Biological control of *Otiorhynchus sulcatus* by insect parasitic nematodes, *Heterorhabditis* spp., at low temperatures; a systems analytical approach

Promotoren: dr. ir. A.F. van der Wal
hoogleraar in de nematologie

dr. ir. R. Rabbinge
hoogleraar in de theoretische produktie-ecologie

Co-promotor: dr. ir. W. van der Werf
universitair docent bij de vakgroep Theoretische productie-ecology

Paula R. Westerman

Biological control of *Otiorhynchus sulcatus* by insect parasitic nematodes, *Heterorhabditis* spp., at low temperatures; a systems analytical approach

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 25 juni 1997
des namiddags te vier uur in de Aula.

Westerman, Paula R.

Biological control of *Otiorhynchus sulcatus* by insect parasitic nematodes, *Heterorhabditis* spp., at low temperatures; a systems analytical approach
Paula R. Westerman.
Thesis Landbouwuniversiteit Wageningen. -With summary in Dutch and Frisian.

ISBN 90-5485-722-6

Cover designed by Marion Westerman.

This thesis contains the result of a research project at the Van Hall Instituut in Leeuwarden (formerly Agrarische Hogeschool Friesland, and Bijzondere Hogere Landbouwschool te Leeuwarden), in collaboration with St. Patrick's College in Maynooth, Ireland and Axis (formerly Agricultural Genetics Company Ltd.) in Cambridge, UK. The project was generously supported by the Integraal Structuur Plan voor het Noorden, the European Union (ECLAIR, project AGRE 0002), Fûns Universitaire Festiging Fryslân, and Stichting Fonds Hoger Agrarisch Onderwijs en Bedrijfsleven Friesland.

Stellingen

1. Voor het verbeteren van de inzetbaarheid van insecten-parasitaire nematoden is het zinvoller plaaginsecten te screenen op hun geschiktheid als doelwit dan isolaten van nematoden te screenen op hun geschiktheid als middel.
   Dit proefschrift

2. Het optimaliseren van productie- en opslagtechnieken om de effectiviteit van insecten-parasitaire nematoden te verhogen wordt verwaarloosd ten gunste van genetische selectie op gedragskenmerken.
   Dit proefschrift

3. Het clusteren van insecten-parasitaire nematoden in gastheren is een belangrijke belemmering voor succesvolle biologische bestrijding van bodeminsecten.
   Dit proefschrift

4. Infectiositeit is niet een intrinsiek vermogen van insecten-parasitaire nematoden maar de uitkomst van een interactie tussen nematoden, insecten en abiotische omstandigheden.
   Dit proefschrift
   Curran, J.1993. In: Bedding, R., Akhurst, R, and Kaya, H. (Eds.), *Nematodes and the biological control of insect pests*. CSIRO, East Melbourne, p. 67-78.

5. Het beste perspectief voor succesvolle toepassing van *Heterorhabditis* spp. tegen taxuskever in de volle grond moet gezocht worden in het ontwikkelen van strategieën die het moment van toepassen in de richting van de zomer verschuiven.
   Dit proefschrift

6. Geen van de termen entomopathogeen, entomogeen, entomofaag, entomofiel of insecten-parasitair, geeft een juiste karakterisering van de relatie die nematoden van het geslacht *Heterorhabditis* hebben met insecten.

7. Inzicht in de werking van insecten-parasitaire nematoden maakt het mogelijk dit biologisch bestrijdingsmiddel succesvol toe te passen en daarmee vaak uitgesproken verwachtingen waar te maken.

8. De bijdrage van genetische modificatie aan de toekomst van biologische bestrijding met nematoden blijft vooralsnog marginaal in vergelijking tot die van populatie-ecologisch onderzoek.
   Burnell, A.M. & Dowds, B.C.A. 1996. *Biocontrol Science and Technology* **6**: 435-447.

9. Een plotselinge toename van overzichtsartikelen op een bepaald vakgebied duidt òf op een snelle ontwikkeling in resultaten en ideeën òf juist op een stagnatie daarvan.

10. Herhalingen van experimenten in ecologisch onderzoek in de nematologie dienen evenzeer om inzicht te krijgen in de variabiliteit als om de betrouwbaarheid van conclusies te vergroten.

11. Communicatie is verraderlijk wanneer men denkt elkaar te begrijpen.

12. De bijdrage van stoplichten voor fietsers aan de verkeersveiligheid is omgekeerd evenredig met de gemiddelde wachttijd.

13. De voorkeur van veel Friezen voor grote open vlakten (agorafilie) is zowel oorzaak als gevolg van het ontbreken van bomen en bos op het Friese platteland.

