

BIOLOGICAL CONTROL OF MOSQUITOES
WITH RHABDITOID NEMATODES

by

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ABSTRACT

The potential for biological control of culicine mosquito larvae with the entomophilic nematodes Neoaplectana carpocapsae Weiser, (Steinernematidae) and Heterorhabditis heliothidis (Heterorhabditidae) was investigated. The relationship between depth of water and the rate of parasitism of Aedes aegypti larvae is described, and the implication for control of Anopheles vectors of malaria is discussed. The differences in susceptibilities of the larval instars and in performances of the parasite species were investigated. Also the effects of host-parasite ratios, duration of exposures and the availability of particulate (organic) food in water on the uptake of pre-parasites, prevalence and intensity of infection were studied.

Damage to infective nematodes, ^{probably} caused by larval mandibles during entry, was identified as a major factor limiting the performance of the parasites. This led to the conclusion that the rhabditoids cannot be utilized as a single effective control method for culicine mosquitoes. Their possible integration with other control agents and methods is discussed.

Nematode survival, development and reproduction within single hosts were investigated, with particular emphasis on effects of the host defence reaction. The influence of parasite intensity (density) within larvae on the

morphometrics of adult worms was also documented. This necessitated development of a standard method for preparing specimens for microscopical examination. A description of the quantitative and qualitative effects of different processing techniques is provided.

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1 INTRODUCTION

Widespread reliance on chemical insecticides for insect pest control is now associated with various problems. Amongst these problems are insecticide resistance in pest populations, environmental pollution, health hazards associated with certain persistent pesticides and increased cost of pesticide production and application (Chapman, 1974; Burges et al. 1981; Service, 1981, 1983, Axtell, 1983). Consequently, research into alternative and supplementary methods has increased in recent years. This study concerns a particular pest, namely culicine mosquitoes, and explores the use of rhabditoid nematodes for their control.

The rhabditoid families Steinernematidae (Chitwood and Chitwood, 1937) and Heterorhabditidae (Poinar, 1975b) contain some of the most promising nematode species for biological control of insect pests (Poinar, 1979; Webster, 1980; Wouts et al. 1982). Members of these families are obligate parasites of insects (Poinar, 1979). However, they can be cheaply produced in vitro (Bedding, 1981; Wouts, 1981) even on a commercial scale (Bedding, 1984). Other useful attributes of these nematodes include environmental safety, especially for vertebrates (including man), and ease of storage, transportation and application. They have demonstrated considerable effectiveness against certain insect pests, particularly in Agriculture and Forestry, where most biocontrol tests have been conducted.

Nematologists and insect pathologists are yet to reach a

consensus on the taxonomy of most rhabditoids. The study of their morphology has been inadequate, and a critical evaluation of the differentiating characters remains to be undertaken (Wouts, et al. 1982). Since Poinar (1979) produced a detailed review of the description and classification of these nematodes, only a brief summary is necessary. The family Steinernematidae (syn. Neoaplectanidae Sobolev) was divided into the genera Neoaplectana Steiner, 1929 and Steinernema Travassos, 1927. This division was based on the number and arrangement of the head sense organs, and of the male copulatory papillae (Poinar, 1979; Wouts et al. 1982). However, studies by Mracek and Weiser (1979), Mracek et al. (1981) and Wouts et al. (1982) revealed that these features are not valid taxonomic characters. While re-describing the original specimens of Aplectana (= Steinernema) kraussei, the type species of Steinernema, Steiner (1929) reported the presence of six labial and six cephalic papillae, but made no mention of pre-anal papillae. Later, during the redescription of this species, Mracek (1977) reported five pre-anal pairs of copulatory papillae. Recently, scanning electron microscope studies by Mracek and Weiser (1979) and Wouts et al. (1982) showed that both Steinernema and Neoaplectana have 6 labial, 4 cephalic (submedian) and 5 pairs of pre-anal papillae. Thus, Wouts et al. (1982) proposed that Neoaplectana Steiner, 1929 be made the junior synonym for Steinernema Travassos, 1927, a proposal that awaits international acceptance. Already, Poinar (1984) has rejected

this proposal on the ground that Wouts et al. (1982) did not conduct hybridization tests of the original species with their isolate from the type host. Poinar (1984) also re-emphasized the variability within the characters discussed by Wouts et al. (1982) and the possible parasitism of the same host by nematodes from different genera. Finally, he objected to the lack of clarity of the micrographs presented by Wouts et al. (1982), which could invalidate their conclusions.

Poinar (1979) and Wouts et al. (1982) provided keys for the identification of species. These keys indicate that the description and nomenclature of the species within the Steinernematidae are not excluded from this taxonomic controversy. Poinar (1979) attributed this lack of consensus to the failure of early descriptions to discuss certain characters that are presently considered of importance for species identification. He also attributed it to the considerable quantitative and qualitative variability within and between these species. This subject is discussed in detail later in the present study. Because of this controversy, the steinernematid used in this study has been called Neoaplectana carpocapsae, as recommended by Poinar (1984) and as it is known in most of the literature.

The entomopathogenic rhabditoids attack a wide range of insects from many orders (Poinar, 1975a, 1979; Bedding et al. 1983). Infection is initiated by the ensheathed 3rd-stage juveniles, termed dauer larvae, which are the transmission

agents (Poinar, 1979). These juveniles actively locate the host (Lindegren et al. 1978, 1981; Poinar, 1979; Gaugler et al. 1980; Gaugler, 1981), and invade the haemocoel via the mouth, anus and spiracles. Infective heterorhabditids also enter the host by penetrating the intersegmental membranes and the tender cuticle of some early instars (Bedding and Molyneux, 1982). Once in the haemocoel, the juveniles commence development, thereby releasing associated bacteria from a specialized vesicular part of the intestine, into the haemocoel of the host. These bacteria proliferate rapidly, killing the host by septicaemia within 24-48 hours of infection. They convert the host haemolymph into suitable nutritional material for the development and reproduction of the nematodes, while suppressing the multiplication of other bacteria within the cadaver. The nematodes grow and develop into males and females of the bisexual generation (steiner-nematids) or into hermaphroditic females (heterorhabditids). The latter **produce eggs which** give rise to young worms that develop into males and females of the bisexual generation. The nematodes complete 2-3 cycles before the dauer larvae emerge from the host cadavers.

Despite a broad host spectrum demonstrated in laboratory tests, the feasibility of utilizing steinernematid and heterorhabditid nematodes for biocontrol of insect pests has only recently started to be demonstrated. This slow pace of progress is attributable to lack of understanding of the

ecological and physiological relationships of the nematodes with the target insects (Webster, 1980). Poinar (1979), Gaugler (1981) and Molta (1982) reviewed the factors limiting survival, performance and persistence of dauer larvae in the pre-parasitic environment. The most significant factor is the availability of adequate moisture (Poinar, 1979; Webster, 1980; Gaugler, 1981). Thus, Webster and Bronskill (1968) and, Simons and Poinar (1973) indicated that foliar applications of these nematodes were ineffective because of rapid and lethal evaporation. Webster and Bronskill (1968) increased the survival time of Neoaplectana carpocapsae (= Steinernema feltiae Wouts et al. 1982) by formulating them in a mixture of Gelgard-M Polymer, Arlatone T and folicote 351 (an evapo-retardant mixture). They observed that this formulation increased the performance of the nematodes against larch sawflies on larch foliage, in the laboratory.

After Moore (1965) reported survival of infective nematodes for one and half hours compared to 24 days on exposed leaf surfaces and in slowly dried soil, respectively, Simons and Poinar (1973) concluded that soil application of these nematodes would be more effective than application on foliage. Further support for this conclusion comes from the reports of Bedding and Miller (1981b) that Heterorhabditis (= Chromonema) heliothidis was highly effective against larvae, pupae and newly-emerged adult black vine weevil (Otiorhynchus sulcatus) in soil in pots. Also, Molyneux et al. (1983) and Molyneux and Bedding (1984) reported high efficacy of heter-

orhabditid nematodes against sheep blowfly larvae (Lucilia cuprina) in the soil. However, there are limiting factors in the soil also. Molyneux and Bedding (1984) noted that soil texture and moisture potential affect nematode parasitism of insect pests. For example, infective nematodes are attracted to carbon dioxide (Gaugler et al. 1980) and insect faeces and other products (Schmidt and All, 1978, 1979). The rate of diffusion of these substances particularly carbon dioxide, in soil depends on the moisture content of the soil. Also the ease of movement of nematodes through soil depends on the available pore spaces (Georgis and Poinar, 1983; Molyneux and Bedding, 1984).

Because moisture is so important for the viability and performance of rhabditoid nematodes, most workers (e.g. Welch and Bronskill, 1962; Webster, 1973; Gaugler, 1981; Poinar and Kaul, 1982) concluded that these entomopathogens had promise for control of insects in the aquatic environment. Thus, the aquatic stages of blackflies and mosquitoes were considered to be potential targets (Gaugler, 1981; Finney and Harding, 1981; Poinar and Kaul, 1982). This hypothesis formed the basis for most of the present research.

Experiments by Welch and Bronskill (1962), Dadd (1971b) and Poinar and Kaul (1982) confirmed that mosquito larvae could be infected by steinernematid and heterorhabditid nematodes. They also indicated that factors such as host-parasite ratios, larval instar, and physiology of the insects, might influence the efficacy of the nematodes.

However, details of the host-parasite interactions and the significance of each factor were not determined. Webster (1973) noted that entomophilic nematodes may be effectively used as control agents of mosquitoes and blackflies only when parameters such as water volume, velocity, temperature and salinity have been taken into account. Thus, the effects of some of these micro-environmental factors on interactions between infective rhabditoids and culicine mosquito larvae were investigated during the present study.

The defence reactions of an insect are a factor that could decrease the effectiveness of nematodes as biological control agents (Sandner and Stanuszek, 1971; Webster, 1973; Poinar and Kaul, 1982; Bedding et al. 1983). Welch and Bronskill (1962) and Poinar and Kaul (1982) reported encapsulation and melanization of N. carpocapsae and H. heliothidis in Aedes aegypti and Culex pipiens larvae, respectively. Bronskill (1962), Poinar and Leutenegger (1971), Andreadis and Hall (1976) and Beresky and Hall (1977) provided details of these reactions. Following penetration of the nematode into the haemocoel, the innermost cells of the body-wall of the host disintegrate, releasing melanin particles which are deposited around the nematode. The nematode cuticle, which contains tyrosine (pre-cursor of melanin), and the cytoplasmic melanin particles, both constitute sources of the pigment which forms an envelope around the parasite. Haemocytes of the larvae, normally concentrated in the last two abdominal segments and anal

papillae, are also mobilized and accumulate rapidly, forming a capsule around the nematode. However, haemocytes from the anal papillae are not absolutely necessary for successful capsule formation (Andreadis and Hall, 1976). In any case, the defence reaction of mosquitoes, particularly its effects on nematode survival, development and reproduction, was also considered during the course of these studies.

The present study was designed primarily to evaluate the potential of steinernematid (N. carpocapsae) and heterorhabditid (H. heliothidis) nematodes for the biological control of mosquito larvae. Little mention is made of the bacteria, Xenorhabdus nematophilus and X. luminescens, associated with the steinernematids and heterorhabditids, respectively. This is not meant to deny the important role they play in causing any pathology, rather, a study of factors affecting the bacteria was beyond the scope of this work. For a discussion of the significance of nematode - bacterium association, the reader is referred to Poinar and Thomas (1967), Poinar et al. (1971, 1977, 1982), Lysenko and Weiser (1974), Thomas and Poinar (1979) and Akhurst (1980, 1982a,b, 1983).

2 INTERACTIONS BETWEEN DAUER JUVENILES AND MOSQUITO LARVAE

2.1 HOST-PARASITIC CONTACT, AND MODE OF INFECTION

2.1.1 Introduction

Contact between host and parasite is an essential requirement for the establishment of a host-parasite system (Kennedy, 1975). This factor determines the rate of acquisition of parasites by their hosts (Anderson, 1982b) and is a function of the behaviour of both parasites and hosts, and the ecological conditions of their habitat (Kennedy, 1975). Contact, as well as the mode of infection of a host by parasites, is an important determinant of the range of hosts that are vulnerable to infection by particular parasites and the prevalence of the parasites.

Infection of culicine mosquito larvae by Neoaplectana carpocapsae Weiser (Welch and Bronskill, 1962; Poinar and Leutenegger, 1971; Dadd, 1971b) and Heterorhabditis bacteriophora Poinar (Poinar and Kaul, 1982) is reported to occur by oral ingestion of the infective stages during larval feeding. Since these mosquitoes are predominantly bottom feeders (Christophers, 1960; Horsfall, 1972) it has been inferred that contact between them and the nematodes takes place on the bottom. However, this hypothesis has not been verified experimentally. Finney and Harding (1981) and Molta (1982) showed that the nature of the substrate influenced the rate of infection of A. aegypti larvae by steinernematids and concluded that an uneven substrate reduced the availability of the nematodes to the larvae. Finney and Harding (1981)

further suggested the use of nematode formulations that might improve availability of the pre-parasites to the mosquitoes, but it remained unclear whether all contact, or only part, occurs on the substrate.

Several workers have demonstrated entry of infective-stage N. carpocapsae into various insects through natural openings, such as the mouth, anus and spiracles (Poinar, 1979). Poinar and Himsworth (1967) reported entry of the nematodes into greater waxmoth (Galleria mellonella) larvae via the mouth and anus. Triggiani and Poinar (1976) sealed the mouth and anus of adult California oakworm, Phryganidia californica and observed infection by the Agriotos strain of N. carpocapsae through the spiracles and trachea. Recently, Molyneux and Bedding (1984) showed the penetration of infective Heterorhabditis sp. into sheep blowfly (Lucilia cuprina) larvae via the anal route. Also, the present worker (unpublished data) sealed the mouth and anus of adult houseflies, Musca domestica, and demonstrated both oral and anal entry of infective N. bibionis into the insects.

Some N. carpocapsae strains can also infect the pupal stages of various lepidopterous (Schmiede, 1963; Kaya and Hara, 1980; Moyle and Kaya, 1981; Kaya and Grieve, 1982) and coleopterous (Schmiede, 1963; Pye and Burman, 1978) species. Invasive nematodes entered the thin-layered and thick, multi-layered cocoons of G. mellonella and the silkworm Bombyx mori, respectively, through the cocoon pores and invaded the pupal haemocoel via the spiracles (Moyle and

Kaya, 1981). However, the inner layer of the cocoons of certain hymenopterous insects (e.g. Hyposoter exiguae and Apanteles medicaginis) is non-porous and prevents entry of the nematodes (Kaya and Hotchkin, 1981). Other, more active modes of penetration into hosts have been reported. For example, Bedding and Molyneux (1982) exposed early instar termite, Mastotermes darwiniensis nymphs and L. cuprina larvae in 1.5% agar, and demonstrated inter-skeletal and intersegmental membrane penetration by H. bacteriophora Poinar. Therefore, understanding the mode of entry of rhabditoid nematodes into insects following contact, is important for explaining host range and vulnerability of insects to infection.

This section describes a series of experiments to elucidate the interactions between rhabditoid nematodes and A. aegypti larvae in water. The region of contact between parasites and host and the mode of infection by the nematodes are described. This leads to an attempt to predict the range of mosquito species against which the nematodes may prove useful.

2.1.2 Materials and Methods

The following materials and methods were used in the present and subsequent experiments, unless otherwise specified.

Mosquito cultures

Aedes aegypti L. Silwood Park and 'Black-eyed' strains were maintained under controlled conditions ($25 \pm 2^{\circ}\text{C}$, ca. 50

- 70% R.H., 12-hour photoperiod). The Black-eyed strain was obtained from Dr.G.Tingley of the London School of Hygiene and Tropical Medicine, London. Larvae were raised on a mixture of Tetramin (6 parts) yeast extract (1 part) and crumbled bread (2 parts), while adults were fed with 4% (w/v) sucrose solution and blood meals from laboratory mice.

Nematode cultures

Heterorhabditis heliothidis and Neoaplectana carpocapsae DD-136 strain, obtained from Mr.P.N. Richardson, Glasshouse Crops Research Institute, Littlehampton, Sussex and Dr.W.J.C. Oswald, formerly of London School of Hygiene and Tropical Medicine, London, respectively were used. Also cultures of N. bibionis (= Steinernema bibionis, Wouts et al. 1982) and H. bacteriophora from Drs.R.A. Bedding and R.J. Akhurst of CSIRO, Hobart, Australia, were maintained. All nematodes were mass reared in vivo, in G. mellonella larvae using the method of Poinar (1979) and ^{W.J.C.} Oswald (pers. comm.). The moth was maintained in 2 colonies, in Silwood and Ashurst, on an artificial diet consisting of 45g of molten beeswax, 225g of honey, 90g of dried brewer's yeast, 306g of middlings, and 157ml of glycerol ^{D.} (Batt, pers. comm.), which was modified from Poinar (1979). Late instar larvae were infected with invasive nematodes at a constant exposure rate of 200 infectives per larva. Dauers were extracted in 0.1% formalin according to the methods described by Dutky et al. (1964) and Poinar (1979) based on White's (1927) technique. Infective N. carpocapsae and H. heliothidis were stored in 1-2cm deep

tap-water or 0.05% formalin, usually in flat-bottomed lunch-pack boxes (width, 11cm; length, 17cm; height, 6cm) at 5° and 10°C, respectively until used. Fluid containing dauer nematodes was replaced weekly to avoid oxygen depletion.

Viability of nematodes and symbiotic bacteria.

Both freshly extracted and stored nematodes were checked for viability before each experiment. Only cultures under 2 months old, with less than 10% worm mortality were used for experimental infections. Occasional checks for primary (1°) forms of the symbiotic bacteria, Xenorhabdus spp. were made. The bacteria were obtained either from infected Galleria larvae or by the 'hanging drop' technique (Poinar, 1979). To isolate bacteria from infected Galleria, moth larvae were surface-sterilized in 0.05% Hibitane (Chlorhexidine) for 3 hours before the insect haemolymph was inoculated onto artificial medium (liver extract, 1g; yeast extract, 2g; Bacto-peptone, 5g; Sodium chloride, 5g; and Agar, 15g per litre) modified from Difco Laboratories (1971) based on Hansen and Yarwood (1968). After 48 hours at 25°C, bacterial colonies formed were transferred onto Tergitol-7 Agar (peptone, 5g; yeast extract, 3g; lactose, 10g; Agar, 15g; Tergitol-7, 0.1ml; Bromothymol blue, 0.025g; and Triphenyl-tetrazolium chloride, 40mg per litre) (Poinar et al. 1971; Akhurst, 1980; Webster and Rutherford, in press). Also the hydrogen peroxide (rapid method) of Dr R.A. Bedding (pers. comm.) was applied to confirm presence of the 1°-form bacteria. This involved addition of 10% hydrogen peroxide to

bacterial colonies and looking for the liberation of hydrogen as "fizzing". Absence of fizzing indicated that bacteria are in the secondary (2^0) form.

2.1.2.1. Region of contact

Both H. heliothidis and N. carpocapsae were used. Exposures were carried out in plastic dishes (diameter = 9cm, height = 4cm). Test dishes had 0.75mm mesh sieves located about 12mm above the bottom of the dishes. These were designed to allow nematodes, applied at the surface, to drop to the bottom, but confined the mosquito larvae to the top portion of the dishes. Control containers had no sieves.

Before each experiment was performed, 200ml of 24 hours old tap-water was placed in each test dish to give an operational volume of 100ml above and below the sieves. Corresponding controls had 100ml of water each. Mosquito larvae[^] (Silwood Park strain) were introduced in groups of 15 3rd-instars into each dish, then 3,000 infective nematodes (ie. 200 per larva) were inoculated into each dish, before ca. 35mg of pulverized food (Tetramin, crumbled bread and yeast extract) was added. Exposures lasted 24 hours at $25 \pm 2^{\circ}\text{C}$. Then, to remove any external pre-parasites, larvae were thoroughly washed on a 0.75mm mesh net, with clean tap-water. They were placed singly in 2.5ml of water, in individual wells of multi-well tissue culture (MWTC) plates, and separately by treatments. Each larva was provided with a pinch of food for a 24 hour post-exposure period. Mortality counts and dissections to

assess haemocoelic infections were then made. Seven experiments (with 1-5 replicates) were conducted with N. carpocapsae and two experiments (with 2 replicates) with H. heliothidis.

2.1.2.2. Mode of infection

Exposures to determine mode of infection of A. aegypti larvae by H. heliothidis and N. carpocapsae were performed in small petri dishes (diameter = 6.8cm, surface area = 36.3cm²) containing 5ml of tap-water (depth ca. 0.5cm). Four dishes were used during each experiment. Each of three dishes was inoculated with 1,258 hand-picked infective nematodes.

Prior to exposures, some 3rd-instar larvae were selected and their mouths were sealed with molten raw wax (melting point, ca. 50°C) on a hot plate. This was accomplished by holding a larva with forceps, blotting it on a tissue, and dipping its head into the wax. These larvae were observed for possible adverse handling effects, for a pre-test period of 30-60 minutes before 15 'normal' ones were transferred into one of the inoculated exposure dishes. The same number of larvae were selected randomly from the rearing bowl and transferred directly into the second exposure dish. No larvae were introduced into the third dish that contained nematodes (control 1) while 15 larvae were placed in the dish without nematodes (control 2).

After 24 hours exposure, the larvae were carefully separated from nematodes in the dishes. They were placed

individually in ca. 2.5ml of tap-water in the wells of MWTC plates, pending dissections. Larvae with unsealed mouths were provided with ground Tetramin/crumbled bread/yeast extract. Meanwhile, the numbers of nematodes in the exposure and control dishes were counted under a stereomicroscope using a 9 x 9 chambered counting dish. Forty-eight hours after the experiments began, the larvae were examined for mortality and dissected in ringer's solution to confirm any haemocoelic infections. This experiment was repeated 5 and 3 times with N. carpocapsae and H. heliothidis, respectively. Data sets were statistically computed using the unrelated sample t-test (Parker, 1973).

2.1.3 Results

2.1.3.1 Host-parasite contact

There was absolutely no infection of A. aegypti larvae by either H. heliothidis or N. carpocapsae when larvae were denied access to the bottom (Table 2.1.1) and no unaccountable mortality occurred. However, considerable mortality due to both parasite species occurred among larvae that had free access to the bottom of the exposure dishes. Generally, larval mortality rates were higher with H. heliothidis than with N. carpocapsae, but variations within treatments were higher with the latter than with the former.

The abundance of N. carpocapsae within the haemocoel varied considerably. Most of these parasites ($\bar{x} = 70.1 \pm 21.9\%$, range = 15.8 - 100%) were either encapsulated or

Table 2.1.1 Percent mortality¹ of 3rd-instar A. aegypti larvae caused by H. heliothidis and N. carpocapsae following contact at bottom of water.

Parasite	Percent mortality of		No. parasites in haemocoel of infected
	Surface contact	Bottom contact	
<u>H. heliothidis</u>	$\bar{x} \pm \text{SD}$	0	78.3 $\pm$ 6.4
(n = 4)	range	0	73.3 - 86.7
<u>N. carpocapsae</u>	$\bar{x} \pm \text{SD}$	0	69.5 $\pm$ 21.8
(n = 14)	range	0	20.0 - 86.7
			2.5 + 3.1
			1 - 8

¹mean $\pm$ standard deviation

melanized.

Interestingly, when a batch of 15 larvae was placed in a dish with a 1mm mesh sieve containing 200ml of tap-water inoculated with 3,000 infective N. carpocapsae for 24 hours, 3 of the larvae penetrated the mesh to gain access to the bottom. All 3 larvae were dead after the exposure period. Both melanized and living parasites were observed in the haemocoel of these larvae. None of the 12 larvae that remained at the surface died of infection. These results confirmed that contact between A. aegypti larvae and the infective rhabditid nematodes occurred at the bottom and was essential for infection of the larvae.

2.1.3.2 Mode of pre-parasite entry into mosquito larvae.

Nematode counts

Remarkably few nematodes were recovered from dishes containing larvae with unsealed mouths (Table 2.1.2. As expected, control dishes without larvae contained the highest numbers of worms after the experimental period. A small number of worms of both species disappeared in exposure dishes with larvae which had sealed mouths, but the means were not significantly different ($p > 0.05$) when compared to the mean for dishes with no mosquitoes. The mouths of 4/15 test larvae that were exposed to N. carpocapsae were found to have been imperfectly sealed with wax. Another larva, submitted to the same treatment, ecdysed and hence discarded the wax on the old cuticle. Also, mouth-blocking was not accomplished

Table 2.1.2 Recovery of infective-stage H. heliothidis and N. carpocapsae from dishes without A. aegypti, with larvae having wax-sealed mouths, and with untreated larvae after 24 hours (initial inoculum = 1,258 nematodes per dish, volume of water = 5 ml).

Nematode species	Mean ($\pm$ SE) no nematodes ¹ recovered from containers		
	Without mosquito larvae (control)	With sealed larval mouths	With free/ unsealed larval mouths
<u>N. carpocapsae</u> (5 expts)	1,116 $\pm$ 77 (813 - 1,225)	1,027 $\pm$ 97 (713 - 1199)	2 $\pm$ 1 (1 - 8)
<u>H. heliothidis</u> (3 expts)	1,008 $\pm$ 52 (956 - 1,059)	897 $\pm$ 80 (817 - 977)	4 $\pm$ 3 (1 - 7)

¹Figures in brackets are ranges.

in 1/45 larvae that were exposed to H. heliothidis. All 6 larvae were dead after 24 hours and dissections revealed nematodes in the haemocoel. Exposure dishes that previously contained these larvae had relatively fewer residual nematodes compared to those with properly-sealed larval mouths. This probably accounts for the slight difference in mean nematode recovery noted above. None of the larvae that had properly sealed mouths showed N. carpocapsae or H. heliothidis parasitization.

Therefore, these results show that all infections of the mosquito larvae by the rhabditoid nematodes in water occurred via the oral route. However, when 5 larvae were placed on 0.8% Agar with about 400 infective-stage H. heliothidis, infection of one of these larvae by 2 nematodes via the anal route was observed. Both parasites penetrated the hindgut wall, entered the haemocoel and triggered a rapid melanization reaction. Complete melanic capsule formation around one parasite occurred within 3 and a half hours.

Generally, higher numbers of N. carpocapsae than of H. heliothidis were recovered from the dishes, but the means were not significantly different.

Larval mortality

A. aegypti larvae, both with sealed and with unsealed mouths, showed substantial mortality (Table 2.1.3). However, only larvae with free mouths exhibited a high mortality that could be attributed to the parasites. Unaccountable mortality rates were high among larvae with wax-sealed

Table 2.1.3 Percent mortality¹ of A. aegypti larvae, with wax-sealed and unsealed mouths, exposed to infective-stage N. carpocapsae and H. heliothidis

Nematode Species	Percent mortality ¹ of larvae			
	Without		With sealed	
	nematodes	mouths	mouths	free mouths
(control) Parasitized Unaccountable Parasitized Unaccountable				
<u>N. carpocapsae</u>	6.7 + 2.1	6.7 + 3.0	43.9 + 11.7	49.3 + 14.1
(5 expts)	(0 - 13.3)	(0 - 13.3)	(20.0 - 86.7)	(0 - 13.3)
<u>H. heliothidis</u>	4.5 + 2.2	2.2 + 2.2	33.4 + 6.7	55.6 + 5.9
(3 expts)	(0 - 6.7)	(0 - 6.7)	(26.7 - 46.7)	(0 - 13.3)

¹Mean + standard error, ranges in parentheses

mouths, while they were comparatively low for the controls and larvae with unwaxed mouths.

2.1.4 Discussion

2.1.4.1 Host-parasite contact

Results of the present investigations into the region of contact between rhabditoid nematodes and A. aegypti larvae strongly support earlier assumptions that contact occurs at the bottom of water. The results further showed that no contact leading to infection occurred at the surface. These findings lead to a consideration of the type of substrate as a factor influencing the performance of the nematodes. Finney and Harding (1981) and Molta (1982) showed that on a porous or uneven sandy substrate, nematodes conceivably disappear into spaces between particles, thus becoming unavailable for contact with browsing larvae. However, a smooth substrate such as the bottom of rock pools and artificial containers, enhances contact.

Although A. aegypti larvae undertake limited pharyngeal filtration at the surface (Christophers, 1960), no infective nematodes were ingested in this region. This is because nematodes settle rapidly to the bottom (Finney and Harding, 1981; Molta, 1982), thus escaping ingestion by surface-confined larvae. Infective N. carpocapsae settle to a depth of 13.5 cm within 5 minutes following placement at the surface (Molta, 1982). The present findings, also demonstrate the poor swimming ability of the infective nematodes, a fact

that was confirmed by microscopical examination.

Death of the three larvae trapped under a 1mm mesh sieve might have resulted from shortage of respiratory oxygen, parasitic infection or both. According to Macfie (1917) and Christophers (1960), a full grown A. aegypti larva undertakes little cutaneous respiration and could only remain alive for a maximum of twelve hours when submerged in water. Since larvae were exposed for twenty-four hours, it is possible that death was caused by lack of oxygen. Unfortunately, the exact duration of submergence of these larvae is not known. The development of bacterial septicaemia leading to death of insect hosts infected by N. carpocapsae, usually occurs within 24 hours of infection (Poinar and Thomas, 1967; Poinar et al. 1971; Poinar, 1979; Thomas and Poinar, 1979). Nematode-induced mortality of A. aegypti can occur within 8 hours (see Section 2.5), thus the observed larval mortality could equally have been caused by infection. However, since post-mortem examinations revealed the presence of parasites in the haemocoel of the three larvae, it is concluded that host-parasite contact occurred at the bottom of the container.

Since, the region of host-parasite contact is restricted to the bottom, this suggests that water depth may influence the parasitism of predominantly surface-feeding mosquito larvae, such as Anopheles spp. (Marshall, 1938; Horsfall, 1972), by these nematodes. The rate of parasitization of such larvae will probably decrease as depth increases.

Laboratory experiments are needed to test the validity of this hypothesis. However, depth is not likely to be an important factor in the interaction between the nematodes and bottom-feeding culicine larvae, since their feeding behaviour forces them to enter the region of contact. This subject is further investigated in a later part (see Section 2.2) of this work. If the present predictions are correct, then a surface formulation of the agents would be required for application against surface-feeders in deep water. Also, a formulation that will prevent nematode disappearance into an uneven substrate might enhance the performance of these entomo-pathogenic agents against bottom-feeding mosquito larvae.

2.1.4.2 Mode of infection

Infective juveniles of both H. heliothidis and N. carpocapsae entered the host through the mouth. Thus, inhibition of feeding by sealing the mouth prevented nematode entry into the larvae. These findings confirm and extend earlier reports by Welch and Bronskill (1962), Poinar and Leutenegger (1971), and Poinar and Kaul (1982) that the nematodes were ingested orally during larval feeding. These results further indicate that no other routes were exploited by the entomopathogens during normal exposure procedures.

The observation that virtually all nematodes inoculated into dishes with uninhibited larval feeding were ingested within 24 hours of exposure indicates that pre-parasite

uptake by the larvae was a rapid process. Thus, the low mortality of larvae exposed in these dishes relative to pre-parasite uptake suggests considerable mortality of worms in the alimentary system of the larvae. Dadd (1971b) observed that many C. pipiens larvae exposed to infective N. carpocapsae were found to contain numerous dead worms in the midgut within 1 hour of exposure. Though he failed to quantify the uptake, he reported damage to nematode cuticles caused by larval mouthparts during entry. Gaugler et al. (1983) also reported injury of infective Steiner nema feltiae (Neaplectana carpocapsae) caused by the mouthparts of mid and late instar Simulium ochraceum larvae during ingestion. Thus, the low ratio of larval infection to pre-parasite ingestion may be attributed to the nematode's poor survival of ingestion. This subject is further investigated and discussed in Section 2.5.

The observation that partial inhibition of feeding, due to unsuccessful application of molten wax to larval mouths, produced some mortality from infection, confirms that nematode entry occurred via the oral route. It is not surprising, therefore, that dishes in which these larvae were exposed showed slight decrease in residual pre-parasite counts. Also, larvae with inhibited feeding exhibited a higher unaccountable mortality when compared to those without blocked mouths. It is believed that this mortality was largely a result of starvation and not adverse effects of wax application since most larvae died during the post-exposure period, at least 24 hours after treatment.

The artificial infection technique of exposing larvae to infective H. heliothidis on agar, raised some interesting points about the host-parasite interaction. Thus, providing a suitable substrate was present, it proved possible for these nematodes to enter through the larval anus. This finding is similar to those reported by Poinar and Himsworth (1967), Poinar (1979), Molyneux and Bedding (1984), and noted by the present worker (unpublished data) for various insect species from several orders. The process apparently involves an active location of the host by the nematodes. Infective stages of N. carpocapsae are attracted to insect faeces (Schmidt and All, 1979), to carbon dioxide (Triggiani and Poinar, 1967; Schmidt and All, 1978; Gangler et al. 1980; Bedding pers. comm.), and to uric acid and xanthine, among other excretory products of insects (Schmidt and All, 1979). It is highly likely that the nematodes found the larvae by a directional attraction to some stimulus, rather than a chance or random contact (Reed and Carnes, 1967). Thus, prior to infection, the larva had a tube of faecal matter surrounded by peritrophic membrane, oozing out of the anus. Nematodes swiftly moved towards and into the anus in a klinotactic fashion, as described by Croll and Matthews (1977), following an 'arcing approach' (Gaugler et al. 1980). Presumably, low motility of the larvae on agar permitted the establishment of a chemical gradient to which nematodes were attracted. Support for this hypothesis comes from Croll (1970) that accumulation of root exudate or diffusate around plants

elicited an orientation of phytophagous nematodes towards the plants. According to this author, the attraction plays an important role in host-parasite contact. Also, Schmidt and All (1978) reported similar responses of N. carpocapsae to relatively immobile insects, including Galleria larvae in agar. The present observation warrants further investigation.

Once in the gut, it is known that nematode penetration into the haemocoel of mosquito larvae occurs in the proventricular (Welch and Bronskill, 1962) and membranous neck (Poinar and Kaul, 1982) regions. However, the present infection on agar demonstrated that nematodes could penetrate the wall of the hindgut into the haemocoel. The hindgut is made up of the ileum, colon and rectum. Except for the colon, whose wall resembles that of the midgut, the walls of the hindgut are thin, composed of small cuboidal cells (Christophers, 1960). The present observation reveals that the hindgut wall did not pose a barrier to penetration of the nematodes. This suggests that some orally ingested worms that survive passage along the intestine may invade the haemocoel through the hindgut. But, further observation is required to confirm this speculation. This is the first report of anal penetration of a rhabditoid nematode into mosquito larvae, and although highly artificial, this bioassay has further illuminated the interaction between the nematodes and the hosts. While entry occurred passively, on the part of the nematodes, in water, the worms actively

invaded the host on agar. Bedding (pers. comm.) remarked that viscosity of medium or substrate is influential to the mode of infection of insect hosts by rhabditoid nematodes. Thus, it is assumed that the observed differences between the infection processes in water and on agar were due, at least in part, to differences in viscosities of the two substances.

