BIONOMICS OF BRITISH STRAINS OF ENTOMOPATHOGENIC NEMATODES (STEINERNEMATIDAE)

by

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ABSTRACT

A survey of British soil resulted in the isolation of many Steinernema populations. Identification of some of the isolates was carrried out. Detailed studies then concentrated on a Tasmanian and two British strains of Steinernema bibionis Bovien.

Morphometric and hybridization studies revealed that both British isolates belong to the species Steinernema bibionis Bovien 1937 (Poinar, 1979). Characteristics of the colonies of the symbiotic bacteria isolated from the two native nematodes assisted the identification.

A number of samplings in $100m^2$ of grassland was carried out to examine the horizontal and vertical distribution of a natural population of S. bibionis (Nashes) in Silwood Park. High numbers of nematodes appeared in a few sites and low numbers or none in others, showing an aggregated pattern common to other soil organisms. Most of the nematodes were found in the top layers of the soil (0-5cm). The numbers declined sharply with increasinig depth, so that few nematodes were present at a depth of 15-20cm.

Fluctuations in the nematode numbers recovered from soil samples taken at different times were observed. However, whether a seasonal effect is involved could not be determined because of the variability in the data. It is hypothesised that the nematode population under investigation may not differ greatly at each season.

Comparison of the Tasmanian strain (T319) and the two British strains (Site 42 and Nashes) of S. bibionis with respect to their infectivity, reproduction and emergence at various temperatures revealed that the Nashes strain favoured cooler temperatures while the Site 42 strain preferred warmer ones in the range of 5 - $30^{o}C$. The numbers of nematodes that established in hosts were used to assess infectivity, instead of using percent mortality as most other workers have done.

Comparison of the two British strains with the T319 strain also revealed that there were other differences in their biological attributes. The Nashes nematodes were superior in their ability to infect, reproduce and even emerge at temperatures as low as 5°C, while the Site 42 nematodes were superior in their capacity to produce large numbers of infective juveniles over a wide range of temperatures.

Studies on the survival of the two British strains in sand at 5°C in the absence of insect hosts showed that the low temperature greatly increased survival of the infective juveniles in sand. Against the expectation of a declining trend for infectivity over time, both strains showed a U-shaped pattern in their infectivity for late instar larvae of Galleria mellonella (L.) after storage in sand at 5°C. The mechanism is unknown, but may be related to low temperature.

There was a direct relationship between the number of nematodes that could be recovered from Galleria cadavers and the number of nematodes available in the habitat. However, the proportion of the nematode population that established in the insects (the level of infection) was independent of dose and ranged between 30 and 40% under optimal conditions. Based on this consistent proportion, the number of nematodes in a soil sample would be estimated as N/P, where N = the mean number of nematodes that established in Galleria cadavers after they were exposed to nematode infection consecutively at least twice and P = 0.3 - 0.4.

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CHAPTER 1

INTRODUCTION

Nematodes of the rhabditid families Steinernematidae and Heterorhabditidae are among the most extensively studied groups of insect pathogenic nematodes. They contain some of the most promising nematode species for biological control of pest insects (Poinar, 1979). Members of these families are characterized by having a non-feeding, free-living (usually soil-dwelling), infective stage juvenile (dauer larva) and a symbiotic association with a specific bacterium (Xenorhabdus spp.) monoxenically in the venticular region of the intestine (Akhurst, 1980; Bird and Akhurst, 1983; Poinar, 1979). The infectives are attracted to insects (Bedding and Akhurst, 1975; Byers and Poinar, 1982; Gaugler et al., 1980; Schmidt and All, 1978, 1979) and invade via the mouth, anus, and/or spiracles (Poinar, 1979) and in Heterorhabditis species, via intersegmental membranes as well (Bedding and Molyneux, 1982). Following host penetration, the infective stage nematodes move to the haemocoel where the bacterium is released. The bacterium proliferates, suppressing the multiplication of other microorganisms, causing death of the host by septicaemia and establishing suitable conditions for nematode growth and reproduction. After about two weeks, thousands of infective juvenile nematodes emerge from the host cadaver into the soil, where they might start another infection (Poinar, 1979).

Interest in entomogenous nematodes, especially steinernematids and heterorhabditidis as biological control agents of agricultural and forest pests in recent years is demonstrated by the number of review articles (e.g. Finney, 1981; Gaugler, 1981; Kaya, 1985; Poinar, 1986; Webster,

1980). Because of their broad host range (Poinar, 1979) and non-adverse effects on the environment (Obendorf et al., 1983), they have considerable potential for use as biological control organisms. In nature, these nematodes have been isolated from most continents and steinernematids have been found in soil-inhabiting insects, in insects on tree trunks and free-living in soil (Kaya, 1985). Their wide geographical distribution may provide considerable opportunities to obtain new species or strains differing in virulence, infectivity and physical tolerance to environmental limiting factors which usually narrow the effective host range in the field.

The taxonomy of entomopathogenic rhabditids is not settled and many controversies have arisen. Only the genus Steinernema has been studied in detail (Wouts, et al., 1982) and the results are not universally accepted (Poinar, 1984). There are many problems at both the generic and the specific level. A detailed review on the taxonomic status of the species in the genus Steinernema is presented in Chapter 3.

At the generic level, the classification within the family Heterohabditidae is settled as there is only the genus Heterorhabditis Poinar (1979). There are four species currently described. H. bacteriophora Poinar, 1976; H. heliothidis (Khan, Brooks and Hirschmann, 1976) Poinar, Thomas and Hess, 1977; H. hoptha (Turco) 1970; H. hambletoni (Pereira) 1937. However, the taxonomy at this level is difficult because the first generation is hermaphroditic. Hence, it is impossible to confirm species identification by conventional cross-breeding techniques. Studies using DNA analysis techniques (Curran and Webster, 1989) and isozyme analysis (Akhurst, 1987) will hopefully resolve this problem.

Each Heterorhabditis and Steinernema species has been found mutualistically associated with a bacterial species belonging to the genus Xenorhabdus which was erected in the family Enterobacteriaceae (Akhurst, 1982a, 1983a; Thomas and Poinar, 1979). These bacteria are carried monoxenically within the ventricular portion of the intestine of infective-stage juveniles (Akhurst, 1982a, Poinar, 1966; Poinar and Leutenegger, 1968). In Steinernema spp., the bacteria are restricted to an intestinal vesicle (Bird and Akhust, 1983). In Heterorhabditis, the bacterial cells have been found to be in the ventricular portion of the intestine (Poinar et al., 1977) or in the anterior portion of the intestine (Wouts, 1979). The specificity of the nematode/bacterium association has been discussed by Akhurst (1983b, 1986a).

The significance of the nematode/bacterium association has been demonstrated by the number of papers dealing with the associations (e.g. Akhurst, 1980, 1982b; Götz et al., 1981; Poinar, 1966, 1972, 1979; Poinar and Thomas, 1966). The symbiotic bacterium has been shown to be able to inhibit growth of other bacteria and establish favourable conditions for nematode reproduction (Akhurst, 1982b; Poinar and Thomas, 1966). However, Jarosz (1986) doubted the inhibitory role of the bacterium because he found that Xenorhabdus spp. had a low biostatic activity and narrow spectrum of antibacterial action. On the other hand, Couche and Gregson (1986) isolated metabolites from Xenorhabdus in vitro cultures which had antibiotic activity. An endotoxin was also thought to be detected (Sandner et al., 1977, in Akhurst, 1986b). It still remains to be proven whether Xenorhabdus spp. produce toxins.

There are phase variations for Xenorhabdus bacteria. This affects growth and reproduction of the nematodes, because the primary phase

provides better growth conditions for the nematodes than does the secondary phase (Akhurst, 1980). The cause of the variation is still unknown. However, the shift from primary to secondary form occurs frequently, especially in vitro. A shift from secondary to primary form has not been found.

Entomopathogenic rhabditids attack a wide range of insects from many orders (Poinar, 1979; 1986). This broad spectrum has been determined mainly from filter paper tests in the laboratory. The feasibility of using steinernematids and heterorhabditids for biological control of insect pests has been tested in many field trials, mainly with S. feltiae strains. Much of the early research concerning field applications has been summarized by Benham and Poinar (1973), Gaugler (1981) and Poinar (1979).

Most of the attempts to control field populations of insect pests with rhabditid nematodes gave inconsistent results. Possible explanations for the failures have been presented by a few authors. Gaugler (1981) pointed out that the improper choice of target insect would account for many failures. For example, the poor or negative results for controlling foliage-feeding insects with steinernematids have been attributed to desiccation, which reduces nematode activity severely (Bedding, 1976; Kaya, 1986; Webster and Bronskill, 1968; Welch and Briand, 1961). Also, solar radiation has been shown to contribute to low host mortality (Gaugler and Boush, 1978). Temperature extremes are another limiting factor for the use of the nematodes on foliage (Kaya, 1986).

Soil inhabiting insects are considered logical targets for control by steinernematids and heterorhabditids. Although using nematodes against these insects has been advocated and results are sometimes

encouraging, success has not been consistent (Lewis and Raun, 1978). This emphasizes the importance of thoroughly studying environmental factors that influence the survival and infectivity of the nematodes in soil.

Once the infective juveniles leave their host cadavers, they will be subjected to various environmental stresses, including biotic and abiotic factors. Until recently, most observations on survival of these nematodes in soil were recorded during field trials (Georgis and Haque, 1981; Glaser and Farnell, 1935; Harlan et al., 1971; Hoy, 1954; Saunders and All, 1982; Welch, 1965), where conditions were not controlled, so that effects of specific environmental factors could not be determined. This led to carefully controlled laboratory experiments which examinied the survival of infective juveniles in soil, especially in relation to soil temperature, moisture and over time, in the absence of hosts (Gray and Johnson, 1983; Ishibashi and Kondo, 1986a; Molyneux, 1985). The influences of soil type, moisture and temperature on the behaviour of the infective juvenile nematodes in the presence or absence of a range of insect hosts were documented in a notable laboratory study by Molyneux (1983). The details of the influence of the environment on these nematodes will be reviewed separately in Chapters 4, 5 and 7.

Fully understanding the effectiveness of entomopathogenic rhabditids for biological control depends on a knowledge of their biological and ecological attributes. Distribution of the nematodes in the field is one of the ecological attributes which should be considered. Entomopathogenic nematodes of the family Steinernematidae were first discovered in the European ecosystem in 1923 (Poinar, 1979). Since then, numerous insect species have been found to be naturally infected with steiner-

nematids, though at low prevalence (Poinar, 1979). Since Bedding and Akhurst (1975) developed the Galleria trap for isolating entomopathogenic rhabditids from soil samples, the geographical distribution of steinernematids and heterorhabditids has been investigated in various countries (Akhurst and Brooks, 1984; Akhurst and Bedding, 1986; Beavers et al., 1983; Burman et al., 1986; Hominick and Briscoe, in prep.; Mráček, 1980, 1982a; Poinar, 1979). The baiting technique has shown that these nematodes are widely distributed in Britain (Hominick and Briscoe, in prep.), in Czechoslovakia (Mráček, 1980), in cool, temperate and tropical regions of Australia (Akhurst and Bedding, 1986), in North Carolina (Akhurst and Brooks, 1984), in Florida (Beavers et al., 1983) and in Sweden (Burman et al., 1986). The relationship between habitat type and distribution of the nematodes was also investigated. Authors from different countries found the nematodes favoured different habitats. They attributed the differences in distribution to the distribution of suitable host insects in various countries, to the species of nematodes found and perhaps to soil types.

Most studies on the geographical distribution of entompathogenic rhabditid nematodes are qualitative surveys. Sometimes, the abundance of infective juveniles in soil samples was inferred from the insect mortality observed in the bioassay. However, the objectives of such surveys were to discover and isolate new species or strains of the nematodes rather than to quantify nematode populations in the field. Reliable quantitative information on nematode populations must be obtained from studies over a small area in relation to horizontal and vertical planes, as well as over time.

A great deal of work has been done on estimating the numbers and distribution of plant-parasitic nematodes and soil-inhabiting free-living nematodes. Yeates (1981) presented an excellent review of the work, with special emphasis on nematode populations in relation to soil environmental factors. However, from the literature to date, it appears that the distribution of entomopathogenic rhabditids has not been extensively studied. An estimate of the population size in the field is even more rare either by a one-time inventory or a time series of measurements to determine patterns of change and response to various environmental influences. One exception was the study by Bednarek and Mráček (1986). They reported their results on quantification of Steinernema infective juveniles in eight habitats in Poland by analysis of the intensity of infections in G. mellonella larvae in the Galleria trap. However, they extrapolated the results from small samples of soil and a few bioassays to cover extensive areas. This unrealistic procedure is discussed in Chapter 4.

To achieve an estimate of size of a nematode population, selection of an appropriate extracting method for the types of nematode anticipated is important (Goodwell, 1982). A wide range of extraction methods is available for populative studies of soil nematodes and each has its biases and efficiency (Southey, 1985). Whatever extraction method is used, there is always the problem of assessing whether or not the nematodes are active in the soil at the sampling time (Yeates, 1981). The "Whitehead tray" technique (Whitehead and Hemming, 1965), which was derived from the Baermann funnel method, has been used to extract entomopathogenic rhabditids from soil (Poinar and Hom, 1986; Renn, 1985). Since these nematodes have a dauerlarva stage in soil, their similarity

to other soil-dwelling rhabditids makes their direct identification difficult. Even if the entomopathogenic rhabditids are extracted, it is still necessary to show that the extracted nematodes are able to infect insects. Therefore, the Galleria baiting method, developed by Bedding and Akhurst (1975), for detecting entomopathogenic rhabditids in a soil sample has been widely used. Though rarely done, the number of nematodes which have penetrated and developed inside a G. mellonella larva can be determined by exposing the insects to infective juveniles, allowing some time for the growth of the invaded nematodes and dissecting to reveal the numbers (Johnson, 1986, unpublished). Counts of the nematodes which had entered after a post-mortem examination of the dead larvae provides a means of estimating the nematode population in the soil sample. However, large numbers of dissections are involved. Perhaps for this reason, few such quantitative works have been conducted. The vast majority of authors simply assess the percent of larvae killed in a bioassay and assume that it is related to the nematode population. As will be shown in Appendix II, this assumption is unsatisfactory for studies of population dynamics.

A recent survey of British soil using Bedding and Akhurst's Galleria trap method (Hominick and Briscoe, in prep.) resulted in the isolation of nematodes later identified as S. bibionis from 197/403 sites sampled. This species has never been documented in Britain and therefore its biology has never been studied. This thesis concerns identification of some of the nematodes, their distribution in a small area of grassland and evaluation of the effect of temperature on their infectivity, reproduction and survival.

CHAPTER 2

GENERAL METHODS AND MATERIALS

2.1 G. mellonella CULTURE

Cultures were maintained using methods modified from Poinar (1979). Larvae were reared on an artificial diet consisting of 45g of molten beeswax, 225g of honey, 90g dried brewer's yeast, 306g of middlings and 157ml of glycerol. They were contained in sandwich boxes (27.5 x 15.5 x 8.5cm) on one receipe of diet and kept in a 25°C incubator. Some cultures were at 30°C to accelerate growth and development. The moths were netted and placed in boxes with fresh diet to allow eggs to be laid.

2.2 NEMATODE CULTURES

The T319 strain of Steinernema bibionis was isolated from the slopes of Mt Wellington near Hobart, Tasmania in 1978. This strain was isolated by the method of Bedding and Akhurst (1975) using Galleria larvae as bait. The climate in Hobart is temperate, a little milder than would be expected from its latitude because of the ameliorating effect of the sea. Mt Wellington rises more or less directly from the sea to 1300m. The isolation site of T319 is about 700m above sea level and therefore a few degrees colder than Hobart. This strain is of particular interest because it is active at lower temperatures than other Tasmanian isolates of S. biobionis. It has been cultured routinely in Galleria larvae and in vitro (Bedding's methods) at 23°C (Akhurst, pers. comm.).

Two other strains of the same species were isolated from British soil using Bedding and Akhurst's Galleria trap method (Table 3.1). All nematodes were reared in vivo in G. mellonella larvae. Late instar

larvae were exposed individually to infective juvenile nematodes in sterilized sand in 30ml universal tubes at a rate of 20 - 40 infectives per larvae at 15 - 20°C. Dauerlarvae were extracted two weeks later in tap water according to the methods described by Poinar (1979), based on White's technique (namely "White trap"). Infective juvenile nematodes were cleaned by allowing them to pass through two layers of Kleenex tissue lining in a funnel. They were stored in 2cm deep water in 250ml Pyrex conical flasks at 10°C as stocks. Clean tap water was added monthly to replace the old.

2.3 CONTROLLED INFECTION OF WAX MOTH LARVAE

This method was based on that of Molyneux (1985) with some modifications. Approximately 25ml of clean sand (previously autoclaved and oven-dried, then passed through a 1.18mm aperture sieve) was mixed with 1ml of tap water and then placed in a plastic universal tube (diameter 2.2cm, height 8.0cm). Infective nematodes in 1ml of water were introduced into a centrally-made hole (0.5cm in diameter, 4.0cm deep), which was then obliterated by shaking the tube. Addition of a total of 2ml of water to 25ml of dry sand made the water content 8% (v/v). A Galleria larva was placed on the sand surface, the lid was screwed and the tube inverted. Dosages of 1-10 nematodes were individually counted while dosages greater than 10 were estimated by a dilution technique. After the required time, the tubes were examined for dead insects.

2.4 DILUTION OF NEMATODE SUSPENSION AND COUNTING

For nematode counting, the suspension was diluted in a 250ml conical flask and mixed by bubbling air from a Mystere "twin" air pump

for 5 minutes. The nematodes in 3 or 5 1ml aliquots were counted in a dish marked with a grid. The average number of nematodes per aliquot was calculated and used to estimate the total number of nematodes in the suspension or the volume of suspension required to give specific dosages.

2.5 DISSECTION OF *G. mellonella* CADAVERS

The *G. mellonella* cadavers recovered from soil or sand were cleaned in three rinses with tap water. Dissections were in Taylor and Baker's (1978) Ringer solution (NaCl 6.75g, KCl 0.09g, $CaCl_2$ 0.115g and $NaHCO_3$ 0.215g in 1 litre of distilled water). Each cadaver was dissected in Ringer's solution in a plastic dish (2.5cm in diameter) and left for at least 30 minutes to allow the nematodes to emerge into the saline. After careful examination of insect tissues for possible trapped nematodes, the nematodes present were counted and the numbers were recorded. Males and females were separately recorded whenever possible.

2.6 EXTRACTION OF JUVENILES FROM SAND

Two methods of extraction were used to recover nematodes from sand:

1. Modification of Whitehead and Hemming's (1965) method (Fig.2.1).

The small amounts of sand used in the controlled infections required special 'mini' extraction procedures. No.11 petri dishes (9.5cm in diameter) with lids were the basic materials. The centre of the dish was removed and the outer rim was punctured with a number of holes by melting the plastic with heated steel tubing. A nylon mesh (aperture 0.09cm) was glued to the bottom of the dish with Bostic general purpose glue. This was used as the inner basket required for the Whitehead tray

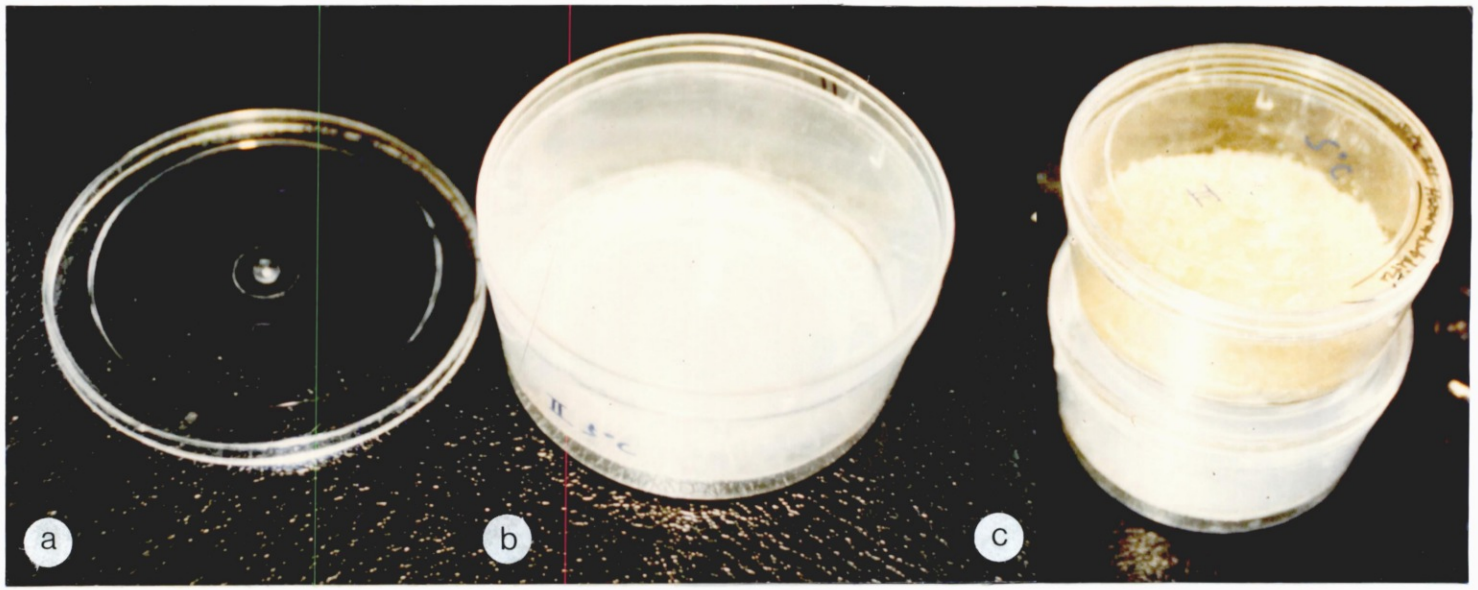


PLATE 2.1 Modified Whitehead and Hemming sieves for extracting insect - parasitic rabditids from sand. a: lid; b: empty sieve; c: working sieve with sand.

method. In use, the meshed dish containing one layer of a circular piece of kimwipe tissue, was suspended inside a similar dish with an intact bottom. The sand was evenly distributed on the tissue paper and then 25ml of tap water was poured into the bottom dish, so that the surface of the water just touched the tissue. The lid was then placed on the top dish to prevent excess evaporation. The unit was undisturbed and left for 24 hours in a $20^{o}C$ incubator. To count the nematodes, the bottom dish was replaced by another containing clean tap water. The contents were poured into a counting dish and the numbers were recorded.

2. Modification of centrifugal flotation method.

This method was adapted from that described by Hooper (in Southey, 1986). Granular cane sugar was used to make a solution of sp. gr. 1.18. When required, 50ml of the solution were added to a 300ml beaker containing the sand (25ml) to be extracted. The contents were shaken by hand for 2 minutes. After sitting for a few seconds, the clean supernatant was poured into another beaker containing 250ml of tap water. The nematodes were allowed to settle for at least two hours and then the excess water was slowly poured off. After settling, this was searched for nematodes and the numbers were recorded.

CHAPTER 3

ISOLATION AND IDENTIFICATION OF THE ENTOMOPATHOGENIC NEMATODES USED IN THIS STUDY

3.1 INTRODUCTION

The simple method of Bedding and Akhurst (1975) for detecting and isolating entomopathogenic nematodes from soil samples has enabled workers all over the world to collect new strains/species which can be evaluated for their use against pest insects (Akhurst and Bedding, 1986; Akhurst and Brooks, 1984; Beavers *et al.*, 1983; Burman *et al.*, 1986; Finney, 1985; Hominick and Briscoe, in prep; Mracek, 1980, 1982a; Wang and Li, 1987). In Britain, a large scale survey for new strains/species, especially cold-tolerant strains/species, has been carried out (Hominick and Briscoe, in prep.). The identification of these isolates and evaluation of their effectiveness under British conditions for further use are essential.

The taxonomy of steinernematids is controversial at both the generic and specific level. Travassos erected a new genus *Steinernema* for the species of *S. kraussei* (= *Aplectana krasssei*, Steiner, 1923) in 1927 (in Poinar, 1979). Two years later, in 1929, Steiner established *Neoaplectana*, with the type species *N. glaseri* (in Poinar, 1979). Recently, Wouts *et al.* (1982) proposed that the two genera were synonyms and that *Steinernema* therefore had pecedence. However, Poinar (1984) disagreed with the opinion. He questioned whether Mráček (1977) in actuality did re-isolate the original *S. kraussei* which was used by Wouts *et al.* (1982) as type specimens of the genus *Steinernema* to compare with the other species from the genus *Neoaplectana*.

Similarly, there are problems at the specific level. Some 16 species have been described in the genus Neoaplectana, but Poinar (1985) recognized only N. carpocapsae (Weiser), N. bibionis (Bovien) and N. glaseri (Steiner) and the new species N. intermedia (Poinar, 1985). Others (for example, Wouts et al., 1982) recognized S. kraussei as well, and consider that S. feltiae is the senior synonym of N. carpocapsae. N. anomali is a recently-described species (Poinar and Kozodoi, 1988).

Most workers have adapted the proposal of Wouts et al. (1982) and recognize the generic name Steinernema and consider S. feltiae to be the senior synonym of N. carpocapsae. This is the convention that has been adopted in the present study.

Like the difficulties which have arisen for the taxonomy of insect-parasitic rhabditids, identification of the nematodes has also been difficult. Although a critical evaluation of the differentiating characters remains to be undertaken, several authors did try to provide keys for classification of these nematodes, based on morphological features of males and infective juveniles. The introduction of scanning electromicroscopy has greatly improved studies on the taxonomy of insect parasitic nematodes (Mráček, 1986). Use of DNA restriction enzyme techniques (Curran and Webster, 1989; Curran et al., 1985) will prove even more useful.

Distinguishing between the families Steinernematidae and Heterorhabditidae can be easily achieved by observing the colour of cadavers and examining the first generation adults. The difficulty arises for the identification of species within Steinernematidae, because of quantitative and qualitative variations occurring within the same species and lack of detailed information on the extent of such variation. How-

ever, Poinar's (1985) key for identification of steinernematids can be used to some extent to identify species. For diagnostic purposes, the shape of the capitulum, the shape of the tips of the spicules, and the presence of a cuticular tail spine in males were used. The quantitative measurements of the total length and distance from head to excretory pore of infective stage juveniles can also be used for specific diagnosis, because these features are fairly constant (Poinar, 1985).

Hybridization studies under laboratory conditions based on genetic compatibility of individuals of the same species should be used as an important means of confirming the identity of Steinernema species and as an indicator of those characters which show greatest divergence between species (Akhurst and Bedding, 1978). To conduct hybridizations, Poinar (1979) reared nematodes from infective stage to adults in hanging drops of G. mellonella blood. Pairs were then examined for mating in 24 hrs. Akhurst and Bedding (1978) described a simpler cross-breeding technique to facilitate identification of Steinernema species by injecting G. mellonella larvae with one infective stage from a known stock culture and another from the population being tested. Whether or not fertile offspring are produced must be tested as well. Although this technique is useful in deciding Steinernema species, identification of Heterorhabditis to species level is impossible using the same technique, because these nematodes are hermaphroditic.

Entomopathogenic rhabditids are mutualistically associated with bacteria Xenorhabdus spp. (Poinar, 1979). Recent research (Akhurst, 1983a, 1986b, 1986c) confirmed four subdivisions of the species Xenorhabdus nematophilus which is associated with steinernematids:

nematophilus (symbionts of S. feltiae), bovienii (S. bibionis), poinarii (S. glaseri) and beddingii (two underscribed Steinernema spp. from Australia). Each bacterial isolate has a specific association with the nematode species from which it was isolated and not with other species. Therefore, isolating the associated bacteria and comparing the colony morphology and biochemical characteristics may help in classifying the nematode species.

Biotechnology has started to be applied to this field. Curran et al. (1985) used genomic DNA restriction fragment length differences to identify Steinernema species, but not strains of the same species. Examination of isozymes using starch gel electrophoresis was used by Akhurst (1987) to define groups within the genus Heterorhabditis. The possibility of using specific DNA probes to identify species and strains or geographical isolates is under investigation (Reid, pers.comm.). Protein profiles obtained from symbiotic bacteria associated with different species or strains of the entomopathogenic nematodes also showed clear divergence between species (Hand, personal communication). Employment of these advanced techniques shows promise for stabilizing the taxonomy of these insect-parasitic nematodes.

This chapter describes morphological characters of the nematodes and the cross-breeding technique of Akhurst and Bedding (1978) to identify some nematodes isolated from British soil.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of entomopathogenic nematodes from soil

A survey for entomopathogenic rhabditids in British soil was conducted in 1986-1987. Sites were sampled randomly. At each site, 6 soil

samples were taken each to a depth of 10cm and having a volume of approximately 250cc. The 6 samples were bulked in plastic bags and transferred to the laboratory. Bedding and Akhurst's (1975) Galleria trap method was used to detect entomopathogenic nematodes from the samples. For each volume of soil, 3-5 late instar larvae of G. mellonella were exposed. The larvae were examined for any nematode parasitization 1 week later at 20°C. The cadavers were placed on White traps (in Poinar, 1979) for a further week to allow emergence of infective stage juveniles.

3.2.2 Identification of the nematodes

Morphological features and behaviour

The infective stage juveniles from different sources were used to infect last instar wax moth larvae and the dosage of infective juveniles was kept at 10-20 dauers per insect. The larvae were then incubated at 20°C. First generation adults were collected by dissection 5 days after the exposure and second generation adults nine or ten days after the exposure. The infective stage nematodes were later collected and used for identification. The adults as well as infectives were killed by pouring hot TAF (Courtney, Polley and Miller, 1955, in Southey, 1985: formalin 7ml, triethanodamine 2ml and distilled water 91ml) solution (65°C) into the watchglass where they were contained. The fixation process took at least 24 hrs. The fixed nematode specimens were then processed to glycerol using Seinhorst's glycerol-ethanol method (in Southey, 1985). The fixative in the watchglass was removed with a pipette and solution I (96% ethanol 20 parts; glycerol 1 part and distilled water 79 parts) added. The watchglass was placed in a desiccator

containing about 1/10 of its volume as 96% ethanol and left in this saturated atmosphere for 24 hrs in an oven at 38°C. Solution II (glycerol 5 parts and 96% ethanol 95 parts) was then used to top up the watchglass which was placed in a petri dish partly closed by its lid. This was incubated at 40°C until the nematodes were in pure glycerol. Males were examined and infective stage juveniles were measured. Morphological dimensions and photographs were taken with a Leitz Dialux microscope equipped with differential interference contrast optics. The data recorded were compared with those given by Poinar (1979, 1985). A known species of nematode, S. bibionis (T319), was also treated in the same manner as a standard. The morphological characters recorded for this species were used as a control.

Cross-breeding experiments

A cross-breeding technique (Akhurst and Bedding, 1978) was used to confirm the morphological identification. The test and stock populations (in this case, S. bibionis T319 strain) were surface sterilized by immersing them into 0.1% merthiolate for 30 minutes. After rinsing the populations with sterilized Ringers 3 or 4 times in watchglasses, they were finally transferred into clean sterile Ringers. Injection was made by means of a 10µ syringe with a 0.4mm diameter needle and plunger which goes to the needle tip to ensure that both nematodes were ejected into the G. mellonella larva. For each of 60 Galleria larvae, one infective juvenile nematode from stock S. bibionis (T319) culture and another from the population being tested were injected together. Controls which consisted of 60 Galleria larvae each injected with two infective juveniles from the test population were also made. Injected larvae were incubated

at 20°C on filter paper in a plastic Petri dish, and after 7 days, they were placed individually in plastic containers for extraction of dauer-larvae. The infectivity and reproductive capability of infective juvenile nematodes collected were tested by exposing live G. mellonella larvae to them in sterilized sand (host : parasite = 1:10), and dissecting cadavers on the 5th and the 8th day at 20°C.

Characters of symbiotic bacterial colonies

The techniques described by Akhurst (1980) were used to isolate bacterial associates from the nematodes by sampling the haemocoel of G. mellonella larvae infected with infective juveniles. Samples were streaked on nutrient agar (NA) and NA containing 0.004% (wt/val.) Triphenyltetrazolium Chloride (TTC) and 0.0025% Bromothymol Blue (BTB) (NBTA medium). The cultures were incubated at 15°C. Colony characteristics were recorded from 2-day-old NA and NBTA medium. The three bacterial isolates were compared for their colony characters. The relevant information presented in the literature for Xenorhabdus spp. was used as a key.

3.3 RESULTS

3.3.1 Description of the isolates chosen for the study

Nematodes were recovered from many sites sampled in the survey. Thriving nematode juveniles extracted from two sites in which exposed Galleria larvae were heavily infected were used for further studies.

These two isolates and the corresponding sites are described in Table 3.1.

TABLE 3.1: Description of the isolates chosen for this study.

Isolate	Location (Grid Ref.)	Habitat	Vegetation	Designation
1	SU 843 407	Field Hay	Hay, at edge overhung by oak	Site 42
2	SU 945 692	Permanent grassland	Grass, uncut young oaks.	Nashes

3.3.2 Identification of the nematodes

Morphological features and behaviour

The nematode specimens isolated from the 2 localities in Britain were firstly identified to be rhabditid nematodes, because the pharynx of the nematode possessed a basal bulb and lack of a stylet. The infective juveniles of these nematodes can penetrate into G. mellonella larvae and cause their death, showing that they are entomopathogenic rhabditids (Poinar, 1979).

Infective stage juveniles of Steinernema are less variable in size and shape than the adults and quantitative measurements can be used to identify species. Measurements of heat-killed infective juveniles of the two isolates, together with measurements made for S. bibionis (T319) from a stock population available in this laboratory, and for a New Zealand population described by Wouts (1980) are presented in Table 3.2. The male tail and the shape and characteristics of the spicules for the two isolates along with those of S. bibionis (T319) are presented in Plate 3.1.

Observation of these two isolates in water after storage revealed that the infective juveniles are coiled (Plate 3.2), showing the typical behaviour of S. bibionis (Bedding, per. comm.).

