift the curve away from that typical of an

overdispersed distribution.

Calculation of the variance : mean ratios shows that the values for all the controls (apart from the medium-injected control in experiment 2) are considerably greater than 1.0, indicating an aggregated distribution of *H.diminuta* within the host population. For *M.moniliformis*-parasitised hosts in experiments 1 and 3, the ratio is much less than 1.0, indicating a tendency of *H.diminuta* towards under-dispersion rather than normal distribution.

A unique observation was made during this work, where a small proportion of *H.diminuta* larvae were found to have developed inside the envelopes of *M.moniliformis* (Holt, 1989). Apart from the free cysticercoids (Table 5.3) found in the haemocoeles of 50 cockroaches that had been injected with both *H.diminuta* and *M.moniliformis*, over 100 *H.diminuta* were recovered from within the envelopes of *M.moniliformis* cystacanths, there being 1 or 2 *H.diminuta* larvae inside the envelopes of *M.moniliformis* in most cases, but occasionally as many as 4 (see Figure 5.2). Host haemocytes were found adhering to small patches of the envelope surface on those *M. moniliformis* harbouring *H.diminuta* cysticercoids, but did not seem to effect the development of the larvae within.

The viability of the combined parasites was tested by administering 60 of them to 8 Wistar strain rats and later examining the rat's faeces for parasite eggs (see 2.2.3.2). *H.diminuta* eggs were found 24 days post-infection and were found together with *M.moniliformis* eggs by 7 weeks post-infection. When the rats were later dissected and their small intestines examined, the *M.moniliformis* were always found anteriorly to the position of the *H.diminuta* as in sections 2.3.1 and 2.3.2. Of the 60 parasites of each species administered, 31 *H.diminuta* and 44 *M.moniliformis* were recovered.

TABLE 5.1 COMPARISON OF THE ABILITY OF DIFFERENT LABORATORY STRAINS OF *HYMENOLEPIS DIMINUTA* TO INFECT COCKROACHES

Strain	Percentage prevalence (pooled data)	Mean parasite number (pooled data)	Total number parasites	Total number hosts
Exeter	32.26%	2.19	68	31
Texas	18.75%	0.91	29	32
Japanese	6.06%	0.48	16	33

TABLE 5.2 SUMMARY OF DATA OF *HYMENOLEPIS DIMINUTA* INFECTIONS OF *PERIPLANETA AMERICANA* (FROM 72 SEPARATE EXPERIMENTS)

Insect	Passage	Total N°. of Hosts	Overall Prevalence	Mean N°. Parasites/host	Highest* Prevalence	Highest Mean N° Parasites/host *
P.americana						
	1	137	7.30%	0.71	47.05%	5.50
	2	89	15.73%	0.82	54.50%	4.18
	3	121	3.31%	0.24	27.27%	2.54
	4	28	0.00%	0.00	0.00%	0.00
P.australasiae	1	56	7.14%	14.30	50.00%	100.25
	2	48	8.33%	8.00	16.66%	19.08
	3	108	5.55%	0.28	40.00%	1.36
S.gregaria	1	33	100.00%	111.58	100.00%	190.20
	2	19	100.00%	100.74	100.00%	152.75
	3	20	100.00%	104.55	100.00%	129.40

* Highest values given for infections using single batches of eggs.

TABLE 5.3 THE PREVALENCE OF *HYMENOLEPIS* IN UNPARASITISED AND *MONILIFORMIS*-INFECTED COCKROACHES

		Expt.1	Expt.2	Expt.3
Unparasitised control (medium)	prevalence	3/11 (27%)	6/11 (55%)	1/9 (11%)
	mean intensity ± s.d.	2.5 ± 4.4	3.5 ± 4.9	0.6 ± 1.7
	variance : mean	2.88	1.86	7.98
Unparasitised control (ovariole)	prevalence	2/12 (17%)	3/9 (33%)	4/10 (40%)
	mean intensity ± s.d.	1.3 ± 3.0	8.6 ± 21.4	2.5 ± 6.5
	variance : mean	0.38	4.15	0.17
Parasitised (40 M.m)	prevalence	11/11 (100%)	11/11 (100%)	7/7 (100%)
	mean intensity ± s.d.	13.5 ± 8.7	*26.3 ± 56.1	6.1 ± 2.7
	variance : mean	0.38	4.15	0.17

* 1 cockroach contained 194 *H.diminuta*.

FIGURE 5.1 FREQUENCY DISTRIBUTION OF NUMBERS OF *HYMENOLEPIS DIMINUTA* LARVAE DEVELOPING, UNENCAPSULATED, IN PARASITISED AND *MONILIFORMIS MONILIFORMIS*-INFECTED (40 LARVAE) COCKROACHES.

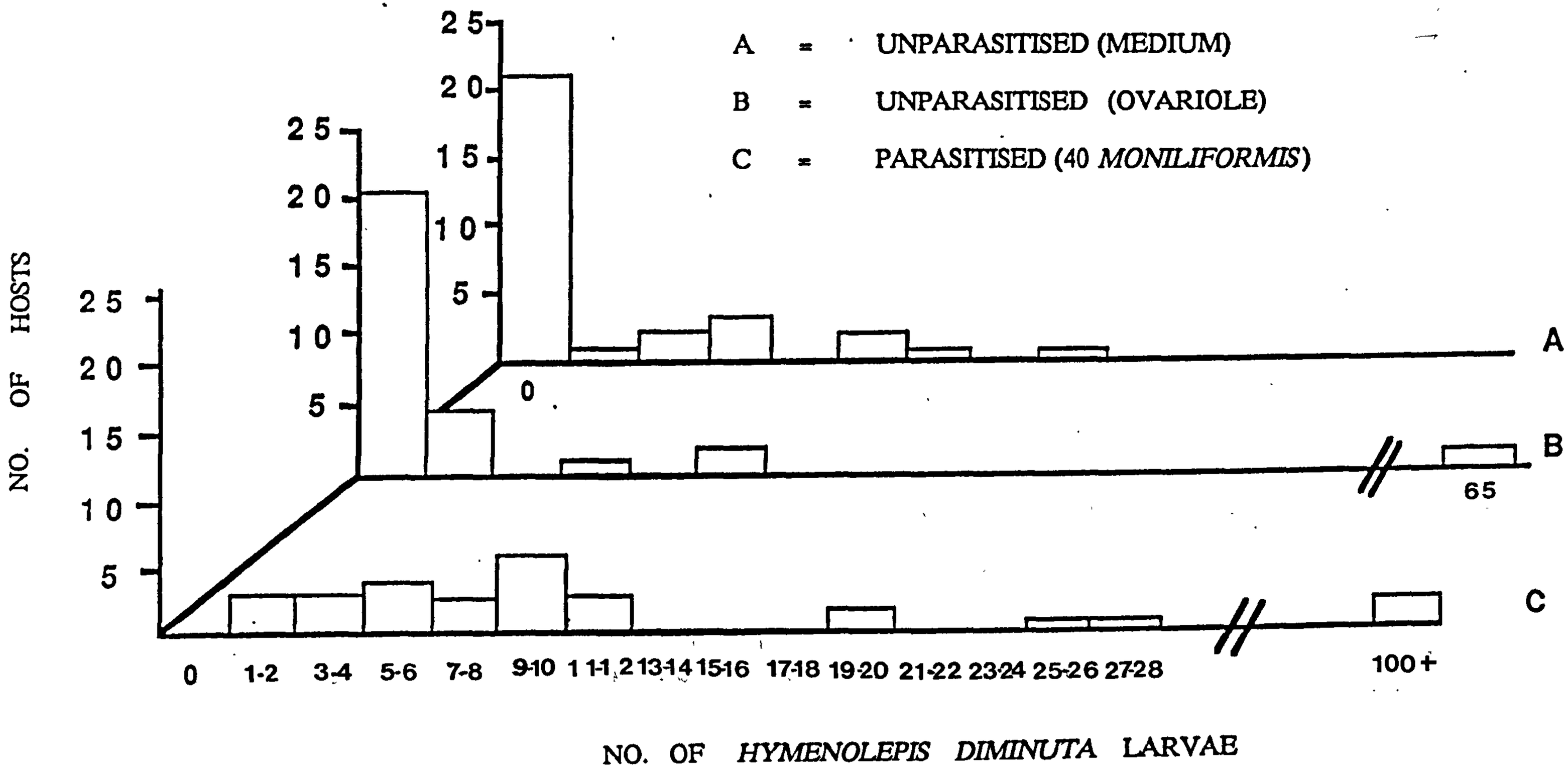


FIGURE 5.2 *HYMENOLEPIS DIMINUTA* UTILISES THE ENVELOPE SURROUNDING *MONILIFORMIS MONILIFORMIS* IN ORDER TO SURVIVE IN THE COCKROACH HOST

The photomicrographs show the unique occurrence of the cysticercoid larvae of *Hymenolepis diminuta* inside the envelopes of the acanthella and cystacanth stages of *Moniliformis moniliformis.* During the two species infection of *Periplaneta americana* with *Moniliformis* and *Hymenolepis* some of the injected oncospheres burrowed through the developing envelope and continued their growth within. In some cases the only *Hymenolepis* to develop in experimentally infected cockroaches were those inside the envelope (see section 5.3.2).

C2 = Stage 2 cysticercoid

C5 = Stage 5 cysticercoid (after Voge and Heyneman, 1957)

A = Stage IV acanthella

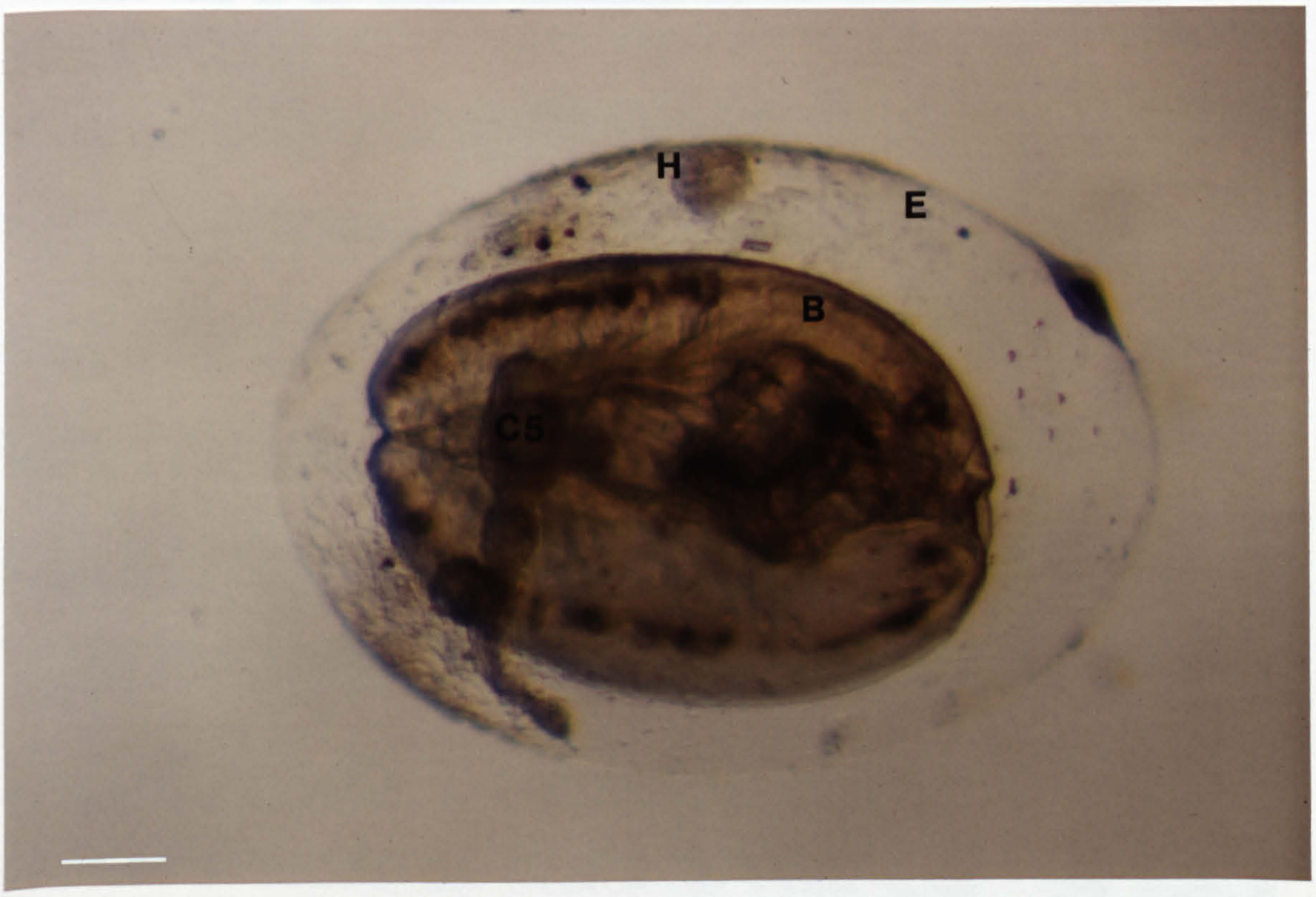
B = Cystacanth (after King and Robinson, 1967)

E = Envelope

H = Host haemocytes

Scale bar = 0.25mm


E
C2
A
H


H
E
B
C5

5.4 DISCUSSION

Infectivity, based on the data for prevalence and intensity of infection was greatest in the Exeter strain of *H.diminuta* used in section 5.3.1. As Exeter strain is a "wild-type", not having been maintained in the laboratory for long, it is possible that it has greater genotypic variation, resulting in more cysticercoids surviving in the cockroach than those of Texas and Japanese strains of *H.diminuta* In the intermediate host, both Texas and Japanese strains seemed better adapted to living in their own particular species of beetle (Kino and Kennedy, 1987), possibly as a consequence of repeated passaging in the laboratory and the effects of inbreeding. However, in all the strains used to infect cockroaches, oncosphere survival was low, the majority were found later encapsulated and melanised, adhering to the inner lining of the haemocoele. This is perhaps surprising when considering the wide variety of insect species in which *H.diminuta* has been found (Burt, 1980).

The basis of the selection programme was that those few oncospheres which survived were presumed to be in some way pre-adapted to conditions in the haemocoele of the cockroach and could therefore pass on this trait to subsequent generations. Not only was the infectivity of the parasite important in considering strain selection but also the susceptibility of the host; the following examples show how host susceptibility can affect the success of a parasite. The infectivity of *Hymenolepis citelli* was shown to decrease in response to selection pressure from its host, *Tribolium confusum* (Schom *et al*, 1981). In this case the parasites of high infectivity tended to kill the hosts, therefore leaving only those parasites which had a less pathological effect. Sage *et al.*, (1986) studied hybrid mice in South Germany, and found that they were more susceptible to infections of worms (nematodes and cestodes) than either of the original speces. They suggested that the hybrids might have lost genes for resistance to the parasites.

In many of the cockroaches, haemocytes apparently successfully encapsulated all of the injected oncospheres although there were a few cases where large numbers of cysticercoids were recovered from *P.australasiae*. It is possible that the cockroaches in which parasites could develop, especially those with large numbers of cysticercoids, were in some way immunosuppressed, therefore allowing the "fittest" parasites to develop in the "weakest" hosts.

Lackie (1976) suggested that the few surviving cysticercoids escaped encapsulation by taking refuge in "privileged sites" within the cockroach. These sites included areas of the thoracic musculature, especially the coxae, from which many of the larvae were recovered in this work. Presumably the densely packed muscle tissue restricted haemocyte access to the developing parasite larvae which then could grow normally. However this was not the only way in which *H.diminuta* was found to survive in the cockroach, as cysticercoids were also recovered from the abdominal haemocoele even when oncospheres were injected directly into the thoracic region of the insect's body.

For the success in selection of a strain of *H.diminuta* infective to cockroaches, the selection pressure to adapt must come from the immune system and physiology of the insect host. However, a problem with the above method used to infect cockroaches is that the *in vitro* hatching process may itself select for certain oncospheres or even change them in some way so that only a very small proportion are potentially infective. As most of the oncospheres are recognised immediately, and very few stage 3-5 larvae (Voge and Heyneman 1957) are found melanised later, it is possible that the hatching and injection processes damage the surface of the oncospheres, rendering them immediately susceptible to immune recognition by the cockroach haemocytes but not to those of the locust. However, if a "dirty" suspension of oncospheres is used, containing fragments of egg shell and cytoplasmic layer, none of the larvae develop and therefore it is critical that the high standard of

preparation of the oncosphere suspension is maintained. On 72 separate occasions, using thoroughly cleaned suspensions, attempts to select a strain of cockroach-infective *H.diminuta* by sequential passaging were made. But each time, after the 3rd or 4th passage the selected line died out as no cysticercoids were recovered.

Selection pressure, from whatever source, must be applied at an optimal level for adaptation or evolution of a strain to take place. Too much, and too few parasites, if any, will survive to allow the continuation of the selection programme; not enough, and very little change in phenotype will occur (Falconer, 1964). Lackie (1976, 1979, 1981, 1986b, c, d, e), has shown the immune system of cockroaches to be more "efficient" at recognising non-self than that of locusts, and therefore it is possible that cockroaches exert a higher selection pressure on *H.diminuta* to adapt than do locusts. For *H.diminuta* to have the scope to adapt to the cockroach, it possibly requires a high degree of genetic variability to allow for physiological or morphological changes. As an alternative to the methodological failings of the selection programme it is possible that the inherent genetic variability of the parasite is too low, especially if self-fertilisation occurs, for a large enough number of oncospheres to adapt under high selection pressure. This would result in only very few parasites surviving and the eventual extinction of the strain. The cysticercoids which did survive were possibly in naturally immunosuppressed hosts.

The situation in the locust may be interpreted in a similar manner. Selection pressure for *H.diminuta* to adapt to the locust is presumably much lower than in the cockroach, as large numbers of cysticercoids always develop. However, although there was no increase in success of the parasite after each passage, this was not necessarily as a result of a lack of selection pressure; low genetic variability would also result in similar findings.

Instead of selecting for an existing genotype of *H.diminuta* capable of infecting cockroaches, this selection programme possibly required the mutation

of a gene so that many more parasites become infective. As this does not occur frequently (Falconer, 1964; Smyth and Smyth, 1964) in most systems under normal conditions, many more generations of the parasite would have to be passaged through the cockroach / rat cycle before any adaptation would possibly occur. In the wild this occurs naturally, so that not only new strains of *H.diminuta* develop but also speciation occurs (Mongomery *et al.*, 1987) where the parasite may utilise different available host species.

Suggestions were put forward for the raising of a strain of cockroaches susceptible to parasitisation by *H.diminuta* However, a method for assessing whether or not cockroaches in a breeding programme are parasitised without killing the insects has not been devised. It would be possible to dissect parasitised female cockroaches which were carrying fully developed ootheca, which could be hatched, and the offspring infected in a similar manner. Due to the lengthy period of development before sexual maturity, this process would be very time consuming, and could take many years for only a few passages.

It is already known that both laboratory and wild-type strains of *H.diminuta* cannot infect cockroaches by the oral route and from the failure of the strain selection programme using injected larvae, it appears that *H.diminuta* is also unable to survive under "normal" conditions in the haemolymph. This must be considered as conclusive evidence for the inability of *H.diminuta* to infect cockroaches and therefore comparative work using adapted and non-adapted strains has had to be abandoned. However, in this chapter (and Chapters 4 and 6) *M.moniliformis* interactions with the host and *H.diminuta* have been studied and from these some new and interesting information has been gathered on the host immune system and host/parasite interactions.

As a small proportion of *H.diminuta* larvae survive in the uninfected cockroaches and also the larvae can develop *in vitro* in a medium formulated

for cockroach haemocytes (Lackie, 1976, see also Huxham and Lackie's medium in Crompton and Lassiere, 1987) it would seem that the barrier to infection of *P.americana* by pre-hatched oncospheres is immunological rather than physiological. It is likely that in the cockroaches many of the oncospheres fail to develop because they are incorporated within haemocyte aggregates found later adhering to the dorsal diaphragm. In many cases there were melanised particles found in the coxae, which approximated to the size and form of what could have been the remains of oncospheres or partially developed cysticercoids. The ability of *H.diminuta* larvae to survive in locusts may stem from the apparent inability of the locust's immune system to distinguish them as non-self. Thus they survive at high prevalence and intensity of infection.

As a known dose of *H.diminuta* oncospheres was injected directly into the insect haemocoele, the overdispersed distribution of *H.diminuta* in previously naive cockroaches may have resulted from host heterogeneity in immune responsiveness. Alternatively, as discussed by Crompton *et al.* (1984), this overdispersion may have resulted from the interaction of 2 randomly-distributed variables, such as the variation in the burrowing activity of the larvae and the variation around the mean number of oncospheres injected. However, since the distribution of *H.diminuta* in *M.moniliformis*-infected cockroaches tends towards underdispersion, which in itself may be a result of altering the host's immunological processes (Anderson, 1982), this suggests strongly that overdispersion in the naive hosts was derived from immunological causes.

The results, in which the presence of *M.moniliformis* enhances the success of an infection of *H.diminuta* in *P.americana* contrasts with the results found by Lackie (1986d) in similar experiments. The reasons for the difference can only be speculated upon, but may result from the different infection regimes and doses used in the 2 sets of experiments. Since Lackie first attempted these experiments, the quality of the oncosphere preparation has been refined

so as to produce a "cleaner" suspension of larvae.

In a few cases *H.diminuta* cysticercoids were discovered inside the envelopes of *M.moniliformis* larvae (Holt, 1989), thus providing further evidence that the acanthocephalan envelope protects its enclosed parasite against the immune response (Lackie and Lackie, 1979). Larvae of *H.diminuta* inside the envelopes were found to develop normally. In some cases, small aggregates of host cells were seen adhering to a small portion of the envelope's outer surface, possibly marking the point at which *H.diminuta* larvae damaged the envelope on entry. This unique association has clearly shown that the envelope is acting as a barrier that protects developing larvae by excluding haemocytes, yet allows entry of nutrients, since both parasites developed without a decrease in normal growth rate.

In the introduction to this chapter it was reasoned that the presence of *M.moniliformis* in the same cockroach host might enhance cestode survivorship if *M.moniliformis* reduced the effectiveness of the immune system. Immunosuppression in acute infections by insect pathogens such as parasitoids (Davies *et al.*, 1987; Rizki and Rizki, 1984; Stoltz and Guzo, 1986) has been recorded before, but the observation that a chronic infection such as *M.moniliformis* exerts an immunosuppressive effect is surprising since the parasite requires a minimum of 6 weeks for development and its cockroach host lives in an environment offering great potential for microbial invasion.

J.M. Lackie (1972) attempted to superimpose infections of *M.moniliformis* in the cockroach and found that an existing infection did not seem to cause an increase in the numbers of larvae developing in subsequent infections. This has been taken as evidence for immunosuppression not having taken place, as it was suggested that immunosuppressed cockroaches would allow more *M.moniliformis* larvae to develop within the haemocoele. However, if the processes leading to parasitisation of cockroaches by *M.moniliformis* are examined it would appear that the initial barrier to infection is probably

physical rather than immunological. The gut prevents the majority of the larvae from reaching the haemocoele and therefore only a small number, independent of dose size (J.M. Lackie, 1975), develop to the cystacanth stage. Any further attempts to infect the cockroaches would therefore result in similar numbers of acanthors reaching the haemocoele, on each occasion their numbers being regulated by the gut rather than the immune system. It would be more relevant, therefore, to determine whether pre-infected cockroaches are more susceptible to directly injected acanthors rather than those which reach the haemocoele via the gut.

Previous evidence has also suggested that *M.moniliformis* survives by immune evasion, whereby the envelope protects the parasite from immune recognition through looking similar to host "self" (J.M. Lackie, 1975; Lackie and Lackie, 1979; Lackie 1986d). The envelope, like the tissue surfaces lining the cockroach haemocoele, contains a non-fibrillar collagen and glycosaminoglycan-like molecule (O'Brien, Kusel and Lackie, unpubl. O'Brien, 1988). It seems therefore that there is evidence to support both immune evasion and immunosuppression by *M.moniliformis* in the cockroach. The evidence for the immunosuppressive action of *M.moniliformis* on its host is supported by further work to assay the responsiveness of the cockroach haemocytes to immune stimulants (see Chapter 6).

5.5 SUMMARY

1. An attempt was made to raise a cockroach-infective strain of *H.diminuta* by injecting hatched larvae directly into the haemocoele, thereby avoiding the gut-barrier.

2. Of the strains of *H.diminuta* maintained in the laboratory, the Exeter wild-type strain appeared to be the most successful when injected into cockroaches. However attempts to increase the

infectivity of the strain to cockroaches by repeated passaging through the *P.americana* / rat system failed for a number of possible reasons:-

a. The inherent genotypic variation within the strain was possibly not great enough for adaptation to occur under the high selection pressure exerted by the cockroaches' immune system.

b. The method for the *in vitro* hatching of oncospheres in some way predisposed them to recognition and encapsulation by the cockroach haemocytes, but not by locust haemocytes.

3. However, when *H.diminuta* was injected into *M.moniliformis*-infected cockroaches, prevalence and intensity of infection were significantly greater than in naive controls, indicating a possible immunosuppressive action from *M.moniliformis* which facilitated *H.diminuta* development. In some cases the larvae of *H.diminuta* were found to have burrowed through the envelope surrounding *M.moniliformis*, and continued normal development unmolested by the host's haemocytes. Parasites developing in this way were found to be infective to rats.

CHAPTER SIX

THE EFFECTS OF PARASITES ON THE IMMUNE SYSTEM OF INSECTS AS DETERMINED BY ASSAYS FOR HAEMOCYTE BEHAVIOUR

6.1. INTRODUCTION

Information gathered in the previous chapters has demonstrated that *H.diminuta* and *M.moniliformis* interact with the intermediate host to alter the insect's susceptibility to parasitic infection. In Chapter 4, when eggs of both species of parasite were fed simultaneously to cockroaches, *H.diminuta* in some way adversely affected the success of *M.moniliformis*. This was likened to a similar situation in which *Raillietina cesticillus* affected *H.diminuta* in *T.confusum* (Gordon and Whitfield, 1985). Conversely, in Chapter 5, *M.moniliformis*-infected cockroaches were found to support elevated numbers of *H.diminuta* compared to naive controls.