2.2 DEPTH OF WATER

2.2.1 Introduction

Experimental infections of culicine mosquito larvae with rhabditoid nematodes almost always occur in shallow water. For example, during infectivity tests of the DD-136 strain of Neoaplectana carpocapsae for Aedes aegypti, Welch and Bronskill (1962) conducted exposures in 1cm deep tap-water. Similarly, while investigating the processes of encapsulation and melanization of N. carpocapsae by A. aegypti larvae, Bronskill (1962) exposed larvae to infective nematodes in the same (1cm) depth of water. A further study of this defence reaction was conducted by Poinar and Leutenegger (1971) using the nematodes against Culex pipiens larvae. During this work, larvae were exposed to invasive nematodes in 6.0cm diameter petri dishes containing 24ml of water (depth, <1cm). Likewise, Finney and Harding (1981) and Poinar and Kaul (1982) used very shallow depths (less than 2 and 1cm, respectively) of water in studies on the effects of substrate on rate of parasitism, and on the infectivity of a new nematode species for mosquito larvae, respectively. Perhaps the shallowest water (ca. 3mm) was used by Dadd (1971b) during a study of the inter-instar susceptibility of Culex pipiens larvae to N. carpocapsae. Although most laboratory experiments are designed to conserve working space and materials (Anderson, 1982b; Anderson and Gordon, 1982), these experiments imply that shallow water is critically important in the interactions between the nematodes and mosquitoes. If

so, the relationship between depth and the rate of parasitism by these nematodes must be quantified. This section describes some laboratory experiments on the relationship between depth of water and parasitism of A. aegypti larvae by H. heliothidis and N. carpocapsae.

2.2.2 Materials and Methods

Laboratory cultures of H. heliothidis and N. carpocapsae were tested against the Silwood Park strain of A. aegypti L. in these experiments. Stock suspensions of H. heliothidis and N. carpocapsae in 0.1% formalin, were stored at 10⁰ and 5⁰C, respectively. Storage was never longer than 4 weeks.

To perform the test, 95, 120, 232, 465, and 945ml of dechlorinated tap-water were placed into each of five 1,000ml glass beakers (diameter, 8.5cm; height, 18cm; area of bottom, 56.8cm²) to give depths of 1,2,4,8, and 16cm, respectively. Then, 15 randomly selected 3rd-instar larvae were introduced into each beaker, before five lots of 2,625 nematodes each, were inoculated into the respective beakers. Prior to inoculation the nematodes were washed twice in tap-water, to remove formalin. They were reconcentrated by centrifugation at 500 r.p.m. (5g). About 30 mg of larval food was added to each beaker before they were covered with transparent lids to minimize evaporation. The control utilized 15 larvae in 100ml of water, in a similar beaker, without nematodes.

Exposures lasted for 24 hours at 25 ± 2⁰C. Then, the larvae were separated from nematodes, and kept for a 24-hour

post-exposure period as described in Section 2.1.2. Dead larvae were dissected between 48 and 60 hours following the commencement of the exposures. Mortality caused by parasites was recorded, and the experiment was repeated 3 and 9 times with H. heliothidis and N. carpocapsae respectively, using a constant parasite-host ratio of 175 nematodes to 1 larva.

2.2.3 Results

As expected, there was no significant correlation ($p > 0.05$) between depth of water and the rate of parasitism of A. aegypti larvae by either H. heliothidis or N. carpocapsae (Table 2.2.1). Regression analysis of the data (Fig. 2.2.1) showed a poor negative correlation ($r^2 = -4.7\%$ and -2.0% for parasitism by H. heliothidis and N. carpocapsae, respectively) between larval mortality and depth. Thus, there was only a very slight decrease in mortality as depth increased from 1 to 16cm. The analysis of variance performed on the data (Table 2.2.2) confirmed no significant difference ($p > 0.05$) in mortality between the various depths, with either of the entomopathogens.

The individual data-sets for each treatment varied considerably (Fig. 2.2.1). Variations (expressed as 95% confidence intervals, but not standard deviations or ranges) were much higher in tests conducted with H. heliothidis than those with N. carpocapsae, at all depths. Similarly, the average mortalities obtained at all depths were higher with H. heliothidis than with N. carpocapsae. However, 5 larvae

Table 2.2.1 % Mortality of Aedes aegypti larvae caused by
H. heliothidis and N. carpocapsae at five
depths of water.

Parasite species	Depth of Water (cm)	Per cent mortality of larvae caused by infection		
		Mean	SD	Range
<u>Heterorhabditis</u>	1	47.9	13.5	31.1 - 58.9
<u>heliothidis</u>	2	56.9	13.7	43.1 - 75.0
(n = 4 expts)	4	49.9	16.4	26.6 - 63.4
	8	54.3	28.5	21.4 - 90.0
	16	47.0	11.5	35.3 - 58.9
<u>Neoaplectana</u>	1	39.1	23.3	0.0 - 90.0
<u>carpocapsae</u>	2	41.3	21.2	14.9 - 75.0
(n = 10 expts)	4	41.9	16.5	21.4 - 68.6
	8	38.0	25.5	0.0 - 75.0
	16	38.9	19.7	14.9 - 75.0

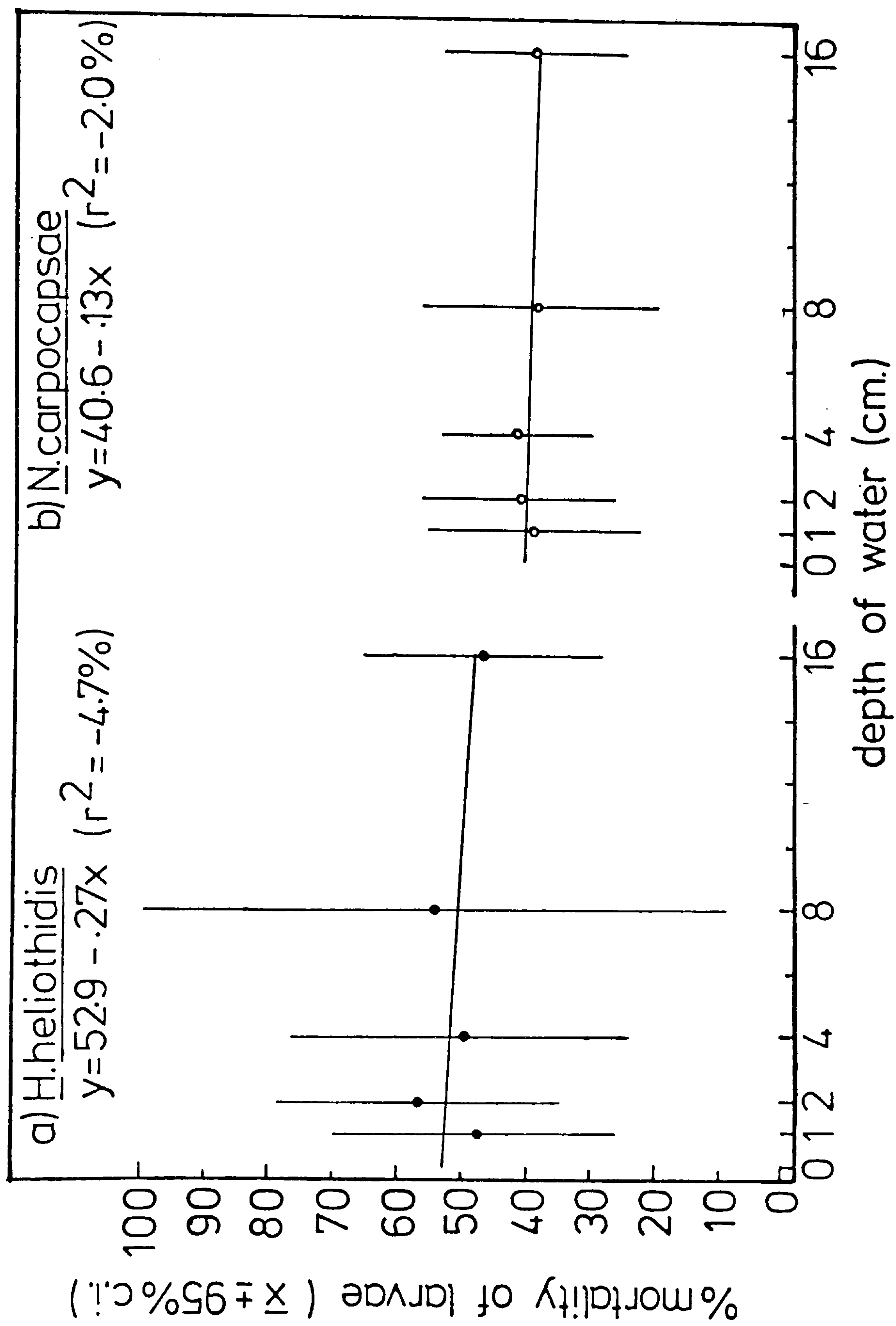


Figure 2.2.1 Mean mortality rates of A. aegypti larvae infected by H. heliothidis and N. carpocapsae at various depths of water. (Bars indicate 95% confidence intervals)

Table 2.2.2 One-way analysis of variance on data in Table 2.2.1, after angular transformation

Parasite	Source	DF	SS	MS	F.Ratio	P ¹
<u>H. heliothidis</u>	Treatment	1	42.8	42.8	0.154	>0.05
	Error	18	4995.4	277.5		
	Total	19	5038.2			
<u>N. carpocapsae</u>	Treatment	1	24.3	24.3	0.056	>0.05
	Error	48	20789.7	433.1		
	Total	49	20814.0			

¹Significance of difference; p>0.05, not significant

died in 2 experiments with H. heliothidis and dissections revealed no parasites.

The dissections of dead larvae revealed varied numbers of parasites within the haemocoel (Table 2.2.3). However, the mean parasite abundance was not significantly ($p>0.05$) correlated with the depth of water. Generally, H. heliothidis showed slightly greater abundance than N. carpocapsae within mosquitoes infected at all depths.

2.2.4 Discussion

Within the range tested, depth of water was unimportant in determining the rate of parasitism of A. aegypti larvae by both H. heliothidis and N. carpocapsae. The abundance of parasites in infected larvae was similarly unaffected. The results were not affected, for it is known that the nematodes settle rapidly to the bottom when applied to an aquatic system (Finney and Harding, 1981; Molta, 1982). Contact between nematodes and mosquitoes occurs when A. aegypti larvae dive to the bottom. Since the larval stages of most Aedes and Culex species are predominantly bottom feeders, browsing on bacteria, yeast, algae and other organic and inorganic particulate matter (Marshall, 1938; Christophers, 1960; Horsfall, 1972) it may be that, within the range of water depths normally inhabited by these mosquitoes, the depth is unimportant for the rate of parasitism. On the other hand, Anopheles larvae dwell almost entirely at the surface (Marshall, 1938; Horsfall, 1972) and it seems likely

Table 2.2.3 Abundance of Heterorhabditis heliothidis and Neoaplectana carpocapsae within the haemocoel of Aedes aegypti larvae infected at various depths of water.

Depth of water (cm)	Abundance, within haemocoel of infected larvae, of:			
	<u>H. heliothidis</u>		<u>N. carpocapsae</u>	
	$\bar{x} + S.D.$	Range	$\bar{x} + S.D.$	Range
1	3.2 $\pm$ 1.8	1 - 8	1.6 $\pm$ 0.3	1 - 3
2	2.2 $\pm$ 1.4	1 - 6	2.0 $\pm$ 0.5	1 - 4
4	2.5 $\pm$ 1.4	1 - 5	1.2 $\pm$ 0.4	1 - 2
8	1.8 $\pm$ 1.0	1 - 4	1.5 $\pm$ 0.5	1 - 3
16	2.4 $\pm$ 0.8	1 - 4	1.3 $\pm$ 0.4	1 - 3

that the rate of parasitism will be affected by the depth of water.

Apparently, little is documented in the literature on water depth preferences of bottom-feeding mosquitoes. however, general indications are that the larvae dwell in fairly shallow water-bodies of temporary nature. Christophers (1960) and Horsfall (1972) provide extensive reviews of the breeding habitats. According to these and many other authors, the breeding sites are very diverse and include, for A. aegypti, artificial containers, water receptacles of a wide range, domestic receptacles, uncovered cisterns, traps of drains, flush tanks and pans of water closets, to mention only a few. The rural forms breed in rock pools. The shallow water in these breeding places would not significantly influence the level of parasitism by rhabditoid nematodes, since the larval feeding behaviour ensures their entry into the region of contact with the pre-parasites. Consequently, the rate of host-parasite contact and the subsequent uptake of pre-parasites by the larvae is limited by the nature of the substrate (Finney and Harding, 1981; Molta, 1982) but not depth of water.

Investigating the effects of water volume on the levels of infection of C.p. quinquefasciatus by the mermithid Romanomermis culicivorax, Petersen and Willis (1970) observed that small volumes had no differential effects on parasitism. However, they noted that infection decreased considerably at much higher volumes. They explained that increase in volume

reduced the rate of contact between the actively swimming pre-parasites and the larvae. Presumably, volume increase was associated with increase in surface area as well as depth. The surface area was not tightly controlled. Since the larvae intermittently disappeared from the region of contact (in this case, surface) (Petersen and Willis, 1970) to feed at the bottom, host-parasite contact was significantly reduced or at least delayed. However, during the present investigations in which only depth was varied, there was almost no 'spatial dilution' of the infective nematodes applied. This observation agrees with the one by Petersen (1973), that depths of 10 to 30 cm produced no significant differences in the mean parasitism of C.p. quinquefasciatus by Reesimermis nielsenii (= Romamomermis culicivorax Ross and Smith, 1976). Thus, it is highly unlikely that depths beyond the present ones might produce any significant changes in the rate of parasitism. However, the present results would have been different if changes in depth were accompanied by changes in area of the bottoms of the exposure arenas. This assumption needs experimental demonstration.

It seems likely that depth may be critically important in determining the levels of parasitism of surface-feeding mosquito species by the nematodes. With these species, it is highly probable that contact with nematodes will be maximum in extremely shallow water, and will decrease with increase in depth. In deep water, the nematodes, having settled to the bottom will be completely separated from the larvae, with

the result that little or no infection will occur. This prediction may not hold true in certain habitats of the larvae where turbulence or currents keep the water mixed, so that the infective worms are continually brought into the vicinity of the larvae. Experimental data are required to support these theories.

The mean mortality of larvae caused by H. heliothidis during this study, was higher than that by N. carpocapsae at each depth. This observation is probably attributable to the higher efficacy of the heterorhabditid compared to the neoaplectanid, and may be related to uptake and penetration of the nematodes into the larvae. This assumption is supported by the observation that the mean abundance of H. heliothidis in the haemocoel of infected larvae was slightly greater than that of N. carpocapsae at each exposure depth. These subjects are further investigated later.

2.3 EFFECT OF FOOD ON NEMATODE-INDUCED MORTALITY

2.3.1 Introduction

The lethal action of some control agents against the aquatic stages of mosquitoes and blackflies depends on their ingestion by the insects. The efficacy of these agents is partly determined by the presence and concentration of particulate food in the environment (Ramoska and Pacey, 1979; Gaugler and Finney, 1982). Ramoska and Pacey (1979) reported significant alteration of the efficacy of Bacillus sphaericus against mosquitoes and attributed it to the presence of suspended food particles in the water. On the basis of this report, Mulla et al. (1982) thought that the presence of high levels of organic and inorganic particulates in polluted lagoons accounted for the reduced efficacy of B. thuringiensis (H-14) against the stagnantwater mosquitoes in these habitats. Also, Aly (1983) demonstrated that the presence of food particles in water significantly influenced the effectiveness of B.t. var. israelensis against Aedes vexans larvae. He noted that increased concentration of food particles yielded decreased larval mortality. Conversely, Ignoffo et al. (1981) observed that the presence of small amounts of pulverized Tetramin as A. aegypti larval food enhanced the performance of B. t. var. israelensis against the mosquitoes. These investigators concluded that food acted as a stimulant to increase the rate of ingestion of the pathogens.

Additional evidence for the influence of food particles

on the efficacy of pest control agents comes from work conducted on blackfly larvae (Undeen and Nagel, 1978; Gaugler and Molloy, 1980; Gaugler and Finney, 1982). Gaugler and Molloy (1980) observed that inhibition of feeding in Simulium vittatum larvae was accompanied by increased mortality when B. t. var. israelensis was applied before feeding inhibition. Conversely, they observed a decrease in mortality when Bti was applied after feeding inhibition. However, Undeen and Nagel (1978), citing unpublished data, reported a decrease in the performance of B. thuringiensis on Simulium larvae in the presence of food. According to these workers, the extent of decrease in the effectiveness of the pathogens increased as the food concentration increased. But, on the contrary, Lacey et al. (1978) observed that the efficacy of B. thuringiensis ^{var. israelensis} against S. vittatum larvae was not affected by food concentration.

Larval diets have often been added to experimental units involving late instar larvae of mosquitoes to avoid starvation and the associated chances of unwanted cannibalism of the larvae (Christophers, 1960). Since rhabditoid nematodes exert their lethal actions on culicine mosquito larvae following oral ingestion during larval feeding, (Welch and Bronskill, 1962; Poinar and Leutenegger, 1971; Poinar and Kaul, 1982; also see Section 2.1) it is of interest to determine whether the presence of suspended particulate food in the aquatic system might influence the efficacy of these agents. This section describes the effects

of the presence of a single concentration of food particles on the rate of infection of A. aegypti by Heterorhabditis heliothidis and Neoaplectana carpocapsae (DD-136 strain).

2.3.2 Materials and methods

The Silwood Park strain of A. aegypti, maintained at $25 \pm 2^{\circ}\text{C}$ and 12 hours photoperiod was used in these investigations. Eggs were hatched twice a week to obtain the required 3rd-instar larvae. H. heliothidis and N. carpocapsae (DD-136 strain) were cultivated in-vivo in Galleria mellonella larvae. The infective-stage juvenile nematodes were extracted in 0.1% formalin solution and stored in dechlorinated tap-water at 10° and 5°C , respectively, until used. Storage times never exceeded 8 weeks.

The larval diet was a finely ground mixture of Tetramin Tropical Fish Food (TTFF, 6 parts), crumbled bread (CB, 2 parts) and yeast extract (YE, 1 part), used at a concentration of 10 mg per larva.

Tests were conducted on batches of 15 3rd-instar larvae per standard exposure dish containing 100 ml dechlorinated tap-water (ca. 2 cm deep). The diet and 3,000 infective-stage nematodes were added to each test dish almost simultaneously. The controls were identical, but without food. Exposures lasted for 24 hours. Then, the larvae were separated from the nematodes by washing and rinsing them with clean water. They were placed singly in the wells of MWTC plates. Then, the individuals were provided with pinches of

the food mixture for a post-exposure period of 24 hours. A total of 16 paired tests were conducted with each parasite species before the results were analysed using one-way ANOVA following angular transformation of the raw data.

2.3.3 Results

The rates of larval mortality attributable to infection by the nematodes (i.e. nematodes revealed by dissection) varied considerably within and between treatments (Table 2.3.1 and Fig 2.3.1). Generally, such mortalities were higher in the absence of food. With H. heliothidis, the presence of food produced a significant decrease ($p < 0.001$) in the rate of larval mortality (Table 2.3.1). However, the influence of food on the rate of mortality caused by N. carpocapsae was not significant ($p > 0.05$).

In the absence of food, both nematodes produced similar mortalities. However, when food was present, N. carpocapsae caused a significantly **greater** mortality than H. heliothidis ($p < 0.01$).

The dissections revealed a negligible influence of food on the numbers of parasites observed within the haemocoel of infected larvae. The mean numbers of H. heliothidis in parasitized larvae exposed in the presence and absence of food particles were 1.3 ± 0.6 (range = 1 - 3) and 1.9 ± 1.0 (1 - 5), respectively, compared to 1.3 ± 0.5 (1 - 3) and 1.4 ± 0.7 (1 - 4) for N. carpocapsae.

Occasionally, dissections of dead mosquitoes failed to

Table 2.3.1 Mortality of A. aegypti larvae due to infection by H. heliothidis and N. carpocapsae exposed with and without food

Treatments (N = 16)		Percent mortality caused by:	
		<u>H. heliothidis</u>	<u>N. carpocapsae</u>
With food	$\bar{x} \pm SD$	22.9 $\pm$ 21.2	46.3 $\pm$ 19.7
	Range	0.0 - 80.0	6.7 - 86.7
Without food	$\bar{x} \pm SD$	60.8 $\pm$ 27.6	57.5 $\pm$ 24.9
	Range	13.3 - 93.3	13.3 - 93.3

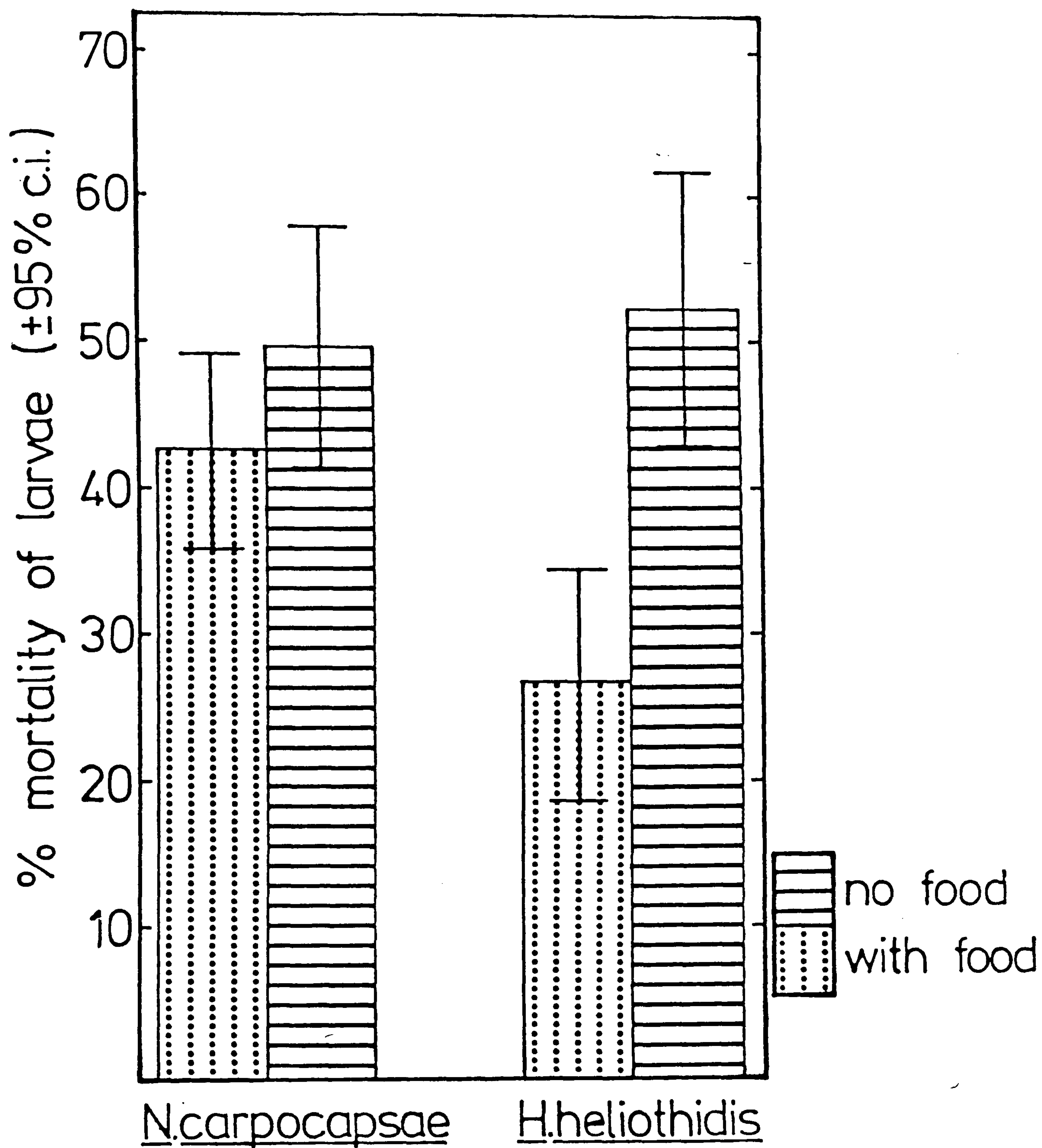
Table 2.3.2 Results of one-way ANOVA on the arcsine transformed data comparing the effects of the presence and absence of food on the mortality of A. aegypti larvae caused by H. heliothidis and N. carpocapsae.

Treatment	$\bar{x} \pm SD$ for arcsine transformed data for % mortality caused by:			
	<u>H. heliothidis</u> ¹	<u>N. carpocapsae</u> ²		
With food	26.7 $\pm$ 14.9	42.6 $\pm$ 12.5		
Without food	52.4 $\pm$ 17.8	49.6 $\pm$ 15.8		

Source	df	ss	ms = ss/df	f-ratio~
Food ¹	1	5285	5285	19.65 ***
Error	30	8107	270	
Total	31	13391		
Food ²	1	395	395	1.95 NS
Error	30	6079	203	
Total	31	6474		

~ns, not significant; ***, very highly significant

Figure 2.3.1 Mortality of 3rd-instar A. aegypti L caused by H. heliothidis and N. carpocapsae in the presence and absence of larval food.



reveal nematodes. In the presence of food and with H. heliothidis 4/16 experiments produced 1 unaccountable mortality each and 2/16 produced 2 such mortalities each. For the same species, but in the absence of food, 2/16 experiments produced 1 unaccountable mortality each and 2/16 produced 2 each. Mortalities that could not be attributed to N. carpocapsae never occurred.

2.3.4. Discussion

Ingestion of infective-stage juvenile H. heliothidis and N. carpocapsae during feeding is prerequisite for infection of culicine mosquito larvae. The rate of ingestion by filter feeders is a function of the concentration of particulates in the environment (Dadd, 1970, 1971a; Mulla and Lacey, 1976). Also, the size of suspended particles may enhance or inhibit ingestion by mosquito larvae (Dadd, 1970, 1971a) and blackfly larvae (Mulla and Lacey, 1976; Gaugler and Molloy, 1980). Therefore, it is perhaps not surprising that the presence of food in the environment of 3rd-instar A. aegypti larvae influenced the rate of infection by the nematodes. Generally, the prevalence of the infections decreased in the presence of food while the abundance of the parasites was unaffected. The observed reduction in prevalence with food is similar to that reported for Culex restuans (Ramoska et al. 1981), Aedes vexans (Aly, 1983) and a number of Simulium spp. (Gaugler and Molloy, 1980; Gaugler and Finney, 1982) exposed to various Bacillus species/strains. The

effect of food particles on parasitism of the mosquitoes probably reflects changes in pre-parasite uptake rate and the rate of voidance of ingested materials by the larvae. Presumably, food availability increased the attainment of satiation, thus reducing browsing and ingestion time. Consequently, fewer infective nematodes would be ingested in the presence of food than in its absence. Support for this hypothesis comes from work by Dadd (1970, 1971a). He observed that the overall rate of particulate ingestion by mosquito larvae in a constant environment was changed by alterations in the speed of movement of the larval mouthparts or the actual time spent browsing. He also observed that particulates present in water slightly reduced the filtering rate of Culex pipiens larvae.

Alternatively, food particles that were bigger than the average body lengths (542 ± 63 and $654 \pm 38 \mu\text{m}$) of infective N. carpocapsae and H. heliothidis, respectively, probably reduced the availability of the nematodes to the hosts. Finney and Harding (1981) observed that the presence of sand particles and leaves in containers in which A. aegypti larvae were exposed to infective N. carpocapsae markedly reduced the availability of infective-stage nematodes to the mosquitoes.

However, the present results disagree with the findings of Ignoffo et al. (1981) that the presence of food (pulverized Tetramin) enhanced the performance of B.t. var. israelensis against Aedes aegypti larvae. The reason for this discrepancy is not known. Certain solutes, such as

water-soluble yeast extract, adenylic acid and sperm nucleic acid (Dadd, 1970, 1971b), lactalbumin hydrolysate (Poinar and Leutenegger, 1971) and other hydrolysates (Poinar, pers. comm.) are known to stimulate mosquito larval feeding. However, if yeast extract, present in the larval food used in this study, stimulated feeding, it clearly did not enhance the performance of the nematodes. Mulla and Lacey (1976) suggested that the efficacy of an insecticide may be influenced by the rates of larval feeding and voidance. Perhaps, yeast extract induced an increase in larval ingestion rate which was compensated for by an increase in the rate of voidance. Obviously, this could have minimized the chances of nematode penetration of the larval gut-wall into the haemocoel, thus leading to the observed decrease in prevalence. The relationship between phagostimulant components of larval food and the efficacy of these control agents requires further investigation.

The level of reduction in the efficacy of control agents is known to increase with increase in concentration of food particles (Ramoska and Pacey, 1979; Gaugler and Finney, 1982; Aly, 1983). Aly et al. (in press) observed a preference by Aedes vexans larvae to ingest particles with nutritional value and they utilized these findings to produce a more efficacious formulation of B. t. var. israelensis. These factors were not investigated during the present study, but could be of interest in determining the potentials of these nematodes for controlling natural populations of culicine

mosquitoes in aquatic habitats with varying concentrations of organic and inorganic particulates. Since particulate food affects parasitism by these nematodes, then a relationship between the concentration of food particles in water and the rate of parasitism of the larvae would be expected.

Considerable variations were observed in the rates of infection of the mosquitoes by the nematodes, both with and without food. This variability contributed to the lack of a statistically significant effect of food on the prevalence of N. carpocapsae infections. Nevertheless, the presence of food reduced the rate of larval infection by these nematodes.

2.4 INSTAR SUSCEPTIBILITY OF A. AEGYPTI L. LARVAE TO RHABDITOID NEMATODES.

2.4.1 Introduction

Various laboratory and field experiments have demonstrated that the susceptibility of insect pests to control agents depends, to a large extent, on the instar (age) of the insects. This observation is well documented for chemical insecticides (Busvine, 1971). Boucias and Nordin (1977) reported that the susceptibility of lepidopterous larvae to insect-pathogenic baculoviruses decreases as the instar increases. Similarly, there is abundant evidence from the literature that susceptibility of a wide range of insect species to bacterial pathogens, notably B. thuringiensis strains (McGaughey, 1978; Molloy et al. 1980a; Mulla et al. 1982; Gaugler et al. 1983) is inversely correlated to the age of the instars. That is, the susceptibility of the insects to these pathogens decreases as the instar increases.

For macroparasite infections, Petersen and Willis (1970) and Petersen (1981) reported decreases in susceptibility of some culicid mosquito larvae to the entomophilic mermithid nematode, Romanomermis sp. as the larvae developed. The opposite pattern of inter-instar susceptibility has been reported following experimental trials of Neoaplectana carpocapsae against both blackfly (Molloy et al. 1980b; Gaugler and Molloy, 1981a, 1981b; Gaugler et al. 1983) and Culex pipiens larvae (Dadd, 1971b). Dadd (1971b) reported

that first- and second-instar larvae were not susceptible to parasitism by the nematodes.

However, lack of correlation between instar and susceptibility to control agents has also, been reported. For example, Pye and Burman (1978) conducted infection studies of the large pine weevil, Hylobius abietis using N. carpocapsae and reported no differences in susceptibility between the instars.

In previous experiments (Molta, 1982) the inter-instar susceptibility of Aedes aegypti larvae to the DD-136 strain of N. carpocapsae was quantitatively investigated. First-instar larvae were not affected while the older instars demonstrated varied degrees of susceptibility. Third-instars were slightly more susceptible than fourth-instars, which were much more susceptible than the second. This section describes additional experiments which were conducted to investigate factors responsible for different susceptibilities. It consists of two major parts. The first is a behavioural study of the vertical distribution of the larval instars in water, since previous investigations (see Section 2.1) revealed that all host-parasite contact leading to infection occurred at the bottom. Therefore, this study was aimed at determining whether the more susceptible late instars spent more time browsing at the bottom than the less susceptible early instars. The second part is a morphometric study of the mosquito larval instars and of the invasive juvenile nematodes. This was geared towards determining

whether 'physical exclusion' of the infective worms from early instars (Dadd, 1971b) was the cause of insusceptibility of these larvae, and the relatively low susceptibility of the second instars.

2.4.2 Materials and methods

2.4.2.1 Larval distribution in water.

Eggs of the Silwood Park strain of the mosquitoes used in these experiments were induced daily to hatch. The batches of larvae obtained were reared separately, at $25 \pm 2^{\circ}\text{C}$, until all 4 larval instars became available for the experiments. Then, 6-12, 24-48, 48-72, and over 72 hours old 1st, 2nd, 3rd, and 4th-instar larvae, respectively were used.

For each larval instar, 10 larvae were selected and placed in 25 ml dechlorinated tap-water (depth = 3.5 cm) contained in a flat-sided sterilin bottle (capacity = 50 ml, width = 2cm, length = 4cm, surface area = 8 cm^2). Two bottles containing different instars were focused upon with a Hitachi video camera (Hv.16S) with a close-up lens and a 'timer' device attached to the camera. The camera was placed 1.5 m away from the larvae to minimize interference with their normal behaviour. Because of depth of field limitations, only 2 bottles, each containing a different instar, were observed at a time. Thus, the 4 bottles with the appropriate larval instars were randomly changed, in pairs. The movements of the larvae up and down the columns of water were tape-recorded using a National 'Time-Lapse' VTR

(NV-8030) linked to a Shibaden black/white television.

Before each observation period of 30 minutes, in a room with natural light, a pinch of powdered larval food was sprinkled onto the water in each bottle. Then, the set-up was allowed a pre-observation period of about 15 minutes before recording was commenced. At the end of the observation period, the VTR was stopped and the bottles with the larvae were replaced. The activity of the new set of larval instars was, also recorded then the numbers of larvae of each instar, that were at the surface, on the bottom or in between were counted after every 3 minutes of the 30 minutes observation period (= 10 sub-samples). The experiment was repeated six times.

The distribution of the larval instars in the three locations was, then analysed using a Genstat analysis of variance (ANOVA) programme.

2.4.2.2 Morphological measurements of mosquito larvae and invasive nematodes.

A. aegypti larvae for morphological measurements were killed and fixed in fresh Baker's formaldehyde-calcium (40% formaldehyde, 10 ml.; 10% anhydrous Calcium chloride, 10 ml.; distilled water, 80 ml.; powdered Calcium carbonate, slight excess) (Pantin, 1948). This fixative offered the advantage of slowly killing the larvae and more importantly preventing morphological distortion (osmotic, lipoid, etc) of the specimens. The larvae were fixed for 6 hours before

measurements were made.