On the basis of the morphological characters evaluated in the present study, the key given by Poinar (1985) and the behaviour of the infective stage in water, these two isolates from British soil were identified as S. bibionis.

TABLE 3.2: Measurements (mean and range) of the infective stages of the strains of S. bibionis.

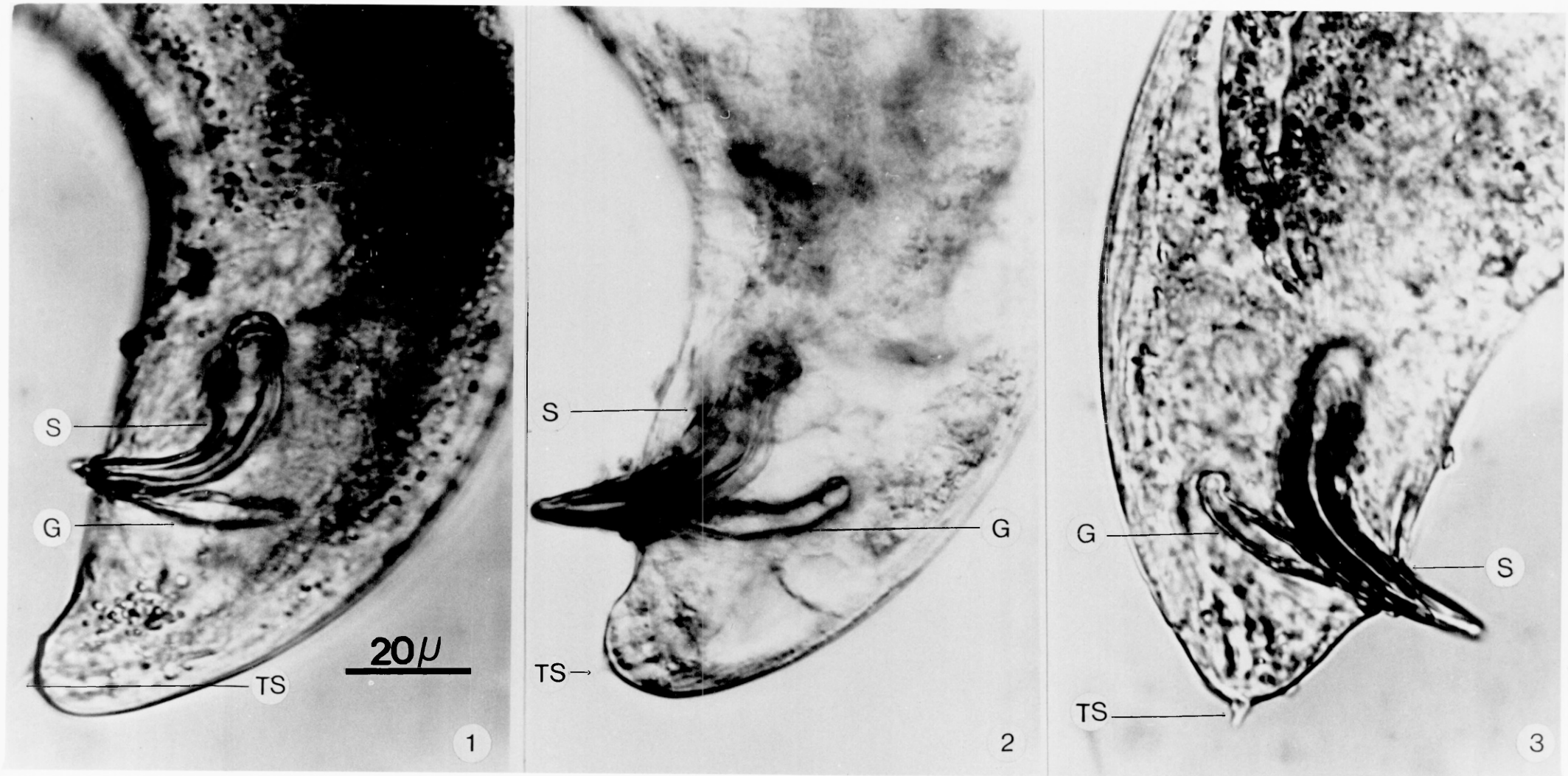
Character	NZ strain (N=20) (μm) (Wouts, 1980)	T319 strain (N=25) (μm) (present study)	Site 42 (N=25) (μm) (present study)	Nashes (N=25) (μm) (present study)
Total length	880 (750-950)	844.2 (785.4-928.2)	831.8 (737.8-934.2)	830.8 (737.8-904.4)
Greatest width	25 (22-27)	27.7 (26.2-29.8)	27.8 (26.2-29.8)	27.2 (25.0-29.8)
Distance: head to excretory pore	62 (63-67)	58.4 (53.8-65.8)	57.6 (53.8-62.8)	56.2 (50.8-62.8)
Distance: head to pharynx base	135 (115-150)	135.6 (125.6-140.5)	138.9 (125.6-143.5)	138.0 (131.6-146.5)
Length tail	83 (71-92)	75.8 (68.8-83.7)	79.8 (71.8-86.7)	82.1 (75.6-88.2)
Anal diameter	16 (15-17)	16.4 (15.0-17.9)	17.1 (15.0-19.4)	16.8 (15.0-17.9)
Ratio E = Distance:head to E.P. / Length tail	-	0.77 (0.69-0.85)	0.72 (0.65-00.83)	0.69 (0.61-0.77)

PLATE 3.1 Mail tails of *Steinernema bibionis*. All photos at the same magnification. G: gubernaculum, S: spicule, Ts: tail spine.

FIGURE 1 *S. bibionis* Nashes strain.

FIGURE 2 *S. bibionis* Site 42 strain.

FIGURE 3 *S. bibionis* T319 strain.


S
G
TS
20μ
1
S
G
TS
2
G
S
TS
3

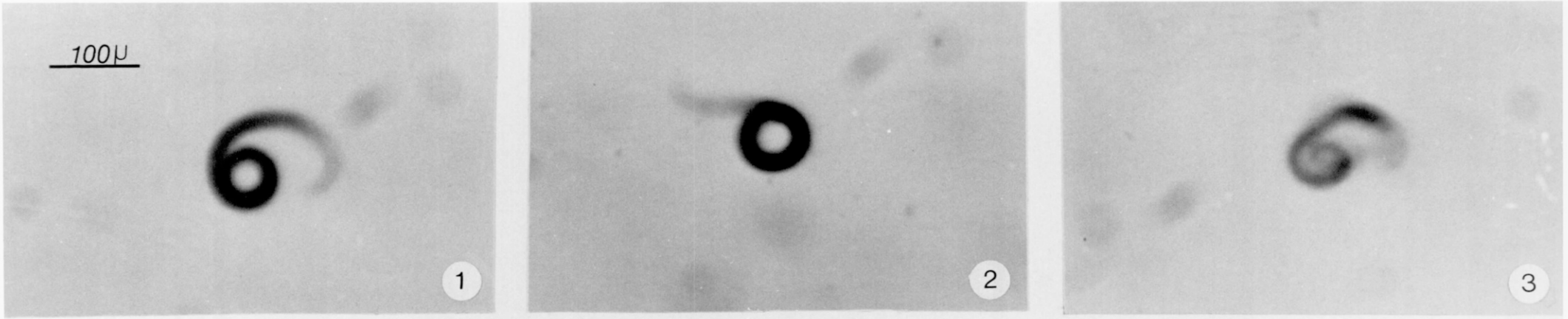


PLATE 3.2 Coiled posture of Steinernema bibionis infective juveniles after storage in tap water after 4 weeks at 5 C. All photos at same magnification.

Figure 1 The T319 strain infective juvenile nematode.

Figure 2 The Site 42 strain infective juvenile nematode.

Figure 3 The Nashes strain infective juvenile nematode.

Cross-breeding experiments

The cross-breeding experiment between the site 42 isolate and S. bibionis (T319) was positive. At 20°C, fourteen days after the infection, infective juveniles started emerging. A week later, infective stages had emerged from 20% of the G. mellonella cadavers. Forty-five or 30% of the cadavers injected with either two Site 42 nematodes or 2 T319 nematodes had released the infective nematodes. The infective juveniles obtained from the cross-breeding experiment can parasitize G. mellonella larvae and cause their death within 48 hours. Dissection of Galleria cadavers on the 5th day revealed the presence of females with fertile eggs. In some of the cadavers, J1S could be seen in the insect tissue. By the 7th day, hundreds of 2nd generation males and females were observed. This showed that the offspring produced by hybrids of S. bibionis (T319) and Site 42 nematodes were able to infect a host and reproduce.

Hybridization between the Nashes isolate and S. bibionis (T319) was also positive, although at lower numbers (2/53). The controls using 2 Nashes nematodes or 2 T319 nematodes were successful at the rate of 22% or 30%. Infective juveniles were obtained thirteen days later from the cadavers injected with one Nashes nematode and one T319 nematode. They were able to infect late instar larvae of greater wax moths in sand and develop and reproduce normally inside the insect cadavers.

Characters of symbiotic bacterial colonies

On NA, the colonies of the primary form of X. nematophilus, the S. bibionis symbiont, were yellow, convex and circular with slightly irregular margins. The diameter of the colony ranged from 1mm to 2 mm

after two days at 15°C. On NBTA medium, the primary form produced blue colonies and a red core could be seen if the blue colony was examined under the microscope. These characters confirm with the description presented in the literature (Thomas and Poinar, 1979; Akhurst, 1980). Therefore, the bacterial symbiont isolated from S. bibionis (T319) was regarded as a standard to which the bacterial colonies from the other two isolates were compared.

The bacterial colonies formed on NA from Site 42 nematodes and Nashes nematodes were similar to those of S. bibionis (T319) in their characters. The bacterial colonies were shown to Dr. Hand (IHR-Littlehampton) who confirmed that their characteristics were those expected from the S. bibionis bacteria.

3.4 DISCUSSION

The two British isolates of Steinernema were compared with a living population of S. bibionis (T319) and a documented S. bibionis (NZ) (Wouts, 1980). The comparative measurements for the infective juveniles showed clear similarities between the T319 strain of S. bibionis and both of the British isolates. Although statistical analysis of morphological measurements between the documented New Zealand strain of S. bibionis and the studied isolates were not carried out because of the absence of appropriate data from the NZ strain, the mean and range of each feature presented in Table 3.2 showed obvious similarities.

In the present study, the total length of infective juveniles of S. bibionis (T319) was 785-928μm compared to 738-935μm (Site 42) and 738-904μm (Nashes). Comparing these to the length of S. bibionis (NZ)

(750-950μm) given by Wouts (1980), this character remained fairly constant for the four populations. This agrees with Poinar (1985) that the length of the infective stage is a fairly reliable character for distinguishing infective juveniles of Steinernema species.

For diagnosing S. bibionis, as it is presently known, a tail projection (or spine) possessed by males and the colour of the spicules are the two major diagnostic features in addition to the length of infective juveniles (Poinar, 1986). In this case, the spicules of both British isolates were yellow in colour and the majority of the males possessed spines, which showed that both isolates were likely to belong to the species of S. bibionis. Successful production of hybrids in the cross-breeding experiments between the known strain of S. bibionis (T319) and the two British strains confirmed the diagnosis.

Each Steinernema species has its own particular bacterial subspecies of X. nematophilus (Akhurst, 1983b, 1986b). Although nematodes of one species may carry the symbiotic bacterium of another species by experimental feeding on artificial media, the best development and reproduction only occurred when the nematode was associated with its own symbiont (Dunphy et al., 1985). Therefore, the symbiotic bacteria were isolated from S. bibionis (T319) and the other two British populations of nematodes to provide supporting evidence for the previous identification. The colonies grown on NA and NBTA media could not be distinguished. A detailed examination of bacterial cells and other microbiological tests were not carried out, as there was no doubt that the bacteria were those expected to be associated with S. bibionis.

CHAPTER 4

POPULATIONS OF *Steinernema bibionis* IN A NATURALLY INFESTED FIELD

4.1 INTRODUCTION

Steinernematids have been isolated from the soil in many countries in the world. Because of their importance as parasites of insects, many biological and ecological attributes have been studied and these were reviewed by many authors such as Gaugler, 1981; Kaya, 1985; Poinar, 1979, 1986 and Webster, 1980. Most studies were made under carefully controlled laboratory conditions in order to assess various factors which may influence performance of these nematodes in their natural environment. Both biotic and abiotic influences were shown to play important roles in persistence, infectivity and movement of these nematodes in their habitats and also in their developmental processes inside hosts.

In general, temperature, moisture and soil type are regarded as the three most important abiotic factors which affect these nematodes. Detailed information on temperature effects on infectivity, development and reproduction of the nematodes are presented in Chapter 5 and its influence on survival of the nematodes in the terrestrial environment is assessed in Chapter 7.

Moisture is also important for the activities of these nematode juveniles outside hosts, although in soil, moisture is usually adequate for their movement and persistence. Laboratory studies (Dutky, 1959; Welch and Briand, 1961; Schmiege, 1963) have shown that high mortality of infective juveniles of *S. feltiae* is caused by rapid desiccation, and the nematodes of *S. feltiae* Agriotos strain could not survive well on

foliage (Kaya, 1986). However, a study on survival of S. feltiae in relation to soil moisture (Gray and Johnson, 1983) showed that a soil moisture level between 10 and 30% did not significantly affect nematode survival. These results indicated that the nematodes can tolerate a fairly wide moisture range, once they are in soil. Gray and Johnson (1983) also showed that the influence of moisture is determined by the accompanying temperature, suggesting that the temperature is even more important in the environment.

Apart from the effect of soil moisture on survival of the nematode juveniles, any change in moisture will immediately influence their movement which, in turn, will influence their capacity to seek and infect insect hosts (Molyneux, 1983). Molyneux (1983) also showed that the moisture limits for nematode infection varied between different soil types. Nevertheless, the nematode (S. glaseri KG strain) parasitized Lucilia cuprina larvae over a wide range of moisture potentials in different soil types. In loamy sand, the nematodes parasitized L. cuprina larvae even at moisture potentials equivalent to and below the permanent wilting point of plants (Molyneux and Bedding, 1984). Therefore, in a temperate and moist climate such as that of Britain, moisture would be ideal for the nematodes' movement and infectivity.

Movement, survival and infectivity of steinernematids in relation to soil types have also been investigated. The presence of clay and silt resulted in less dispersal than in sand alone (Georgis and Poinar, 1983a). In moist sandy soil, the majority of S. feltiae remained at the point of placement if placed on the surface, although some migrated up to 14cm laterally and 12cm vertically in the absence of hosts (Moyle and Kaya, 1981). However, when the nematodes were placed 15cm below the

surface, the majority remained at that location but dispersal tended to be upwards rather than downwards. Presence of a host increased the number of nematodes that dispersed (Georgis and Poinar, 1983a, 1983b; Moyle and Kaya, 1981). When there were no hosts present, S. feltiae survived best in silty soils and worst in sandy soils (Ishibashi and Kondo, 1987). This result suggests that nematodes move least in the silty soils so that large numbers survive with sufficient energy to be extracted.

Biotic factors are another important consideration when assessing persistence of nematodes in the field. Fungi pathogenic to nematodes and predation by other nematodes and by Collembola probably have a significant impact on populations of insect-parasitic rhabditids in soil (Bedding, pers.comm.). Ishibashi and Kondo (1986a) showed that infective stages of Steinernema spp. persisted longer in sterilized soil than in nonsterilized soil in a laboratory test and later (1987) in a field test, they recovered more DD-136 J3S from the nematicide 1,3-D (Telone II) treated soil than from non-treated soil. Although this implicated biotic factors in affecting persistence of entomopathogenic nematodes in soil, identification of the biotic factors and documentation of their effects are required.

In addition to the above studies, distribution of the nematodes in the field as one of their ecological attributes has been studied by a number of workers (e.g. Akhurst and Brooks, 1984; Akhurst and Bedding, 1986; Hominick and Briscoe, in prep.) using the Galleria trap method (Bedding and Akhurst, 1975). Because these studies were mainly designed to isolate the nematodes for biological control purposes, they are qualitative rather than quantitative. If "prevalence" was mentioned, it

usually represented the percent mortality of insects in the bioassay. Also, in most studies, the distribution of the nematodes over time was not investigated.

Localized distribution of steinernematids and their seasonal changes are rarely studied. An exception was that of Mráček (1982a) using a natural population of S. kraussei. He found that S. kraussei were only present in humus (3-7cm) and organo-mineral (5-10cm) layers. Two peaks of insect mortality were observed for S. kraussei during the year when assessed by Galleria traps. In contrast, a study on a natural population of S. feltiae (Georgis and Hague, 1981) showed no evidence of seasonal variation in prevalence of these nematodes in British soil. Although these studies investigated nematode distribution and their variations over time, they did not document actual nematode numbers in the soil. They also assumed that insect mortality in the bioassay is related to the number of nematodes in the soil.

In an attempt to document the number of steinermatids in 8 habitats located on the southern and western slopes of the Mountain complex Radziejowa in Poland, Bednarek and Mráček (1986) counted the number of nematodes in Galleria larave in successive bioassays. They found that the nematode population was very low. The highest number they recovered was 39 nematodes in a sample of 2000cc soil. Unfortunately, the authors extrapolated the results and multiplied the numbers in 2000cc of soil by the surface area of the habitat to obtain an estimate of the nematode population in this area. This is unrealistic because the nematodes, like most organisms in soil, probably have a patchy or aggregated distribution, though until now no one has documented localized distributions.

In Britain, surveys of steinernematid distribution have been made both on a large scale (Hominick and Briscoe, in prep.) and on a small scale (Renn, 1985). S. bibionis has been shown to be the dominant species in British soil. Although the persistence and seasonal dynamics of the nematodes in 15 natural habitats were studied over a 28 month period (Hominick and Briscoe, in prep.), their data did not provide information on the number of nematodes present and their variations. For population studies, quantifying the numbers is essential.

This chapter presents the results from samples taken in a naturally infested grassland from 1986 to 1988. A plot of $100m^2$ was used as a base and the horizontal and vertical distribution of the nematodes were investigated. The aim of this study was to determine how a natural population of S. bibionis is distributed, the number of nematodes, and whether changes could be documented over time.

4.2 MATERIALS AND METHODS

4.2.1 Experimental site

The field site was located on Nashes field (Grid ref. SU 945 692) at Silwood Park, Imperial College's Field Station. A previous survey (Renn, 1985) documented the presence of a Steinernema sp. in this permanent grassland. Preliminary field sampling conducted in 1986 (see 4.2.2) confirmed the occurrence of Steinernema bibionis Bovien in an area of approximately $80m^2$ within the Nashes field.

To study the distribution of the nematodes in a limited part of a natural habitat, a plot of $100m^2$ was measured out and divided systematically by a 2x2m network of gridlines (Plate 4.1). This created 25, 2x2m sites. The sites were distinguished by tying string around the four





PLATE 4.1 Sampling sites in Nashes field.

A: Entire site, measuring 10 x 10m, viewed facing west ;

B: Portion of the site, showing 2×2m divisions of the site.

wooden stakes firmly positioned at the four corners of each site. Three young oaks were present in Site 1 (measuring 2m in height), Site 16 (measuring 1.6m in height) and Site 18 (measuring 4m in height) respectively. The site is a natural mixed grassland that is not cut. The soil under the grass is predominantly a well-drained loamy sand (Avery, 1953).

4.2.2 Sampling procedures

Preliminary sampling, September 1986

A plot of grassland in Nashes field (approximately 9x9m in size) was sampled by choosing 6 random sites of $1m^2$. Five soil cores were collected randomly within each site to a depth of 20cm with a cylindrical sampling tube 4.2cm in diameter. The five cores were carefully collected at 5cm deep intervals and combined according to each soil depth in polythene bags. These bags were transferred to a $20^{o}C$ C.T. room in a dark, heavy plastic bag. They were stored overnight before the soil was assessed by Galleria traps.

Systematic samplings

A 5.0cm diameter, manually operated soil auger was used for taking all the soil samples. Two sampling methods were employed.

Method A: Within each of the 25 labelled sampling areas, four soil cores were taken randomly to a depth of 20cm. The cores were bulked in a polythene bag according to the soil depth, which was separated carefully at 5cm intervals. The polythene bags were transferred to the laboratory and storesd at $20^{o}C$. Usually, extraction of the nematodes began within 48 hrs. The plot was sampled twice in 1987 (June 20 and

October 1) and once in 1988 (April 26), to determine summer, autumn and spring populations. The mean temperatures 11cm below the soil surface were 14.8 (13.8-16.4°C), 12.9 (12.4-13.5°C) and 10.3 (8.9-11.5°C) respectively on the three dates.

Method B: This method was used in addition to Method A and was designed to assess variabilities within the 2x2m sites. Only 13, 2x2m sites were sampled. At each site, three soil cores were taken to a depth of 10cm. Each individual core was placed in its own polythene bag and transferred to the laboratory. Using this method, samples were taken on November 27, 1987; April 21, 1988 and June 20, 1988, when the average soil temperatures were 6.6 (5.8-7.0°C), 10.7 (10.1-11.3°C) and 17.0 (16.0-17.5°C) respectively.

Extraction and estimation of nematode numbers

Extraction of entomopathogenic rhabditids was carried out in a 20°C C.T.room. After large stones were discarded, the soil in the bag was mixed well and approximately 200cc was removed and placed in a plastic container (350cc in volume) which had a tight-fitting lid. Five late instar *G. mellonella* larvae were placed on the soil surface and were examined 4-6 days later. Fresh larvae were added every 4-6 days until no further insect deaths occurred. The cadavers were dissected and the number of nematodes recorded (2.5).

4.3 RESULTS

4.3.1 Horizontal distribution of *S. bibionis* (Nashes)

To examine the distribution of the nematodes horizontally within the $100m^2$ plot of grassland, the data obtained from the three samplings

using Method A and the three using Method B were analysed. In Method A, 4 soil samples from each of 25 sites were taken at 5cm intervals to a depth of 20cm. A sub-sample of 200cc of soil was taken from the bulked samples of 4 to represent the particular depth at that particular site. Because five Galleria larvae were used in each 200cc of soil, the numbers of nematodes recovered from the 5 Galleria larvae after 4-6 days exposure were summed. To assess the horizontal distribution, the number of nematodes per site was obtained by adding the numbers from the 4 depths. Thus, the total number of nematodes per site recovered after 96 hr exposure was obtained from a total of 20 Galleria larvae in 800cc of soil. In Method B, three soil samples were taken from each of 13 sites to a depth of 10cm. The number of nematodes in each sample was recorded from the total dissected from the 5 Galleria larvae exposed to that sample of about 200cc after 4-6 days. Therefore, the mean number of nematodes to a depth of 10cm per site can be calculated from the three samples taken within each site and used to compare with the other sites.

The total number of nematodes recovered from the 25 sites for the three samplings using Method A are presented in Table 4.1. Because the intention was to quantify the infective nematodes at each site, the extraction procedure using Galleria larvae did not stop after the first exposure. Groups of 5 Galleria larvae were continuously replaced until infections ceased. Thus the total nematodes recorded after the first exposure (Table 4.1(A)) and after infection ceased (Table 4.1 (B)) are listed separately in Table 4.1

The nematodes showed an aggregated distribution pattern within the area sampled on each of the three sampling dates, with the nematode density very high in some sites and very low, even zero, at other sites

TABLE 4.1: Total number of nematodes recovered from 20 Galleria larvae in a total volume of 800cc soil taken from 25 sites on 3 occasions. A: exposure of 4-6 days at 20°C; B: consecutive exposures of 4-6 days each until infections ceased.

Sites	Time of sampling					
	20/6/1987		1/10/1987		26/4/1988	
	A	B	A	B	A	B
1	11	15	20	2814	7	23
2	5	32	53	139	224	573
3	21	369	32	911	611	62
4	14	16	106	196	1	2
5	4	46	47	54	0	0
6	14	23	128	150	1	5
7	1	3	4	6	12	47
8	6	11	9	49	0	1
9	18	929	60	99	15	17
10	21	529	37	233	0	0
11	9	14	0	2	3	3
12	16	46	37	45	10	10
13	9	72	1	1	2	3
14	3	889	37	70	12	73
15	15	2495	10	57	152	571
16	36	112	3	3	4	38
17	1	1384	6	1240	1	1
18	10	168	33	39	19	26
19	16	57	9	9	10	16
20	0	3	0	0	0	0
21	2	6	9	15	1	1
22	5	14	8	13	1	1
23	15	35	45	538	0	0
24	73	829	9	15	1	2
25	17	96	11	173	34	52
Mean ± se	14±3	328±120	29±7	275±124	23±11	61±32

(Table 4.1(B)). Because of the variability and because only three samplings were made, it is impossible to determine whether the nematode population changes with time. However, the average number of nematodes recovered from the 25 sites showed more nematodes present in June and early October than in April. This is reflected in the number of sites which had different population densities for the three samplings (Table 4.2). Thus, April was the sample in which most of the sites had low numbers of nematodes. Similarly, comparing the highest number for a particular site, it can be seen that it reached 2495 nematodes in June (Site 15) and 2814 in October (Site 1), compared to only 573 (Site 2) in April. Therefore, if there is a trend, it would appear that more nematodes are present in the early summer and autumn than in spring.

Comparison between the numbers recovered after the first exposure to Galleria larvae (Table 4.1A) and the total number recorded after infections ceased (Table 4.1B) showed that it was common for substantial numbers of nematodes to remain in the soil after one exposure to Galleria larvae. Therefore, conclusions based on the results after only one bioassay can be misleading. For example, 23 ($\pm$ 11) nematodes were recovered after the first exposure for the samples of April 1988, compared to 14 ($\pm$ 3) nematodes for the samples of June 1987. These means are not significantly different (t-test, $t = 0.839$, $p = 0.409$). However, the means for the total number of nematodes eventually recovered from the samples taken at these two times were completely different, with 61 ($\pm$ 32) nematodes for April 1988 and 328 ($\pm$ 120) nematodes for June 1987.

The results for the samplings using Method B (November 1987, April and June 1988) are presented in Table 4.3. The mean number of nematodes

TABLE 4.2: Number of sites having different nematode densities. The number of nematodes/site was obtained by extracting to exhaustion a particular sample using *Galleria* larvae.

Density Level	Time of sampling		
	20/6/1987	1/10/1987	26/4/1988
>100	16	16	23
100-500	3	5	0
500-1000	4	2	2
>1000	2	2	0

TABLE 4.3: Mean (± se) number and range for nematodes recovered from three soil samples (200cc each) within sites measuring $4m^2$ by exposing 5 Galleria larvae to each sample until infection stopped.

Sites	Time of sampling								
	27/11/1987			21/4/1988			20/8/1988		
	$\bar{x}$	se	range	$\bar{x}$	se	range	$\bar{x}$	se	range
1	14	15	1-39	6	5	1-14	8	9	0-23
2	2	2	0-5	0	0	0	180	221	0-541
3	4	5	0-12	1.3	0.8	0-2	3	2	0-7
4	19	23	0-57	10	9	1-25	0.3	0.4	0-1
5	106	120	0-301	166	203	0-498	1	1	0-3
6	0.7	0.4	0-1	12	14	0-34	5	3	0-9
7	2.7	2.7	0-7	818	872	3-2237	5	1	3-7
8	0	0	0	19	21	1-53	12	9	0-26
9	0.3	0.4	0-1	0	0	0	87	10	0-256
10	5	5	1-14	19	10	10-36	0	0	0
11	59	57	0-151	0	0	0	0	0	0
12	88	104	0-1629	14	17	0-41	0	0	0
13	113	138	0-823	0	0	0	0	0	0
TOTAL	31.8	12.5	0-113	81.9	65.1	0-818	23.2	15.2	0-180

at each site showed similar results to those from Method A in that the nematodes were distributed in an aggregated fashion within the sampling area. The mean and standard error for each site (Table 4.3) indicated that the nematodes had a patchy distribution within the sites as well. The nematode population at most of the sites changed dramatically with time, with no obvious seasonal trend for the changes. Not a single site with a high nematode population retained its high density on all sampling occasions.

In an attempt to determine whether there is any seasonal trend for the nematode population in soil, data obtained by Method A and Method B were assessed after making them comparable. Thus, the total number of nematodes recovered in 200cc of soil taken to a depth of 10cm from 13 sites was calculated. The mean (and range) number of nematodes from the 13 sites for each of the 6 sampling times is presented in Table 4.4.

The large standard errors show that there were large variations between sites for each sampling date. Therefore, it is still impossible to tell whether there were significant differences between the 6 sampling dates. Although the means may suggest that nematodes were present in large numbers in October 1987, the ranges indicated that they were all overlapping. Indeed, the samples on April 21 and 26, 1988, though separated by only five days, showed dramatic differences between the numbers of nematodes extracted. Whether there is a time difference or as a result of the normal variability that occurs due to the procedure used to estimate nematode populations, cannot be determined.

TABLE 4.4: Total number of nematodes per site calculated from samples taken from 13 sites to a depth of 10cm on 6 sampling dates. Volume of each sample is about 200cc.

Sampling date	mean	se	range
20. 6.1987	89.6	54.5	0.5 - 692
1.10.1987	113.3	56.8	0 - 604
27.11.1987	31.8	12.5	0 - 113
21. 4.1988	81.9	65.1	0 - 818
26. 4.1988	4.3	2.2	0 - 24
20. 6.1988	23.2	15.2	0 - 180

4.3.2 Vertical distribution of S. bibionis (Nashes)

The data obtained from the preliminary sampling and the systematic samplings using Method A were analysed for the vertical distribution of the nematodes. In the preliminary sample (September 26, 1986), only 6 sites were assessed, with 5 samples taken at each site. Five Galleria larvae were exposed to a sub-sample of 250cc of soil from each of 4 depths for each site. In the systematic samplings using Method A, 25 sites were assessed, with 4 samples taken at each site. Five Galleria larvae were exposed to a sub-sample of 200cc soil from each of 4 depths for each site.

The results for the mean number of nematodes recovered from each of 4 depths from the 6 or 25 soil samples are presented in Table 4.5. The results were consistent in that for all four sampling dates, most of the nematodes were found in the upper 5cm of soil. Sometimes, nearly all the nematodes were found in the 0-5cm depth (April, 1988) and sometime, considerable numbers were found in the 5-10 and 10-15 layers (June, 1987). Between 15-20cm, nematodes were never found in significant numbers. The absolute nematode density in 0-5cm of soil also changed greatly from one sample to another. The highest mean number of nematodes was found in September, 1986, with 438 nematodes per 250cc of soil. The lowest mean density was found in April, 1988, with 48 nematodes/200cc soil.

The standard error shown in Table 4.5 shows that large variabilities existed between sites. Figure 4.1 presents a breakdown of the results for the September, 1986 samples by site. Although a high density of nematodes was always found in the upper 5cm of soil, only one site (Site 2) had a nematode population over 2000 (2146 nematodes/250cc

TABLE 4.5: Mean (± se) number of nematodes recovered from each of 4 depths from the 6 (26.9.1986) or 25 (the rest) soil samples after consecutive exposures of 4 - 6 days each to *Galleria* larvae until infection ceased.

Depth (cm)	Sampling date			
	26.9.1986	20.6.1987	1.10.1987	26.4.1988
0 - 5	438 ± 376	212 ± 97	247 ± 124	48 ± 28
5 - 10	16 ± 8	50 ± 27	17 ± 11	1 ± 0.5
10 - 15	2 ± 2	61 ± 28	14 ± 6	0.6 ± 0.3
15 - 20	1.3 ± 0.7	2 ± 1	0.4 ± 0.2	2 ± 2

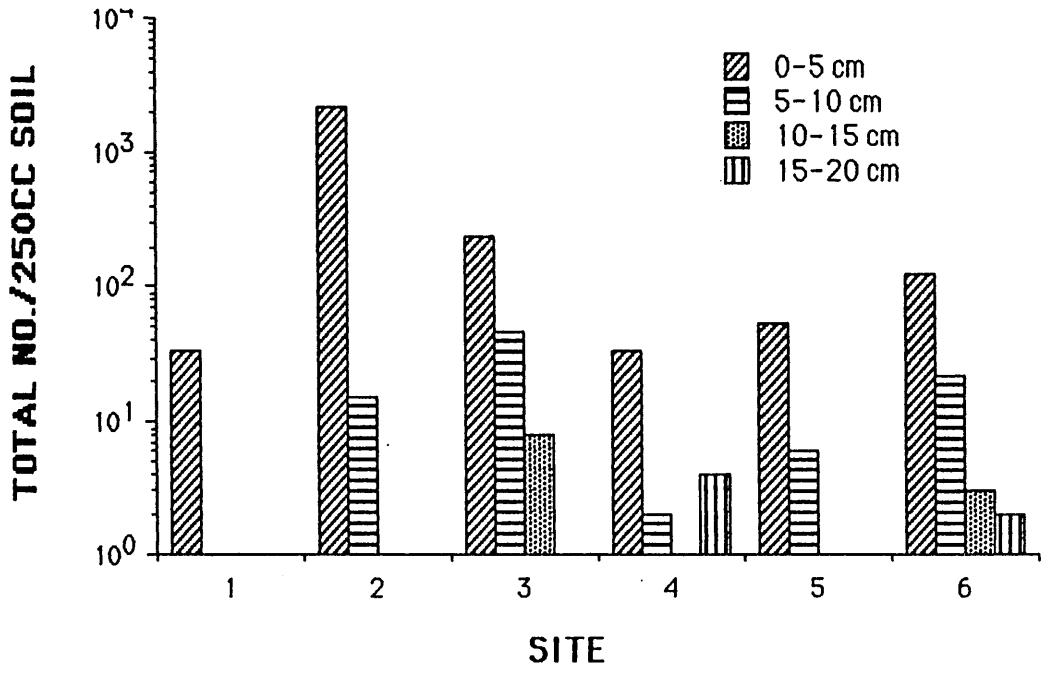


FIGURE 4.1 Total number of nematodes recovered from soil samples taken at 4 depths from 6 sites in September, 1986.

soil) at that depth. The other sites had a population from 33 to 234 nematodes/250cc soil (0-5cm), which was much below the overall mean (438 nematodes/250cc soil) calculated for the 6 sites. Similar results applied for the other depths as well. Therefore, it is important to assess individual sites in order to know how different these sites are. The overall mean from the sites obscured the large variability between sites.