The aim of the work presented in this chapter was to investigate, using quantitative assays for the haemocytic response, how both the above situations are linked to the state of responsiveness of the immune system of the insect host. The principles behind these assays, for haemocytic nodule formation and phenoloxidase-positive haemocytes, are described below.

Nodule formation in response to injected foreign particles (bacteria) has previously been described by Ratcliffe and Gagen (1977) and Ratcliffe and Walters (1983) in *Galleria melonella*, and also in *P.americana* by Ryan and Nicholas (1972) where haemocyte aggregates formed around degranulated coagulocytes at the surface of foreign bodies.

Immune reactivity can be stimulated non-specifically for up to 3 days post-infection by injecting abiotic particles, such as Sepharose beads into the haemocoele. Dularay and Lackie (1987), showed that the immune system of *P.americana* could be stimulated in this manner to recognise xenografted tissues of *Blatta orientalis* which were previously treated as "self". More specific activators, such as injected Zymosan supernatant, containing β1,6- and β1,3-glucans, stimulated measurable changes in haemocytic behaviour *in vivo* (Gunnarsson and Lackie, 1985), thus both phagocytic index and ability to form haemocytic nodules in response to unrelated stimuli are enhanced for up

to 48 hours after injecting Zymosan supernatant (Gunnarsson, 1988a).

Once the immune system of the insect has been activated, the number of nodules formed in response to injections of saline alone increases dramatically. It appears that this may be due to an increased responsiveness of the immune system to wounding the cuticle and epidermis during injection (Lackie, 1988a). Thus the effect of saline injection on stimulating nodules can be used to determine whether *M.moniliformis* or *H.diminuta* have had an affect on the host; ie. in an unstimulated host, few nodules are formed; in an immune-stimulated host, large numbers will be formed.

Numbers of nodules forming in response to immune stimulation can be quantified, and used to assay the status of an insect's immune system (Gunnarsson and Lackie, 1985). Lackie (1986e) has also used this assay for assessing the immune response of *P.americana* and *S.gregaria* to haemolymph from allogeneic and xenogeneic sources, where the assay has been shown to be sensitive to changes in the immune status of the insect.

The second assay used in this work is based on the production of a phenoloxidase (PO) by activated granular haemocytes. PO oxidises tyrosine and DOPA to form the black pigment, melanin, so that under defined conditions the cells which are associated with the active enzyme can be visualised as black dots. Schmit *et al.* (1977) used this as a histological method before the initial studies on phenoloxdase activation. The precursor of PO, prophenoloxidase (proPO), can be activated by β1,3-glucans (eg. Laminarin, or those in Zymosan supernatant, as described above), which, through a sequence of enzymic reactions known as the phenoloxidase cascade (Söderhäll, 1982), produces PO as the end product. Huxham and Lackie (1986) developed an assay using the above system to test for stimulatory and inhibitory products of an insect pathogenic fungus, *Metarhizium anisopliae*, the effects of which were related to changes in number of black-stained cells found *in vitro* As the assay had proven effective for use with *S.gregaria* haemocytes, only slight modifications in the density of cells in culture had to be made to adapt the

assay to *P.americana* haemocytes.

Now that suitable media for the handling of cockroach and locust haemocytes are available (Huxham and Lackie's medium, see Crompton and Lassiere, 1987) it has provided scope for a method of assessing haemocytic encapsulation ability *in vitro*

Davies and Vinson (1986) were able to induce the haemocytes of larval *Heliothis virescens* to clump around targets *in vitro* to form a capsule similar to that formed *in vivo* From this they were able to establish the nature of encapsulation-promoting factors by isolating the haemocytes from the plasma and then adding these factors to the cells in the *in vitro* assay.

The advantage of an *in vitro* encapsulation assay is that insect haemocytes can be handled in the absence of plasma or serum factors. Cells can be labelled, fixed or separated into sub-populations as appropriate (Huxham and Lackie, 1988) and therefore specific activities of the cells studied. However it is important that the appropriate positive and negative controls are used in such an assay. For example, Davies and Vinson (1986) used cotton fibres as positive and *H.virescens* nerve cord as negative controls so as to ensure that firstly, the cells would encapsulate if they were presented with an appropriate target, and secondly, to gauge that the cells discriminated between "self" and "non-self" surfaces.

The response of haemocytes to foreign particles *in vivo* is graded according to the type of surface. Vinson (1974), using *H.virescens* larvae, Dunphy and Nolan (1982) using *Christoneura* larvae, and Lackie (1983, 1986b) using adult *P.americana* and *S.gregaria* have found that differently charged ion-exchange beads are encapsulated to different extents. It has been proposed (see Lackie, 1988a) that surface electrostatic charge may influence haemocytic adhesion, indirectly by effecting the adsorption of soluble intermediaries necessary for adhesion or by directly attracting or repelling haemocytes according to their own charge. Takle and Lackie (1985) found that

locust cells were significantly more negatively charged than those of the cockroach which may explain why locust haemocytes appear to have difficulty in making contact with experimental biotic implants and parasites, which in general, carry a negative charge. Lackie and Vasta (1988) have also shown that the biochemical composition of the surface of a target also influences the response of encapsulating haemocytes *in vitro* They found that a galactose-rich surface provokes a strong haemocytic response, possibly mediated by lectins associated with the haemocytes or soluble in the haemolymph.

By observing the ability of *P.americana* haemocytes to encapsulate the parasites *H.diminuta* and *M.moniliformis in vitro* some basic principles about how the parasites avoid or succumb to the immune system may be answered.

6.2 MATERIALS AND METHODS

6.2.1 ANIMALS

6.2.1.1 INSECTS

Cockroaches *P.americana* and locusts *S.gregaria* were bred and maintained in the department's insectary as described in sections 2.2.1.3 and 3.2.1.

6.2.1.2 PARASITES

H.diminuta were maintained in *T.confusum* and Wistar rats as described in section 2.2.1.4.

Egg suspension in 60% sucrose solution was used for the initial experiments involving *H.diminuta* (see section 3.2.4.1) but was replaced, for ease of handling and administration, by using mature sections of tapeworm containing 800-1000 eggs in approximately 15mg of proglottid (blotted weight). *P.americana* were fed on these sections of worm which were eaten readily after being dipped in 60% sucrose solution. Sections of worm containing non-viable

(immature) eggs were used as controls. Cockroaches, held in one hand by the wings, could be fed by presenting them with pieces of sucrose-dipped proglottid held in fine forceps in the other (See Figure 6.1).

H.diminuta cysticercoids were obtained from stock cultures in *T.confusum*

M.moniliformis were maintained in Wistar rats and cockroaches as described in section 2.2.1.4. For experiments requiring a standard dose of *M.moniliformis*, cockroaches were given 40 stage I acanthellae by injection with a micropipette and then used in the experiments 1 week later (see section 5.3.2); otherwise stock cockroaches which had been fed previously with *M.moniliformis* eggs in 60% sucrose were used 2-3 weeks post infection.

M.moniliformis cystacanths were obtained from stock infections of cockroaches maintained in the Department's insectary.

6.2.2 MEDIA AND SALINES

Cockroach cell culture medium (CM) - see section 5.2.3.

HEPES-buffered balanced salt solution, pH 7.2 (HBS) - see 2.2.1.5.

Zymosan supernatant (Zs) was obtained by suspending 1% (weight/volume) Zymosan (Sigma) in HBS; the suspension was agitated on a Whirlimix and centrifuged several times to produce a particle-free supernatant (Gunnarsson and Lackie, 1985).

Laminarin solution was prepared by dissolving Laminarin (Sigma) in warm cockroach medium (CM) to a final concentration of 1mg ml^{-1}.

Anticoagulant for cockroach haemolymph - 2.5% disodium-ethylenediaminotetraacetic acid (EDTA) in calcium/magnesium free (CMF) or citrate buffered (CIT) Hanks' saline, pH 7.0 (Gunnarsson and Lackie, 1985).

6.2.3 EXPERIMENTAL PROCEDURES

6.2.3.1 EFFECTS OF *HYMENOLEPIS DIMINUTA* ON THE INDUCTION OF HAEMOCYTIC NODULES BY SALINE INJECTION *INVIVO*

To ascertain whether *H.diminuta* oncospheres were causing a stimulatory effect from within the cockroach midgut, haemocytic nodule formation was induced in the haemolymph by an injection of saline (see section 6.1) at a specific time post-infection. If the cockroach immune system had been activated, then an elevated number of nodules forming in response to the saline was expected.

Cockroaches were fed with *H.diminuta* proglottid sections as in section 6.2.1.2 containing either viable (experimental), non-viable eggs (control 1) and naive (control 2), handled as with the other groups of cockroaches but not fed with parasites. They were housed in plastic containers at 28±1°C with food and water provided *ad libitum*

The insects were divided into groups, so that after certain time intervals, between 1 and 18 days, a group of insects could be used to assay for nodule formation. All the insects in each group (including naive control insects) were each injected with 10μl of HBS into the abdominal haemocoele. Three hours later, haemolymph was flushed out of each insect by injecting approximately 1ml of CMF-EDTA into the abdominal haemocoele, and the diluted haemolymph exuded from the stumps of 3 severed legs was added directly to a further 1ml CMF-EDTA in a 3cm diameter Petri dish. The number of haemocyte aggregates in each dish was counted under x 40 magnification on a Leitz Ortholux microscope. This was repeated 3 times using fresh batches of *H.diminuta* eggs in proglottid sections.

Other controls were included in a second series of experiments to compare the different feeding techniques used for dosing the cockroaches with

H.diminuta. Using *H.diminuta* egg suspensions in 60% sucrose, cockroaches were fed as above, and nodule numbers recorded at 3 days post feeding. Cockroaches were dosed with either viable egg suspension, non-viable egg suspension, egg suspension (viable and non-viable) homogenate and the supernatants from both. This was also repeated 3 times using 7-10 cockroaches in each group.

6.2.3.2 EFFECTS OF *HYMENOLEPIS DIMINUTA* ON LAMINARIN-STIMULATED PHENOLOXIDASE PRODUCTION BY *PERIPLANETA AMERICANA* HAEMOCYTES *IN VITRO*

Cockroaches were fed with viable eggs in sucrose-dipped proglottid sections (experimental) and sucrose, as above. Three days after feeding, the cockroaches were CO_2-anaesthetised and each received a 200μl dose of CIT EDTA by intrahaemocoelic injection. Exuded diluted haemolymph, used as a source of intact haemocytes, was then collected with a Pasteur pipette from the stump of a severed hindleg and pooled for each group of 8 insects into an excess of CIT EDTA held on ice.

The assay for activation of pro-phenoloxidase by Laminarin, and hence the production of PO-positive cells, was modified slightly from the assay described by Huxham and Lackie (1986) as follows:-

The cell suspensions were washed twice in CIT EDTA by centrifugation at 5^{o}C, 250 x g for 5 minutes and resuspended in a fixed volume of the same (2ml CIT EDTA for the haemolymph from 8 cockroaches). The total number of haemocytes in suspension was determined by counting a 10 x diluted (10μl cell suspension + 90μl CIT^{-} EDTA) aliquot using a Fuchs Rosenthal haemocytometer. After re-centrifuging, the anticoagulant was removed by aspiration and the cells re-suspended in CM on ice to a concentration of $2x10^6$ cells ml^{-1} at which they could be handled for 5-10 minutes without clumping. $5x10^5$ cells (in 0.25ml of suspension) were dispensed into 3cm diameter (Nunc) culture dishes, containing 2ml of CM, using 6 dishes per suspension to control for

slight heterogeneity between aliquots. The cell suspensions were pipetted gently to avoid clumping, then the dishes rocked from side to side to ensure the formation of an even monolayer of haemocytes. The dishes were left for 15-30 minutes to allow the haemocytes to adhere and spread.

The culture medium was then replaced with CM + 10% foetal calf serum (FCS) and then haemocyte monolayers in half the dishes were stimulated by the addition of Laminarin (Sigma; final concentration 1mg ml^{-1} in CM; dissolved by warming) for 15-30 minutes at room temperature, leaving the other monolayers as controls. To visualise the PO-positive cells, 100μl of 5mg ml^{-1} L-DOPA (Sigma) in HBS (dissolved by heating to approximately 80^{o}C, then cooled before use) was added to each dish. The dishes were then left for 18-24 hours at room temperature to develop.

After aspirating the supernatant from the cultures, the cells in each dish were fixed with 1ml of 2.5% glutaraldehyde in HBS for 1 hour, then the fixative replaced with fresh HBS. Black-stained cells in 12 different fields of view per dish were counted under x 50 magnification on a Wild dissecting microscope and results expressed as number of PO-positive cells per mm^2 (ie. mean number of black-stained cells per field of view divided by 6.26).

6.2.3.3 EFFECTS OF *MONILIFORMIS MONILIFORMIS* ON THE INDUCTION OF HAEMOCYTIC NODULES BY ZYMOSAN SUPERNATANT *IN VIVO*

Cockroaches that had been injected with CM + 10% FCS or 40 stage I acanthellae *M.moniliformis* larvae (see 5.2.4.2) one week previously, received an intra-abdominal injection of 20 1 HBS (control) or 20μl of 1% Zs (to stimulate nodule formation *in vivo*). Three hours later, haemolymph was flushed out of each insect, as above, into a 3cm diameter Petri dish. The number of haemocyte aggregates in each dish was counted under x 40 magnification on a Leitz Ortholux microscope. This experiment was repeated 3

times.

The assay was also carried out using insects infected by the oral route. Cockroaches were fed with *M.moniliformis* eggs in 60% sucrose (see section 3.2.4.1) and haemocyte aggregation assays performed 1, 2 and 3 days post infection. Similarly, assays were performed using cockroaches with older infections of *M.moniliformis* at 4 weeks post infection by which time the larvae had reached stage II-III acanthellae. Once haemolymph had been extracted from the above insects, they were dissected in HBS and the numbers of larvae recovered recorded along with numbers of nodules per insect.

6.2.3.4 EFFECTS OF *MONILIFORMIS MONILIFORMIS* ON THE LAMINARIN-STIMULATED PHENOLOXIDASE PRODUCTION BY HAEMOCYTES *IN VITRO*

Groups of control and *M.moniliformis*-infected cockroaches were set up as above. One week after injection, the haemolymph from each cockroach was collected in CIT EDTA as above, and for groups of 8 insects, pooled on ice. Cell cultures were set up as in section 6.2.3.2, stimulated with laminarin, and then PO-positive cells visualised by the addition of DOPA. The results were expressed as the number of PO-positive cells per mm^2.

6.2.3.5 THE EFFECTS OF ZYMOSAN STIMULATION ON THE SUCCESS OF *HYMENOLEPIS DIMINUTA* IN LOCUSTS

Four groups of *S.gregaria* were treated with injections of either saline or Zs to stimulate haemocytic aggregation *in vivo* (Gunnasson, 1985) and then injected with pre-hatched *H.diminuta* oncospheres as follows.

	Injections			Dissections
Group	0 Hours	12 Hours	14 Hours	8 Days
1	10μl 1% Zs	10μl 1% Zs	*Hymenolepis*	Dissect
2	10μl 1% Zs	10μl HBS	"	"
3	10μl HBS	10μl 1% Zs	"	"
4	10μl HBS	10μl HBS	"	"

Several locusts were dissected approximately 24 hours post infection and the contents of the haemocoele examined under a dissection microscope (at x25-50 magnification) for the presence of larvae and haemocytic capsules.

At 8 days post-infection, the locusts were CO_2 anaesthetised, their heads, legs and wings removed, and the body cavity opened with a circumventral incision. *H.diminuta* larvae were flushed out of the haemocoele with HBS and then counted, taking note of the number of larvae at each stage of development (after Voge and Heyneman, 1957). The experiment was repeated 3 times using different batches of eggs in each case.

6.2.3.6 THE DURATION OF ZYMOSAN EFFECTS ON THE SURVIVAL OF *HYMENOLEPIS DIMINUTA* IN LOCUSTS

To determine whether the effects of the Zs on the host reaction to *H.diminuta*, as found in part 6.2.3.5 had caused mortality of the injected oncospheres, 6 groups of locusts were treated as follows.

	Injections:-			Dissect groups 1-6 at :-		
Groups	0 Hours	12 Hours	14 Hours	8 Days	10 Days	12 Days
1,2,3	10μl Zs	10μl Zs	Hymenolepis	1	2	3
4,5,6	10μl HBS	10μl HBS	"	4	5	6

At each dissection, as above, numbers of larvae at each stage of development were recorded for the 3 replicated experiments.

6.2.3.7 A METHOD FOR ASSESSING THE ABILITY OF *PERIPLANETA AMERICANA* HAEMOCYTES TO ENCAPSULATE PARASITES *IN VITRO*

The main aim in devising the haemocyte encapsulation assay was to compare the ability of *P.americana* haemocytes to encapsulate the intermediate host-stages of *H.diminuta* and *M.moniliformis in vitro* with the situation *in vivo*.

Initial attempts at finding a suitable protocol for an *in vitro* encapsulation assay were made using the haemolymph collected from individual cockroaches, with the problem that cell concentration varied between insects. However, it was found that cells collected in an excess of anticoagulant, after washing and transferring to CM could be pooled on ice and used in the assay as follows.

Haemolymph was collected from cockroaches by injecting 200μl CIT EDTA then collecting the diluted haemolymph with a Pasteur pipette from the stump of a severed hindlimb. Haemolymph from approximately 8 insects was pooled in an excess of CIT EDTA on ice. The suspension was then treated in the same manner as that used in the phenoloxidase assay (see 6.2.3.2) resulting in a haemocyte suspension at a concentration of $2x10^6$ cells ml^{-1} in CM on ice.

Following Davies and Vinson's (1986) method (and Davies, personal communication) for *in vitro* encapsulation using *Heliothis virescens* haemocytes, small siliconised (Sigmacote, Sigma) glass vials (approximate volume, 370μl) were used as containers. Targets for encapsulation, such as 2mm diameter silk loops (prepared from sterile surgical sutures; Mersilk, Ethicon Ltd.), monofilament nylon loops, human hair loops and various stages

of *H.diminuta* and *M.moniliformis* larvae were placed in the bottom of the encapsulation vials after having been wetted with CM to ensure they would not float at the surface. Cold haemocyte suspension (approximately 365μl) was then added to each vial, and the top sealed with Parafilm leaving a small air bubble (Figure 6.2).

Each vial was agitated by hand between thumb and index finger immediately after sealing to tumble the target in the cell suspension and prevent the growing capsule from sticking to the air bubble or to the sides of the vial. This also allowed the contents of the vial to warm while observing the first stages of capsule formation under a dissection microscope (x12-25 magnification, Wild). If large single clumps of coagulated cells formed, the contents of the vial were discarded.

After approximately 5 minutes handling the vials were transferred onto a rotating wheel (an adapted kymograph (Palmer, England) held on its side, with small clips attached to the drum), revolving at 17 r.p.m, mounted in such a way so that at each half revolution the bubble moved along the vial and tumbled its contents. After approximately 1-2 hours the capsules became tighter, more cohesive and smooth, although a positive or negative reaction could usually be judged within 5-10 minutes from the start.

Initially, attempts were made to find targets which would act as a strong positive or negative control for encapsulation. By presenting the haemocytes *in vitro* with positive control targets, this would ensure that the cells ability to encapsulate was not impared. Silk loops, human hair, monofilament nylon loops were used as positive controls. Negative controls were used to show cells had retained some powers of discrimination and were not adhering to any surface they came in contact with. Ovarioles dissected from female *P.americana* were used for this purpose.

Parasite larvae and control targets were placed together in the same encapsulation vials to compare capsule formation around the targets as follows:-

3 vials containing parasites only

3 vials containing parasites plus ovariole sections

3 vials containing parasites plus 1-2 silk loops

3 vials containing ovarioles only

3 vials containing silk loops only

The smaller larval stages of *H.diminuta* and *M.moniliformis*, ie. oncospheres and acanthors, were added to the encapsulation vials in batches of approximately 250 in a drop of CM. Cysticercoid and cystacanth stages were added in groups of 2-5.

FIGURE 6.1 FEEDING COCKROACHES WITH SUCROSE-DIPPED *HYMENOLEPIS DIMINUTA* PROGLOTTID SECTIONS

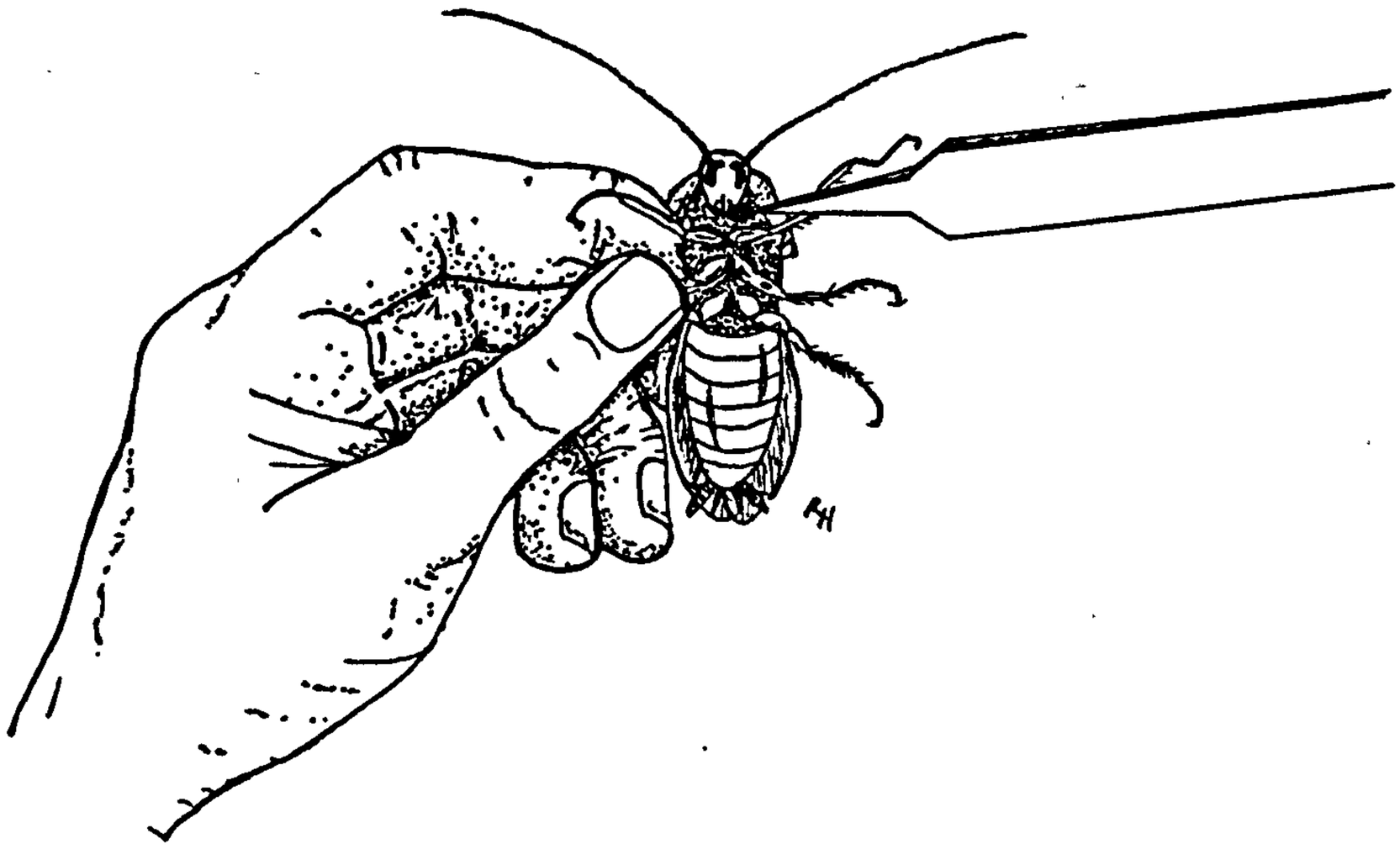
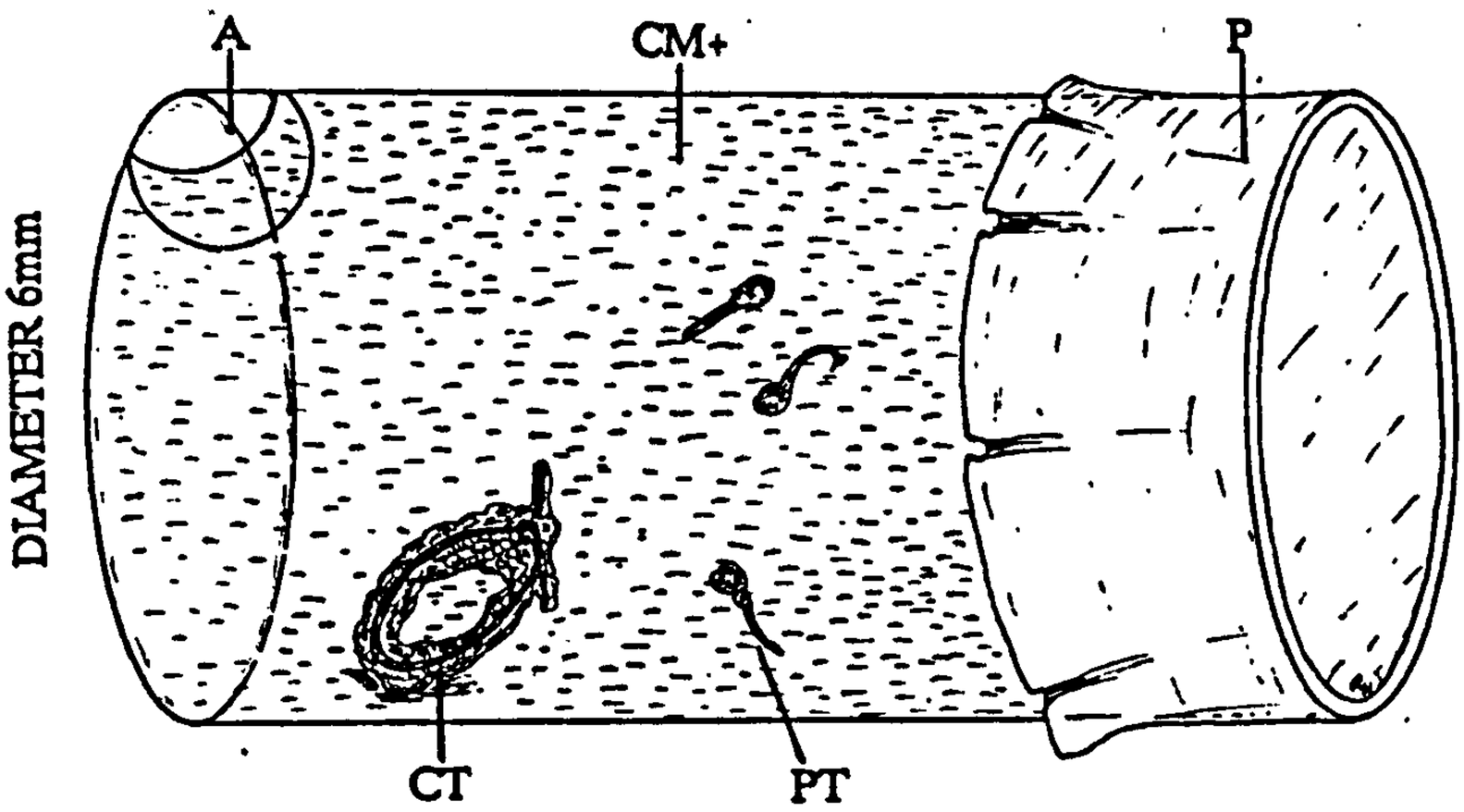


FIGURE 6.2 VIAL USED IN *IN VITRO* ENCAPSULATION ASSAY



LENGTH 20-25mm

A	Air bubble
CM+	D73 cockroach medium + cockroach haemocytes
P	Parafilm seal
CT	Control target
PT	Parasite target

6.3 RESULTS

6.3.1 EFFECTS OF *HYMENOLEPIS DIMINUTA* ON THE INDUCTION OF HAEMOCYTIC NODULES BY SALINE *IN VIVO*

The results are expressed graphically in Figures 6.3-6.5.