Stellingen behorende bij het proefschrift van Paula Westerman: " Biological control of *Otiorhynchus sulcatus* by insect parasitic nematodes, *Heterorhabditis* spp., at low temperatures; a systems analytical approach".
Wageningen, 25 juni 1997

Abstract

The black vine weevil, *Otiorhynchus sulcatus*, is an important pest in ornamentals and nursery stock in The Netherlands. The larvae, which feed on the root system of the plant, can be controlled by insect parasitic nematodes, *Heterorhabditis*. However, the presently available isolates of the nematode are ineffective at temperatures below 12-13 °C, causing problems in black vine weevil control in open cultures. In this study, options to improve control by *Heterorhabditis* are explored, using a systems analytical approach. First, the nematode behavioural processes involved in host finding and control were studied and characterized. These processes are nematode movement, immobilization and remobilization near the soil surface, accumulation near an attractive insect (arrestment), penetration and aggregation of nematodes among insect hosts. The influence of temperature (9 and 20 °C) and host species (*O. sulcatus* or the more attractive and susceptible *Galleria mellonella*) on nematode behaviour was assessed to determine the contribution of these factors to control success. Knowledge of behavioural processes was integrated into a systems simulation model that relates the control success to the underlying behavioural processes. The model simulates movement of nematodes in space and time from the moment of application on a sand column until penetration into a host. The model for *O. sulcatus* at 9 °C was most sensitive to changes in the parameters characterizing aggregation and arrestment. Parameters characterizing penetration, the proportion infectious nematodes and the relative penetration rate, had a moderate effect on model outcome. Options for improvement were evaluated by relating the sensitivity of the model to genetic and phenotypic variation found in the nematodes. The amount of variation was assessed by comparing behavioural traits between and within *Heterorhabditis* isolates. Aggregation and arrestment are host related and there is little variation in *Heterorhabditis* for these traits. There is phenotypic variation in the proportion infectious nematodes. The most promising option to enhance control of *O. sulcatus* by *Heterorhabditis* at low temperatures is, therefore, to improve production and storage conditions to increase and stabilize the proportion infectious nematodes.

key-words: *Otiorhynchus sulcatus*, *Heterorhabditis* spp., insect parasitic nematode, biological control, low temperatures

Voorwoord

Dit proefschrift is het resultaat van onderzoek dat is uitgevoerd aan een instelling voor Hoger Agrarisch Onderwijs in Leeuwarden (Van Hall Instituut, voormalige Agrarische Hogeschool Friesland, voormalige Bijzondere Hogere Landbouwschool te Leeuwarden). Graag wil ik de instelling bedanken voor de gastvrijheid en het ter beschikking stellen van voorzieningen en diensten die noodzakelijk waren voor het onderzoek. Onderzoek was (en is) een vreemde eend in de onderwijs bijt van het HBO. Deze uitzonderingspositie bracht vele voordelen met zich mee, maar helaas ook nadelen.

Graag wil ik van de gelegenheid gebruik maken om iedereen te bedanken die op de één of de andere manier een bijdrage heeft geleverd aan de totstandkoming van dit proefschrift. Omdat het onderzoek dat ten grondslag ligt aan het proefschrift zich uitstrekt over een periode van bijna tien jaar (1986 - 1996) zijn er heel veel mensen betrokken geweest. Een aantal van deze mensen wil ik met name noemen.

Marieke van Zeeland, Martzen ten Klooster, Monique van de Gouw en Hanneke Jousma, bedankt voor jullie onvermoeibare inzet bij het plannen, opzetten en uitvoeren van experimenten en het kweken van de levende have, en voor jullie betrokkenheid en suggesties. Ik hoop, Marieke, dat je gevonden hebt wat je zocht. Het spijt me dat Hanneke Jousma de voltooiing van dit werk niet meer heeft mogen meemaken, want ze had graag bij de promotie aanwezig willen zijn. Verder wil ik Hanneke Godthelp, Jacqueline Wijbenga, Pieter Tolsma en Kerstin Jung bedanken voor hun inbreng en voor de samenwerking. Kerstin, bedankt voor de vele discussies.

Siem Simons bedankt voor de ondersteuning ter plaatse en voor het gestelde vertrouwen. Het is mede jouw verdienste geweest dat het onderzoek aan insecten-parasitaire nematoden in Nederland en in Leeuwarden gestalte heeft gekregen. Beide promotoren, prof. dr ir A.F. van der Wal en prof. dr ir R. Rabbinge, hartelijk dank voor de discussies, de kritische opmerkingen op de teksten, de tijd en aandacht, en voor de gastvrijheid aan huis en op de vakgroep. Co-promotor Wopke van der Werf, mijn oprechte dank voor je geduld en begeleiding tijdens de laatste fase van het onderzoek. Jouw hulp was onmisbaar bij de voltooiing van dit proefschrift.

Vele studenten kwamen in de loop der jaren het leven in Leeuwarden opvrolijken en hadden dan vaak verrassende ideeën; Vincent de Kok, Yvonne Peereboom, Sjouke Rinsma, Eddie Fennema, Lieme Lautenbach, Ge van der Vliet, Ineke Adriaanse, Herre van der Sloot,

Sadegh Zarza, Jan Buwalda, Lieuwe Bierma, Jolanda de Boer, Geppie van der Wal, Nelly Tensen, Caroline Bus, Olivier Poitevin, Anthony Froger en Ennio Venuto. Het ga jullie goed.

It was a pleasure to co-operate with Christine Griffin, Ann Burnell, Martin Downes, and Barbara Dowds (St. Patrick's College, Maynooth, Ireland) and Paul Rodgers (Axis Ltd., former AGC Ltd. Cambridge, UK) in a joint research project. Christine, thanks for valuable suggestions and corrections of manuscripts. Ann, thanks for the hospitality and showing more than just a professional interest.