4.4 DISCUSSION

The number of S. bibionis (Nashes) in a plot of natural grassland showed an aggregated distribution pattern. This occurred in both a $100m^2$ plot and on a smaller scale 2x2m sites within the larger plot (Table 4.1 and Table 4.3). This aggregated distribution occurred on all the sampling dates, which confirms the pattern generally found for other soil nematodes.

The vertical distribution of this nematode was documented to a depth of 20cm below the grass. It is the first study to date in which the actual nematode numbers were used to show how this nematode was distributed vertically in soil. Generally, most of the nematodes were found in the top 10cm of soil with 0-5cm having the majority of the population. The numbers declined rapidly with depth and only a few nematodes were present at a depth of 15-20cm.

Soil is a heterogenous environment with a variety of physical and biological characters which affect nematodes and their distribution (Yeates, 1981). An explanation for the nematodes being more abundant in some sites than in others is not easy to provide because there are so many environmental components that could influence their numbers. In

general, soil physical factors such as texture, temperature and moisture are considered to have some kind of association with the distribution and abundance of soil nematodes (Wallace, 1973). Since infective juveniles of S. bibionis are soil-dwelling nematodes, they would also be expected to be influenced by these factors. However, the area sampled in the present study is limited to a small plot and a change in soil physical factors is not likely to play an important role in determining the distribution of this nematode within this area. Therefore, this aggregated pattern is assumed to be mainly due to the interaction of the insect-parasitic nematode and its hosts.

It is well known that once infective juveniles in soil have made contact with a host, they are able to penetrate through natural openings and reproduce, yielding thousands of infective juveniles which emerge into the soil (Poinar, 1979). The capacity to reproduce and release a large number of nematodes from a single host makes it possible for a single insect to act as a spot source for nematodes. The comparatively low mobility of entomopathogenic rhabditids (Georgis and Hague, 1981; Moyle and Kaya, 1981; Poinar and Hom, 1986) would tend to preserve this aggregation. Although the distribution of possible hosts was not studied, particular attention was paid towards finding insects during the process of soil examination. Insects were found in the soil within the sampling area and most often in the top 5cm. A few earthworms were present in the soil below 5cm as well. It is generally believed that insects are obligate hosts for these entomopathogenic nematodes (Poinar, 1979), while earthworms which have been injured may provide a substance to support nematode development, although nematode-induced earthworm mortality is unusual (Capinera et al., 1982).

The response of the nematodes to certain gradients might have played a contributory role to their vertical distribution. Just as plant-parasitic nematodes are attracted to roots from which exudates are released (Klinger, 1965), insect-parasitic rhabditids have been reported to respond to the carbon dioxide produced from G. mellonella larvae (Gaugler et al., 1980). A large amount of roots were found in the top 5cm of soil, and the high concentration of nematodes in this layer might have been due to CO_2 produced by the roots. These roots may also harbour more insects which can produce more nematodes after infection. The root system may also act as traps which retard nematode downward movement, resulting in lower numbers found in the deeper soil.

Despite the fact that the number of nematodes recovered from soil samples changed from time to time, presence of this nematode in the $100m^2$ plot continued over the period from 1986 to 1988, although the population was highly variable between sites. It has been shown in a longitudinal study of natural populations of steinernematids in Britain that populations of the nematodes persisted for 28 months in some locations as indicated by parasitization of Galleria larvae exposed to the soil samples (Hominick and Briscoe, in prep.). These results for persistence of the nematodes in natural environments are different from the results for the nematodes that had been introduced to soil. In the latter case, nematodes usually present for no more than several months (e.g. Ishibashi and Kondo, 1987; Poinar and Hom, 1986). This has been attributed to various unfavourable factors which are likely to be encountered in soil. However, these factors would also influence the natural nematode population. Therefore, although these abiotic factors are important for persistence of nematodes, there are other factors

which may also play a part. For example, the natural nematodes may have stronger tolerance or resistance to unfavourable environmental conditions than artificially cultured ones. Also, natural grassland is likely to have a large variety of insect species compared to an agricultural field. Therefore, it would be easier for the nematodes to reproduce and continue their generations through these species of hosts in the grassland.

It is impossible to conclude from the present study whether there is a seasonal affect on the nematode population because the total number of nematodes recovered from samples taken at each site was highly variable. This is best shown by the samples on April 21 and 26, 1987 which were taken within a few days of each other, but were very different in the number of nematodes recovered (Table 4.4). There have been suggestions that there are seasonal variations in the incidence of steinernematids, but the trend is different in different climates or localities (Akhurst and Bedding, 1986). Differences between nematode species may also be importnat (Hominick and Briscoe, in prep.). Thus, some evidence suggests that the prevalence of S. feltiae does not vary with season (Georgis and Hague, 1981). The present study supports the hypothesis that S. bibionis is not affected by seasons. It is in my opinion that host availability rather than season is the most important factor influencing the nematode population. Seasonality varies between countries and areas. Under British conditions, it is usually temperate and moist. The temperature measured in summer (20/6/1988) was 17^{o}C on average at 11cm below soil surface and at the beginning of winter the temperature was 6.6^{o}C (27/11/1987). Within this temperature range, S. bibionis infective nematodes in soil can infect and reproduce in their

hosts (Molyneux, 1985, Chapter 5). Therefore, these nematodes in the field may reproduce year-around to continue their population whenever there are hosts present. Also, in the bioassays, the number of nematodes was accumulated and the results were obtained at 20°C. If some soil samples which were collected in winter contained nematode-infested insects, even though at very low numbers, the rapid reproduction of the nematodes at the higher temperature will cause significant numbers in the soil sample. This could bias severely the results on a particular sampling occasion. For example, on April 21, 1988, the overall mean number of nematodes was 82 compared to 4 on April 26 (Table 4.5). This was because at Site 7, one of the 3 samples produced 2237 nematodes while the other two had 3 and 214 nematodes respectively. Therefore, a true seasonal trend could be obscurred by one sample that biased the results greatly. In any case, the variability observed, even in samples taken within a 2x2m plot, prevented an analysis of seasonal effects on the nematode population.

CHAPTER 5

TEMPERATURE EFFECTS ON INFECTIVITY, DEVELOPMENT AND REPRODUCTION OF *S. bibionis*

5.1 INTRODUCTION

Soil is the natural habitat for the free-living dauerlarvae of entomopathogenic rhabditids. Once outside their insect hosts, the nematodes will be subjected to various environmental stresses which can influence a variety of activities. Three of the most important factors influencing soil-dwelling nematodes are soil type, moisture and temperature (Wallace, 1961). In the population study (Chapter 4), which was conducted in a small area of natural grassland, variations in soil type would be expected to be relatively unimportant. Similarly, the grass-layer covering the soil moderates moisture content. However, soil temperature fluctuates markedly on a daily and annual basis as well as with depth. Therefore, in any particular locality, temperature is probably the environmental variable which has the greatest influence on the nematodes.

The effects of temperature on the activity, survival and development of soil-dwelling nematodes have been described in the literature (for example, Wallace, 1963). Considerable differences in responses between nematode species from different climatic regions, and even between nematode species from the same locality, may occur (Dao, 1970). For steinernematids, different responses to temperature have been shown between various species, between different geographical isolates of the sampe species, and even between isolates from the same geographical area

(Molyneux, 1986). Infectivity, development, reproduction and emergence of infective juveniles can each have different temperature optima and ranges (Molyneux, 1984). Most of the work on the effects of temperature on development, reproduction and emergence of Steinernema nematodes involved S. feltiae (Kaya, 1977; Molyneux, 1986; Pye and Burman, 1978), although one study included S. glaseri (Molyneux, 1986). Similar studies on S. bibionis and its various strains have yet to be made.

Some studies compared development of different species at the same temperature. Thus, Poinar (1979) reported variations in development rates among several Steinernema species and Molyneux (1986) compared different species of both Heterorhabditis and Steinernema. He found that S. glaseri KG strain developed at the fastest rate at each temperature tested (12, 15, 20, 23, 25, 30 and 32^{o}C). However, the development rates of different strains of the same species have not been studied, especially for S. bibionis strains.

Reproductive rate, defined as the number of infective juveniles produced per G. mellonella larva, has been estimated for S. feltiae and S. glaseri (Kaya, 1977; Molyneux, 1983). These studies referred to "numbers produced by at least one pair of adults". However, the number of dauer larvae produced per G. mellonella larva depends on the number of adults present inside the insect (Pye and Burman, 1978). Since the number of adults per host was not recorded by either Kaya (1977) or Molyneux (1983), their estimates are unsatisfactory.

Differences in temperature responses of various Steinernema nematodes may reflect the climate of the locality from which they were originally isolated (Akhurst and Brooks, 1984). Therefore, it should be

possible to find cold-adapted strains or species by assessing and comparing the various activities of different isolates at low temperatures. Finney (1985) followed this rationale when she attempted to isolate cold-tolerant entomopathogenic nematodes from soil. The temperature responses of the nematodes which she isolated have not been reported.

In reading the literature, it was found that different authors confused different processes or used different terms interchangeably. For example, "development and reproduction", "reproduction", and "production". In fact, they are different processes as distinguished by Molyneux (1983). "Development" means that the penetrated infective juveniles grow to the adult stage; "Reproduction" means that the developed nematodes (females and males) mated and the females produced eggs; "Production" means that the infective juveniles can finally emerge from the insect cadavers. "Development and reproduction" may include the meaning of both developing and reproducing processes, but it does not include production of infective juveniles, which most authors included in their meaning of "development and reproduction". Therefore, throughout this study, the terms "development", "reproduction" and "production" are used as defined above so that there is no ambiguity in meaning.

This chapter describes a series of experiments designed to compare the infectivity, development, reproduction and production of different strains of S. bibionis over a range of temperatures. Such information does not exist for nematodes of this species. Moreover, because some of the strains originated from British soils, the results would indicate whether any adaptations to low temperatures had occurred.

5.2 MATERIALS AND METHODS

Nematodes used were S. bibionis (Nashes), S. bibionis (Site 42) and S. bibionis (T319) (see 2.2 for their origins). In all experiments, infective juveniles were obtained from in vivo culture 7 days after emergence began at 20°C. They were stored at 10°C for two weeks before they were used.

5.2.1 Comparision of three strains of S. bibionis

Effect of temperature on infectivity

Late instar larvae of G. mellonella were exposed individually to nematode infection within plastic tubes filled with moist sand (see 2.3). A dose of 40 infective juveniles per larva was used with 20 replicates for each nematode/temperature combination. Nematode infectivity was studied at 5, 10, 15, 20, 25, and 30°C.

After an exposure time of 48 hrs at each temperature, the Galleria larvae were placed on moist filter paper and incubated at 20°C for a further 48 hrs when the dissections started. The number of nematodes which had successfully entered the haemocoel was recorded.

Effect of temperature on production of juveniles

Late instar larvae of G. mellonella were infected in the sand the same way as above with a dose of 10 S. bibionis infective juveniles from one of the three sources. There were 20 replicates for each nematode strain. The sand tubes were maintained at 20°C for 48 hrs to allow infection to occur. Then the dead insects were placed individually in plastic tubes (diameter 3.5cm, height 5.0cm) containing 10ml of tap water. Each cadaver was supported above the water level by a piece of

filter paper (length 5.0cm, width 1.5cm) as described by Kaya (1977) but with some modifications. The filter paper was folded so that its edges rested on the bottom of the tube while the flat centre portion supported the cadaver. Nematodes that emerged moved down the filter paper into the water. The tubes were covered with parafilm and incubated at the required temperature.

Emergence of the nematodes was observed daily and allowed to proceed until no further emergence occurred. The nematode suspension was diluted and the nematodes in five 1ml samples were counted. The mean of the 5 counts was used to estimate the number of juveniles that emerged per cadaver. Cadavers which did not produce juveniles were dissected and examined under a dissecting microscope to ascertain the reasons for their failure to produce nematodes.

Infection and development of infective juveniles at different temperatures

Late instar larvae of G. mellonella were exposed individually to nematodes within sand tubes. A dose of 40 infective juveniles was used for each nematode strain with 40 replications at temperatures of 5-30^{o}C at 5 degree increments. There were 20 nematode-free controls at each temperature to see if the low and high temperatures in themselves were lethal. After the tubes were incubated at each of the 6 temperatures for 48 hrs, the Galleria larvae were removed from the sand and rinsed with water. For the temperatures from 15 to 30^{o}C, the insects killed by nematodes could be identified by the typical ochre colour of the cadavers. The dead insects were divided randomly into two groups. Those in Group 1 were placed on moist filter paper and then incubated at

the infection temperature for 4 more days when they were put on "white traps". They were examined for emergence of infective juveniles daily. Those in Group 2 were placed on moist filter paper and incubated at the infection temperature for 14 more days when they were dissected to reveal the developing stages in each cadaver. The Galleria larvae infected at 5 and 10°C were usually immobile after 48 hrs and the colour indicating a successful infection was not apparant. Because insects killed by the nematodes could not be identified, the 40 insects were divided into two groups of 20 and treated the same as those infected at higher temperatures. Because of the absence of colour, mortality of the insects at 5 and 10°C was calculated from the results of the dissections on day 14.

Effect of temperature on nematode production from a founder female

Late instar larvae of G. mellonella were weighed and those between 120 to 140mg were used in this study.

Each of 60 larvae was injected with 2 infective juveniles from a stock population of either S. bibionis (T319) or S. bibionis (Site 42) by the method described in 3.2.2. The larvae were placed in a plastic Petri dish lined with moistened filter paper and incubated at 10, 15 or 20°C. Four days later, the dead Galleria larvae were placed in plastic tubes individually on a piece of folded filter paper. Fecundity was assessed by counting the total number of infective-stage juveniles that emerged per cadaver.

5.2.2 Development of S. bibionis (Nashes) at low temperatures

The Nashes strain of S. bibionis was used in this study and the

temperatures chosen reflected those that usually occur in soil under British weather conditions. Two separate experiments were carried out.

Development at 5°C

Three infecting methods were used to ensure enough successful infections for further studies. One hundred and twenty late instar larvae of G. mellonella were exposed individually to 50 infective juveniles in sand at 5°C. Two days later, another 120 larvae were infected in the same way at 15°C. On the 4th day, 120 larvae were individually injected with 10 infective juveniles. On the same day, the larvae that were subjected to nematode infection at 5°C for 3 days and at 15°C for 1 day were recovered from the sand. The insects obtained from these three treatments were placed on moist filter paper and maintained at 5°C. They were dissected at two-day intervals until the 6th day. After that, they were dissected on day 15, 30, 60 and 90. At least 10 insects from each treatment were dissected on each day. The developing stages and the colour change of the cadavers were observed and recorded.

Development at 10°C

One hundred late instar larvae of the wax moth were exposed individually to 40 infective juveniles in sand at 10°C. After 7 days, the insects were recovered from the sand, rinsed with clean water and treated as follows:

a. for those that were dead, the cadavers were placed in a plastic Petri dish lined with moist filter paper and incubated at 10°C,

b. for those that were still alive, half were maintained at 10°C on

filter paper while the other half were kept at 15°C on filter paper. This was to ascertain whether the hosts had been infected, but had not died because of the low temperature.

Dissections to reveal the stage of development of the nematodes began 24 hrs later for the three treatments.

5.2.3 Effect of sudden transfer to low or high temperatures

Sixty late instar larvae of G. mellonella were exposed at 12°C individually to 40 infective juveniles in sand. After 8 days at 12°C, the insects were recovered and the dead were divided into 2 groups. One was maintained at 12°C to reveal the time required to complete the life cycle at this temperature. To see how an 8-day infection would be affected by sudden transfer to different temperatures, most of the second group was transferred to 5°C and dissections were carried out on the 4th, 8th and 15th day after transfer to 5°C. On the 15th day, the 5 remaining cadavers were transferred back to 12°C and dissected 4 days later. Ten cadavers infected at 12°C were transferred to a higher temperature (15°C) for 4 days before being dissected.

5.3 RESULTS

5.3.1 Temperature effects on three strains of S. bibionis

Effect of temperature on infectivity (Figs. 5.1 and 5.2)

In this experiment, infectivity was assessed by allowing the nematodes to infect at different temperatures and then removing the cadavers to an optimal temperature to allow development. The three strains of S. bibionis could infect G. mellonella late instar larvae throughout the

temperature range of 5 to 30°C. The percent mortality and the mean numbers of nematodes which successfully infected the insects at the temperatures tested are presented in Figures 5.1 and 5.2.

In general, S. bibionis (Site 42) was similar to the T319 strain in its ability to parasitize Galleria larvae at the six temperatures tested (Fig. 5.1). However, the Nashes strain was much more lethal at 5°C and much less lethal at 30°C. High mortalities caused by the T319 and Site 42 strains maintained throughout the temperature range of 10 to 30°C, whereas the Nashes strain produced high mortalities between 5 and 25°C.

Data for mean abundance (mean numbers of nematodes in all 20 insects, ie. including uninfected hosts) of nematodes recovered from the haemocoel (Fig. 5.2) revealed that temperature affected the nemtodes more than the mortality of the hosts suggests. That is, mortality of the hosts has no relation to the numbers that penetrated. At 15 and 20°C, there were no significant differences between the mean numbers of nematodes which had entered for the three strains (ANOVA, $F=1.5$, $p>0.15$, $p>0.05$, respectively). However, at temperatures outside this range, the abundance showed a clear tendency to decline. At 10°C, although the insect mortalities were 90-95% (Fig. 5.1), the abundance of the three strains had declined to a mean of about 4 nematodes per insect. At 25°C, the mean abundance of Site 42 nematodes was as high as it was at 15 or 20°C, whereas the Nashes and T319 strains had decreased to a mean of about 6 per host.

Data for the numbers of nematodes per host at 5 and 30°C confirmed the observation based on mortality of the hosts that the Nashes strain

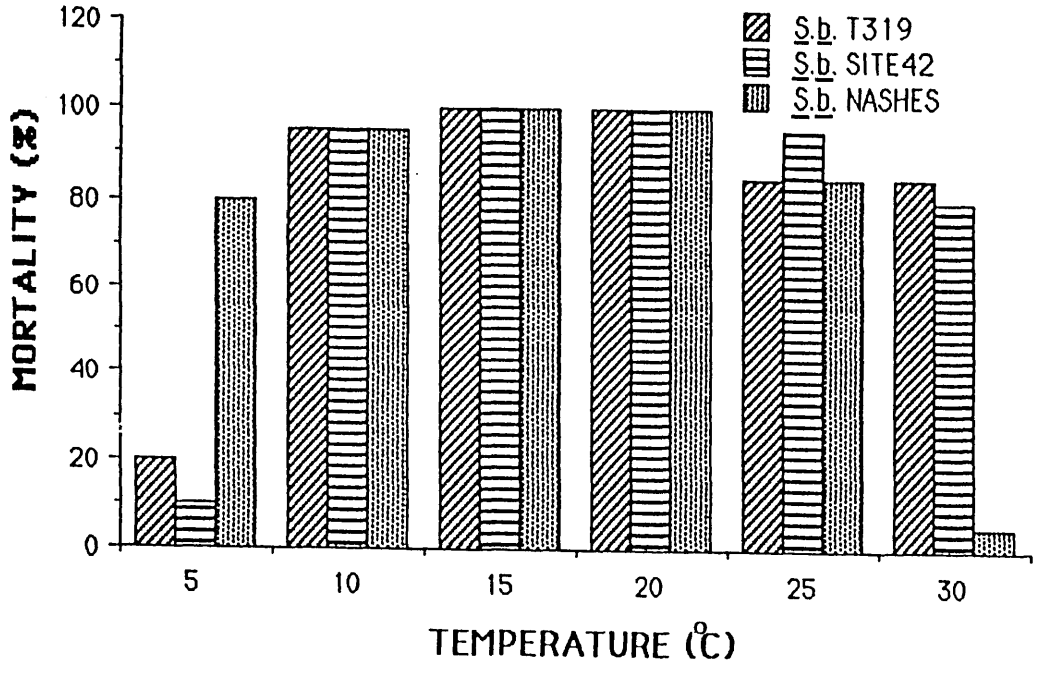


FIGURE 5.1 Mortality of late instar larvae of Galleria mellonella (L.) exposed individually to doses of 40 juveniles of three strains of Steinernema bibionis in sand at different temperatures (N = 20). After 48 hrs, hosts were incubated at 20°C.

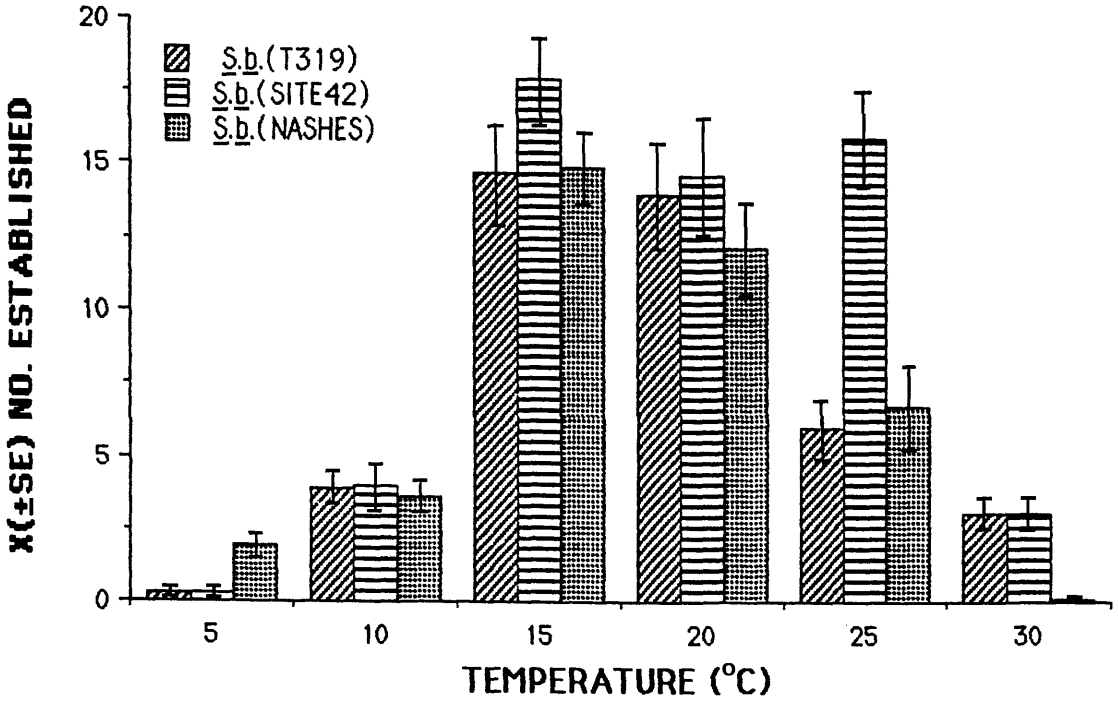


FIGURE 5.2 Mean abundance* (± se) of nematodes in haemocoel of 20 Galleria larvae exposed individually to doses of 40 infective juveniles of three strains of Steinernema bibionis in sand at different temperatures. After 48 hrs hosts were incubated at 20°C

*Abundance = mean number recovered from 20 Galleria larvae.

is adapted to low temperatures. Thus, at 5°C, 16/20 hosts harboured the Nashes strain compared to only 4/20 with the T319 strain and only 2/20 with Site 42 nematodes. The mean abundance figures reflected these differences. By contrast, at 30°C, only one insect was infected by Nashes nematodes compared to 16 by Site 42, and 17 by T319 worms. Again, data for the mean abundance at this temperature reflected these differences.

The conclusion from Figure 5.2 is that the Site 42 strain has the widest temperature range for infectivity, being able to infect with more nematodes at temperatures of 15-25°C. The Nashes strain is better adapted to low temperatures.

None of the Galleria larvae was killed by any of the S. bibionis strains within 48 hrs at 5 and 10°C. In contrast, the nematodes caused the insects to die within 48 hrs at temperatures above 10°C. This probably reflects the effect of temperature on the release and multiplication of the symbiotic bacteria inside the Galleria larvae.

Effect of temperature on production of juveniles (Table 5.1)

In this experiment, production of juveniles was assessed by allowing the nematodes to infect the hosts at an optimal temperature and then placing the cadavers at different temperatures. The optimal temperatures for production of juveniles were 15 and 20°C (Table 5.1) for the three strains tested. The large variability was probably caused by an inability to control the number of reproducing females in each insect. At 20°C, the Site 42 strain produced significantly more nematodes than the others at the same temperature (ANOVA, $F=10.51$, $p<0.01$). At 15°C, production of nematodes was not significantly different for the

TABLE 5.1: Effect of temperature on number of juveniles emerging from Galleria larvae after exposure to 10 infectives per larva in sand for 48 hrs at 15°C. The numbers are means and standard errors calculated from those larvae that produced infectives.

Strains	Temperature (°C)					
	5	10	15	20	25	30
S. bibionis T319	0 (n = 20)	7946 ± 2942 (n = 5)	38875 ± 7637 (n = 8)	30729 ± 5445 (n = 7)	18864 ± 5152 (n = 5)	0 (n = 0)
S. bibionis Site 42	0 (n = 20)	18047 ± 4278 (n = 6)	34585 ± 6279 (n = 15)	53289 ± 4643 (n = 10)	29145 ± 6492 (n = 6)	0 (n = 20)
S. bibionis Nashes	A few (n = 1)	13304 ± 3338 (n = 5)	25740 ± 7442 (n = 9)	16560 ± 10420 (n = 3)	0 (n = 20)	0 (n = 20)

three strains (ANOVA, $F=0.81$, $p>0.05$). Similarly, there were no significant differences between the number of nematodes producted at $10^{o}C$ for the three strains (ANOVA, $F=1.72$, $p>0.05$).

At $5^{o}C$, only Nashes strain was able to produce a few infective juveniles. This required 3 months (compared to almost one month at $10^{o}C$) and was unexpected, so the samples were amalgamated before the nematodes were noticed which prevented accurate counts. Emergence of infective juveniles was observed from one cadaver and four more cadavers were full of well developed nematodes (2nd generation adults and their progeny) when they were dissected. Dissection of the cadavers infected with S. bibionis (Site 42) and S. bibionis (T319) revealed that these nematodes could develop to 1st generation giant females and males and some females contained eggs.

The nematodes were also differently affected at the upper end of the temperature range. At $25^{o}C$, the Nashes strain produced no nematodes while the other two produced large numbers. Cadavers with the Nashes strain went fetid, which occasionally occurred for the T319 strain and rarely for the one from Site 42. However, at $30^{o}C$, none of the strains produced nematodes and all cadavers went fetid.

Infection and subsequent development of infective juveniles at different temperatures (Table 5.2)

Infection and subsequent development was assessed by allowing both infection and development to occur at the same temperature. The three strains of S. bibionis displayed the same optimal temperature range (15 - $20^{o}C$) for infection and subsequent development and reproduction. By day 14, infective juveniles emerged from most of the dead insects in the

optimal temperature range. At 25 and 30°C, the three strains showed some differences in their infectivity and subsequent development. S. bibionis (T319) and S. bibionis (Site 42), in general, showed a similar ability to infect G. mellonella larvae while infectivity of the Nashes strain was much lower, especially at 30°C. There were also substantial differences between the three strains in their development and reproduction. At 25°C, the T319 and Site 42 strains could develop and reproduce, but at different rates. All the cadavers infected with S. bibionis (Site 42) were full of active nematode juveniles and dauerlarvae started emerging from one cadaver on the 14th day. A few cadavers infected with S. bibionis (T319) contained 2nd generation adults, but most contained pre-J3 stages. At 25°C, the Nashes strain developed into 1st generation adults but then died. Large numbers of infective juveniles emerged from all cadavers infected with Site 42 nematodes after three weeks, a few emerged from cadavers with T319 nematodes after 4 weeks and none emerged from the ones infected with the Nashes strain. At 30°C, S. bibionis (Site 42) and S. bibionis (T319) were able to infect the insects and developed to 1st generation adults but they failed to reproduce S. bibionis (Nashes) rarely infected a host and could not be found on the 14th day, probably due to decay of the dead nematodes.

The three strains were also affected differently at low temperatures. At 10°C, S. bibionis (Site 42) showed higher infectivity than either S. bibionis (T319) or S. bibionis (Nashes). After 14 days, some nematodes from the three strains had developed to 1st generation adults with young males predominating. Infective juveniles emerged from a few cadavers infected with S. bibionis (Site 42) and S. bibionis (Nashes) after 5 weeks compared to 7 weeks for S. bibionis (T319). At 5°C, only

TABLE 5.2: Effect of temperature on infection (exposure to 40 juveniles for 48 hrs) and subsequent development of S. bibionis in Galleria larva.

Strains	Temperature °C											
	5		10		15		20		25		30	
	A*	B**	A	B	A	B	A	B	A	B	A	B
S. bibionis (T319)	0	none infected pre-J3	30	alive giant	100	J3 emerge	100	J3 emerge	92.5	most formed	90	dead giant
S. bibionis (Site 42)	0	none infected	80	alive giant	100	J3 emerge	100	J3 emerge	95.0	all nematodes pre J3	90	dead giant
S. bibionis (Nashes)	40	alive 4th	30	alive giant	100	J3 emerge	100	J3 emerge	82.5	dead giant	5	nematode not found

A* percent of the insects infected with nematodes. 5 - 10°C, n = 20; 15 - 30°C, n = 40.

B** developing stages by day 14 after infection of the hosts (n < 20)

S. bibionis (Nashes) could infect the larvae. The nematodes inside the cadavers developed to young males or 4th stage females within 14 days.

Generally, S. bibionis (Nashes) is better adapted for infection and development at lower temperatures than either S. bibionis (Site 42) or S. bibionis (T319). Of the three strains, Site 42 has the fastest average rate of development over a wider range of temperatures compared to the other two strains.

Effect of temperature on nematode production from one founder female (Fig. 5.3)

Because the population of nematodes may be affected by density-dependent processes, the number of juveniles produced by a single founder female was assessed. The numbers produced by one female of S. bibionis (Site 42) were significantly higher than the numbers produced by S. bibionis (T319) at the three temperatures tested (Fig. 5.3. t-tests, $p<0.05$). There were no significant differences between the mean number of juveniles produced by S. bibionis (Site 42) at the three temperatures. However, the mean number produced by S. bibionis (T319) at $12^{o}C$ was significantly lower than that produced at 15 and $20^{o}C$ (t-test, $t = 3.082$ $p = 0.08$ and $t = 3.439$ $p = 0.004$ respectively).

The highest number of infective juveniles arising from a single female was 103,320 (S. bibionis Site 42 strain at $20^{o}C$).

5.3.2 Development of S. bibionis (Nashes) at low temperatures

Development at $5^{o}C$ (Table 5.3)

The results of development of S. bibionis (Nashes) at $5^{o}C$ in late instar larvae of G. mellonella after infection by 3 different methods

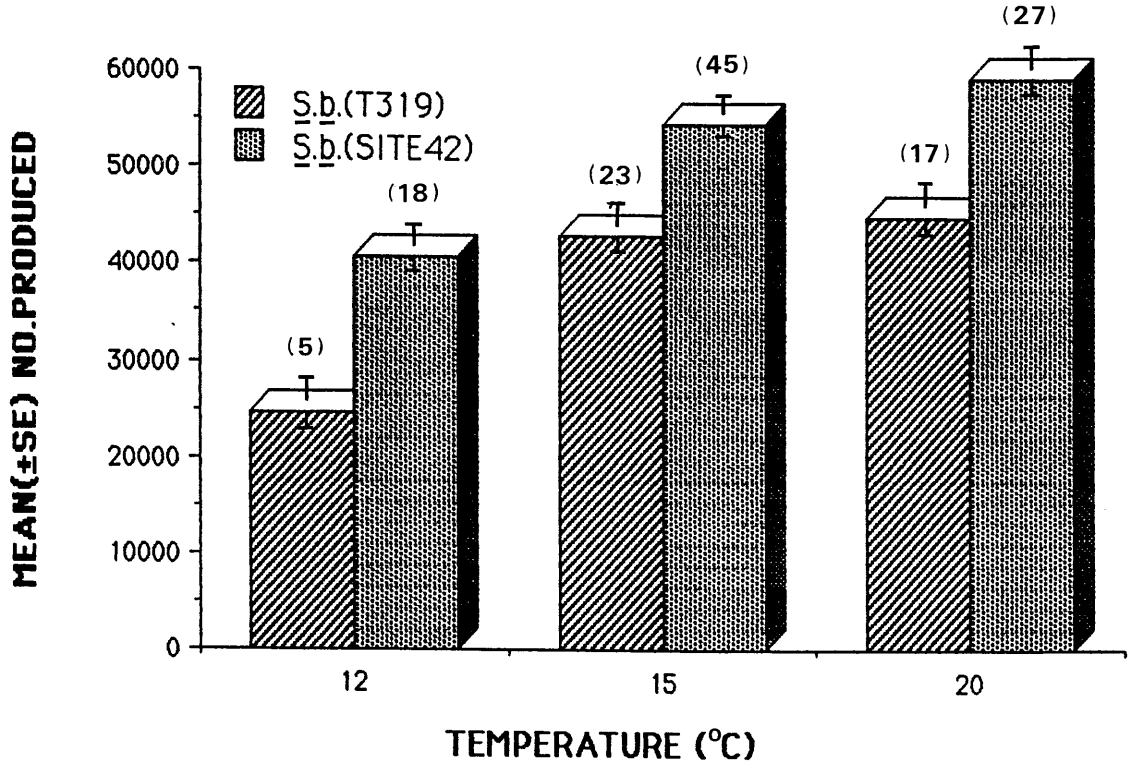


FIGURE 5.3 The mean (± se) number of infective juveniles produced by one pair of adults of Steinernema bibionis.
Number in brackets = sample size.

are presented in Table 5.3. The different methods of infection were used because it was not possible to predict which would yield enough infections for assessments. Treatments B and C proved more satisfactory than A (Table 5.3).