The most important feature of the results is the highly significant ($p < 0.001$) increase in nodule count 3 days after feeding viable *H.diminuta* eggs, compared to the nodule counts from the control insects. This occurred in all of the experimental replicates, although most notably in experiment 2 (Figure 6.4). There was no difference in trends found in data from cockroaches fed on either egg-suspension or eggs in proglottids.

No stimulation of nodule numbers was seen when saline was injected into *H.diminuta*-fed cockroaches 24 hours post infection, but when saline was injected at 48 hours nodule numbers per cockroach began to rise to reach a maximum at 3 days, after which values tailed off gradually until, after 8 days, they returned to control levels (Figures 6.3-6.5)

No stimulation of nodule formation was seen after the cockroaches received the homogenised egg suspension and supernatants (Figure 6.6).

6.3.2 EFFECTS OF *HYMENOLEPIS DIMINUTA* ON THE LAMINARIN-STIMULATED PHENOLOXIDASE PRODUCTION BY HAEMOCYTES *IN VITRO*

Laminarin activates the proPO pathway to produce PO which converts DOPA to melanin; the number of blackened cells or small cell aggregates produced in haemocyte monolayers from control (unparasitised) animals was very similar to that found by Huxham and Lackie (1986). However a highly significantly ($p < 0.001$ in all cases; Students t-test) greater proportion of the haemocytes from

H.diminuta-infected insects, 3 days post infection, were PO-positive after Laminarin stimulation (Figure 6.7).

There are significant differences between the *H.diminuta*-stimulated peaks in each experiment, which are possibly due to variations in conditions in the cell culture *in vitro* Also differing rates of passage of the oncospheres into the midgut of the insect may affect the results as discussed in Chapter 3.

6.3.3 EFFECTS OF *MONILIFORMIS MONILIFORMIS* ON THE INDUCTION OF HAEMOCYTIC NODULES BY ZYMOSAN SUPERNATANT *IN VIVO*

Injections of saline stimulated the production of only a small number of nodules as in controls in section 6.3.1; there was no significant difference between the effect HBS had on controls and on the cockroaches with well established infections that had not been injected with Zs.

Zymosan supernatant stimulated large numbers of nodules in the control cockroaches, but significantly fewer ($p < 0.001$, Students t-test) in the *M.moniliformis*-infected insects (Figure 6.8).

The situation was found to be reversed when cockroaches had been recently (2-3 days post-feeding) fed with *M.moniliformis* eggs; saline injections stimulated significantly more nodules than in control insects, but not to the same extent as that found in *H.diminuta*-fed cockroaches (Figure 6.9-6.11).

Cockroaches, containing *M.moniliformis* from orally-presented infections, were also found to produce fewer nodules in response to Zs stimulation (Figure 6.12). Once the assay had been performed, the infected cockroaches were dissected and the number of *M.moniliformis* larvae noted. Figure 6.13, a scatter graph of these 3 sets of pooled data ($\log_{10}$ nodules versus $\log_{10}$ parasite number + 1), shows the best, although small, negative correlation between the number of nodules and the number of parasites per host. The 95%

confidence limits for the slope (-0.2970±0.2717) of the regression curve ($\log_{10}$ y = -0.2970 x $\log_{10}$x + 2.1595) show that there is a tendency towards 0, ie. the depression in number of nodules is almost independent of numbers of parasites, and seems to be brought about by only very few larvae in the haemocoele. However, the highest numbers of nodules were found in those cockroaches with no *M.moniliformis* in the haemocoele (which were also similar to controls), and this alone would bias the linear regression curve towards a negative gradient.

6.3.4 EFFECTS OF *MONILIFORMIS MONILIFORMIS* ON LAMINARIN-STIMULATED PHENOLOXIDASE PRODUCTION BY HAEMOCYTES *IN VITRO*

As in section 6.3.2 the numbers of PO-positive cells or cell aggregates from the control cockroaches were found to be similar to those found by Huxham and Lackie (1986).

However, a highly significantly smaller proportion ($p < 0.001$, students t-test, in all cases) of the haemocytes from *M.moniliformis*-infected insects, 7-10 days post injection, were PO-positive after Laminarin stimulation (Figure 6.14).

6.3.5 AN *IN VITRO* ENCAPSULATION METHOD USING THE HAEMOCYTES OF *PERIPLANETA AMERICANA*

The following targets were found to be encapsulated readily (presented here in order of capsule quality and thickness formed around each material).

Positive controls

Silk loops - Haemocytes adhere over all of the surface within the first few minutes and later adhere to each other and spread to form a closely packed capsule after approximately 45-60 minutes. Silk loops were therefore chosen

as positive control targets for use in further experiments.

Monofilament nylon - Haemocytes adhere over much of the surface of the nylon within the first few minutes, to form thick but patchy capsules after 30-60 minutes of tumbling.

Human hair - Encapsulated readily in most cases, but capsule formation tended to vary considerably depending on the method used to clean the hair before use in the assay.

Similarly, cotton loops, catgut suture-loops and wool filaments were found to be encapsulated, but the structure of the capsules tended to be loose and amorphous and therefore difficult to compare between replicate experiments.

Negative control

Only one suitable negative control was found; pieces of ovariole dissected from female *P.americana* as used in section 5.2.4.2. Sections of ovariole remained free of haemocytes, except at the cut ends and at any region where the surface of the ovarioles had been damaged. This agrees with observations made *in vivo* and thus provides a very strong indication that the assay is reliable and that haemocytes used in the assay are able to dicriminate between different surfaces.

Encapsulation of parasites

Observations made during encapsulation *in vitro* of the above target materials and parasites are shown below. In all cases the haemocytes reacted to controls in the presence of parasites to the same degree as to control targets on their own.

H.diminuta oncospheres - Encapsulation was very rapid, followed by the conglomeration of individually encapsulated oncospheres into larger units.

Eventually, after 60-90 minutes the capsules appeared (using x100 magnification, Leitz Ortholux microscope) smooth. All movement of the oncospheres ceased after this time, although it was not determined whether death had occurred.

H.diminuta cysticercoids - Only very few cells were found adhering to the surface of the cysticercoids, possibly on areas where some damage had occurred during handling. This was most noticeable around the posterior end of the cysticercoid especially if the tail section had been lost.

M.moniliformis acanthors - As with *H.diminuta* oncospheres, encapsulation was rapid and eventually resulted in smaller capsules joining to form larger conglomerates containing many acanthors.

Stage I acanthellae and cystacanths - Very few cells adhered to the surface of the envelopes of the more advanced larvae, apart from small areas which may have been damaged through handling.

M.moniliformis with *H.diminuta* cysticercoids within the envelope (see section 5.3.2) - a small number of *M.moniliformis* cystacanths with *H.diminuta* cysticercoids inside their envelopes were used as targets and, as with stage I acanthellae and cystacanths, very few cells adhered to the envelope surface. However just after the parasites had been dissected from the cockroach it was noted that there were already some cells adhering to the envelope which were added to during tumbling in the cell suspension. This probably marked the point at which the oncospheres originally penetrated the envelope when injected into the cockroaches.

6.3.6 THE EFFECT OF ZYMOSAN STIMULATION ON THE SUCCESS OF *HYMENOLEPIS DIMINUTA* IN LOCUSTS

The mean number of larvae recovered from locusts injected with Zs was

significantly lower ($p < 0.001$ in all cases, Students t-test) than those found in control insects injected with HBS at 8 days post infection (Figure 6.15). The majority of larvae recovered from the experimental animals were at stage 1-2 (Voge and Heyneman, 1957), compared with stage 3-5 cysticercoids found in controls.

Gunnarsson (1988a) found that nodule formation *in vivo* peaked when locusts were injected with HBS 12 hours after injecting with Zs (as in condition 2, section 6.2.2.5), but in this work there was no significant difference in numbers of larvae recovered between the different Zs treatments. This suggests that the indirect effect Zs has on the development of oncospheres may be stimulated by the process of the injection itself in a similar manner to that found when nodule numbers increase after injections of saline in cockroaches.

6.3.7 THE DURATION OF ZYMOSAN SUPERNATANT EFFECTS ON THE SUCCESS OF *HYMENOLEPIS DIMINUTA* IN LOCUSTS

Examination of the contents of the haemocoele approximately 24 hours post-infection revealed many small non-melanised haemocytic capsules containing what appeared to be oncosphere larvae, although only the hooks could be seen through the layers of cells when viewed at 100x magnification (Leitz Ortholux). The reduction in numbers of *H.diminuta*, as found above, was later found to be a temporary phenomenon. Zs-treated locusts, dissected at 10 and 12 days post-infection, were found to be infected with similar numbers of larvae as those in control groups (Figure 6.16) (null hypothesis rejected at $p > 0.05$ in all cases), although at 8 days post infection the results were similar to those in the control groups. Development of the larvae in the experimental locusts at days 8 and 10 was found to be delayed compared with that of the controls. As in section 6.3.6, at 8 days post infection, the majority of the larvae were at stage 1-2 and by 10 days had reached stage 3-4

whereas in controls the majority were fully developed by this time (stage 5). It appears therefore that Zs injection resulted in a 3 day lag in development of the cysticercoid compared with the larvae in control insects, without resulting in an increase in mortality.

Figure 6.3 Stimulation of nodule-formation by saline injections into naive, *Hymenolepis*-fed or non-viable *Hymenolepis*-fed *Periplaneta americana*. Experiment 1

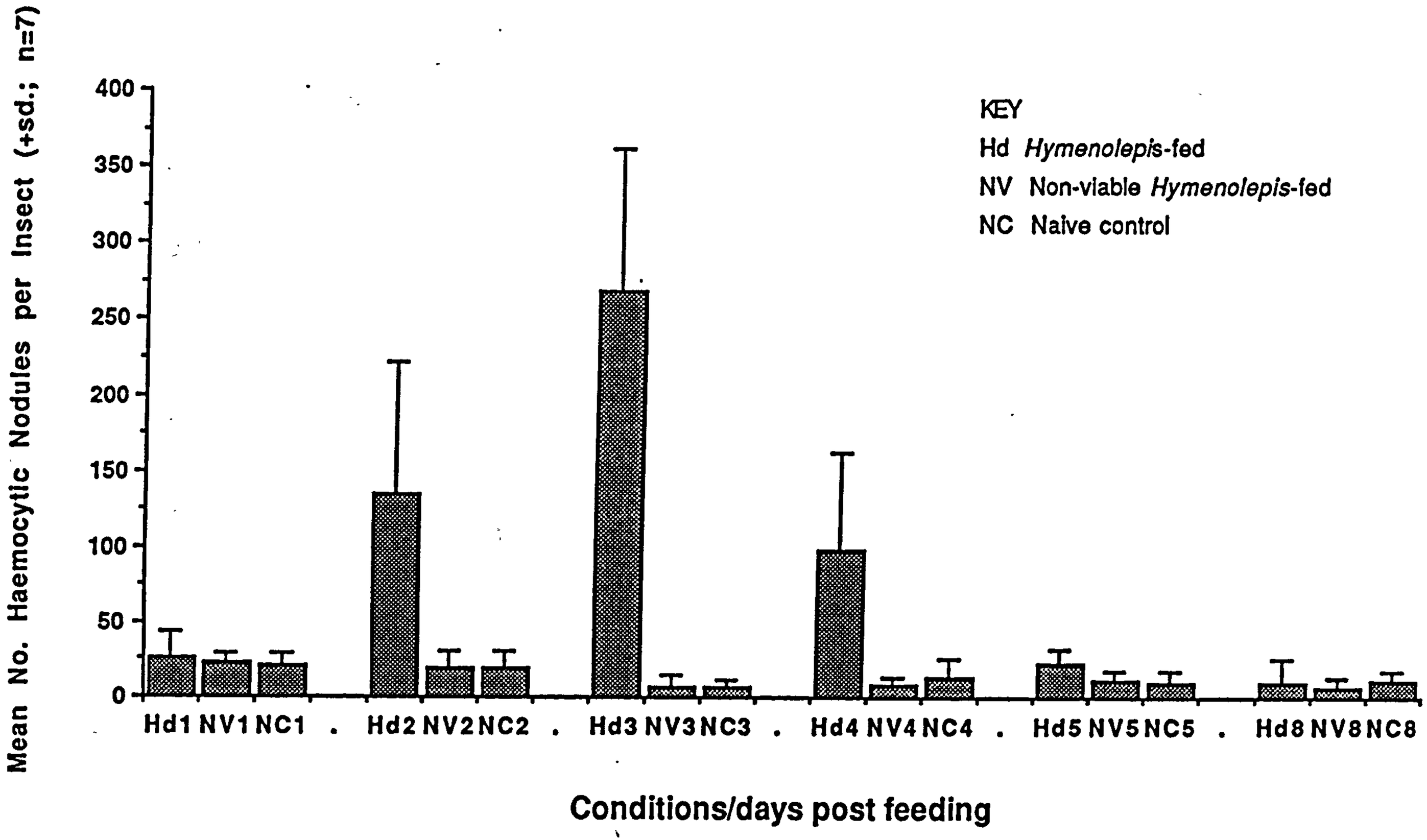


Figure 6.4 **Stimulation of nodule-formation by saline injections into naive or *Hymenolepis*-fed *Periplaneta americana*. Experiment 2.**

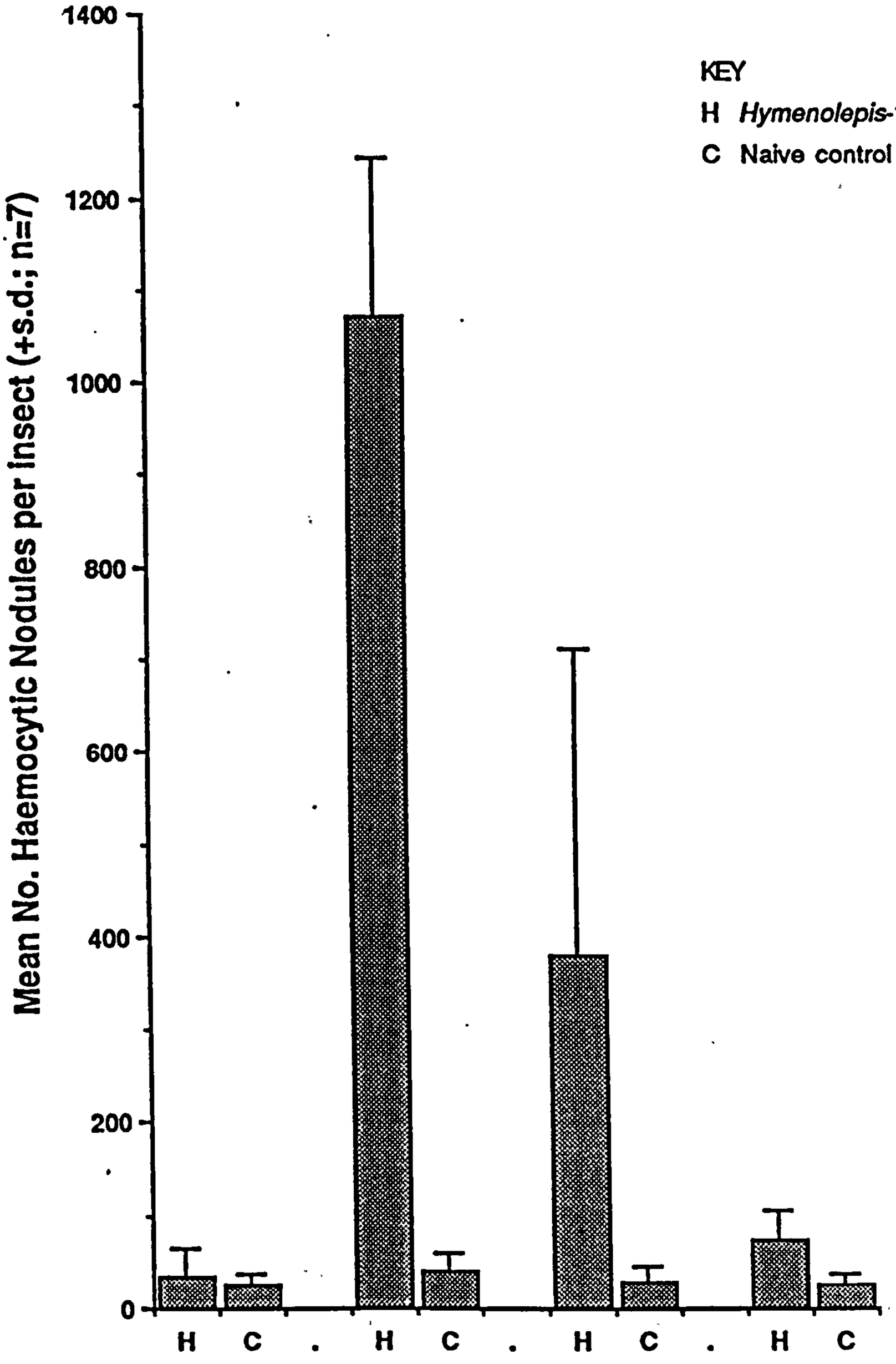


Figure 6.5 **Stimulation of nodule-formation by saline injection into naive and *Hymenolepis*-fed *Periplaneta americana*. Experiment 3.**

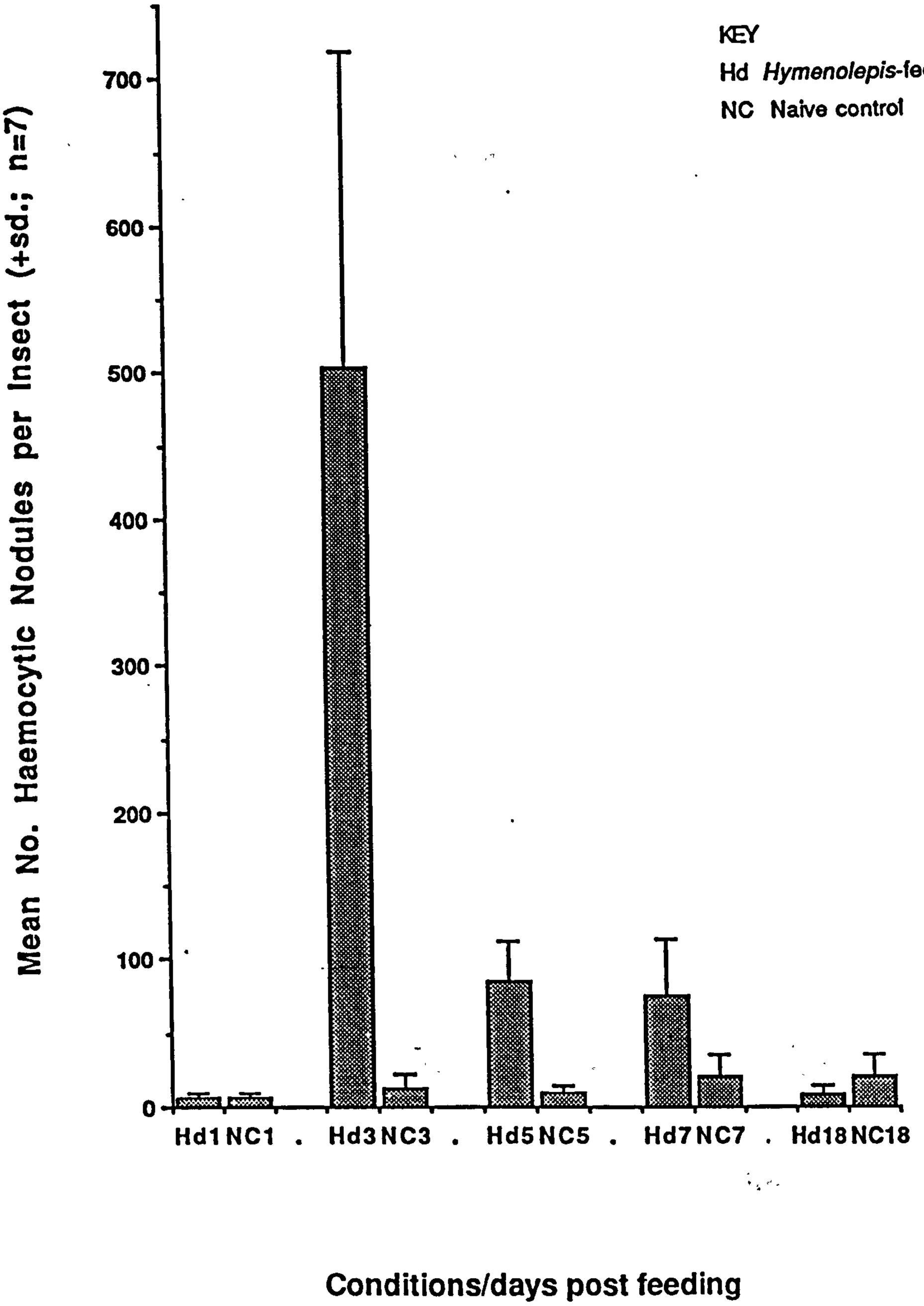


Figure 6.6 **Stimulation of nodule-formation by saline injection into control and *Hymenolepis*-fed *Periplaneta americana* 3 days post-feeding.**

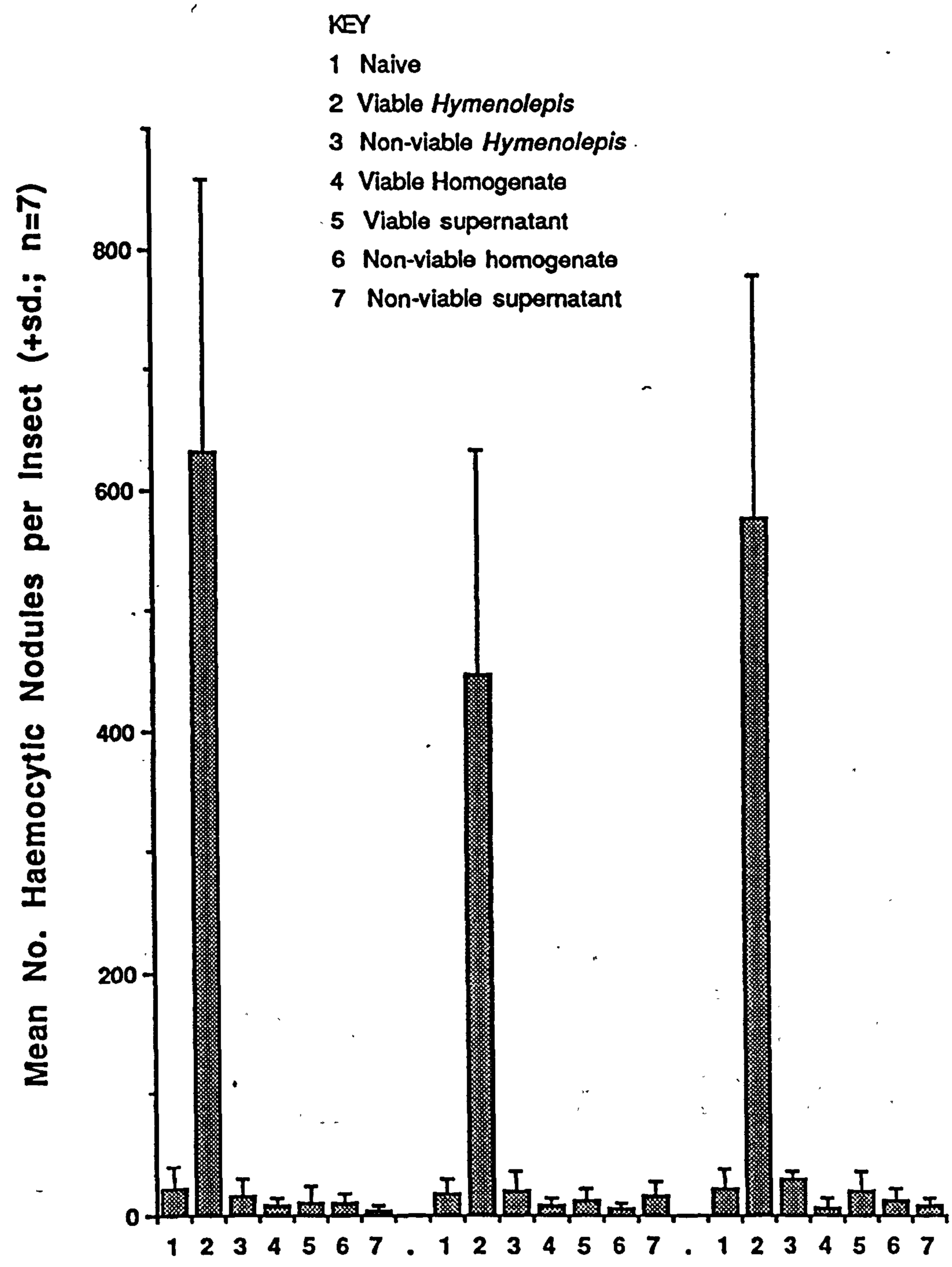


Figure 6.7 **The effect of *Hymenolepis diminuta* on the Laminarin stimulation of PO-positive cockroach haemocytes *in vitro*.**