Rob van Tol (Proefstation voor de Boomkwekerij) bedankt voor het herhaaldelijk leveren van taxuskever-eitjes en literatuuroverzichten. Gerrie Wiegers e.a. (IPO DLO) bedankt voor het leveren van wasmotlarven als er problemen waren met de eigen kweek. Doeke van der Schaaf en Rick van der Pas (Koppert B.V.), Niek Steeghs en Theo Cuijpers (Brinkman B.V.), Peter Smits (IPO DLO), Rob van Tol (Proefstation voor de boomkwekerij) en Joke Fransen (Proefstation voor de Bloemisterij), bedankt voor de samenwerking.

Sietze Bouma en Gerjo Groeneveld hielden de financiën van het onderzoek in de gaten. Jaap Groeneveld, Doeke Slagter en Otto Huitema verrichten allerlei hand- en spandiensten en hielpen bij problemen van technische aard. Eeltje Osinga onderhield de tuin en de kassen. De mensen van informatica-afdelingen hielpen wanneer er problemen waren met computers; o.a. Peter Busschers, Martin Haagsma, Gerard Smink, Wim Has en Rob Dierkx (TPE LUW). Eus Drent, Margriet Stapel (GLW DLO), Aad van Eynsbergen en Albert Otten (Wiskunde, LUW) zorgden voor onmisbare hulp bij problemen van statistische aard. Gon van Laar (TPE-LUW) tekende Figuur 6.3 en hielp bij de eindafwerking van het proefschrift. Idwerd Hania zorgde voor de Friese samenvatting. Bert van Amersfoort hielp bij de PE aanvraag.

De mensen van de vestiging aan de Antillenweg en met name de buurmannen, waaronder Sjoerd Gerbrandy en Herke Fokkema, wil ik bedanken voor de plezierige sfeer aldaar. Verder heb ik genoten van de goede sfeer en collegialiteit onder medewerkers en gastmedewerkers van de vakgroep Theoretische productie-ecologie. Hartelijk dank daarvoor en voor de gastvrijheid. De promovendi van de PE onderzoeksschool bedankt voor de discussies.

Familie, vrienden en kennissen bedankt voor jullie belangstelling en betrokkenheid. En tenslotte, Mart, bedankt voor je nimmer aflatende steun en goede zorgen.

Contents

Account

Parts of this thesis have been included, in part or in whole, in the following publications:

Chapter 2 Comparative vertical migration of twenty one isolates of the insect parasitic nematode *Heterorhabditis* spp. in sand at 20 °C. *Fundamental and applied Nematology* **18** : 149-158.

Chapter 3 Comparative vertical migration of insect parasitic nematodes *Heterorhabditis* spp. and *Steinernema* spp. in sand at 9 °C. *Fundamental and applied Nematology* **20** (accepted).

Chapter 4 Penetration of the insect parasitic nematode *Heterorhabditis* spp. into host insects at 9 and 20 °C. Submitted to *Journal of Invertebrate Pathology*.

Chapter 5 Aggregation of insect parasitic nematodes, *Heterorhabditis* spp. and *Steinernema* spp., among host insects at 9 and 20 °C. Submitted to *Journal of Invertebrate Pathology*.

Chapter 6 Exploring the scope for improving biocontrol of black vine weevil, *Otiorhynchus sulcatus*, with *Heterorhabditis* spp. at low temperatures; a simulation study. Submitted to *Agricultural systems*.

Chapter 1

General introduction

Black vine weevil, *Otiorhynchus sulcatus*: injury, damage and control

Larvae of the black vine weevil, *Otiorhynchus sulcatus* F., and related species of *Otiorhynchus* are causing major problems to growers of soft fruits, ornamentals, nursery stock vines and hops, throughout the temperate zone. Over the past few decades, damaging infestation levels with *O. sulcatus* have become more common, probably due to changes in cropping system (intensification) and the abandonment of persistent pesticides, such as aldrin and dieldrin (e.g. Klingler, 1959; Zimmerman and Simons, 1986; Moorhouse *et al.*, 1992). In the Netherlands, *O. sulcatus* is one of the major pests in nursery stock and ornamentals. Adult weevils cause cosmetic injury to the leaves. The most important damage, however, is caused by the larvae, which feed on the roots, starting on fine roots and progressing to coarse roots and stems. Reduction of the root system, girdling or stripping of coarse roots and boring of larvae into the stem, result in lower production and unsalable products. Plant characteristics affect injury and damage, e.g. shape and size of the root system, and the ability of the plants to recover from injury by vegetative growth, as does the culture system, temperature and soil volume. Economic injury levels have been estimated for some crops, but it is unfeasible to determine them for all, because many (small) crops are involved.

The adult females of *O. sulcatus* reproduce parthenogenetically and oviposit from May - June to the second half of September (Figure 1.1), when temperature drops below 11 °C. During this period, a female can lay several hundreds of eggs (Evenhuis, 1978). The eggs hatch when temperatures are above 6 °C. Early instars are present during summer, while late instars are present during autumn, winter and spring. Under Dutch conditions, the majority of the insects overwinters as larvae (Evenhuis, 1978), while a small proportion overwinters as adults or inactive pre-pupae. During winter, development is retarded, but not stopped. Late instar larvae (stage 6 - 7) cause most feeding injury, especially in spring, when the overwintering larvae resume feeding (2 - 6 °C). Adults appear from May onward (Klingler, 1959; Moorhouse *et al.*, 1992). They start laying eggs after a pre-oviposition period of several weeks.