Drawing of the mouths and head-capsules of 10 larvae of each instar were made using a Leitz camera lucida, at x100 magnification, as described by Hooper (1970). The morphological dimensions measured were the longitudinal opening and maximum width of the mouth, and the width of the headcapsule of each larval instar. The longitudinal opening of the mouth was measured between the epipharynx (below the post-palatal lobe) and the suspensorium (chitinous bar between the two mandibles). The maximum width of the mouth was determined as the distance along a straight line, between the two lateral apices of the oral opening, lying between the black-spot areas and the anterior portions of the tentoria. The width of the head was measured through the positions of the ocelli. The dimensions of 3 lots of 10 larvae each, drawn from different (temporal) cultures of the strain were determined.

The lengths and widths of 30 dauer juveniles of N. carpocapsae were, also measured. The body width was determined at the position of the genital primordium. Prior to measurements, the juvenile nematodes were heat-relaxed using hot water at 65°C. The nematodes were mounted in tap-water.

Differences between the larval morphometrics of the different instars were tested for statistical significance using one-way analysis of variance.

2.4.3 Results

2.4.3.1 Larval distribution in water

Larval stages of A. aegypti were not distributed uniformly (Table 2.4.1 and 2.4.2, Figs 2.4.1 and 2.4.2). For each instar, the distribution of the larvae at the surface, bottom and in between was significantly uneven ($p < 0.05$). There was also a highly significant difference between the numbers of the 4 different instars in the 3 regions ($p < 0.001$). These observations were not affected by the time factor incorporated into the experiments (that is the uneven larval distribution was observable at all times).

The 4th-instar larvae were most abundant at the surface, while the 3rd and 4th-instars were the most dominant at the bottom (Figs 2.4.1 and 2.4.2). The 1st-instar larvae were most common in between the surface and the bottom.

2.4.3.2 Morphometrics of A. aegypti larval instars

Linear measurements of the mouth and head-capsule of the 4 larval instars of A. aegypti are shown in Table 2.4.3. As expected, the dimensions of the characters increased as the instar of the larvae increased. For each of the characters, differences were highly significant ($p < 0.001$) between the measurements of the different instars. Little overlap occurred between the character measurement ranges (Fig. 2.4.3.). The size differences between the 2nd, and 3rd-instars were smaller than those observed between any other pair of larval instars (Fig 2.4.3)

Table 2.4.1 Average score for 6 experiments for
location of 10 Aedes aegypti larvae in 3.5
cm of water, observed at 3 minute intervals
for 30 minutes (SE of mean = 0.18, derived
from ANOVA residuals). The data are
plotted in Fig 2.5.1.

Location in water	Larval instars				
		1	2	3	4
Surface	mean	2.43	3.82	4.05	4.48
	range	0 - 6	0 - 6	1 - 6	2 - 8
In between	mean	5.23	3.93	2.75	2.38
	range	2 - 9	1 - 7	1 - 5	0 - 5
Bottom	mean	2.33	2.25	3.20	3.18
	range	0 - 5	0 - 7	0 - 8	0 - 6

Table 2.4.2 Analysis of variance (Genstat ANOVA) table for the distribution of A. aegypti larval instars in water (depth = 3.5cm; temperature = 25 + 2°C)

Source of variation	DF	SS	SS%	MS	VR	P ¹	ESE
Experiment stratum	5	0.0	0.0	0.0	0.0	NS	
Experiments . Units . Stratum							
Location	2	133.158	6.86	66.579	34.210	*	0.090
Instar	3	0.0	0.0	0.0	0.0	NS	0.104
Time	9	0.0	0.0	0.0	0.0	NS	0.164
Location . Instar	6	486.275	25.07	81.046	41.643	***	0.180
Location . Time	18	57.342	2.96	3.186	1.637	NS	0.285
Instar . Time	27	0.0	0.0	0.0	0.0	NS	0.329
Location . Instar . Time	54	105.225	5.42	1.949	1.001	NS	0.569
Residual (sampling error)	595	1158.0	59.69	1.949			
Total	714	1940.0	100.0	2.717			
Grand total	719	1940.0	100.0				
Grand mean		3.333					
Total number of observations		720					

¹ NS = not significant at 5% significance level; * = significant (p<0.05); *** = very highly significant (p<0.001).

Figure 2.4.1 Diagram of data in Table 2.4.1
(Bars indicate effective standard error derived from ANOVA residuals).

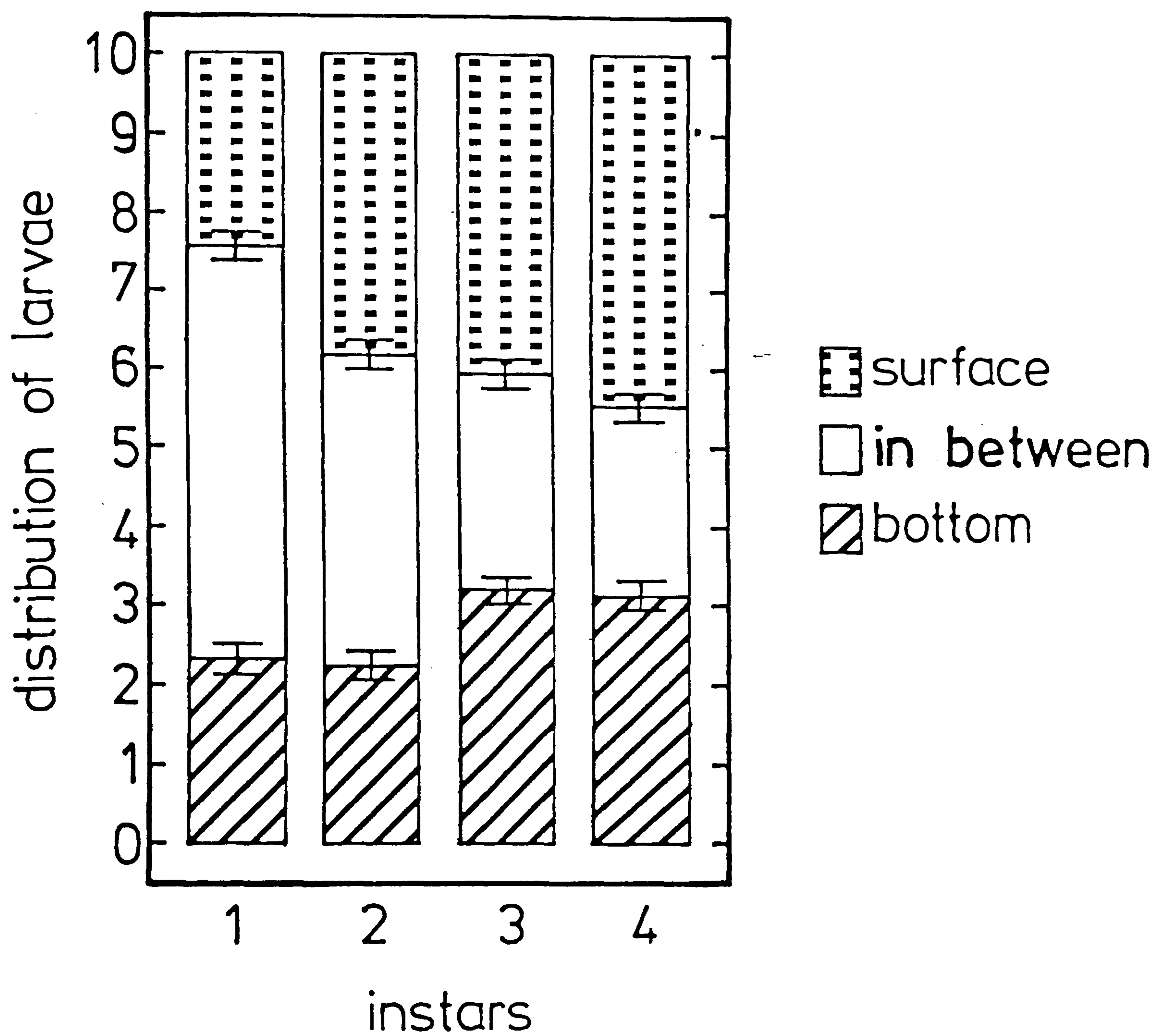


Figure 2.4.2 Diagram of data in Table 2.4.1
(see Figure 2.4.1 for explanation)

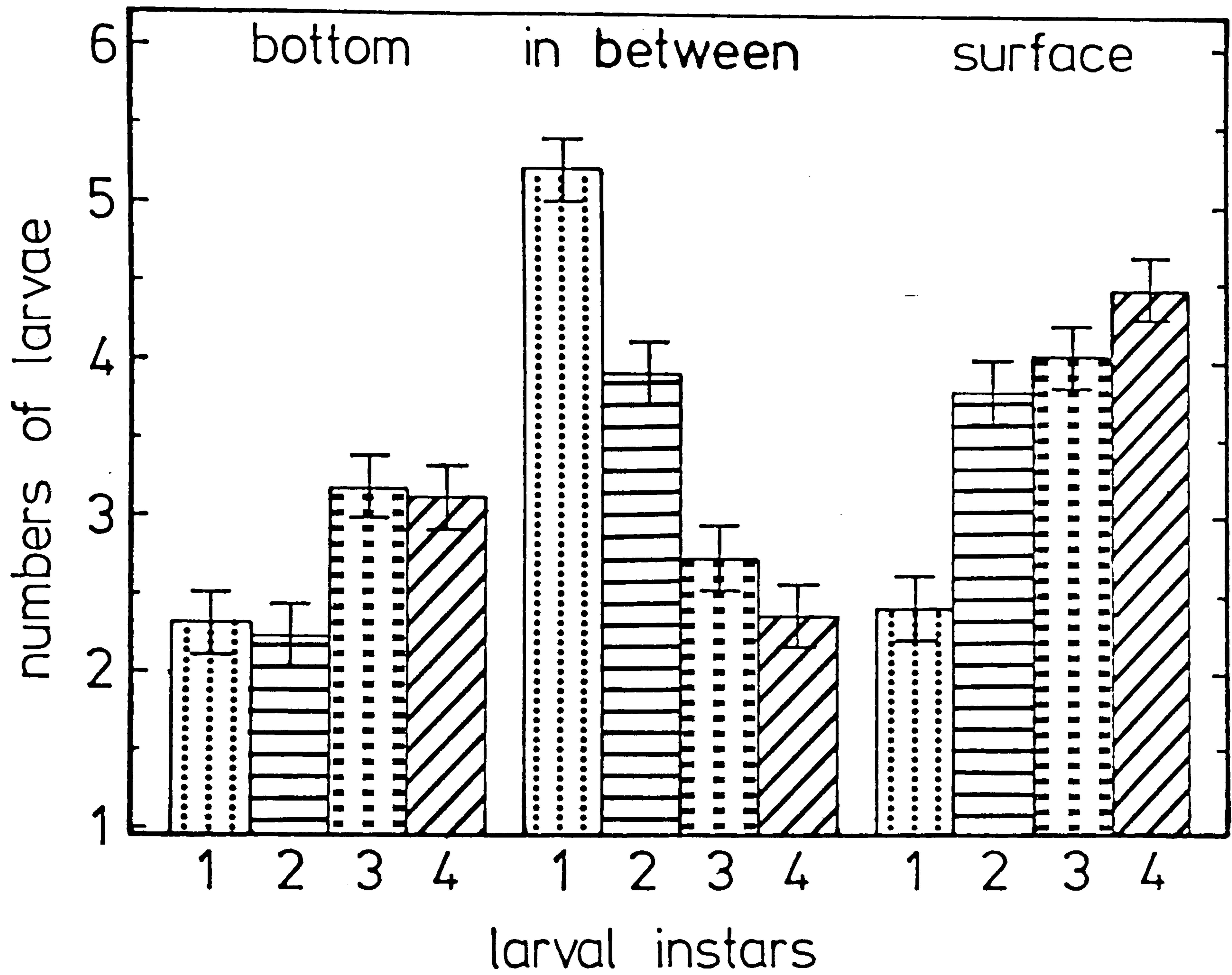
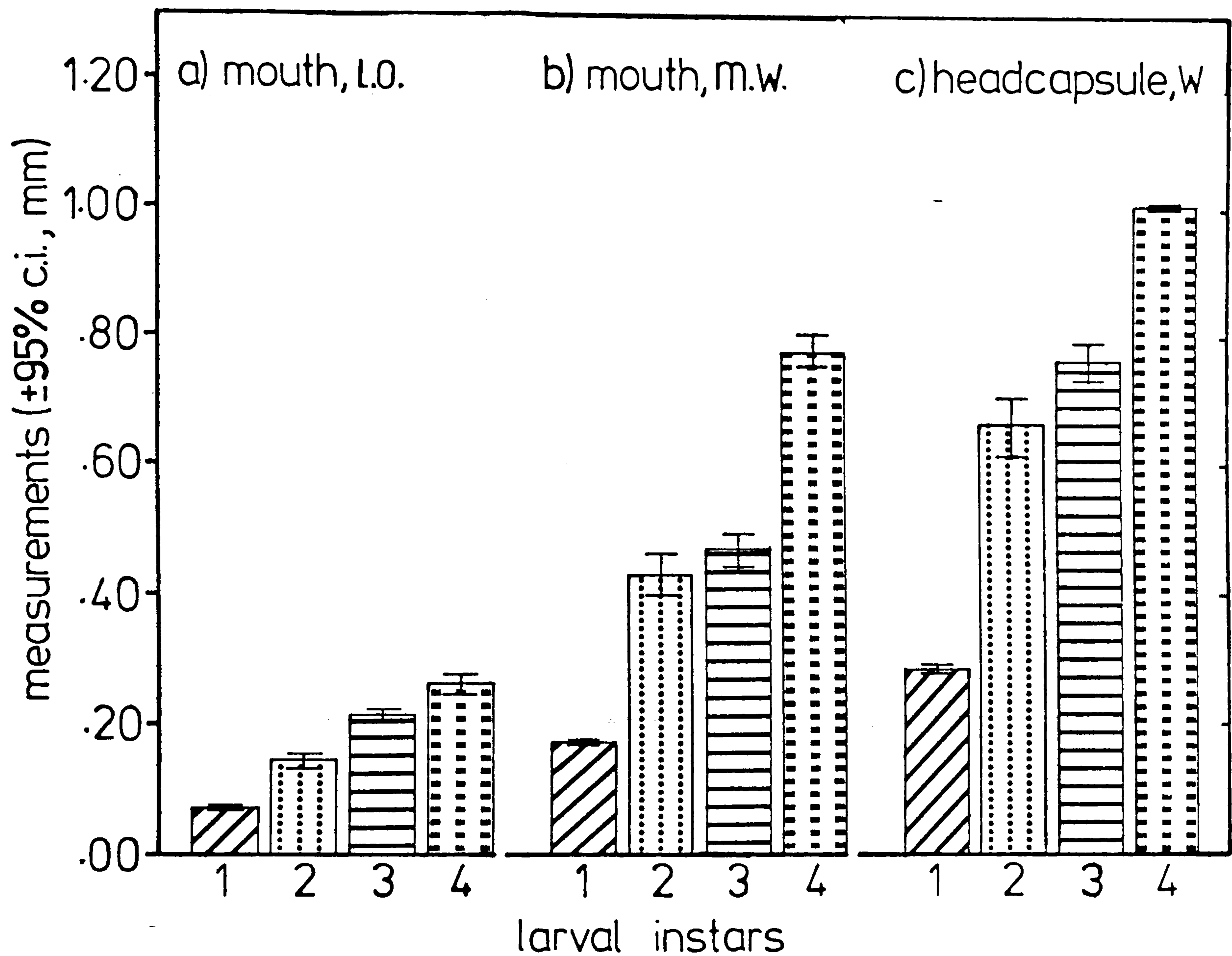


Table 2.4.3 Dimensions of the mouth and headcapsule of Aedes aegypti L. larval instars, cultured at $25 \pm 2^{\circ}\text{C}$.

Measurements in millimetres (n = 30)				
Larval instar				
		Longitudinal opening of mouth	Maximum width of mouth	Width of headcapsule
1	$\bar{x} \pm \text{S.D.}$	0.07 ± 0.01	0.17 ± 0.01	0.29 ± 0.02
	range	$0.06 - 0.09$	$0.14 - 0.19$	$0.25 - 0.30$
2	$\bar{x} \pm \text{S.D.}$	0.15 ± 0.03	0.43 ± 0.09	0.67 ± 0.12
	range	0.07 ± 0.20	$0.25 - 0.58$	0.44 ± 0.84
3	$\bar{x} \pm \text{S.D.}$	0.21 ± 0.03	0.47 ± 0.07	0.78 ± 0.08
	range	$0.18 - 0.27$	$0.38 - 0.60$	$0.65 - 0.88$
4	$\bar{x} \pm \text{S.D.}$	0.26 ± 0.04	0.78 ± 0.07	1.04 ± 0.05
	range	$0.18 - 0.32$	$0.65 - 0.89$	$0.93 - 1.12$
Difference ¹ (p)		p<0.001	p<0.001	p<0.001

¹Significance of differences tested by one-way analysis of variance; p<0.001, very highly significant

Figure 2.4.3 Mean measurements of the longitudinal opening (l.o.) and maximum width (m.w.) of the mouth, and width (w) of the headcapsule of *A. aegypti* L. larval instars cultured at $25 \pm 2^{\circ}\text{C}$ (N = 30 larvae, killed and fixed in Baker's formaldehyde-calcium; bars indicate 95% fiducial limits).



2.4.3.3 Measurements of the infective juvenile N. carpocapsae

The average infective-stage juvenile nematode was $27 \pm 3 \mu\text{m}$ wide at the genital primordium, and $545 \pm 67 \mu\text{m}$ long. The width of the nematode was much less than either oral dimension of any larval instars. However, only the width of the mouth of the 4th-instar exceeded the length of the nematode.

2.4.4 Discussion

Factors which account for differences in susceptibility between different instars are often related to the behaviour and/or morphology of the insect species and the control agent in question. Thus, the observation that the larval instars of the mosquito were present, in different numbers, in the three different regions in water, shows that all four instars spent some time in the region of contact with infective nematodes (see Section 2.1). Therefore, the absolute insusceptibility of the first-instar larvae to the nematodes, observed in previous experiments, cannot be attributed to lack of contact between these larvae and the infective nematodes. Also, the numbers of third-instars at the bottom hardly exceeded those of fourth-instars, hence there must be other factors involved in the slightly higher susceptibility of third-instars than fourths. Perhaps, cessation of feeding of fourth-instars, which normally precedes pupation (Christophers, 1960) reduced the ingestion of nematodes by

these larvae. This hypothesis is supported by the observation that the fourth-instars were more dominant at the surface compared to the third. Furthermore, Dadd (1971b) noted that the effectiveness of penetration (measured as the number of parasites per host) of N. carpocapsae (DD-136 strain) into third-instar Culex pipiens was higher than into the fourth. It is, therefore surprising that, despite the greater effectiveness of nematode penetration into third-instars, Dadd (1971b) found the fourth-instars of Culex pipiens more susceptible (ie. more died) than the third. He explained that third-instars ingested nematodes poorly compared to the fourth. However, his experimental techniques make valid comparisons between the different larval instars rather difficult. Although, mixed instars were exposed, the dosage of infective nematodes Dadd (1971b) administered to the larvae, duration of exposures, as well as numbers of the four instars, were not strictly controlled. These factors could affect the instars in a differential manner. Also, only 3mm of water were used, which means that late instars were continuously in contact with infective nematodes (see Section 2.1) while the early instars only made intermittent contact with the pre-parasites during the exposure periods. But, the reason for the reported responses of the third and fourth-instars remains unclear. It may involve damage to nematodes during entry. This factor is further investigated in Section 2.5. The lower numbers of second-instar larvae compared to the third and fourth at the bottom, may partly

explain the low susceptibility of this early instar relative to the older ones.

The observed pattern of larval distribution was probably influenced by nutritional and respiratory requirements of the larvae. The larval stages of most Aedes (Christophers, 1960; Aly, 1983) and Culex (Dadd, 1971a, 1971b) species dive to the bottom of water in search of particulate food and return to the surface for atmospheric air. These aquatic stages undertake varying degrees of cutaneous respiration. The extent to which the larval instars take in dissolved oxygen from the water is dependent on the permeability of their cuticles (Macfie, 1917; Wigglesworth, 1930; Christophers, 1960). The first-instars have the most permeable cuticles and are capable of spending several days away from the water surface (Macfie, 1917), relying entirely on cutaneous respiration for their oxygen requirements. By contrast, the fourth-instars could only survive for a maximum of 12 hours without atmospheric oxygen. The present observations showed that the first-instars were by far the most dominant larvae in between the surface and bottom, while few occurred at the surface. The relatively high atmospheric oxygen demand and the low particulate food requirements of the fourth-instars, particularly the pre-ecdysis stage (Christophers, 1960) would explain their numeric domination of the surface, and the lower numbers recorded at the bottom during the present experiments.

Attempts by Aedes aegypti larvae to browse on the

surface film are often induced by starvation (Christophers, 1960). This behaviour of the larvae, particularly late instars, was observed during the present experiments, but it is highly improbable that it was triggered by starvation, since the larvae were provided with food. This aspect of their behaviour needs further investigation, since it could possibly be exploited in designing surface formulations of the larval control agents.

Results of the present morphometric studies showed that the instars were discrete size groups with little or no morphometric overlaps. Dimensions of the mouths in all four larval instars exceeded the body width of the infective-stage nematodes. However, only the mean width of the mouth in fourth-instars was greater than the body length of the average-sized infective nematode. Also, except for the first-instars, the width of the headcapsules of all the instars was much greater than both the length and width of the pre-parasites. It is clear, therefore that there was no direct correlation between the dimensions of the mouths of the larval instars and their different susceptibilities to the nematodes on the one hand, and the dimensions of the infective-stage nematodes on the other. Hence, the previously observed differences in susceptibility of the instars to parasitism by the nematodes could not be simply attributed to the observed larval mouth measurements.

On the other hand, it is perhaps of some significance that the mean widths of the headcapsules of the three

susceptible larval instars (second, third, and fourth) were greater than both the mean length and width of the nematodes. It seems likely that the size of the headcapsule imposed a limit on the maximum aperture of the mouths of the larvae during ingestion of materials. If so, it is easy to appreciate the relative difficulty of ingesting the invasive nematodes by the first-instars in which the mean width of the headcapsule barely exceeded half the average length of the pre-parasites.

Similar relationships between instar size and rates of parasitism of Culex pipiens (Dadd, 1971b) and Simulium verecundum and S. vittatum (Molloy et al. 1980b; Gaugler and Molloy, 1981a, 1981b.) by N. carpocapsae have been reported. Also, Poinar and Kaul (1982) reported that size of Culex pipiens influenced susceptibility to Heterorhabditis bacteriophora. They observed that first-instars were not susceptible to the heterorhabditid, while Dadd (1971b), Molloy et al. (1980b), and Gaugler and Molloy (1981a, 1981b) reported lack of susceptibility of first and second-instars to the neoaplectanid. These authors suggested that the early instars physically excluded the infective nematodes from ingestion due to size limitations. On the basis of present observations, the same explanation cannot be offered without qualification. The insusceptibility, and/or low susceptibility of the early instars relative to the older ones, might involve a behavioural component as opposed to a simple size factor. Each instar probably prefers different sizes of food

particles. Also, the rate of damage to infective nematodes, caused by the mandibular teeth of larvae (Dadd, 1971b; Poinar and Kaul, 1982) may be different for different instars. Further work is required to confirm these speculations, and Section 2.5 provides more discussion on the subject.

The present mean widths of the headcapsules of the larval instars slightly exceeded those of 0.3, 0.45, 0.65, and 0.95mm given by Christophers (1960) for the first to the fourth-instars, respectively. They were also slightly higher than Christophers' (1960) values for the respective pre-ecdysis stages. Presumably, the observed differences were due to differences in rearing conditions.

These results of investigations into the inter-instar susceptibility of A. aegypti larvae and understanding the differential responses of the larval stages to the nematodes is important for attempts at biological control. Both the timing of application and the quantities of the agents used would obviously be influenced by the stage of development of the larvae, among other factors.

2.5 HOST-PARASITE RATIOS AND EXPOSURE TIME

2.5.1 Introduction

Quantitative assessments of dose- and time-response relationships between pest control agents and target insects offer vital information on the potential for effective pest control. The slopes of dose-response curves have been widely used as indicators of potency of the control agents or as measures of susceptibility of pest insects (Finney, 1964; Busvine, 1971; Hewlett and Plackett, 1979; Bedding et al. 1983; Molyneux et al. 1983; Hughes et al. 1984). Such information is critically important for the selection of control agents for experimental and field application against specific insects (Bedding et al., 1983) and for commercial interests (Hughes et al. 1984).

Welch and Bronskill (1962) reported the ability of the DD-136 strain of N. carpocapsae to infect late instar larvae of some Aedes and Culex spp. During their pioneering work, they applied specific numbers of nematodes against known numbers of mosquito larvae, but there is no indication that a critical dose-response analysis was conducted. Whilst experimentally infecting fourth-instars of Culex pipiens in the laboratory, Dadd (1971b) observed an increase in infection rate with increased concentration of the DD-136 strain applied. However, the experimental conditions were not strictly controlled and the results were not critically assessed. Recently, Poinar and Kaul (1982) observed that the concentration of infective H. bacteriophora was one of

several factors which governed the degree of infection of Culex pipiens. More recent studies conducted on the infectivity of a number of species of Heterorhabditis and Neoaplectana for various insects (Bedding et al., 1983) have further emphasized the significance of dose-response bioassays for determining the potentials of the nematodes for control of specific pest insects. From these reports, it seemed important to conduct a dose-response bioassay for the rhabditoids against mosquito larvae.

There are three possible ways to conduct time-response bioassays. These involve varying dose (i.e. varying time of exposure to a given dosage), monitoring time of response, and varying dose as well as monitoring the time of response (Hewlett and Plackett, 1979). The present study was primarily centered around the first application of time-response assessments. Already, information has been gained from the works of Welch and Bronskill (1962) and Dadd (1971b) that the infection of Aedes and Culex spp. larvae by N. carpocapsae occurs as early as one and half and 2 to 4 hours respectively, after exposure to the parasites. Bronskill (1962) recorded the shortest infection time of 12 minutes, while Poinar and Leutenegger (1971) reported infection of Culex pipiens larvae by N. carpocapsae within 15 minutes. This raised the question of what proportion of larval parasitism occurs with varying durations of exposures to the infective nematodes.

This section describes bioassays conducted to determine

the responses of 3rd-instar A. aegypti L. to varying dosages of infective-stage nematodes, and to different times of exposure of the larvae to H. heliothidis and N. carpocapsae. It also provides the first comparison of the efficacies of the heterorhabditid and neoaplectanid against mosquitoes.

2.5.2 Materials and Methods

The DD-136 strain of N. carpocapsae and H. heliothidis that had been continuously cultured in-vivo, on G mellonella larvae, and the black-eyed strain of A. aegypti obtained from Dr G. Tingley of the LSHTM, London (see Section 2.1.2) were used.

2.5.2.1 Dose-response bioassay

These experiments were conducted at $25 \pm 2^{\circ}\text{C}$. Each test consisted of 24 10cm diameter plastic dishes containing 15 third-instar mosquito larvae. Nematode dosages were 15, 150, 300, 750, 1500, 3,000 and 15,000 per dish (i.e host-parasite ratios of 1:1, 1:10, 1:20, 1:50, 1:100, 1:200, and 1:1000). Each dosage had 3 replicates and each set of replicates had 1 control without nematodes.

Exposures were carried out in 100ml of dechlorinated tap-water (ca. 1cm deep). Test dishes were inoculated with infective nematodes from suspensions of known nematode concentrations. The worms in these suspensions were uniformly mixed by bubbling in air from a Mystere 'twin' air-pump for at least 3 minutes. For the lowest dosage,

nematodes were hand-picked from a shallow watchglass using a piece of hair mounted on a wooden stalk. Then, a batch of 15 3rd-instar larvae was introduced into each dish and provided with 40mg powdered mixture of larval food. Exposures lasted for 24 hours. Then, the larvae were separated from infective nematodes. They were kept singly in the depressions of MWTC plates and separately according to treatments. Each larva was fed on a pinch of food mixture for another 24 hours. Dissections of dead larvae were made between 48 and 72 hours after the start of the experiment. The experiment was repeated 3 times for the two species of nematode. Data obtained for each nematode species were pooled and statistically analysed using probit analysis (Finney, 1964, 1971; Hewlett and Plackett, 1979) after a test for correlation between nematode dosage and larval response.

2.5.2.2 Time-response bioassay

A. aegypti larvae were exposed to H. heliothidis or N. carpocapsae for periods ranging from 15, through 30, 60, 120, to a maximum of 240 minutes. Fifteen standard exposure containers, (10cm diameter plastic dishes), plus three nematode-free controls were used in each experiment. Each dish had 15 3rd-instar larvae in 100ml of tap-water.

The containers were divided into 3 groups of 6. One inoculum of ca. 3,000 ($\pm$ 80) infective nematodes was added to each of the 5 test dishes in the first group. Exposures in these dishes were terminated after 15, 30, 60, 120, or 240

minutes after introduction of the nematodes. In the second group, the same dose of nematodes was inoculated into each dish and exposures in the 1st and 2nd dishes were stopped after 15 and 30 minutes, respectively. Then a second dosage of 3,000 nematodes was added to each of the remaining 3 test dishes 30 minutes after exposures began. These exposures were stopped 60, 120, and 240 minutes respectively, after introduction of the first dosage. The third group was treated as the second, but all exposures were terminated after 240 minutes. No larval food was provided during these experiments.

After the exposures, larvae were washed in tap-water and placed in sterilin plates. They were fed on Tetramin/crumbled bread/yeast extract mixture during post-exposure periods of up to 60 hours.

Meanwhile, the water in each exposure dish was examined for residual infective nematodes. The numbers of the worms were estimated indirectly, (except where very few were present, in which case total counts were made), by counting worms in 3 1ml samples. The mean number of worms from the 3 samples was multiplied by the total volume of water in each dish. The methods of mixing nematode suspensions by alternate inversion of the container was used throughout these experiments, since it was found to be more reliable than the rotation methods, as confirmed by the results of Townshend (1964).

The rates of infection of the larvae were assessed

between 60 and 96 hours post-exposure. Dead larvae were dissected in Ringer's solution to confirm parasitism, and the numbers of active and encapsulated/melanized parasites in the haemocoel were recorded. This experiment was repeated 5 and 2 times with DD-136 and H. heliothidis, respectively.

2.5.3 Results

2.5.3.1 Dose-response bioassay

Prevalence of infection

Results of the dose-response experiments are summarized in Table 2.5.1. The prevalence of both H. heliothidis and N. carpocapsae was positively correlated with the dosage of infective-stage nematodes applied. The probit-transformed mortality of the larvae gave a significant ($p < 0.05$) linear regression against the logarithm of dosage of nematodes (Table 2.5.2 and Fig. 2.5.1). A chi-square test for the goodness of fit performed on the data showed that heterogeneity between the observed and predicted larval responses to the various nematode dosages was not significant ($p > 0.05$). A likelihood ratio test for parallelism indicated that the data did not contradict a hypothesis for parallelism ($p > 0.05$), although the slope of the line for H. heliothidis was slightly higher than that for N. carpocapsae. Thus, the median lethal dosage (LD_{50}) of H. heliothidis and N. carpocapsae predicted from the probit analysis was 141 and 1,263 infective-stage juveniles respectively, per mosquito larva. The same equations predicted that 95% mortality of

Table 2.5.1 Effects of dosage of N. carpocapsae and H. heliothidis on the rates of parasitism of 3rd-instar A. aegypti

Host-parasite ratio (dosage)	No. of larvae used ¹	<u>N. carpocapsae</u>			<u>H. heliothidis</u>		
		No. Parasitized larvae ²	No. Parasites in larvae	No. Melanized parasites	No. Parasitized larvae ²	No. Parasites in larvae	No. melanized parasites
1:1000	135	68	246	179	118	408	283
1:200	135	28	75	65	74	161	148
1:100	135	12	29	29	57	117	109
1:50	135	11	21	21	31	56	50
1:20	135	9	11	11	24	35	35
1:10	135	2	3	3	19	23	22
1:1	135	1	1	1	9	9	9

¹ data based on 3 experiments each with 3 replicates

² Same as mortality of parasitized larvae

Table 2.5.2 Probit analysis of the dose-mortality-response data in Table 2.5.1

Aspect	Larval mortality caused by	
	<u>H. heliothidis</u>	<u>N. carpocapsae</u>
Heterogeneity	$\chi^2(5) = 9.567, \text{NS}$	$\chi^2(3) = 5.876, \text{NS}$
Regression (g)	$t(5) = 0.016 *$	$t(3) = 0.033, *$
Intercept	2.497	1.997
Slope ($\pm$ S.E.)	1.164 ($\pm$ 0.0740)	0.968 ($\pm$ 0.0901)
LD ₅₀	141	1,263
(95% c.l.)	(116 - 175)	(837 - 2,204)
LD ₉₅	3,665	6.3×10^4
(95% c.l.)	(2,292 - 6,638)	$(2.4 \times 10^4 - 2.5 \times 10^5)$
Difference of slopes: $\chi^2(1) = 2.829, \text{NS}$ (lines parallel)		

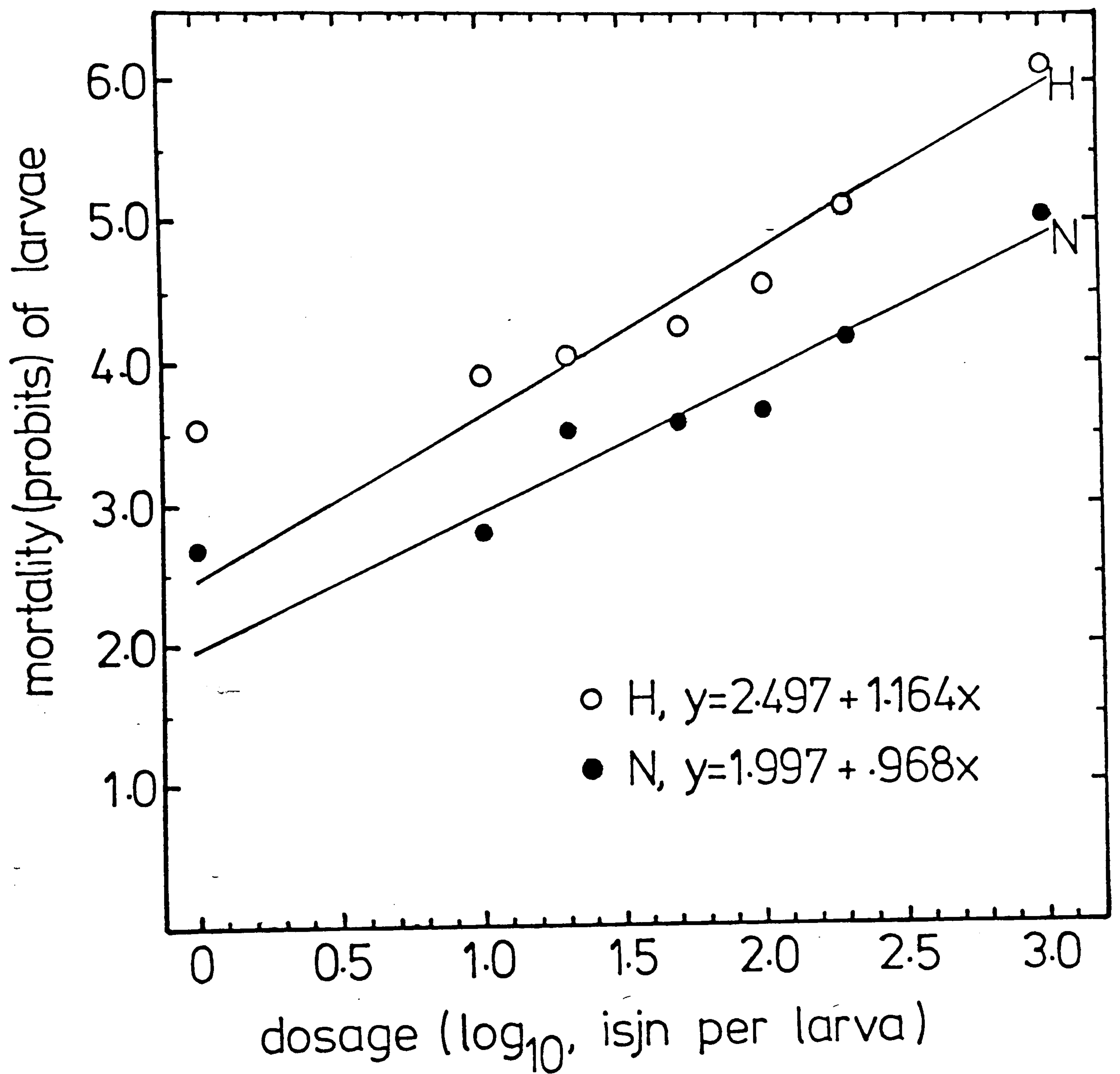
c.l. = confidence limits

Significance of differences : NS, not significant

($p > 0.05$);

* significant.