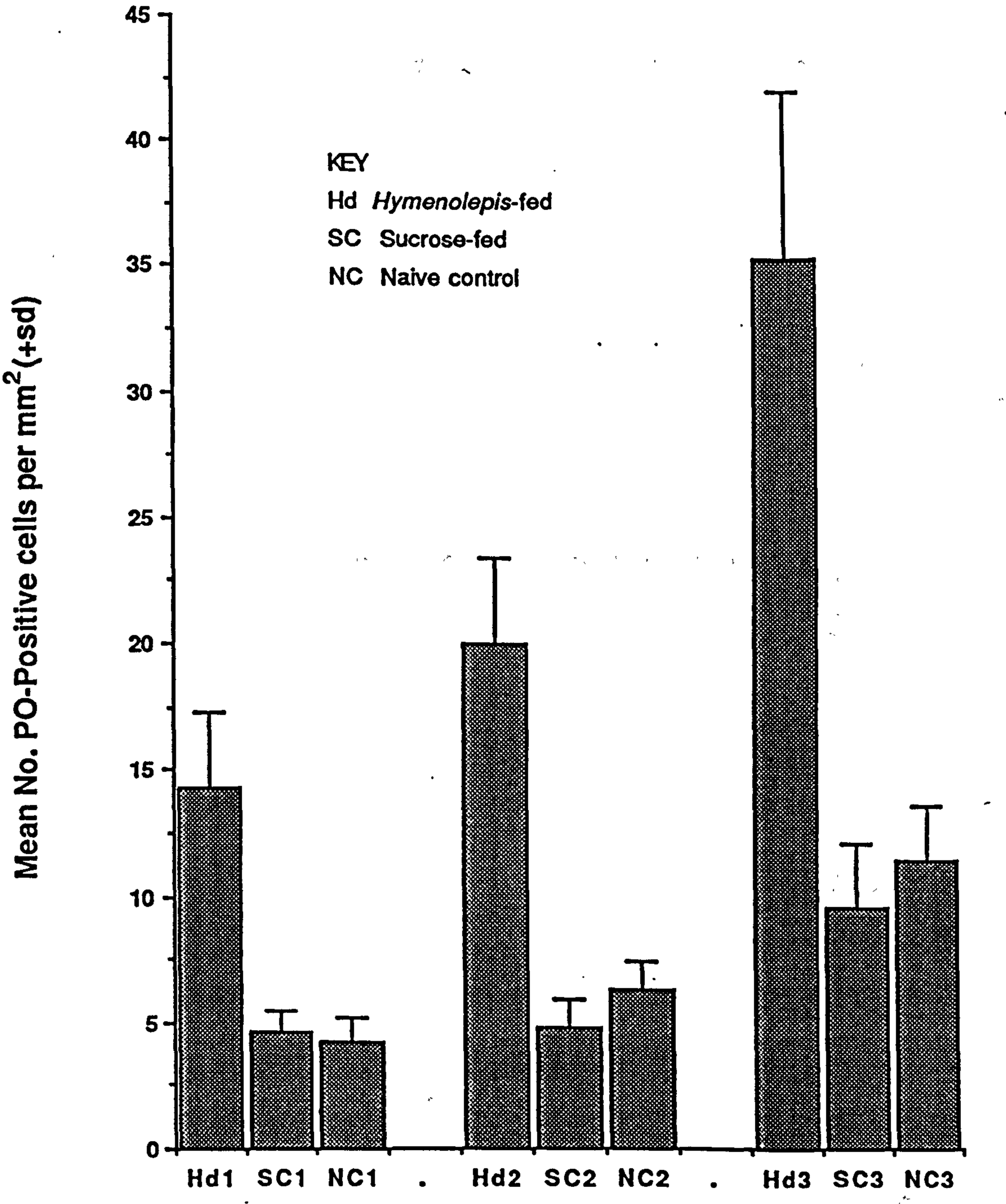


Figure 6.8 The effect of *Moniliformis moniliformis* on the induction of haemocytic nodules in vivo.

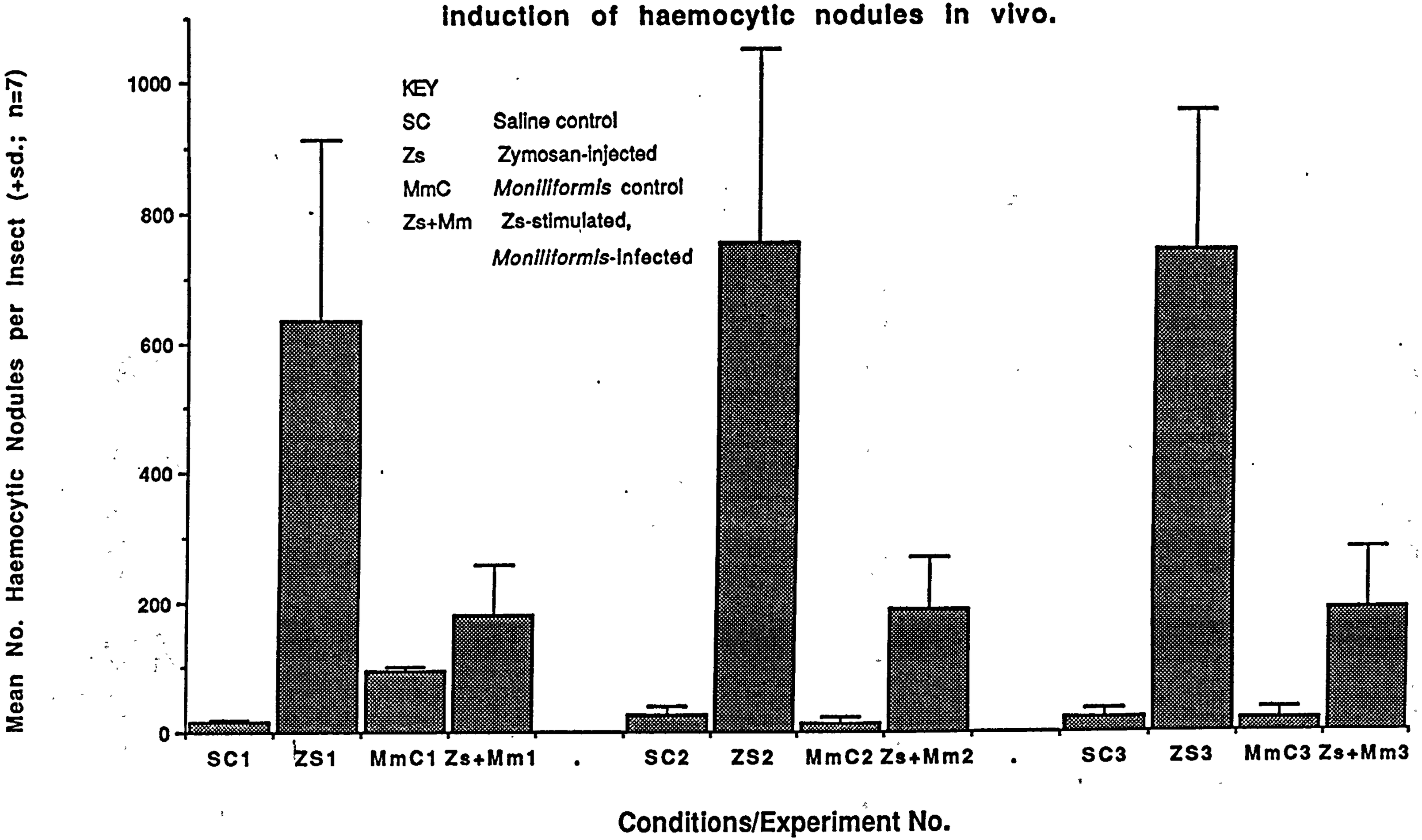


Figure 6.9 **Stimulation of nodule formation in *Periplaneta americana* by orally administered *Moniliformis* acanthors. Experiment 1.**

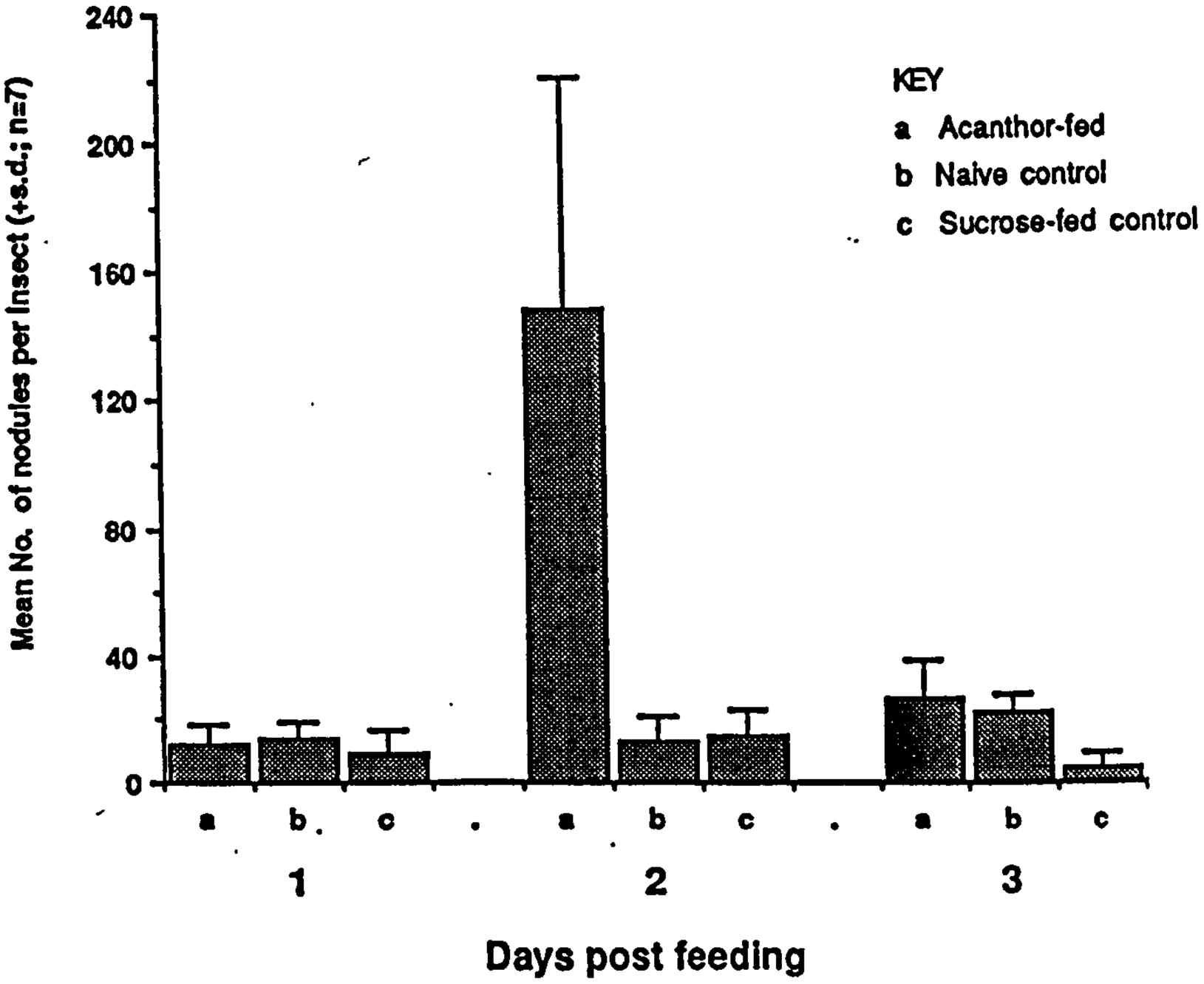


Figure 6.10 **Stimulation of nodule formation in *Periplaneta americana* by orally administered *Moniliformis* acanthors. Experiment 2.**

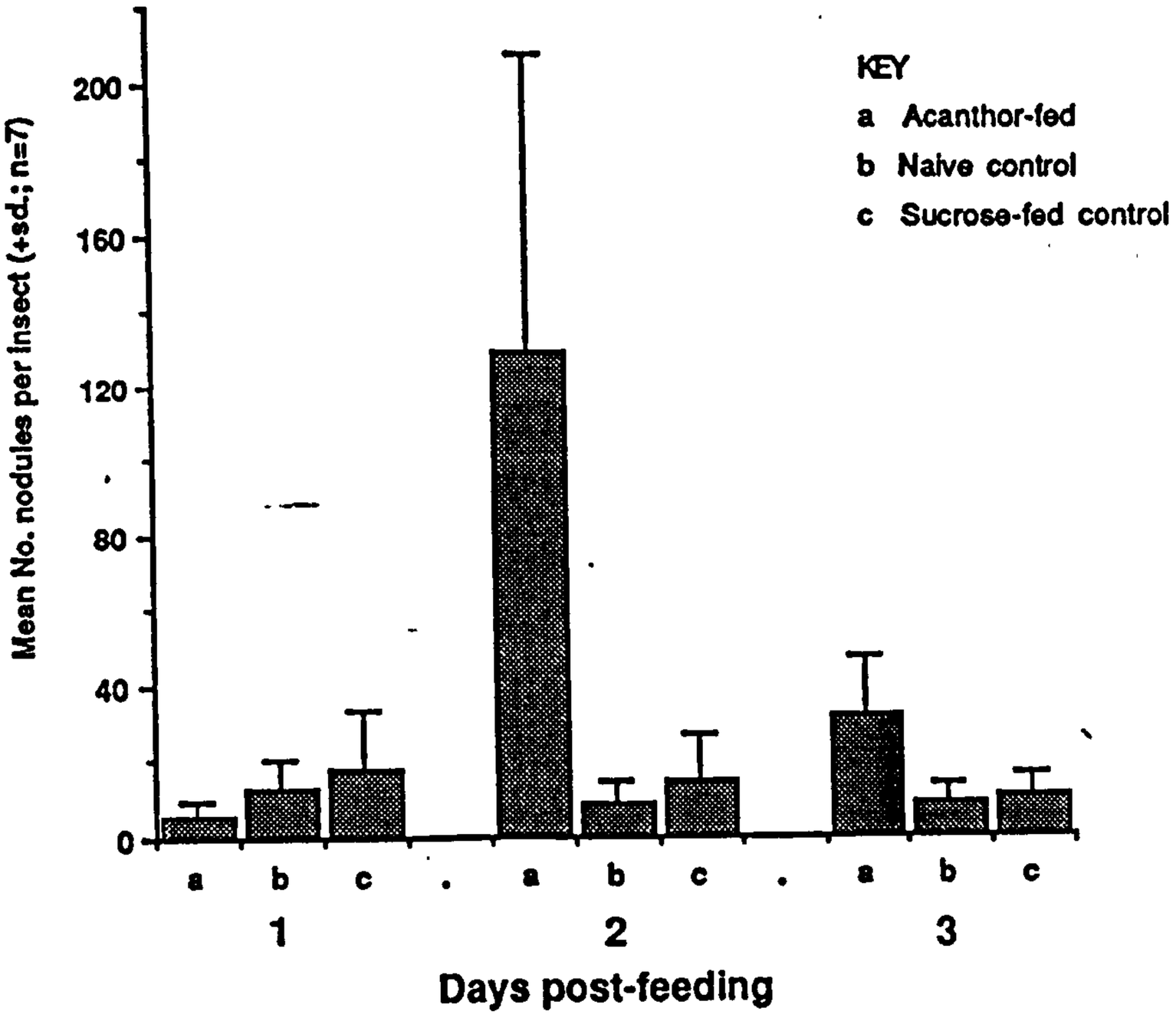


Figure 6.11 Stimulation of nodule formation in *Periplaneta americana* by orally administered *Moniliformis* acanthors. Experiment 3.

Figure 6.12 The effect of variable numbers of established *Moniliformis moniliformis* in cockroaches on the induction of haemocytic nodules by Zymosan in vivo.

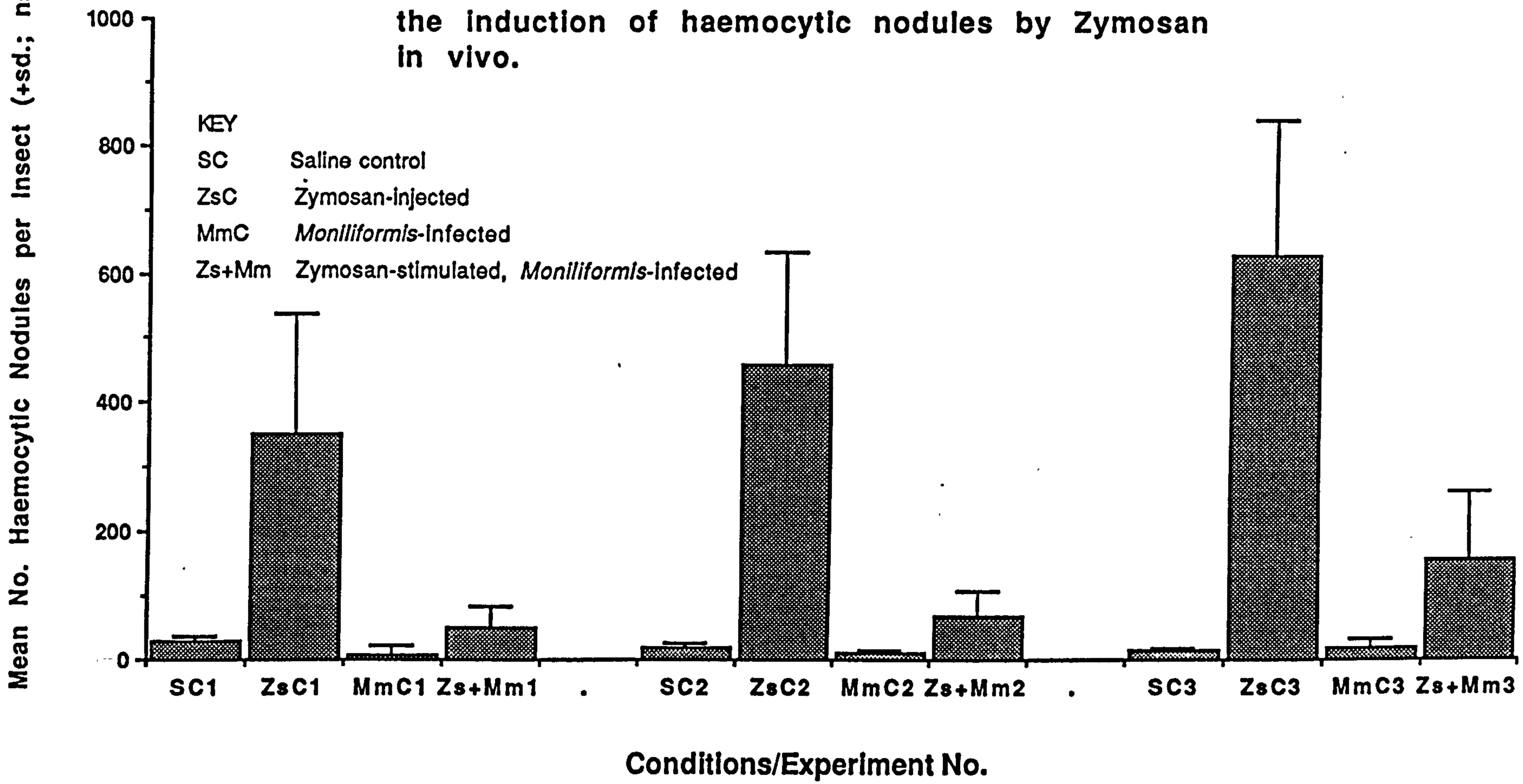


Figure 6.13 The relationship between haemocytic nodule count in Zymosan-stimulated cockroaches and numbers of *Moniliformis moniliformis* recovered 4 weeks pi.

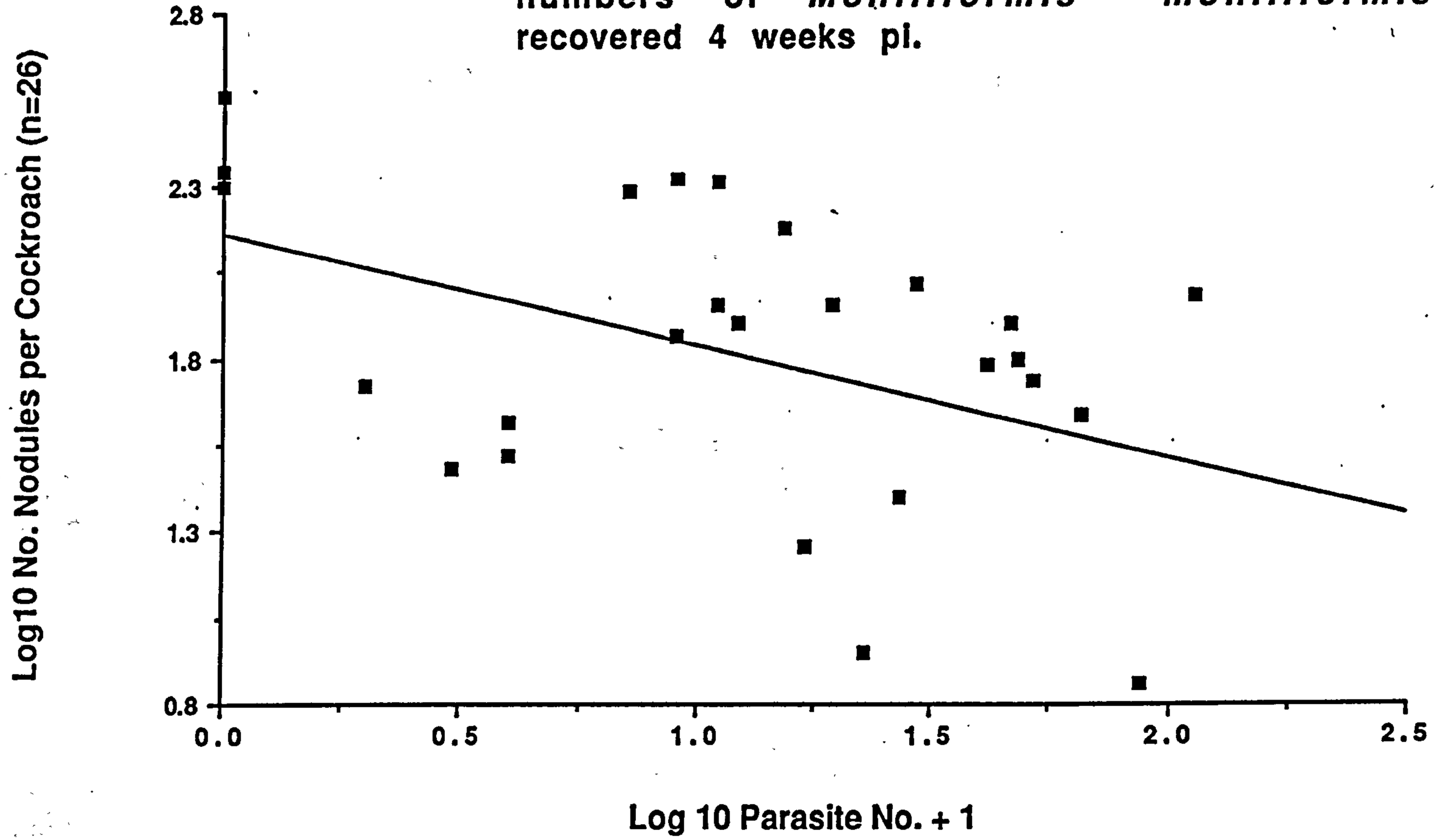


Figure 6.14 Effects of *Moniliformis moniliformis* on the Laminarin stimulation of PO-producing haemocytes *in vitro*.

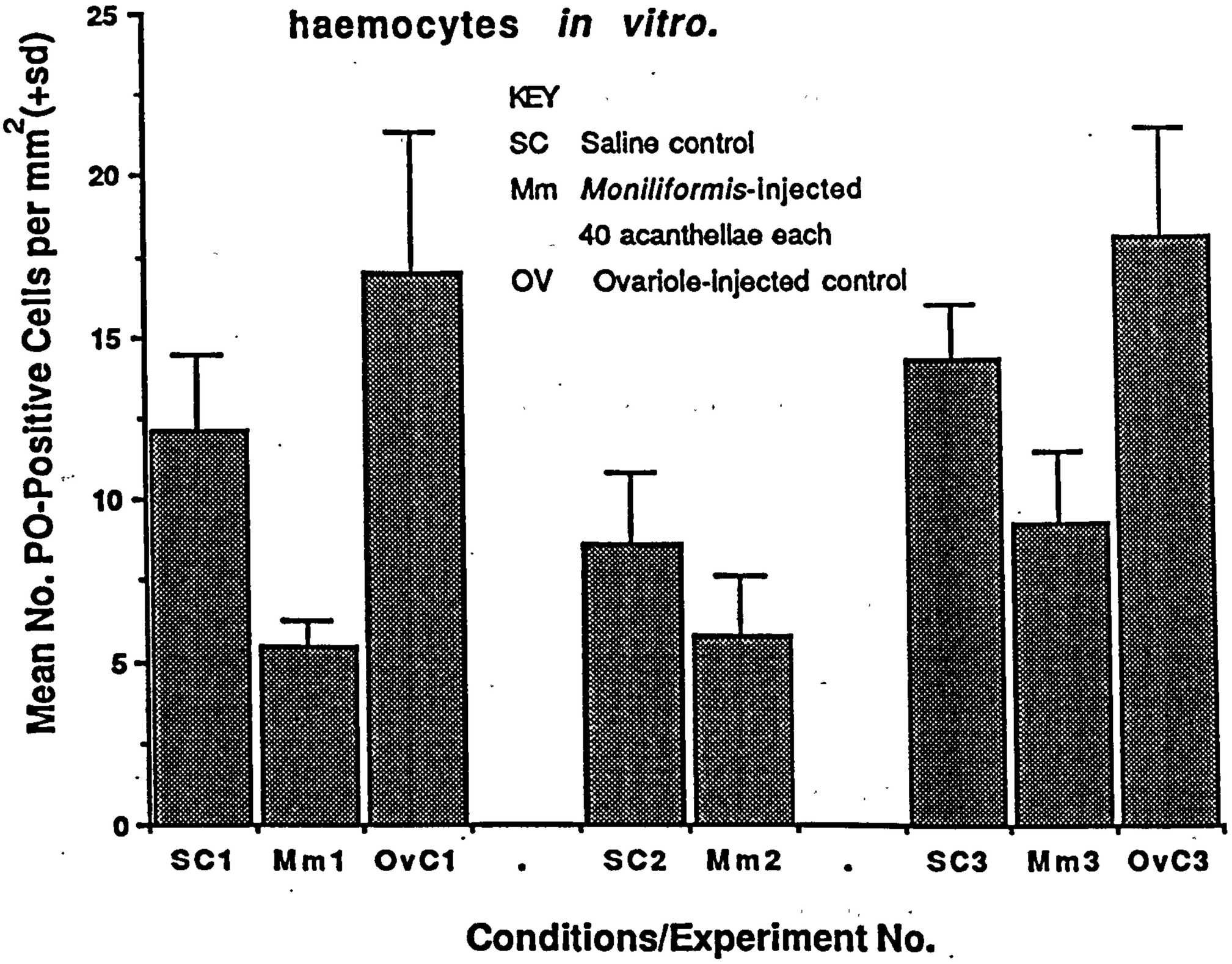


Figure 6.15 The effects of Zs stimulation on the success of *Hymenolepis diminuta* in locusts.