The whole developmental cycle of the population of S. bibionis (Nashes) at 5°C was estimated to be a minimum of 90 days. Within 6 days, the nematodes had moulted into the young J4 stage. By day 15, most of the nematodes were 1st generation adults and some of the females were gravid. After 30 days most of the females were gravid and a few of the eggs had hatched. On the 60th day, the nematode juveniles had developed to 4th stage larvae and some had developed into 2nd generation adults. The last dissections and observations on day 90 revealed that infective stage juveniles were formed or forming and some were emerging from the cadavers.

The colour change of the cadavers, indicating successful development of the symbiotic bacteria, was also noted. Most of the cadavers showed a typical bacterial-induced ochre colour after 15 days when the nematodes were at the adult stage. Dissection of the cadavers without this colour revealed no nematodes or nematodes at much earlier stages of development.

Development at 10°C

When the Galleria larvae were recovered from the sand with infective juveniles after 7 days at 10°C, 50/100 of the insects were motionless and appeared dead while the rest were still moving. Half of the mobile insects were retained at 10°C and the other half moved to 15°C

TABLE 5.3: Stages of development of S. bibionis (Nashes) in Galleria larvae at 5°C after infection by 3 different procedures.

Post exposure at 5°C (Days)	Treatment A (Infection at 5°C for 72 hrs)	Treatment B (Infection at 15°C for 24 hrs)	Treatment C (Injection with 10 infective juveniles)
2	*(3/10) J3	(9/10) J3	(9/10) J3
4	(3/10) most J3	(6/10) some young J4	(10/10) most J3
6	(6/10) young J4	(9/10) young J4	(8/10) young J4
15**	(6/30) giant gravid	(23/25) giant gravid	(9/14) giant gravid
30	(5/10) most alive some — eggs + J1	(9/10) all alive some — eggs	(8/10) all alive some — eggs + J1
60	(4/10) most juvenile dead, some — J4	(9/10) most alive J1-J4 (some dead)	(6/10) all alive — 2nd generation
90***	(2/10) pre - J3	(6/22) pre - J3 (some dead)	(30/38) 10/30 J3 emerging 13/30 dead J3 7/30 reproducing

* Numbers in brackets indicate proportion of successful infections

** On day 15, the cadavers which did not show the typical colour indicating an infection were dissected and examined.

*** On day 90, the remaining cadavers were dissected and examined.

for 24 hrs. Most of the insects at 10°C died on the 8th day and those at 15°C died and showed the typical ochre colour induced by the associated symbiotic bacterium. These results showed that most of the insects had been infected.

At 10°C, colour development in dead insects was slow. On day 7, when the insects were removed from the sand, none of the dead insects showed the colour. Nine days after the insects were placed on filter paper at 10°C, 45/55 of the cadavers were yellow. When dissecting the 10- cadavers without the colour, it was found that 5 contained small nematodes while the other 5 were uninfected. The nematodes inside the cadavers with the obvious colour had developed to mature females and males. On day 17, 10 cadavers which had been maintained at 10°C were put on "White traps". When they were examined two weeks later, infective juveniles had emerged. The whole life cycle at 10°C for the Nashes strain took about 40 days.

5.3.3 Effect of sudden transfer to low or high temperatures

The life cycle of the Nashes strain (J3-J3) could be completed in about 20 days at 12°C. As expected, raising the temperature to 15°C enhanced development of the nematodes. Thus, after 8 days at 12°C and a further 4 at 15°C infective juveniles emerged from 5/10 of the cadavers, while at 12°C, 2/10 of the cadavers contained only eggs and Jls from the 1st generation adults. Reducing the temperature to 5°C decreased the rate of development but did not kill the nematodes which had already developed to adults within 15 days. When they were placed back to 12°C for a further 4 days, all 5 contained pre-infective stage juveniles, showing that the nematodes which had gone through chilling at 5°C could develop normally when they were returned to higher temperatures.

5.4 DISCUSSION

This is the first study to document the intensity of infection of S. bibionis at different temperatures. The ability of steinernematids to move and to locate and infect an insect is influenced by temperature (Molyneux, 1986). The lower temperature limits for movement and infectivity have been ascribed to the climate in which the nematodes are genetically selected through natural selection. Thus different strains of the same species from different geographical locations have been found to possess different temperature ranges for infectivity (Molyneux, 1984, 1986). Comparison of the infectivity of the three strains of S. bibionis showed that this species was affected similarly. Although the three strains could infect G. mellonella larvae throughout the range of 5 - 30°C, optimal infectivity differed between them (Figs. 5.1 and 5.2). In general, S. bibionis (T319, Tasmanian) and S. bibionis (Site 42, British) displayed a similar optimal temperature range for infestation of the Galleria larvae (10 - 30°C), whereas the other British strain (Nashes) favoured the colder end of the range (5 - 25°C). This result is surprising because the climate at the three localities is similar, especially for the two British strains. Perhaps the Nashes strain is (or has) adapted to low temperatures because of genetic drift, for example, change in the temperature sensitivity of the gene or genes for infectivity.

The results demonstrated that the effect of temperature on the number of nematodes which could penetrate and establish was greater than the effect on the percent of the insect hosts which were parasitized. For example, S. bibionis (Site 42) attained maximum numbers within the hosts between 15 and 25°C, but there was no difference over the range of

10 to 30°C for its infectivity. S. bibionis (T319) had the same temperature range for optimal infectivity, but displayed a more restricted temperature range (15 and 20°C) for maximum abundance of the nematodes. These results are important because percent mortality or parasitization is often used to assess and compare infectivities of different steinernematids at various temperatures. While this assessment does give an indication of nematode ability to parasitize insect hosts, it does not contribute to an understanding of the number of nematodes which can parasitize the hosts. In fact, for a similar death rate at different temperatures or by different strains of the nematode at the same temperature, the abundance of the parasites may vary a great deal. For example, from 10 to 25°C, the percent mortalities caused by S. bibionis (T319) ranged from 95 to 100%, while the mean numbers of nematodes ranged from 3.9/larva to 17.9/larva (7.8 to 35.8% of the initial dose applied). Similarly, at 25°C, the mortalities caused by T319, Site 42 and Nashes strains were 85, 95 and 85% respectively, while the mean numbers of nematodes ranged from 6.0 to 15.9 per larva (12 to 31.8% of the initial doses applied). Therefore, to understand the population dynamics of the nematodes, the numbers of parasites per host is the important variable while the percent mortality caused by the parasites is less important.

The much lower number of nematodes in larvae at temperatures below 15°C may be a result of reduced mobility of the infective juveniles. Those nematodes which could penetrate may have certain differences in their ability to adapt to a cold environment. Although there may be possibilities for finding or selecting for strains active at low temperatures (Finney, 1985), the study on intensity of nematode infestation

indicated that it is unlikely that all the nematodes isolated from a cold climate will have the same ability. The relationship between nematode activity and attraction of G. mellonella larvae may also be a factor which influenced the numbers that penetrated. At temperatures below 11 - 12°C (Blackshaw and Newell, 1987), Galleria larvae were usually immobile. The release of CO_2 or other excreted materials which are usually regarded as attractants to infective juveniles (Gaugler et al., 1980) would undoubtedly be affected by the low metabolic rate of the Galleria larvae at the low temperatures. Therefore the effectiveness of metabolites in attracting the nematodes to the hosts could be greatly reduced.

At low temperatures, the growth of the nematode and the associated bacterium was much slower than at higher temperatures and the number of parasites per host was low. Perhaps Galleria larvae which are colonised quickly at higher temperatures, attract more nematodes to enter. This kind of attraction, the result of rapid development of the nematodes and bacteria which produce substances that increase penetration by more infective juveniles, would not be achieved at the low temperatures. This possibility is examined in experiments documented in Chapter 6.

Development and reproduction of infective juveniles in larvae of G. mellonella were also influenced by temperature. Generally, the three strains were able to develop and reproduce from 5 to 25°C. However, the degree varied for strains and temperatures. In particular, the Nashes strain favoured colder temperatures while the T319 and Site 42 strains were better adapted to higher temperatures.

In agreement with previous observations (Molyneux 1986), parasitization of G. mellonella by S. bibionis occurred in a temperature range

that was greater than that permitting nematode development and reproduction. The ranges for infectivity, development and reproduction of the three strains show some similarities and some differences when compared with the two Australian strains that Molyneux tested. In the present study, the three strains possessed the same temperature range for infectivity (5 to 30°C) whereas Molyneux reported that the temperature range for infectivity of the T335 and N60 strains were from 3 to 30°C. Because 5°C was the lowest temperature tested in the present study, it may be that the three strains are able to infect Galleria larvae at temperatures below 5°C. The temperature ranges for development and reproduction were also the same for the three strains (5 - 25°C), while the ranges for production of infective juveniles were different and narrower. Similar results were also obtained for the T335 and N60 strains (Molyneux, 1986) in that the ranges for production of infective juveniles were narrower than those for development and reproduction. However, the lower temperature limit supporting production of the T335 (15°C) and N60 (12°C) strains was different from that of the T319 (10°C), Site 42 (10°C) and Nashes (5°C) strains examined in the present study. The higher limit for the T335, T319 and Site 42 strains was the same, but the 23°C limit for the N60 and 20°C for the Nashes strain shows that they were adapted to lower temperatures. The differences in temperature ranges for production of infective stages as shown by the Site 42 (10 - 25°C) and Nashes (5 - 20°C) strains supported the finding (Molyneux, 1986) that different strains of the same species may have different temperature ranges for production, even if they were isolated from the same geographical region.

Generally, the temperature range allowing development and reproduction can be associated with the temperatures usually encountered in the localities from which the nematodes were isolated (Molyneux, 1983). Site 42 and Nashes nematodes were isolated from Britain while the T319, T335 and N60 strains were isolated from a milder temperate area of Australia (Molyneux, 1983). The differences documented for the strains from the different areas may have reflected influences of the temperatures experienced locally. S. kraussei, which was isolated from Poland which has a more extreme continental climate, possessed a temperature range of 6 - 20°C for its development and reproduction (Molyneux, 1983). Although S. bibionis (Nashes) and S. kraussei are two different species, they have a similar temperature range for development and reproduction which supports further Molyneux's statement of association of temperature limits with the usually encountered local temperatures. On the other hand, these data add weight to the suspicion that S. kraussei is actually S. bibionis. Hand (pers. comm.) isolated the bacteria from S. kraussei which was identical to that of S. bibionis.

S. feltiae is another steinernematid which has been studied over a wide temperature range (Kaya, 1977; Molyneux, 1986; Pye and Burman, 1978). As in the present study for S. bibionis, different temperature limits for development and reproduction of different strains of S. feltiae were discovered. Thus, Molyneux (1986) showed that the Agriotos (Russian) strain did not reproduce at temperatures above 30°C whereas Kaya (1977) reported that S. feltiae DD-136 (American) strain did not reproduce at temperatures above 28°C. Sometimes, differences for the same strain were reported. Thus, the lower limit for reproduction of S. feltiae Agriotos was found between 13 - 15°C (Pye and Burman, 1978)

while Molyneux (1983) obtained infective juveniles from G. mellonella larvae infected with the same strain of S. feltiae at 12°C. One explanation for this difference may be that these two authors used different techniques during their studies. Molyneux (1983) left the cadavers at 12°C for 70 days whereas Pye and Burman (1978) left the cadavers at 13°C for only 30 days. Longer time may be required for the nematodes to emerge. It is also possible that genetic drift or adaptations to culturing procedures in different laboratories may have occurred. The Agriotos strain of S. feltiae was first isolated in 1970 (Poinar and Veremchuk, 1970) and has undergone countless generations in different laboratories since then. Similaraly, the DD-136 strain was originally isolated by Dutky in 1955 (Dutky, 1955).

Comparing S. feltiae with S. bibionis, these two steinernematids showed different preferences to temperature. S. bibionis is a species which is adapted to lower temperatures while S. feltiae is a species which is adapted to higher temperatures. Again, the temperatures usually encountered in their natural habitats probably contributed to the differences between these two species. As mentioned before, the T319, T335 and N60 strains of S. bibionis were isolated from temperate areas of Australia with a mild climate (Akhurst, 1983b; Molyneux, 1983). However, the temperature limit for reproduction can be as low as 5°C, though no infective stage juveniles emerged (Molyneux, 1983 and present study). According to Bedding (cited in Molyneux, 1986), it is very likely that S. bibionis was introduced accidentally to Australia, most probably from Europe. Therefore, it is not surprising that the climatic influence may have been retained, although temperature adaptation can happen. The T335 strain, which was mobile at 4°C and could develop and

reproduce at 28°C (Molyneux, 1983) may be an example of temperature adaptation.

The number of infective juveniles produced by the three strains also differed as did the temperature range for maximum productivity. The Site 42 strain generally produced the highest number of infective juveniles whereas the Nashes strain produced the lowest. The Maximum number of infective juveniles produced by S. bibionis (Site 42) occurred between 15 and 25°C, whereas the T319 strain produced the maximum number over a narrower temperature range (15 - 20°C). This is the first documentation for the effect of temperature on the numbers of infective juveniles produced by S. bibionis. However, there is information for other steinernematids. S. feltiae (Agriotos) has been reported to produce maximum numbers of infective juveniles between 23 and 25°C and S. glaseri (KG strain) produced the most between 23 and 30°C (Molyneux, 1983). Kaya (1977) showed that the DD-136 strain of S. feltiae produced the maximum number of juveniles at 25°C. These results show once again that S. bibionis is better adapted to lower temperatures than S. feltiae.

The decline in production of infective juveniles at higher temperatures has been explained by a lack of nutrition and/or oxygen for nematode maturation as a result of competition with its own bacterial symbiont (Molyneux, 1986). A change of quality of the bacterial symbiont may also be an important factor. Both British strains were isolated from soil where the temperature rarely exceeds 20°C. The nematodes and their symbiont bacterium may have adapted to the cool temperature. Therefore, at higher temperatures, the bacteria may not be able to grow efficiently to provide the best environment for nematode production.

Contamination by fast growing foreign bacteria brought in during the process of infection may also affect production of infective juveniles at higher temperatures (Molyneux, 1986). Contaminated Galleria cadavers infected with S. bibionis (Nashes) were often observed at temperatures above 20°C while the cadavers infected with the Site 42 strain were usually clean. This may explain the difference between these two British strains for production of infective juveniles in the upper temperature ranges. The nematodes may also have an innate production potential which varies with strains. The decline in production of infective juveniles at lower temperatures could reflect slow growth of the symbiotic bacteria and/or the nematode's ability to produce eggs.

The results obtained in the experiment which assessed development and reproduction after infection at the same temperature (Table 5.2) supported and confirmed the results obtained in the earlier experiments where infectivity was separated from development and reproduction. The differences in the effects of temperature on development and reproduction were, if anything, more pronounced for the three strains. The Nashes strain was again shown to be capable of developing at 5°C. There were some differences between the mortalities obtained in this experiment and the first infection experiment, in particular at the lower end of the temperature range tested. This variability can be explained partly in terms of the way the two experiments were conducted. In the first experiment, the insects were exposed to the various temperatures for 48 hrs and then immediately transferred to 20°C for further development. In the second experiment, the insects were incubated and infected at the same temperatures and the percent infection was assessed by dissecting on day 14 after infection. It was possible, in this case, that

some insects were infected, but the nematodes died after penetration because they were unable to develop at low temperatures. Also difficulties in recognizing dead young nematodes in the insect may have resulted in a lower record for the percent of the insects infected. In contrast, when the insects were transferred to 20°C after the infection period, those nematodes which penetrated at the low temperatures would have better chances to resume development. Also growth of the symbiotic bacterium at 20°C supported development of the nematodes and recognition of insect death from infection became much easier and more reliable.

For large scale use of nematodes, it is important to know whether they can be produced economically and whether they can reproduce in the environment. One aspect which must be considered is fecundity of the nematodes, defined as the number of infective stages arising from a single mated female. Until now, such information was unavailable for S. bibionis. Georgis and Hague (1981) counted the number of eggs in 1st and 2nd generation females of S. feltiae. However, these were natural infections in sawfly (Cephalica lariciphila) and they could not control the number of females present in a host. Nor is it known whether all the eggs in a female represents her fecundity or whether she will reproduce more eggs as she oviposits. Others (Kaya, 1977; Molyneux, 1986; Pye and Burman, 1978) also failed to control the numbers of females in a host when estimating fecundity. This problem was overcome in the present study by injecting one pair of nematodes into each host. Then, temperatures usually experienced in British soil were examined for their effect on production of dauerlarvae by a single female. The Site 42 strain produced more infectives than the Australian T319 strain at all temperatures tested. Indeed, the number produced by the Site 42 strain

at 12°C was nearly twice that produced by the T319 strain. Thus, the British strain is capable of better production than the Australian one at temperatures that would be expected in British soil. The reasons for the superior performance of the Site 42 strain at the higher temperatures are unknown. One possibility is that the T319 strain has been in culture for a comparatively long time, while the Site 42 strain has been recently isolated from the soil. It is possible that over long periods, cultural practices may have reduced the genetic variability of nematode stocks and their bacteria and thus changed their ability to adapt to different temperatures (Dunphy and Webster, 1986). Genetic adaption of the T319 strain to warmer Austrlian temperatures could be another possibility.

It was interesting that the Nashes strain developed and reproduced at 5°C and that dauerlarvae could even emerge at this temperature two months after infection. Until now, these are no reports that *Steinernema* nematodes can develop, reproduce and emerge at a temperature as low as 5°C, although they can infect insects at this temperature or even lower ones (Molyneux, 1986). Therefore, the more detailed study aimed at examining the developing stages of the nematode inside *Galleria* larvae at 5°C was carried out. The results confirmed that *S. bibionis* (Nashes) developed in *G. mellonella* larvae at 5°C, though at very low rates. The time taken to complete its life cycle at this temperature would require at least 90 days (Table 5.3).

Sudden transfer of *Galleria* larvae infected with *S. bibionis* (Nashes) from 12 to 5°C did not harm those nematodes which had already developed in the insect cadavers within 15 days. Similarly, when the temperature was raised suddenly, the nematodes continued to develop.

This reflects the ability of the nematodes to cope with changes in temperature, as would be expected in the field, although there the change will not be as sudden.

The present study also relates to the question of whether low temperatures could have a beneficial effect on dissemination of the nematode. Molyneux (1986) mentioned that some steinernematids may remain in the insects haemocoel as infective juveniles without killing the host until the temperature rises enough to allow bacterial growth and nematode reproduction. Similar results were obtained in the present study in that the Galleria larvae did not die soon after being infected at 5 or 10°C. A rise in temperature (to 15 or 20°C) resulted in rapid death of the infected Galleria larvae. However, contrary to Molyneux's (1986) observations, the infected Galleria larvae eventually died at the same low temperature (10°C), suggesting that the bacterium can grow and support nematode development, although at slow rates. During this prolonged period of development of the infection, the insects carrying the nematodes are not likely to migrate too far, unless adult insects (i.e. moths) are infected as shown by Timper et al. (1988).

In conclusion, the results documented in this chapter have emphasized the importance of temperature for infection, development and reproduction of steinernematids. Not only do different strains of the same species have different abilities to tolerate the cold, but also individuals vary in their tolerance. Therefore, the intensity of nematode infestation (number of nematodes penetrated) instead of percentage of hosts infected should be emphasized when attempting to evaluate or select cold-tolerant strains.

CHAPTER 6

ESTIMATION OF NUMBER OF NEMATODES IN A SOIL SAMPLE

6.1 INTRODUCTION

The infective juveniles of *Steinernema spp.* locate and parasitize insect hosts in the soil environment. Although there are many studies on the behaviour of plant parasitic nematodes, there are relatively few for entomopathogenic rhabditids. Nevertheless, attraction of steinernematids to *G. mellonella* larvae in soil has been demonstrated and used to isolate the infective juveniles from a soil sample (Bedding and Akhurst, 1975). The nature of the attractant(s) is unknown. Infective juveniles of *S. feltiae* respond to chemical gradients diffusing from *G. mellonella* larvae (Pye and Burman, 1981) and Gaugler *et al.* (1980) demonstrated orientation of this species to carbon dioxide. A thermosensory response has also been postulated (Byers and Poinar, 1982).

The *Galleria* trap (Bedding and Akhurst, 1975) has been widely used to investigate the distribution of steinernematids in various countries. In most cases, soil samples were bioassayed once and the insect mortality in the trap was used to estimate the prevalence. However, there is no evidence that shows that the number of insects which die in the *Galleria* trap is related to numbers of nematodes in the soil. Extracting the nematodes in one soil sample by using a succession of *Galleria* larvae and dissecting each insect would give the number of nematodes which had infected the insects. The question that remains is whether all the nematodes in the soil sample are recovered by this procedure. The limited evidence to date suggests that they are not all able to infect the insects. Thus, Bednarek and Nowicki (1986) showed

that no more than 50% of the infective juveniles of S. feltiae in contact with G. mellonella larvae in Petri dishes penetrated into them. Johnson (1986, unpublished) showed a similar phenomenon with infective juveniles of S. feltiae (DD-136) and S. bibionis (T319) in sand. In addition, if the nematode population is to be analysed quantitatively, it is important to know whether the population that could be extracted in the bioassay is predictable after a limited number of bioassays.

The experiments described in this chapter were designed to investigate the efficiency of Galleria traps for extracting S. bibionis from soil samples. The relationship between the nematode numbers recovered from insect cadavers and the number of infective larvae in sand samples was examined under various conditions. The results led to a discussion on the suitablity of a method for estimating the number of nematodes in a sample.

6.2 MATERIALS AND METHODS

6.2.1 Efficiency of Galleria trap

Recovery of nematodes from naturally infested soil

The data bases analysed were from the vertical distribution study in October 1986 and 1987 (Chapter 4). The soil samples were randomly chosen from different sites. Four to five cores from each site were combined to provide about 250cc (1986) or 200cc (1987) of soil for extracting infective nematodes by burying 5 G. mellonella larvae in the soil. The larvae were replaced at 4 - 6 day intervals until no further insect deaths occurred. This procedure was applied for soil samples taken at 4 depths. The numbers of nematodes dissected from the Galleria larvae could then be grouped by depth, site and times. The percentage

recovery on any particular occasion was calculated by taking the total accumulated number of nematodes recovered up to that time, divided by the total number of nematodes accumulated by the end of the assay, which represented the infective nematode population in the samples.

Recovery from "spiked" sand

Infective juveniles of S. bibionis (Nashes) whose age was no more than 14 days, and which had been stored at 10°C in water, were introduced in 1ml of water into sand-filled tubes (2.3) at doses of 10, 50, 100, 150 and 300 per tube. A Galleria larvae was placed on the sand surface and the tubes were incubated at 15°C for 1 week. The insects were changed weekly for a total of 6 times and at each time, the dead ones recovered were dissected. The number of nematodes present was recorded. There were 15 replicates for each of the 5 doses.

6.2.2 Nematode infection in living vs dead insects

Ninety sand tubes were prepared as usual and 60 were inoculated with a nematode suspension of 50 infective juveniles per tube (2.3). Each of 60 late instar larvae of G. mellonella was injected with 5 infective juveniles (3.2.2) and incubated at 15°C for 48 hrs. When they had died, 30 cadavers were placed on the surface of the sand in tubes with the nematodes and the others were placed on sand in tubes without nematodes (Control I). Thirty uninfected live Galleria larvae were placed on the sand in the remaining 30 of the tubes containing nematodes (Control II).

The tubes were incubated at 15°C. After 24, 48 and 72 hours, 10 tubes from each of the three treatments were removed and the Galleria

larvae were recovered and dissected. The nematodes inside the cadavers were counted.

6.2.3 Time course of infection

Two hundred tubes, each containing 50 infective juveniles of S.bibionis (Nashes), were set up. A late instar larva of G. mellonella was placed on the sand surface of each tube, which was then placed randomly into one of 4 groups with 50 tubes in each group. At the following times, the Galleria larva were recovered and replaced with fresh ones.

Group 1	24 hrs (a)	24 hrs (b)	24 hrs (c)
Group 2	24 hrs (a)	24 hrs (b)	
Group 3	48 hrs (a)	24 hrs (b)	
Group 4	72 hrs (a)	72 hrs (b)	72 hrs (c)

Those larvae which had been exposed to infection for 24 or 48 hrs were incubated on filter paper at 15°C for a further 1 or 2 days before dissection and examination for nematodes.

6.2.4 Dose-response analysis

Influence of exposure time

Late instar larvae of G. mellonella were exposed to S. bibionis (Nashes) juveniles in sand tubes at a dose of 50 nematodes per insect (2.3). The tubes were incubated in a plastic bag containing water-saturated tissue at 15°C for 24, 48 or 72 hrs. At the required time, 50 tubes were removed and the Galleria larvae were rinsed with tap water

and placed on moistened filter paper for a further 1 or 2 days at the same temperature. They were then dissected and the number of nematodes recorded.

Influence of dose and times of bioassays

Doses of 10, 50, 100, 150 and 300 infective nematodes of S. bibionis (Nashes) were introduced into sand-filled tubes. After addition of G. mellonella larvae, the tubes were incubated at 15°C for 72 hrs, when the first set of dissections started. The insects, both dead and alive, were removed and replaced with fresh ones without disturbing the sand. The second examination started 72 hrs later. This process was repeated until no more insects were infected. The Galleria larvae were rinsed, dissected and the numbers of nematodes were recorded. There were 15 replicates for each of the five doses.

Influence of different strains

The data analysed here were from the experiment described in Chapter 5. Infective juveniles of S. bibionis (T319) and the two British strains (Nashes and Site 42) were used. The dose applied was 40 juveniles per Galleria larva. There were 20 replicates for each of the three strains. The experiment was conducted at 15°C and the incubating time was 48 hrs.

Influence of temperature

The data analysed here were also from the experiment described in Chapter 5, in which Galleria larvae were exposed to nematode infections at 5, 10, 15, 20, 25 or 30°C in sand tubes, containing 40 S. bibionis

(Nashes) juveniles in each tube. After 48 hrs, the insects were transferred to moistened filter paper in Petri dishes at 20°C. Dissections and counts were made 4 days later. There were 20 replicates for each temperature treatment.

Influence of group and individual infections

This experiment was to assess the effect of using large containers which were similar to those used in the Galleria trap. Thus, 300ml plastic containers were filled with moistened sand (water content = 8.0%, v/v). Doses of 400, 600, 800 and 1000 nematodes of S. bibionis (Nashes) per container were applied. Five late instar larvae of G. mellonella larvae were placed on the surface. There were five replicates for each of the four doses. The containers were incubated at 15°C and the same sand was assessed a second time after 144 hrs by replacing the old insects with fresh ones. This was repeated until infections ceased. The rest of the experimental procedure was the same as described for individual infections.

6.3 RESULTS

6.3.1 Efficiency of Galleria trap

Recovery of nematodes from naturally infested soil

Without knowing the number of infective nematodes in a sample of soil, it is possible to estimate the efficiency of their recovery. This can be done by exposing Galleria larvae to the soil continuously until nematodes cease to infest. Then, the numbers of nematodes recovered weekly at each site can be expressed as a percent of the total eventually recovered from that site. Figure 6.1 presents these data as

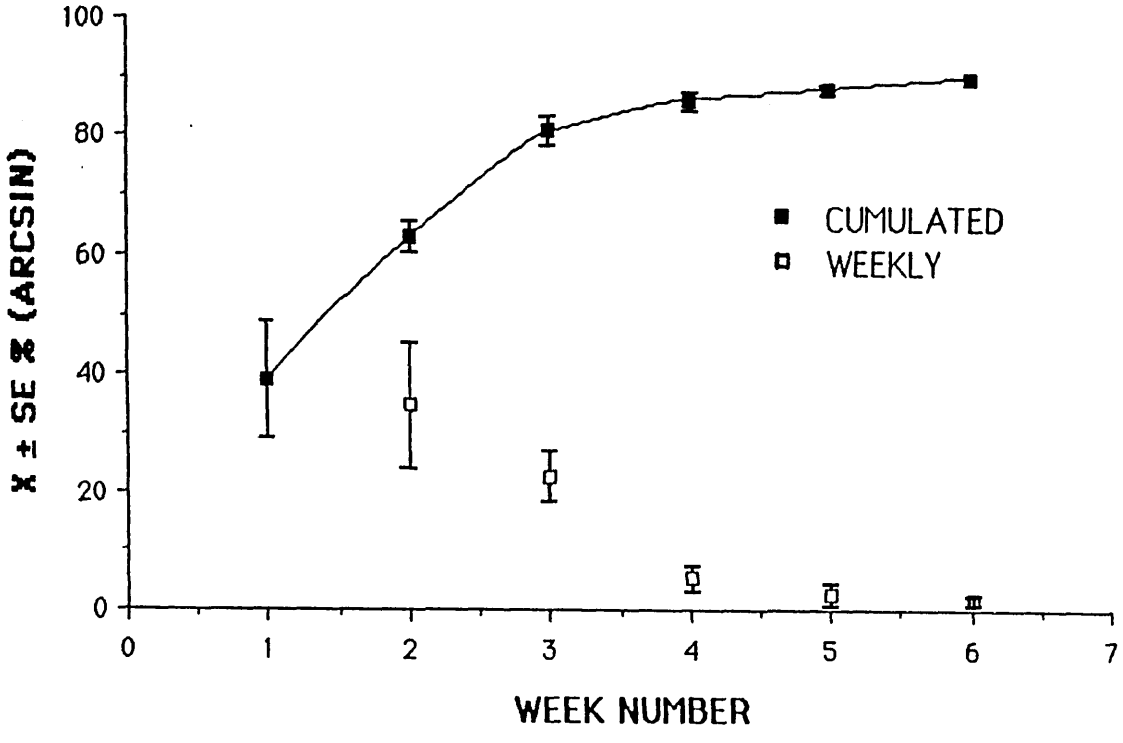


FIGURE 6.1 Weekly (white) and cumulative (black) mean percentage (arcsin transformed data) of extraction from the 6 soil samples taken in October 1986. Five Galleria larvae were used in each dish.

the mean of six sites, for both weekly and cumulative percentages.

Six consecutive exposures were required before nematodes ceased to infest fresh larvae. The largest proportion was obtained in the first exposure and the proportion decreased thereafter (Fig. 6.1). In the first week, 39.8% of the nematodes were recovered (coefficient of variation 24.5%) and the standard error bars show that large variations existed between the samples for the first two exposures. However, when the accumulated mean percent recovery was calculated, the variability was much reduced. In other words, when Galleria larvae were exposed to the same soil twice at one-week intervals, the accumulated percent recovery reached a fairly consistent level, regardless of the nematode density.

To test whether this relationship was consistent, a second assay was carried out a year later on soil collected from the same area. Figure 6.2 shows the mean percentages from 24 sites, for both weekly and cumulaticve data. Although 16 consecutive exposures were required to recover all the nematodes, the shape of the cumulative curve in Figure 6.2 is similar to that in Figure 6.1. More importantly, the mean accumulated percent recovered in the second year again reached a fairly consistent level (mean 81.4%, C.V. = 6.9%) after the second exposure. This compares favourably with the results from the October, 1986 samples. Thus, up to 16 consecutive bioassays may be required to recover all the nematodes in a soil sample. However, two consecutive assays will extract about 80% of the infective nematode population.

Recovery from "spiked" sand

To simulate the conditions of naturally-infested soil samples, where the densities of nematodes are unknown and variable, 25ml samples

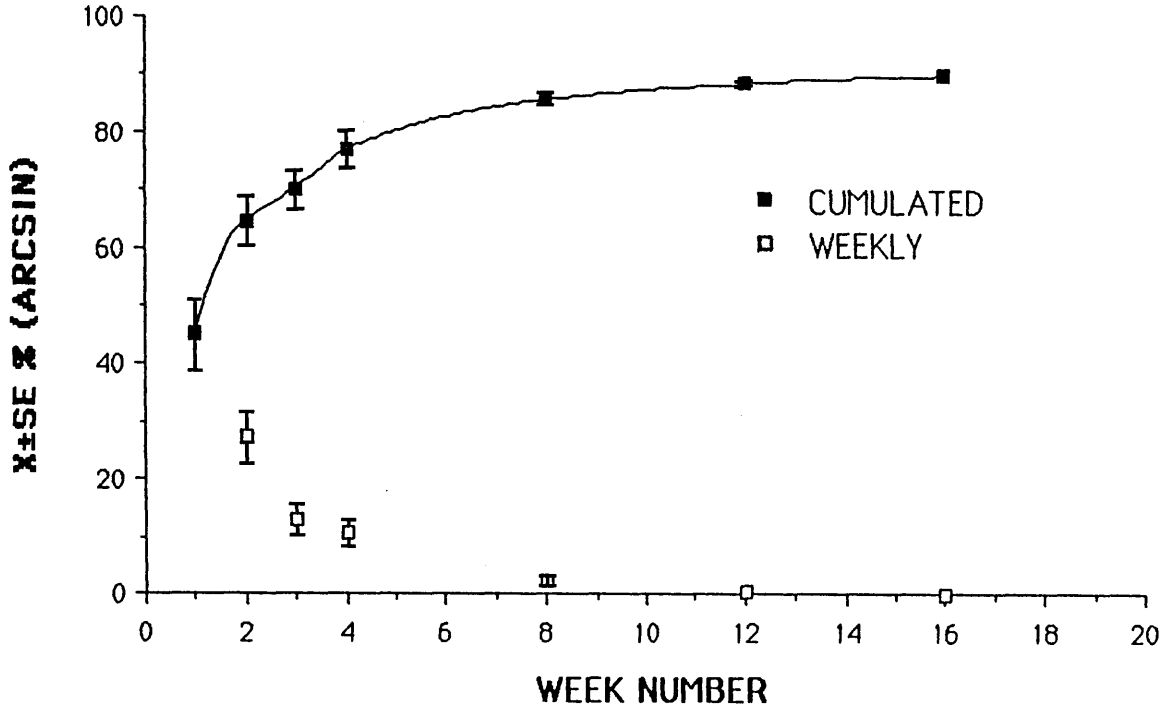


FIGURE 6.2 Weekly (white) and cumulative (black) mean percentage (arcsin transformed data) of extraction from 24 soil samples taken in October 1987. Five Galleria larvae were used each dish.

of sand were "spiked" with 10, 50,100, 150 and 300 nematodes. Table 6.1 presents the data for both the mean number of nematodes recovered in 6 consecutive weekly bioassays and the percent of the total eventually recovered for the 5 densities. Figure 6.3 presents the mean percentage recovery from all the 5 densities as weekly and accumulated ones.