Figure 2.5.1 Probit mortality of 3rd-instar A. aegypti larvae parasitized by N. carpocapsae (●N) H. Heliothidis (○H) at varying worm dosages



the mosquito larvae required 3,665 H. heliothidis and 63,130 N. carpocapsae infective-stages per mosquito larva. Graphical estimation (Fig. 2.5.1) of potency revealed that H. heliothidis was 5.2 times more potent than N. carpocapsae, against the mosquito larvae.

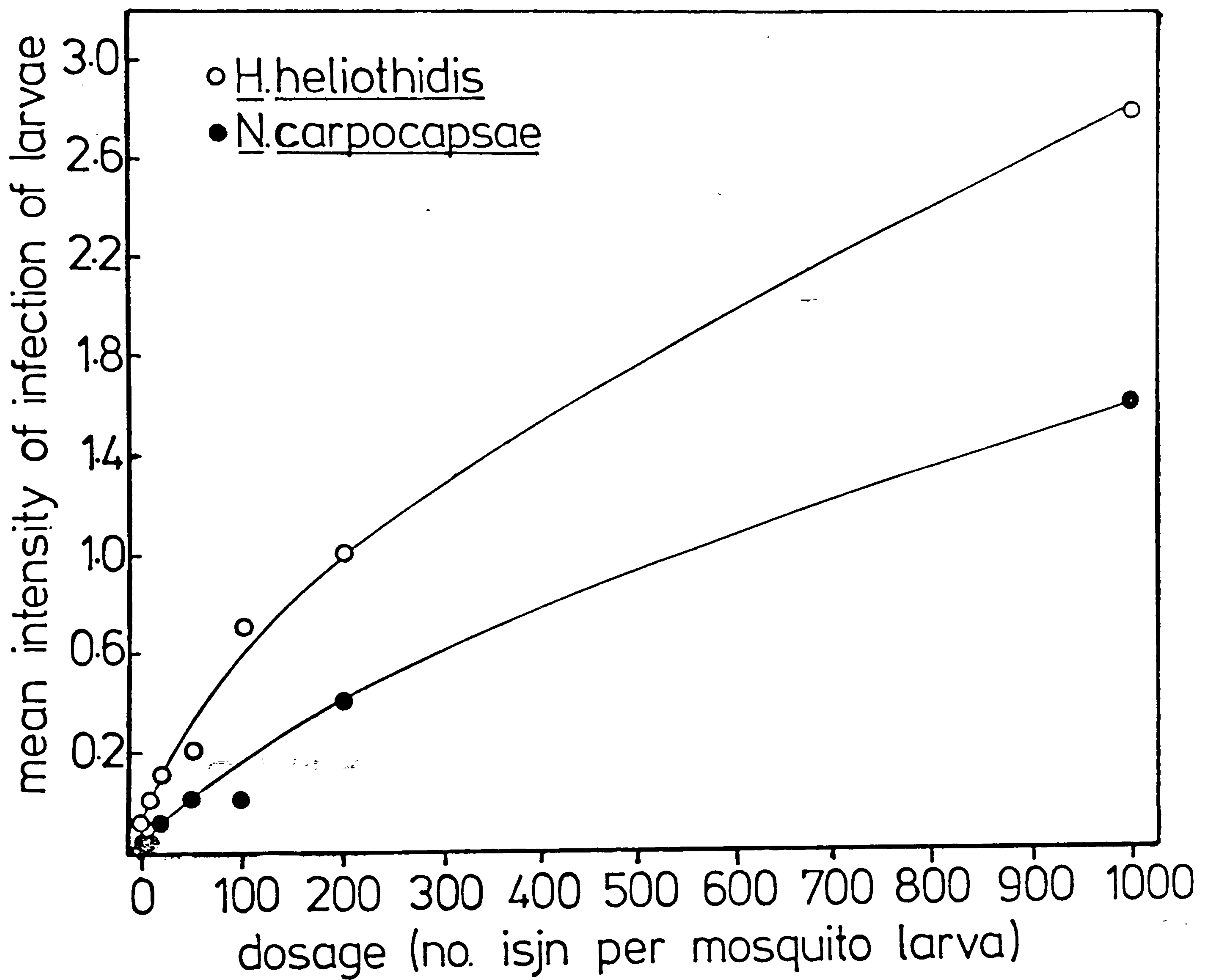
Mean intensity of infection

Apparently, there is no agreement amongst parasitologists, on the definition of the term 'mean intensity' of infection. The definition by Anderson (1982b) (the total number of parasites observed in infected larvae divided by the total number of larvae exposed) was adopted during the present work. The mean intensity was chosen because, unlike the mean abundance it takes into account those larvae that did not respond to treatment, which agrees with the procedure for a dose-response analysis.

The mean intensity of infection of the mosquitoes by both Heterorhabditis and Neoaplectana species increased as the dosage of infective-stage nematodes increased (Fig. 2.5.2). For each parasite species, there was a convex curvi-linear pattern of response of the mean intensities with change in dosage of the nematodes.

The mean intensity of infection of the larvae by both parasite species was similar at dosages below 100 infective-stage juveniles per larva. But, at a dosage of 100 infectives per larva and above, the mean intensity of infection by H. heliothidis was considerably higher than that

Figure 2.5.2 Mean intensities of N. carpocapsae and H. heliothidis in A. aegypti larvæ exposed to varying dosages of infective nematodes



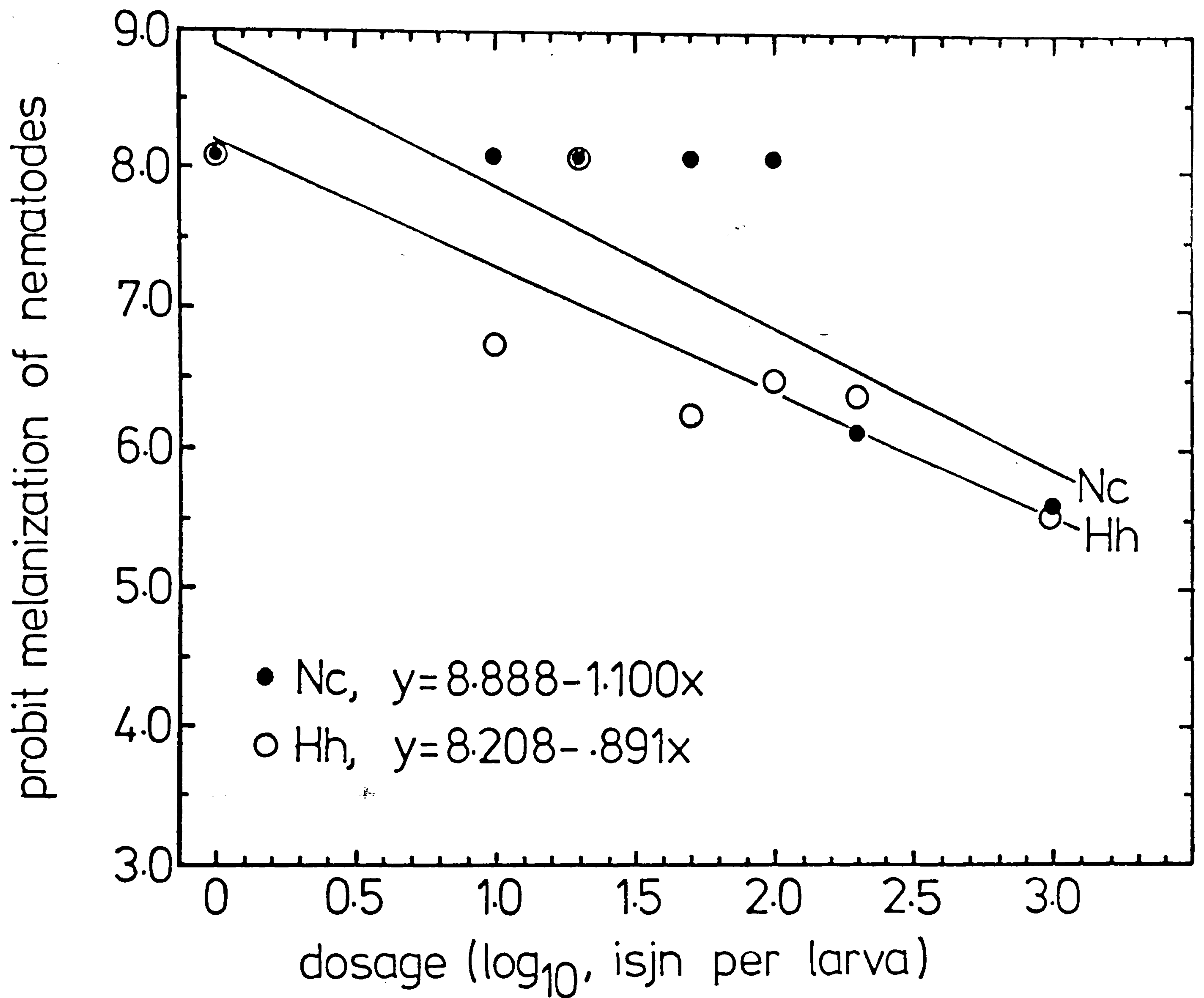
by N. carpocapsae. The highest numbers of H. heliothidis and N. carpocapsae recorded per mosquito larvae were 47 and 22, respectively.

Melanization of parasites

The rates of melanization of both H. heliothidis and N. carpocapsae in the haemocoel of infected mosquito larvae (Table 2.5.1), showed a negative correlation with the dosages of infective stages. This relationship gave a significant linear regression (Fig. 2.5.3) when the proportions of melanized parasites were transformed into probits and regressed against the log-dosages of nematodes. For each of the parasites, the observed rates of melanization fluctuated considerably from the best fit lines. However, differences between the observed and the expected rates of melanization were not significant ($p > 0.05$, $\chi^2(1) = 3.455$ and $\chi^2(5) = 3.964$, for H. heliothidis and N. carpocapsae, respectively). The general patterns of responses were similar for both parasite species, as confirmed by likelihood ratio test for parallelism ($p > 0.05$, $\chi^2(1) = 0.7004$).

Interestingly, increases in nematode dosage resulted in decreases in the proportion of melanized parasites within infected larvae. That is, the proportions of nematodes that survived the host defence reactions increased with an increase in dosage of infective juveniles. Thus, 95% melanization of the parasites in the haemocoel occurred when 75 (95% c.i. = 25 - 95) and 109 (30-197) infective-stages of

Figure 2.5.3 Probit analysis of melanization of N. carpocapsae and H. heliothidis in A. aegypti exposed to varying dosages of infective nematodes.



H. heliothidis and N. carpocapsae respectively, were applied per larva.

The data in Fig. 2.5.3 imply that low numbers of parasites in the haemocoel are melanized, but only a proportion are melanized if there are large numbers present. To verify this, the data from Table 2.5.3 showed that significant melanization of both H. heliothidis and N. carpocapsae occurred when there were less than 7 nematodes per larva, while low melanization occurred with more than 7 parasites. The proportions of both nematode species melanized at each parasite abundance were similar.

2.5.3.2 Time-response bioassays

Uptake of infective nematodes

The mean uptake of infective N. carpocapsae and H. heliothidis by the mosquitoes was positively correlated ($p < 0.001$ and $p < 0.05$ for the two species, respectively) with the duration of exposure. There was a remarkable ingestion of both nematode species even within the first 15 minutes of inoculation (Tables 2.5.4 and 2.5.5). The uptake of H. heliothidis was generally greater than that of N. carpocapsae after each exposure time. Half the infective juveniles in a dose of 3,000 were ingested within 30 minutes of inoculation. Nearly all of 3,000 infectives of this parasite were devoured after 4 hours. Thus, the exposure containers were almost depleted of H. heliothidis before the termination of the experiments. The addition of a second inoculum of the worms

Table 2.5.3 Distribution and melanization of N. carpocapsae and H. heliothidis within parasitized A. aegypti larvae

No. parasites per host	<u>N. carpocapsae</u> infections			<u>H. heliothidis</u> infections		
	No. parasitized	No. melanized	Percent melanized	No. parasitized	No. melanized	Percent melanized
	hosts	parasites	parasites	hosts	parasites	parasites
1	40	39	97.5	123	119	96.8
2	34	65	95.6	81	150	92.6
3	24	71	98.6	70	192	91.4
4	12	36	75.0	31	101	81.5
5	6	22	73.3	16	70	87.5
6	4	21	87.5	3	16	88.9
7	4	21	75.0	3	12	57.1
8	1	0	0.0			
9	4	19	52.8			
10	1	1	10.0			
11						
12				2	9	37.5
22	1	4	18.2			
47				1	1	2.1

Table 2.5.4 Effects of exposure time on the interactions between N. carpocapsae and 3rd-instar A. aegypti L. larvae.

Time of exposure (mins)	Dosage (isjn) ^a	No. larvae used	Mortality caused by infection	Uptake ^b of isjn per 15 larvae ($\bar{x} \pm SE$)	No. parasites haemocoel	No. parasites melanized	% Parasites melanized	$\bar{x}$ Parasite intensity	$\bar{x}$ Parasite Abundance
15	1 x 3000	150	35.3	504 $\pm$ 109 (135 - 1130)	130	56	43.1	0.9	2.5
30	1 x 3000	150	32.0	604 $\pm$ 82 (193 - 942)	167	64	38.3	1.1	3.5
60	1 x 3000	75	34.7	665 $\pm$ 145 (337 - 1016)	81	23	28.4	1.1	3.1
	2 x 3000	75	37.3	1338 $\pm$ 284 (497 - 1860)	100	35	35.0	1.3	3.6
120	1 x 3000	75	41.3	637 $\pm$ 129 (400 - 1091)	87	41	47.1	1.2	2.8
	2 x 3000	75	46.7	1620 $\pm$ 370 (255 - 2505)	162	42	25.9	2.2	4.6
240	1 x 3000	225	48.0	1178 $\pm$ 114 (225 - 1578)	282	149	52.8	1.3	2.6
	2 x 3000	300	57.0	1891 $\pm$ 184 (255 - 3180)	504	237	47.0	1.7	2.9

Legend : a, isjn = infective-stage juvenile nematodes; b, uptake ranges in parentheses;

Table 2.5.5 Effects of exposure time on the interactions between H. heliothidis and 3rd-instar A. aegypti L. larvae

Time of exposure (min)	Dosage (isjn)	No. larvae used in tests	Mortality caused by infection (%)	Uptake ^b of isjn per 15 larvae ($\bar{x} \pm SE$)	No. parasites in haemocoel	No. parasite melanized	% parasites melanized	$\bar{x}$ Parasite intensity	$\bar{x}$ Parasite Abundance
15	1 x 3000	60	15.0	1943 $\pm$ 120 (765 - 1260)	62	58	93.5	1.0	6.9
30	1 x 3000	60	51.7	1500 $\pm$ 76 (1365 - 1695)	65	61	93.8	1.1	2.1
60	1 x 3000	30	63.3	2168 $\pm$ 38 (2130 - 2205)	43	40	93.0	1.4	2.3
	2 x 3000	30	80.0	1710 $\pm$ 855 (855 - 2565)	57	55	96.5	1.9	2.4
120	1 x 3000	30	70.0	2441 $\pm$ 20 (2421 - 2460)	47	40	85.1	1.6	2.2
	2 x 3000	30	83.3	2858 $\pm$ 203 (2,655 - 3060)	68	65	95.6	2.3	2.7
240	1 x 3000	90	70.0	2830 $\pm$ 53 (2625 - 2966)	138	125	90.6	1.5	2.2
	2 x 3000	120	89.2	4262 $\pm$ 258 (2700 - 4890)	277	263	94.9	2.3	2.6

Legend: a, isjn = infective-stage juvenile nematodes; b, uptake ranges in parentheses;

replenished the system and led to increased ingestion by the mosquitoes. With 3,000 N. carpocapsae, only one fifth and one third infectives were ingested after half and 4 hours respectively, post-inoculation. Addition of a second dose of 3,000 resulted in 'swamping' the system. Consequently, there was about 2-fold increase in the rate of ingestion of the parasites with time.

The addition of a second dose of infective nematodes 30 minutes after the start of exposures, resulted in a much larger uptake of nematodes than was achieved by merely leaving the smaller dose for an equivalent period of exposure. The second inoculum had a more significant effect on the uptake of N. carpocapsae compared with H. heliothidis, especially after 60 and 120 minutes.

Since time was used to monitor nematode dosage during these experiments, the data obtained with 1 and 2 inoculations of each nematode species were amalgamated before they were subjected to a probit analysis. The results of this analysis (Table 2.5.6) showed a significant ($p < 0.05$) heterogeneity between the observed and predicted ingestion rates for both nematodes (Fig 2.5.4). Nevertheless, regression analysis of the probit uptake against the logarithm of time revealed a significant ($p < 0.05$) regression for N. carpocapsae ingested with time, while the regression for H. heliothidis was not significant ($p > 0.05$). The slopes of the probit lines for the two species were significantly different ($p < 0.05$). Thus, the lines were not parallel, indicating that the pro-

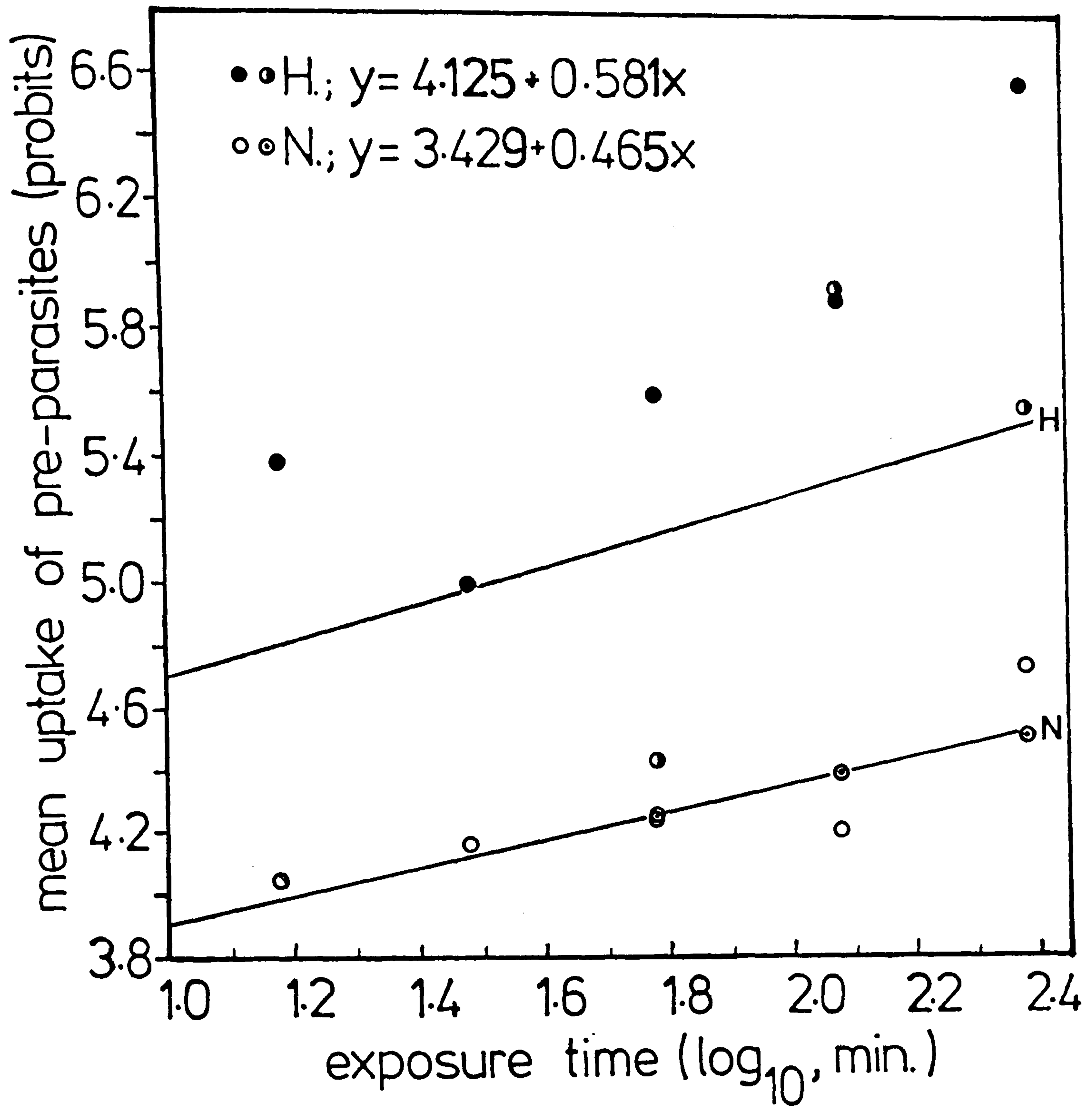
Table 2.5.6 Probit analysis of the time-uptake of infective nematode data in Tables 2.5.4 and 2.5.5

Aspect	Larval uptake (ingestion) of	
	<u>N. carpocapsae</u>	<u>H. heliothidis</u>
Heterogeneity	$\chi^2(6) = 137.1, *$	$\chi^2(6) = 4736, *$
Regression (g)	$t(6) = 0.276, *$	$t(6) = 5.051 \text{ NS}$
Intercept	3.429	4.125
Slope ($\pm$ SE)	0.465 ($\pm$ 0.100)	0.581 ($\pm$ 0.534)
LT ₅₀	2,370	32
(95% c.l.)	(739 - 8.9×10^4)	--
LT ₉₅	8.1×10^6	2.2×10^4
(95% c.l.)	(1.6×10^5 - 2.4×10^{12})	-
Difference of slopes	$\chi^2(1) = 16.756, *$ (lines not parallel)	
	Legend : As in Table 2.5.2	

Table 2.5.7 Probit analysis of the time-mortality response data in Tables 2.5.4 and 2.5.5.

Aspect	Mortality of larvae parasitized by	
	<u>N. carpocapsae</u>	<u>H. heliothidis</u>
Heterogeneity	$\chi^2(5) = 5.306, \text{NS}$	$\chi^2(6) = 30.54, *$
Regression (g)	$t(5) = 0.134, *$	$t(6) = 0.384, *$
Intercept	3.610	2.913
Slope ($\pm$ SE)	0.611 ($\pm$ 0.114)	1.294 ($\pm$ 0.328)
LT ₅₀	188	41
(95% c.l.)	(139 - 286.3)	98.9 - 77.9)
LT ₉₅	9.2×10^4	766
(95% c.l.)	(1.5×10^4 - 4.2×10^6)	(284.2 - 3.3×10^4)
Difference of slopes	$\chi^2(2) = 13.771, *$ (lines not parallel)	
	Legend : As in Table 2.5.2	

Figure 2.5.4 Probit uptake of infective N. carpocapsae (N) and H. heliothidis (H) by 3rd-instar A. aegypti during different exposure times (o● = 1 dosage of 3,000 nematodes, ●● = 2 dosages of 3,000 nematodes each).



portions of the 2 parasite species ingested with time, were not constant.

The median uptake times (LT_{50} s) predicted by the regression equations are 32.1 and 2,370 minutes for H. heliothidis and N. carpocapsae respectively, while the corresponding LT_{95} s are 2.18×10^4 and 8.12×10^6 , respectively. It was not possible to estimate graphically the order of difference in uptake of the 2 parasites since the regression lines were not parallel. Nevertheless, the results showed that the rate of ingestion of the heterorhabditid was much greater than the rate for the neoaplectanid.

Mortality of parasitized larvae

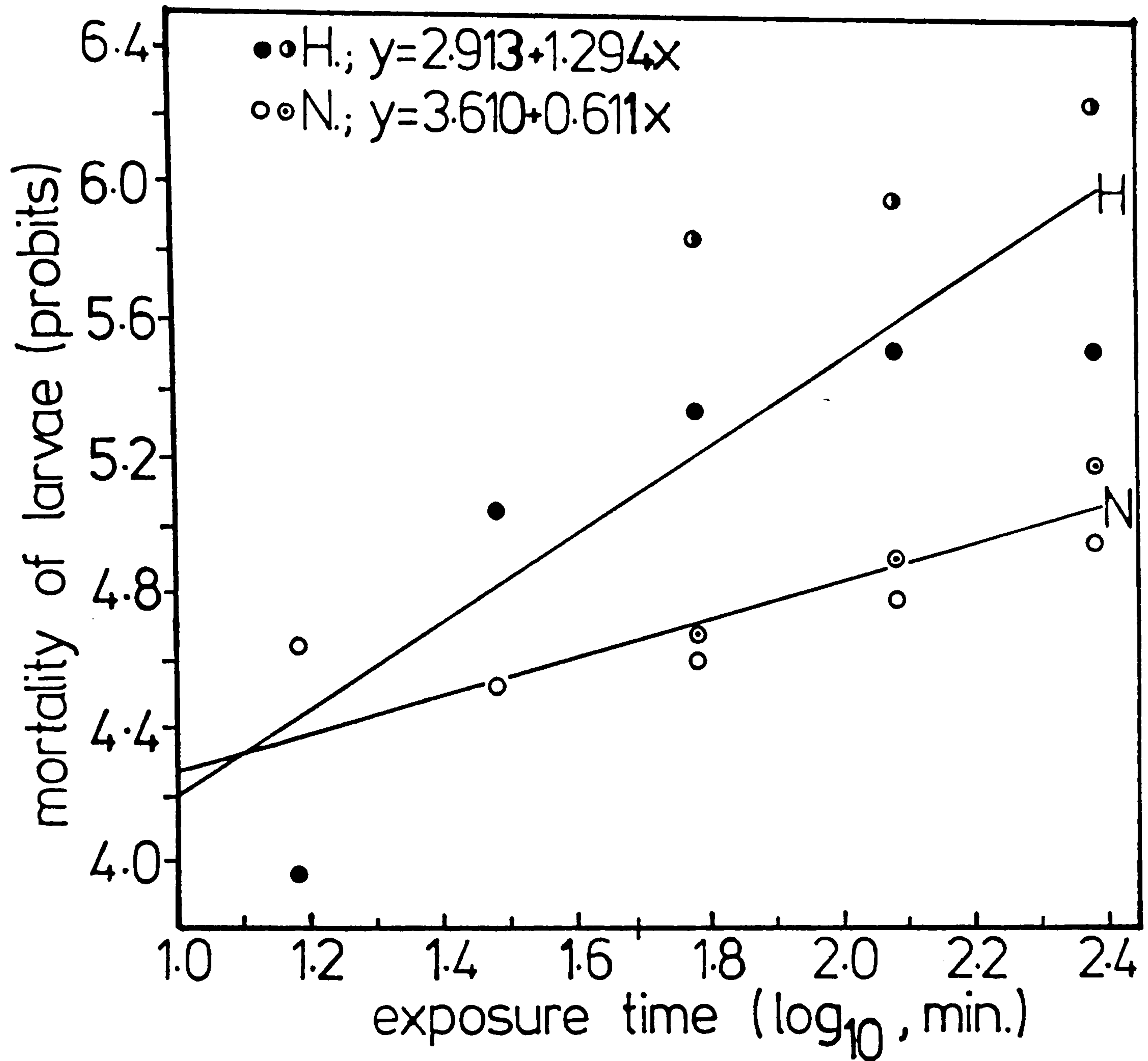
The proportions of larvae parasitized by N. carpocapsae and H. heliothidis increased with time of exposure to one and two dosages of the nematodes (Tables 2.5.4 and 2.5.5). As for the rate of ingestion, addition of a second inoculum of both parasites, 30 minutes after the beginning of exposures, increased the prevalence of infection. Generally, the prevalence of H. heliothidis was higher than that for N. carpocapsae after each exposure time. Also, addition of a second inoculum of pre-parasites yielded higher increases in prevalence of the heterorhabditid, compared to the results of single dosage treatments, than it did for N. carpocapsae. However, 'doubling' of nematode dosage did not double the mortality caused by either parasites at any exposure time. Thus, the increase in prevalence, especially that of N.

carpocapsae, produced by addition of a second dose of infectives, was not commensurate with the increased consumption of nematodes. The difference in mortality caused by 1 and 2 dosages of both parasite species increased with time. Thus, the highest difference of 9.0 and 19.2% mortality of larvae parasitized by N. carpocapsae and H. heliothidis respectively, occurred after exposures for 240 minutes.

Probit analysis of the data (Table 2.5.7) revealed a significant ($p < 0.05$) regression for larval mortality caused by both parasite species with time, and a chi-square test showed heterogeneity was significant ($p < 0.05$) only for mortality caused by H. heliothidis. A comparison of the slopes of the 2 lines using the maximum likelihood test disclosed that the lines were not parallel.

Larval parasitization by N. carpocapsae, after the shortest exposure time of 15 minutes, was higher than the level induced by H. heliothidis, while the reverse occurred with longer exposure. The prevalence of both parasites was equal after 11.2 minutes exposure (Fig 2.5.5). The equations of the regression lines predict LT_{50} s of 41.0 and 187.5 and LT_{95} s of 766.3 and 9,200 minutes for H. heliothidis and N. carpocapsae, respectively. Thus, mortality of the mosquitoes caused by H. heliothidis occurred much more rapidly than that by N. carpocapsae, an observation which agrees with the uptake results.

Figure 2.5.5 Probit mortality of A. aegypti larvae parasitized by N. carpocapsae (N) and N. heliothidis (H) at varying exposure times (description as in Fig.2.5.4).



Mean parasite intensity and larval defence reactions.

The mean parasite intensity (Tables 2.5.4 and 2.5.5) was correlated ($p < 0.05$) with exposure time. However, the intensities of both parasites showed only slight increases as the duration of exposure increased from 15 to 240 minutes. Infection by H. heliothidis produced a slightly higher intensity than by N. carpocapsae for equivalent exposure times. Not surprisingly, doubling the nematode dosages yielded a slight increase in the mean intensities of both parasite species.

There was no correlation between the mean abundance of either parasite and the duration of exposure (Tables 2.5.4 and 2.5.5). Also, the abundance of both parasites showed only slight increases with the addition of the second inoculum of nematodes. Generally, N. carpocapsae was more abundant in the haemocoel of larvae than H. heliothidis, indicating greater clumping of the former parasite.

As for the mean parasite abundance, there was no correlation ($p > 0.05$) between the rate of melanization of either rhabditoid and the duration of exposure to infective nematodes (Tables 2.5.4. and 2.5.5). Similarly, the proportions of invading parasites melanized were not affected by increasing the dosage of pre-parasites. But, a Spearman's rank correlating analysis of the data (summarized in Tables 2.5.4 and 2.5.5) revealed a significant ($p < 0.05$) correlation between the rates of melanization and the intensities of infection by both parasites. Thus, melanization increased as the intensities of N. carpocapsae and H. heliothidis increas-

ed under the conditions of the present time-response study.

Contrary to the results of the previous dose-response experiments, percent melanization was much greater for H. heliothidis than N. carpocapsae. For equivalent exposure times, there was approximately a 2-fold melanization of H. heliothidis over that of N. carpocapsae.

2.5.4 Discussion

Dose-response bioassays

A positive correlation between insect control levels and the dosage of entomogenous rhabditoids is almost always encountered during dose-response bioassays (e.g. Sandner and Stanuszek, 1971; Kaya, 1978; Simons, 1981; Bedding and Miller, 1981a, 1981b; Bedding et al., 1983; Molyneux et al. 1983). Thus, it is not surprising that mortality of 3rd-instar A. aegypti larvae parasitized by H. heliothidis and N. carpocapsae increased as the dosage of infective nematodes increased. However, it is interesting that the two nematode species demonstrated differential infectivities for the larvae. This is the first quantitative comparative assessment of the rhabditoids against mosquito larvae. Most bioassays for rhabditoids against mosquitoes (e.g. Welch and Bronskill, 1962; Dadd 1971b; Finney and Harding, 1981) used N. carpocapsae, which is now revealed to be inferior to the Heterorhabditis species.

The observed differences between the performances of the nematodes are in agreement with other findings by Molyneux et

al. (1983) and Bedding et al. (1983) that appreciable differences occur in the infectivities of various heterorhabditid and steinernematid species and strains for different insect species. These results also agree with their observation that heterorhabditids are generally more effective than the steinernematids and indicate that the former are more promising biological control agents than the latter. However, the reason for the differential infectiousness of the two parasite species for the mosquitoes is not clear. Bedding et al. (1983) suggested attraction, penetration mechanisms, rates of host and nematode movements, and the defence reactions of hosts as possible factors responsible for the differential levels of infectivity of entomopathogenic rhabditoids. It is possible that some of these factors contributed to the observed differences in infectivities of H. heliothidis and N. carpocapsae for A. aegypti larvae. However, it is doubtful that the differences can be attributed to attraction and speed of nematode movement in water, because of viscosity limitations discussed in Section 2.1 and the inability of these nematodes to swim in water. Attraction was not a problem since the mosquitoes avidly devoured large numbers of parasites. However, remarkably few managed to enter the haemocoel. Also, although the rates of melanization of the parasites in mosquitoes differed slightly, it is unlikely that this factor influenced the levels of control, since melanization of invading juveniles rarely saved larvae from death. As Beresky and Hall (1977)

noted, the mere presence of nematodes in the haemocoel of mosquito larvae causes 'physical damage' which leads to host mortality. Also, melanization is probably too slow to prevent liberation of the pathogenic bacteria from the nematode intestine into the haemocoel.