The most effective strategy for preventing damage is preventive spraying against the adults. It is difficult to control adult *O. sulcatus* with chemical pesticides because they hide during daytime. Larvae can be controlled either preventively or curatively (Moorhouse *et al.*,

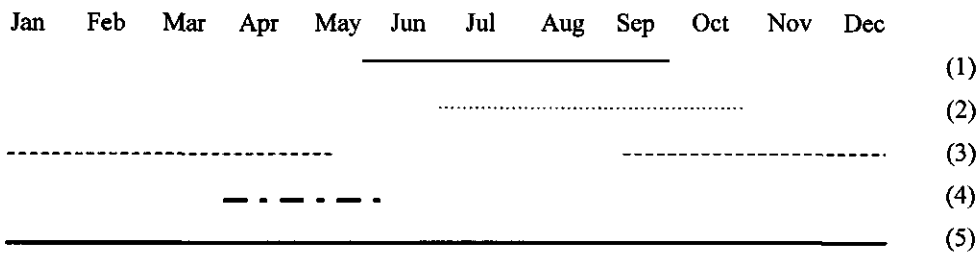

Figure 1.1. Life cycle of *O. sulcatus*: 1, eggs; 2, young larvae (larval stage 1 - 5); 3, older larvae (larval stage 5 - 7); 4, pupae; 5, adults.

1992). For preventive control of the larvae a persistent insecticide is mixed through the soil before the start of a crop. The most effective preventive pesticides, aldrin and dieldrin, have been banned, because of their persistence. They accumulate in the soil and contaminate ground- and surface water. New or better preventive pesticides are not expected on the short term, because it is difficult to recover the expenses for registration of new pesticides in small crops. For curative control of the larvae, a fast-working, well-penetrating insecticide is applied as a spray or drench to the soil during the crop. Timing is important here because the curative pesticides have limited persistence. When applied during summer, they will not kill later appearing larvae; oviposition continues until the second half of September. Early instar larvae are not a target for curative control, because they do not cause much injury, they are difficult to monitor, and natural mortality of the early instars can be high anyway as a result of competition or unfavourable conditions (60 - 94%). Therefore, curative control usually aims at the late instar larvae in autumn (September - November) and spring (March - May).

On an estimated total area in the Netherlands of 3000 ha field nursery and 750 ha nursery stock in containers, approximately 3.3 million Dutch guilders were spent in 1996 on chemical pesticides for control of black vine weevil. The largest part (approximately 2.4 million guilders) was used for control of the larvae (Van Tol, pers. comm., based on Ministry of Agriculture, Nature Management and Fisheries, 1990). The situation is similar in most other North West European countries. For example, injury by *O. sulcatus* was reported on 96% of the nurseries in Schleswig-Holstein and Lower Saxony in Germany (1800 ha). Control was carried out on 90% of the nurseries; 78% of the nurseries used repeated chemical treatment (Reibnitz *et al.*, 1993).

Growers tend to control *O. sulcatus* whenever it is reported because the pest is so difficult to control, because monitoring is difficult, because damage may occur unnoticed for a long time, and because they want to prevent the build up of the pest population over the

years. As a result, chemical usage and dependency is high. The multi-year crop protection plan (Ministry of Agriculture, Nature Management and Fisheries, 1990) sets out to break and reverse this trend. Improved pest monitoring and biocontrol options are the most promising means to this end.

Biological control of *O. sulcatus* by means of *Heterorhabditis*

Insect parasitic nematodes, also referred to as entomopathogenic nematodes, of the genera *Steinernema* Travassos and *Heterorhabditis* Poinar, are frequently used to control black vine weevil larvae. They are very effective when temperature is above 12 - 13 °C, as is the case in greenhouses, pots and containers. The nematodes possess several attributes that make them better suited for control of soil pests than chemical pesticides. They are mobile in the soil and search for their hosts, kill them fast, and they are harmless to non-insects (Kaya, 1985). Furthermore, they can be mass-produced at relatively low cost, and they are relatively easy to apply in the field. The nematodes are mainly applied as an inundative treatment against the larvae by applying large numbers for immediate control, as a biological pesticide. This is because ornamentals and crops in nurseries generally last a single season. Long-lasting regulation of the pest and reproduction of the nematodes in the field (inoculative control) are not necessary, although nematodes may reproduce in insect cadavers.

The largest part of the nematodes life cycle takes place inside the insect (cadaver). The third stage juvenile (J3) is the only stage that occurs outside the host and that is adapted to long term survival in the soil. During their stay in the soil the J3 juveniles do not feed and survive on internal food stores. It is capable of moving in the soil and penetrating new hosts. After penetration the juveniles develop into adults and two generations later new J3 juveniles are produced that leave the cadaver in search of new hosts. The main difference between *Heterorhabditis* and *Steinernema*, with respect to life cycle, is that in *Heterorhabditis* the first generation adults are hermaphrodites and the second generation amphimictic males and females, while in *Steinernema* both generations are amphimictic males and females.