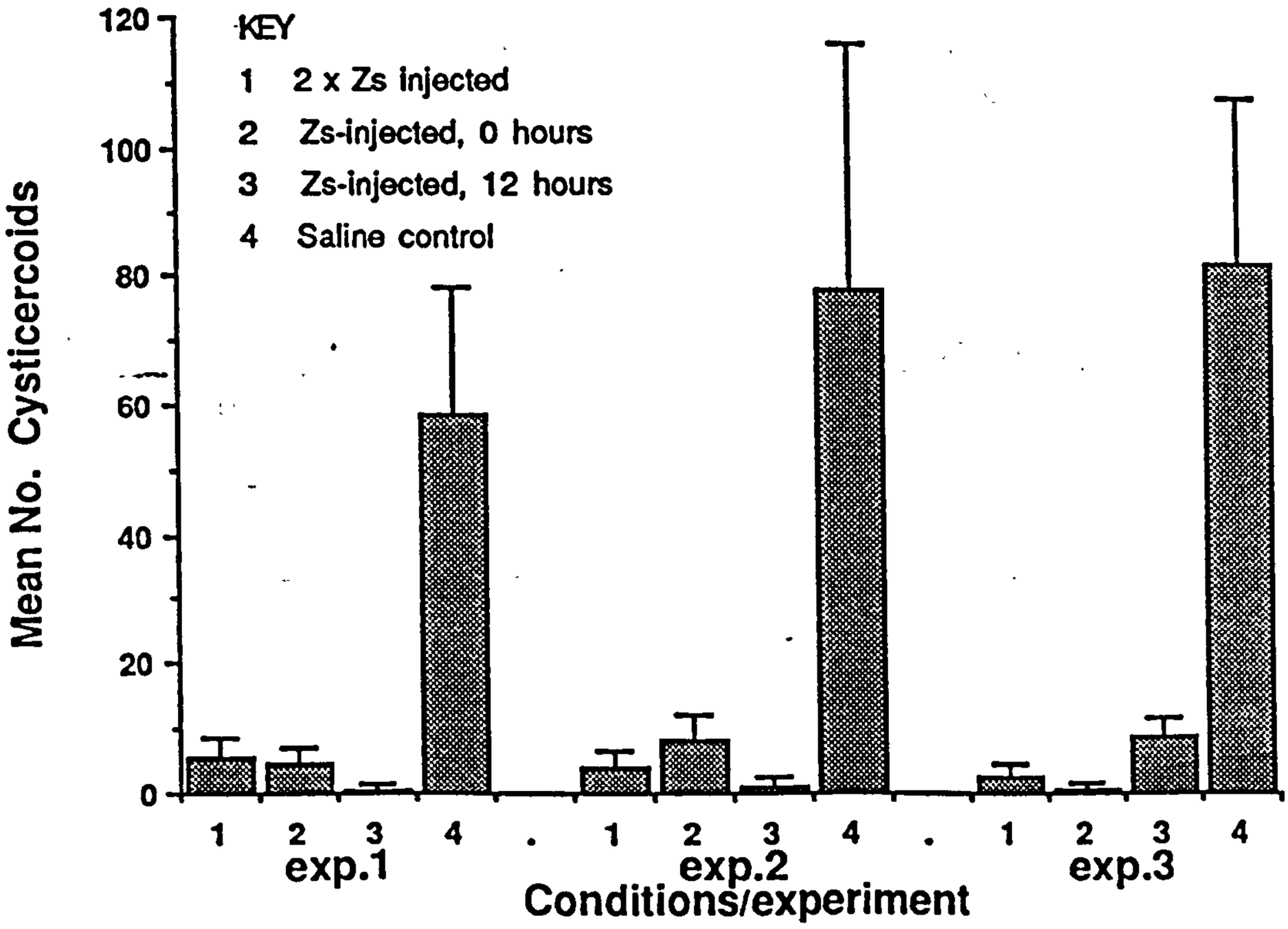
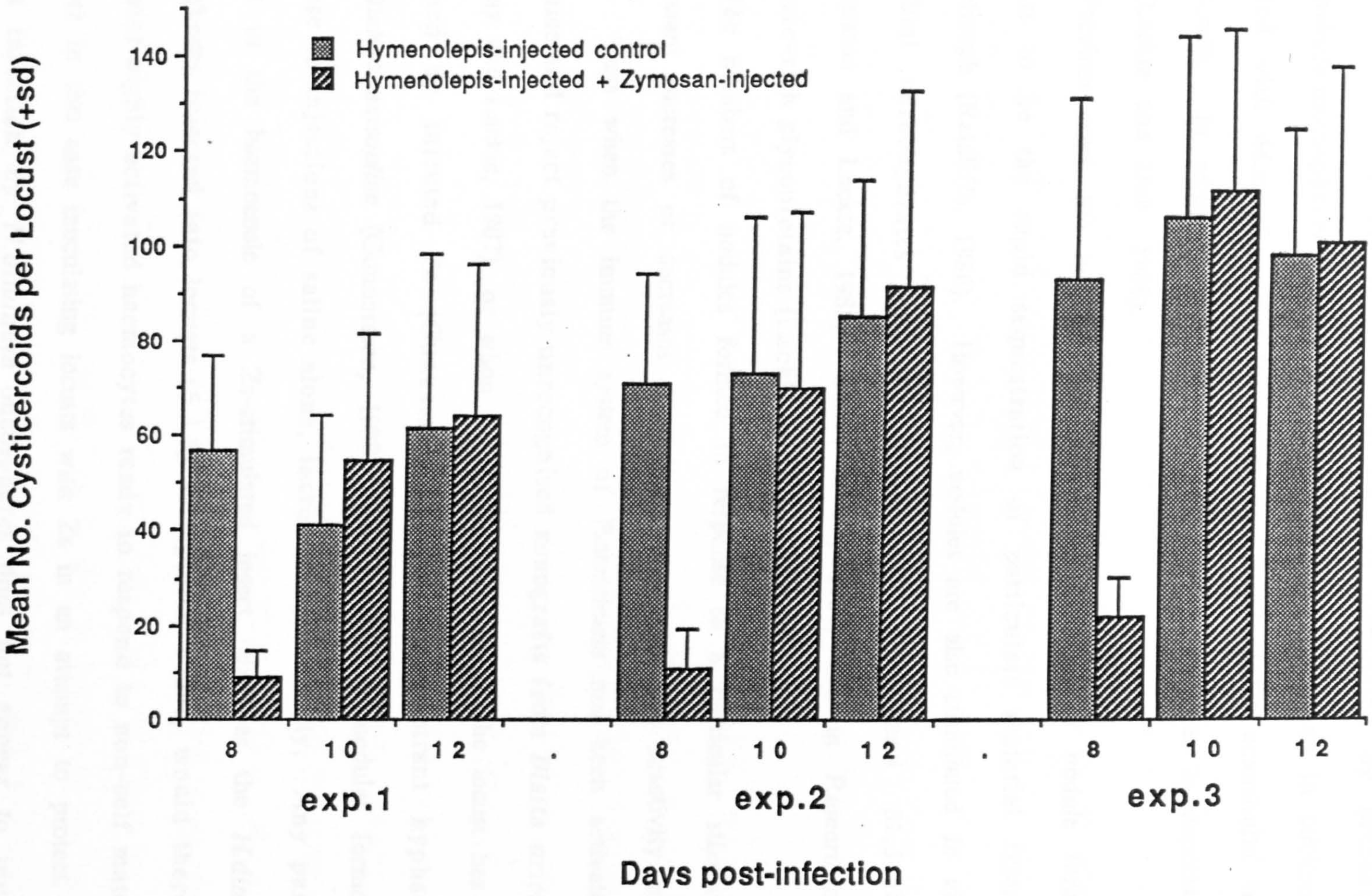


Figure 6.16 The duration of Zs effects on the survival of *Hymenolepis diminuta* in locusts.



6.4 DISCUSSION

The results of the assays for haemocytic behaviour indicate that certain aspects of the cellular immune response are stimulated by presence of *H.diminuta* oncospheres, in the cockroach gut, but depressed in cockroaches infected with *M.moniliformis* larvae at the stage II/III acanthella in the haemocoele. In addition, phagocytosing ability of haemocytes is depressed *in vivo* (Lackie and Holt, 1988).

Previous work has indicated that the main function of nodule formation appears to be the rapid sequestration of particulate material from the haemolymph (Ratcliffe, 1986). However, nodules are also stimulated *in vivo* by microbial carbohydrates such as lipopolysaccharides and β1,3-glucans (Gunnarsson and Lackie, 1985; Smith *et al.*, 1984) and in *P.americana* by galactose-rich glycoproteins (Lackie and Vasta, 1988).

The numbers of nodules formed in response to a particular stimulus or suppressant increases or decreases depending on the immune reactivity of the insect. Thus when the immune system of *P.americana* has been activated to recognise and reject previously unrecognised xenografts from *Blatta orientalis* (Dularay and Lackie, 1987), or when the immune system of the locust has been activated by injected Zs (Gunnarsson, 1988a) or penetrant hyphae of *Metarhizium anisopliae* (Gunnarsson, 1988b), the number of nodules formed in response to injections of saline alone, increases dramatically. Any parasite present in the haemocoele of a Zs-stimulated insect, such as the *H.diminuta* oncospheres injected into locusts (6.3.6) as reported above, would therefore encounter highly activated haemocytes ready to respond to non-self material. However in this case inoculating locusts with Zs in an attempt to protect them against infection by *H.diminuta* oncospheres does not appear to increase parasite mortality but merely delays their development by approximately 3 days. Microscopic observation of capsules recovered from the locust haemocoele, 24 hours post infection, revealed that the oncospheres had been

partially encapsulated but not melanised, thus for development to occur the cells forming these capsules must be lost at some point. This has led to the suggestion that temporary encapsulation results in the slowing of development, either through haemocyte damage to the oncospheres or prevention of the oncospheres absorbing nutrients. Dularay and Lackie (1985) demonstrated that β1,3-glucans (from Zymosan) caused a dramatic increase in phenoloxidase production in locust haemolymph lysate supernatant. This was similar to the affect seen by Takle (1985) in *P.americana*, Pye (1974) in *Galleria mellonella* larvae and Soderhall and Smith (1983) in various crustaceans. Why the oncospheres remained non-melanised in immune-stimulated locusts is unknown, perhaps they are not recognised as non-self as in the non-stimulated locusts or the oncospheres themselves possess the ability to block or avoid melanisation.

An alternative explanation for the arrest in *H.diminuta* development in the above experiment is that injected Zs perturbs the physiology of the locust so that the physiological environment becomes "unsuitable" for normal *H.diminuta* development. However, the finding of large numbers of partially encapsulated larvae soon after injection, which could not be found in these capsules at a later date, suggests that the first hypothesis was more likely.

The above situation is comparable to that found when acanthors of *M.moniliformis* burrow through the cockroach gut and emerge into the haemocoele where they are temporarily encapsulated by haemocytes (Lackie and Lackie, 1979). The cells forming the capsule disappear after larval development results in production of the protective envelope. The stimulus for this temporary encapsulation has been suggested to be due to damage of the surface of the acanthor as it burrows through the midgut wall, thus enabling the parasites to be recognised by the haemocytes (Brennan and Cheng, 1975). However the stimulus would seem to be more likely to arise from possible burrowing action of the larvae wounding the midgut tissues; as suggested in section 6.3.3, as orally administered acanthors significantly increase

haemocytic nodulation in response to saline injections. Wound factors may be released from the damaged tissues in the midgut to alert the haemocytes to encapsulate the penetrating larvae. Wound factors, such as "hemokinin" (Cherbas, 1973) have already been shown to change haemocyte morphology *in vitro* and may act at a distance away from the wound site. Certainly, results of assays for nodule formation and PO-positive haemocytes show that *H.diminuta* has a highly stimulatory effect on the immune system of *P.americana* from within the gut, although the mechanism of the stimulatory effect is unknown. This may explain the phenomenon seen in Chapter 4, where the presence of *H.diminuta* in the midgut adversely affected the survival of *M.moniliformis* acanthors as they penetrated to the haemocoele. The haemocytes may have been reacting to a wounding stimulus initiated by the *H.diminuta* oncospheres, and therefore their threshold for recognition of foreignness could have been lowered (Lackie, 1988a). If the thickness of a capsule formed around a foreign body is related to the degree of stimulation of the immune system, one would expect a tissue-destructive parasite to become encapsulated. This may be the case in this work, where *M.moniliformis* appears to initiate a small response and is therefore weakly encapsulated, whereas the response to *H.diminuta* is much greater, resulting in the encapsultion of any perasite entering the haemocoele.

Further work is required to study the state of the internal tissues of the midgut after an oral infection of *H.diminuta*, possibly by using scanning electron microscopy (Spies and Spence, 1985). So far studies of the gut at the light microscope level have not disclosed whether tissue damage has occurred which could stimulate the immune system. However there is no evidence suggesting anything other than the activity of the oncospheres is causing the immune stimulation; dead oncospheres, homogenates and supernatant preparations of oncospheres have no affect on the cockroach immune system, and only the presence of live, motile oncospheres initiates this response.

Gunnarsson (1988b) found that the hyphae of *Metarhizium anisopliae*, which penetrate the cuticle of insects in the initial stages of infection, caused haemocytic recruitment at the subepithelial basement membrane at the site of infection on the cuticle. This occurred before hyphal penetration was accomplished, possibly because the stimulus passed through the epidermal cells into the haemocoele.

Transmission of stimuli across tissues may also account for the stimulatory effect of *H.diminuta* in the cockroach gut. Midgut cells may be sensitive to physical disruption (wounding) or chemicals released by the oncospheres; alternatively soluble molecules pass into the haemocoele via intercellular junctions (Lane and Skaer, 1980), and thus the immune system may be alerted to the presence of the oncospheres and initiate an immune reaction.

The insect gut appears to show little uptake-selectivity, so that almost all solutes in the gut lumen readily pass through into the haemolymph (Treherne, 1967). As a consequence of this, haemolymph composition and volume (in the case of sap- and blood-feeding insects) may change considerably over a short period of time (Madrell, 1981). Lane and Skaer (1980) proposed that cells, in certain systems where consistancy of the extracellular fluids is critical to their function, are protected from possible fluctuations in their composition by tight junctions. However, penetration gland secretions or metabolites from the oncospheres or wound factors from damaged gut cells might leak through the gut wall and eventually make contact with the circulating haemocytes which may then lead to the observed change in haemocyte behaviour.

Littleis known about the effect a parasite may have on the insect immune system when burrowing through the gut wall. Glinski and Jarosz (1986) attempted to induce a defence response in *Galleria mellonella* larvae by feeding with pathogenic bacteria (*Pseudomonas aeruginosa*, *Serratia marcescens*, and *Xenorhabdus nematophilus*). Apart from a non-significant increase in blood lysozyme activity they found no reaction unless the bacteria were injected into the haemocoele (see also Jarosz and Spiewak, 1979). However, Chippendale

and Kilby (1969) suggested that the midgut pericardial cells, amongst other tissues, in *Pieris brassicae* may be involved in haemolymph protein biosynthesis and that proteins may cross the intestinal epithelium to reach the midgut lumen or haemolymph. It is also possible that unknown substances produced by *H.diminuta* could cross the gut wall in the cockroach.

Dow (1986 and personal communication) has suggested that the endocrine cells in the epithelium of the midgut of *P.americana* may alert the immune system if the midgut tissues are damaged and thereby alert immune and tissue repair functions (see also Endo and Nishiitsutsuji-Uwo, 1980, 1982). Indeed, it has been shown that extensive damage to the cells of the midgut of *Manduca sexta* larvae caused by orally administered *Bacillus thuringiensis* crystal endotoxin (Spies and Spence, 1985) was rapidly repaired so that the larvae did not suffer any prolonged ill effects.

In section 6.2.2.3 the cockroaches contained a standard number of *M.moniliformis* larvae, chosen because it approximated the mean number of larvae found after oral administration of eggs during standard laboratory maintenance of the parasite. However, it was found that *M.moniliformis* exerted a suppressive effect, almost totally independent of the number of parasites present. Thus, in orally infected cockroaches, showing a wider range of infection intensity, as few as 2-3 larvae appeared to depress the numbers of nodules formed. This compares well with data presented in Chapter 5 (section 5.4.2) where *M.moniliformis*-infected cockroaches with low numbers of *M.moniliformis* permitted increased numbers of injected *H.diminuta* to develop.

When considering how parasites may survive in the haemocoele by immunosuppressing the host, even one parasite must be capable of causing a similar immunosuppressive effect to that produced by several together, otherwise single parasites would be unable to resist encapsulation. Consequently, the result indicating that hosts infected with even very few

M.moniliformis were immunosuppressed to a similar level as those infected with many parasites was perhaps not surprising. This effect does not seem to render the host open to secondary unrelated infections (as discussed in Chapter 5), especially in the case of cockroaches which tend to thrive in bacteria-rich surroundings. Instead, the immunosuppressive effect could be acting in the immediate vicinity of the parasite in the haemolymph, ie. on or near the surface of the envelope of *M.moniliformis*, as suggested by Brennan and Cheng (1975). They hypothesised that polyanionic mucins associated with the glycocalyx of the envelope inhibit tyrosinase preventing the production of toxic quinones and therefore melanin. Thus the host could continue routine immune functions without affecting the parasite. However the assays used in this set of experiments suggest that the effect is a general one and the mechanism by which *M.moniliformis*-infected cockroaches remain free from unrelated infections is not clear.

The assays used in this work are all based on assessing changes in the proportions of cell types which have responded to various agents (stimulatory or suppressive). The changes in proportions of cells responding to a stimulus or displaying certain characteristics are then interpreted to the affects these agents might be having on the immune system *in vivo*

Previous work has shown that *in vivo* stimulation of the immune system of *S.gregaria* by a localised trans-cuticular invasion of *Metarhizium anisopliae* (Gunnarsson *et al.*, 1988) causes the number of PO-positive haemocytes produced in response to Laminarin stimulation *in vitro* and the number of competent phagocytes *in vivo* to increase. The total haemocyte count decreased steadily during the fungal infection, inversely corresponding with the development of the inflammation reaction and haemocytic involvement at the site of infection. There was also a marked increase in the proportion of circulating cells capable of phagocytosing latex beads by 25 hours after application of the fungus.

Similarly the initial stages of an oral infection of *P.americana* with

H.diminuta leads to an elevated number of cells capable of phagocytosis (M. Carr, personal communication) and of PO-positive cells and nodules (nodulation was also stimulated to a lesser extent by the acathors of *M.monilifmormis*). This stimulation must either cause an increase in numbers of responsive cells (or proportions of a responsive cell type) or in the ability of cells to recognise "non-self" or damage. At present the significance of these changes is not clear but are thought to be brought about by the affects both parasite species might have on the midgut wall. Possible roles for the proPO activation sequence in the immune response are discussed by Smith and Söderhäll (1986). Although the evidence is equivocal (Lackie, 1988a, 1988b), it is also possible that PO itself might exert a killing activity comparable to that of the peroxidase of mammalian granulocytes and snail haemocytes (Dikkeboom *et al*, 1987).

In *P.americana*, *M.moniliformis* appears to suppress the immune system, as in the assay for phagocytosing ability (Lackie and Holt, 1989), nodule production in response to Zs and numbers of PO-positive cells, cell proportions or absolute numbers of cells are reduced. This suggests that *M.moniliformis* may cause a reduction in numbers of cells (perhaps of a sub-population) either through delayed maturation (Peake, 1979) or changes in adhesion properties to haemocoelic surfaces (Chain and Anderson, 1983; M. Carr, personal communication), or to a decrease in their sensitivity to stimulation of the pro-PO pathway (Huxham and Lackie, 1986; discussed in Lackie, 1988a). These types of changes are an alternative to the mechanism of immunosuppression described by Brennan and Cheng (1975) as above.

The tendency for *P.americana* haemocytes to aggregate and form capsules in saline and various media *in vitro* has been successfully manipulated to occur under controlled conditions around defined targets. In this way, it has been demonstrated that the cells will encapsulate many materials, but more importantly react in different ways to larval stages of *H.diminuta* and

M.moniliformis. It appears that the haemocytes retain some ability to discriminate between different surfaces in this assay, which itself is an important aspect when considering other applications for its use. It has also shown that there are differences in the surface properties between the recently hatched larval stages and the older larvae of both parasite species taken from the insect haemocoele. Both the gut-penetrant stages of *M.moniliformis* and *H.diminuta* are readily encapsulated by *P.americana* haemocytes *in vitro* as *in vivo* but the cystacanths and cysticercoids are not. In the other work in this chapter it has been shown that these gut-penetrant stages stimulate the insect's immune system where as the older stages established in the haemocoele do not. This implies that compatibility with the host is established over a period of time and not necessarily immediately on entering the host at the start of an infection.

Davies and Vinson (1986) also found that the eggs of the braconid wasp, *Chardiochiles nigriceps*, remained free of cells in a similar *in vitro* encapsulation assay as that described above. They found that a fibrous layer on the egg's surface, which consisted of a neutral glyco- or mucoprotein, seemed to prevent encapsulation. This situation was also found to occur *in vivo*. Protoplasts of the fungal pathogen, *Entomophthora egressa*, evade encapsulation *in vivo* (in *Lambdina fiscellaria*) and *in vitro* (Dunphy and Nolan, 1980). The immune system of the host was still able to cope with unrelated invading microorganisms *in vivo* and would encapsulate nylon fibres *in vitro* The authors suggested that the protoplasts relied on electrostatic repulsion to evade the immune system. Söderhäll (1981) suggested a different mechanism for the survival of fungal pathogens in arthropods, which involved changes in the cell wall chemistry. He proposed that β 1,3-glucans, which stimulate prophenoloxidase and hence immune recognition were replaced by α-glucans which are non-stimulatory.

M. Carr (personal communication) has now used the same assay technique as described in section 6.2.2.7 to compare the ability of *H.diminuta*-stimulated

cockroach haemocytes to encapsulate latex beads *in vitro* and *in vivo* From both systems it has been found that haemolymph from cockroaches 3 days post-feeding with *H.diminuta* produces thinner capsules. This may be interpreted as further evidence for a change in the numbers and proportions of cells involved in immune stimulation.

6.5 SUMMARY

1. The assays used both show that *H.diminuta* larvae, and to a lesser extent *M.moniliformis* larvae when present in the gut, stimulate aspects of haemocytic behaviour of *P.americana.* This is presumed to be due to wounding of the midgut tissues by the larvae penetrating or attempting to penetrate the midgut wall. Suggestions have been put forward for the mechanism by which the stimulus activates the haemocytes.

2. The phenomenon seen in chapter 4 whereby *H.diminuta* adversely affected the success of *M.moniliformis* when fed simultaneously to cockroaches appears to be a direct consequence of the stimulatory effect of *H.diminuta* on the immune system.

3. The developing larvae of *M.moniliformis*, from stage II acanthella onwards have been shown to depress haemocytic activity in all the assays used. This may explain the elevated numbers of *H.diminuta* surviving in *M.moniliformis*-infected cockroaches.

4. Zymosan stimulates the immune system of locusts. Oncospheres of *H.diminuta* injected into these Zs-stimulated locusts are partially encapsulated by haemocytes, resulting in a slowing in the development of the parasites. However, this effect was temporary, did not involve a

melanisation reaction and the parasites eventually developed normally.

5. An assay has been devised to observe the encapsulation of target materials *in vitro* by the haemocytes of *P.americana.* It has been used to show differences in the haemocytic encapsulation of the different larval stages of *M.moniliformis* and *H.diminuta.* Suggestions for further applications of the assay have been put forwards.