Contrary to the present observations, Kaya (1978) conducted comparative infectivity tests of H. heliothidis and N. carpocapsae for Apanteles militaris in cocoon clusters of armyworm and reported higher performance of the neoaplectanid than the heterorhabditid. This author suggested higher activity of N. carpocapsae than of H. heliothidis as possible explanation for the differential efficacies, a suggestion that appears questionable from the results of Bedding et al. (1983), Molyneux et al. (1983) and Akhurst (pers. Comm). It remains to be demonstrated experimentally which of the two nematode species is more active than the other.

Results for the defence reactions of A. aegypti larvae against the nematodes confirm earlier reports by Welch and Bronskill (1962), Bronskill (1962), Salt (1963) and Pionar and Kaul (1982), of a breakdown in host defences as the numbers of invading parasites increase. Nearly, all nematodes of both species, when alone in the haemocoel, were melanized. However, multiply-infected larvae had fewer nematodes killed by defence mechanisms. These findings suggest the existence of a threshold of infection below which the probability of worm survival in the haemocoel is minimal, and above which virtually all nematodes survive host

defences. This subject needs further investigation since it affects the chances of parasite development and reproduction (further discussed in Chapter 4.0) within the hosts.

According to Poinar and Kaul (1982), it is only when the maximum number has been melanized that additional nematodes develop freely in the hosts. Obviously, the numbers of parasites which escape encapsulation and melanization increase as the exposure dosage of pre-parasites increases, as demonstrated by the present results. However, the existence of a threshold means that the rate of melanization need not be directly and consistently correlated with dosage of nematodes. This fact is supported by the observation (see Fig 2.5.3) that melanization of N. carpocapsae was close to 100% with dosages up to 100 nematodes per larva while the regression between log-dose and probit-melanization was statistically significant. Obviously predictions (LD_{50} s and LD_{95} s) based upon regression equations must be made cautiously. Also, the degree of aggregation or clumping of the worms within some individuals of the larval subpopulation will be important in determining the rate of reproduction of the parasites.

Results for the present dose-mortality studies appear to fit an exponential model (Hughes et al. 1984) in which the LD_{50} s of 141 and 1,263 infective H. heliothidis and N. carpocapsae respectively, do not represent the mean tolerance doses, but provide estimates of the dosages of infective nematodes necessary for 50% of the 3rd-instar

larvae to acquire the lethal dose under the conditions of this study. Thus, these values are likely to change when the experimental conditions are altered, particularly in the field. The observation that considerable mortality of singly-infected larvae occurred during this work, supports an hypothesis of independent as opposed to cooperative action (Hughes et al. 1984). This hypothesis assumes that the infective nematodes act independently of one another to produce mortality, and that a mean probability exists that each inoculated infective worm is capable of producing a fatal infection. The observation that even the host-parasite ratios of one-to-one yielded larval mortality supports this hypothesis. Also, results of infectivity tests of a range of species and strains of Heterorhabditis and Neoaplectana for different insect species (Bedding et al., 1983; Molyneux et al., 1983; Poinar and Kaul, 1982; Welch and Bronskill, 1962) give additional support to this hypothesis.

Although N. carpocapsae has received much more attention than any rhabditid in both laboratory and field experiments aimed at determining potentials for biological control of insect pests (Poinar, 1979; Bedding and Miller, 1981a; Gaugler and Kaya, 1983) it is clear from the LD₅₀ and LD₉₅ values obtained in this study that H. heliothidis is more effective than N. carpocapsae against A. aegypti larvae. Obviously, equivalent levels of control will require far more infective N. carpocapsae than H. heliothidis and, although both agents can be cheaply produced on artificial media

(Bedding 1976, 1981, 1984; Poinar, 1979; Wouts, 1981) large scale application will be more economical with the latter. This indicates the necessity for screening several species and strains of rhabditoids against any particular insect target before choice is made of an appropriate candidate for biological control.

Time-response bioassays

It is interesting that the rate of ingestion of H. heliothidis by the larvae was much higher than the uptake of N. carpocapsae. Together with the slightly higher mean intensity of the heterorhabditid compared with the neoaplectanid at all exposure times, this suggest that differences in effectiveness between the two species are partly due to differences in entry and penetration of the nematodes into the mosquitoes. Thus, H. heliothidis is more efficient than N. carpocapsae in entering the larval gut and penetrating into the haemocoel. However, the poor correlation between the rates of pre-parasite ingestion and the subsequent intensities of both nematode species, suggests a remarkable loss of nematodes in the gut of the larvae. This loss is most probably attributable to damage or injury to infective nematodes caused by larval mouthparts during entry, as suggested by Dadd (1971b) and Poinar and Kaul (1982). Gaugler et al. (1983) have also reported injury of Steinernema feltiae caused by blackfly larval mouthparts during ingestion.

According to Dadd (1971b) such injured pre-parasites underwent enzymatic digestion in the gut and were subsequently voided. Hence, the ingestion of infective nematodes does not guarantee infection of larvae, as confirmed by results of subsequent exposures of both parasites to the mosquitoes (see Appendix 1). These results showed that 45/45 and 44/45 of the larvae ingested H. heliothidis and N. carpocapsae respectively, during exposures that lasted for 60 minutes, as indicated by the presence of cuticles, damaged and undamaged worms in the gut. However, mortality due to infection by the heterorhabditid and neoaplectanid amounted to only 48.9 and 51.1% respectively, and very few nematodes penetrated into the haemocoel. None of the data explains the poorer uptake of N. carpocapsae compared to H. heliothidis. Nematode entry into mosquitoes in water is thought to be a passive process, which depends entirely upon active ingestion by the larvae (Welch and Bronskill, 1962; Dadd, 1971b, Finney and Harding, 1981; Poinar and Kaul, 1982). If so, then the differences in ingestion rates can not be attributed to differences in activities of the heterorhabditid and neoaplectanid. Nor can the differences be attributed to size, since the infective-stages of H. heliothidis and N. carpocapsae are nearly similar ($\bar{x}$ lengths = 654 and 545 μm , and $\bar{x}$ widths = 28 and 27 μm , respectively). This subject of ingestibility warrants further investigation.

The mortalities of larvae exposed to a single dose of

H. heliothidis for durations longer than 60 minutes were clustered (that is, occurred within a narrow range). However, addition of infective nematodes to the system increased mortality. These data confirm the depletion of the first inoculum and indicate that prolonged exposure at this host-parasite ratio would have little or no additional lethal effects on the larvae. With this heterorhabditid, as opposed to the neoaplectanid, the exposure system was virtually depleted of infective worms after 60 minutes exposure. Hence, inundation was necessary for a further increase in larval mortality. These findings imply that effective control of mosquitoes with H. heliothidis might require repeated application. However, inundation with N. carpocapsae may not be cost-effective, since only slight improvement would occur in the relatively low control levels. The data also indicate the rapidity of the process of infection by the heterorhabditid, which is confirmed by the low LT_{50} -value of 41 minutes, compared to 188 minutes for N. carpocapsae.

Not surprisingly, both ingestion of infective N. carpocapsae and H. heliothidis and prevalence of the parasites increased with prolongation of exposures. Also, the mean intensity of infection by both parasites showed slight increases with exposure time. Thus, as Finney and Harding (1981) suggested, a formulation that can increase the length of time that the nematodes remain available to the larvae, will improve their performance against the mos-

quitos. Field applications of these nematodes will obviously require longer exposures than the present LT_{50} -values, as suggested by Frommer et al. (1980) for bacterial pathogens against blackfly larvae. However, the practicability of these predictions remains to be determined.

It is puzzling that melanization of N. carpocapsae was much lower than the rate for H. heliothidis at each equivalent exposure time and dosage. This observation contradicts the results obtained during the dose-response assays, that melanization of the neoaplectanid was slightly higher than that for the heterorhabditid. The reason for this discrepancy may be related to the number of parasites in each host. For both dosages of nematodes, there were more N. carpocapsae than H. heliothidis in the haemocoel of the mosquitoes after each exposure time, except the first 15 minutes. Thus, the number of N. carpocapsae per host usually exceeded the number that could be melanized, while H. heliothidis numbers were low enough so that most could be melanized. The relatively high aggregation of N. carpocapsae will be an advantage for survival, development and reproduction, but a disadvantage for biocontrol of the mosquitoes as indicated by the low parasitization levels.

The probit-mortality-log-time regression equations predict that 95% larval mortality would require 12.8 and 1533.3-hour exposures to H. heliothidis and N. carpocapsae, respectively. These predictions are unrealistic, especially for N. carpocapsae. However, they indicate the significant

difference between the efficacies of the 2 nematodes against the mosquitoes. It is clear therefore, that H. heliothidis is, by far, a better candidate for control of these vectors which have a short life cycle. This revelation is supported by the dose-response data, which show that the heterorhabditid is 5.2 times more potent against the mosquitoes, than the neoaplectanid.

3 EFFECTS OF PREPARATION TECHNIQUES ON ADULT MORPHOMETRICS

3.1 Introduction

A wide range of methods of relaxing, killing and fixing nematodes has been used by many workers to make whole specimens suitable for light microscopical examination. Anaesthetization of worms using various anaesthetics or narcotics, notably propylene phenoxetol and dichlorodiethyl ether has been employed prior to making temporary mounts (Ellenby and Smith, 1964); Hooper, 1970; Stone, 1971; Curran and Hominick, 1980). Hooper (1970) outlines some of the common methods of killing nematodes by heat. One approach involves placing specimens in a drop of water, on a plain or cavity glass slide which is then heated over a small flame for the shortest period necessary to cause death. Alternatively, worms are often placed in a drop of water in a concave watchglass, followed by the addition of hot water onto the specimens. Poinar (1975a) suggests the use of hot saline (1% NaCl) as an alternative to hot-water for killing insect parasitic nematodes, freshly extracted from their hosts. Golden (Hooper, 1970) placed nematodes in a watchglass containing 2-3 ml of water, in an oven maintained at 43°C, for 12 minutes as a preliminary step towards fixation. Also, Zuckerman (1960) killed freshly extracted soil nematodes by putting them in water, into an oven at 60°C for 15 minutes. Hooper (1970) considers heating nematodes in a tube of water as a safer killing method. Seinhorst (1962) and Netscher and Seinhorst (1969) among other

prominent workers have used more sophisticated techniques to achieve heat killing and fixation simultaneously.

Some of the available methods cause significant alterations in certain nematode groups. For example, Lamberti and Sher (1969) showed the differential effects of various preparation techniques on taxonomic characters of Longidorus africanus Merny (Longidoridae). Similarly, significant effects of various killing, fixing and mounting methods were demonstrated by Stone (1971) and Curran and Hominick (1980) for Heterodera rostochiensis (Heteroderidae) and Romanomermis culicivorax and Gastromermis sp. (Mermithidae) respectively.

Apparently, little or no importance has been attached to the influence (if any) of preparation techniques on the taxonomic characters of rhabditid nematodes, especially the steinernematids whose taxonomy has been a subject of great controversy in recent years. Thus, virtually no information (quantitative or qualitative) is retrievable from the literature on the subject. Various workers employed different killing, fixing and mounting techniques for steinernematids. For example, while describing the DD-136 strain of N. carpocapsae Weiser, Poinar (1967) killed saline-suspended nematodes by gentle heat before measuring them in 3% formalin. Sandner and Stanuszek (1974) mounted nematodes in physiological saline during their work on the same strain of nematodes but did not provide details of the relaxing method that they used. Two percent formalin and glycerin were used in semi and permanent mounts, respectively, of

Heterorhabditis (= Chromonema) heliothidis during the description of this species (Khan, Brooks and Hirschmann, 1976). It is, therefore clear that workers on steiner-nematid nematodes have used varied preparation techniques and that the choice of technique, probably was not based on findings of a discriminatory screening procedure.

This study has two primary objectives. First, it is designed to assess the possible quantitative and qualitative morphological changes in adult N. carpocapsae (DD-136 strain) attributable to different relaxing, killing and fixing methods. Second, it aims to identify a practical technique to be applied in a subsequent investigation on the influence of the environment on morphometrics of adult female parasites.

3.2 Materials and Methods

3.2.1 Animals and laboratory infection procedures

The Silwood Park strain of Aedes aegypti L. cultured in the laboratory at $25 \pm 2^{\circ}\text{C}$ and 12 hours night-day cycles, were used in these experiments. Third-instar larvae were exposed to infective-stage juveniles of the DD-136 strain of Neoaplectana carpocapsae (= N. feltiae) for 4 hours, during each experimental infection. A constant host-parasite ratio of one mosquito larva to 200 infective worms was adopted, and 100 larvae were exposed in each experiment. After the exposure period, the larvae were washed free of external infective nematodes and were transferred singly into the

wells of MWTC plates. The individual larvae were provided with powdered TTFF/YE/BC mixture and the plates were kept under the same laboratory conditions.

Mature male and female parasites were dissected out of their infected hosts between 48 and 96 hours post-infection. The dissections were made in Ringer's solution which consisted of NaCl(6.75g), KCl (0.09g), CaCl_2 (0.115g), and NaHCO_3 (0.215g) in 1 litre of deionized distilled water (Baker and Taylor, 1978). Parasites for measurements were subsequently treated by one or more of the following procedures.

3.2.2 Anaesthetization of parasites

Each adult parasite was hand-picked on a mounted needle and transferred into a deep watchglass containing 1 ml of 0.35% (v/v) propylene phenoxetol in saline solution. The worm was narcotized for 25-30 minutes before it was mounted in 2 drops of 0.125% (v/v) propylene phenoxetol in the depression of a cavity glass slide for measurement, drawing and/or photography. This combination of concentrations of the anaesthetic appeared to enhance post-anaesthetization recovery of the worms.

To minimize variability of measurements caused by surface-curvature of the mounting fluid (Fenwick and Franklin, 1942) a coverslip was placed on the fluid containing the specimen. Excess fluid was drained off to maintain a constant working depth of the solution (mountant). Morphological characters of the worms were measured using a

camera lucida as described in Section 2.4.2. Specimens were photographed using a Leitz Dialux Interference Contrast microscope.

3.2.3 Resuscitation of anaesthetized parasites.

Nematodes were removed from the slide and transferred into fresh ringer's solution in clean watchglasses to resuscitate at room temperature. Resumption of 'normal' movements of both anterior and posterior ends of the body was used as an indicator of complete resuscitation.

3.2.4 Hot saline relaxation

The method of Poinar (1975a) was modified to compare the effects of anaesthetization with killing/relaxing using hot ringer's solution at 80°C. A beaker of the solution was maintained in a water-bath at 80°C and used to heat-relax a set of 24 adult female worms that had previously been anaesthetized, measured and resuscitated. Each worm was placed in about 0.2 ml of ringer's solution at room temperature in a deep watch-glass. Then, hot saline was added using a multi-range micro pipette (Oxford Laboratories), adjusted to give samples of 1.11 ± 0.01 ml (N=10). The pipette tip was allowed 20 seconds in the hot solution before it was withdrawn. The solution was immediately transferred onto the worm in the watchglass. The watchglass was covered with a lid and the material left to cool to room temperature before the parasite was mounted

in 3% formalin in ringer's solution. It was then measured and/or photographed.

3.2.5 Hot water relaxation

The method of Hooper (1970) and Curran and Hominick (1980) was slightly modified for the present investigation. Male and female parasites were transferred from the dissection or resuscitation watchglasses into a small plastic tube (diameter = 1.0 cm., depth = 2.20 cm) with a sieve (pore size = 50 μ m) glued to the bottom and standing in a watchglass containing ringer's solution. The worms were treated singly. Each was allowed to settle onto the sieve under gravity before the tube was removed from the glass and excess solution was drained off with a tissue. The tube was then held in a beaker containing distilled water at 65°C for 3 seconds before it was removed and returned to the watchglass containing saline. The worm was mounted in saline and measured and/or photographed as previously described.

3.2.6 Fixation

Twenty-five adult female nematodes that had been anaesthetized and heat-relaxed in water were divided into five groups of five nematodes per group. The groups were randomly allocated to one of the following five fixatives: a) FA 4:1 (40% formaldehyde, 10 ml; glacial acetic acid, 1 ml; and saline solution, up to 100 ml), b) FAA (95% ethanol, 20 ml; 40% formaldehyde, 6 ml; glacial acetic acid, 1 ml; and

saline solution, 40 ml), 10% (v/v) formalin (40% formaldehyde, 10 ml; and saline solution, 90 ml), Kale's fluid (96% ethanol, 17 ml; 40% formaldehyde, 6 ml; glacial acetic acid, 2 ml; and saline solution, 28 ml), and TAF (40% formaldehyde, 7 ml; triethanolamine, 2 ml; and saline solution, 9 ml).

Individual worms were placed in a watchglass containing 3 ml of the appropriate fixative. To prevent evaporation, Silicone High Vacuum grease was applied to the top edges of the watchglasses before they were covered with glass lids. The nematodes were fixed for 1 week, at room temperature before they were measured and photographed **using phase contrast technique.**

3.2.7 Statistical analysis

The non-parametric Wilcoxon's matched-pairs signed-ranks test (Siegel, 1956) and the parametric student's t-test for paired or related samples (Parker, 1973) were used. In both tests, each worm was used as its own control so that comparisons were made between measurements taken before and after treatments. Since the results from both tests were similar, the paired sample t-test was finally adopted for its convenience and greater power-efficiency. The mean per cent change between pairs of treatments (Curran and Hominick, 1980) was determined using the equation:

$$\text{Mean \% change} = \frac{\text{mean difference } (\bar{z}) \text{ between treatments}}{\text{overall mean of measurements from both treatments}} \times 100$$

3.3 Results

3.3.1 Anaesthetic, followed by hot saline.

Five of the characters of nematodes measured in propylene phenoxetol demonstrated significant dimensional changes following relaxation in hot saline (Table 3.1). Significant increases occurred in the total length, length to the base of oesophagus and the vulva, and the body widths measured at the vulva and anus.

Generally, specimens killed in hot saline were more robust (longer and thicker) than those narcotized and measured in propylene phenoxetol. It is interesting that the taxonomic ratio of excretory pore to pharynx[^], and the per-
(length, head to excretory pore/length of pharynx)
(length, head to vulva/total body length)
cent vulva[^] were not significantly altered by the hot saline treatment. Both values showed only slight decreases after this treatment.

When the specimens were compared to living untreated nematodes some qualitative changes were apparent. About half of the anaesthetized worms had abnormally rounded or blunt anterior ends. At the posterior end, the normal tapering shape was considerably reduced (Fig. 3.8). Also, localized wrinkles (transverse folds) appeared in the normally smooth cuticle, especially at the base of oesophagus and the caudal region of the same specimens. The stoma, procorpus and excretory duct and its pore remained clearly visible (Figs. 3.7, 3.15) while the nerve ring and terminal bulb of the oesophagus were obscure in about 40% of the specimens. In virtually all specimens killed in hot saline, both anterior

Table 3.1 Measurements¹ of adult N. carpocapsae females in anaesthetic, followed by relaxation in 80°C saline

Character (N = 24)	Anaesthetized		Hot saline-relaxed		Difference	
	x ± SD	Range	x ± SD	Range	% Change	Significance
Total length (mm)	3.33 ± 0.81	1.98 - 4.65	3.90 ± 0.97	2.01 - 5.78	+ 15.8	* * *
Length of stoma	3 ± 0	3	3 ± 0	2 - 3	+ 4.3	NS
Width of stoma	4 ± 1	3 - 5	4 ± 1	3 - 5	+ 2.3	NS
Length head to excretory pore	69 ± 13	50 - 100	72 ± 12	50 ± 10	+ 3.5	NS
Ratio of excretory pore to pharynx	0.31 ± 0.05	0.19 ± 0.38	0.30 ± 0.04	0.24 ± 0.36	- 1.8	NS
Length, head to nerve ring	138 ± 24	100 - 200	144 ± 20	95 - 175	+ 4.0	NS
Length, head to base of oesophagus	226 ± 34	175 - 290	238 ± 31	175 - 300	+ 4.9	*
Width at base of oesophagus	126 ± 15	105 - 155	130 ± 18	100 - 180	+ 2.6	NS
Length, head to vulva (mm)	1.85 ± 0.44	1.10 - 2.77	2.09 ± 0.45	1.12 ± 2.90	+ 12.5	* * *
Percent Vulva	55.6 ± 2.8	51.2 - 61.9	54.1 ± 3.5	42.9 - 61.3	- 2.7	NS
Width at vulva	155 ± 25	125 - 210	164 ± 27	130 - 220	+ 5.6	* *
Width at anus	80 ± 10	60 - 100	86 ± 11	75 - 115	+ 6.5	*
Length of tail	47 ± 11	30 - 60	52 ± 9	40 - 70	+ 8.8	NS

¹ measurements to the nearest whole micrometre, except where otherwise indicated,

² NS, not significant; *, p<0.05; **, p<0.01; ***, p<0.001; (+) increase; (-) decrease.

of nematodes

Figure 3.1 - 3.4 Specimens anaesthetized in 0.35% propylenephenoxytol, resuscitated, then killed in water at 65°C (3 seconds)

Figure 3.1 Head, fixed in FAA

Figure 3.2 Tail, fixed in FAA

Figure 3.3 Head, fixed in FA 4:1

Figure 3.4 Tail, fixed in FA 4:1

Figure 3.5 Head, heat-relaxed in Ringer's saline at 80°C

Figure 3.6 Tail, heat-relaxed in Ringer's saline at 80°C

Figure 3.7 Head, anaesthetized in 0.35% propylene phenoxytol

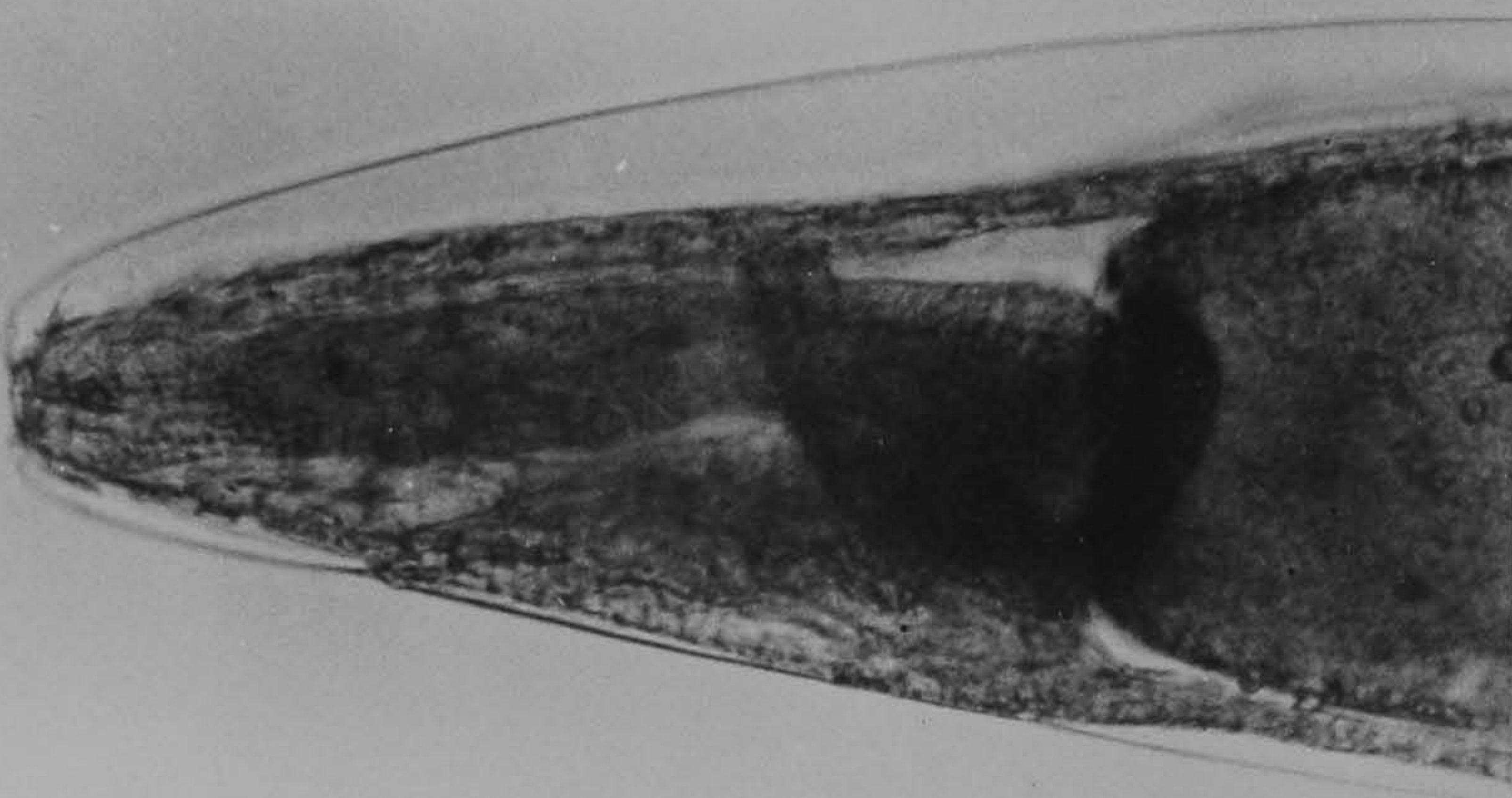
Figure 3.8 Tail, anaesthetized in 0.35% propylene phenoxytol