Although there is a considerable overlap in characteristics between isolates of *Steinernema* and *Heterorhabditis*, *Heterorhabditis* is preferred for black vine weevil control. Isolates of this genus tend to be more pathogenic to the larvae than *Steinernema* (e.g. Rutherford *et al.*, 1987; Van Tol, 1993), they are more effective against early instars (Georgis and Poinar, 1984), and they are more mobile, making them more suitable as a curative means. Despite the advantages of *Heterorhabditis*, isolates of *Steinernema* are sometimes used for black vine weevil control due to the poor availability of *Heterorhabditis* based products on

the market. Steinernematids are more easily produced in fermentors and they can be stored for longer periods. In this study attention is focused on *Heterorhabditis*.

The presently available isolates of insect parasitic nematodes are ineffective when soil temperature is below 12 - 13 °C (e.g. Simons and Van der Schaaf, 1986; Rutherford *et al.*, 1987; Van Tol, 1993). Soil temperatures may vary between 5 and 15 °C during Dutch autumn and spring, when effective biocontrol is required to prevent injury by the larger larvae that are present during these periods of the year. Consequently, there is only a narrow temperature and time window for control of black vine weevil larvae in the open field. Due to the temperature dependence, the level of control in the field is unpredictable and frequently insufficient. To strengthen the basis of integrated pest management (IPM) in nurseries and to further reduce pesticide usage, it is essential that the performance of the insect parasitic nematodes at low temperatures is improved. A reduction of the threshold temperature for activity from 12 - 13 °C to 9 °C would be sufficient for black vine weevil control in the open, because temperatures in the critical periods are 50% of the time or more above 9 °C (e.g. Van Tol, unpubl.; Simons and Van der Schaaf, 1986; Westerman and Van Zeeland, 1989).

Problem definition and research objectives

A chain of events has to take place before the insect host is successfully penetrated and killed by the nematodes. After application the J3 larvae of the nematodes have to move into the soil, locate the hosts, and penetrate them. Penetration takes place either directly via soft spots in the integument or through natural openings such as stigmata, mouth and anus, followed by penetration of the wall of the intestine. Next, they release insect pathogenic bacteria, *Xenorhabdus* Thomas and Poinar (*Steinernema* spp. or *Photorhabdus* Boemare, Akhurst and Mourant (*Heterorhabditis* spp.) from their intestine into the haemocoel. When enough bacteria have been introduced in the haemocoel, they will kill the insect host, usually in a few days after release.

It is not clear why control is poor at low temperatures. Target species is an important factor in the biocontrol success of nematodes. The larvae of *O. sulcatus* are more difficult to kill by the nematodes than other insect species, such as *Galleria mellonella* (L.) (e.g. Bedding *et al.*, 1983). Temperature is another important factor. Effects of temperature on efficacy and behaviour of the nematodes have been documented in many studies (for review see: Griffin, 1993), but these relationships were mainly descriptive. They do not identify which effect(s) is (are) most limiting for black vine weevil control. It is difficult to predict the influence of temperature and other factors on insect mortality by the nematodes, because it is unknown

what happens to the nematodes in quantitative sense between application and insect death. Temperature will affect any of these processes although sensitivity to temperature may differ between processes.

Given the variation among isolates of *Heterorhabditis* spp. and *Steinernema* spp. in their efficacy at low temperatures (e.g. Simons and Van der Schaaf, 1986) and their ability to kill *O. sulcatus* (e.g. Bedding *et al.* 1983), it is very well possible that more effective isolates exist or can be reared. A number of potentially cold effective isolates, mainly steinernematids, have been identified (e.g. Wright and Jackson, 1988), although understanding of why they are more cold effective is lacking. A proportion of the observed variation may not have a genetic basis, but is induced during pre-treatment of the nematodes (e.g. via size, stored energy reserves, conditioning and acclimation). The resulting variation in behaviour has an environmental basis. This will make it difficult to identify or select more cold effective nematode genotypes.

The objective of this study is to highlight possibilities for improving biological control of *O. sulcatus* with *Heterorhabditis* at low soil temperatures, i.e. to identify nematode traits and strategies that can best be used for improvement. To assess whether performance of the nematodes at low temperatures can be improved, the relationship between nematode dose and insect mortality has to be defined quantitatively, as well as the impact of temperature on this relationship. Possible strategies are screening for more appropriate nematode species or isolates, selective breeding, improving production and storage conditions, or defining or controlling environmental conditions after nematode application. A systems analytical approach was followed comprising three steps:

1) gaining insight into and quantifying the processes that lead to infection and death of insects by *Heterorhabditis* spp.;
2) constructing a simulation model that integrates the results at the process level, such that the observations at the systems level can be explained;
3) use the model to explore possibilities at the systems level.

Research approach and thesis outline

Much effort was put into understanding how processes underlying biocontrol work and which factors contribute to success or failure at low temperature. Experiments were carried out at the population level to quantitatively describe the behavioural processes that lead to insect infection and death. The available knowledge on the processes was integrated to construct a model, using the state variable approach (e.g. Rabbinge *et al.*, 1989). Sensitivity analysis was

used to identify the most influential parameters for infection at low temperatures. Sensitivity was compared with the available genetic and environmental variation. Once the major processes and factors contributing to the biological control of *O. sulcatus* and sensitive to low temperatures were identified a strategy for enhancement could be selected.