This makes Figure 6.3 comparable to Figures 6.1 and 6.2. Like the nematodes in soil from the field, those applied to sand could not be recovered by one exposure to a Galleria larva. The higher the density, the greater the number of assays required to recover the active nematodes (Table 6.1). Unlike the nematodes in field soil, those in sand were recovered more efficiently. Regardless of nematode density, a mean of 97.25% (C.V. = 2.98%) of the nematodes which could be recovered were recovered with two consecutive bioassays.

6.3.2 Nematode infection in living compared to dead hosts

Some Galleria larvae were killed by injecting 5 nematodes 48 hrs before the experiment began. For these, the number of nematodes which subsequently penetrated and established was obtained by subtracting the mean number in the Control I group (injected but not exposed to infection) from the number recorded in each host. These were then compared with the number of nematodes which had penetrated into Galleria larvae which were living and uninfected at the start of the experiment. The results (Fig. 6.4) showed that significantly more nematodes were found in nematode-killed cadavers than in the living larvae after an exposure time of 24 hrs (t-test, $t=2.702$, $p=0.01$). After 48 hrs, there were no significant differences between the number of nematodes which had penetrated into the nematode-killed (N = 15.7) and fresh (N = 11.4)

TABLE 6.1: Number (mean ± se) and percent (calculated on the basis of the total which penetrated the hosts) of Steinernema bibionis (Nashes) penetrating each time and the accumulated totals from sand (duration of each exposure = 6 days; temperature = 15°C; n = 15 Galleria larva per tube).

Nematode density (Juveniles/25ml sand)			Time of extraction						Total recovered
			1st	2nd	3rd	4th	5th	6th	
	10	N	3.4 ± 0.5	0.3 ± 0.1	0.1 ± 0.1	0	0	0	3.9 ± 0.6
		%	87.28	7.69	3.33	0.60	0.00	0.00	100
	50	N	25.2 ± 1.9	2.3 ± 0.8	0.2 ± 0.1	0	0	0	27.8 ± 1.7
		%	90.65	8.27	0.72	0.00	0.00	0.00	100
	100	N	40.1 ± 2.6	1.2 ± 0.6	0.3 ± 0.1	0	0	0	41.4 ± 2.6
		%	96.86	2.90	0.72	0.00	0.00	0.00	100
	150	N	56.5 ± 2.4	11.9 ± 1.3	1.5 ± 0.3	0	0	0	68.4 ± 2.6
		%	82.60	17.40	2.19	0.44	0.00	0.00	100
	300	N	108.1 ± 7.6	18.3 ± 1.8	5.5 ± 1.2	4.1 ± 2.7	0.1 ± 0.1	0.1 ± 0.1	136.1 ± 8.0
		%	79.43	13.45	4.04	3.01	0.07	0.05	100

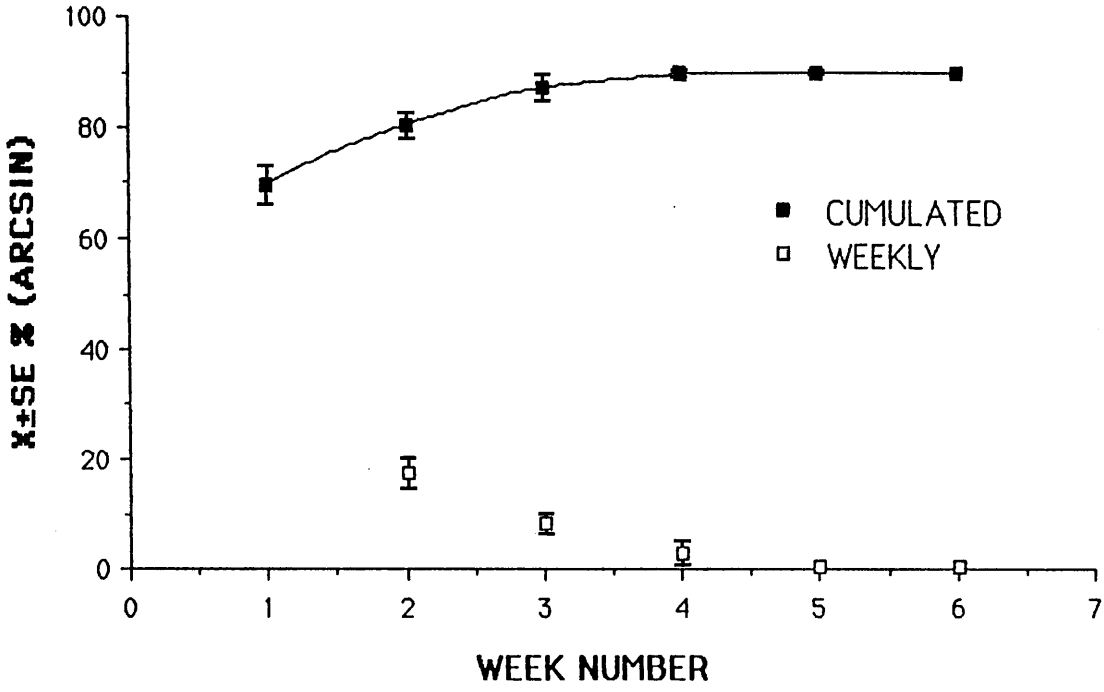


FIGURE 6.3 Weekly (white) and cumulative (black) mean percentage (arcsin transformed data) of extraction from sand samples with 5 different densities.

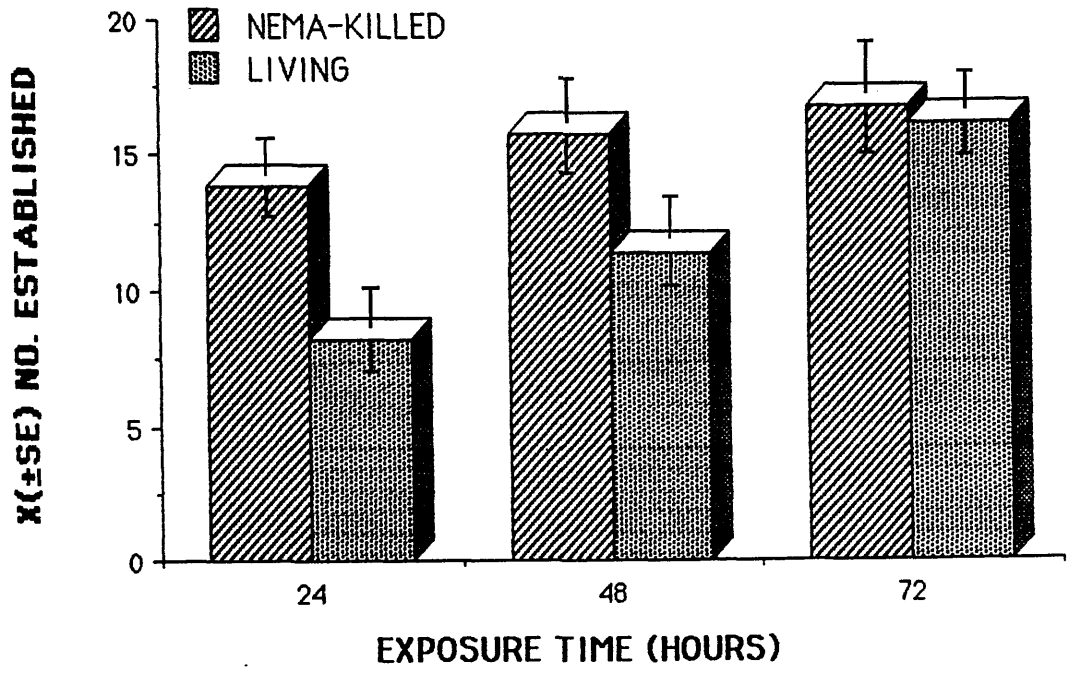


FIGURE 6.4 Number of Steinernema bibionis (Nashes) which penetrated into live Galleria mellonella larvae and nematode killed insects at the three exposure times. Each larva was exposed to 50 infective juveniles in sand (n = 10).

Galleria larvae (t-test, 7=1.862, p=0.07). The differences disappeared after 72 hrs exposure (t-test, t=0.142, p=0.89). At this time, the mean percentage of the initial dose recovered was 33.0% for the nematode-killed cadavers and 32.4% in the initially alive hosts. The mean number of nematodes in the nematode-killed cadavers was not significantly influenced by the increase in exposure time (ANOVA, F=0.82, p>0.05). However, the numbers of nematodes which penetrated into the living Galleria larvae were significantly different at each exposure time (ANOVA, F=5.25, p<0.01), especially between 24 and 72 hrs (t-test, t=3.167, p=0.003) (Fig. 6.4). Therefore, more nematodes penetrate into a recently-infected host than a living host.

6.3.3 Time course of infection

The results for the number of nematodes which infected the Galleria caterpillars after various durations of exposure are presented in Table 6.2 and Figure 6.5. A single exposure failed to extract all the nematodes from the sand, even if the duration of exposure was extended from 24 to 72 hrs (Table 6.2). Diagrammatic presentation of the results (Fig. 6.5) shows that the nematodes continued to penetrate and establish in the caterpillars in successive extractions. However, the proportion of the nematodes which established in the successive exposures was influenced by the duration of exposure in the first extraction.

The accumulated numbers of nematodes which infected Galleria larvae added to the same tube are also shown in Figure 6.5. The highest percentage of nematode penetration occurred in 4a, in which each Galleria larva was exposed to the nematodes in sand for 72 hrs. In this

TABLE 6.2: Effect of duration and sequence of exposure to Galleria larvae on the number of Steinernema bibionis (Nashes) that penetrated. Temperature : 15°C; 50 insects per group; 50 nematodes per tube.

Group	Treatment	Duration of exposure (hrs)	Number of nematodes per insect ($\bar{x} \pm$ se)	Accumulated duration of exposure (hrs)	Accumulated numbers of nematodes/insect ($\bar{x} \pm$ se)*
1	a	24	8.0 ± 1.0	24	8.0 ± 1.0
	b	24	3.4 ± 0.3	48	11.4 ± 1.1
	c	24	0.9 ± 0.2	72	12.3 ± 1.2
2	a	24	7.5 ± 0.7	24	7.5 ± 0.7
	b	48	5.8 ± 0.7	72	13.3 ± 0.8
3	a	48	18.5 ± 1.0	48	18.5 ± 1.0
	b	24	1.2 ± 0.3	72	19.7 ± 0.8
4	a	72	20.4 ± 0.7	72	20.4 ± 0.7
	b	72	3.1 ± 0.4	144	23.2 ± 0.8
	c	72	0.3 ± 0.02	216	23.5 ± 0.8

*each tube monitored separately, so numbers of nematodes that established in sequential exposures could be summed.

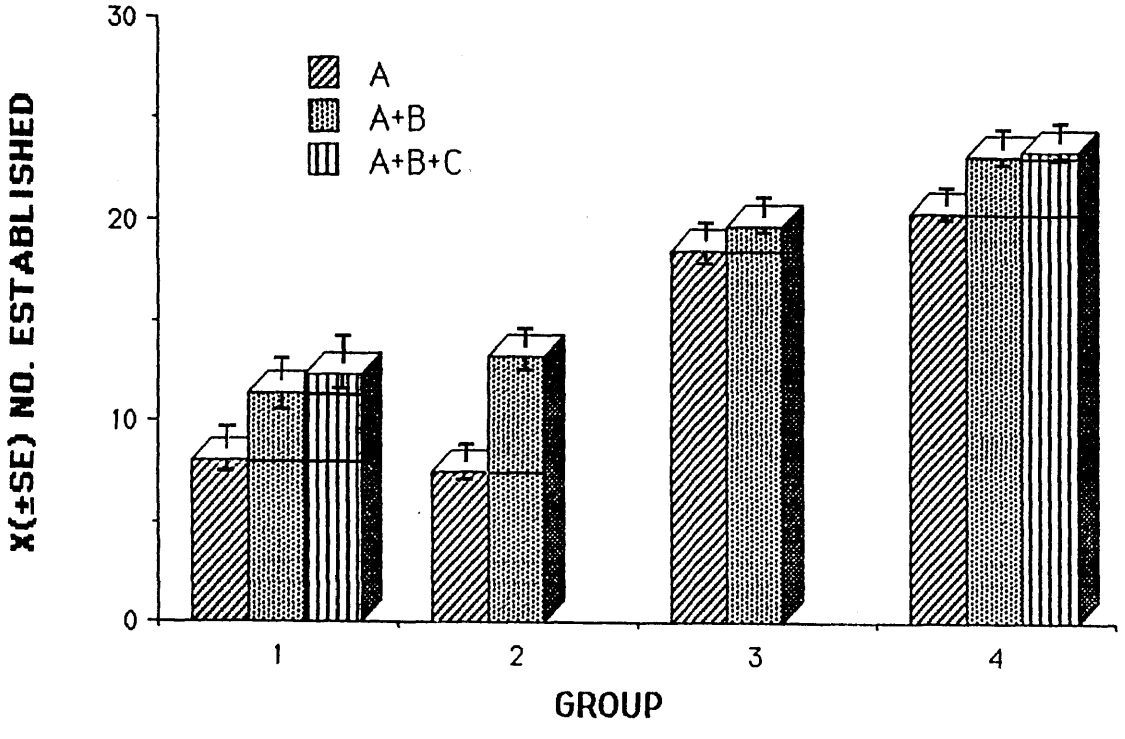


FIGURE 6.5 Number and percent of infective dose of Steinernema bibionis (Nashes) which established in Galleria larvae after various sequential exposure times totalling 72 hrs at 15°C.

1A,B,C = 3 consecutive exposures of 24 hrs each.
2A & B = exposure for 24 hrs followed by 48 hrs.
3A & B = exposure for 48 hrs followed by 24 hrs.
4A = exposure for 72 hrs.

case, 40.8% of the nematodes penetrated into the caterpillars. The lowest accumulated percent of nematode penetration (24.6%) occurred in Group 1 in which the Galleria larvae were changed every 24 hrs. This is statistically the same as Group 2, in which 26.6% of the nematodes were extracted after a 24 then 48 hr exposure (t-test on arcsin transformed data, $t=0.847$, $p=0.40$). However, if the first exposure was 48 hrs followed by 24 hrs (Group 3), more nematodes (39.4%) were extracted, and most penetrated in the first 48 hrs. The cumulated percentage recovered in Group 3 is the same statistically as the continuous exposure for 72 hrs (t-test on arcsin transformed data, $t=0.512$, $p=0.61$).

The results of this experiment show that the duration of exposure between 24 and 48 hrs played a crucial role in determining the number of nematodes which could penetrate. The most active period for infection is a continuous exposure of 48 hrs. After that, infection by the nematodes begins to decrease and although some nematodes can still penetrate, the number is small after 72 hrs. This supports the results from the previous experiment (6.3.2) in which nematode-killed larvae were parasitized by more nematodes than living larvae.

6.3.4 Dose-response analysis

Influence of exposure time

Groups of 50 Galleria larvae were exposed individually to 50 infective nematodes of S. bibionis (Nashes) for 24, 48 or 72 hrs. Figure 6.6 shows the number of nematodes which established, expressed as a percent of the dose applied. It shows that there was a significant increase between 24 and 48 hrs and that only a slight increase occurred thereafter. Analysis of variance on the arcsin transformation of the

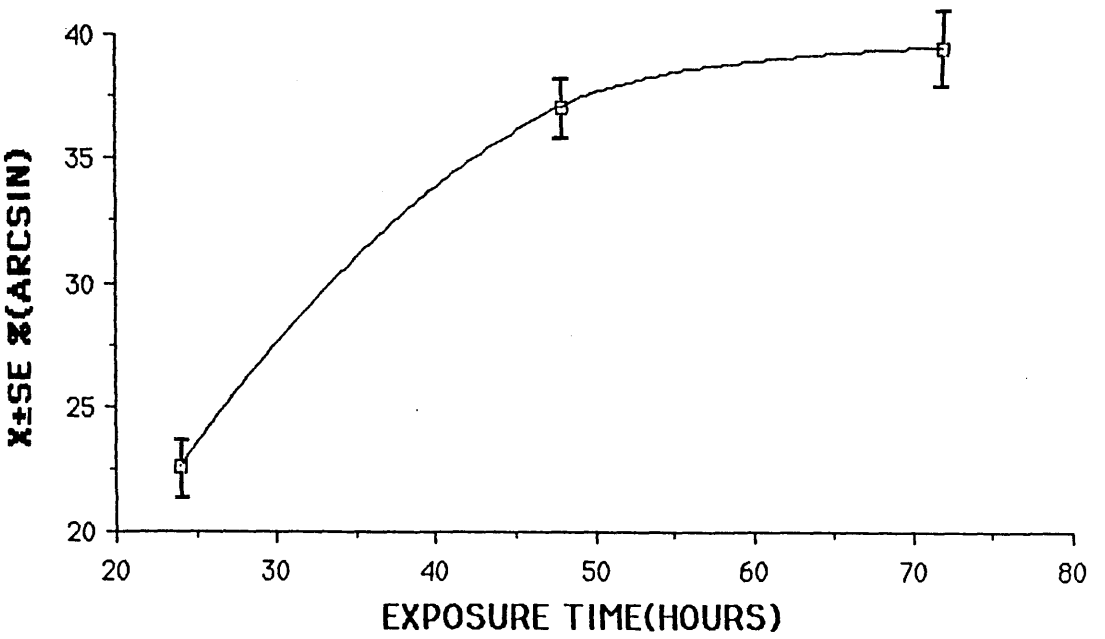


FIGURE 6.6 Influence of exposure time on the percent of Steinernema bibionis (Nashes) which established from a dose of 50 infectives per Galleria larva at 15°C (n = 50 for each exposure time).

percentage establishment values indicated that they were significantly different ($F=64.3$, $p<0.01$). However, the percent establishment after 72 hrs was not significantly different from that after 48 hrs (t-test, $t=1.405$, $p=0.16$). Thus, a range of 36-40% of the initial dose of infective juveniles penetrated and established in the Galleria larvae.

Influence of dose and times of bioassay

The mean numbers of nematodes recovered from insects exposed to different doses of nematodes for 72 hrs are plotted in Figure 6.7. The results show that the mean number of nematodes recovered increased as the dose of infective juveniles increased. Within the range of doses tested in this experiment (10-300), the increase was linear, with the relationship $y = -2.214 \pm 0.363x$. This implied that the proportion of the nematodes which could penetrate and establish in the host was the same (the slope of the line = 36.3%) for each dose tested.

Because previous results showed that a single exposure failed to extract all the nematodes, a fresh insect was added to each tube every 72 hrs until no further nematodes penetrated. The results for the percent of establishment in 1, 2 or 6 exposures are presented in Figure 6.8. When the insects were exposed once, only the dose of 10 showed a significantly smaller recovery than the others (ANOVA, $F=5.35$, $p<0.01$). After the second exposure (Fig. 6.8B), considerable variability for dose 10 still occurred, but the analysis of variance performed on the arcsin transformed data showed no significant differences (ANOVA, $F=0.76$, $p>0.05$). An additional 4 exposures resulted in little change (Fig. 6.8C). Thus, the overall mean percent extracted after two exposures for all the doses was 36.9% compared to 37.6% after 6 exposures. Therefore, regardless of the dose size, less than 40% of the initial dose could

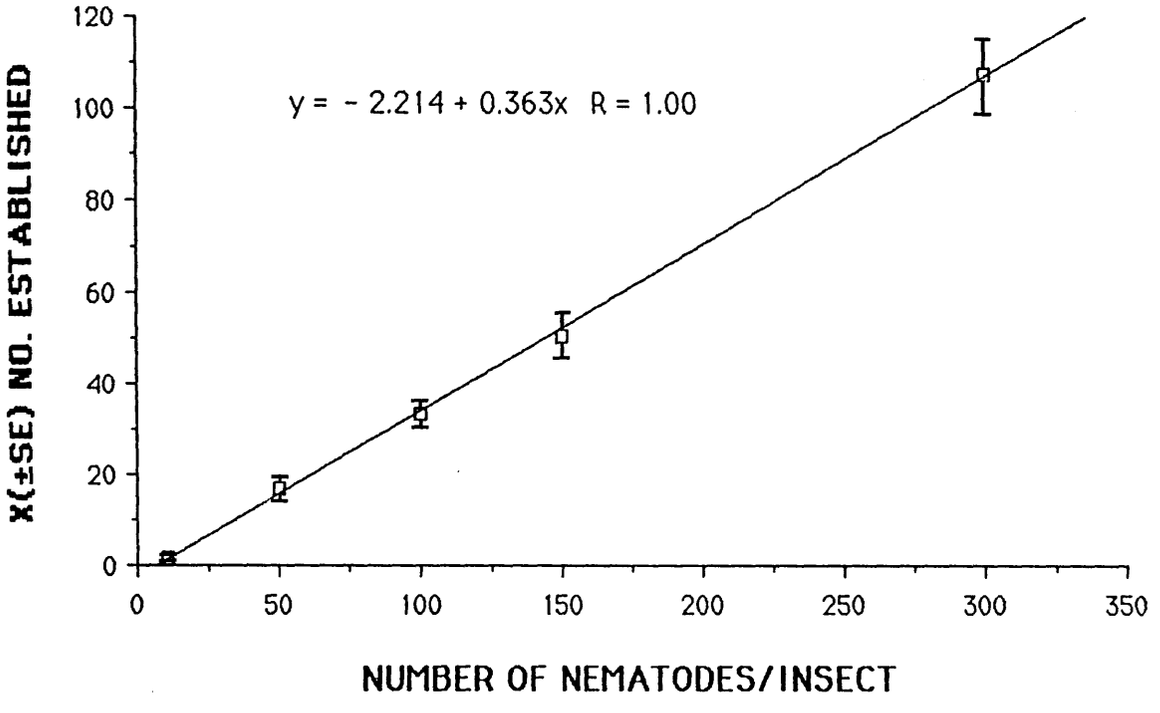


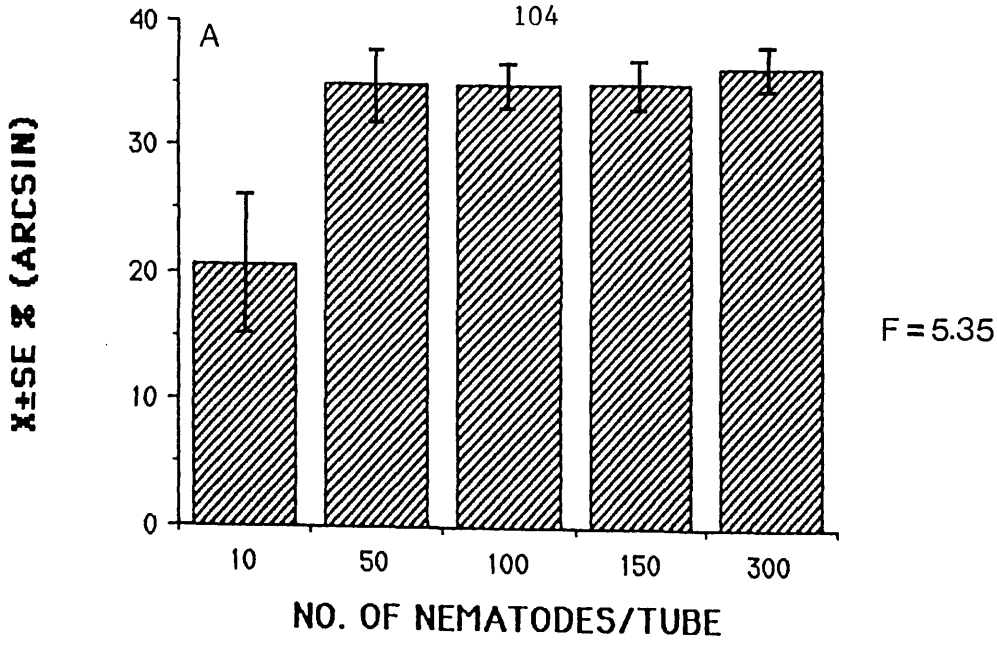
FIGURE 6.7 Number of Steinernema bibionis (Nashes) which established in Galleria larvae after 72 hrs at 15°C (n = 20).

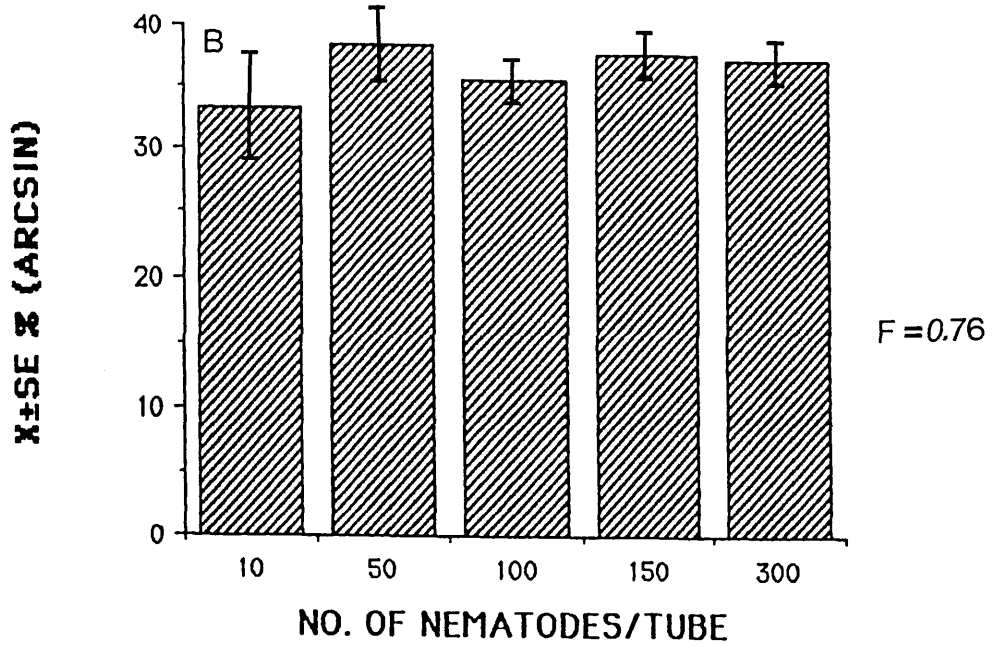
FIGURE 6.8 Influence of dose and times of extraction on percent of Steinernema bibionis (Nashes) which established in Galleria larvae at 15°C (n = 15).

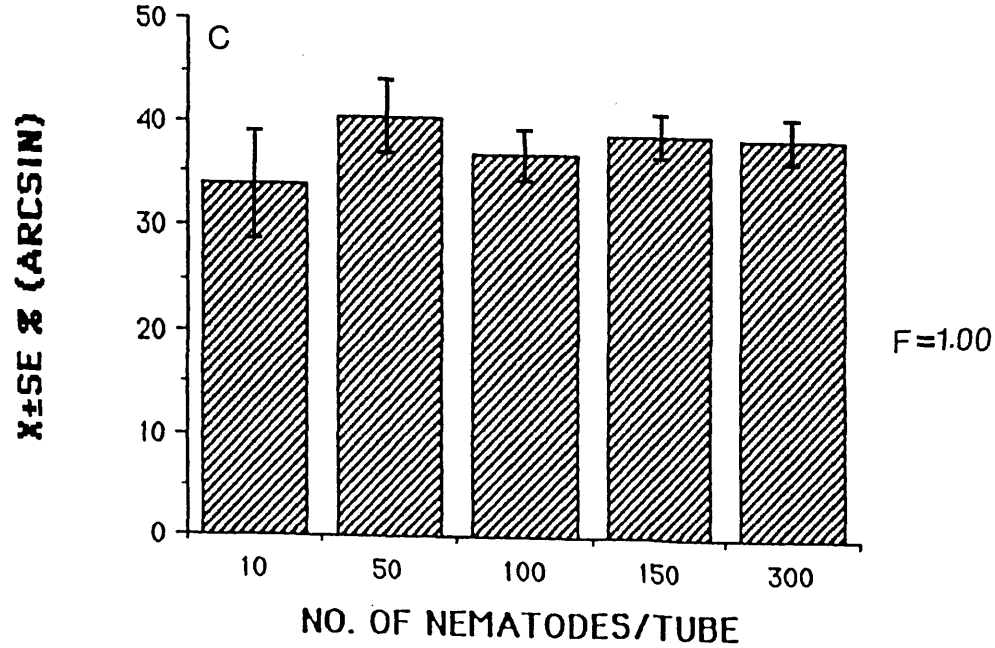
A: 72 hr exposure.

B: 2 x 72 hr exposures.

C: 6 x 72 hr exposures.


A
40
30
20
10
0
X±SE % (ARCSIN)
10
50
100
150
300
NO. OF NEMATODES/TUBE
F = 5.35


B
40
30
20
10
0
X±SE % (ARCSIN)
10
50
100
150
300
NO. OF NEMATODES/TUBE
F = 0.76


C
50
40
30
20
10
0
X±SE % (ARCSIN)
10
50
100
150
300
NO. OF NEMATODES/TUBE
F = 1.00

penetrate and establish in the Galleria larvae. Two successive extractions were enough to estimate the eventual percent recovery to an accepted level.

Influence of different strains

These data are from the experiment documented in Chapter 5. The percent establishment of a dose of 50 nematodes after 72 hrs at 15°C is shown in Figure 6.9. There was no significant difference between the establishment of the three strains tested (ANOVA, $F=1.25$, $P>0.05$). Again, the percent establishment was in the region of 40% of the initial dose applied.

Influence of temperature

Figure 6.10 presents the results of the experiment described in Chapter 5, analysed to show establishment of S. bibionis (Nashes) at different temperatures. In this experiment, 40 nematodes were present in each tube. At 15 and 20°C, the optimal temperature for infection and development of S. bibionis (Nashes), the percentage of the dose that established reached a maximum of 30 - 40%. At the temperatures outside this range, the percentage which established never exceeded 20% of the initial dose.

Influence of group and individual Galleria infection

The number of Galleria larvae in a bioassay can affect the resulting level of mortality (Bedding et al., 1983). To test whether the number of insects in a bioassay can also influence recovery of the nematodes from the soil, 5 Galleria larvae in 300cc of sand were exposed

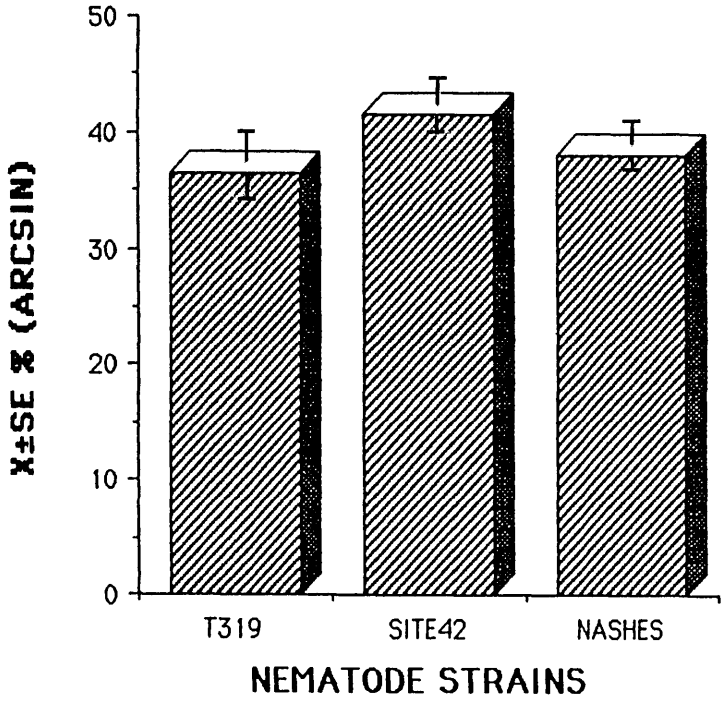


FIGURE 6.9 Establishment of three strains of Steinernema bibionis at 15°C after exposure to Galleria larvae for 72 hrs (n = 20, dose = 40 nematodes per insect).

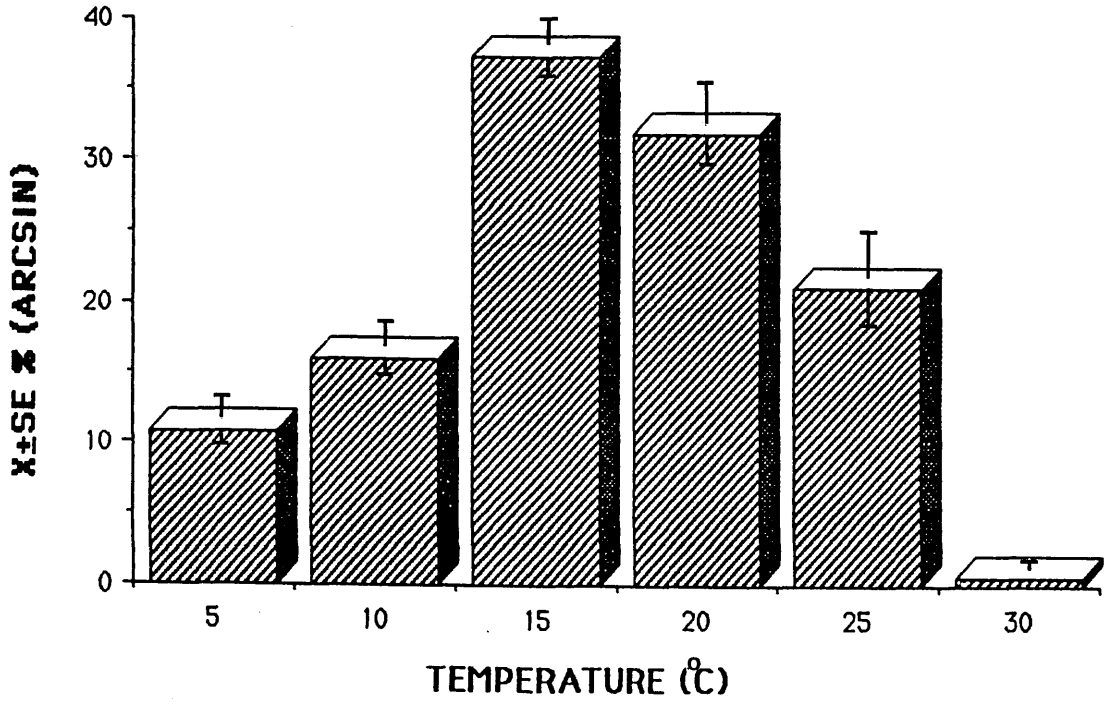


FIGURE 6.10 Influence of temperature on establishment of Steinernema bibionis (Nashes). Dose = 40 infective juveniles per insect, Duration = 72 hrs and n = 20.

to different doses of nematodes. They were exposed for 144 hrs to maximise infections. The total number of nematodes recovered in the 5 Galleria larvae were recorded for each dose. The percentage recovery was calculated as usual and the mean value for the 5 replicates was calculated.