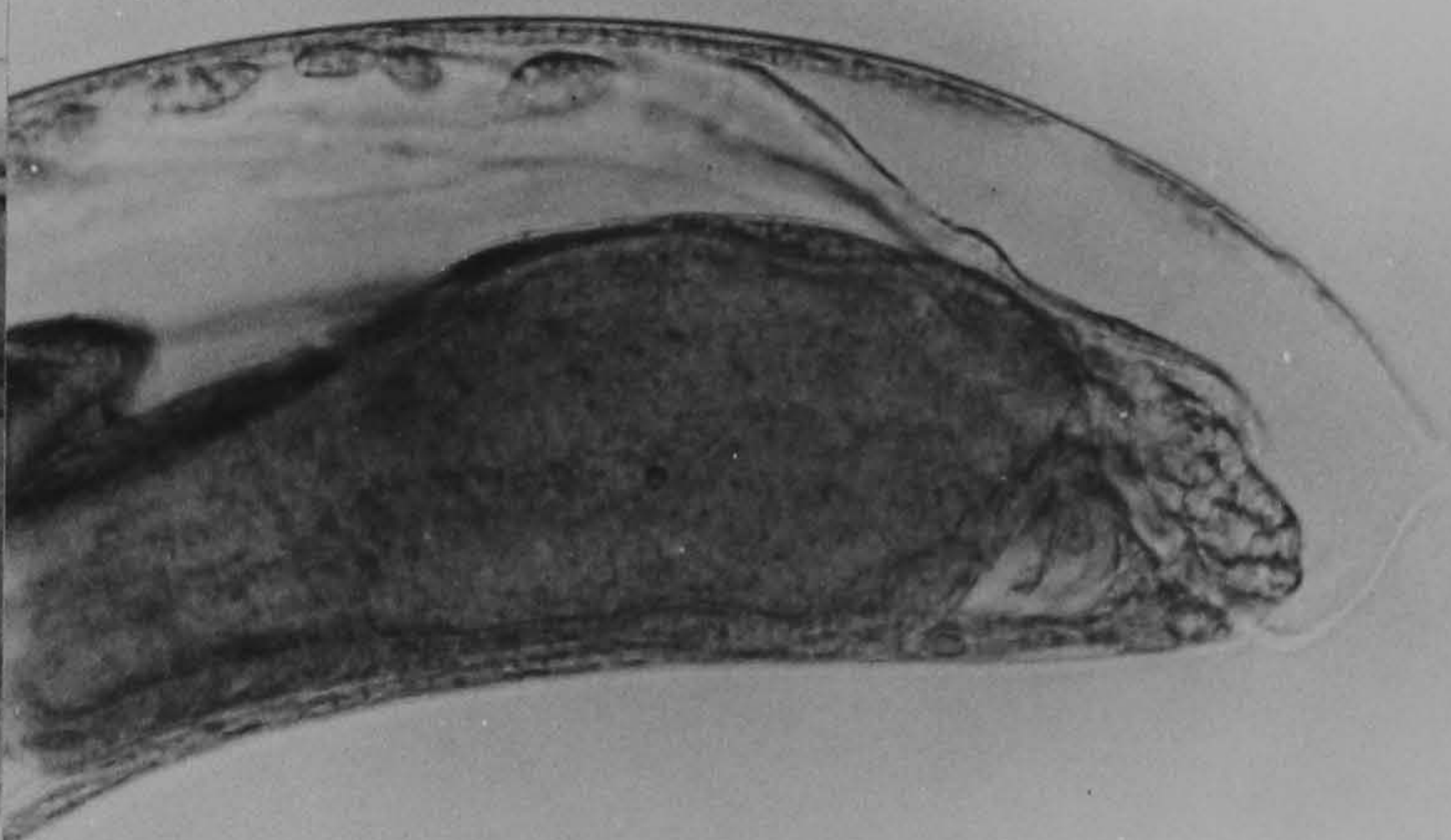
1



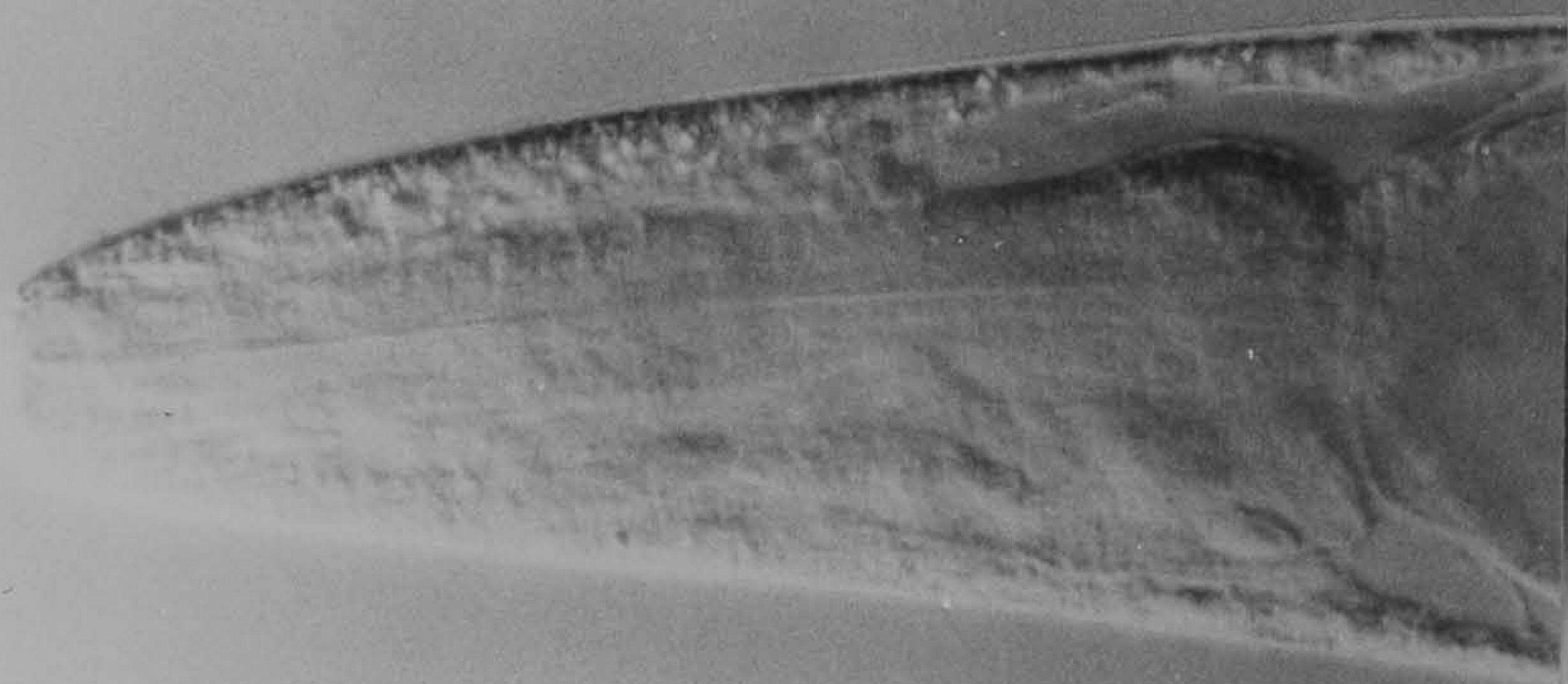
2



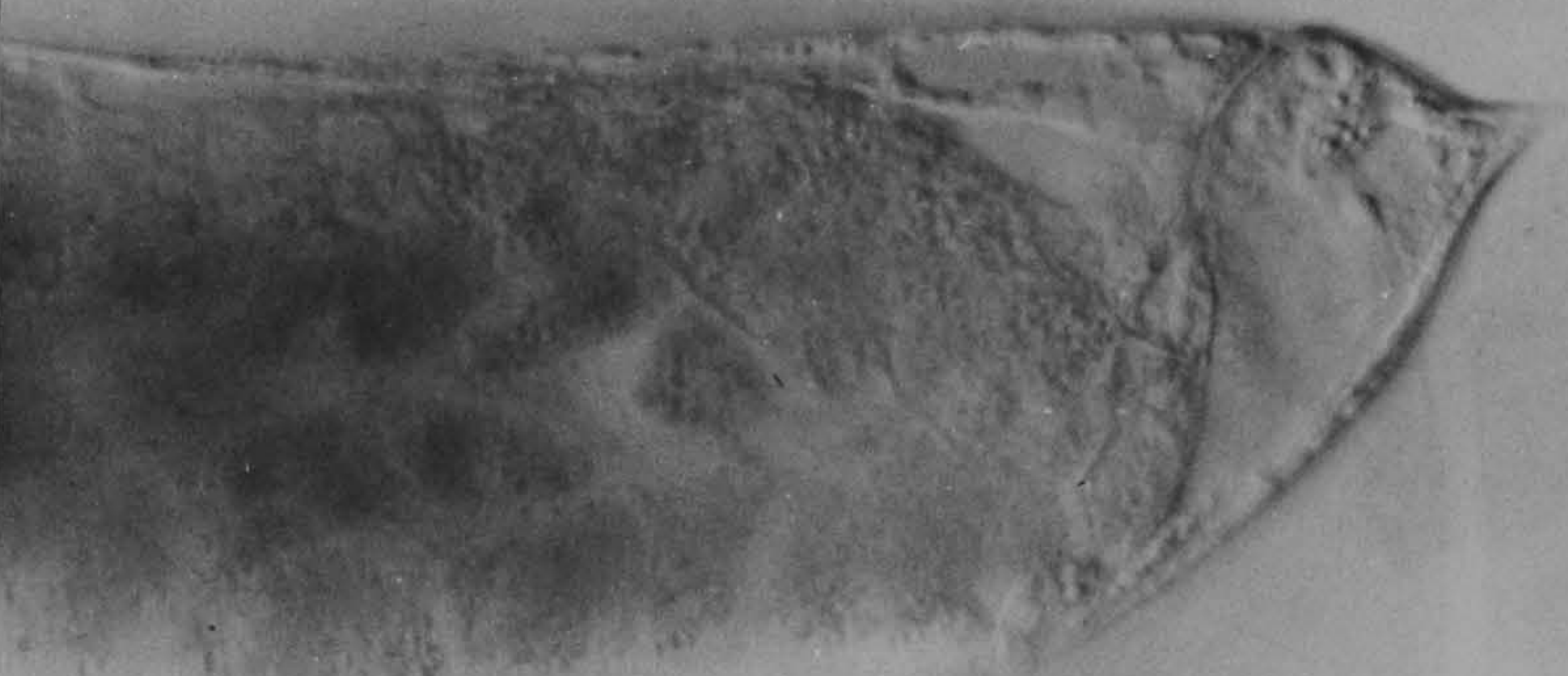
3



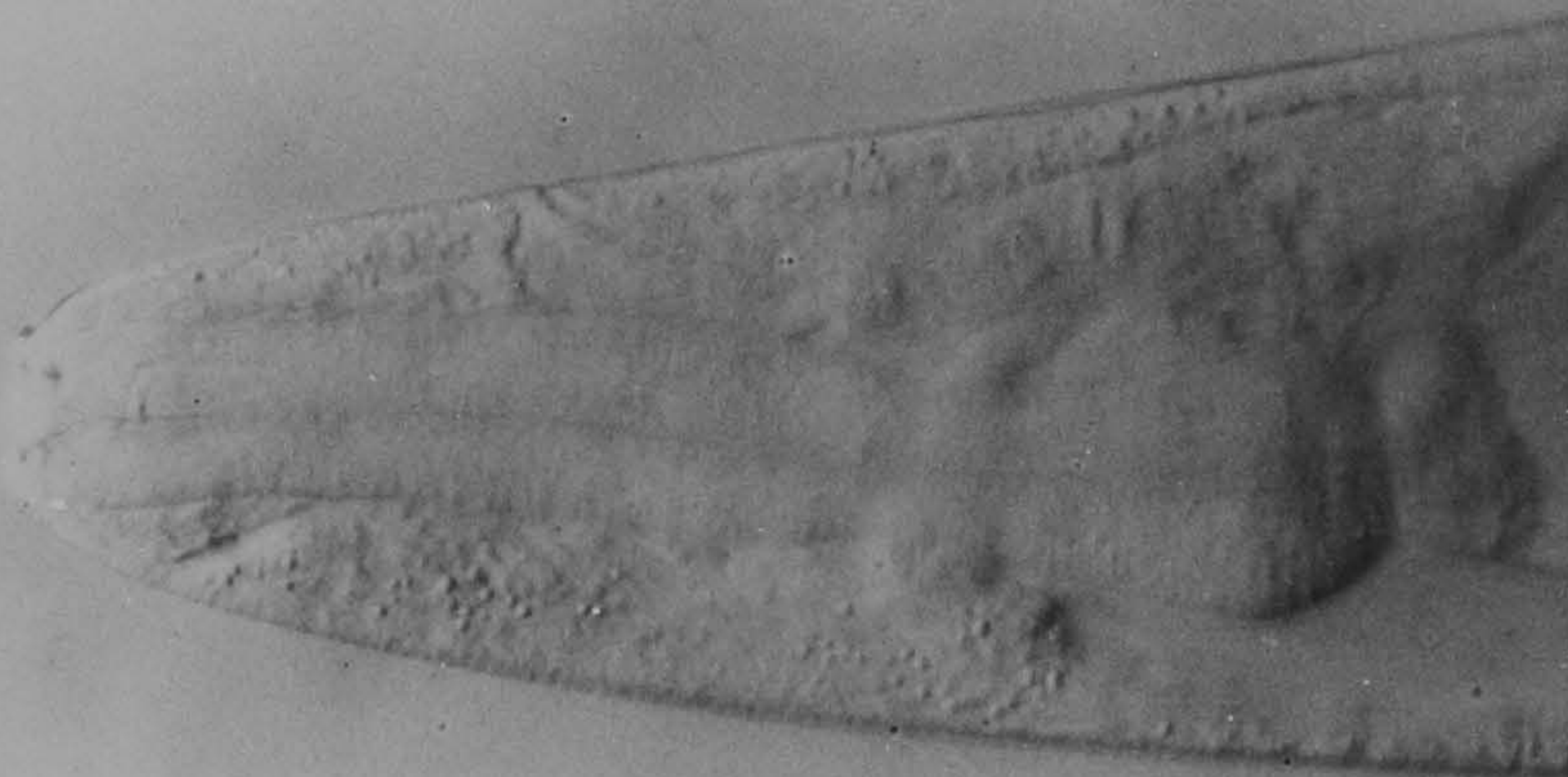
4



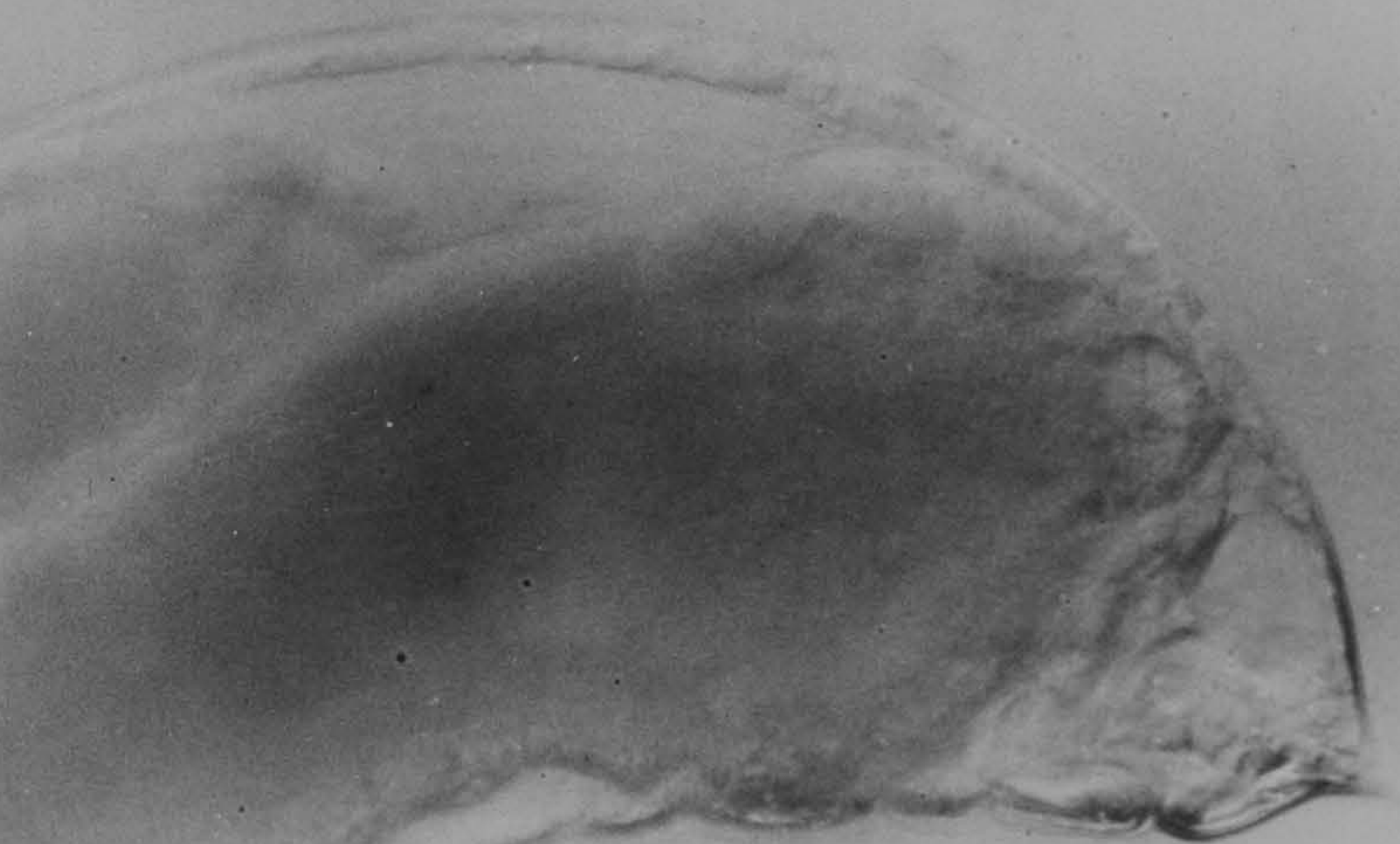
5



6



7



8

50 μ m

of nematodes
Figure 3.9 - 14 Specimens, anaesthetized in 0.35%
propylene phenoxetol, resuscitated,
then killed in water at 65°C
(3 seconds)

Figure 3.9 Head, fixed in TAF

Figure 3.10 Tail, fixed in TAF

Figure 3.11 Head, fixed in Kale's fluid

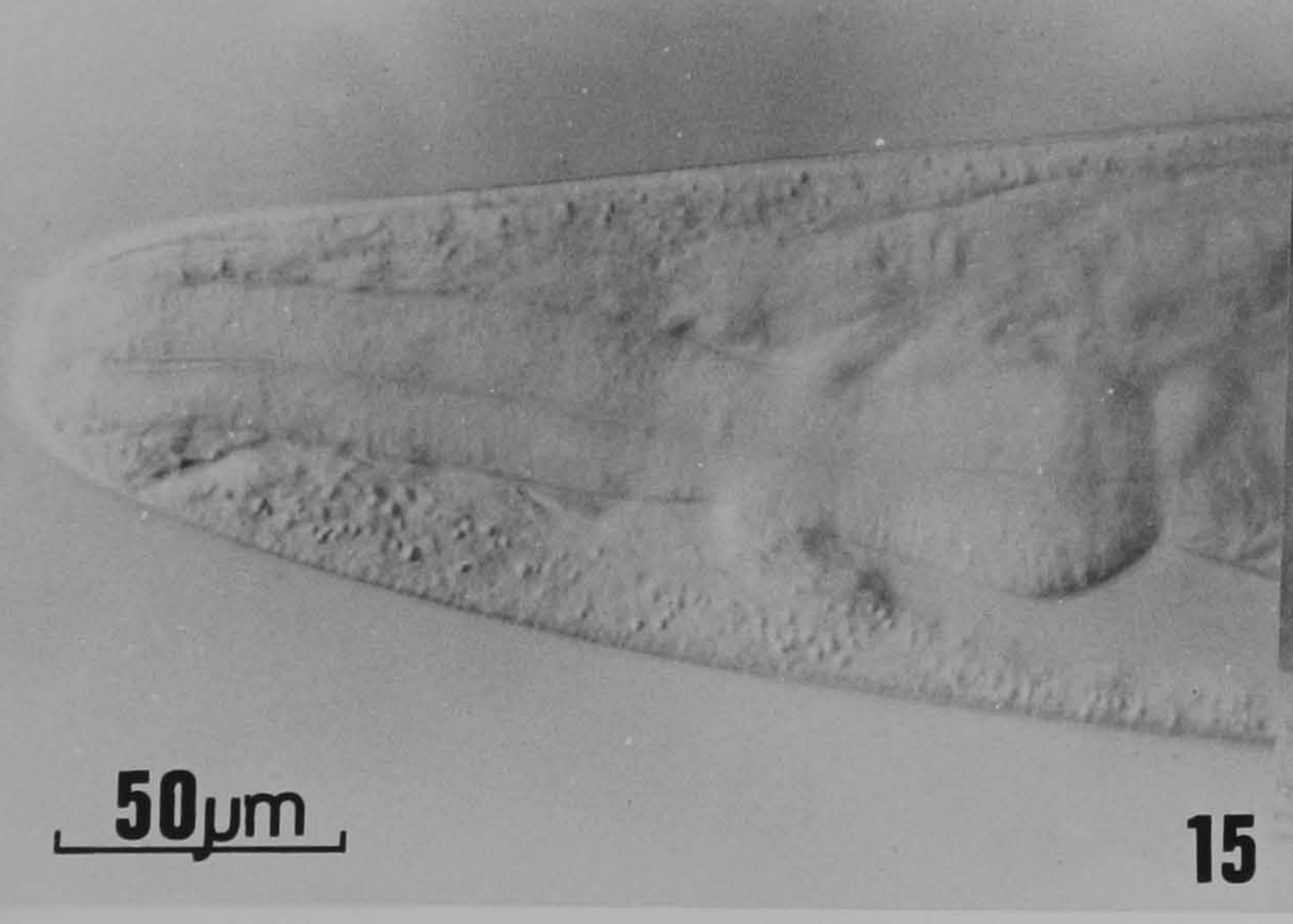
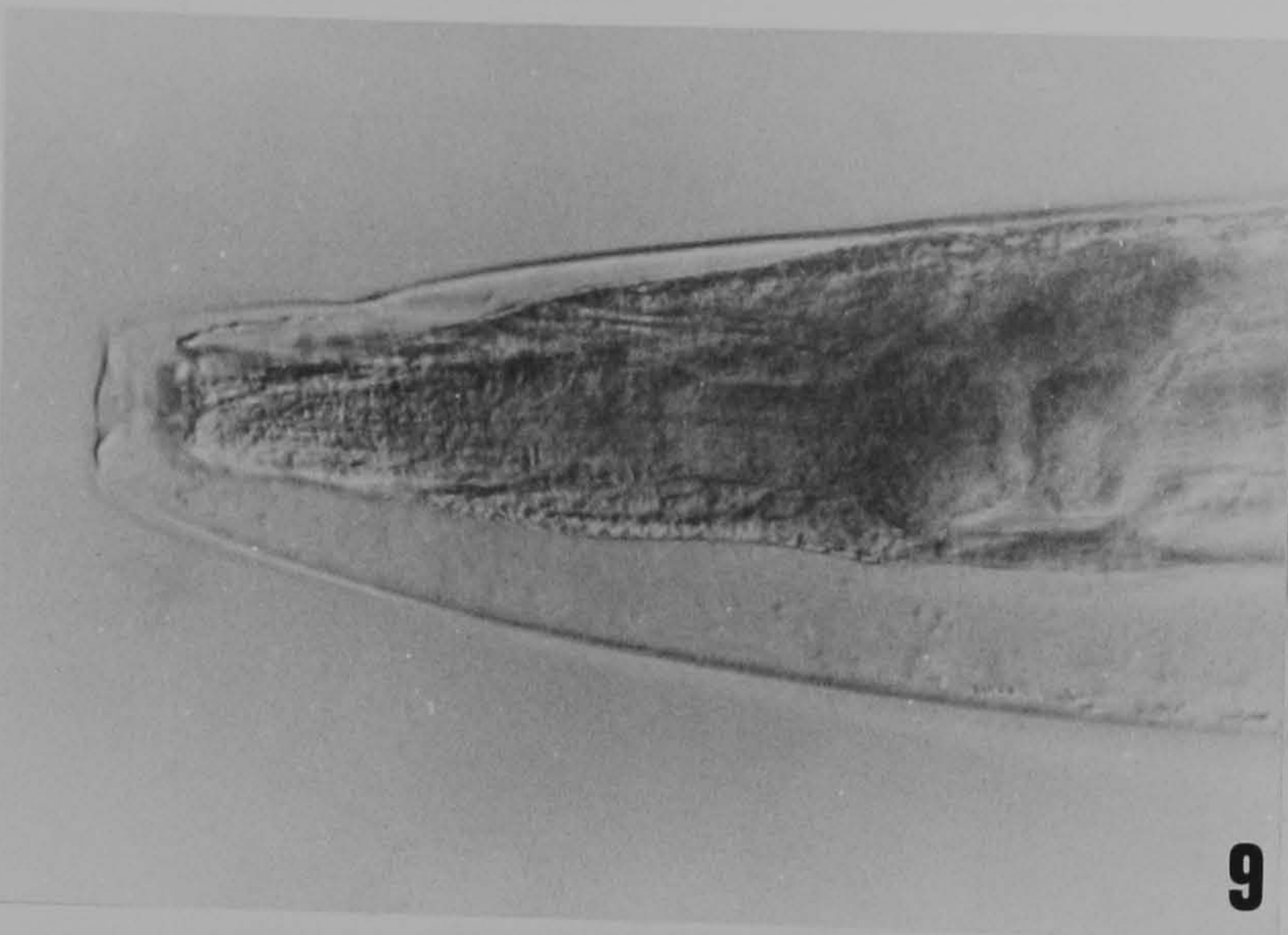
Figure 3.12 Tail, fixed in Kale's fluid

Figure 3.13 Head, fixed in 10% formalin

Figure 3.14 Tail, fixed in 10% formalin

Figure 3.15 Head, anaesthetized in 0.35% propylene
phenoxetol

Figure 3.16 Tail, anaesthetized in 0.35% propylene
phenoxetol



50µm

and posterior terminals were rather pointed (Figs. 3.5, 3.6). Cuticular folds were rarely seen and the terminal bulb of the oesophagus and the nerve ring became clearly visible. However, most of these specimens exhibited some loss of contrast between the internal organs. They displayed increased 'turbidity' behind the base of oesophagus and in the tail region.

3.3.2 Anaesthetic, followed by hot water

Most female measurements were not changed significantly by the hot water treatment (Table 3.2). Generally, there was a slight increase in size, but only the body length, length to the vulva, and width at the anus changed significantly. As in the saline-relaxed specimens, the ratio of excretory pore to pharynx and the percent vulva were unaffected. Qualitatively, these specimens showed the same changes as those treated with hot saline. However, there was a greater loss of definition of the internal structures after hot water treatment.

For males, hot water relaxation produced significant morphometric changes in four of the 15 characters of specimens previously measured in propylene phenoxetol (Table 3.3). The most significant change was in the body length, while the lengths from head to nerve ring and to the base of oesophagus, and the distance from tip of testis to tail, showed less significant changes. Generally, hot-water relaxed specimens were larger than the anaesthetized

Table 3.2 Measurements¹ of females of N. carpocapsae Weiser, in anaesthetic followed by relaxation in 65°C water.

Character (N = 25)	Anaesthetized		Hot water relaxed		Difference	
	x + SD	Range	x + SD	Range	% Change	Significance
Total length (mm)	3.34 + 0.92	1.97 - 6.52	3.59 + 0.96	2.16 - 6.93	+ 7.2	* *
Length of stoma	3 + 0	2 - 3	3 + 0	3	+ 4.1	NS
Width of stoma	5 + 1	3 - 7	5 + 1	3 - 7	+ 5.8	NS
Length head to excretory pore	65 + 12	40 - 90	68 + 10	50 - 80	+ 5.1	NS
Ratio of excretory pore to pharynx	0.28 + 0.05	0.19 - 0.38	0.29 + 0.04	0.21 - 0.40	+ 4.6	NS
Length , head to nerve ring	137 + 18	110 - 170	145 + 26	110 - 210	+ 4.8	NS
Length, head to base of oesophagus	233 + 28	180 - 300	263 + 31	180 - 280	+ 1.4	NS
Width at base of oesophagus	122 + 15	80 - 150	124 + 11	100 - 155	+ 0.7	NS
Length, head to vulva (mm)	1.83 + 0.45	1.15 - 3.27	1.99 + 0.47	1.23 - 3.63	+ 8.5	* * *
Percent vulva	55.1 + 2.9	50 - 61	55.1 + 1.8	52 - 60	- 0.3	NS
Width at vulva	146 + 26	100 - 210	149 + 23	100 - 180	+ 1.6	NS
Width at anus	68 + 13	50 - 100	78 + 12	55 - 100	+ 11.5	* *
Length of tail	40 + 11	25 - 65	45 + 8	30 - 60	+ 12.8	NS

¹ as in Table 3.1

Table 3.3 Morphometrics¹ of adult males of N. carpocapsae Weiser, in anaesthetic, followed by water at 65°C for 3 seconds.

Character (N = 10)	Anaesthetized		Hot water relaxed		Difference	
	x ± SD	Range	x ± SD	Range	% Change	Significance
Total length (mm)	1.12 ± 0.11	0.95 - 1.28	1.28 ± 0.15	1.00 - 1.47	+ 13.5	* * *
Length of stoma	3 ± 1	3 - 4	4 ± 1	3 - 4	+ 5.7	NS
Width of stoma	3 ± 0	2 - 3	3 ± 0	2 - 3	+ 11.3	NS
Length, head to excretory pore	54 ± 8	40 - 60	57 ± 12	40 - 75	+ 5.9	NS
Ratio of excretory pore to pharynx	0.32 ± 0.05	0.23 - 0.38	0.32 ± 0.06	0.23 - 0.41	+ 0.9	NS
Length, head to nerve ring	101 ± 10	80 - 113	118 ± 16	90 - 140	+ 15.2	*
Length, head to base of oesophagus	168 ± 5	160 - 175	180 ± 16	140 - 200	+ 6.8	*
Width at base of oesophagus	64 ± 7	60 - 80	66 ± 6	60 - 75	+ 3.5	NS
Length, tip of testis to tail	799 ± 66	675 - 905	957 ± 133	705 - 1170	+ 18.0	* *
Length of spicule	63 ± 5	55 - 70	61 ± 4	50 - 65	- 2.3	NS
Width of spicule	13 ± 1	10 - 15	14 ± 1	13 - 15	+ 5.3	NS
Length of gubernaculum	45 ± 6	35 - 55	45 ± 8	40 - 60	+ 0.5	NS
Width of gubernaculum	10 ± 0	10	10 ± 0	10	0.0	NS
Width at anus	47 ± 5	40 - 55	47 ± 10	40 - 70	+ 1.1	NS
Length of tail	26 ± 7	15 - 40	27 ± 7	20 - 40	+ 6.8	NS

¹ As in Table 3.1

specimens. However, the ratio of excretory pore to pharynx and, not surprisingly, the lengths and widths of the spicule and gubernaculum, were unaffected.

Qualitative changes induced by anaesthetization of males were mainly the loss of clarity of internal structures. The outline of the oesophagus, the tip of testis and the position of the nerve ring were indistinct in half the specimens. Part of the intestine moved anteriorly and obscured the basal bulb and the nerve ring. This phenomenon was reversed by subsequent treatment of the specimens in hot water. Also the extent of protrusion of the spicules in 4/10 hot water-treated specimens increased over that of anaesthetized worms. As observed in females, the terminal parts of most narcotized males were bluntly rounded, but cuticular wrinkles were not common. Rounding of the posterior end reduced the visibility of the terminal spine in some specimens.

3.3.3 Treatment with fixatives: Preamble.

There were significant quantitative and qualitative changes when the nematodes were treated with anaesthetic, hot water and fixatives. However, the same character did not always show statistically significant changes when a treatment was repeated on another group of specimens. Therefore, data obtained from each group of specimens that were submitted to exactly the same treatments (Tables 3.4 to 3.8) were analysed separately for each group. Then, an overview of the morphological and morphometric responses of the

nematodes to all the treatments was attempted and is provided in the summary of results (Section 3.3.9).

3.3.4 Anaesthetic, followed by hot water and FA 4:1.

Relaxation in hot water caused a significant increase in the dimension of head to vulva compared to measurements obtained in propylene phenoxetol. No other character was significantly affected by the hot water treatment (Table 3.4). The FA 4:1 fixed specimens were considerably smaller in size than the hot water relaxed worms. This decrease was significant in the body length, distance of vulva from head, and the width at vulva. Generally, relaxation in hot water increased the size of the measurements, while fixation in FA 4:1 caused them to decrease. Thus, when measurements after fixation were compared to those from anaesthetized specimens the only significant change was in the increased width at the anus.

The qualitative appearance of some specimens was fairly well preserved following fixation in FA 4:1. However, gross morphological distortions were not uncommon. In 5 out of 11 specimens, the somatic muscles were detached from the hypodermis and cuticle, especially at the anterior and posterior ends of the nematodes (Figs. 3.3, 3.4). These specimens had abnormal hyaline areas at the points of muscular detachment. Also, a noticeable neck constriction appeared in a few specimens. Most specimens developed a brownish colour in this fixative but details of the internal structures were easily

Table 3.4 : Measurements of N. carpocapsae, Weiser in anaesthetic followed by 65°C water and FA 4:1

Character (N = 5)	Anaesthetized			Heat-relaxed			FA 4:1 Fixed			Difference		Overall	
	x ± SD	Range	x ± SD	Range	% Change	Range	x ± SD	Range	% Change	% Change	% Change	% Change	% Change
Total length (mm)	3.10 ± 0.45	2.71 - 3.80	3.55 ± 0.70	3.02 - 4.37	+ 13.6	3.02 - 4.37	3.00 ± 0.46	2.41 - 3.48	- 16.9	*	- 3.4	NS	NS
Length of stoma	3 ± 1	2 - 3	3 ± 0	3	+ 14.3	3	3 ± 1	2 - 3	- 6.9	NS	- 7.4	NS	NS
Width of stoma	5 ± 1	3 - 6	5 ± 2	3 - 7	+ 3.9	3 - 7	4 ± 1	3 - 5	- 2.13	NS	- 17.4	NS	NS
Length, head to excretory pore	61 ± 13	45 - 70	66 ± 12	50 - 80	+ 7.9	50 - 80	63 ± 15	40 - 80	- 4.7	NS	+ 3.2	NS	NS
Ratio of excretory pore to pharynx	0.28 ± 0.05	0.21 - 0.33	0.29 ± 0.07	0.21 - 0.40	+ 5.6	0.21 - 0.40	0.29 ± 0.05	0.21 - 0.33	- 2.1	NS	+ 3.5	NS	NS
Length, head to nerve ring	141 ± 23	120 - 170	149 ± 26	125 - 190	+ 5.5	125 - 190	125 ± 5	120 - 130	- 17.5	NS	- 12.0	NS	NS
Length, head to base of oesophagus	217 ± 16	200 - 240	230 ± 30	190 - 270	+ 5.8	190 - 270	218 ± 24	190 - 250	- 5.4	NS	+ 0.5	NS	NS
Width at base of oesophagus	122 ± 17	110 - 150	125 ± 11	110 - 140	+ 2.4	110 - 140	117 ± 10	100 - 125	- 6.6	NS	- 4.2	NS	NS
Length, head to vulva (mm)	1.71 ± 0.22	1.50 - 1.95	1.94 ± 0.34	1.64 - 2.35	- 12.6	1.64 - 2.35	1.60 ± 0.21	1.28 - 1.77	- 19.1	*	- 1.3	NS	NS
Percent vulva	55.3 ± 0.6	51.3 - 59.6	54.8 ± 1.6	53.1 - 57.0	- 1.1	53.1 - 57.0	53.4 ± 2.2	51.3 - 56.5	- 2.2	NS	- 3.3	NS	NS
Width at vulva	145 ± 25	115 - 170	150 ± 24	130 - 180	+ 3.4	130 - 180	130 ± 15	115 - 150	- 14.3	**	- 10.9	NS	NS
Width at anus	63 ± 10	50 - 75	77 ± 9	65 - 90	+ 20.0	65 - 90	78 ± 5	75 - 85	+ 1.3	NS	+ 21.3	*	*
Length of tail	33 ± 7	25 - 40	43 ± 10	30 - 55	+ 26.3	30 - 55	39 ± 8	30 - 50	- 9.8	NS	+ 16.7	NS	NS

1 heat-relaxed compared to anaesthetized; 2 fixed compared to heat-relaxed; 3 fixed compared to anaesthetized;

NS, not significant; *, p<0.05; **, p<0.01; ** p<0.002; (+), increase; (-), decrease.

resolved in most specimens.

3.3.5 Anaesthetic, followed by hot water and FAA.

In this experiment, none of the morphometric alterations caused by the treatments was statistically significant (Table 3.5). The FAA-fixed specimens generally showed a small size decrease compared to the anaesthetic-treated ones. However, the hot water-relaxed worms showed both increases and decreases compared to anaesthetized specimens.

Most specimens fixed in FAA remained undistorted. The lips and the mouth were clearly visible, as were the excretory pore, pharyngo-intestinal junction, anterior demarcation of the ovary, rectum and the anal opening (Figs. 3.1, 3.2). However, in 2 of the 10 specimens, there was a slight increase in cuticular thickness at the head-end. Also, the outline of the oesophagus was indistinct in many specimens. Like FA 4:1, FAA fixation produced a slightly brownish appearance in the nematodes.

3.3.6 Anaesthetic, followed by hot water at 65°C and 10% formalin.

As in previous experiments, the treatments produced changes, but when dimensions measured after fixation were compared with those obtained after anaesthetization, none of the changes were significant (Table 3.6)

Qualitatively, structural details of the oesophagus, lips, excretory duct and pore, and the caudal region of the

Table 3.5: Measurements of N. carpocapsae, Weiser females in anaesthetic followed by 65°C water, and FAA

Character (N = 4)	Anaesthetized			Heat-relaxed			Difference ¹			FAA - Fixed			Difference ²			Overall ³		
	x ± SD			Range			x ± SD			Range			x ± SD			Range		
Total length (mm)	4.14 ± 1.73	2.73 - 6.52	4.35 ± 1.85	2.74 - 6.93	NS	3.79 ± 1.38	2.64 - 5.69	NS	3.79 ± 1.38	2.64 - 5.69	NS	- 13.7	NS	- 9.0	NS			
Length of stoma	3 ± 0	3	3 ± 0	3	NS	3 ± 0	3	NS	3 ± 0	3	NS	0.0	NS	0.0	NS			
Width of stoma	5 ± 1	5 - 6	5 ± 0	5	NS	5 ± 2	3 - 7	NS	5 ± 2	3 - 7	NS	+ 4.9	NS	0.0	NS			
Length head to excretory pore	71 ± 8	60 - 75	65 ± 7	60 - 75	NS	69 ± 11	55 - 80	NS	69 ± 11	55 - 80	NS	+ 5.6	NS	- 3.6	NS			
Ratio of excretory to pharynx	0.28 ± 0.06	0.20 - 0.34	0.28 ± 0.01	0.27 - 0.30	NS	0.30 ± 0.02	0.28 - 0.33	NS	0.30 ± 0.02	0.28 - 0.33	NS	+ 5.2	NS	+ 6.1	NS			
Length, head to nerve ring	140 ± 18	125 - 160	134 ± 18	120 - 160	NS	135 ± 17	120 - 160	NS	135 ± 17	120 - 160	NS	+ 0.9	NS	- 3.6	NS			
Length head to oesophagus	263 ± 39	220 - 300	233 ± 25	220 - 270	NS	234 ± 34	200 - 280	NS	234 ± 34	200 - 280	NS	+ 0.5	NS	- 11.6	NS			
Width at base of oesophagus	124 ± 19	100 - 145	123 ± 7	115 - 130	NS	118 ± 17	100 - 140	NS	118 ± 17	100 - 140	NS	- 4.2	NS	- 5.2	NS			
Length head to vulva (mm)	2.16 ± 0.80	1.55 - 3.27	2.32 ± 0.93	1.52 - 3.63	NS	2.07 ± 0.70	1.46 - 3.00	NS	2.07 ± 0.70	1.46 - 3.00	NS	- 11.5	NS	- 4.4.	NS			
Percent vulva	52.9 ± 2.9	50.2 - 56.8	53.8 ± 1.4	52.4 - 55.0	NS	54.9 ± 1.5	52.7 - 55.9	NS	54.9 ± 1.5	52.7 - 55.9	NS	+ 1.9	NS	+ 3.3	NS			
Width at vulva	148 ± 24	125 - 175	148 ± 17	130 - 170	NS	144 ± 19	120 - 165	NS	144 ± 19	120 - 165	NS	- 2.6	NS	- 2.6	NS			
Width at anus	73 ± 16	50 - 85	74 ± 14	60 - 90	NS	79 ± 30	50 - 120	NS	79 ± 30	50 - 120	NS	+ 6.6	NS	+ 8.3	NS			
Length of tail	45 ± 6	40 - 50	46 ± 5	40 - 50	NS	40 ± 14	30 - 60	NS	40 ± 14	30 - 60	NS	- 14.5	NS	- 11.8	NS			

Legend as for Table 3.4

Table 3.6: Measurements of N. carpocapsae Weiser, females in anaesthetic, followed by 65°C water and 10% formalin

Character (N = 5)	Anaesthetized			Heat-relaxed			Formalin Fixed			Difference ²			Overall ³		
	$\bar{x} \pm \underline{\hspace{0.5cm}}$	SD	Range	$\bar{x} \pm \underline{\hspace{0.5cm}}$	SD	Range	$\bar{x} \pm \underline{\hspace{0.5cm}}$	SD	Range	% Change	% Change	% Change	% Change	% Change	
Total length (mm)	3.30 $\pm$ <u> </u>		2.94 -	3.39 $\pm$ <u> </u>		2.44 -	3.66 $\pm$ <u> </u>		3.06 -	+2.7 NS	+ 7.6 NS	+10.4 NS			
	0.33		3.69	0.76		4.48	0.61		4.63						
Length of stoma	3 $\pm$ <u>0</u>		3	3 $\pm$ <u>0</u>		3	3 $\pm$ <u>0</u>		3	0.0 NS	0.0 NS	0.0 NS			
Width of stoma	5 $\pm$ <u>1</u>		4 - 7	5 $\pm$ <u>2</u>		4 - 7	4 $\pm$ <u>1</u>		3 - 5	+3.8 NS	-20.4 NS	-16.7 NS			
Length, head to excretory pore	66 $\pm$ <u>19</u>		45 - 90	70 $\pm$ <u>13</u>		50 - 85	68 $\pm$ <u>8</u>		60 - 75	+5.9 NS	- 2.9 NS	+ 3.0 NS			
Ratio of excretory pore to pharynx	0.29 $\pm$ <u> </u>		0.20 -	0.30 $\pm$ <u> </u>		0.27 -	0.29 $\pm$ <u> </u>		0.24 -	+4.7 NS	- 6.1 NS	- 1.4 NS			
	0.06		0.38	0.04		0.37	0.06		0.39						
Length, head to nerve ring	140 $\pm$ <u>28</u>		110 - 170	133 $\pm$ <u>31</u>		110 - 175	136 $\pm$ <u>11</u>		120 - 150	-5.1 NS	+2.2 NS	- 2.9 NS			
Length, head to base of oesophagus	228 $\pm$ <u>28</u>		180 - 250	234 $\pm$ <u>47</u>		180 - 280	244 $\pm$ <u>40</u>		180 - 290	+2.6 NS	+4.2 NS	+ 6.8 NS			
Width at base of oesophagus	125 $\pm$ <u>9</u>		120 - 140	130 $\pm$ <u>14</u>		120 - 155	124 $\pm$ <u>4</u>		120 - 130	+3.9 NS	-4.7 NS	- 0.8 NS			
Length, head to vulva (mm)	1.83 $\pm$ <u> </u>		1.66 -	1.95 $\pm$ <u> </u>		1.68 -	2.02 $\pm$ <u> </u>		1.74 -	+6.2 NS	+3.4 NS	+ 9.6 NS			
	0.21		2.12	0.31		2.47	0.36		2.61						
Percent vulva	55.5 $\pm$ <u> </u>		53.4 -	54.2 $\pm$ <u> </u>		52.7 -	55 $\pm$ <u>2</u>		53.2 -	-2.2 NS	-1.5 NS	- 0.7 NS			
	2.4		59.1	1.1		55.4			56.9						
Width at vulva	159 $\pm$ <u>10</u>		150 - 175	163 $\pm$ <u>16</u>		145 - 180	158 $\pm$ <u>8</u>		145 - 165	+2.5 NS	-3.1 NS	- 0.6 NS			
Width at anus	74 $\pm$ <u>4</u>		70 - 80	78 $\pm$ <u>6</u>		70 - 85	83 $\pm$ <u>8</u>		70 - 90	+5.3 NS	+6.9 NS	+ 11.5 NS			
Length of tail	49 $\pm$ <u>16</u>		30 - 65	43 $\pm$ <u>10</u>		35 - 60	44 $\pm$ <u>10</u>		30 - 55	-13.0 NS	+2.3 NS	- 10.8 NS			

Legend as for Table 3.4

specimens were well preserved by fixation in formalin (Figs. 3.13, 3.14). The thickness of the cuticle was unchanged, but cuticular wrinkles occasionally appeared, especially in the region posterior to the basal bulb. Also, in some specimens, the position of the posterior intestine shifted relative to the anus (Fig. 3.14). Generally, formalin-fixed nematodes were darker and slightly more granular in appearance, than those measured in narcotic or heat-relaxed in hot water.