Experimental work was carried out for three purposes: 1) to understand and quantify the behavioural processes that lead to insect infection and death; 2) to quantify the impact of host species and temperature on these processes; and 3) to quantify genetic and environmental variation in *Heterorhabditis*. The influence of temperature on nematode behaviour was tested by using low (9 °C) and high (20 °C) temperature conditions during the experiments. The influence of host species was tested by comparing the behaviour of *Heterorhabditis* in the presence of *O. sulcatus* larvae with that in the presence of larvae of the highly susceptible lepidopteran larvae of *Galleria mellonella* (L.) (occasionally *Spodoptera exigua* (Hb.) or *Agrotis segetum* Schiff.). The amount of genetic and environmental variation in behavioural traits was assessed by comparing the behaviour of nematodes between isolates and within isolates. Experiments were conducted under simplified laboratory conditions compared to the field situation, to avoid interference with factors other than temperature, e.g. soil type, moisture content, micro fauna of the soil. All experiments were carried out in sieved, sterile sand of known particle size (approximately 72% 180 - 300 µm, 21% 300 - 425 µm) and moisture content (8% w/w), in petri-dishes (25 ml, ∅ 5 cm; penetration and aggregation in the host) or vertical columns (9 cm high, ∅ 4.5 cm; movement and host attraction). Unless stated otherwise, nematode batches were used within two months from extraction from the insect cadaver, to avoid problems with deteriorating nematode quality.

Experimental work on the process level is presented in Chapters 2 to 5. The distribution of various nematode isolates over the sand column (e.g. Georgis and Poinar, 1983c) in the course of time was used to describe movement and host attraction at 20 °C (Chapter 2) and 9 °C (Chapter 3), and to evaluate genetic and environmental variation for these traits. The behaviour of the nematodes in the sand columns in the time series was quantified using the dynamic simulation and calibration software package SENECA. Description of movement and host attraction was gradually improved by subsequently altering assumptions with respect to nematode movement, altering the corresponding model structure, calibrating parameters, and evaluating the goodness of fit of the model to the observations (Chapter 6). Nematode movement was expressed in the proportion immobile nematodes and the speed of movement, and the description of host attraction required the additional characteristic arrestment. In Chapter 4, the penetration process was studied and

quantified by two parameters, the proportion infectious nematodes and the rate of penetration. Variation in the proportion infectious nematodes was assessed between and within nematode isolates, in the presence of the various insect species, and at low and high temperatures. The same data were used to assess the degree of aggregation of penetrated nematodes among the hosts (Chapter 5). The degree of aggregation, quantified by the parameter k of the negative binomial distribution, was estimated. It was used to describe the relationship between infection pressure (the mean number of penetrated nematodes per insect) and the proportion killed insects in a population. The processes characterized in Chapters 2 to 5 were used to develop a model in the simulation language FST (Rappoldt and Van Kraalingen, 1996) that simulated the dynamics of a nematode population in time and space from the moment of nematode application on the surface of a sand column until penetration into the host. It quantitatively relates nematode dose to insect infection and mortality. Due to its mechanistic nature, the model provided insight in the relative importance of variables and the impact of changing conditions on insect mortality. This information was used for a feasibility study to explore options for enhancing biological control of *O. sulcatus* at low temperatures (Chapter 6). Finally in the last chapter (Chapter 7), some problems and decisions during the study are discussed, as well as the approach, and the significance of this thesis to research and practice.

Chapter 2

Comparative vertical migration of twenty one isolates of the insect parasitic nematode *Heterorhabditis* spp. in sand at 20 °C

Abstract Twenty one heterorhabditid isolates, most likely belonging to six taxonomic groups and originating from ten countries, were tested for downward migration in 9 cm vertical sand columns at 20 °C with or without a larva of the greater wax moth, *Galleria mellonella*, at the bottom. The fractions of nematodes in the top layer (1.5 cm), middle section (6 cm) and bottom layer (1.5 cm) of the columns were determined at intervals during 8 h and occasionally at 48 h. All isolates except *Heterorhabditis zealandica* NZH3 migrated better than isolates of *H. bacteriophora*. Differences in migration were congruent with putative taxonomic groups within the genus. In most cases, the presence of a *G. mellonella* larva increased the downward movement. Nematodes of the North West European isolates were the most mobile, however, migration varied between batches of the same isolate. In the presence of *G. mellonella*, 95% to 99% of the nematodes of a number of Dutch isolates left the top layer of the sand column and were recovered from the bottom layer 6 h later.

Introduction

Insect parasitic nematodes of the genus *Heterorhabditis* Poinar are used commercially in The Netherlands and other North West European countries for control of the black vine weevil, *Otiorhynchus sulcatus* F., in indoor ornamentals. The larvae of this weevil cause serious damage to the root system and base of the plants. The insect parasitic nematodes, which naturally inhabit the soil, favour a dark and moist environment and are eminently suited for control of this type of soil-dwelling pest.

The normal practice in horticulture is to apply nematodes to the soil surface as a curative biological insecticide. Quick movement of the nematodes into the soil is necessary to escape solar radiation and desiccation (Gaugler, 1988). Since the nematodes search actively for their hosts, their use for controlling soil-borne insects has an advantage over chemical pesticides whose contact with the insect depends on diffusion, water percolation or insect movement. However, application of insect parasitic nematodes must result in high levels of parasitism even when the host insects are scarce in order to be economically attractive. This makes exacting demands on the ability of the nematodes to search for and reach the host. A high host searching ability is generally regarded as the prime requisite of an effective natural

enemy (Doutt and DeBach, 1964). Westerman and Stapel (1992) found that migration of the Dutch heterorhabditid HFr86 was related to the degree of efficacy against the black vine weevil. No data are available yet to allow extrapolation of this relationship to other heterorhabditid species and isolates.