The results for group infections (Fig. 6.11) were similar to those for single infections. Thus, several exposures were required before the nematodes ceased to infect. Analysis of variance showed no significant differences between the percent establishment at the different doses. This applied for 1, 2 or 5 exposures (Fig. 6.11) (ANOVA, $F=1.19$, 0.99 and 0.84 respectively, $p>0.05$).

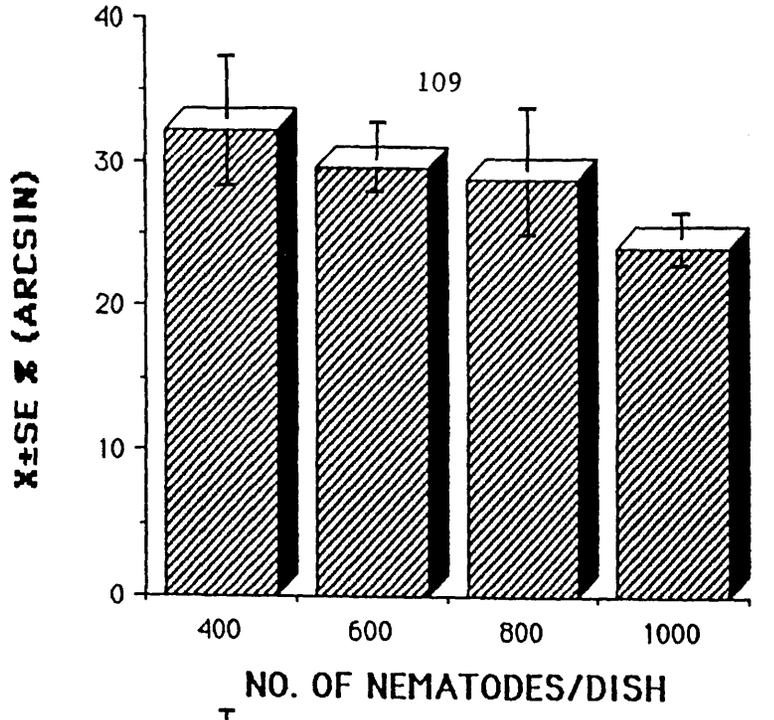
To compare the percentage recovery from single or multiple host exposures, Figure 6.12 was constructed. Statistical tests cannot be performed because of the different methods used in the two experiments. Nevertheless, the histograms (Fig. 6.12) show that most of the mean percent establishment figures were in the range 30 - 40% and that the percent establishment was independent of the dose applied.

6.4 DISCUSSION

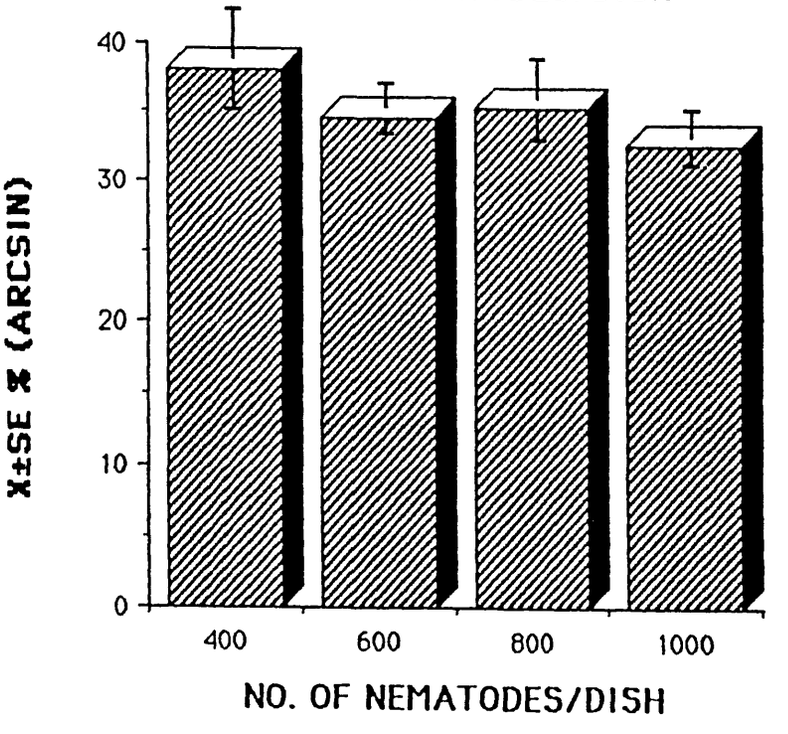
To achieve the purpose of estimating the population density of nematodes in the field with an acceptable degree of precision, the number of nematodes in a soil sample must be estimated. Galleria traps (Bedding and Akhurst, 1975) have been used to extract entomopathogenic rhabditids from soil by numerous authors. However, the results were usually treated qualitatively and the efficiency of this method has not been assessed until this study. The results showed that a single exposure to Galleria larvae was not sufficient to extract all the active

FIGURE 6.11 Establishment of Steinernema bibionis (Nashes) applied in different doses to dishes with 5 Galleria larvae in 300cc of moist sand (temperature = 15°C, n = 5 replicates/dose).

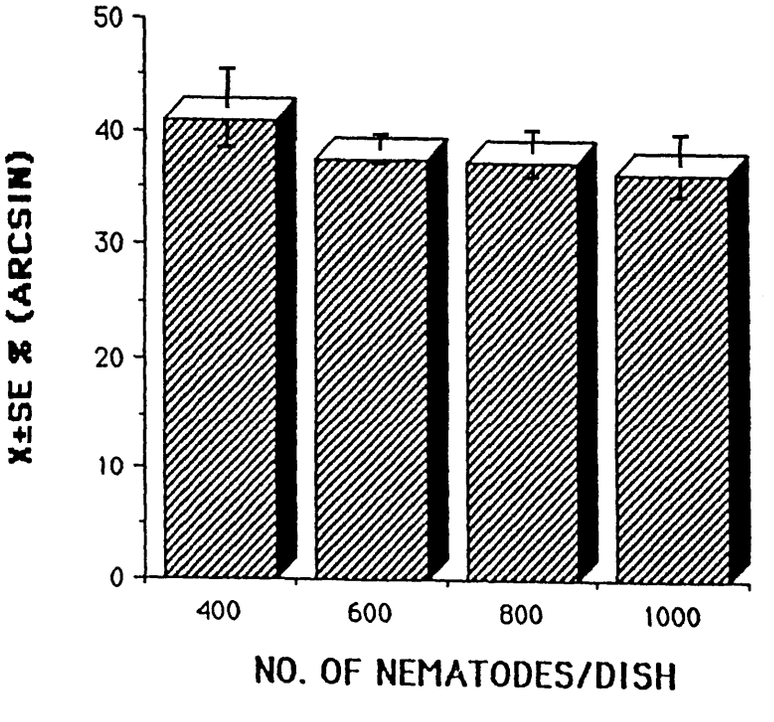
A = 144 hr exposure.
B = 2 x 144 hr exposures
C = 5 x 144 hr exposures.


40
30
20
10
0
X±SE % (ARCSIN)
400
600
800
1000
NO. OF NEMATODES/DISH

F=1.19


40
30
20
10
0
X±SE % (ARCSIN)
400
600
800
1000
NO. OF NEMATODES/DISH

F=.99


50
40
30
20
10
0
X±SE % (ARCSIN)
400
600
800
1000
NO. OF NEMATODES/DISH

F=.84

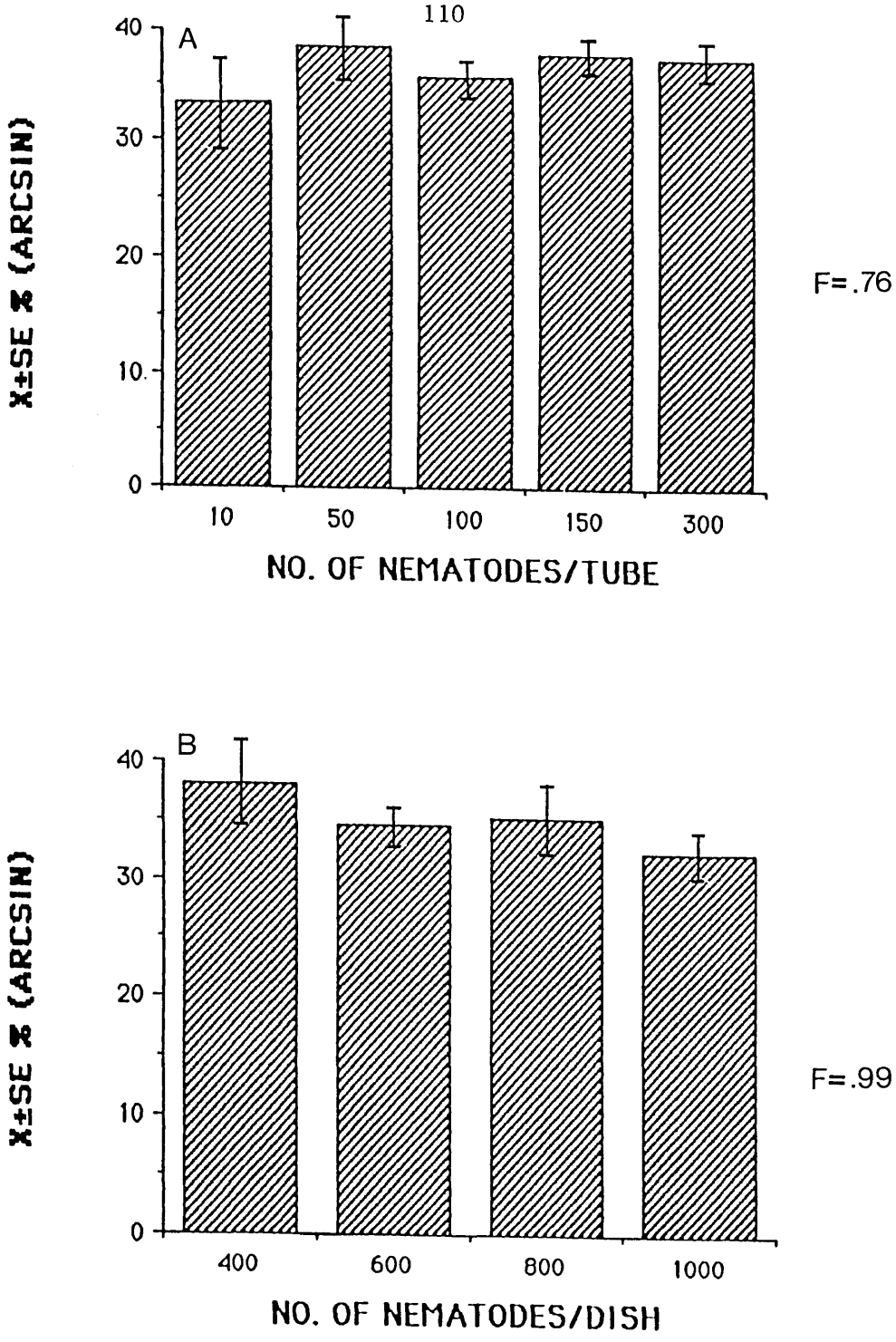


FIGURE 6.12 Comparison of the percent of Steinernema bibionis (Nashes) that established in Galleria larvae at 15°C after two exposures.

A = single larva in a tube, each exposure 72 hrs.

B = 5 larvae in a dish, each exposure 144 hrs.

nematodes or a stable proportion of the infective nematodes from a soil sample. A number of successive extractions was required to recover all the infective nematodes from the sample. However, this process was very tedious and labour-consuming in practice. Instead of extracting to exhaustion, the results obtained from the present study showed that two successive extractions were sufficient for reliably estimating the number of nematodes which would be extracted eventually (Fig. 6.1 and Fig. 6.2). From the analysis, a fairly constant mean level of extraction efficiency (E) was achieved by successively exposing G. mellonella larvae to the same soil sample twice at intervals of at least 3 - 4 days, no matter what the nematode density was in the sample. The number of nematodes that can eventually be extracted could then be estimated by applying the coefficient (E). In the present study, a mean of 79% (c.v. = 3.9%) of the infective nematodes was recovered from 6 field samples in 1986 and 81.4% (c.v. = 6.9%) from 24 field samples taken in 1987 after the soil samples had been assessed twice. A test using "spiked" sand samples showed similar results in that two exposures produced a mean recovery with little variation even though the doses of nematodes varied more than 10-fold. The higher recovery in sand may reflect its suitability as a medium for nematode infection.

These results suggested the feasibility of using the mean percent of nematodes recovered after two consecutive assays as a coefficient to estimate the numbers of infective nematodes present in the sample. It would first be necessary to calibrate the assay for soil type and nematode species. Then if tests confirm that 80% (for example) of the nematodes can be extracted from a soil sample by exposing 5 G. mellonella to 200-250cc soil at 4 - 6 day intervals twice, and the

number of nematodes recorded from the Galleria larvae are (N_1+N_2), then the number of infective nematodes would be estimated as $(N_1+N_2)/0.80$.

Even if a Galleria assay is not performed for purposes of estimating the number of nematodes in a soil sample, a second assay is recommended when estimating prevalence of the nematodes. It was observed in the present study that some soil samples yielded no infections after the first assay but did give infections in the second one. Thus, a second assay would prevent low estimates of the prevalence (i.e. positive sites) when nematode distributions across large areas are investigated. For example, the results obtained by Hominick and Briscoe (in prep.) showed that 48.6% (196/403) sites were positive when the soil samples were bioassayed twice, compared to 39.4% (159/403) positive sites after only one exposure. Thus, nearly 10% of the sites were false negatives if the soil samples were only assessed once.

While successive bioassays eventually extract all the infective nematodes, there is still a question of whether those nematodes represent all the nematodes present in the soil sample. Experiments conducted by Johnson (1986) in this laboratory revealed that when the infective juveniles of S. bibionis (T319) were applied in sand, only a proportion of the initial dose could be obtained from the G. mellonella larvae. Similar results were also obtained when S. feltiae was used to infect insects in sand (Johnson, 1986) or on filter papers (Bednarek and Nowicki, 1986). Both studies showed that less than 40% of the original population was recovered from the insect cadavers. The present studies confirm and extend their results.

The reasons for many nematodes remaining in the sand or on filter papers are unknown and require further investigations. However, the

significance of these experiments lays in the similar proportion of the initial dose that was recovered from the insects. Based on these experiments, the present study related the number of nematodes that established in Galleria larvae to the initial dose applied. The rationale behind this was to examine whether this relationship is consistent under various conditions. It was revealed that the percent of the initial dose recovered in Galleria larvae did show a fairly consistent value (30-40%) which was not influenced by nematode dose, strain or manner of infections. Therefore, the nematodes recovered from insects only represent a proportion of the total nematode population.

Two factors which did influence recovery were temperature and exposure time. Temperature has been reported to influence many aspects of the biology of entomopathogenic nematodes (Dunphy and Webster, 1986). It is then not surprising that the number of nematodes that established inside insects can also be affected by temperature. Possible reasons for the different abundance of nematodes at various temperatures were discussed in Chapter 5. Because most studies in the literature only document insect mortality resulting from parasitization by the nematodes, little information is available on the actual numbers that entered. The present study provides such information which is useful not only for our understanding of nematode abundance at different temperatures, but also for the favourable conditions for maximal establishment of the nematodes. This is very important for conducting bioassays which reliably estimate the number of nematodes in a sample.

Exposure time influenced the number of nematodes which infected the hosts (Fig. 6.5 and Fig. 6.6). In general, the longer the nematodes are in contact with the insects, the more nematodes that can penetrate.

However, there is a time limit for the number of nematodes which establish. The present study shows that within 48 hrs, the numbers which established is dependent on an increase in the exposure time. After this time, the increase in the number of nematodes recovered from the insects is not significant. A positive relationship between increased mortality of insects (number of nematodes were not recorded) and increased exposure time has been reported for various species of insect-parasitic rhabditids (eg. Finney et al., 1982; Kaya and Grieve, 1982; Toba et al., 1983). Infectivity of three Steinernema species, S. feltiae, S. bibionis and S. glaseri to Spodoptera litura larvae, in respect of the number of nematodes that invaded, also showed an increased trend with increasing exposure time of the insects to the nematodes within 48 hrs (Kondo and Ishibashi, 1986). These authors also showed differences between nematode species.

To invade and kill insects, the infective juveniles have to contact a suitable host. S. feltiae has been shown to respond to carbon dioxide (Gaugler et al., 1980), thermal gradients (Byers and Poinar, 1982) and excretory products of insects (Schmidt and All, 1978; 1979). Nevertheless, in the present study, living Galleria larvae were nearly motionless because they were restrained by the sand, and few nematodes penetrated within 24 hrs. If nematode-killed larvae were used, then many more nematodes penetrated within the same period. Danilov (1987) in a less carefully controlled experiment, found many S. feltiae infective juveniles also penetrated the previously infested insect larvae. This suggests that attractants such as carbon dioxide and other metabolites are less effective than attractnats produced by dead hosts that already harbour an infection. The nature of these substances is unknown.

The differences in number of nematodes found in nematode-killed insects and fresh insects after 48 and 72 hrs disappeared, suggesting that the fresh insects parasitized within 24 hrs actually acted as the nematode-killed insects to obain higher infection in the following 24 or 48 hrs. The higher number of nematodes recovered in the insects which had been exposed continuously for 72 hrs than in the insects that had been exposed for three 24 hr periods showed once again the attractiveness of the nematode-infested insect cadavers.

It is also interesting to note that even though the dose-response study (6.3.4) showed that a host could accommodate infections of over 100 nematodes, the total number of nematodes recorded from the nematode-killed cadavers and the freshly infected cadavers after 72 hrs did not exceed 40% of the initial population of 40 infective nematodes. It is possible that the longer time the nematodes are in contact with hosts, the fewer individuals are able to settle in the insects because of increasing energy depletion of individuals and/or weakening attaction of the hosts (Woycik and Grabiec, cited in Bednarek and Nowicki, 1986). Unfortunately, the original paper was not in English so that the evidence supporting the above statement cannot be assessed here. However, Bednarek and Nowicki (1986) did mention that when excreta of G. mellonella larvae were used to treat infective juveniles of S. feltiae, "following in succession in a short time nematode stimulations were weaker and weaker". It was also mentioned that the original authors related "the observed decreases of activity of invasive larvae in time with a growing energy degradation of individuals, which is the greater, the longer lasts the contact of nematodes with host faeces". In the present study, when the nematode-killed cadavers were used, infection

occurred rapidly and then a decrease in infection occurred. These dead insects do not produce faeces , therefore it is unlikely that the insect faeces play an important role in the relationship. It is also unlikely that energy depletion would account for the difference, because the effect was observed over a matter of hours, not days.

In the present study, it was shown that only 30-40% of the initial nematode population will penetrate and establish in Galleria larvae. Similar studies by Bednarek and Nowicki (1986) and Danilov (1987) showed a maximum of 35% of the invasive juveniles can be found in insects. This value falls well within the range obtained in the present study, although different numatode species, doses and experimental procedures were used. In their studies, insects were placed on moist filter paper containing large numbers of nematodes. The results obtained in the present study using sand would be more representative of the natural environment.

To summarize the results from these experiments, the following points can be made: (a) not all the applied nematodes could infect the insects, even under continuous optimal conditions; (b) a 72 hr exposure time was sufficient for assessing infectivity of the nematodes; (c) an optimal temperature of 15-20^{o}C gave minimal invasion of infective juveniles of S. bibionis; (d) at the optimal conditions (15-20^{o}C, 72 hr exposure), the percent of the initial dose that established in G. mellonella larvae was fairly consistent (30-40%) regardless of the dose applied. If the present results are also representative of the nematode infection in soil samples from the field, a coefficient representing infectivity can be introduced (P=0.3 - 0.4). As discussed earlier, two

exposures of Galleria larvae to the same soil sample will allow an estimate of the numbers of infective nematodes present ($\frac{N_1 + N_2}{E}$). However, this estimate is only 30 - 40% of the entire nematode population actually present in the soil. Therefore, by using the infectivity coefficient P, the nematode population in a soil sample would be estimated as $N = \left(\frac{N_1 + N_2}{(E)(P)} \right)$ where N_1 and N_2 = number of nematodes recovered from Galleria larvae in the first or second exposure; E = efficiency of Galleria trap; and P (infectivity coefficient) = 0.3 - 0.4. A critical question that remains is "Does the 60% of the nematode population that remain ever become infective, or do the nematodes lose their population?"

CHAPTER 7

SURVIVAL AND INFECTIVITY OF THE BRITISH STRAINS AT LOW TEMPERATURES

7.1 INTRODUCTION

Survival of infective juveniles of steinernematids and heterorhabditids is influenced by various factors including moisture and temperature. A review by Kaya (1985) gives a general idea of how survival of these nematodes is related to moisture, temperature, sunlight, oxygen and chemicals when the nematodes are in media other than soil. While these studies are biologically interesting, they cannot be directly related to the natural habitat of the nematodes. The soil environment is a favourable habitat for survival of insect-parasitic rhabditis because adequate moisture is usually present. Thus, the influence of soil temperature and soil type becomes more important.

The survival of two species of Heterorhabditis, S. feltiae (Agriotos) and S. glaseri at 4 temperatures was studied in sterilized sand in the absence of hosts (Molyneux, 1985). Like most non-feeding, free-living stages of parasitic nematodes, these insect-parasitic nematodes rely on their stored food reserves for the energy necessary to locate and infect hosts. Increased longevity was thus found at lower temperatures, suggesting that the rate of utilization of nutrient reserves was lower due to the reduced degree of mobility of the nematode juveniles (Molyneux, 1985). However, it was also found that steinernematids survived longer than did the heterorhabditids at all the temperatures tested (Molyneux, 1985). In his thesis, Molyneux (1983) attributed the difference to the original habitats associated with these

nematodes. The Heterorhabditis species, which are from tropical and subtropical areas, are able to infect hosts all year round so that long-term survival of infective juveniles is not necessary. However, the Steinernema species/strains are from temperate and cold climates and long-term survival of infective juveniles during winter months would be an advantage (Molyneux, 1983).

The type of medium also influences persistence of the infective juveniles. Ishibashi and Kondo (1986a) studied the survival of S. feltiae (DD-136) and S. glaseri in soil and bark compost at 25°C in the laboratory. Both species survived longer in bark compost than in soil, although fewer numbers of DD-136 were recovered than S. glaseri. The nematodes in bark compost caused higher Galleria mortality than did those in soil, though the number of invading J4s was not investigated. The poor soil biota and favourable physical conditions in bark compost were suspected to support better persistence of the nematodes (Ishibashi and Kondo, 1986a). Biotic factors are important in affecting persistence of nematodes in soil. These were discussed in Chapter 4.

For most studies concerning persistence of infective juveniles, mobility of the juveniles is usually regarded as an indicator of nematode survival (Saunders and All, 1982; Whitehead and Hemming, 1965). However, infectivity (defined as mortality of the test insects) is a more specific property than mobility through a Whitehead sieve (Molyneux, 1985). Molyneux and other authors such as Ishibashi and Kondo (1986a) used mortality to measure vigour of the surviving nematodes. As shown in previous chapters, mortality is not a good indicator for nematodes which can infect. It is necessary to assess the numbers which are able to penetrate into a host. Studies of this type are rare.

Most of the work on steinernematid survival concerns species other than S. bibionis. Therefore, the British strains, Site 42 and Nashes, were tested for their ability to survive in sand at temperatures that resembled British conditions. Their ability to infect a host after they had survived for different lengths of time was also assessed. Because the numbers of nematodes that establish in Galleria larvae (i.e. intensity of infection) may vary greatly while the mortality rate of the hosts can be similar, intensity of infection was used to assess survival ability, in addition to mobility and percent mortality as used by others.

7.2 MATERIALS AND METHODS

7.2.1 Survival and infectivity of S. bibionis (Site 42) at 5°C

The method used was based on that of Molyneux (1985) with a few modifications. Sand tubes were prepared as usual (2.3), each containing a mean of 215 (range 200-226) infective juveniles and sealed with a screw cap. The tubes were divided into 5 groups, each with 15 replicates. Each group was wrapped with water-saturated tissue in a plastic bag and then placed in a 5°C fridge. At the required intervals (Day 0, 7, 14, 21 and 28), one group was taken out and treated as follows: (1) Five Galleria larvae, one per tube, were placed in 5 tubes and were incubated at 15°C. Every 4 days, a fresh larva was added to each tube until no more insects were infected. (2) Another 5 tubes were emptied individually into five modified Whitehead sieves (2.6). At 24 hr intervals at 20°C, the water in the bottom dish was poured into a plastic counting dish and the number of nematodes recorded. The procedure was repeated until nematodes ceased to appear. (3) The sand in the remaining

5 tubes was emptied into five beakers and the nematodes were extracted using the sucrose extraction method (2.6).

7.2.2 Survival and infectivity of S. bibionis (Nashes) at 5 and 15°C

A method similar to that above was used in this experiment. Three hundred sand tubes containing a mean of 250 (239-258) infective juveniles of S. bibionis (Nashes) per tube were divided into 14 groups of 20 in plastic bags. Seven groups were incubated at 5°C and 7 at 15°C. The additional group of 20 served as Day 0 controls. At the required intervals (Day 1, and Week 2, 4, 6, 8, 12 and 16), one plastic bay was removed from each of the two temperatures. Then, 10 replicates from each temperature were exposed to Galleria larvae individually at 15°C. Fresh larvae were added every 4 days until no more were killed. Five replicates were extracted by the modified Whitehead sieves (2.6) at 20°C and the remaining 5 replicates were subjected to sucrose extraction (2.6).

7.3 RESULTS

7.3.1 Survival and infectivity of S. bibionis (Site 42) at 5°C

The results for survival of S. bibionis (Site 42) at 5°C are presented in Figure 7.1. Immediately after inoculation (Day 0), approximately 80% of the nematodes were recovered by the modified Whitehead sieve, which indicates the efficiency of the technique. On Day 7, a significant reduction (t-test, $t=4.207$, $p=0.004$) in the number of nematodes recovered was discovered. Thereafter, there was no significant difference between the number recovered on Day 7 and the numbers on Day 14, 21 and 28 (ANOVA, $F=0.37$, $p>0.05$).

The number of nematodes recovered by sucrose extraction was also assessed (Fig. 7.1). In general, the trend of recovery follows a similar pattern to that obtained by the modified Whitehead sieve, but lower numbers were recovered. The largest difference between the methods occurred on the day of inoculation (Day 0).

The mean numbers of nematodes recovered from the 5 G. mellonella larvae after one exposure to the insects are shown in Figure 7.2. All the insects were parasitized in every assessment, so the mortality of the hosts remained 100% throughout the experiment. However, the number of nematodes per host was very different. Thus, on the day of inoculation, 54 $\pm$ 13 nematodes per host (25% of the applied nematodes) were recovered. Then, a remarkble decrease in the number of nematodes per host was recorded after the nematodes had been stored in sand at 5^{o}C for one week. Only 7.4% of the applied dose was able to infect the insects. These low numbers were recorded for a further 2 weeks. Then a significant and surprising rise in the number of nematodes per host was recorded after the infective juveniles had been stored in sand for 4 weeks (t-test, t=3.986, p=0.016). Thus, on Day 28, the same number of nematodes per host (53 $\pm$ 10) was recorded as on Day 0 (t-test, t=0.069, p=0.947). Therefore, nematode activity, if expressed as the mean number of nematodes which were able to infect G. mellonella larvae after storage, was different from nematode activity expressed as the numbers that could be extracted by the Whitehead sieve and from nematode activity expressed as the percent mortality of the Galleria larvae.

Figure 7.2 also shows the accumulated mean numbers of nematodes which were able to infect Galleria larvae after 6-11 consecutive exposures of 4 days each to fresh larvae. There was much more

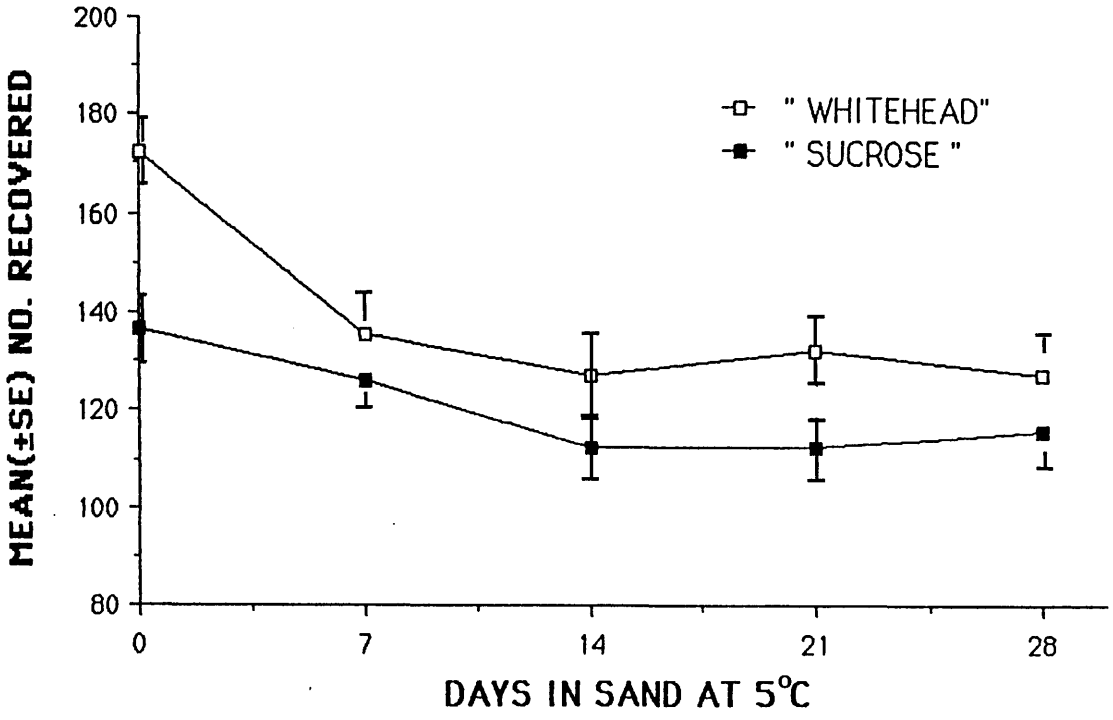


FIGURE 7.1 Recovery of infective juveniles of Steinernema bibionis (Site 42) after storage in moist sand at 5°C, applied at a dose of 215 nematodes per tube (n = 5).

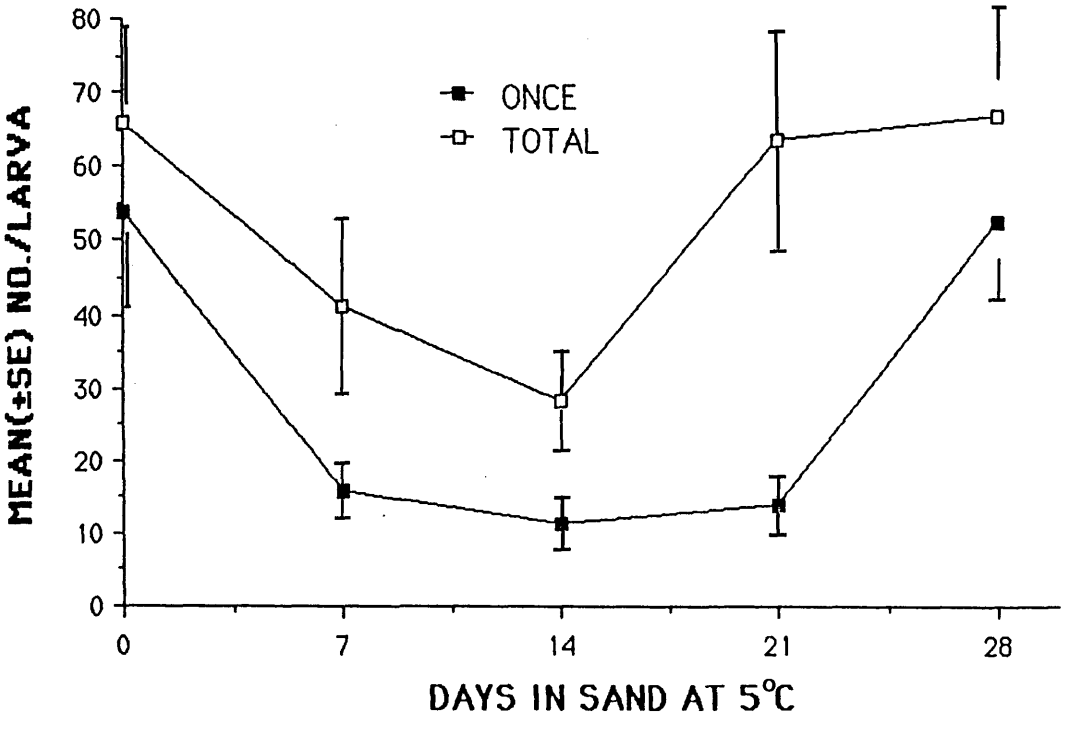


FIGURE 7.2 Number of Steinernema bibionis (Site 42) established in a Galleria larva after increasing storage time in sand at 5°C (n = 5, dose = 215 juveniles/tube).

variability in the results, but the same trend was observed. There was no significant difference between the means in the single and consecutive exposures on Day 0 or on Day 28 (t-test, t=0.76, p=0.472; t=0.888, p=0.404, respectively). Thus, most of the nematodes that could penetrate did so on the day of inoculation and after 28 day's storage. By contrast, a significant number of nematodes on Day 7, 14 and 21 failed to penetrate on the first exposure to Galleria larvae. On Day 14, the least variability occurred and the smallest number of parasites per host was observed, even though the nematodes were allowed access to hosts on 40 days, after which time no further infection occurred.