3.3.7 Anaesthetic, followed by 65°C water and Kale's fluid.

Fixation of nematodes in Kale's fluid produced a significant dimensional change in one of the 13 characters compared to the anaesthetized specimens (width at the base of oesophagus) (Table 3.7). Most other characters exhibited decreases that were not statistically significant.

Observable structural changes induced by this fixative included loss of contrast between the gonads and intestine in the mid-region. At the posterior ends, the somatic muscles were sometimes slightly detached from the hypodermis. In 2 of 9 specimens, the cuticle was detached from the hypodermis between the head and base of the oesophagus (Fig. 3.11). However, details of other internal structures, such as the pharynx, nerve ring and excretory duct, as well as those of the tail region (Fig. 3.12) were well preserved.

3.3.8 Anaesthetic, followed by 65°C water and TAF

Nematodes measured in anaesthetic and compared after

Table 3.7: Measurements of N. carpocapsae Weiser, females in anaesthetic, followed by 65°C water, and Kale's fluid

Character (N = 4)	Anaesthetized			Heat-relaxed			Difference ¹			Kale's - Fixed			Difference ²			Overall ³		
	$\bar{x} \pm \text{SD}$	Range	$\bar{x} \pm \text{SD}$	Range	$\bar{x} \pm \text{SD}$	Range	% Change			$\bar{x} \pm \text{SD}$	Range	% Change				% Change		
Total length (mm)	3.58 $\pm$ 1.14	2.64 - 5.17	3.77 $\pm$ 0.86	2.94 - 4.82	3.34 $\pm$ 0.69	2.69 - 4.21	+ 5.2 NS			3.34 $\pm$ 0.69	2.69 - 4.21	- 12.1 *			- 6.9 NS			
Length of stoma	3 $\pm$ 1	2 - 3	3 $\pm$ 0	3	3 $\pm$ 1	2 - 3	+ 8.7 NS			3 $\pm$ 1	2 - 3	- 8.7 NS			0.0 NS			
Width of stoma	4 $\pm$ 1	3 - 4	4 $\pm$ 1	3 - 6	4 $\pm$ 1	3 - 6	0.0 NS			4 $\pm$ 1	4 - 5	+ 19.4 NS			+ 6.1 NS			
Length head to excretory pore	55 $\pm$ 12	40 - 65	64 $\pm$ 11	50 - 75	64 $\pm$ 6	55 - 70	+ 14.7 NS			64 $\pm$ 6	55 - 70	0.0 NS			+ 14.7 NS			
Ratio of excretory to pharynx	0.25 $\pm$ 0.04	0.19 - 0.30	0.27 $\pm$ 0.02	0.25 - 0.28	0.31 $\pm$ 0.03	0.28 - 0.36	+ 14.0 NS			0.31 $\pm$ 0.03	0.28 - 0.36	+ 15.5 NS			+ 29.4 NS			
Length, head to nerve ring	136 $\pm$ 13	120 - 150	139 $\pm$ 10	125 - 150	128 $\pm$ 15	110 - 140	+ 1.8 NS			128 $\pm$ 15	110 - 140	- 8.5 NS			- 6.6 NS			
Length head to oesophagus	234 $\pm$ 26	210 - 270	240 $\pm$ 34	200 - 280	206 $\pm$ 11	195 - 220	- 7.5 NS			206 $\pm$ 11	195 - 220	- 15.1 NS			- 12.5 NS			
Width at base of oesophagus	124 $\pm$ 5	120 - 130	121 $\pm$ 14	100 - 130	109 $\pm$ 12	100 - 125	- 2.0 NS			109 $\pm$ 12	100 - 125	- 10.9 NS			- 12.9 *			
Length head to vulva (mm)	1.99 $\pm$ 0.60	1.43 - 2.75	2.07 $\pm$ 0.43	1.66 - 2.58	1.83 $\pm$ 0.33	1.52 - 2.23	+ 3.6 NS			1.83 $\pm$ 0.33	1.52 - 2.23	- 12.3 *			- 8.7 NS			
Percent vulva	55.8 $\pm$ 3.4	53.2 - 60.7	55.0 $\pm$ 1.2	53.6 - 56.5	54.9 $\pm$ 1.5	53.0 - 56.5	- 1.8 NS			54.9 $\pm$ 1.5	53.0 - 56.5	0.0 NS			- 21.5 NS			
Width at vulva	151 $\pm$ 40	125 - 210	151 $\pm$ 28	125 - 180	131 $\pm$ 27	110 - 170	0.0 NS			131 $\pm$ 27	110 - 170	- 14.2 NS			- 14.2 NS			
Width at anus	68 $\pm$ 24	50 - 100	81 $\pm$ 14	70 - 100	71 $\pm$ 17	55 - 90	+ 18.5 NS			71 $\pm$ 17	55 - 90	- 13.1 NS			+ 5.4 NS			
Length of tail	38 $\pm$ 7	30 - 45	46 $\pm$ 10	40 - 60	34 $\pm$ 5	30 - 40	+ 20.9 NS			34 $\pm$ 5	30 - 40	- 31.3 NS			- 10.2 NS			

Legend as for Table 3.4

fixation in TAF showed no morphometric changes that were statistically significant (Table 3.8).

Considerable structural distortion was induced by fixation with TAF (Figs. 3.9, 3.10). In 10/15 specimens, the somatic musculature was detached from the hypodermis at localized positions, particularly in the anterior and posterior regions. Also, 2 of 15 specimens showed increased granulation, especially in the region between the basal bulb and vulva. The intestine and ovary were easily distinguished and the smoothness and thickness of the cuticle were unaffected. Also, some specimens demonstrated exceptional clarity of the oesophagus, lips and excretory pore.

3.3.9 Summary of results

The results of the present investigations showed that all processing/preparation techniques employed in the morphometric studies on adult females of the DD-136 strain of N. carpocapsae, produced some measurement changes. The most sensitive characters were total body length, tail length, distance from head to vulva, and from head to the base of oesophagus, and widths of the body measured at the base of oesophagus, the vulva and the anus. Much less affected were length and width of the stoma, distance from the head to excretory pore, and to the nerve ring, and lengths and widths of the spicule and gubernaculum in males.

The processing after anaesthetization, caused broadly two different types of effects. Heat relaxation generally

Table 3.8: Measurements of N. carpocapsae Weiser, females in anaesthetic followed by 65°C water, and TAF

Character (N = 5)	Anaesthetized			Heat-relaxed			Difference ¹		TAF Fixed			Difference ²		Overall ³	
	$\bar{x} \pm \text{SD}$	Range	$\bar{x} \pm \text{SD}$	Range	$\bar{x} \pm \text{SD}$	Range	% Change		$\bar{x} \pm \text{SD}$	Range		% Change		% Change	
Total length (mm)	2.74 + 0.55	1.97 - 3.31	3.02 + 0.57	2.16 - 3.60	3.02 + 0.57	2.16 - 3.60	+ 9.7 **		2.69 + 0.56	1.99 - 3.51		- 11.7 NS		- 2.0 NS	
Length of stoma	3 + 0	3	3 + 0	3	3 + 0	3	0.0 NS		3 + 1	2 - 3		- 6.9 NS		- 6.9 NS	
Width of stoma	4 + 1	3 - 5	5 + 1	4 - 6	5 + 1	4 - 6	+ 21.3 NS		5 + 1	4 - 5		- 8.0 NS		+ 13.3 NS	
Length, head to excretory pore	67 + 7	60 - 75	71 + 60	65 - 80	71 + 60	65 - 80	+ 5.8 NS		65 + 9	50 - 75		- 8.8 NS		- 3.0 NS	
Ratio of excretory pore to pharynx	0.30 + 0.05	0.25 - 0.37	0.29 + 0.02	0.27 - 0.31	0.29 + 0.02	0.27 - 0.31	- 3.4 NS		0.32 + 0.07	0.21 - 0.39		+ 7.9 NS		+ 4.5 NS	
Length, head to nerve ring	128 + 11	110 - 140	164 + 31	125 - 170	164 + 31	125 - 170	+ 24.7 NS		137 + 14	120 - 150		- 96.4 NS		+ 6.8 NS	
Length, head to base of oesophagus	225 + 25	200 - 260	243 + 16	225 - 270	243 + 16	225 - 270	+ 7.7 NS		210 + 28	180 - 240		- 14.6 *		- 6.9 NS	
Width at base of oesophagus	115 + 22	80 - 140	116 + 11	100 - 130	116 + 11	100 - 130	+ 0.9 NS		109 + 19	80 - 125		- 6.2 NS		- 5.4 NS	
Length head to vulva (mm)	1.53 + 0.32	1.15 - 1.92	1.73 + 0.35	1.23 2.17	1.73 + 0.35	1.23 2.17	+ 12.5 *		1.50 + 0.31	1.12 - 1.91		- 14.4 NS		- 1.9 NS	
Percent vulva	55.8 + 3.7	50.2 - 59.3	57.2 + 1.9	55.6 - 60.3	57.2 + 1.9	55.6 - 60.3	+ 1.8 NS		55.8 + 2.4	52.9 - 59.1		+ 2.1 NS		+ 0.4 NS	
Width at vulva	125 + 22	100 - 160	132 + 29	100 - 180	132 + 29	100 - 180	+ 5.5 NS		121 + 18	100 - 140		- 8.7 NS		- 3.3 NS	
Width at anus	63 + 12	50 - 75	74 + 16	55 - 100	74 + 16	55 - 100	+ 16.1 NS		67 + 8	60 - 80		- 9.9 NS		+ 6.2 NS	
Length of tail	34 + 10	25 - 50	43 + 5	40 - 50	43 + 5	40 - 50	+ 23.4 NS		37 + 8	30 - 50		- 15.0 NS		+ 8.5 NS	

Legend as for Table 3.4

caused an increase in measurements, while fixation resulted in some shrinkage. Consequently, the smallest changes relative to anaesthetized worms was produced by FAA, while FA 4:1 produced the largest.

Frequently, (75% of all cases) decreases in tail lengths were accompanied by increases in body width at the anus. However, in every case in which the tail length increased with treatment, there was an accompanying increase in body width at the anus.

The taxonomic ratios a, b, and c were calculated and their means were found to show wide variations between treatments (Table 3.9). These were independent of the processing techniques used in killing or fixing. However, the overall average of the mean b-values for all the treatments showed very little variation relative to a and c.

By contrast, the mean percent vulva and the mean ratio of excretory pore to length of pharynx showed relatively small changes, both within and between treatments or preparation methods.

3.4 Discussion

The occurrence of intraspecific morphometric variation due to preparation techniques has been reported for many nematode species (e.g. Fenwick and Franklin, 1942; Seinhorst, 1954, 1962; Lamberti and Sher, 1969; Hooper, 1970; Stone, 1971; Curran and Hominick, 1980). However, little is known of the effects of processing methods on insect-parasitic

Table 3.9: Mean morphometric ratios of adults of the DD-136 strain of N. carpocapsae Weiser

Treatment	Mean $\pm$ S.D.				
	Percent Vulva	Excretory pore/ length pharynx	a	b	c
3.1 Anaesthetic (n=24) Hot saline	55.6 $\pm$ 2.8 54.1 $\pm$ 3.5	0.31 $\pm$ 0.05 0.30 $\pm$ 0.04	23.6 $\pm$ 4.5 26.5 $\pm$ 5.0	14.6 $\pm$ 2.3 16.4 $\pm$ 3.2	72.3 $\pm$ 26.6 76.9 $\pm$ 20.9
3.2 Anaesthetic (n=25) Hot water	55.1 $\pm$ 2.9 55.1 $\pm$ 1.8	0.28 $\pm$ 0.05 0.29 $\pm$ 0.04	24.6 $\pm$ 4.2 26.2 $\pm$ 5.4	14.3 $\pm$ 3.2 15.4 $\pm$ 4.6	88.7 $\pm$ 28.2 81.4 $\pm$ 22.6
3.4 Anaesthetic (n=5) Hot water	55.3 $\pm$ 0.6 54.8 $\pm$ 1.6	0.28 $\pm$ 0.05 0.29 $\pm$ 0.07	23.3 $\pm$ 0.9 25.7 $\pm$ 2.5	14.3 $\pm$ 1.5 15.7 $\pm$ 3.7	98.1 $\pm$ 28.5 85.5 $\pm$ 21.1
FA 4:1	53.4 $\pm$ 2.2	0.29 $\pm$ 0.05	24.2 $\pm$ 2.1	13.9 $\pm$ 2.9	78.5 $\pm$ 14.5
3.5 Anaesthetic (n=4) Hot water	52.9 $\pm$ 2.9 53.8 $\pm$ 1.4	0.28 $\pm$ 0.06 0.28 $\pm$ 0.01	29.7 $\pm$ 7.9 31.7 $\pm$ 11.5	15.4 $\pm$ 4.4 19.0 $\pm$ 9.0	94.8 $\pm$ 47.4 92.2 $\pm$ 31.8
FAA	54.9 $\pm$ 1.9	0.30 $\pm$ 0.02	28.9 $\pm$ 9.2	16.2 $\pm$ 5.8	104.4 $\pm$ 58.7
3.6 Anaesthetic (n=5) Hot water	55.5 $\pm$ 2.4 54.2 $\pm$ 1.1	0.29 $\pm$ 0.06 0.30 $\pm$ 0.04	23.2 $\pm$ 2.1 23.0 $\pm$ 3.2	14.6 $\pm$ 2.0 14.6 $\pm$ 2.8	74.4 $\pm$ 29.5 82.4 $\pm$ 26.9
10% formalin	55.0 $\pm$ 1.6	0.29 $\pm$ 0.06	33.0 $\pm$ 14.7	15.3 $\pm$ 3.3	89.1 $\pm$ 37.4
3.7 Anaesthetic (n=4) Hot water	55.8 $\pm$ 3.4 55.0 $\pm$ 1.2	0.25 $\pm$ 0.04 0.27 $\pm$ 0.02	25.7 $\pm$ 3.8 27.5 $\pm$ 2.3	15.6 $\pm$ 6.1 15.9 $\pm$ 3.5	95.5 $\pm$ 24.3 83.6 $\pm$ 24.7
Kale's fluid	54.9 $\pm$ 1.5	0.31 $\pm$ 0.03	27.7 $\pm$ 2.1	16.4 $\pm$ 4.0	99.2 $\pm$ 16.0
3.8 Anaesthetic (n=5) Hot water	55.8 $\pm$ 3.7 57.2 $\pm$ 1.9	0.30 $\pm$ 0.05 0.29 $\pm$ 0.02	22.8 $\pm$ 2.1 24.3 $\pm$ 2.4	12.2 $\pm$ 2.1 12.3 $\pm$ 1.8	84.1 $\pm$ 21.7 71.3 $\pm$ 17.7
TAF	55.8 $\pm$ 2.4	0.32 $\pm$ 0.07	23.3 $\pm$ 2.5	12.7 $\pm$ 1.5	76.5 $\pm$ 25.5

Overall mean and (95% c.i.) 55.0 (54.5-55.4) 0.29 (0.28-0.30) 26.1 (24.6-27.5) 15.0 (14.2-15.8) 85.7 (81.1-90.4)

rhabditids. It is perhaps not surprising that all eight procedures tested in this work produced different quantitative and qualitative effects. As Stone (1971) remarked, each method has its own advantages and disadvantages, which presumably, explains the lack of agreement between workers on the best techniques for preparing nematode specimens for microscopical examination.

Since the nematodes were dissected from cadavers, where presumably the environment had a higher osmotic pressure than water, it was surprising that ringer's solution at 80°C produced more significant morphometric increments than distilled water at 65°C. The reason for this observation is not clear. It might be due to the short exposure (3 seconds) to hot water, relative to ringer's solution. Previously, it was found that female nematodes placed in deionized distilled water at room temperature, burst (for which reason all fixatives and anaesthetic were prepared in ringer's) after a minimal period of 4 minutes. The present hot water exposure period was, conceivably too brief for the osmotic pressure factor to exert a significant influence on the observed results. It would be interesting to determine the effects of longer exposures of these nematodes to hot water at 65°C, and the effect of killing using hot ringer's at 65°C on the morphometrics of these worms.

Temperature for temperature, the present observation was similar to that of Curran and Hominick (1980) but these workers used water at the higher temperature of 80°C. This

finding was contrary to the observation by Fenwick and Franklin (1942) that higher killing temperatures had a shortening effect on the measurements of H. rostochiensis larvae, which led them to recommend the adoption of 70°C as worm relaxing temperature. The reason for this discrepancy is not known, but both species and developmental stage factors offer possible explanations.

Of all the fixatives used, 10% formalin produced the least amount of shrinkage. Hooper (1970) noted that fixatives which contained ethanol were liable to produce shrinkage. Therefore, the morphometric reductions in specimens that were fixed in FAA and Kale's fluid can probably be attributed to the alcohol in these fixatives. Also, dimensional reductions in some morphological characters of Longidorus specimens have been attributed to the effects of acetic and/or propionic acid components of fixatives (Lamberti and Sher, 1969). Thus, acetic acid could have been partly responsible for the observed reduction in body lengths of specimens that were fixed in FA 4:1 and Kale's fluid, and the slight shrinkage in FAA-fixed specimens. It is surprising that, though it contains both alcohol and acetic acid, FAA produced the smallest morphometric changes compared to FA 4:1, Kale's fluid and TAF. There is no clear explanation for this observation. If it can be accepted that ethanol produced negligible amount of shrinkage relative to acetic acid, then the differential amounts of measurement decreases caused by these fixatives can, probably be explained by their

proportions of acid. FAA, which has the least amount of acid, therefore, caused the smallest degree of shrinkage of the specimens. The hygroscopic triethanolamine in TAF (Hooper, 1970) probably exerted some exosmotic pressure on the tissues of specimens, which led to the observed shortening. It might be interesting to know the effects of Ringer's solution in the fixatives on their effects on the observed measurements of fixed specimens.

The present results agree with the findings of several authors (e.g. Barraclough and Blackith, 1962; Geraert, 1968, 1978a, 1978b; Turco et al. 1971; Curran, 1981) that de Man's a: (body length/body width), b: (body length/oesophagus length), and c: (body length/tail length) values vary extensively within groups of a wide range of nematodes. This variation renders these values useless for diagnosing the species. Turco et al. (1971) found the de Man's a-, b-, and c- ratios useless for distinguishing between species of the genus Neoaplectana, a conclusion supported by the present work. The respective grand means of these ratios, however, showed less variability compared to the different treatment averages. This observation, indicates the need for taxonomists working with these nematodes to examine large numbers of specimens in order to draw valid conclusions. Moreover, the present results strongly support the use of per cent vulva and ratio of excretory pore to pharynx length as valid taxonomic characters of N. carpocapsae. Both characters demonstrated minimal variations over many different treat-

ments and agree closely with figures supplied by Poinar (1967, 1979) and Turco et al. (1971). Likewise, the lengths and widths of the spicule and gubernaculum of the males showed only small variations between specimens, and were unaffected by the different preparation methods used. They are undoubtedly of taxonomic importance as observed by Poinar (1967, 1979), Turco et al. (1971), and Stanuszek (1974) for this strain of Neoaplectana carpocapsae.

Measurements of adult males and females of the DD-136 strain of N. carpocapsae, documented by Poinar (1967, 1979) and Sandner and Stanuszek (1974) were compared with the present results. Poinar (1967) killed saline-suspended adult nematodes with gentle heat and mounted them in 3% formalin. Size differences between his female measurements and the present hot saline-relaxed ones were greater than the differences between his results and those of hot (65°C) water-relaxed females documented in the present work. The average measurements of most adult male characters examined by the same worker were different from the present ones but the dimensions of the spicule and gubernaculum were not significantly different. Sandner and Stanuszek (1974) reported measuring isolated living nematodes in physiological saline, but gave no information on their relaxing or killing method. The total body lengths and greatest widths of specimens recorded by these workers were remarkably different from the present measurements. There is little doubt that these morphometric differences were attributable to both differences

in rearing host/conditions and in preparation techniques.

The dimension of the oesophagus is a stable character in most nematodes (Geraert, 1968, 1979). It is, therefore interesting that the measurements of this character underwent considerable changes following heat relaxation in ringer's saline and water, and fixation in Kale's fluid and FAA. However, this finding does not question the validity of this taxonomic character. Rather, it emphasizes the need for a critical assessment of nematode preparation techniques used in specific taxonomic situations.

Ideally, narcotized worms should be measured (Stone, 1971) but the practical application of this technique is difficult. Ellenby and Smith (1964) noted that specimens relaxed and mounted in a mixture of the narcotic, propylene phenoxetol and Bovine Ox plasma for microscopical examination gave the working advantage that the refractive index of the mounting medium (mixture) matched that of the body cuticles of Panagrellus redivivus and Heterodera rostochiensis. This enhanced clarity of images of the specimens. Indeed, the life-like appearance of some phenoxetol-anaesthetized specimens examined during the present investigation was appreciable. However, the anaesthetic had the disadvantage of producing very significant morphometric and qualitative changes in many specimens. This method induced 'rounding' of the head and caudal region in many specimens. In some nematodes, considerable distortion occurred in the organization of the internal organs, particularly in the

mid-region of the body but these disappeared when worms were resuscitated.

Relaxation of living worms in 65°C distilled water for 3 seconds was the preparation method of choice. Subsequent fixation in FAA or in 10% formalin for one week produced satisfactory results. Both fixatives caused minimal morphometric changes. Although, one FAA-fixed specimen had some cuticular distortion at the anterior end of the body, the general appearance of most specimens was well preserved. Gentle heat treatment using water at 65°C produced no drastic morphological alterations, although there was a slight loss of contrast between the internal tissues. This killing technique is a practical method, especially for handling large quantities of specimens/samples. A slightly-increased granulation was evident in specimens fixed in 10% formalin, but this qualitative change could be minimized or prevented by adding calcium carbonate to the fixative (Hooper, 1970). This compound neutralizes any free formic acid in the material. TAF is another fixative worthy of consideration for morphometric studies on adults of this rhabditid nematode. Anterior structures were fairly well preserved in this fixative, but considerable shrinkage and distortion of cellular and tissue organization, especially in the caudal region, occurred in some specimens. There was, also a slight increase in granulation in 13 per cent of specimens (N = 15) that were treated with this fixative. This observation is contrary to expectation since the fixative contains triethan-

olamine which was expected to neutralize any free formic acid produced during the fixation process, thereby preventing any increase in granulation (Hooper, 1970). Perhaps, the observed granules were mere artifacts produced in the mature female parasites but it seems doubtful that excess free formic acid was formed in the materials, leading to incomplete neutralization by the available triethanolamine. Some morphological characters were fairly well preserved by fixation in FA 4:1 and Kale's fluid, but both fixatives caused enormous amounts of shrinkage. In addition, FA 4:1 produced significant tissue distortion, particularly in the posterior region of some specimens. Thus, both FA 4:1 and Kale's fluid are not suitable for morphometric studies of the adult stages of N. carpocapsae Weiser.

The results of this study demonstrated clearly that the different preparation methods had different effects on the nematodes. Obviously, the choice of method for employment in any investigation will depend on the specific aspects of the nematodes to be studied, and the size of sample(s) to be handled. Doubtlessly, the observations described in this study could offer useful hints for choosing suitable techniques.

4 PARASITE DEVELOPMENT AND REPRODUCTION

4.1 Introduction

The development of parasites within their hosts depends upon the availability of nutritional resources, among other factors such as defence reactions in the hosts. The amount of resources available to any individual parasite is a function of the number of parasites competing with it for the same finite resources (Kennedy, 1975; Anderson, 1982b). Hence, pressure on the limited resources increases proportionally to the parasite population within the host. Such intra-specific competition in host-parasite systems is extensively discussed in many textbooks (e.g. Kennedy, 1975, Hassell, 1976, 1978; Poinar, 1979; Gradwell et al. 1980; Anderson, 1982a; Cox, 1982) so that another detailed review is unnecessary. It is enough to say that competition has a wide range of consequences for the parasite, including reduction in individual fitness or performance (establishment, growth rate, egg production), alteration of sex ratios in some species, and increased mortality (Kennedy, 1975; Gradwell et al., 1980) and some of them are examined in this section.

Among mermithid nematodes, high mortality rates, plus increased formation of males of the mosquito parasite, Romanomermis culicivorax Ross and Smith, 1976 have been reported in multiply-infected hosts (Petersen and Willis, 1975; Westerdahl et al., 1982; Hominick and Tingley, 1984). A disadvantageous consequence for these biocontrol agents is

their possible extinction as the level of parasitism increases (Hominick and Tingley, 1984).

Recently, Golden and Riddle (1982) conducted a study of the density-dependent mechanisms regulating the larval development of Caenorhabditis elegans. They reported that a specific 'crowding' pheromone, as well as food supply influenced the formation of the dauer larval stages. Also, Schmiede (1964), Poinar (1967, 1979), Sandner and Stanuszek (1971, 1974), and, Danilov and Veremtschuk (1981) reported some effects of density-dependent constraints on the development and reproduction of some steinernematid parasites of insects. According to these authors, the maximum body size of the parasites and production of dauer larvae are influenced by crowding. Furthermore, Poinar (1979), citing Stanuszek's obscure publication reported size changes, in individuals of subpopulations of N. carpocapsae in relation to parasite density and food resources. All these studies used relatively large hosts, often late-instar Galleria larvae. This posed the question as to what type and degree of response might be elicited in a relatively small host such as the mosquito larvae. Therefore, the effects of increasing densities of nematodes in 3rd-instar A. aegypti on the morphometrics of adult worms, and on the production of dauer larvae was investigated.

4.2 Materials and methods

4.2.1 Parasite density and morphometrics of adult female N.

carpocapsae

Successive lots of 100 3rd-instar A. aegypti larvae were exposed to infective nematodes at a constant host:parasite ratio of 1:200, for a period of 4 hours. Exposures were conducted in 100ml of 24 hours old tap-water contained in 10cm diameter plastic dishes. During each exposure, a pinch of yeast extract was added into the container as feeding stimulant (Dadd, 1971b; Poinar, pers. comm.). After the exposures, larvae were separated from external infective nematodes by washing and rinsing with clean tap-water. They were placed singly in the individual wells of MWTC plates containing 2-3ml of water, and provided with powdered Tetramin/bread/yeast, before incubation at $25 \pm 1^{\circ}\text{C}$ and 12-hour light cycles.

The larvae were dissected in ringer's solution between 60 and 90 hours post-infection. Female parasites isolated from the larvae were heat-relaxed by immersing into distilled water at 65°C , for 3 seconds. Then, each worm was mounted in saline and measured using a camera lucida. The morphometric data for each of the parasite burdens were pooled together, before the results were analysed for correlation between the degree of crowding and female measurements using Spearman's rank correlation test.

4.2.2 Parasite density and dauer production

Infection and rearing procedures were the same as those outline above. Infected larvae were examined for parasites

starting 48 hours after infection. Larvae that contained both parasite sexes were selected for further investigations. To minimize variability of parasite densities caused by the presence of males, infected larvae that contained at most 3 males were selected for this study. Then, the development and reproduction of the parasites were monitored in vivo. Dauer larvae that emerged from the cadavers were removed and counted 2-3 times per week, until emergence was completed. These juveniles were stored together in 0.05% formalin solution in tap-water, at 5°C.

After emergence stopped, 30 infective juvenile nematodes were randomly selected from the stored suspension. They were heat-relaxed in 65°C distilled water and measured with a camera lucida, at x400 magnification.

4.3 Results

4.3.1 Distribution of N. carpocapsae

The distribution of adult N. carpocapsae (1st Generation) within the 5 subpopulations of A. aegypti larvae was clumped (Table 4.1), with k-values ranging from 0.109 to 3.022. Thus, the degree of aggregation of the parasites in each larval subpopulation was different. The majority of infected larvae harboured few parasites, while a few harboured large numbers of worms. The highest number of living parasites recorded in a host was 17. Differences between the observed and expected parasite numbers per host, when fitted to a negative binomial distribution were

Table 4.1 Data from 5 experiments in which 100,
3rd-instar A. aegypti larvae were exposed to
20,000 infective N. carpocapsae

	Experiment number				
	1	2	3	4	5
Number of					
parasites per					
host (range)	0 - 8	0 - 7	0 - 17	0 - 8	0 - 6
Mean ($\bar{x}$)	1.470	2.020	1.410	1.970	1.780
Variance (S^2)	4.939	3.010	10.992	3.221	2.517
($S^2/\bar{x}$)	3.360	1.490	7.796	1.635	1.414
Calculated χ^2	0.487	3.022	0.109	2.619	2.882
Chi-square for					
fit to negative					
binomial	1.939	10.416	6.854	17.831	12.80
degree of					
freedom	6	6	4	6	5
probability	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.01$	$p < 0.05$

significant ($p < 0.05$) for 2/5 experiments. Experiments 2, 4, and 5 had the largest chi-square values (i.e. deviated more from the negative binomial model). As the k-value increases, the dispersion approaches a Poisson (random) distribution with variance/mean ($s^2/\bar{x}$) close to 1. However, a chi-square test for a fit to a Poisson distribution showed significant departure ($p < 0.001$) from the expected frequencies for the numbers of parasites within the mosquitoes. The chi-square values were 24.3, 28.6 and 27.3, with 5 degrees of freedom each, for the 2nd, 4th and 5th experiments, respectively.

As expected from results of previous experiments (see Chapter 2.0) the numbers of melanized parasites decreased with an increase in the worm burden of the larvae. Thus, singly-infected larvae had more melanized parasites than multiply-infected ones. Apparently, male and female nematodes were randomly distributed in individual larvae, but no effort was made to document this observation.

4.3.2 Morphometrics of female parasites

The dimension of most characters of females (Table 4.2) were inversely correlated with density of the females in mosquito larvae. The widths at base of oesophagus and anus showed the best correlation with parasite numbers, while tail length, width of stoma, percent vulva, and ratio of excretory pore to pharynx were not correlated ($p > 0.05$) with the parasite burden of the hosts.

Generally, worms from lower parasite densities per

Table 4.2 Morphometrics of adult females of N. carpocapsae reared at different parasite densities in 3rd-instar A. aegypti larvae

Character	Number of female parasites per host											Cor-
	1 (N=17)	2 (N=18)	3 (N=21)	4 (N=8)	5 (N=10)	6 (N=10)	7 (N=12)	8 (N=8)	10 (N=20)	11 (N=11)	relation	
Total length (mm))	$\bar{x} + SD$ 4.15+ 1.16	$\bar{x} + SD$ 3.35+ 0.87	$\bar{x} + SD$ 3.08+ 0.60	$\bar{x} + SD$ 2.30+ 0.65	$\bar{x} + SD$ 3.36+ 0.97	$\bar{x} + SD$ 2.84+ 0.93	$\bar{x} + SD$ 2.37+ 0.43	$\bar{x} + SD$ 3.45+ 0.92	$\bar{x} + SD$ 1.94+ 0.32	$\bar{x} + SD$ 2.48+ 0.58	*	
Length of stoma	3 + 0	3 + 0	3 + 0	3 + 1	3 + 0	3 + 1	3 + 1	3 + 1	2 + 0	2 + 1	*	
Width of stoma	5 + 1	5 + 1	4 + 1	3 + 1	5 + 1	4 + 1	4 + 1	4 + 1	4 + 1	3 + 1	NS	
Length, Head to excretory pore	73 + 8	70 + 12	66 + 13	54 + 13	69 + 13	61 + 12	58 + 12	66 + 15	51 + 10	51 + 12	**	
Excretory pore/ pharynx ratio	0.31+ 0.04	0.30+ 0.06	0.30+ 0.06	0.28+ 0.05	0.29+ 0.04	0.32+ 0.05	0.28+ 0.05	0.30+ 0.07	0.20+ 0.05	0.27+ 0.04	NS	
Length, Head to nerve Ring	148 + 20	141 + 24	134 + 19	115 + 24	142 + 29	123 + 22	121 + 18	139 + 9	106 + 26	116 + 18	*	
Length Head to base of oesophagus	238 + 23	235 + 21	219 + 27	197 + 39	236 + 38	191 + 26	203 + 26	219 + 22	177 + 30	191 + 22	*	
Width at base of oesophagus	130 + 9	125 + 16	122 + 12	117 + 17	120 + 22	113 + 31	95 + 7	119 + 16	85 + 12	88 + 12	***	
Length, head to vulva	2.26+ 0.59	1.84+ 0.47	1.67+ 0.34	1.28+ 0.38	1.84+ 0.50	1.62+ 0.60	1.32+ 0.24	1.89+ 0.47	1.07+ 0.20	1.38+ 0.29	*	
Percent vulva	55 + 2	55 + 2	55 + 2	56 + 3	55 + 2	57 + 5	56 + 2	55 + 2	55 + 2	56 + 2	NS	
Width at vulva	156 + 24	153 + 30	147 + 16	133 + 15	145 + 31	151 + 30	126 + 5	149 + 17	121 + 14	116 + 23	**	
Width at anus	86 + 14	76 + 15	69 + 8	67 + 10	71 + 15	68 + 20	63 + 11	67 + 8	59 + 9	59 + 11	***	
Length of tail	49 + 11	43 + 12	41 + 8	43 + 5	48 + 8	40 + 8	39 + 7	44 + 6	39 + 7	42 + 8	NS	
a - value ²	28.6 +	24.0 +	22.7 +	18.2 +	25.0 +	21.2 +	21.4 +	25.6 +	18.8 +	24.2 +	NS	
	5.5	3.6	3.7	4.1	4.0	2.9	3.8	5.1	2.1	2.2		

Measurements to the nearest whole micrometre unless otherwise indicated

1 Spearman's Rank Correlation; NS, not significant; * p<0.05; **, p<0.01; ***, p<0.001.

2 de Man's index, a = total length/(widths at vulva and base of oesophagus/2).

host were more robust than those from more crowded conditions. However, the pattern of morphometric changes relative to increased crowding was not consistent. The trend was interrupted at female parasite burdens of 5 and 8, where the average measurements of most characters exceeded those of the immediately preceding parasite burdens (4 and 7, respectively). Nevertheless, the overall trend showed smaller sizes of most characters as the numbers of females per host increased.