In the present study, downward movement by twenty one *Heterorhabditis* isolates originating from ten countries, including a large number of isolates from NW Europe (the Netherlands, U.K., Germany and Ireland), were measured in sand columns at 20 °C in the presence and absence of a larva of the greater wax moth, *Galleria mellonella* (L.).

Materials and methods

Nematode cultures

The isolates of *Heterorhabditis* tested (Table 1.1) were grouped according to their currently proposed taxonomic status (Smits *et al.*, 1991; Dix *et al.*, 1992; Joyce *et al.*, 1994a; 1994b). All nematodes were propagated on *G. mellonella* at 20 °C and harvested in modified White traps (Poinar, 1975). Nematode suspensions were cleaned by decanting and adding fresh tap water, after which the nematodes were allowed to migrate overnight through a cotton-wool filter into a water film below. The infective juveniles were stored in 500 ml water in 1 l bottles in a dark cold room at 4 - 5 °C under constant aeration with sterile-filtered air. Eight isolates (B1, NC1, V16, HDa, HP88, HI82, NZH3 and M198), however, were stored at 9 - 12 °C, because 4 - 5 °C proved lethal to them (Westerman, unpubl.). Three days before the beginning of the experiments the nematodes were transferred to 20 °C to let them adjust to the test temperature.

Migration

Nematode migration was assessed in 9 cm high PVC cylinders (diam. 4.5 cm), made of six, 1.5 cm rings, connected with adhesive tape. The bottom of the cylinder consisted of a petri-dish (5.5 cm diam.) fixed to the bottom ring with synthetic modelling clay. The cylinders were filled with fine sterile sand (particle size; 72% 180 - 300 mm, 21% 300 - 425 mm), moistened with demineralized water (8% w/w), and kept at 20 °C. Immediately after preparation, cylinders with and without a last instar larva of *G. mellonella* at the bottom, were inoculated on top with approximately 2000 living nematodes in 0.5 ml water and covered with a petri-dish lid. After 2 - 8 h or 48 h at 20 °C the rings were separated and the sand of each ring was rinsed in 50 ml water. The number of nematodes in ring 1, rings 2 - 5 and ring 6 were

Table 1.1. Origin of the nematode isolates.

Isolate	Code	Location	Source *
H. zealandica	NZH3	Auckland, New Zealand	Akhurst
H. bacteriophora	B1	Brecon, South Australia	Kaya, Smits
NC1	NC1	Clayton, NC, USA	Burnell, Brooks
V16	V16	Geelong, Victoria, Australia	Burnell, Brooks
Hda	HDa	Darmstadt, Germany	Ehlers, Bathon
HP88	HP88	Logan, Utah, USA	Glazer
HI82	HI82	Italy	Deseö
H. megidis	HO1	Ohio, USA	Akhurst, Griffin
Heterorhabditis sp. (Irish group)			
IRL-H-K122	K122	Wexford, Ireland	Griffin
IRL-H-M145	M145	Wexford, Ireland	Griffin
IRL-H-M198	M198	Cork, Ireland	Griffin
Heterorhabditis sp. (NW European group)			
NL-H-W79	HW79	Wageningen, The Netherlands	Vlug
NL-H-L81	HL81	Limburg, The Netherlands	Galle
NL-H-F85	HF85	Flevoland, The Netherlands	Simons, Van Zeeland
NL-H-Fr86	HFr86	Friesland, The Netherlands	Simons, Van Zeeland
NL-H-Nb87	HNb87	N-Brabant, The Netherlands	Simons, Van Zeeland
NL-H-B87.1	HB1'87	Bergeyk, The Netherlands	Smits
NL-H-E87.3	HE87	Eindhoven, The Netherlands	Smits
UK-H-UK211	HUK211	United Kingdom	Rodgers
D-H-HSH	HSH	Schleswig Holstein, Germany	Ehlers
USSR-H-Kem	Hkem	Kemerovo, Siberia, Russia	Wijbenga

* R. Akhurst, CSIRO, Canberra, Australia; H.K. Kaya, Univ. California, Davis, USA; P.H. Smits and H. Vlug, Research Institute for Plant Protection, Wageningen, The Netherlands; A.M. Burnell and C.T. Griffin, St. Patrick's College, Maynooth, Ireland; W.M. Brooks, NC State Univ., Raleigh, NC, USA; R.U. Ehlers, C.A. Univ., Kiel, Germany; H. Bathon, Inst. biologische Schädlingsbekämpfung, Darmstadt, Germany; I. Glazer, The Volcani Center, Bet Dagan, Israel; K.V. Deseö, Univ. Bologna, Italy; F. Galle, De Groene Vlieg Company, Nieuwe Tonge, The Netherlands; W.R. Simons, M.G. van Zeeland, J. Wijbenga, Van Hall Instituut, Leeuwarden, The Netherlands; P.B. Rodgers, Agricultural Genetics Company Ltd, Cambridge, UK.