7.3.2 Survival and infectivity of S. bibionis (Nashes) at 5 and 15°C

The results for long term survival of S. bibionis (Nashes) at 5 and 15°C are presented in Figure 7.3. On the day of inoculation, a mean of 211 (± 11) nematodes was recovered using the modified Whitehead sieve, which gave an efficiency of approximately 84%. The survival as assessed by the Whitehead sieve showed that the Nashes nematode juveniles survived much better at 5°C than at 15°C. A significant decrease in the number of nematodes recovered after 1 day at 15°C was recorded at 15°C (t-test, t=11.518, $p<0.001$). Only 94 (± 4) nematodes were able to move actively through the sieve into the water. By contrast, the same number of nemtaodes was recovered on Day 1 (211 ± 4) for the nematodes stored at 5°C as the numbers recovered on Day 0 (211 ± 11). Thereafter, survival was very different at the two temperatures. Thus, at 5°C, a mean of 162 (± 11) nematodes was still extracted from the sand stored at 5°C for 16 weeks, while only 6 (± 2) nematodes survived at 15°C for the same length of time.

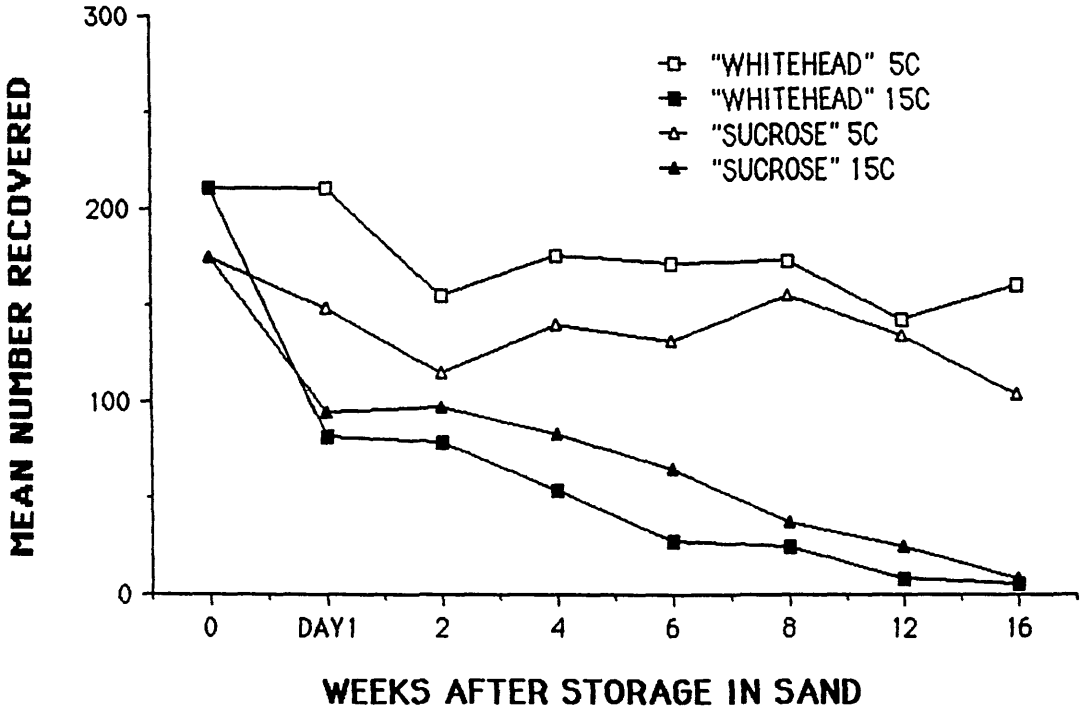


FIGURE 7.3 The effect of temperature on recovery of Steinernema bibionis (Nashes) after storage in moist sand, applied at a dose of 250 nematodes per tube (n = 5).

-□- at 5°C assessed by modified Whitehead sieve.
-■- at 15°C assessed by modified Whitehead sieve.
-△- at 5°C assessed by sucrose extraction.
-▲- at 15°C assessed by sucrose extraction.

The numbers of nematodes recovered by sucrose extraction are also shown in Figure 7.3. In general, the trend of recovery followed a similar pattern to that obtained by the modified Whitehead sieve. Slightly lower numbers were recovered compared to the sieve technique for the nematodes stored at 5°C and higher numbers for the nematodes stored at 15°C. Significant differences were found for the numbers recovered by the two extraction techniques on Week 16 at 5°C (t-test, $t=4.556$, $p=0.003$), but no significant difference was found for those at 15°C (t-test, $t=1.358$, $p=0.223$).

Percent mortality of the Galleria larvae caused by the nematodes after they were stored for various lengths of time at the two temperatures are presented in Figure 7.4. A larger difference between the number of surviving nematodes (see Fig. 7.3) and mortality of the insects was found at 15°C than at 5°C. Thus, 40% of the Galleria larvae were still infected by the nematodes after storage for 16 weeks at 15°C, although only a mean of 6 infective juveniles was recorded from the sand. The mortality caused by the surviving nematodes after storage at 5°C for various lengths of time did not show any trend to decrease with time. Considerable varibility existed so that the lowest mortality was recorded on Week 4 and Week 6 (70%) and the highest on Day 0 and Day 1 (100%). Ninety percent of the Galleria larvae were parasitized on Week 16.

As for the Site 42 nematodes, nematode infectivity was measured for S. bibionis (Nashes) in terms of the mean number of nematodes recovered from the 10 G. mellonella larvae after one exposure to the insects. Significant differences in both numbers established and in the pattern of recovery with time were found for the nematodes at the two

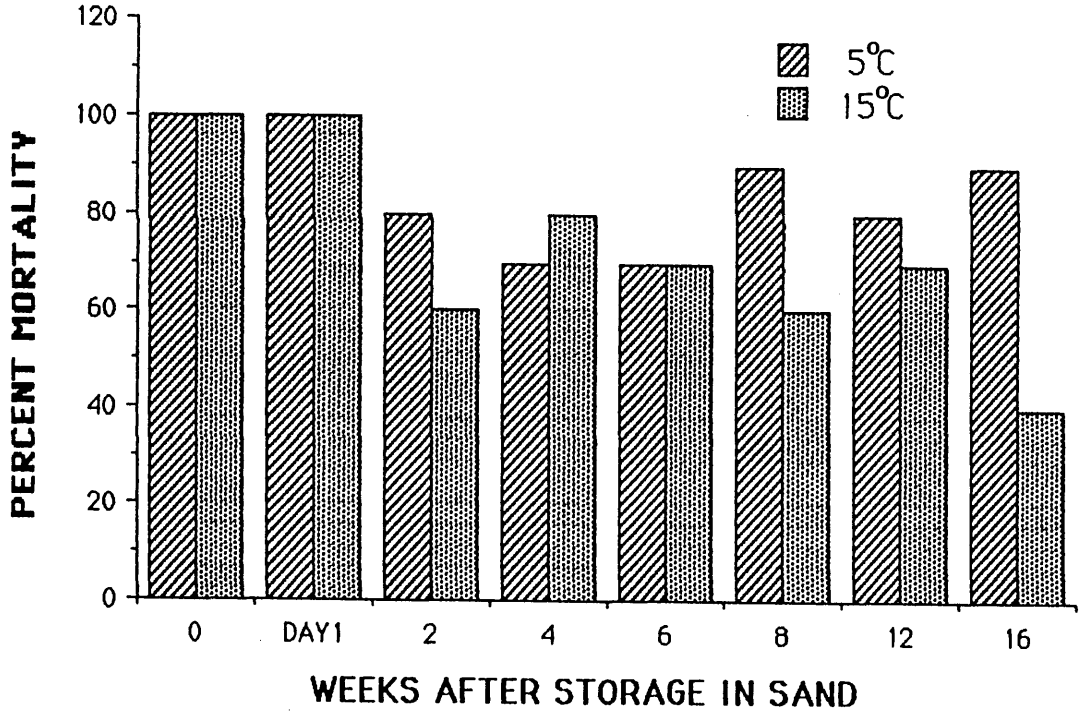


FIGURE 7.4 Mortality of Galleria mellonella larvae due to parasitization of Steinernema bibionis (Nashes) infective juveniles after storage in moist sand for various lengths of time at 5°C or 15°C. Ten Galleria larvae were exposed to the nematodes at 15°C for 96 hrs.

temperatures (Fig. 7.5). Nematode infectivity decreased rapidly at 15°C and by Week 16, only 2 infective juveniles on average were recorded from each Galleria larva, which was less than 3% of the nematodes established in Galleria larva on Day 0. In contrast, nematode infectivity at 5°C displayed a u-shaped trend, just as it did for the Site 42 nematodes. Thus, infectivity decreased rapidly to 10 nematodes/larva after 4 weeks and then gradually increased to 70 nematodes/larva after 16 weeks. Consequently, infectivity of the nematodes after 16 weeks returned to the same level as on the Day 0 (80 nematodes/larva).

Figures 7.6 and 7.7 show the accumulated mean number of nematodes which were able to infect Galleria larvae after several consecutive exposures of 4 days each to fresh larvae for the two storage temperatures respectively. The mean numbers recovered after one exposure for the two temperatures are also plotted in the Figures. The same trend for each particular temperature was observed. In general, the nematodes stored at 15°C for various lengths of time penetrated in the first exposure to the Galleria larvae and additional exposures did not result in significantly higher infections. However, for the nematodes which survived at 5°C, only Day 0, Day 1 and Week 16 bioassays showed no significant differences between the numbers established in Galleria larvae in the first exposure and the cumulative total numbers. Thus, when nematodes were stored at 5°C, their infectivity decreased rapidly but after 16 weeks, it returned to the level observed for "fresh" nematodes. Furthermore, after Day 1 and before Week 16, nematodes gradually recovered their infectivity if allowed extended exposure times to Galleria larvae. However, over weeks 2-12, the nematodes never attained the infection levels of Day 0 and 1 or Week 16.

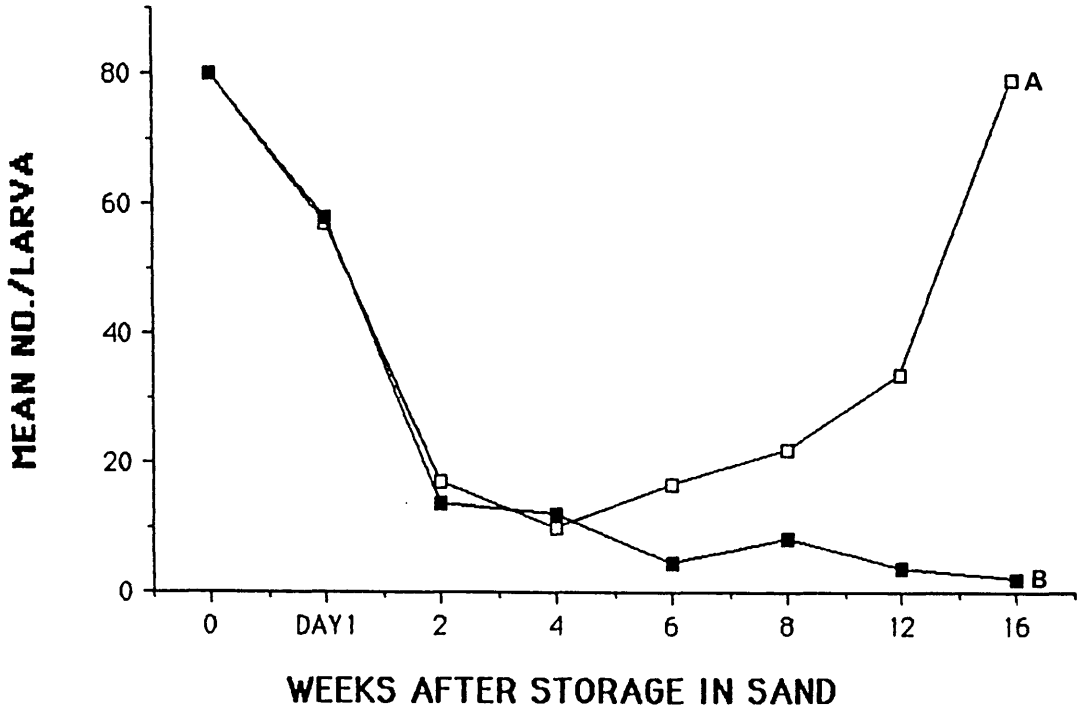


FIGURE 7.5 Number of Steinernema bibionis (Nashes) established in a Galleria larva after increasing storage time in sand (n = 10). A dose of 250 nematodes per tube was applied.

A: stored at 5°C and assessed after one exposure at 15°C for 96 hrs.

B: stored at 15°C and assessed after one exposure at 15°C for 96 hrs.

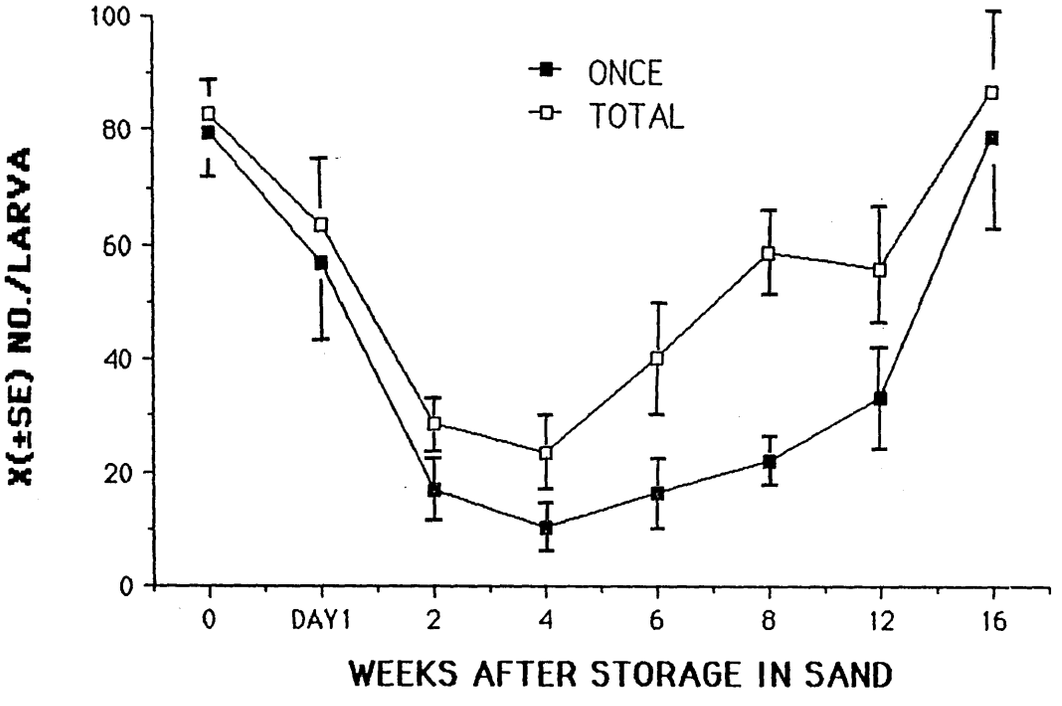


FIGURE 7.6 Number of Steinernema bibionis (Nashes) established in a Galleria larva after increasing storage time in sand at 5°C (n = 10, dose = 250 juveniles/tube).

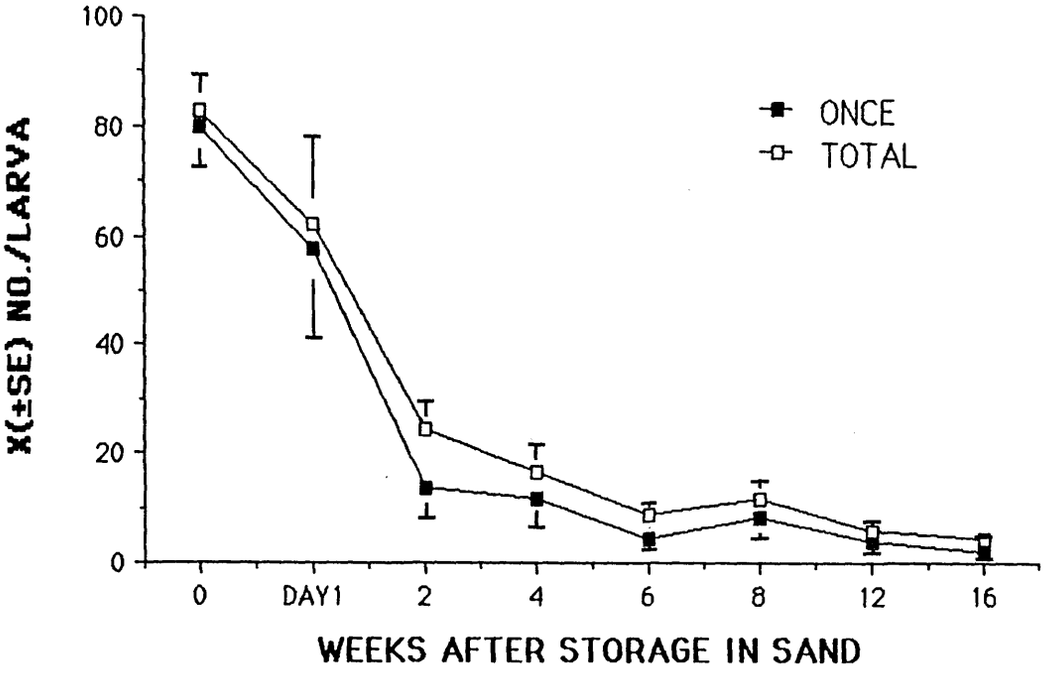


FIGURE 7.7 Number of Steinernema bibionis (Nashes) established in a Galleria larva after increasing storage time in sand at 15°C (n = 10, dose = 250 juveniles/tube).

7.3.3 Assessment of infectivity based on surviving nematodes

When the nematodes were used to infect G. mellonella larvae on Day 0, 33.1% (Nashes strain) and 30.7% (Site 42 strain) of the initial dose was eventually recovered from the cadavers, although the initial doses applied were different. These results confirmed the earlier ones (Chapter 6), which showed that the percent of nematodes that established in Galleria larvae was within the range of 30 - 40%, which was independent of nematode strain and the dose applied. However, the present experiments have gone further in that they have assessed the number of surviving nematodes in the sand over time. Hence, the cumulative numbers of nematodes that were infected can be expressed as a percentage of the surviving nematodes (i.e. those extracted from the Whitehead sieves) after storage at 5 or 15°C. Figures 7.8 and 7.9 present the data.

There was little difference between the percent of the nematodes that survived and infected on the day of inoculation for the two strains. Then, for both strains at 5°C, there was a drop in infectivity after several day's or week's storage at 5°C, followed by a steady increase. Thus, by Week 4, 52.5% of the surviving Site 42 nematodes were infective. The rate of increase for Nashes nematodes was slower, but by Week 16, 54% of the survivors were infective. Therefore, it appears that storage of the nematodes at 5°C does not dramtically affect survival (Fig. 7.1 and 7.3), but it does significantly affect the infectivity of the survivors (Fig. 7.8 and 7.9).

Infectivity of the surviving Nashes nematodes after storage at 15°C had a similar pattern compared to that obtained at 5°C, except for the unexpected abnormality on Day 1 (Fig. 7.9). However, while survival

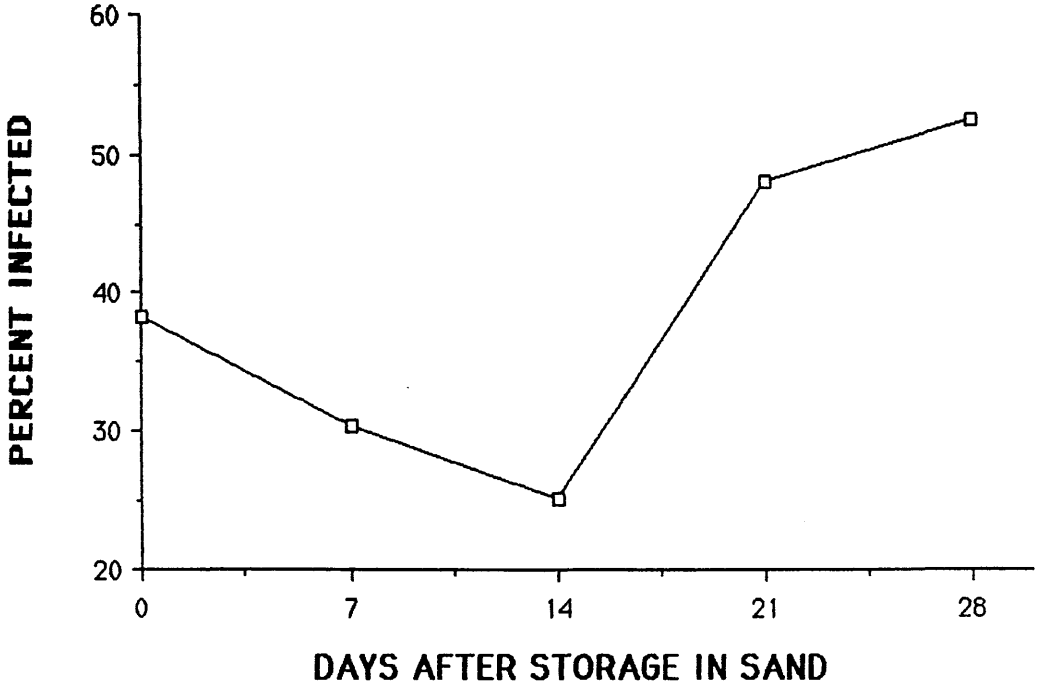


FIGURE 7.8 Mean number of Steinernema bibionis (Site 42) nematodes that infected as a percentage of the mean numbers extracted by modified Whitehead sieves.

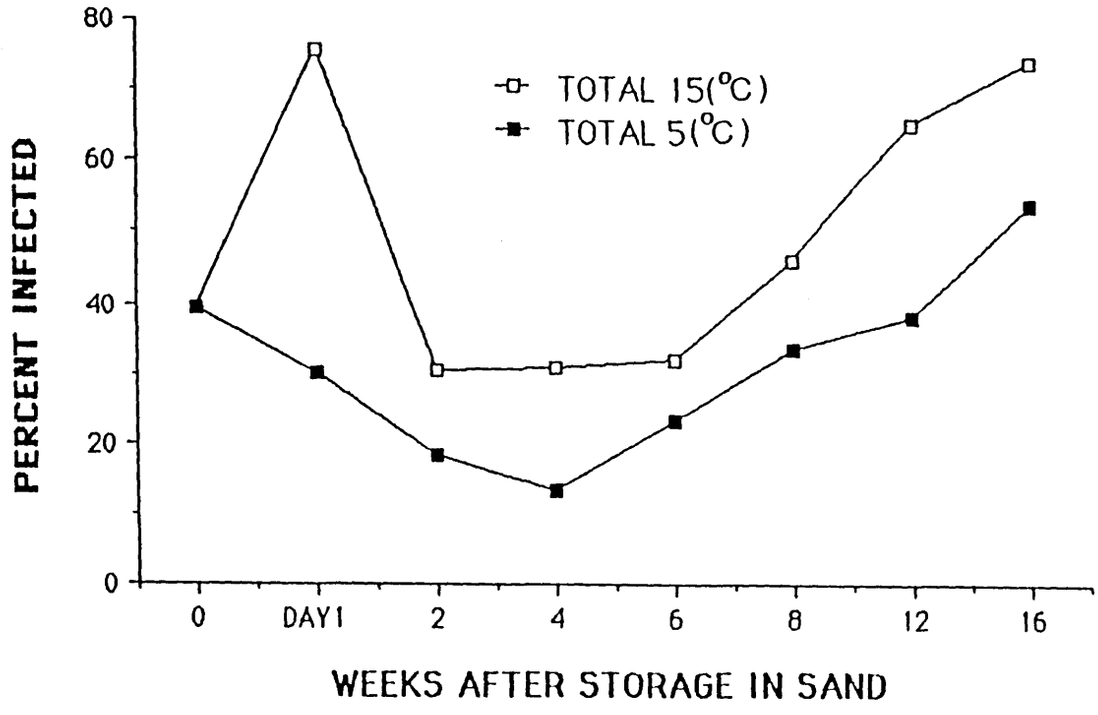


FIGURE 7.9 Mean numbers of Steinernema bibionis (Nashes) nematodes that infected as a percentage of the mean numbers extracted by modified Whitehead sieves.

at 15°C was much poorer compared to 5°C (Fig. 7.3), those nematodes that did survive were highly infective. Thus, after 6 weeks storage at 15°C, infectivity of the survivors began to increase and eventually reached 74.1% on Week 16.

7.4 DISCUSSION

The most important discovery in the present study was that the survival of infective juveniles at 5°C as measured by the numbers which could infect a host showed a u-shaped pattern with passing time instead of an expected decreasing trend. This u-shaped pattern was found for both the Site 42 and Nashes strain after they were stored in sand at 5°C for some time. The only difference was the time lapse between the movement when the nematodes showed reduced infectivity and the moment when they regained it (Fig. 7.2 and 7.6). This possibly represents a biological difference between the strains. This u-shaped pattern was not observed for the nematodes that had been stored in sand at 15°C (Fig. 7.5), indicating the special effect of 5°C on these nematode juveniles. It appears that the cold temperature induces the nematodes into a state in which they lose their ability to parasitize hosts, although they can move normally. For example, after 4 weeks at 5°C, a mean of only 24 nematodes was finally recorded from the insect cadavers while at this time, most of the infective juveniles migrated through the Whitehead sieve. It is unlikely that the sudden introduction of the juveniles into 5°C weakened or damaged them and caused the low infectivity, because the infectivity returned to the high level after storage at this temperature for a longer period.

The reasons for the loss and then regaining of infectivity at 5°C are unknown. One possibility is that some infective juveniles in the population lose their infectivity when subjected to low temperatures and require a period to adapt and regain it. Another possibility is that the infective juveniles that emerge from a cadaver become infective at different rates. This would extend the infectivity of the population over time and hence increase the probability that some of the nematodes would contact and infect a host.

The present study clearly indicated that the nematodes survived well at 5°C and retained their infectivity. If this period of storage at 5°C is similar to the winter season which these nematodes experience, the u-shaped pattern for infectivity may be an advantage for the nematodes. Instead of wasting energy to find and infect limited hosts in mid-winter, which would result in slow development or no development, it may be better for the nematodes to stay in the soil as infective stages until the winter has come to an end, when they can invade and reproduce. Observations during the experiments on low temperature effects on development of Nashes nematodes showed that some nematodes that had penetrated into the Galleria larvae died during the prolonged period at 5°C. Also, at cold temperatures, fewer individuals can infect and hence any hosts that are infected will harbour few or even single nematode. Though these nematodes can develop slowly to adults, the chances for them to reproduce will be small because of the absence of both sexes. As far as the population cycling is concerned, these infected hosts will make no contribution. However, if the nematodes remain in the soil in sufficient numbers and wait for warmer temperatures, then the chances for many nematodes to infect one host will be higher. The probability

of reproduction and release of new infective juveniles into the environment will then be much higher than at low temperatures.

The present study also shows that temperature is a very important factor affecting nematode survival. As measured by their mobility through Whitehead sieves, a greater number of S. bibionis (Nashes) that had been stored at 5°C survived than if they were at 15°C (Fig. 7.3). These results are similar to those of Molyneux (1985) who studied survival of S. feltiae and S. glaseri at different temperatures. Although the present study tested 2 temperatures, compared to 4 temperatures tested by Molyneux (1985), the inverse relationship between nematode survival and temperature is still valid.

It has been shown in laboratory studies (Georgis and Poinar, 1983a, b; Moyle and Kaya, 1981) that when infective juveniles were added to soil, they tended to move both vertically and horizontally from the point of application. The rate of movement depends on soil texture (i.e. most rapidly in sandy soil, Poinar, 1986) and temperature (Molyneux, 1983). Thus, the significant decrease in the number of nematodes recovered at 15°C (even just after 24 hrs at 15°C) is likely to be related to the rapid movement of the Nashes nematodes in the sand at this higher temperature, resulting in rapid utilization of their lipid reserves. Energy degradation was also thought to be a reason for poor survival of S. feltiae (Agriotos) infective juveniles at higher temperatures (Molyneux, 1985).

Other reasons for the different survival ability of various species of entomopathogenic nematodes have been proposed. Molyneux (1983) stated that differences in survival of species/strains at various temperatures are likely to be the result of their original geographical

habitats. Thus, species such as S. feltiae and S. glaseri that are from temperate or cold climates had long-term survival ability so that the infective juveniles can survive the winter months when insect hosts are likely to be scarce. Like S. feltiae (Molyneux, 1985), S. bibionis (Nashes) can survive over 16 weeks at 15°C, although only a few nematodes can be recovered after that time. Different from the above two species, S. glaseri (Molyneux, 1985) survived extremely well at high temperatures and even after 32 weeks in sand at 28°C, half of the applied nematodes could be recovered. The reason for this was that the S. glaseri K.G.strain became quiescent in sand as characterized by a distinct "coiled" posture even at 23°C. S. bibionis nematodes have been observed to become coiled in water when they were stored at temperatures below 10°C. Nematode juveniles extracted at 15°C by the Whitehead sieves after storage at 5°C, also adopted coiled postures. However, this coiled state was never found for the nematodes stored at 15°C. Furthermore, when the water containing the coiled nematodes was brought to room temperature (20°C), the nematodes were observed to uncoil gradually. Therefore, better survival of S. bibionis nematodes at 5°C may be because they became coiled and reduced their activities.

To measure survival of infective juveniles, Whitehead sieves (Ishibashi and Kondo, 1986a, 1987; Molyneux, 1985), sucrose extraction (centrifugal-flotation) (Ishibashi and Kondo, 1986a,b, 1987) and parasitization of insects by nematodes (Molyneux, 1985) have been used. Saunders and All (1982) showed that the Baermann funnel extraction (original form of Whitehead sieve) was superior to centrifugal-flotation in recovering N. carpocapsae (= S. feltiae) from sand. By contrast, Ishibashi and Kondon (1986b) reported that more nematodes of this

species could be extracted by the centrifugal-flotation technique than the Baermann funnel method. On the basis of these results, they claimed that S. feltiae was probably in a quiescent state during their experiments. In the present study, the number of nematodes recovered by the sucrose extraction method was generally lower than by the Whitehead sieve method, which supports the results obtained by Saunders and All (1982), presumably at room temperature. However, it was also found that more nematodes were extracted by the sucrose method than by the Whiteheead sieve after they had been stored at 15°C. At this temperature, the S. bibionis nematodes recovered from sand appeared transparent and the degree of transparency appeared to correspond with increasing storeage time. Also, more transparent nematodes were recovered when the sucrose extraction method was used. Therefore, at higher temperatures, the lower number of nematodes recovered by Whitehead sieves was likely to be related to the decreased mobility or inability to migrate through sieves, due to depleted energy reserves. However, the nematodes that were not able to move through the Whitehead sieve could be extracted by the sucrose solution which is not dependent on nematode activity. Because the extractions made by Ishibashi and Kondo (1986b) were after the nematodes had been stored in soil at 25°C, their claim for a state of quiescence in S. feltiae seems to be questionable for explaining the higher number they recovered by the sucrose extraction technique.

In the present study, infectivity of juveniles as measured by percent parasitization of G. mellonella larvae did not show a close relation to survival of the nematodes with time as assessed by their mobility through a Whitehead sieve. However, Molyneux (1985) showed that parasitization of Lucilia cuprina declined with time in a similar

manner to their survival as assessed by mobility. This difference may be because the testing hosts were different. Galleria larvae are well-known for their high susceptibility to entomopathogenic rhabditids and even at low dosages, high percent mortality can be achieved (Molyneux, 1983). In the present study, more than 200 nematodes were applied and the temperature of 5°C was also favourable for survival of the majority of nematodes. Thus, it is not surprising that 100% mortality could always be recorded after storage at 5°C, regardless of the period of storage (i.e. Site 42 nematodes). At 15°C, though the nematodes did not survive well, high mortality still occurred (Fig. 7.4).

Infectivity indicated by percent mortality has been accepted and used as a more specific property than mobility for measuring nematode "vigour" (i.e. Ishibashi and Kondo, 1986a; Molyneux, 1985). However, the percent mortality of Galleria larvae did not give a realistic indication of nematode vigour. Also, percent mortality of hosts cannot give the numbers of nematodes which established. To assess the number of nematodes with "vigour", a post-mortem of an infected host, preferably one as highly susceptible as larvae of G. mellonella, must be carried out.

In conclusion, differences in survival and infectivity of S. bibionis infective juveniles in sand were found at 5 and 15°C. The nematodes were capable of surviving over 16 weeks at 15°C. The U-shaped pattern for their infectivity may suggest that these nematodes have a means of regulating their over-winter survival and synchronizing their activity with host availability.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Extensive surveys for entomopathogenic rhabditid nematodes in the U.K. have yielded predominantly one species, S. bibionis Bovien (Blackshaw, 1988; Hominick and Briscoe, in prep.). The wide distribution of this species makes it an important insect-parasitic nematode to be considered for future biological control programmes in Europe. However, the information that existed on the infectivity, reproduction, survival and other biological attributes of this nematode was far less than for other Steinernema species. In particular, there was no information available for the British strains of S. bibionis. Therefore, this study was initiated to investigate the bionomics of a population of S. bibionis native to grassland in Silwood Park. Because it is well-known that biological strains of steinernematids are common, another British isolate and a standard Tasmanian strain were included in the study for comparative purposes.