4.3.3 Length and width of females

The body width (= average between width at base of oesophagus and that measured at vulva) was approximately linearly correlated with the length of female N. carpocapsae (Fig. 4.1). The mean widths expressed as proportions of the average lengths of the parasites from different burdens were between 3.5 and 5.4%.

A regression analysis of the data showed that width (μm) = $0.018 \text{ length} + 71.7$ when the parasite densities were disregarded (solid line in Fig. 4.1). This analysis gave a good correlation ($r^2 = 69.1\%$) between the dimensions of the 2 characters. The analysis showed a better correlation between worm length and width ($r^2 = 74.7\%$) when the densities of the parasites within the larvae were taken into consideration. In this case, the upper broken line in Fig 4.1 represents lower worm densities of 1-6 females per host, while the lower broken line is for higher burdens of 6-11 females. The 2

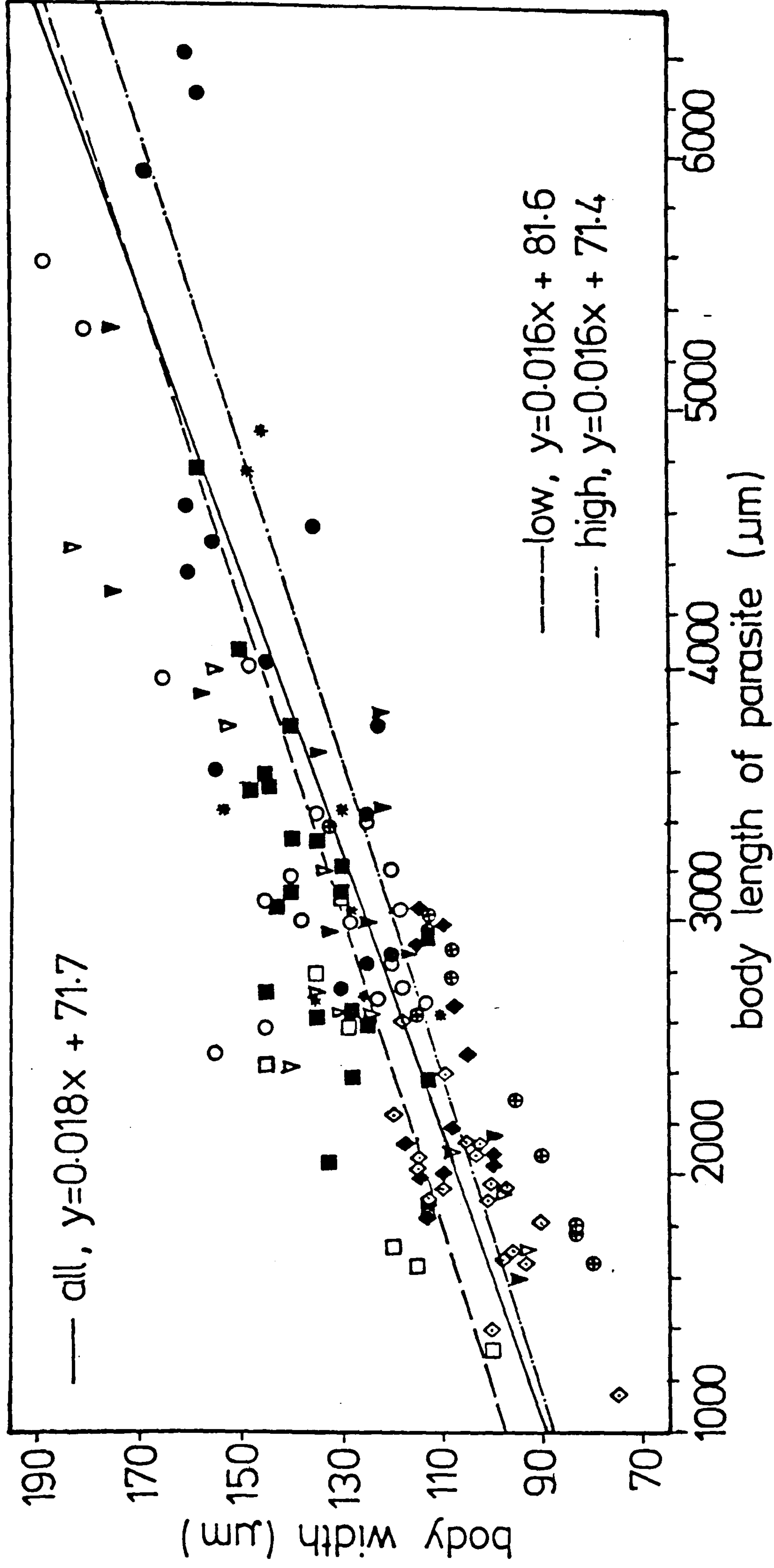


Figure 4.1 Relationship between body length and width of adult females of N. carpocapsae reared in 3rd-instar A. aegypti, at various parasite densities per larva, ($\bullet$ = 1, $\circ$ = 2, $\blacksquare$ = 3, $\square$ = 4, $\blacktriangledown$ = 5, $\triangledown$ = 6, $\blacklozenge$ = 7, $\ast$ = 8, $\diamond$ = 10, and $\oplus$ = 11 females/host; Low density = 1-6, high = 7-11 parasites per host).

lines are parallel, hence the dynamic relationships between worm lengths and widths for both lower and higher parasite densities of larvae, (expressed by their slopes), were similar. The respective equations for the 2 lines for lower and higher worm burdens are $\text{width } (\mu\text{m}) = 0.016 \text{ length} + 83.6$, and $\text{width } (\mu\text{m}) = 0.016 \text{ length} + 71.4$. Thus, for any given length of the parasites, the width of worms from lower densities exceeded that for those from higher burdens by a factor of about 12 micrometres.

None of the regression lines passed through the origin. Thus, the relationship between length and width can not be expressed as a simple ratio (de man's a-value). Nevertheless, the lines showed that the lengths of adult N. carpocapsae were strongly correlated, in a near linear manner, to their widths. The results indicate that the body volume decreased as the degree of crowding within mosquito larvae increased. Thus, the number of nematodes within individual mosquito larvae constituted a density-dependent constraint on female body size.

4.3.4 Production of infective-stages

female body

If Δ volume and fecundity are correlated, then the reproductive capacity of the females would also be density-dependent. Therefore, production of infective-stages after various degrees of crowding was assessed. As expected, the mean numbers of dauer-stages of N. carpocapsae produced per A. aegypti larva increased as the number of female parasites

per host increased, up to a density of 5 females (Table 4.3, and Fig.4.2a). Thereafter, dauer production declined sharply as the number of females increased within the larvae. A rank correlation analysis of the data revealed a significant ($p < 0.05$) decrease in numbers of dauers produced per host as the female worm burden of the larvae increased.

The numbers of infective nematodes produced per female showed an exponential decline with increase in parasite burden (Fig. 4.2b). Thus, each female at the optimum density (5 females) for dauer production per host, contributed fewer infective juveniles to the observed totals, than females that occurred singly within the larvae. Thus, there is a strong density-dependent constraint operating on the fecundity of these nematodes.

4.3.5 Measurements of infective juveniles

The size of infective juveniles from 3rd-instar A. aegypti was compared with that of individuals from late-instar G. mellonella larvae (Fig. 4.3). Dauers from mosquito larvae were significantly shorter ($\chi^2_{(1)} = 15, p < 0.001$) than those from moth larvae, but the average widths (measured at the position of the genital primordium) were not significantly different ($\chi^2_{(1)} = 0.267, p > 0.05$). There was little variation in size between individuals extracted from the same host species. Since dauers from mosquitoes came from hosts with 1-11 females, this suggests that the degree of crowding of females within A. aegypti larvae did not produce a signi-

Table 4.3 Infective-stage (Dauer) production of N. carpocapsae Weiser, at different female parasite densities in A. aegypti larvae

Female parasite burden of host	Number of hosts examined	Dauer larvae produced per host		Dauer larvae produced per female parasite	
		Mean + SD	Range	Mean + SD	
1	36	310 + 269	19 1,352	310 + 269	
2	23	365 + 223	98 953	183 + 112	
3	12	412 + 293	77 1,177	137 + 98	
4	7	448 + 257	127 898	112 + 64	
5	2	464 + 222	307 621	93 + 44	
6	8	158 + 177	22 546	26 + 30	
7	1	61		9	
8	1	72		9	
9	3	258 + 336	25 643	28 + 21	
10	1	62		6	
Correlation ¹		*		**	

¹ Significance of correlation between dauer production and female burden of host tested by Spearman's rank correlation test;
*, significant (p<0.05); **, highly significant (p<0.01).

Figure 4.2 Mean ($\pm$ SE) numbers of infective-stage of N. carpocapsae produced per host (a) and per female parasite (b) in A. aegypti larvae, at various parasite densities of the mosquitoes.

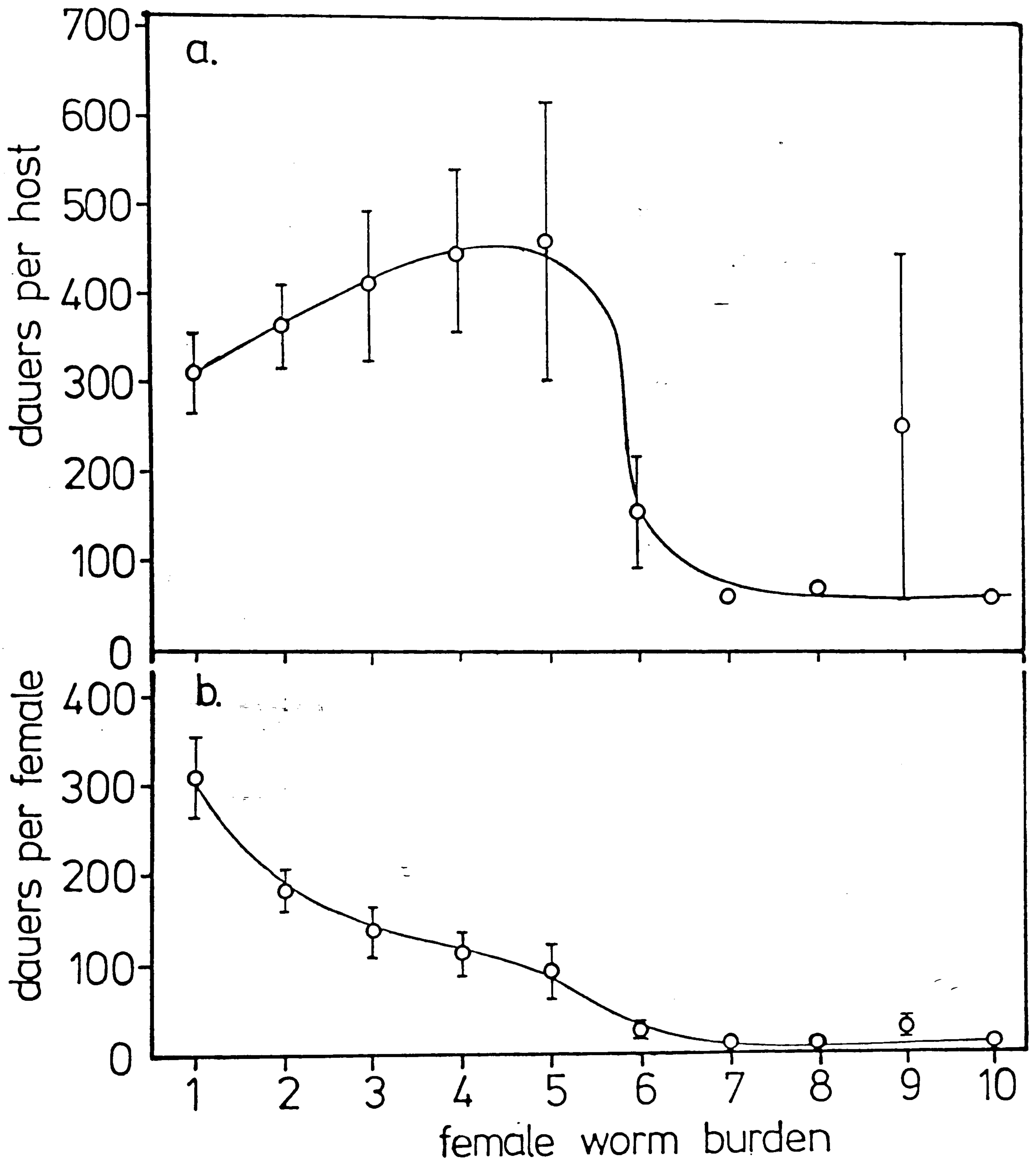
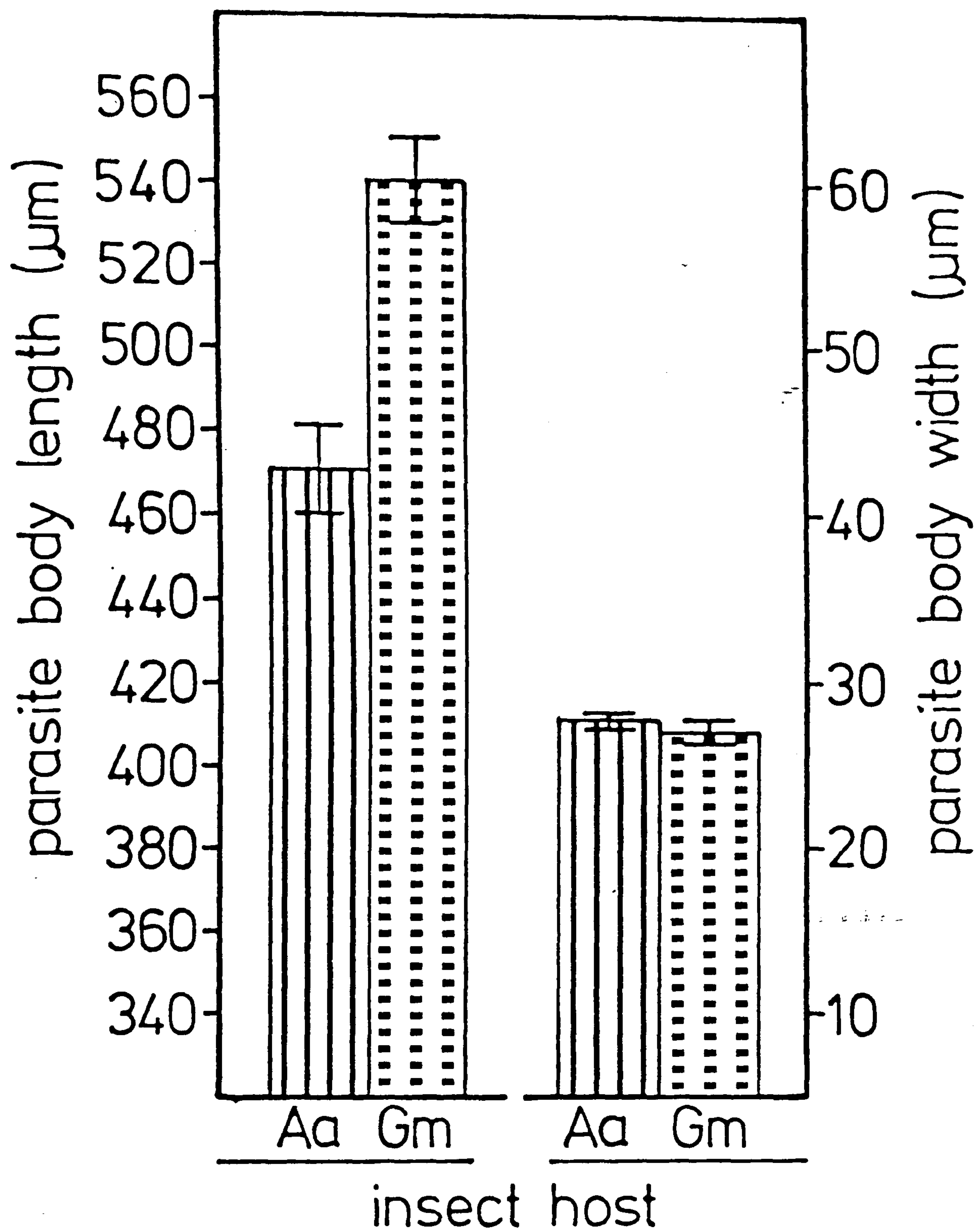


Figure 4.3 Measurements of 30 infective N. carpocapsae produced in A. aegypti (Aa) and 30 in G. mellonella (Gm) larva, at $25 \pm 1^{\circ}\text{C}$. (Bars represent standard error about the mean).



ficant effect on the size of dauer larvae.

4.4 Discussion

Investigations into the ability of potential biocontrol agents to reproduce within their target hosts are often incorporated into screening programmes. For these agents, the ability to replicate and recycle within the host population(s) is important for determining the frequency of inundative applications (Webster, 1980; Gaugler, 1981; Batra, 1982; Service, 1983; Hominick and Tingley, 1984). Thus, it is of some importance that the results of the present study confirmed and extended earlier reports by Welch and Bronskill (1962) and Molta (1982) on the reproduction of N. carpocapsae in A. aegypti larvae. The present results have shown, for the first time, that development and reproduction of the parasites were influenced by density-dependent constraints, in addition to a strong host defence reaction. Thus, although multiple-infection increased the probability of nematode survival of host defence reactions, it could retard parasite development and reproduction.

The measurements of adult females raised at different densities in the mosquitoes showed that some density-dependent constraints operated on growth and development of the parasites. There was an inverse correlation between the measurements of most characters and the parasite burdens of the hosts. This indicates that crowding and the associated intra-specific competition affected the parasite in a

density-dependent manner. Widths of the worms measured at the base of oesophagus and anus provided the strongest indication of density-dependent limitations on growth of the females. Since, the width of the stoma and length of the tail were unaffected, they are not indicators of the effects of crowding on the nematodes.

The results revealed a direct correlation between body length and width of the female parasites. Geraert (1979) observed a similar relationship between length and width in the rhabditids, Rhabditis teres Schneider, and Acrobeliodes nanus de Man. The measurements of these two characters, which determine body volume (Geraert, 1979), decreased as density of N. carpocapsae increased within the larvae. Hence, females from less crowded conditions had greater body volumes than those from higher densities per host. Among nematodes Geraert (1979) noted that a large body is utilized for the production of large numbers of eggs. He (Geraert, 1978a) observed a high correlation ($r = +0.984$) between the absolute measurements of body-width and female reproductive system for the tylenchid, D. destructor. A positive correlation between body size and female reproductive output is also observable among most other invertebrate parasites (Hoagland and Schad, 1978). Thus, the present observations imply that small females produced at high parasite burdens had a lower reproductive potential than those harboured at lower densities. This was confirmed when the number of dauers produced per female were counted at different parasite

densities. There was no apparent increase in mortality of the nematodes which could be attributed to increased density of these parasites. Hence, it is concluded that worm density influenced fecundity rather than survival of the parasites. This conclusion agrees with Anderson's (1982a) observation for most helminth parasites.

Geraert (1979) described three types of growth which produce large body volumes in nematodes. These include primary, secondary and tertiary growth processes. According to this worker primary growth is associated with excessive growth in width, producing short, thick females, while excessive growth in length occurs during secondary growth, producing long, thin females. During tertiary growth, proportional increases in length and width produce the same body proportions. Evidence from the present data supports the predominant occurrence of tertiary growth in females raised at both low (1-6) and high (7-11) parasite densities per host. However, there is a slight indication of secondary growth, since it was observed that the long females obtained from hosts with low parasite burdens were not as thick as expected from a strictly proportionate (absolute tertiary) growth. The observed relationships between length and width was probably necessitated by the maintenance of a vermiform shape for motility of the parasite, as suggested by Geraert (1979).

It is interesting that the production of infective N. carpocapsae decreased as the parasite burden of the

mosquitoes increased. This indicates the operation of a density-dependent constraint on the growth of the parasite population. Although, the absolute numbers of dauers produced per host increased as the female worm burden increased to the optimum density of 5 per host, maximum dauer production per female occurred in larvae with only one female, each. The dauer production per female declined as the degree of crowding of the adult worms increased. Sandner and Stanuszek (1971) also reported density-dependent limitations on the production of infective juveniles of N. carpocapsae in the waxmoth, G. mellonella. They observed that the density of first generation (giant) females of this nematode in pupae and late-instar larvae exerted a strong influence on production of the infectives, and noted that 50 females per host was optimum for dauer production from an individual host. Minimum production occurred with 200 females per host. Their data also indicate that maximum production per female occurred in hosts that harboured 10 females. Thus, intra-specific competition acted as an effective feedback (population control) mechanism for 10 or more females per host, but not at lower parasite densities. Also, overdispersion, which resulted in aggregation of the parasites in some individuals within the larval subpopulations, had advantageous effects on parasite reproduction and population growth when female densities were below and up to the optimum numbers per host. Above the optimum number, intensive competition^{for food and space} between the parasites significantly

reduced their reproductive outputs. Thus, aggregation of these nematodes above the optimum numbers per host constituted a disadvantageous influence for population growth.

The production of infective juveniles was unusually high at the density of 9 females per host. A reason for this exceptional occurrence might be that a few of the females commenced development earlier than the others in the same larvae. Consequently, the former suppressed the development of the latter. So, the numbers of infective juveniles recorded at this density may have been contributed by a few females. The extremely high standard deviation (± 336 juveniles) of the numbers of dauers produced at this female density of the larvae supports this argument.

The present study provides some insight into the statistical distribution of these nematodes in experimentally infected mosquitoes. Thus, although infection procedures were strictly controlled, an overdispersed or heterogeneous pattern of distribution occurred in most (3/5) experiments. Some experiments gave very low k -values, which indicates a high degree of aggregation of the parasites (Elliot, 1977; Anderson, 1982b). This clumped distribution is typical of most parasites in host populations (Kennedy, 1975; Anderson, 1980; 1982a,b; Anderson and Gordon, 1982). It indicates the variability within and between host-parasite systems. Overdispersion may arise from slight differences in behaviour and susceptibility of the host or from differences in infectivity and spatial distribution of the parasites (Anderson, 1982b).

Unfortunately, this distribution pattern does not indicate the exact cause of variability. Thus, the present pattern could have resulted from slight differences in susceptibility of individual larvae and/or the spatial distribution of the infective nematodes in the exposure containers. The significant departures from the negative binomial model observed in two experiments may be attributed to extreme variability in the performance of these nematodes against the larvae. Thus, in one of these experiments, the last 13 frequency classes had to be pooled for the chi-square test.

Measurements of body length and width of infective nematodes showed that the juveniles produced in A. aegypti larvae were significantly shorter, but not wider than dauers produced in Galleria larvae. The present measurements particularly those of juveniles raised in Galleria larvae, are similar to those recorded by Poinar (1967, 1979) and Sandner and Stanuszek (1974). The mean lengths differ slightly, but their ranges show varied degrees of overlap. This comparison shows that dauers produced in mosquito larvae were shorter than those from second generation females in Galleria larvae. The longest infective worms were those produced by giant first generation females in Galleria. These differences may be associated with differences in the infectivity of the dauer nematodes. Thus, Alikhan et al. (1985) reported higher infectivity of smaller juveniles of N. carpocapsae, after rearing in Sitophilus granarius compared to the larger infectives which emerged from Galleria larvae.

These authors predicted that the former would be viable for longer periods than the latter because of a more gradual development of catalytic (redox potential) activity in juveniles from S. granarius, compared to those from G. mellonella. Maximum activity was reached after 60 and 10 minutes, for juveniles from the two host species, respectively. Although interesting, their results must be interpreted with caution since replication and statistical analysis were inadequate. The implications of their report for the present results would be that infective N. carpocapsae from mosquito larvae would be more infectious and longer-lived, than those from Galleria larvae. This speculation requires supportive experimental data.

Sandner and Stanuszek (1974) observed a significant reduction in body size of third generation infective juveniles compared with the first and second, in Galleria larvae. They considered this reduction to have arisen from overcrowding of the parasites and the associated depletion of previously available food resources in the larvae. The further reduction in size of the juveniles produced in mosquito larvae was most probably in response to more severe overcrowding in the much smaller host. While discussing energy-size constraints on parasite reproduction, Hoagland and Schad (1978) remarked that the number of offspring can be maximized by a reduction in offspring size, alongside an increase in reproductive effort. Therefore, N. carpocapsae may have achieved greater numbers of dauers, for higher

success of transmission, by reducing the sizes of the juveniles while responding to density-dependent constraints in the mosquito larvae.

It is not surprising that few second generation juveniles of N. carpocapsae died within the mosquitoes in which reproduction occurred. These juveniles rapidly developed into dauers, rather than second generation adults, and emerged into the surrounding fluid, in as early as 48 hours of dauer formation. Poinar (1979) remarked that the dauer stage is not obligatory, but forms in response to unfavourable environmental conditions. He added that these juveniles are resistant to various environmental stresses. Thus, by forming dauers and emerging out of the host cadavers, these juveniles avoided the risks of mortality due to overcrowding. By contrast, Sandner and Stanuszek (1971) reported substantial mortality of juvenile N. carpocapsae in larvae and pupae of G. mellonella and imagines of L. decemlineata under crowded conditions within the hosts. They attributed this mortality principally, to oxygen deficiency within the cadavers.

Apart from the question of whether mosquito cadavers will survive for the minimum 7 days (for parasite development and reproduction) in the natural habitat with predators, scavengers, and water currents, it is apparent that recycling of the rhabditoids will be hampered by larval defence reactions and density-dependent constraints. Thus, the control of mosquitoes using these nematodes, even in the most

favourable environment for the parasites, will depend upon repeated application of the agents.

5 GENERAL DISCUSSION

The potential for biological control of *culicine* mosquito larvae with rhabditoids has been discussed by several workers on entomophilic nematodes (e.g. Welch and Bronskill, 1962; Briand and Welch, 1963; Webster, 1973; Gaugler, 1981; Finney and Harding, 1981; Poinar and Kaul, 1982). Generally, they agree that the aquatic habitat of the pests, unlike the terrestrial environment, provides nearly optimal conditions for the survival of the invasive nematodes. However, few experimental studies (e.g. Dadd, 1971b; Finney and Harding, 1981) have been conducted into the factors that might affect the interactions between the nematodes and the mosquitoes. Hence, this question was addressed during the present study. Since, the subject of host-parasite interactions is complex and multifactorial, the scientific approach of studying one factor at a time (Webster, 1980) was adopted to evaluate the contribution of each component of the interactions.

Much of the present work demonstrated interspecific difference in the effectiveness of the heterorhabditids and neoaplectanids used against the mosquitoes. The most important differences were exhibited in the uptake and entry of the different parasite species, and in the level of parasite-induced larval mortality. This is the first assessment of its kind carried out on this host-parasite system. However, studies by Bedding et al. (1983), Molyneux et al. (1983), and Molyneux and Bedding (1984) have also demonstrated inter-specific differences in infectivity between various

rhabditoids for specific soil-inhabiting insects. Their results support the conclusion from the present study, that the heterorhabditids are generally more efficacious than the neoaplectanids. It is implicit from these results that there is a need for extensive screening of a wide range of nematodes to find an appropriate candidate for application against mosquitoes. This is a pre-requisite for the full realization of their potential.

It is remarkable that a large number of pre-parasites are ingested by the mosquitoes, but only a few manage to penetrate into the haemocoel. The considerable loss of nematodes was attributed to damage or injury to the invasive worms caused by larval mouthparts. This conclusion was also reached by Dadd (1971b), Poinar and Kaul (1982) and Gaugler et al. (1983), working with various rhabditoids against mosquitoes and blackflies. However, they did not provide a scale of the loss to the parasite population. Thus, ingestion of pre-parasitic nematodes by mosquito larvae does not guarantee infection. Together with the knowledge that the larval instars are differentially susceptible to the nematodes (Dadd, 1971b; Molta, 1982), these results preclude the use of entomophilic rhabditoids as a single control agent for culicine mosquitoes.

It seems, therefore, that the prospect for the utilization of these nematodes and their associated bacteria against mosquitoes lies in their integration with other control agents. The presence of two pathogens could have synergistic effects

on an insect population (Webster, 1973). For example, laboratory experiments conducted by Lam and Webster (1972) showed that the combination of N. carpocapsae and B. thuringiensis var. thuringiensis improved the performance of the nematodes alone against leatherjacket (Tipula peludosa) larvae. Possible candidates for an integrated approach include fungal (Culicinomyces, Coelomomyces, Lagenidium, Metarhizium), bacterial (Bacillus thuringiensis, B. sphaericus) and nematode (Romanomermis sp.) pathogens of mosquitoes (Chapman, 1974; Laird et al., 1981; Service, 1981, 1983; Washino, 1981). A possible innovation would be the application of infective rhabditoids as carriers of larval stomach/gut poisons, apart from being entomopathogens in their own right. Also, infective-stages of N. carpocapsae have demonstrated considerable resistance to some insecticides and carbamide herbicides (Fedorko et al., 1971a,b,c). Thus, there is a need to conduct compatibility studies of rhabditoid nematodes with effective chemical larvicides (e.g. temephos) and insect growth regulators (e.g. methoprene, diflubenzuron).

The physiology of an insect especially defence reactions, may decrease the effectiveness of nematodes as biological control agents (Webster, 1973; Bedding et al., 1983). The present results, and those of other workers (e.g. Welch and Bronskill, 1962; Dadd, 1971b; Poinar and Kaul, 1982) provide ample evidence that mosquito larvae are capable of mounting strong defence reactions against invasive rhabditoids. The

mechanisms involve encapsulation, melanization (Welch and Bronskill, 1962; Bronskill, 1962; Andreadis and Hall, 1976; Beresky and Hall, 1977) and possibly humoral defence reactions (Poinar and Leutenegger, 1971). Single nematode infections usually resulted in parasite mortality caused by these reactions, although the host also usually died. While, multiple infection increased survival of the parasites in the haemocoel, it was shown for the first time, that development and reproduction of the nematodes was influenced by density-dependent factors. These findings, added to other ecological considerations, reduce the possibility of recycling of the rhabditoids in the aquatic environment. Briand and Welch (1963) came to the same conclusion based on their studies of host reactions against the nematodes. Therefore, the use of rhabditoid nematodes against mosquitoes would depend on inundative application of these agents. This, in turn, depends on a cheap production system. Fortunately, Bedding (1981, 1984) developed an in-vitro technique that meets these requirements. This technique enables the production of 50,000 million infective nematodes per person/week, at a cost of less than 1cent (Australian) per million (Bedding, 1984). Although, progress has been made towards developing reliable transportation and storage methods (Dutky et al., 1964; Bedding, 1981, 1984; Wouts, 1981), the shelf-life of these nematodes under different field conditions remains to be established.

The correct identification and classification of any

nematode depends on an accurate description from specimens prepared by standard methods. Thus, the need for a reliable technique for processing specimens for microscopical examination can not be over-emphasized. This need arises because of the considerable variability within and between populations of these nematodes (Turco et al., 1971; Poinar, 1979; Wouts et al., 1982). As documented in this work, different preparation methods showed different quantitative and qualitative effects on the nematodes. Hence, the existing confusion in the taxonomy of these nematodes (Poinar, 1979; 1984; Wouts et al., 1982) might be resolved, or at least minimized, by standardization of rearing and preparation techniques.

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APPENDIX 1: Infection of Aedes aegypti L. (3rd-instar)

larvae exposed for 1 hour to infective

H. heliothidis and N. carpocapsae,

(Host-parasite ratio = 1:200).

Experiments were conducted to determine whether ingestion of Neoaplectana carpocapsae Weiser, and Heterorhabditis heliothidis by 3rd-instar A. aegypti larvae always led to infection by the entomopathogens. In each experiment, 2 groups of 15 larvae each were exposed nematodes at a host:parasite ratio of 1:200, for 1 hour. Then, larvae in one of the 2 groups were killed in Baker's formaldehyde-calcium and dissected in Ringer's solution. Gut contents and the haemocoel of the larvae were examined for nematodes or parts of nematodes, under a stereoscope and an interference contrast microscope. The second group of larvae exposed to each parasite species was separated from the pre-parasites and kept for a period of 48 hours post-exposure, as described in Section 2.1 of Chapter 2.0.

The results (Table 1 below) showed that 100 and 97.7% of larvae ingested H. heliothidis and N. carpocapsae respectively, although only half the larvae died of infection. There was a decrease in larval mortality as the numbers of larvae with pieces of cuticles, but without undamaged nematodes, increased. Thus, the greater the rate of worm damage, the lower the levels of parasitism by both rhabditoids. Nematodes were probably injured by larval mouthparts during

Appendix 1 - contd

ingestion as suggested by Dadd (1971b), Poinar and Kaul (1982) and Gaugler et al. (1983) for various experimental hosts. Since, percent mortality caused by the parasites was much less than the proportion of larvae confirmed to have ingested nematodes, it is inferred that ingestion of the rhabditoids by A. aegypti larvae does not guarantee infection.

Table 1: Infection of Aedes aegypti L. (3rd-instar) larvae exposed for 1 hour to infective H. heliothidis and N. carpocapsae.
(Host-parasite ratio = 1:200)

Mosquito larvae (n = 15)	Exposures to infective					
	<u>H. heliothidis</u>			<u>N. carpocapsae</u>		
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
No. with nematodes* in gut/haemocele	15	15	15	15	14	15
No. with undamaged nematodes in gut	13	8	2	15	9	1
No. with pieces of worm cuticles only.	2	5	11	0	4	13
No. with parasites in haemocoel	8	4	3	11	5	1
Mortality ⁺ caused by infection	11	7	4	12	10	1
Unaccountable mortality	0	0	0	0	2	0

Footnote: *, damaged and undamaged/intact nematodes
+, mortality assessed 48 hours post-exposure