CHAPTER SEVEN

GENERAL DISCUSSION

Figure 7.1 SCHEMATIC REPRESENTATION OF THE POSSIBLE PATHWAYS LEADING TO THE INFECTION OF THE INSECT INTERMEDIATE HOST BY *HYMENOLEPIS DIMINUTA*

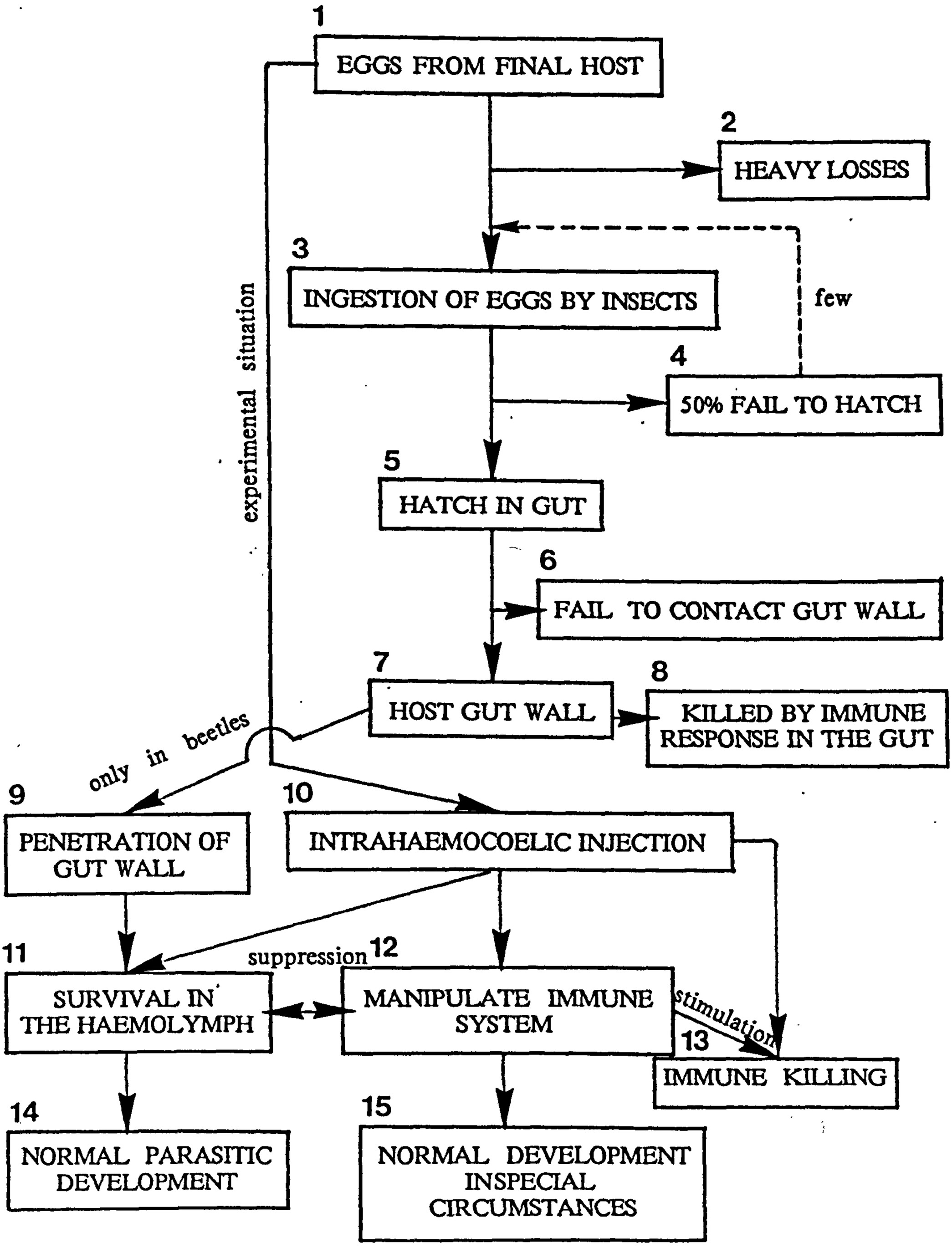
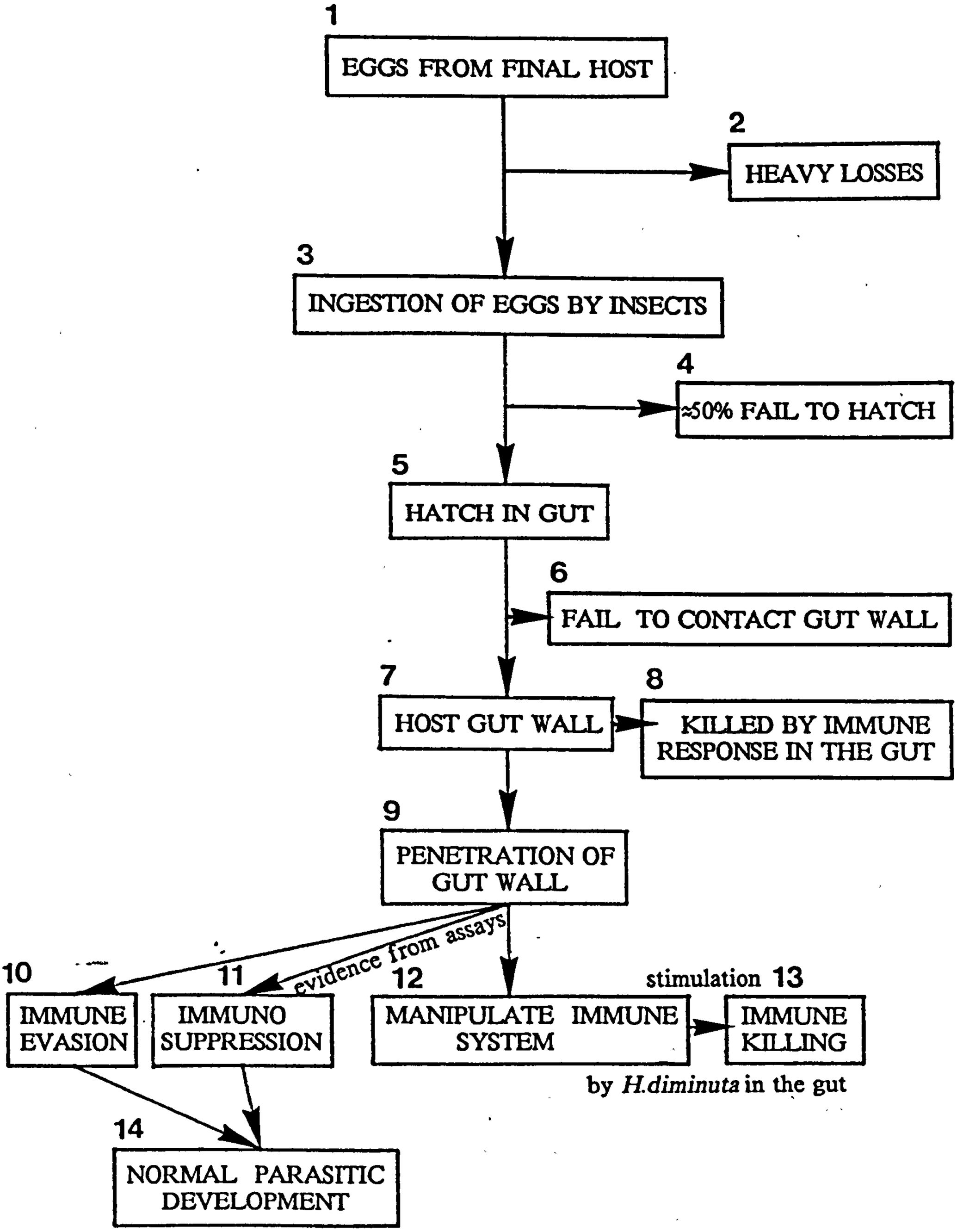


Figure 7.2 SCHEMATIC REPRESENTATION OF THE POSSIBLE PATHWAYS LEADING TO THE INFECTION OF THE INSECT INTERMEDIATE HOST BY *MONILIFORMIS MONILIFORMIS*



7.1 PARASITE ADAPTATIONS TO THE INTERMEDIATE HOST

The faeces of rats harbouring concurrent infections of *Hymenolepis diminuta* and *Moniliformis moniliformis* may contain the eggs of both helminth species for approximately 6-11 weeks (see Chapter 2). Factors affecting egg output in the definitive host have been discussed in section 2.4; for the purposes of the following discussion it will be assumed that, during this period of concomitant patent infections, it is possible for a potential intermediate host to ingest eggs of both host species at the same time. Figures 7.1 and 7.2 show a schematic representation of the processes involved in the parasitisation of the cockroach by *H.diminuta* and *M.moniliformis* respectively and will be referred to throughout this chapter.

7.1.1 HATCHING IN THE HOST GUT

By assessing the success rate in hatching of eggs of both *H.diminuta* and *M.moniliformis* within the guts of various insect species, it appears conditions are conducive to normal hatching (ie. approximately 45-50% of the ingested eggs hatch, see Lethbridge, 1971a and Figure 7.1 parts 1-5) in a wide range of insect species (sections 3.1.2 and 3.1.3). However, the mechanisms of egg-hatching are different for each parasite species as discussed in section 3.4. *H.diminuta* egg shells require physical cracking by the insect's mouthparts to allow water and enzymes to act on the subshell layers. This action causes swelling and digestive breakdown of the subshell layers, resulting in emergence of the active oncospheres after 15 to 30 minutes (Berntzen and Voge, 1965; Lethbridge, 1971a; Ubelaker, 1980; Voge and Berntzen, 1961). The eggs of *M.moniliformis* do not require physical disruption; exposure to a suitable concentration of an ionic solution stimulates acanthor activity and eventually hatching approximately 4 hours later (section 3.3.3, see also Edmonds, 1966).

Conditions in guts of both beetles and cockroaches fulfil the

requirements for the hatching of both parasite species, but the hatching success of *H.diminuta* in *Schistocerca gregaria* was low (section 3.4, and Lethbridge, 1971c). On close investigation it was found that the majority of eggs were passing through the mouthparts of the locust without being cracked. This was probably because the mechanics of the locust mouthparts are adapted for chewing and biting large fragments of plant material therefore allowing smaller items such as *H.diminuta* eggs to pass into the crop undamaged.

7.1.2 CO-ORDINATION OF PARASITE HATCHING WITH DIGESTIVE PROCESSES IN THE INSECT GUT

By following the progress of the parasite eggs along the insect gut (section 3.3.3) and observing hatching, some insight into the reasons for host specificity of the two parasite species has been gained. The co-ordination of digestion, ie. the timing of food movements along the gut and the release of enzymes, has been shown to be an important consideration in the success of a parasitic infection. The intima, the cuticular lining of the crop and hindgut, forms a physical barrier to the penetration of larvae in these regions of the gut. The parasites must therefore reach the relatively unprotected midgut in a state in which they will be capable of penetrating the gut wall. Transit times for food passing down the gut vary considerably between species of insect. Cockroaches tend to store food in the crop before it is passed, in a near-digested state (Chapman, 1985b), into the midgut. For example, *Leucophaea maderae* has been shown to store food in the crop for as long as 50 hours after ingestion (Day and Powning, 1949; Davey and Treherne, 1963a, b, 1964; Engelmann, 1968). In beetles, food travels along the gut relatively quickly (approximately 5 hours in adults, 2 hours in larvae), which in the past has been considered as a reason for the inability of *H.diminuta* oncospheres to penetrate larval beetle gut as they may be swept

along the gut too quickly to begin penetration (Voge and Graiwer, 1964).

The processes involved in the co-ordination of digestion are subject to variation depending on a number of factors including nutritional status of the host; this has been discussed in Chapter 3 and also in the review by Chapman (1985b).

Thus the two species of parasite seem to be adapted to the different rates at which they reach the midguts. A hypothesis has been put forward (section 3.4) that the co-ordination of digestion in *P.americana* and other cockroach species, involving the storage of food items in the crop, prevents the majority of *H.diminuta* larvae, hatched by the insect's mouthparts, from reaching the midgut. The larvae are retained in the cuticular-lined crop along with food undergoing digestion. Here the oncosphere's limited glycogen supplies eventually are used up (Lethbridge, 1971a) so that when the larvae are released into the midgut they are incapable of penetration (Figure 7.1 part 6). *M.moniliformis* acanthors, on the other hand, require several hours exposure to crop-like conditions (Edmonds, 1966) before hatching is completed, and therefore pass into the midgut recently emerged and capable of tissue penetration. *H.diminuta* larvae appear to be better adapted to the relatively quicker digestive processes in beetles, where they penetrate the anterior portion of the midgut soon after hatching.

Not all active *H.diminuta* larvae are prevented from reaching the midgut. A small number of hatched and motile *H.diminuta* oncospheres have been found in the cockroach midgut 8 hours post-feeding. Presumably these have been cracked while passing through the teeth of the proventriculus and not by the cockroach's mouthparts during mastication. Whole eggs would eventually be passed out of the insect in a viable state with the faeces, but for the grinding action of the proventriculus (see section 3.4). The small numbers of hatched larvae found in the midgut of cockroaches have been implicated as being responsible for changes in the immune response of the insect host (see Chapter 6) which will be discussed in further detail below. Any intact,

viable eggs passing out of the cockroach in the faeces may infect other insects; in this way the cockroaches may act as a paratenic host for *H.diminuta* (Lethbridge, 1971a; Figure 7.1 part 4).

7.1.3 PARASITE PENETRATION OF THE INSECT GUT

The next step in the life cycles of both parasites, once hatching has occurred, is to penetrate the gut to continue development in the insect haemocoele (Figure 7.1, parts 7, 9 and 11; Figure 7.2, parts 9, 10, 11 and 14). Comparison of insect gut penetration *in vivo* and *in vitro* by the two species of parasite (Chapter 3) has demonstrated further the differing abilities of *H.diminuta* and *M.moniliformis* to infect cockroaches. Previous studies on the morphology and histology of beetle guts has shown how structural differences between larval and adult gut may prevent oncospheral penetration of larval gut tissues (Voge and Graiwer, 1964; Lethbridge, 1971a). There is some evidence from this work to support the suggestion that morphological differences in gut structure might affect penetration, as oncospheres were observed penetrating the midgut *in vivo* and *in vitro* via the midgut papillae in adult *Tenebrio molitor* (Figure 3.12.1 and 3.12.2).

Cavier and Leger (1965) and later Leger and Cavier (1970) investigated penetration of the insect gut by oncospheres of *Hymenolepis nana.* Their findings were similar to those using *H.diminuta* in this work; the larvae hatched in the guts of cockroaches (*Periplaneta americana, Leucophaea maderae* and *Blaberus fusca*) and beetles (*Tenebrio molitor*), but could not burrow through the gut wall of cockroaches. The difference in penetrability was attributed to variations in the structure and morphology of the peritrophic membrane in different species of insect. Insects with a robust, continuous peritrophic membrane with a small pore size, as in *P.americana*, prevented parasite penetration, whereas insects with a less robust peritrophic membrane allowed the oncospheres to gain access to the gut wall tissues. *H.nana*

oncospheres have a diameter of approximately 20μm (similar to *H.diminuta*) and although they can elongate to fit through narrower spaces, the above authors concluded that the small pore size (0.15-0.20μm) in the meshwork of the peritrophic membrane of *P.americana* and its thickness prevented passage of the parasites.

In this work very few oncospheres penetrated *Schistocerca gregaria* gut, and even when the peritrophic membrane was removed from *P.americana* guts *in vitro* no larvae managed to penetrate the exposed gut wall contrary to results expected on the basis of Leger and Cavier's work (1970). Evidently not only the peritrophic membrane presents a barrier to infection but also the gut wall.

Only *M.moniliformis* were found to penetrate *P.americana* midgut *in vitro* and *in vivo* as well as the guts of the other insect species studied. This difference in tunnelling ability may be explained by the differences in cutting and penetration mechanisms utilised by each species of parasite as discussed in sections 3.1.3, 3.1.5 and 3.4. The oncosphere larvae of *H.diminuta* possess 3 pairs of hooks which are used in a clawing fashion to tear through the tissues of the gut whereas *M.moniliformis* cuts through the tissues using the rostellar blades (see Ogren, 1969; Lethbridge, 1971a; Lethbridge and Gijsbers, 1974; Moczon, 1977; Holmes and Fairweather, 1982 for *H.diminuta* , and Moore, 1946; Robinson and Strickland, 1969; Whitfield, 1971, J.M. Lackie, 1973; for *M.moniliformis*).

7.2 PARASITE INTERACTIONS WITH THE HOST IMMUNE SYSTEM

7.2.1 STIMULATION OF THE IMMUNE RESPONSE FROM WITHIN THE GUT

This section is devoted to examining the possible link between activities of *H.diminuta* and other parasites and pathogens in the insect gut, and the

stimulation of the immune response. The origins of this stimulus have been considered under 2 sub-headings:

1) Actual physical damage due to parasite burrowing and penetration of the tissues causing alterations in the permeability of midgut cell membranes. This could lead to release of "wound factors" or stimulation of a putative neurosecretory link.

2) "Foreign" molecules present in the gut lumen, reaching the haemocoele, possibly via intercellular junctions.

7.2.1.1 PHYSICAL DAMAGE

The evidence presented so far implies that the cockroach gut acts as an effective barrier to penetration and infection by *H.diminuta*, even when the eggs are presented in unnaturally high doses, so that natural infections of cockroaches with *H.diminuta* would seem impossible.

It had been initially suggested (Chapter 4, section 4.1) that the cutting action of *M.moniliformis* on the insect gut wall *in vivo* might facilitate *H.diminuta* penetration if the integrity of the gut wall was affected by the burrowing action of the first parasite. Thus both parasites were fed simultaneously to a range of insect species. In beetles, although both species penetrated the gut wall, *H.diminuta* developed normally and *M.moniliformis* only developed as far as early stage I acanthellae. In locusts, both parasites developed normally, although somewhat more slowly than in normal hosts (see Lethbridge, 1971c for *H.diminuta* development in *Schistocerca gregaria* and section 4.4 for *M.moniliformis* in *Schistocerca gregaria*).

The results of the cockroach infections were especially interesting in so far as the results initiated a new area of research. None of the ingested *H.diminuta* developed, and there was also a significant reduction, compared with controls, in the numbers of stage I acanthellae *M.moniliformis* found 2

weeks post-infection. This effect could not be explained by the "dilution" in number of *M.moniliformis* eggs presented to the insects (ie. the egg suspensions of *H.diminuta* and *M.moniliformis* were mixed, therefore each insect received approximately half the number of eggs of each parasite), and was only found when viable *H.diminuta* eggs were administered along with *M.moniliformis*.

Gordon and Whitfield (1985) found that the presence of *Raillietina cesticillus* in *Tribolium confusum* caused a reduction in numbers of *H.diminuta* developing in a challenge infection. The above authors could not explain these results in terms of competition for space and nutrients. Instead, they speculated that *R.cesticillus* may stimulate changes in the immune response, which, without affecting the stimulator species, may have resulted in the decrease in numbers of *H.diminuta* present in the haemocoele. However, they did not investigate the affect of this two-species infection on the insect immune system. Although in these beetles both parasites were found developing together in the haemocoele, the situation may bear some similarities to that of *M.moniliformis* and *H.diminuta* in the concurrent infections of cockroaches.

In the work described in this thesis, assays for haemocyte behaviour were adapted to determine the affects *H.diminuta* and *M.moniliformis* had on the immune system during the initial stages of infection (Chapter 6). These assays were also used to investigate the affects of established infections of *M.moniliformis* on the cockroach immune system (see below).

The assays indicated that ingested *H.diminuta* oncospheres caused a stimulation of the cockroach immune system which reached a peak 3 days post-feeding (see section 6.4). Orally introduced *M.moniliformis* were also shown to cause some immune stimulation, but not to the same degree as *H.diminuta* The question remained; where and how did this stimulus originate? One hypothesis has been put forward in this work which states that the insect's immune system is responding to wounding of the tissues in the gut lumen, caused by oncospheres attempting to tear into the gut cells to penetrate the

gut wall using their hooks. The difference between the sweeping tearing action of the oncospheral hooks (Moczon, 1977) and the cutting action of the acanthor's rostellar blades (Whitfield, 1971) may also explain the difference in levels of stimulus on the immune system. Thus if it could be shown that *H.diminuta* causes more damage to the gut tissues than *M.moniliformis* it would explain in part the differences in immune stimulation caused by the 2 species (see section 6.4).

However, scanning electron microscopical and histological investigations (section 4.3.4) were unable to provide any evidence of mechanical disruption of the gut wall by the parasites. Although tissue damage is difficult to locate in the gut by any method, *H.diminuta* oncospheres were not observed penetrating or attempting to penetrate the gut wall. It was originally thought that the perturbation of the immune response might have originated from oncospheres trapped in the tissues of the gut wall but there were no traces of melanised or encapsulated oncospheres in the gut wall tissues.

The stimulation of the immune system by *H.diminuta* from within the gut lumen or tissues may help to explain why *M.moniliformis* were found in reduced numbers when compared to controls in the two-species infections of cockroaches. Under normal conditions, acanthors are temporarily encapsulated by a thin layer of cells as they enter the haemocoele; the cells eventually disappear as the larvae begin development (J.M. Lackie and Rotheram, 1972). In the two-species infection experiments any *M.moniliformis* acanthors reaching the haemocoele might have been encapsulated more thickly than normal and melanised, resulting in a decrease in numbers surviving compared with controls (Figure 7.2 parts 12 and 13). Indeed, when the gut-haemocoele interface was examined, there was a greater number of melanised particles adhering to the haemocoelic surface of the gut of the cockroaches concurrently fed with *H.diminuta* and *M.moniliformis* compared to *M.moniliformis*-only infections. Many of these particles, when examined microscopically, were found to be the remains of melanised acanthors, made visible by the rostellar hooks.

The following gives examples of the immunological consequences of damage to the luminal surfaces of the gut. Poinar and Hess (1974) described the response of *Blatella germanica* to the nematode *Abbreviata caucasica* which penetrates the epithelial cells of the colon (see also Schell, 1952). The parasite caused the breakdown of the epithelial cells so that they formed a "giant cell" syncytium in which the nematodes developed. In this case the above authors did not believe that the response was a defence or wound healing reaction. Later, Cawthorne and Anderson (1977) demonstrated that *B.germanica* mounted a successful rejection response causing melanisation of the nematode *Physaloptera maxillaris* within the gut cells.

Unsuccessful attempts were made to induce haemocoelic defence responses in *Galleria mellonella* larvae by feeding them with pathogenic bacteria (*Pseudomonas aeruginosa*, *Serratia marcescens*, and *Xenorhabdus nematophilus*) (Glinski and Jarosz, 1986). It was originally proposed that perturbation of the gut wall by an ingested pathogen may lead to stimulation of the immune system as determined by measuring lysozyme (an inducible antibacterial protein, Boman and Hultmark, 1987) levels in the haemolymph. A non-significant increase in lysozyme levels was found and only a direct intrahaemocoelic injection of foreign or soluble particulate matter (derived, in this case, from cultures of *Enterobacter cloacae*) produced a significant elevation in lysozyme levels (see also Jarosz and Spiewak, 1979). Glinski and Jarosz concluded that the chitinous lining of foregut and hindgut and the peritrophic membrane were the main barriers to bacterial infection. Smirnoff (1972) found that hydrolysis of the chitinous layers of the insect gut by chitinase allowed spores of *Bacillus thuringiensis* to reach the gut epithelium of *Choristoneura fumiferana* larvae more easily. The use of chitinase might also facilitate parasite larvae, such as *H.diminuta* oncospheres, to penetrate the gut of *P.americana*. Evidence to support this comes from Edmonds (1966), who found that the acanthors of *M.moniliformis* secrete chitinase, and

suggested that this was to aid in the process of hatching. Whitfield (1971) added that chitinase was at least equally as likely to weaken the peritrophic membrane, so aiding gut penetration.

Bacillus thuringiensis kurstaki HD-1 crystal endotoxin is known to cause severe swelling and rupturing of goblet and columnar cells in the midgut of *Manduca sexta* larvae when administered orally (Spies and Spence, 1985). The insect appears to suffer no long-term ill effects. The authors did not relate the damage to the gut and changes in gut cell morphology after infection with *B.thuringiensis* to changes in haemocytic behaviour; perhaps a subject for further study in this field.

As the haemocoelic components of the immune system appear to be stimulated by the presence of potentially tissue-disruptive parasites within the gut, it would suggest that the stimulus is in the form of soluble molecules which can pass across the gut wall. These could be wound factors released from the damaged tissues such as Cherbas' (1973) "haemokinin".

It was also suggested (section 6.4) that midgut endocrine cells in *P.americana* (Dow, 1986; Endo and Nishiitsutsuji-Uwo, 1980, 1982) may alert the immune system if they are damaged (Dow, personal communication), although investigations of any link between stimulation of these cells and the immune response has yet to be carried out.

7.2.1.2 SOLUBLE MOLECULES

The insect gut appears to exhibit little selectivity in uptake of molecules, as discussed in section 6.4, and almost all solutes readily pass through into the haemolymph and may significantly alter its composition (Treherne, 1967; Madrell, 1981). Soluble molecules, for example penetration gland secretions (Lethbridge and Gijsbers, 1974), produced by the oncospheres themselves might stimulate the immune system if they leak through into the haemocoele possibly via intercellular junctions (Lane and Skaer, 1980; see

also section 6.4). The precise nature of penetration gland secretions is the subject of much speculation (see Ubelaker, 1980), but if it were shown that the oncospheres released proteases (trypsin-like or serine proteases) to digest the tissues of the gut in order to aid penetration, this might explain the origin of the immune stimulus. These enzymes could potentially activate the prophenoloxidase system in the gut tissues thus leading to stimulation of the immune response. However, homogenates of oncospheres, supernatants from these homogenates and oncosphere-conditioned saline were fed to cockroaches without any observed change in haemocyte behaviour as determined by the assays in Chapter 6.

The evidence so far, indicates that the immune stimulus originates from living oncospheres within the gut, and it would seem unlikely that constituents of the insect's diet could significantly influence the stimulation of the immune system. This is most relevant in the case of cockroaches which live in bacteria-rich conditions. For future studies the use of enzyme isolates from oncosphere penetration glands might give more conclusive results than the undefined oncosphere homogenates used in this work.

7.2.2 PARASITE SURVIVAL AND INTERACTIONS WITH THE INTERMEDIATE HOST

In the above discussion, the immune response of the insect host has been stimulated so that immunocompetence has been increased. Similar examples of increased immunocompetence have been given by other workers. Mohrig *et al.* (1979) stimulated the haemocytes of *Galleria mellonella* larvae with intrahaemocoelic injections of latex beads. Subsequent injections of a normally non-phagocytosable strain of *Bacillus thuringiensis* were then recognised and encapsulated. A similar situation, in which immune stimulated

P.americana rejected normally acceptable tissue implants from closely related *Blatta orientalis* (Dularay and Lackie, 1987), has been discussed in Chapter 6. In this case, *in vivo* phagocytic and nodule-forming ability of the recipient's haemocytes were also enhanced, so that the cells became both more responsive and recognised previously unrecognised surfaces (Lackie, 1988a).