Since the nematodes used were isolated from soil, it was essential to identify them so that comparisons with a known species, in this case, the T319 strain of S. bibionis, could be made. On the basis of morphological characters evaluated in the present study (Table 3.2), the two British isolates were assigned to the species S. bibionis Bovien and this was confirmed by cross-breeding experiments. Assessment of characteristics of the bacterium in the nematode/bacterium complex could also provide valuable information on the identification of the nematode species. Akhurst (1986) showed that Xenorhabdus nematophilus subsp. bovienii was the specific bacterium that was associated with the

nematode S. bibionis. The bacterial isolates of the three strains have been confirmed to be the bibionis-type of bacteria (Hand, pers. comm.). However, characters of the bacteria, morphological measurements and hybridization tests can only distinguish different species of Steinernema nematodes, but not different strains. To achieve strain identification, application of biochemical techniques and DNA analysis might help. Indeed, recent results comparing the DNA of Nashes nematode with other nematode isolates indicate that it is sufficiently different to question its specific status (Reid, pers. comm.).

As reported for other Steinernema spp. (Molyneux, 1986), temperature was an important factor influencing infection, reproduction and emergence of the three strains of S. bibionis tested in this study. The results showed that the three strains have their own optimal temperature range for infection, development, reproduction and emergence, which supported reports by other workers for different species. In general, the Nashes nematodes favoured cooler temperatures while the Stie 42 and T319 strains preferred warmer ones in the range 5 - 30^{o}C.

It is surprising that nearly all the work concerning infectivity of steinernematids used mortality of the hosts (or percent parasitization) as an index of infectivity while the number of nematodes that infected the host was neglected. Much valuable information regarding the population dynamics of the nematodes was, therefore, missed. The present study considered the influences of different temperatures on the number of nematodes that infected and revealed that the effect on the number that penetrated was far greater than the effect on the percent of insect hosts which were parasitized. In this way, a more restricted temperature range for optimal infection by each strain was found. To

evaluate the efficiency of the nematodes, an assessment of the numbers in hosts is more important than insect mortality.

The studies on the effect of temperature on infectivity, reproduction and emergence of the S. bibionis nematodes revealed that the two British strains were very different in their performance, with the Site 42 strain having the superiority in their large capacity for infective juvenile production and the Nashes strain proving superior in its ability to infect and develop at low temperatures. In order to obtain a large number of infectives at lower cost, nematodes such as the Site 42 strain, which always gave higher numbers per host over a wider temperature range should be used. Further tests should be carried out to examine the productivity of the Site 42 nematodes in vitro.

Low temperature influenced the number of nematodes which could penetrate and the rate of development inside cadavers. The insects will not be killed immediately when infected with the nematodes at the low temperatures. Death could be very slow and would only be brought on by a rise of temperature. This is particularly important for dispersal of the nematodes in the field (Molyneux, 1986). However, to disperse and establish a new focus, the nematodes within the cadavers must be able to survive and reproduce. Therefore, those nematodes which can infect insects at low temperatures in the largest numbers will have the best chances to disseminate their populations. This may explain the wide distribution of S. bibionis in British soil. S. feltiae was recorded from British soil once (Georgis and Hague, 1981). Since then, there have been no reports on the discovery of this species in the U.K. The restricted distribution of S. feltiae is likely to be attributed to its biological characters, that is, the warmer temperature requirements for

infection and development. At the same cold temperature, S. bibionis is capable of infection and is then carried away by the infected host while the S. feltiae neamtodes can only wait until temperature rises high enough to start infection. Under British conditions, temperatures higher than 20°C are rare in soil so that the S. bibionis has greater chances to infect and occupy the hosts than the S. feltiae. The dispersal of the nematodes by the infected host is, therefore, likely to be much greater compared to the S. feltiae.

Survival of the S. bibionis nematodes in the absence of hosts was also influenced by temperature. Low temperature greatly increased the survival of the infective juveniles in sand. Not only the survival, but also the infectivty of the surviving nematodes was affected by temperature. Against the expectations of a declining trend for infectivity over time, the Nashes and Site 42 strains displayed a U-shaped pattern in their infectivity to late instar larvae of G. mellonella after they were stored in sand at 5°C. The mechanism for this phenomenon is unknown, but may be related to low temperature because it was not observed at 15°C. In fact, if the survival of the Nashes strain over winter is considered, the U-shaped infectivity pattern will benefit the population. During the winter, few hosts would be expected. Instead of the entire population expecting to locate a host, it would be advantageous if most of the nematodes wait until the end of the winter. Increasing temperatures will favour the chances of finding a host and reproducing in large numbers.

Optimal temperatures allowed maximum infectivity (abundance and intensity) for the three strains of S. bibionis to G. mellonella larvae. However, this maximum infection still represented less than half of the

entire nematode population available in the habitat of the host. Though the number of nematodes that could be recovered from the insect cadaver varied with the number of infective juveniles applied to the medium, the proportion of the nematode population that established in the insect was independent of the dose and ranged from 30-40%, if sufficient contact time (e.g. 72 - 96 hrs) was allowed. There are several factors that account for the fate of the rest of the nematode population. Firstly, some of the nematodes died without penetrating. However, this will only account for part of the population, because the infective juveniles in sand could survive at 15°C. More than 30% of the initial dose applied was left after two week's storage. Secondly, some nematodes may have started to penetrate but died and/or some entered the insects but failed to develop. Traces of dead small cadavers would be difficult to find during the dissection. In the present study, some experiments involved injection of infective juveniles into G. mellonella larvae. It was observed that a mean of 3.5 nematodes could be found to develop to adults when 5 nematodes were injected into one Galleria larva. This may represent the rate of establishment for the nematodes that penetrated. However, possible damage to the nematodes during the injection process should also be considered. Hence, in the process of natural penetration, the rate of establishment should be higher than 70% (3.5/5). Thus, this possibility cannot account alone for the disappearance of 60% of the nematode population. Thirdly, the nematodes may still be alive, but they were not infective at the time of the experiment. In the present study, the nematodes were exposed to insect larvae continuously. By contrast, in a similar experiment by Briscoe (per. comm.), exposure of the nematodes to G. mellonella larvae was broken by intervals of 2 weeks and the

accumulated proportion of the nematode population that established inside the insect larvae was far more than 40%. Therefore, it may be that continuous exposures cause a strong source of stimulation to accumulate. At first, it may help to attract the nematodes (see 6.3.2), but later it may retard further infection. Thus, only part of the population would be attracted when a host appears and is colonized. This would leave the rest to wait for other hosts and would prevent overcrowding in a single individual. This is of obvious importance for sustaining the nematode population, and would prove a fruitful area for further investigation.

Although more research is necessary to provide information on the factors responsible for limiting the level of infection, the stable proportion is certainly useful for predictive purposes. Efforts to estimate the number of nematodes in a soil sample have been made (Mracek, 1982b). However, an estimate of numbers which depends on the mortality of insects is not reliable because the present study has shown that for the same rate of death, the number of nematodes recorded varied greatly. The results suggest that an alternative method for estimating the number of nematodes in a soil sample can be achieved by counting the established nematode adults in the insects. Pre-testing using a particular host would be important because hosts other than Galleria larvae might have different levels of infection.

The population size and distribution of insect-parasitic rhabditids in the field are other questions which should be answered, particularly in a quantitative way. Unlike population studies for plant-parasitic nematodes, in which quantitative analyses can be made based on the nematode population in plants already in the field, studies

of insect-parasitic nematode populations need to use baiting techniques. Many insects and numerous dissections are required if the nematode population is to be quantified. This is probably why few studies on field populations have been carried out.

The present study investigated a natural population of steinernematids in a plot of grassland in an attempt to provide some information on the spacial distribution and numbers of the nematodes. The results were similar to those for other soil organisms in that an aggregated pattern was observed. Thus, high numbers of nematodes appeared in a few sites and low numbers or none in others. In those sites with high numbers of nematodes, it is probable that release of new infective juveniles from an infected host had occurred recently. Indeed, an insect larva containing both males and females of the Nashes nematodes was found in the sample from which 2817 nematodes were finally extracted by the Galleria larvae (Site 1, October 1987). Slow migration of Steinernema nematodes (Georgis and Poinar, 1983a,b) tends to preserve the aggregation. In those sites with low numbers of nematodes, survival rates and migration of the nematodes are the factors that are having the greatest effects on the population size.

In the present study, most of the nematodes were found in the top 5 cm of soil. With increasing depth, the numbers declined sharply, so that only a few nematodes were found in the soil taken at a depth of 15 - 20cm. Here, the importance of hosts in determining the distribution of the nematodes must also be a consideration. It is very likely that nematodes in the lower depths were there because they had dispersed from the top 5cm of soil. In the literature, it has been found that the majority of S. feltiae infectives remained at the point of placement if

placed on the surface, although a few dispersed up to 12cm downwards (Moyle and Kaya, 1981). When deposited at 15cm below the surface, the majority remained at that location, but dispersal tended to be upwards rather than downwards (Moyle and Kaya, 1981). Whether S. bibionis behaves similarly is unknown. Nevertheless, in the field, if a new focus is formed by the death of an infected insect, it is likely that the dissementing process will be carried out near the soil surface rather than deep in the soil. After the release of the infective juveniles, the majority would remain at that place or would seek hosts which frequently appear in the top 5cm of soil. Only a few may move down to the deeper soil. Presence of a host will increase the number of nematodes which disperse (Georgis and Poinar, 1983a,b). However, the chances of encountering suitable hosts in the soil below 15cm are probably small so that the nematodes would rarely reproduce at that depth. Even if they do reproduce occasionally at that depth, the attraction of hosts in the upper soil will probably result in the upward dispersal of these nematodes in large numbers. Thus, the Nashes nematodes were found aggregated in the upper soil rather than in the deeper soil.

The Nashes nematodes were found on all the sampling occasions, even when the soil temperature was 6^{o}C. The number of nematodes obtained on each occasion varied greatly, so no conclusions can be made on whether there is a seasonal variation involved. Even if more samples had been taken more frequently, it is doubtful whether a seasonal effect could be discovered because of the great variability on even limited geographical areas. Actually, it is possible that the nematode has a low but continuous population throughout the year. As expected in

summer, favourable temperatures and plenty of hosts would enhance the production of the nematodes so that large numbers of infective juveniles appear in the field. However, summer temperatures do not benefit the survival of the nematodes and a large proportion will die in a few weeks. This natural mortality will limit the level of the nematode population. In contrast, low winter temperatures tend to favour survival of the nematode juveniles in the field, though reproduction is slow. On balance, the natural nematode population may not differ greatly at each season.

A question arising from the population study is how to assess accurately the size of the nematode population. Percent mortality of the Galleria larvae in the trap indicates the presence or absence of nematodes but not the numbers. Consecutive bioassays using Galleria larvae will show the total number of nematodes in the soil sample. However, this "total number" in some circumstances, contains "potential nematodes" which may not represent the population in the field. For example, if the soil contains nematode-infected insects, the successive exposures at 20°C will enable many infective nematodes to be released, leading to an increase in the number of nematodes in that soil sample. However, if the soil was left in the field, there will be two possibilities. In summer, the warmer soil temperature similar to the temperature in the laboratory will probably give rise to the same results. Then the population estimated by the bioassay is likely to represent the population in the field. Alternatively, if it is winter or the colder months, the population level measured in the laboratory will not be the same as that in the field. Then, the level attained in the laboratory does not represent the nematode numbers in the field, where these "potential

nematodes" may never become the real population because of possible death of the infected juveniles within the host (see Discussion, Chapter 7).

Shortening the exposure time in the bioassay can avoid over-estimating the existing population. However, there is then a larger risk in under-estimating the population, because the present study showed that many nematodes tended to remain in the soil after the first exposure. On balance, it is better to assess the nematode population through successive exposures, because all the nematodes in a sample rarely infect in the first bioassay, while infected hosts are rarely found in a soil sample from the field. In addidion, if seasonal effects are to be studied, special attention should be paid towards the environmental factors such as the temperature around the sampling time. Then appropriate interpretations can be made if exceptionally high populations are encountered.

Correlations between the nematode populations in the field and environmental factors have not been possible in the present study, although the presence of insect hosts is believed to have the greatest effect on the nematodes. Extensive studies are necessary to assess the effect of moisture, vegetation, soil structure and predators or pathogens on the fluctuations of the nematode populations in the field. Such studies should also be carried out to monitor the population distribution and changes following an application of nematodes in control programmes.

S. bibionis was described by Bovien in 1937 and its description, biology and life cycle were elaborated by Wouts (1980). However, use of this nematode in biological control programmes and investigations on its

biology lay far behind the extensive work on S. feltiae . S. bibionis is a wide-spread species in British soil (Blackshaw, 1988; Hominick and Briscoe, in prep.). Although only two British strains have now been studied in some detail, the results show that one strain was cold-adapted and the other was more virulent and productive compared to the Tasmanian T319 strain. Considering that almost half of any soil samples in the U.K. will harbour S. bibionis, there are many opportunities to recover new strains with their own special abilities. This nematode has great potential to be used against pest insects in the U.K. in the future.

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APPENDIX I

EFFICIENCY OF THE MODIFIED WHITEHEAD SIEVE AND SUCROSE EXTRACTION

To extract infective juveniles from sand, modified Whitehead sieves and sucrose extraction methods were used (see General Methods and Materials). To assess the efficiency of the technique in extracting the nematodes from sand, two experiments were carried out.

MATERIALS AND METHODS

Infective juveniles of S. bibionis (Nashes) which were less than 2 weeks old, were introduced into sand-filled tubes (2.3) at doses of 10, 50, 100, 200 and 300 nematodes per tube. There were 5 replicates for each method/dose combination.

The modified Whitehead sieve (see 2.6) was set up. The sand containing various doses of nematodes was evenly distributed on the tissue paper and then 25ml of tap water was poured into the bottom dish. This unit was left undisturbed for 24 hrs in a 20°C incubator. After 24 hrs the bottom dish containing the extracted nematodes was removed and replaced with a new one with 25ml of water. This was repeated at 24 hr intervals to a total of 72 hrs. The nematodes were counted after each extraction.

To assess recovery of the sucrose method, the sand was placed in a 300ml beaker and 50ml of sugar solution (sp.gr. 1.18) was added. The contents were shaken by hand for 2 minutes. After sitting for a few seconds, the clean supernatant was poured into another beaker containing 250ml of tap water. The nematodes were allowed to settle for at least 2 hrs and the excess water was slowly poured off, leaving the nematodes in approximately 30ml of water. The nematodes in the water were counted.

RESULTS AND DISCUSSION

Figure I.1 presents the mean (± se) percent recovery applied at different dosages from sand samples after 24, 48 or 72 hrs using the modified Whitehead sieves. Analysis of variance performed on the transformed data at each of the 5 doses showed no significant differences among the recoveries (or efficiencies) after the three extracting periods (ANOVA F = 0.73, 2.41 or 1.93 respectively, $p>0.05$). Therefore, the pooled mean percentage of extraction after 24, 48 or 72 hrs, regardless of dose, was 54.2, 74.4 and 75.8%. When the data obtained for individual doses were analysed for the influence of extracting time on efficiency, significant differences were found between 24 hrs and 48 and 72 hrs, but a 48 hr extraction produced no significant difference from the 72 hr extraction for doses of 50 to 300. Large variations occurred when there were only 10 nematodes per sand tube so that the longer extracting periods did not yield a significantly better recovery compared to the 24 hr period (ANOVA, $F=1.03$, $p>0.05$). Thus, for extraction of nematodes from sand, the most appropriate extraction time will be between 48 and 72 hrs if the nematode doses are more than 50 infective juveniles/tube. The efficiency will range from 76.5 to 77.8%.

The results for recovery of S. bibionis (Nashes) nematodes from sand samples using the modified sucrose extraction method are presented in Figure I.2. Analysis of variance performed on the transformed data showed that the percent recovery varied with the initial nematode dose applied. Higher recovery was obtained when the doses applied ranged from 100 to 300 infective juveniles/tube than at 10 - 50 infective juveniles/tube. Therefore, two efficiency coefficients were established. One was 28.24% (for doses ranging from 10 - 50) and the other was 53.5% (for doses ranging from 100 - 300).

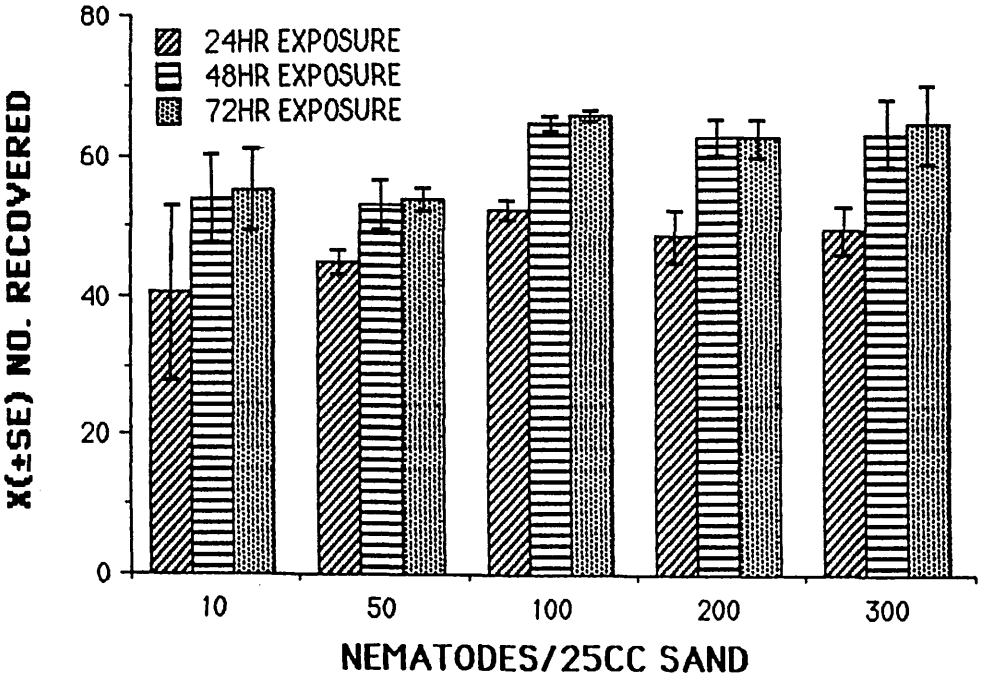


FIGURE I.1 Extraction of Steinernema bibionis (Nashes) from sand samples inoculated with different doses at 20°C using modified Whitehead sieves (n = 5).

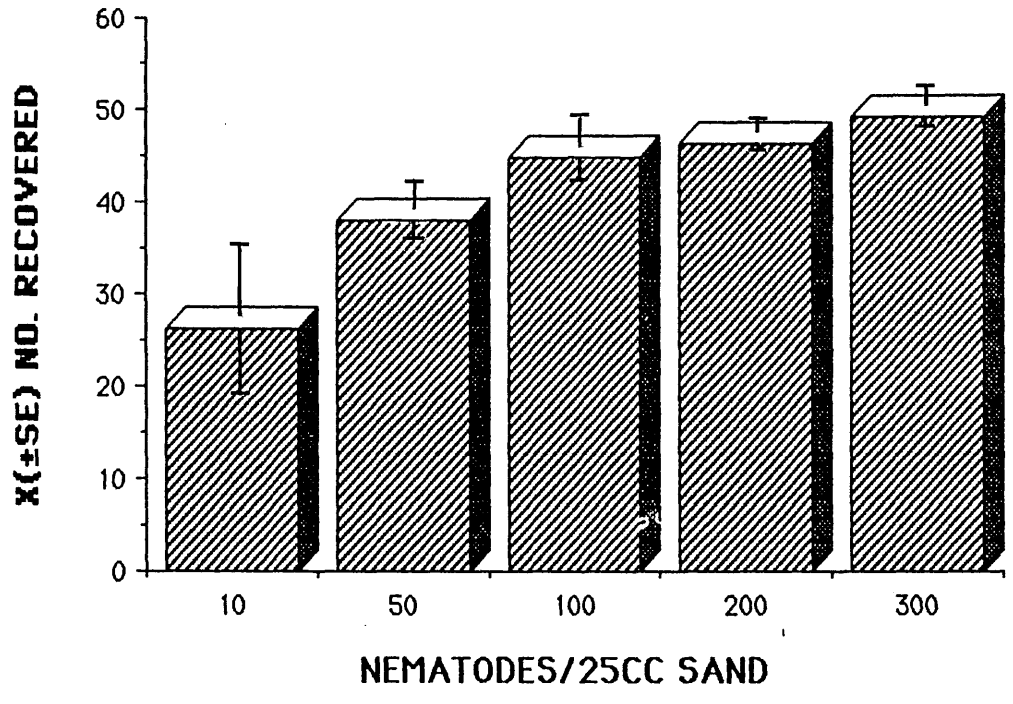


FIGURE I.2 Extraction of Steinernema bibionis (Nashes) from sand samples inoculated with different doses, using a modified sucrose extraction method (n = 5).

There have been a few studies on the efficiency of methods in extracting entomopathogenic nematodes from soil (Ishibashi and Kondo, 1986b; Renn, 1985; Saunders and All, 1982). Because the extracting methods were modified, and the dose of nematodes, the soil or sand volumes or the nematode species/strains used were different, the efficiencies or percent of recovery obtained in each individual study are not comparable. However, these results provide valuable information for various extracting methods.

It was clearly shown that the Whitehead sieves were superior to the sucrose extraction in the present study, which is similar to the results obtained by Saunders and All (1982). Mobility of the nematodes to water may account for the higher recovery of the nematodes in the Whitehead sieve.

The present study assessed the influence of doses and/or extracting time on efficiency of the extracting methods, which is rarely found in the literature concerning extraction efficiency of insect-parasitic rhabditids. The results indicate that the sucrose extraction method was influenced more by nematode dose than the Whitehead sieves. Although extracting time can affect recovery of the nematodes by the Whitehead sieves, the influence disappeared after 48 hrs regardless of dose. To obtain a reasonable efficiency within an acceptable time, the Whitehead sieves are recommended for extraction of the nematodes from sand. In the present setting, approximately 77% of the applied nematodes can be recovered after 72 hrs. This is similar to results of Saunders and All (1982) who recovered 74% of S. feltiae from sand after 48 hrs.

APPENDIX II

RELATIONSHIP BETWEEN PERCENT MORTALITY AND NUMBER OF NEMATODES

Many workers have assumed that the percentage of infected insects in Galleria traps is related to the number of nematodes in a soil sample (e.g. Mráček, 1982b). However, experimental data supporting this assumption is rare.

In earlier studies by the author (Chapter 4), a natural population of S. bibionis was sampled from the soil in grassland. The Galleria baiting technique was used to extract the nematodes from the soil sample. Therefore, each soil sample of about 200cc in volume provided a percentage mortality due to exposure of the insects and also the number of nematodes that were recovered from the infected insects. The experimental studies showed that the number of nematodes recovered from the insect cadavers was closely related to the number of nematodes in a sample (see Fig. 6.7). The results from the grassland sample permitted analysis of the relationship between the percent mortality and the number of nematodes obtained from the infected hosts. Therefore, the percent mortality caused by the S. bibionis nematodes and the number of nematodes obtained from the infected insects were recorded after 5 G. mellonella larvae were exposed for 4 days to soil samples of 200cc taken at a depth of 0-5cm. A two-sample t-test was used whenever possible to see whether there was a significant difference between the mean numbers of nematodes present in the insects for adjacent percentage mortalities.

Table II.1 presents the results. With an increase in the percent mortality, there is a trend for increased mean numbers of nematodes recovered on each of the three sampling occasions. However, when the

TABLE II.1 Mean and ranges for the numbers of S. bibionis (Nashes) recovered from soil samples taken on three different occasions, with corresponding percentage mortalities. Each soil sample was 200cc and was exposed to 5 Galleria larvae for 4 days at 20°C.

Percentage	Sampling time					
	6/1987		10/1987		4/1988	
(%)	Mean	Range	Mean	Range	Mean	Range
0	0	0(2)*	0	0(3)	0	0(11)
20	1.7	1- 3(3)	4	3- 5(3)	1	1(4)
40	3	2- 5(4)	5.8	4- 7(4)	3	3(2)
60	11.4	5-20(5)	11	3-24(4)	11	4- 16(4)
80	12.6	9-21(8)	28.8	7.46(5)	80.3	9-222(3)
100	24.7	14-45(3)	44.3	13-90(6)	152	152(1)

* The number in brackets represents the number of soil samples having that percentage mortality.

mean numbers of nematodes recovered between two adjacent percent values were tested, no significant differences were found because large variability occurred. For example, when the soil samples taken in June, 1987 were assessed, 5-20, 9-21 and 14-45 nematodes were recorded from the insects for mortality rates of 60, 80 and 100% respectively. Although the highest number of nematodes was often recorded from the insects having 100% mortality, the number of nematodes which were found in the insects with the same mortality (100%) usually fell into the ranges for lower mortalities. Therefore, mortality is a poor indication for the nematode population in soil.

Mráček (1982) concluded that the number of infective S. feltiae larvae in the soil can be estimated with the Galleria trap method by evaluating both the percentage mortality and the time at which larvae of G. mellonella died. His results showed a trend in which increasing dosages caused higher mortalities sooner. However, many of the dosages used were high so that from the dose of 500 to 5×10^5, insect mortality was always 100%. There was also considerable overlap in the mortalities between doses and the number of replicates required for different times at the same dose meant that many Galleria larvae were required. Therefore, as the results in this thesis have shown, consecutive exposures to Galleria larvae, together with counting the numbers of nematodes that invade, is the most accurate method for estimating numbers of nematodes in a soil sample.

APPENDIX III

SEX RATIO IN *S. bibionis* POPULATIONS

The structure of steinernematid populations is poorly studied because few workers document numbers of parasites in a host. In the present study, males and females were recorded in several experiments when counting the total number of nematodes that established inside *G. mellonella* larvae. Therefore, the ratio of males to females of *S. bibionis* in the 1st generation could be assessed.

MATERIALS AND METHODS

A. Male/female ratio in experiment 6.2.3

In this experiment, 50 infective juveniles of *S. bibionis* (Nashes) per insect were inoculated into each of 200 sand-filled tubes. After placing *G. mellonella* larvae individually on the sand surface, these tubes were divided into 4 groups with 50 tubes in each group. At the following times, the insects were recovered and new ones were introduced: Group 1, 24 hrs (a), 24 hrs (b) and 24 hrs (c); Group 2, 24 hrs (a) and 48 hrs (b); Group 3, 48 hrs (a) and 24 hrs (b); Group 4, 72 hrs (a), 72 hrs (b) and 72 hrs (c). The recovered insects were dissected in Ringer's solution (see 2.5) and the numbers of males and females were recorded.

B. Male/female ratio in experiment 6.2.1

In this experiment, infective juveniles of *S. bibionis* (Nashes) were introduced into sand-filled tubes at doses of 10, 50, 100, 150 and 300 nematodes per tube respectively. There were 15 replicates for each of the 5 doses. The tubes were incubated at 15^{o}C for 144 hrs and the

Galleria larvae were recovered. This process was repeated five times and 375 Galleria larvae were dissected. The males and females present were recorded.

C. Male/female ratio in experiment 5.2.1

In this experiment, a dose of 40 infective juveniles per larva was used with 20 replicates for each nematode/temperature combination. The nematode strains were S. bibionis (T319), S. bibionis (Site 42) and S. bibionis (Nashes). The temperatures at which the Galleria larvae were exposed for 48 hrs were 5, 10, 15, 20, 25 or 30°C. The Galleria larvae that were recorded were incubated for a further 48 hrs on moist filter paper at 20°C. They were then dissected and the number of males and females were recorded for each Galleria larva.

RESULTS AND DISCUSSION

In these three experiments, the number of males or females recorded from each Galleria larva was summed for each particular group to which it belonged. The ratio of males to females was then calculated for the various groups. The results are presented in Tables III.1, III.2 and III.3 for the three experiments respectively.

In general, the ratio of males to females in a population is approximately 1:1. This ratio does not seem to be affected by temperature (Table III.3), nematode density (Table III.2) or nematode strain (Table III.3). Table III.1 shows the range of the male/female ratio which was calculated for each Galleria larva in a group. These ranges indicate that the sex ratio in individual hosts varies greatly. However, the ratio obtained for the overall population in an experimental group showed roughly equal numbers of both sexes.

TABLE III.1 Sex ratio in an S. bibionis (Nashes) populatin after groups of 50 Galleria larvae were exposed to 50 infective nematodes/insect for various times at 15°C. Each insect was exposed in individual tubes.

Group	Treatment	Number of insects	Number of nematodes		Male/ Female Index	range*
			Female	Male		
1	24 hrs (a)	50	201	198	0.99	0.2 -11.0
	24 hrs (b)	50	83	86	1.04	0.25- 4.0
	24 hrs (c)	50	27	17	0.59	0.33- 2.0
	total (72hrs)	150	313	301	0.96	
2	24 hrs (a)	50	179	198	1.11	0.2 - 4.0
	48 hrs (b)	50	150	139	0.93	0.25- 5.0
	total (72hrs)	100	329	337	1.02	
3	48 hrs (a)	50	427	499	1.17	0.43- 5.0
	24 hrs (b)	50	30	31	1.03	0.33- 2.0
	total (72hrs)	100	457	530	1.16	
4	72 hrs (a)	50	486	524	1.08	0.33- 4.0
	72 hrs (b)	50	18	17	0.94	0.02- 5.0
	72 hrs (c)	50	10	6	0.60	1.0 - 1.0
	total (216hrs)	150	514	547	1.06	

*range: sex ratio calculated for males and females recovered from each individual host within that group. A host which contained a single sex only was not included.

TABLE III.2 Sex ratio of S. bibionis (Nashes) population after a group of 15 Galleria larvae was exposed individually to various doses of infective juveniles for 5 x 144 hrs at 15°C.

	Dose (juveniles/larva)														
	10			50			100			150			300		
	F	M	M/F	F	M	M/F	F	M	M/F	F	M	M/F	F	M	M/F
1st	31	20	0.65	181	197	1.09	305	297	0.97	437	411	0.94	805	812	1.02
2nd	3	2	0.67	20	15	0.75	5	13	2.6	94	85	0.90	143	139	0.97
3rd	1	1	1.00	2	1	0.5	3	2	0.67	11	12	1.09	42	31	0.74
4th	0	0		0	0		0	0		3	2	0.67	27	34	1.26
5th	0	0		0	0		0	0		0	0		1	1	1.00
Total	35	23	0.66	203	213	1.05	313	312	1.0	545	510	0.94	1018	1021	1.0

Key: F = Female; M = Male; M/F = Male/Female

TABLE III.3 Sex ratio of three strains of *S. bibionis* after they were exposed to *Galleria* larvae at 6 temperatures for 48 hrs followed by 48 hrs at 20°C. A group of 20 *Galleria* larvae was used for each strain/temperature combination.

Strains	5°C			10°C			15°C			20°C			25°C			30°C		
	F	M	M/F	F	M	M/F	F	M	M/F	F	M	M/F	F	M	M/F	F	M	M/F
S. bibionis (T319)	2	3	1.5	39	38	0.97	170	124	0.73	153	125	0.82	66	54	0.82	30	32	1.07
S. bibionis (Site 42)	4	2	2.0	44	34	0.77	185	162	0.88	140	153	1.09	154	164	1.06	33	28	0.85
S. bibionis (Nashes)	19	19	1.0	36	35	0.97	133	165	1.24	105	138	1.31	64	70	1.09	1	0	-

Key: F = Female; M = Male; M/F = Male/Female

It was observed that some hosts contained males or females only in these experiments. Table III.4 was constructed to show the number of hosts which contained males or females only and the number of male or female nematodes involved in experiment 6.2.3. The number of hosts which contained male or female nematodes only in the 4 groups varied and there was no evident trend for one sex to predominate in single-sex infectives. In addition, single-sex infectives usually involved few nematodes in a particular host. Therefore, the overall male/female ratio, although in different hosts, was approximately 1:1.

It was also calculated that within the total number of Galleria larvae used (500), 61 insects contained only one nematode (male or female). Of these, 34 were males and 27 were females. If the uninfected Galleria larvae were excluded (500-117=383), nearly 16% of the hosts infected with S. bibionis (Nashes) had only one nematode. In a study of the sex structure of S. feltiae (Bendarek et al., 1986), it was found that 25.1% of all dissected insects contained one nematode, a result similar to the present one. They also found that the hosts with a single male nematode were in a proportion 1:1.6 to the hosts in which single females were found. In the present study, the proportion of 1:0.8 was obtained, but in favour of the males. Unlike the study of Bednarek et al. (1986), another ratio was also calculated. This included all the hosts from which more than one individual of the same sex were recovered rather than only those with single males or females. In this case, 41 hosts contained only females and 40 contained only males, which makes the proportion approximately 1. On the basis of the results of Bednarek et al. (1986), they concluded that S. feltiae population developing within hosts are characterized by the predominance of females over

TABLE III.4 Number of hosts with males or females only and number of male or female nematodes after a group of 50 Galleria larvae was exposed individually to 50 S. bibionis (Nashes)/insect for various hours at 15°C.

Group	Treatment	No. hosts with female only	No. nematodes in the hosts	No. hosts with male only	No. nematodes in the hosts	Male/Female (No. of nematodes)
	24 hrs(a)	3	9	5	12	1.33
1	24 hrs(b)	7	15	7	13	0.87
	24 hrs(c)	10	12	4	6	0.50
	total 72 hrs	20	36	16	31	0.86
	24 hrs(a)	3	7	4	4	0.57
2	48 hrs(b)	2	2	4	4	2.00
	total 72 hrs	5	9	8	8	0.89
	48 hrs(a)	0	0	0	0	
3	24 hrs(b)	6	10	9	10	1.00
	total 72 hrs	6	10	9	10	1.00
	72 hrs(a)	0	0	0	0	
4	72 hrs(b)	3	3	3	3	1.00
	72 hrs(c)	7	8	4	4	0.50

males. The differences observed for the sex ratio between S. feltiae and S. bibionis may be a reflection of species difference. However, possibility of experimental differences cannot be excluded, for example, method of dissection to reveal the males that are easily missed due to their small sizes.

Data from the present study relating to the sex structure of S. bibionis supports an hypothesis that there are similar numbers of males and females in the population. The few hosts with single sexes of parasites probably are chance occurrence. Generally, the larger the nematode numbers in a population the more equal will be the number of males and females. It is likely that the sex of the nematodes is genetically determined before they enter the hosts. The sexes do not appear to differ in their infectivity.