Recent evidence from this laboratory (M. Carr, personal communication) has provided further supportive evidence that immunocompetence of *P.americana* increases when *H.diminuta* oncospheres are present in the gut; total haemocyte count (THC), proportions of phagocytic cells and peroxidase-positive cells increase within 1 day post-ingestion. In the case of *M.moniliformis* infecting the cockroach, the initial stages of the infection appear to stimulate a slight reaction from the immune system, but not on the scale of that initiated by *H.diminuta* However, from similar assays (Chapter 6) for haemocyte behaviour performed on cockroaches harbouring stage I acanthellae onwards the trend is reversed, so that there appears to be a suppression of the immune response (Figure 7.2, part 11). This is most evident in the decrease in reactivity of the cockroach immune system to injected β1,3-glucans (sections 6.3.3 and 6.3.4) and to injected *H.diminuta* oncospheres (section 5.3.2 and Figure 7.1, part 12). The degree of suppression with which *M.moniliformis* seems to control the cockroach host is surprising, when considering the evolutionary implications of such an action. In general terms an immunosuppressed host might be more susceptible to secondary infections which could ultimately kill the insect, resulting in the loss of the host insect and the parasite (Lackie, 1986d; Yoshino and Boswell, 1986).

Brennan and Cheng (1975) suggested that the immunosuppressive action of *M.moniliformis* acts at the parasite's surface by blocking enzyme pathways leading to the production of phenoloxidase. They hypothesised that polyanionic mucins associated with the glycocalyx of the envelope inhibit tyrosinase (a phenoloxidase), thus preventing the production of toxic quinones and eventually melanin (see also section 6.4). In this way the parasite is

able to suppress the host's immune response locally without affecting the routine functions of the immune system. Unfortunately this hypothesis was based on questionable experimental evidence. . The suppressive effect was found after injecting homogenised acanthors into the cockroach haemocoele; an undefined preparation which could produce an immunosuppressive affect for reasons other than blocking of biochemical pathways as suggested above, such as the removal of a sub-population of cells involved in the clearance of the injected acanthor fragments.

Natural occurrences of immunosuppression have been reported in many insect species parasitised by parasitoid wasps (see section 5.4; Nappi, 1975, 1977, 1981; Stoltz and Guzo, 1986; Davies and Vinson, 1986; Rizki and Rizki, 1984). However these are all acute infections which result in the death of the host during emergence of the wasps and are thus not directly comparable to the chronic infection of *M.moniliformis* in *P.americana.*

Another example of a parasite which may immunosuppress the host is the larva of the nematode *Mermis nigrescens*, which renders *S.gregaria* susceptible to experimental infection by two species of protozoan parasite, *Trypanosoma brucei* (procyclics) or *Leishmania hertigi* (promastigotes) (Ibrahim *et al.*, 1986). Locusts infected with *M.nigrescens* were found to have a reduced THC, to approximately one third of control values, a low phagocytic rate and also reduced agglutinating activity. Non-infected locusts could clear the protozoans from the haemocoele within 72 hours, but nematode-infected, immunosuppressed insects suffered 100% mortality by 6 days post-infection.

Destruxins, secondary metabolites of the fungus *Metarhizium anisopliae*, reduce the nodule-forming ability of insect haemocytes in response to other fungal spores (Vey *et al.*, 1988). Huxham and Lackie (1986) found that destruxins also suppressed the phenoloxidase-activating effect of β1,3-glucans on locust and cockroach haemocytes *in vitro* and Zymosan stimulation of nodule formation *in vivo* (Huxham and Lackie, 1986; Huxham *et al.*, 1988). This was

determined by similar assays to those used here to show the immunosuppressive effect of *M.moniliformis* on *P.americana.*

In this work the extraordinary occurrence of *H.diminuta* larvae developing inside the envelopes of *M.moniliformis* larvae (section 5.3.2 and Holt, 1989) may be considered as evidence for the protective nature of the envelope. Once inside the envelope of *M.moniliformis*, *H.diminuta* is protected from haemocytic attack, possibly through a combination of immunosuppressive action from *M.moniliformis* and "cloaking" by the envelope. Haemocytes were found adhering to small areas on the surface of the envelope, possibly marking points at which the envelope had been damaged by penetrant oncospheres. Certainly, the protective nature of the envelope has been demonstrated in the *in vitro* encapsulation assay (section 6.3.5), but as the cysticercoids of *H.diminuta* also remained free of cockroach haemocytes in this assay the mechanisms involved in the avoidance of the immune response by *M.moniliformis* are evidently complex (see O'Brien, 1988).

The effects of *H.diminuta* cysticercoids on the cockroach immune system have not been investigated, primarily because of the great difficulty experienced in obtaining a high enough prevalence and intensity of infection after injection of pre-hatched oncospheres. There have been several mechanisms suggested to explain how *H.diminuta* survives in the intermediate host, involving mimicry of self tissues and active resistance to encapsulation (Heyneman and Voge, 1971, Ubelaker *et al.*, 1970; Collin, 1970; Lackie, 1976). Richards and Arme (1985) noted that during a morphological change between cysticercoid stages III and IV (ie. scolex retraction) parts of the cysticercoid were encapsulated. The above authors regarded this as evidence for the importance of the cyst surface coat in preventing encapsulation.

In this work (section 5.3.1 and also Lackie, 1987), pre-hatched *H.diminuta* oncospheres survive at a very low prevalence and intensity of infection when injected into cockroaches. Similar observations were also made for *H.nana* in *Leucophaea maderae* (Leger and Cavier, 1970). Why only a few

oncospheres survive instead of all-or-none remains unanswered. The following gives some insight to the possible reasons for the success and failure of *H.diminuta* to infect different species of insect.

There is experimental evidence (*in vitro* and *in vivo*) for the immune competence of locusts being poor compared with *P.americana* (see Lackie, 1986d). In this work the intensity and prevalence of infections in locusts injected with pre-hatched oncospheres are both very high, supporting the theory that the two insect species have differences in their immunocompetence. Takle and Lackie (1985) found that locust haemocytes, bearing a greater negative charge than cockroach haemocytes, adhere less readily to negatively charged substrata. Thus one possible reason for such a difference in *H.diminuta* infectivity is that negatively charged oncospheres will, on the basis of surface charge alone, stand a better chance of survival in *S.gregaria* than in *P.americana*. However this still does not explain why some *H.diminuta* are able to survive in cockroaches if the oncospheres are normally recognised as non-self. The total haemocyte count (THC) in *P.americana* can vary considerably between individual insects, therefore some individual cockroaches would appear to have a larger number of cells available to take part in encapsulation than others. Melanised oncospheres were found adhering to the dorsal and ventral diaphragm in many cockroaches (Chapter 5) along with normal, fully developed cysticercoids in the haemocoele. However, there were very few instances of melanised, partially developed cysticercoids being found, suggesting that the critical stage for survival of *H.diminuta* oncospheres in the cockroach appears to be shortly after the time of injection.

If all the available immunocompetent cells in a cockroach with a low THC did not fully encapsulate all the injected oncospheres, then the survivors could possibly develop beyond the critical stage. Indeed, approximately 10% of *H.diminuta*-injected cockroaches yield cysticercoids, the prevalence of

such infections approximating to the percentage of cockroaches with abnormally low THCs (M. Carr, personal communication).

It appears that under certain conditions *H.diminuta* is infective to cockroaches, and genetically pre-adapted larvae or those which survive the *in vitro* hatching process (see. below) may survive if injected into the haemocoele. Any action which could perturb the cockroach immune system and cause immune stimulation, such as the injection procedure itself, may jeopardise the survival of weaker larvae or result in total failure of the infection. However, in locusts, simple penetration of the cuticle and epidermis does not appear to cause an immune response large enough to effect the survival of *H.diminuta*, even multiple injections of β1,3-glucans (Zymosan, a known immune stimulant, see section 6.3.6) only appear to cause an immune response which results in a temporary arrest in parasite development.

The problems involved in the selection of a strain of *H.diminuta* infective to cockroaches have been discussed in Chapter 5. Selection for a new strain of parasite to adapt to newly available hosts may require changes in parasite physiology or morphology. Selection pressure for a parasite to fill a new niche (ie. new host species) has to be applied at an optimal level and on a suitable gene or set of genes which when expressed, enable the parasite to adapt accordingly. In the case of a newly discovered species of *Hymenolepis*, *H.hibernia*, (Montgomery *et al.*, 1987) the physiological requirements of the adult worm were not dramatically dissimilar from other hymenolepids and it was found that this species could be tolerated in species of final hosts in common with *H.diminuta* (ie. rats, and wild caught long-tailed field mouse, *Apodemus sylvaticus*). However, the differences in morphology, physiology and immune systems of the flour beetles compared to those of *P.americana* are possibly much greater and outside the limits of a selection programme such as that used in this work. It might require the selection of an existing genotype (as evidenced by those cysticercoids surviving in cockroaches), or the mutation of a gene or genes (see section

5.4). It is also possible that the selection pressure was applied at the wrong level. For example, if the method for hatching oncospheres *in vitro* was damaging the oncosphere's surface or the technique was selective for a particular "type" of oncosphere, then the components of the cockroach immune system are not necessarily selecting for the most suitably pre-adapted larvae. This may only be remedied by completely changing the *in vitro* hatching method and comparing success rates between the two methods.

One major question remains open; why has *H.diminuta* not already become adapted to utilise the cockroach as the intermediate host? Cockroaches, rats, stored produce feeders (beetles) and the parasites have presumably come into contact frequently in natural situations throughout evolution, their ecological requirements having many similarities.

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Hymenolepis diminuta Utilizes the Envelope Surrounding *Moniliformis moniliformis* in Order to Survive in the Cockroach Host

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ABSTRACT: The acanthocephalan *Moniliformis moniliformis* is surrounded by a membranous envelope that protects the parasite from hemocytic attack in the cockroach host. If injected into a cockroach infected with *M. moniliformis*, hatched oncospheres of the tapeworm *Hymenolepis diminuta* are able to penetrate this envelope and, once inside, utilize its protective function in order to develop. These "double parasites" were infective to rats.

Larvae of the acanthocephalan worm *Moniliformis moniliformis* develop within the hemocoele of their usual intermediate host, the cockroach *Periplaneta americana*. The envelope that surrounds the parasite from the stage I acanthellae onward is largely derived from microvillar outgrowths of the acanthor tegument (Rotheram and Crompton, 1972), and it is this envelope that appears to protect the parasite from hemocytic attack when in the insect's hemocoele (Lackie and Lackie, 1979).

Similarly, the larvae of the cestode *Hymenolepis diminuta* develop in the hemocoele of their intermediate host, normally flour beetles of the tenebrionid family such as *Tenebrio molitor* or *Tribolium confusum*. For both of the above species of parasite, ingested eggs hatch in the gut of their host, and larvae then burrow through the gut wall into the hemocoele. Here development continues to the stage at which the parasite becomes infective to its definitive host.

It has been shown that the definitive hosts, rats, can support concurrent infections with both species; the parasites develop to maturity in the small intestine and for some time simultaneously produce eggs that pass out in the feces (Holmes, 1962).

In a series of experiments designed to investigate this question, attempts were made to infect several insect species naturally (orally) and artificially (intrahemocoelic injection of hatched eggs) with both parasite species. Although preliminary experiments have shown that ingested oncospheres of *H. diminuta* cannot penetrate the gut wall of *P. americana*, a very small proportion of injected oncospheres survive in the hemocoele (Lackie, 1986); it has now been shown that preexistent hemocoelic infections of *M. moniliformis* larvae permit greater survival of injected *H. diminuta*.

Thus, stage I acanthellae of *M. moniliformis*, 21 days postinfection (PI) at 28 C, were removed by dissection from 1 or more *P. americana* and

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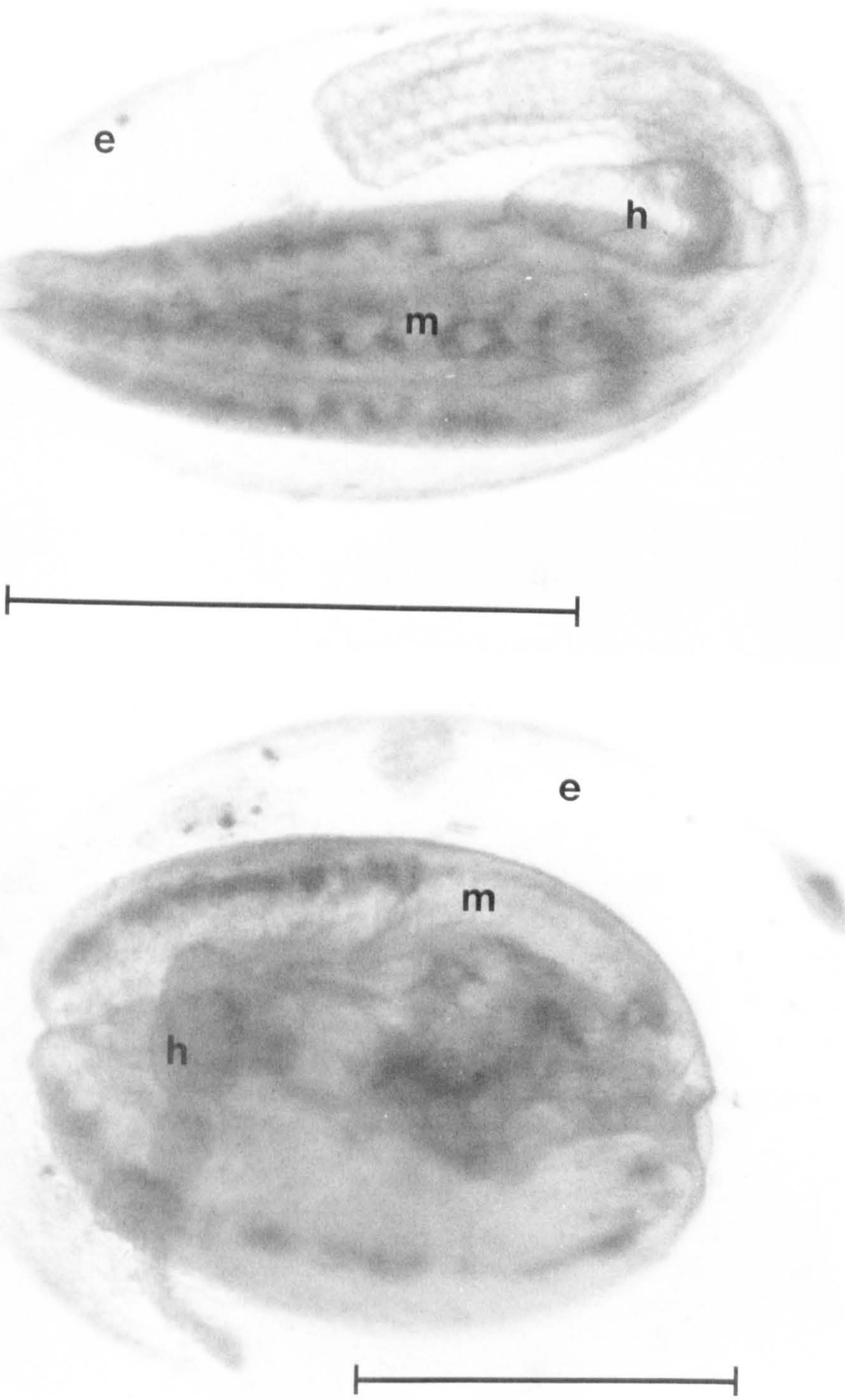
FIGURES 1, 2. *Hymenolepis diminuta*. **1.** (Top). Early-stage *H. diminuta* larva within the envelope of a developing *M. moniliformis* cystacanth. h = *H. diminuta*, e = envelope, m = *M. moniliformis*; scale bar = 1 mm. **2.** (Bottom). Mature *H. diminuta* within the envelope of a fully developed *M. moniliformis* cystacanth. h = *H. diminuta*, e = envelope, m = *M. moniliformis*; scale bar = 1 mm.


e
h
m
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h
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40 larvae were injected into the abdominal hemocoele of each naive cockroach in approximately 20 μl of cockroach cell culture medium with 10% fetal calf serum, using a glass micropipette. Two weeks later a suspension of live hatched *H. diminuta* oncospheres was injected into the same cockroaches at a dose of 500–800/insect (Lackie, 1986), and the parasites were allowed to develop for 10 days at 28 C. The cockroaches were then dissected and the larvae collected for examination (Figs. 1, 2).

Results, detailed elsewhere (Holt and Lackie, 1986), indicated that prior infection of cockroaches with *M. moniliformis* resulted in immunosuppression of the host, thus allowing a greater number of *H. diminuta* to survive free in the hemocoele and to develop fully to the cysticercoid stage in parasitized cockroaches. However, in 5 experiments using 50 cockroaches in total, more than 100 *H. diminuta* were also found to be living within the envelope surrounding the *M. moniliformis* cystacanth, there being 1 or 2 *H. diminuta* larvae inside the envelopes of the *M. moniliformis* in most cases, but occasionally as many as 4.

The viability of the combined parasites was tested by administering 60 of them to 8 laboratory-bred Wistar rats and later examining the rats' feces for parasite eggs. *Hymenolepis diminuta* eggs were found 24 days PI and were found together with *M. moniliformis* eggs by 7 wk PI. When the rats were later dissected and their small intestines examined, *M. moniliformis* were always found anterior to *H. diminuta*. Of the 60 parasites of each species administered, 31 *H. diminuta* and 44 *M. moniliformis* were recovered.

When *H. diminuta* hatches from its egg in the gut of its natural host, it penetrates the gut wall by mechanical means, using 3 pairs of hooks in a "breast stroke"-like swimming action and by enzymatic action with enzymes secreted from penetration glands (Lethbridge, 1971). Presumably, this combination of actions enables oncospheres to cut into and penetrate the cystacanth envelope shortly after being injected into the cockroach hemocoele. Although *H. diminuta* is normally recognized by the cockroach immune system and is readily encapsulated by hemocytes, the parasite is able to develop safely within the envelope that protects the 2 species of developing larvae from hemocytic attack. In some cases, however, small clumps of host cells were seen adhering to a small portion of the envelope's outer wall, and this may mark the point at which *H. diminuta* larva damage the envelope on entry.

This association has clearly shown that the envelope is acting as a barrier that protects developing larvae by excluding hemocytes, yet allows entry of nutrients, as both parasites developed without a decrease in normal growth rate.

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Immunosuppression by larvae of *Moniliformis moniliformis* (Acanthocephala) in their cockroach host (*Periplaneta americana*)

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SUMMARY

The results of 4 different assays for haemocytic immune responsiveness in the cockroach, *Periplaneta americana*, have shown that the presence of larvae of *Moniliformis moniliformis* within the haemocoele depresses the insect's immune reactivity. Thus, the proportion of haemocytes that phagocytose fluorescent latex beads *in vivo*, the proportion of haemocytes that can be stimulated *in vitro* by the β1,3-glucan, laminarin, to produce phenoloxidase, and the number of haemocytic aggregates produced *in vivo* in response to zymosan stimulation, are significantly depressed compared with control, unparasitized cockroaches. Also, when cockroaches are injected intra-haemocoelically with hatched oncospheres of the tapeworm, *Hymenolepis diminuta*, a higher prevalence and intensity of tapeworm larvae are found in insects already parasitized by *M. moniliformis*. All of these assays show that depression rather than total suppression of the immune response occurs; in the wild, this may be sufficient to ensure that the cockroach does not succumb to unrelated infections during the long developmental period of the parasite.

Key words: immunosuppression, haemocytic response, *Periplaneta americana*, *Moniliformis moniliformis*, *Hymenolepis diminuta*

INTRODUCTION

Acanthor larvae of *Moniliformis moniliformis*, after hatching in the cockroach gut and burrowing through the gut wall into the haemocoele, are thinly encapsulated by the host's haemocytes (Rotheram & Crompton 1972). However, within several days, the tegument of the acanthor begins to develop long microvillar extensions and, concomitantly, the encapsulating haemocytes appear to lose interest in the parasite. The microvillar extensions eventually detach from the surface of the parasite and apparently form a continuous, acellular envelope that surrounds the developing larva (Lackie & Rotheram, 1972). There is some evidence from transplantation, cytochemical and biochemical studies (Lackie, 1975; Lackie & Lackie, 1979; O'Brien, 1988; reviewed by Lackie, 1986*a*) that the envelope protects the parasite against the host's immune response by possessing some of the characteristics of host 'self', thus evading recognition.

However, not only the responsiveness of the haemocyte population (Gunnarsson, 1988) but also the acuity of immunorecognition (Dularay & Lackie, 1987) can be enhanced by prior exposure of an insect to specific and non-specific stimuli. Subsequent immune reactivity can be stimulated non-specifically for up to 3 days post-injection by injecting abiotic particles, such as Sepharose beads, into the haemocoele (Dularay & Lackie, 1987). More specific activators, such as injected zymosan supernatant (containing β1,6-and β1,3-glucans) stimulate measurable changes in haemocyte behaviour *in vivo*; thus, both the phagocytic index and the ability to form haemocytic aggregates (nodules) in response to unrelated stimuli are enhanced for up to 48 h after injection of zymosan supernatant.

Because of such potential changes in host immune reactivity, it is possibly dangerous for a parasitic species to rely entirely on evasion of recognition for its survival. On the other hand, where a parasite is also reliant on the longevity of its host in order to reach infectivity or patency, then suppression of the effector arm of the immune response could also be dangerous. The aim of this work was to investigate, using quantitative assays for the haemocytic response, whether or not the larvae of *M. moniliformis* suppressed the immune response of their cockroach hosts. In addition, the effect of *M. moniliformis* on the survival *in vivo* of larvae of the tapeworm *Hymenolepis diminuta* was examined, since only a small proportion of these larvae are able to evade the immune response of naive cockroaches (Lackie 1976); it was reasoned that, if immunosuppression had occurred, cestode establishment within the insect might be increased.

MATERIALS AND METHODS

Insects

Cockroaches, *Periplaneta americana*, were bred and maintained in the Department's Insectary at 28 $\pm$ 1 °C, and fed on rat cake, 'Bemax' and water.

Parasites

Moniliformis moniliformis, obtained from the Molteno Institute, Cambridge, was maintained in albino rats (Wistar strain) and cockroaches. Rats were given 20 cystacanths by intubation and were killed at least 7 weeks later and the worms collected from the small intestine. Parasite eggs (more properly referred to as 'shelled acanthors') were collected from the body cavities of female worms, then were washed in saline, resuspended in 60% sucrose solution (Lackie, 1972), and stored at 4 °C. Cockroaches, that had been starved for 24 h previously, were fed drops of the egg suspension and were then allowed food and water *ad libitum*.

Hymenolepis diminuta was maintained in Wistar rats and flour beetles, *Tribolium confusum*. Rats were given 10 cysticercoids by intubation and were killed at least 3 weeks later to obtain mature worms. Eggs (shelled oncospheres) were teased out of ripe proglottids and were washed at least twice in saline (HBS; see below) by centrifugation at 500 ***g*** for 5 min. The egg suspension was stored at 4 °C and was used within 7 days of collection.

Media and salines

Cockroach cell-culture medium (CM) was that developed and described as Huxham & Lackie's medium in Crompton & Lassière (1987). Medium was supplemented with heat-inactivated foetal calf serum (Flow Laboratories), either at 10% (CMS) or 20% (CMS+).

HEPES-buffered balanced salt solution, pH 7·2 (HBS) was that developed for use with cockroach haemocytes (Huxham & Lackie, 1986). The anticoagulant solution for collecting cockroach haemocytes was a modification of that described by Huxham & Lackie (1986) for locust haemolymph; for cockroach haemolymph the concentration of sodium chloride was increased to 100 mM, to give an osmolality of 350 mosMol.

Zymosan supernatant (Zs) was obtained by suspending 1% (w/v) zymosan (Sigma) in HBS; the suspension was agitated and centrifuged several times to produce a particle-free supernatant (Gunnarsson & Lackie, 1985).

Introduction of parasites into the cockroach haemocoele

Hymenolepis diminuta eggs were mechanically cracked by agitating them with glass beads, and the oncospheres were hatched out in 0·75% α-amylase in HBS at 28 °C (Lackie, 1976). Larvae were washed in HBS, concentrated by centrifugation at 500 ***g*** for 5 min, filtered through 50 μm pore size nylon mesh (Plastoc Associates, UK) to remove unhatched eggs and egg-shells, then washed twice more before being resuspended at 5×10^4/ml. Ten microlitres of this suspension was injected ventrally into the abdominal haemocoele of each adult male cockroach.

In order to obtain hosts containing a standard number of *M. moniliformis* larvae, donor cockroaches that had been infected orally were dissected at approximately 2 weeks post-infection and parasites at the acanthella I/II stage (King & Robinson, 1967) were collected. These larvae were washed in HBS, then washed twice in CMS. Naive recipient cockroaches (adult females), anaesthetized with carbon dioxide, were each injected with 40 larvae in approximately 10 μl of CMS via a sharpened glass micropipette. Control insects received either 10 μl of CMS in which larvae had been temporarily stored or pieces of ovariole, the total size of which was comparable to that of the parasites injected into the experimental hosts. Ovariole was used as a control because transplanted allogeneic tissues, in common with acanthellae and cystacanths, are not encapsulated by haemocytes (Lackie, 1986*b*). The recipient cockroaches were used 6 days post-injection, unless stated otherwise.

Phagocytosis

Control and parasitized cockroaches were each injected with 20 μl of zymosan supernatant (Zs; see above) in order to stimulate the haemocytic response (Gunnarsson & Lackie, 1985; Gunnarsson, 1988). Twenty-four hours later, the cockroaches were injected with 20 μl of a 2% dilution of a stock suspension of fluorescent latex beads (0·2 μm diameter, Polysciences, UK) in HBS, and then left at 28 °C for 3 h to allow phagocytosis to occur; previous results had shown that the highest proportion of haemocytes containing beads was found after this time-interval (B. Dularay & A. Lackie, unpublished results). The insects were then anaesthetized, injected with 200 μl of HBS, and exuded diluted haemolymph was collected with a Pasteur pipette from the stump of a severed hindleg. Haemolymph from each cockroach was added to 600 μl HBS at room temperature and mixed gently, then several drops of the haemocyte suspension were placed on a glass slide that had been pre-coated with CMS. Cells were allowed to settle and spread for 15 min in a humid chamber at room temperature, and the slides were examined by epifluorescence on a Leitz Ortholux microscope.

At least 200 cells were examined per cockroach and the percentage containing fluorescent beads was noted. The proportion of cells that had phagocytosed in the two control groups and the parasitized group was compared using the Mann-Whitney *U* test.

Table 1. Effect of *Moniliformis moniliformis* on Zs-induced phagocytosis of fluorescent latex beads by *Periplaneta americana* haemocytes *in vivo*

	Percentage of cells phagocytic [Median, (95 % confidence limits)]		
	Exp. 1	Exp. 2	Exp. 3
Unparasitized control (medium)	10·5 (9–15) $n = 8$	—	10·5 (5–17) $n = 8$
Unparasitized control (ovariole)	—	10·0 (7–30) $n = 10$	12·0 (7–15) $n = 7$
Parasitized (40 larvae)	3·5 (2–4) $n = 8$ $P = 0·001$	4·5 (3–8) $n = 10$ $P = 0·001$	3·0 (2–5) $n = 10$ $P < 0·005$

The proportion of phenoloxidase (PO)-positive haemocytes in vitro

Control and *M. moniliformis*-infected cockroaches were set up as above. One week after injection, each cockroach was injected with 200 μl of anticoagulant solution, and haemolymph was collected as above and pooled for each group of 8 insects into an excess of anticoagulant held on ice. The assay for activation of prophenoloxidase by laminarin, and hence the production of PO-positive cells, was carried out as described by Huxham & Lackie (1986). Briefly, the cell suspensions were washed twice in anticoagulant by centrifugation at 5 °C, 250 *g*, for 5 min and the cells were finally resuspended at 10^6/ml in CM. Sterile culture techniques were used throughout.

Cells were plated out into sterile 3 cm-diameter tissue-culture dishes (Nunc, Gibco), at a density of 5×10^5/dish (approximately 700/mm^2), and the medium was replaced with CMS+. The cell monolayers were stimulated by the addition of laminarin (Sigma; final concentration 1 mg/ml) for 1 h at room temperature, and the PO-positive cells were finally visualized by the addition of substrate, 4 mg/ml dihydroxyphenylalanine (DOPA; Sigma), overnight; the DOPA was converted to melanin by phenoloxidase. The cells were fixed with 2·5 % glutaraldehyde in HBS, and the number of black-stained cells counted under × 50 magnification on a Wild dissecting microscope. The results were expressed as the number of PO-positive cells/mm^2.

Survival of H. diminuta *in naive and* M. moniliformis*-infected cockroaches*

Cockroaches were injected with medium or ovariole (controls) or 40 *M. moniliformis* larvae and were then injected 7 days later with 500 hatched, washed *H. diminuta* oncosphere larvae. All insects were maintained at 28 °C for 7–10 days and were then dissected in HBS and the numbers of unencapsulated tapeworm larvae counted.

Effect of M. moniliformis *on the induction of haemocytic nodules by Zs* in vivo

Cockroaches that had been injected with CMS or *M. moniliformis* larvae 1 week previously received an intra-abdominal injection of 20 μl of HBS (controls) or 20 μl of 1 % Zs (to stimulate nodule formation *in vivo*). Three hours later, haemolymph was flushed out of each insect with approximately 1 ml of anticoagulant (2·5 % disodium ethylenediaminotetracetic acid in calcium/magnesium-free Hanks' saline, pH 7·0; Gunnarsson & Lackie (1985)) into a further 1 ml of anticoagulant in a 3 cm-diameter Petri dish. The number of haemocytic aggregates in each dish was counted under × 40 magnification on a Leitz Ortholux microscope.

RESULTS

Phagocytosis of latex beads

The results of 3 experiments are shown in Table 1. In all cases, the proportion of haemocytes that had phagocytosed beads in the Zs-injected, parasitized cockroaches was significantly less than in the Zs-injected unparasitized controls.

Nodule formation in response to Zs stimulation in vivo

Injection of saline stimulated production of only a small number of nodules; there was no significant difference in its effect on control and parasitized cockroaches. Zs stimulated large numbers of nodules in the control cockroaches, but significantly fewer in the parasitized insects (Fig. 1).

Laminarin stimulation of phenoloxidase (PO) production by haemocytes in vitro

Laminarin activates the proPO pathway to produce PO which converts DOPA to melanin; the number

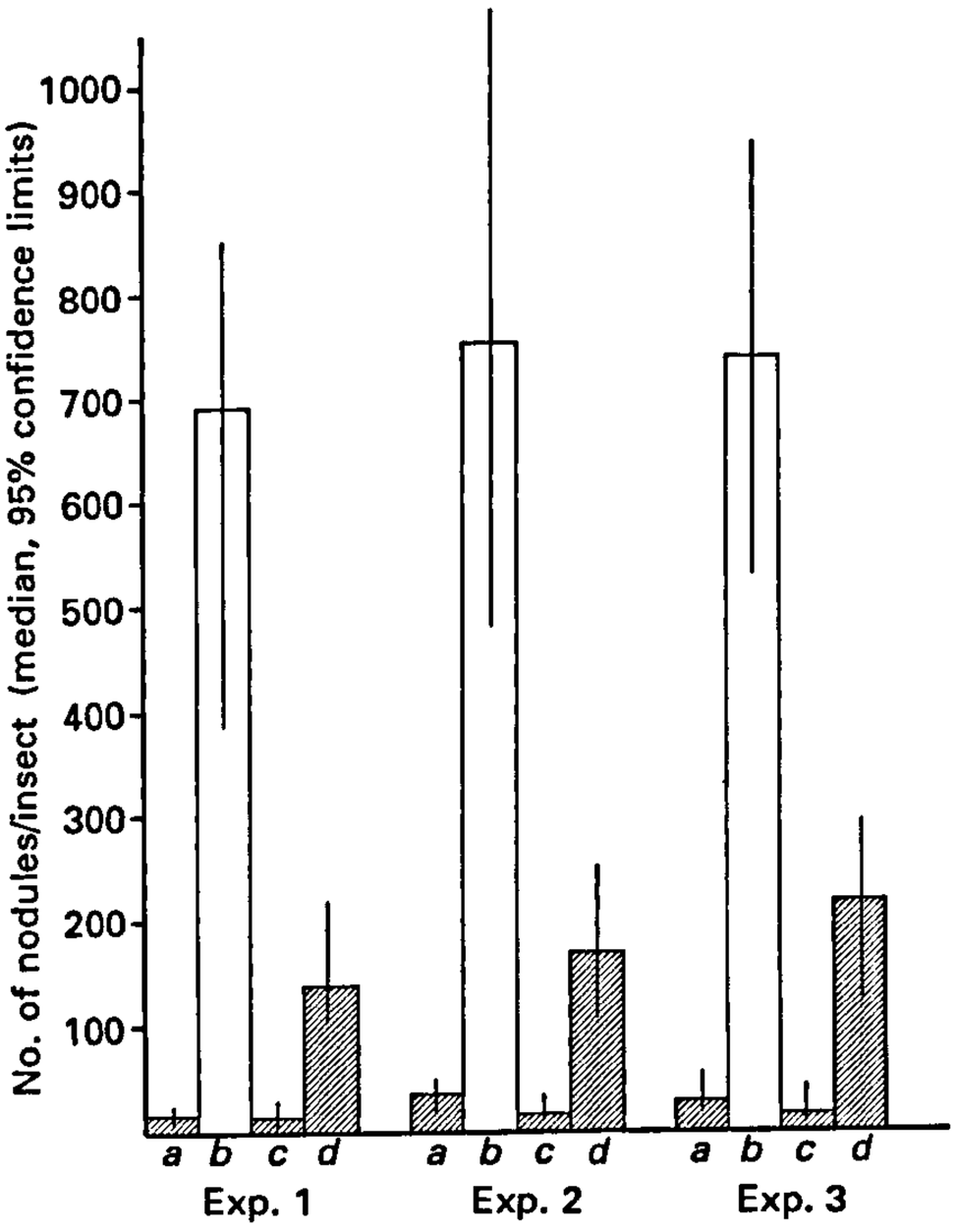

Fig. 1. Nodules formed in response to injection of 1% Zymosan supernatant (Zs) into unparasitized or *Moniliformis moniliformis*-infected (40 larvae) cockroaches. Values represent medians, plus 95% confidence limits, of number of nodules from 7 insects. (*a*) Unparasitized cockroaches, injected with 20 μl of saline (HBS); (*b*) unparasitized cockroaches, injected with 20 μl of 1% Zs; (*c*) parasitized cockroaches, injected with HBS; (*d*) parasitized cockroaches, injected with Zs.

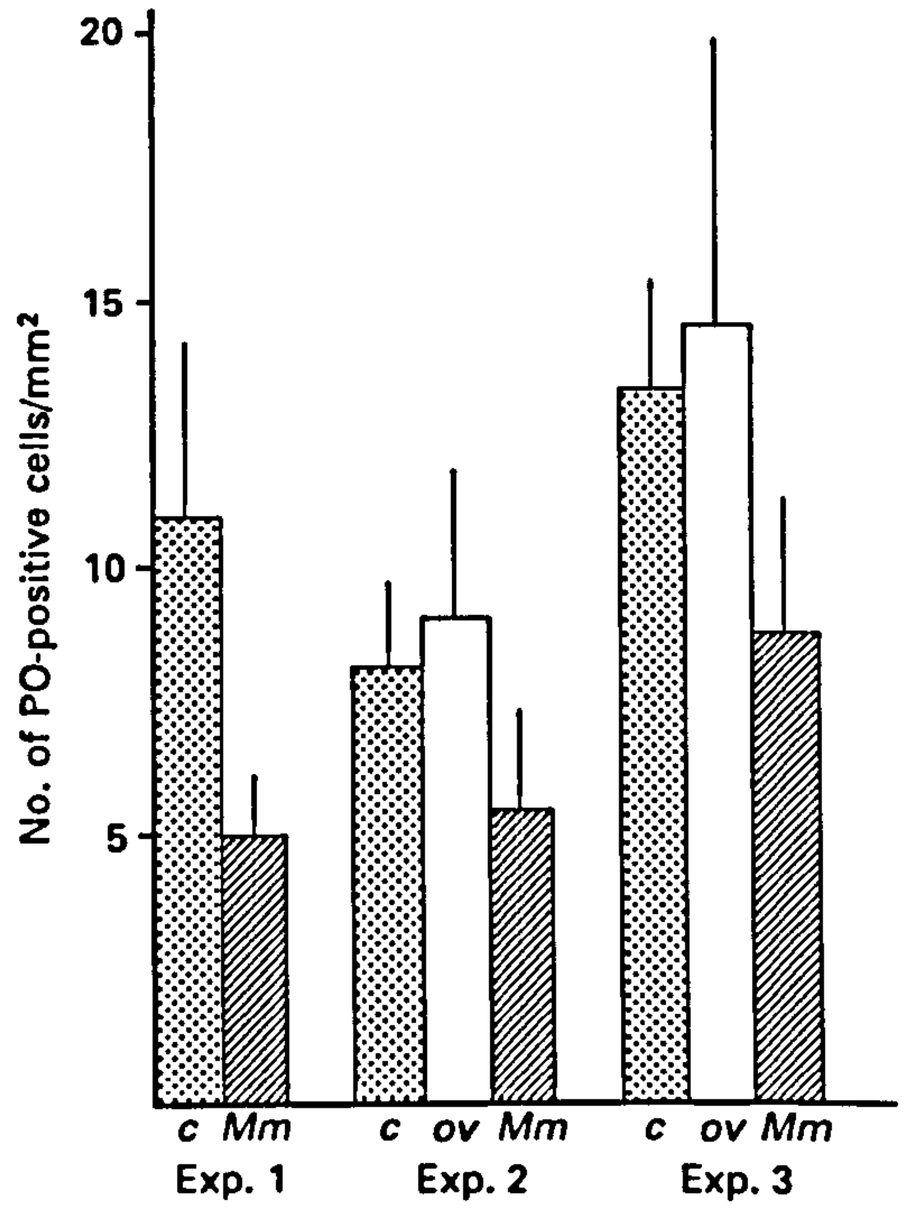

Fig. 2. The number of phenoloxidase-positive haemocytes in unparasitized and parasitized (40 *Moniliformis moniliformis* larvae) cockroaches, as indicated by melanin production after laminarin stimulation of haemocyte monolayers *in vitro*. Values are mean ± SD number of blackened cells in 10 microscope fields from each of 3 dishes. In each experiment, haemocytes were pooled from 8 cockroaches. *c*, Medium-injected control; *ov*, ovariole-injected control; *Mm*, *M. moniliformis* infected. Within each experiment, values for experimental animals (*Mm*) are significantly different ($P < 0.001$) from either *ov* or *c*. Controls are not significantly different from each other ($P > 0.05$).

of blackened cells or small cell aggregates produced in haemocyte monolayers from control animals (Fig. 2) was very similar to that found by Huxham & Lackie (1986).

However, a highly significantly smaller proportion ($P < 0.001$ in all cases) of the haemocytes from *M. moniliformis*-infected insects were PO-positive after laminarin stimulation.

Effect of M. moniliformis *on the survival of* H. diminuta *larvae within the cockroach haemocoele*

Both the prevalence and intensity of infection by *H. diminuta* increased when *M. moniliformis* was already present within the haemocoele (Table 2). Tapeworm larvae that developed to the cysticercoid stage were subsequently shown to be infective to rats. When the data for the 3 experiments are pooled and plotted as a frequency distribution (Fig. 3), it can be seen that the effect of the pre-existent *M. moniliformis* infection is to shift the curve away from that typical of an over-dispersed distribution.

Calculation of the variance: mean ratios shows that the values for all controls (except that of the 'medium' control in Exp. 2) are considerably greater than 1·0, indicating aggregation of the parasite population within the host population. For *M. moniliformis*-parasitized hosts in Exps 1 and 3, the ratio is much less than 1·0, indicating a tendency towards under-dispersion rather than normal distribution.

DISCUSSION

The results for all 4 assays for haemocyte behaviour indicate that the cellular immune response is depressed in cockroaches infected with a standardized number of *M. moniliformis* larvae, at the acanthella II/III stage.

Previous work (Huxham, Lackie & McCorkindale, 1988) has shown that the number of PO-positive haemocytes is decreased by immunosuppressant cyclodepsipeptides, destruxins, produced by the fungal pathogen, *Metarhizium anisopliae*. We have also shown that *in vivo* stimulation of the immune system by invading *Metarhizium* (Gunnarsson, Lackie & Huxham, 1988) or *H. diminuta* oncospheres (R. Holt, unpublished observations) causes the number of PO-positive haemocytes produced in response to laminarin stimulation *in vitro* to increase.

Table 2. The prevalence and intensity of *Hymenolepis diminuta* in unparasitized and *Moniliformis moniliformis*-infected cockroaches

(*H. diminuta* developing inside *M. moniliformis* envelopes (see text) have been excluded.)

	Exp. 1	Exp. 2	Exp. 3
Unparasitized control (medium)			
Prevalence	3/11 (27%)	6/11 (55%)	1/9 (11%)
Mean intensity ±S.D.	2·5±4·4	3·5±4·9	0·6±1·7
Variance:mean	2·88	1·86	7·98
Unparasitized control (ovariole)			
Prevalence	2/12 (17%)	3/9 (33%)	4/10 (40%)
Mean intensity ±S.D.	1·3±3·0	8·6±21·4	2·5±6·5
Variance:mean	5·24	5·57	6·15
Parasitized (40 *M. m.*)			
Prevalence	11/11 (100%)	11/11 (100%)	7/7 (100%)
Mean intensity ±S.D.	13·5±8·7	*26·3±56·1	6·1±2·7
Variance:mean	0·38	4·15	0·17

* 1 cockroach contained 194 *H. diminuta*.

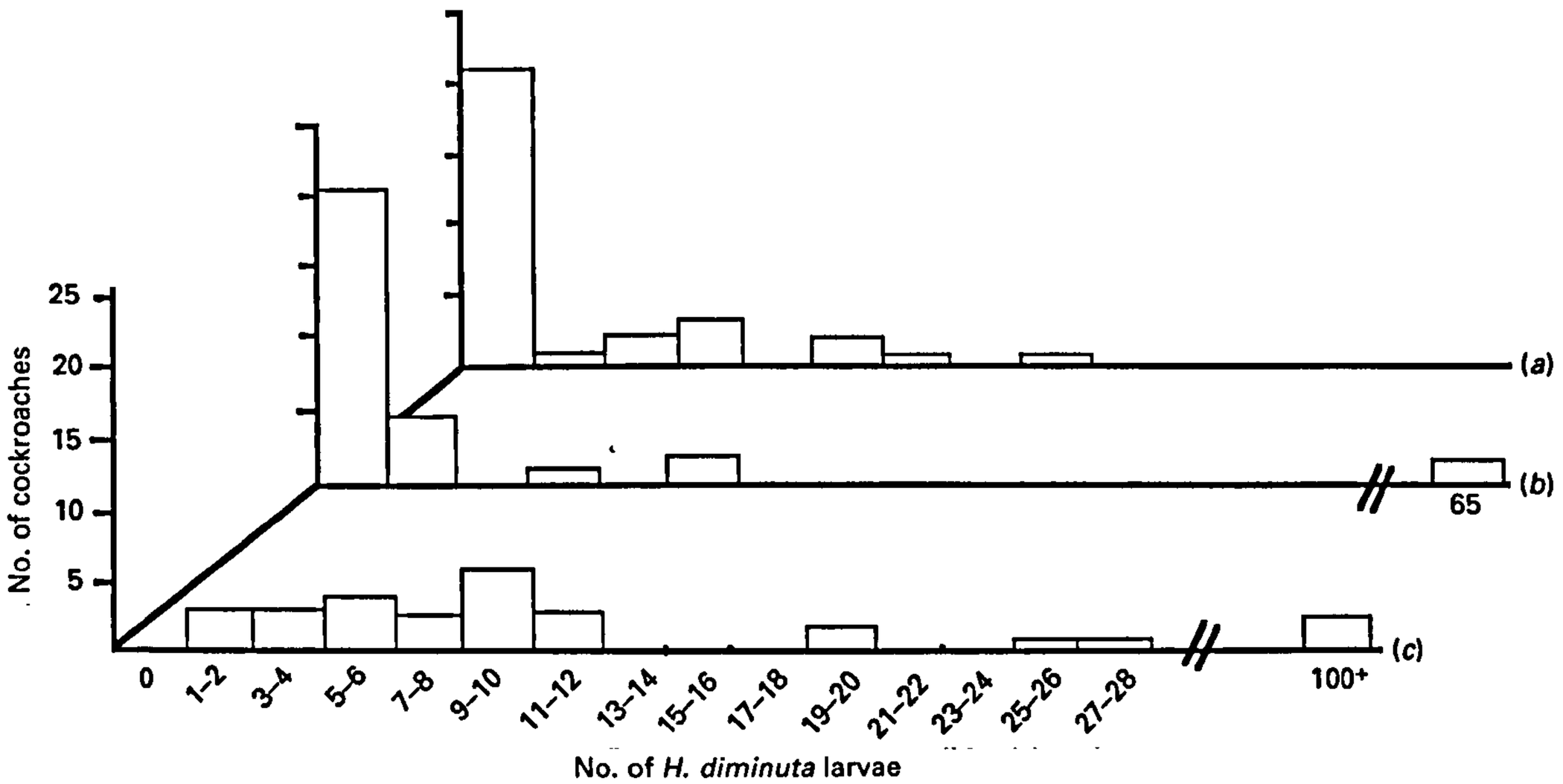

Fig. 3. Frequency distribution of numbers of *Hymenolepis diminuta* larvae developing, unencapsulated, in unparasitized and *Moniliformis moniliformis*-infected (40 larvae) cockroaches. (*a*) Control (medium); (*b*) control (ovariole); (*c*) *M. moniliformis* infected.

Thus, we feel justified in using the potential for proPO activation as an indication of cellular immune reactivity. Whether the observed reduction in the proportion of PO-positive cells is due to a decrease in their number, either through delayed maturation (Peake, 1979) or through increased adhesion to haemocoelic surfaces (Chain & Anderson, 1983), or to a decrease in their sensitivity to stimulators of the proPO-pathway (Huxham & Lackie, 1986), is discussed elsewhere (Lackie, 1988*b*). At present the significance of this reduction is not clear. Possible roles for the proPO activation sequence in the immune response are discussed elsewhere (Smith & Söderhäll, 1986). Although the evidence is equivocal (Lackie, 1988*a*, *b*), it is also possible that PO itself might exert a killing activity comparable to that of the peroxidase of mammalian granulocytes and snail haemocytes (Dikkeboom, Tijnagel, Mulder & van der Knaap, 1987).

The main function of nodule formation appears to be the rapid sequestration of particulate material from the haemolymph (Ratcliffe, 1986). However, nodules are also stimulated *in vivo* by microbial carbohydrates such as lipopolysaccharide and β1,3-

glucans (Gunnarsson & Lackie, 1985; Smith, Söderhäll & Hamilton, 1984) and, in *P. americana*, by galactose-rich glycoproteins (Lackie & Vasta, 1988). As with the number of PO-positive haemocytes, the number of nodules formed in response to a particular stimulus increases or decreases depending on the immune reactivity of the insect. Thus, when the immune system of *P. americana* has been activated to recognize and reject previously unrecognized xenografts from *B. orientalis* (Dularay & Lackie, 1987), or when the immune system of the locust has been activated by injected Zs (Gunnarsson, 1988), the number of nodules formed in response to injections of saline alone increases dramatically; possible reasons for this increase in sensitivity are discussed elsewhere (Lackie, 1988*b*). In the *M. moniliformis*-infected cockroaches used in the experiments reported above, injected Zs had a significantly less stimulatory effect than in unparasitized insects; nevertheless, the response was still significantly elevated above that due to saline alone.

Retention of responsiveness, although significantly depressed compared with control animals, also occurred in cockroaches injected with latex beads for the phagocytosis assay. Despite the marked reduction in the proportion of haemocytes that contain beads after 3 h, there were few free beads left in the haemolymph at this time. Preliminary results suggest that a high proportion of the beads are filtered out of the circulating haemolymph by the dorsal diaphragm underneath the heart (M. Carr, unpublished observations); large numbers of bacteria are removed in this manner in the locust (Hoffmann, Brehélin & Hoffmann, 1974). The low proportion of bead-containing cells seen after 3 h may be due to suppression of the phagocytic ability, or to a reduction in the proportion of phagocytic cells. It is also possible, but less likely, that the peak of clearance activity had occurred earlier and that laden cells had dropped out of circulation and were thus undetectable by this assay. These possibilities are being explored.

Thus, in the population of circulating haemocytes in parasitized insects, there appears to be a decrease in phagocytic-competent cells, a decrease in nodule-initiating cells and a decrease in the proportion of cells that are associated with prophenoloxidase activation and melanin production. Whether all 3 aspects of haemocyte behaviour are dependent upon one particular haemocyte subpopulation is not yet known although in the locust, *Schistocerca*, the subpopulation that is phagocytic *in vitro* is not that which is PO-positive (Huxham & Lackie, 1988).

The effect of a pre-existent *M. moniliformis* infection on the survival of *H. diminuta* in the cockroach haemocoele is particularly interesting. *H. diminuta* cannot penetrate the cockroach gut wall (R. Holt, unpublished observations), but when a clean suspension of hatched, washed oncospheres is injected directly into the haemocoele of naive cockroaches in order to bypass this physical barrier, a small proportion of larvae survive and develop. The remainder of the injected larvae are encapsulated and melanized either immediately or after variable periods of development. That the larvae can develop *in vitro* in a medium specially formulated for cockroach haemocytes (Lackie, 1976; see also Huxham & Lackie's medium in Crompton & Lassière (1987)) suggests that the barrier to development *in vivo* may be immunological rather than physiological, the majority of the parasites being recognized and encapsulated immediately. Even in *M. moniliformis*-infected cockroaches, only a small proportion of the injected larvae is recovered as developing larvae and it is likely that other potential survivors burrow into the tissues and fail to develop, either because the physiological environment within the tissue is unsuitable or because they are incorporated within haemocytic aggregates around the penetration wound. Evidence for the ability of oncospheres to continue burrowing after injection was the discovery, in a few cases, of *H. diminuta* developing inside the envelope of *M. moniliformis* larvae (Holt, 1988), thus providing further proof that the acanthocephalan envelope protects its enclosed parasite against the immune response (Lackie & Lackie, 1979).

Because a known dose of *H. diminuta* oncospheres was injected directly into the insect haemocoele, the over-dispersed distribution of *H. diminuta* in previously naive cockroaches may have resulted from host heterogeneity in immune responsiveness. Alternatively, as discussed by Crompton, Keymer & Arnold (1984), it may have resulted from the interaction of 2 randomly distributed variables, such as the variation around the mean number of oncospheres injected and the variation in the burrowing activity of the larvae. However, since the distribution of *H. diminuta* in *M. moniliformis*-infected cockroaches tends towards under-dispersion, this suggests strongly that over-dispersion in the naive hosts derived from immunological causes.

The cockroaches contained a standard number of *M. moniliformis* larvae, chosen because it approximated the mean number of larvae found after oral administration of eggs during standard laboratory maintenance of the parasite. However, it was also found that *M. moniliformis* exerted a suppressive effect irrespective of the number of parasites present. Thus, in orally infected cockroaches, showing a wide range of infection intensity, as few as 2–3 larvae depressed the number of nodules formed (R. Holt, unpublished observations), and permitted an increased number of injected *H. diminuta* to develop. This latter result contrasts with the situation found in preliminary experiments by Lackie (1986*b*); the

reasons for the difference can only be speculated upon, but may result from the different infection regimes and doses used in the two sets of experiments. Immunosuppression in acute infections by insect pathogens such as parasitoids (Davies, Strand & Vinson, 1987; Rizki & Rizki, 1984; Stoltz & Guzo, 1986) and nematodes (Götz, Boman & Boman, 1981) has been recorded before, but the observation that a chronic infection such as *Moniliformis* exerts an immunosuppressive effect is surprising since the parasite requires a minimum of 6 weeks for development and its cockroach host lives in an environment offering great potential for microbial invasion. Until now, evidence had suggested that the parasite survived by immune evasion; the envelope was protective because it evaded recognition through looking similar to host 'self' (Lackie, 1975; Lackie & Lackie, 1979; Lackie, 1986*a*), possibly because it, like the tissue surfaces lining the cockroach haemocoele, contains a non-fibrillar collagen and glycosaminoglycan-like molecules (V. O'Brien, J. Kusel & A. Lackie, unpublished observations). Whether the depression in the cellular immune response has a significant effect on the ability of the host to become super-infected under natural conditions is not clear. It is possible that infection with *M. moniliformis* could predispose the host to the acquisition of greater than expected intensities of infection on subsequent exposure to parasite eggs; however, Lackie (1972) found that successive oral doses of *M. moniliformis* eggs produced infection intensities within the predicted range.

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