determined by counting the nematodes in four samples of 3 ml of the rinse water. Fifty to 60% of the applied nematodes were recovered, except after a period of 48 h when only 20 to 40% were recovered. The percentage of nematodes in each layer was calculated, based on the

number of recovered nematodes per cylinder. Nematodes found in a ring were considered to have moved halfway through the ring. Accordingly, migration was defined here as the average distance migrated:

$$\text{migration} = \frac{0.75 \times R_1 + 4.5 \times R_{2\text{-}5} + 8.25 \times R_6}{R_1 + R_{2\text{-}5} + R_6} \quad [\text{cm}] \qquad (2.1)$$

where R_1, $R_{2\text{-}5}$, and R_6 are the numbers of nematodes recovered at 4 h in ring 1, rings 2 - 5, and ring 6, respectively. Migration and the average migration rate at 4 h (migration/4 [cm/h]) were used to compare migration and rates of migration among isolates and different batches of isolates tested.

Five isolates (*H. zealandica* NZH3, *H. bacteriophora* B1, NC1, V16, and HDa) were tested at both a 4 h and 48 h incubation period and were inoculated on the same date with the same batch of nematodes. The batch of HDa was also used in the time series (see below). Five more isolates (HI82, HW79, HSH, HKem and M198) were tested at a 4 h incubation period only; M198 was tested twice using different batches. All other isolates, including HDa and batches of NZH3, and B1, were tested in a time series of 2, 4, 6 and 8 h in two to four replicates, with the exception of HL81 and HFr86 (which were tested hourly from 2 to 7 h), and HF85 and B1 (which were not tested at a 2 h period). Of these isolates HF85 and B1, HL81 and HFr86, HI82 and HW79, and HDa and HE87 were tested in pairs on the same date. Additional data on migration at 4 h of HF85, HUK211, and K122 were obtained from experiments conducted for other purposes (partly obtained from K. Jung).

Migration data from the time series were analysed separately for each batch on effects of incubation time and of the absence and presence of the host using linear regression analysis. Standard errors of the mean (S.E.) were calculated about migration at 4 h to permit comparison among isolates. χ^2 - test was used to check for uniform distributions of nematodes in cylinders without *G. mellonella*.

After completion of the experiments the *G. mellonella* larvae from the cylinders were usually kept at 20 °C for another 3 - 4 days and checked for parasitism. Parasitized larvae were recognized by the characteristic colour change induced by the symbiotic bacteria. Occasionally dead larvae were opened after 6 - 7 days under a dissection microscope to count the number of hermaphrodite nematodes in the cadaver (done for NZH3, HDa, HP88, K122, M145, HO1, HUK211 and HE87 in the time series, and NZH3, B1, NC1, V16 and HDa at both 4 h and 48 h). The numbers of nematodes recovered from the insects were usually small ($\leq$ 35 per insect at 8 h) compared to the total numbers recovered (1000 - 1200) and were not

included in calculations of the distance migrated, except for NZH3, B1, NC1, V16 and HDa at the 48 h incubation period and for HE87 in the time series.

Quality

The experiments were performed in the course of several years (1990 - 1994), in which the influence of culturing, time and storage conditions on motility became gradually clear (Westerman, 1992; Westerman and Stapel, 1992). Therefore, from 1991 onwards, the visual appearance of the nematode batches was evaluated using two samples of approximately 300 nematodes as described by Westerman and Stapel (1992) and, except where noted, only batches with a good visual appearance were used in experiments. Fourteen batches and six of the batches that were used in the additional migration experiments were subjected to this test (see Tables 2.2 and 2.3). Criteria used were mortality less than 5%, less than 5% exsheathed nematodes and less than 10% of the nematodes without ample food reserves. Occasionally these criteria were not met, particularly not in case the isolates were stored at 9 °C, viz. NZH3 (72% with ample food reserves and 32% exsheathed in the second batch), B1 (17% mortality, 76% with ample food reserves, 16% exsheathed in the second batch), NC1 (12% exsheathed), V16 (81% with ample food reserves, 19% exsheathed), M198 (13% mortality, 86% with ample food reserves in the first batch; 27% mortality, 71% with ample food reserves in the second batch), and both batches of HUK211 in the additional experiments (17% mortality in the first batch and 27% with ample food reserves and 12% exsheathed in the second batch). Percentage mortality was the only characteristic assessed for the first batch of *H. zealandica* NZH3 (53%). The time of storage from the onset of emergence from the host to the start of the experiments ranged from 10 to 62 days (average 28 days).

Results

H. zealandica NZH3

In the time series 15% of the recovered NZH3 had migrated from the first layer in the absence of an insect at 8 h; 23% in the presence of *G. mellonella* (Figure 2.1A). Downward movement was very slow (0.22 - 0.25 cm/h at 4 h) and the first nematodes reached the bottom layer no earlier than 8 h (≤ 1% of the recovered nematodes). Both test insects at 8 h contained 2 hermaphrodites. Migration was not significantly increased by the presence of *G. mellonella* (e.g. Table 2.2). The other batch of NZH3 yielded similar results at both 4 h (Table 2.2) and 48 h (1.0 ± 0.0 cm ($\bar{x}$ ± S.E.) without insect and 1.1 ± 0.1 cm in the presence of *G. mellonella*). None of the test insects was parasitized.

H. bacteriophora B1, NC1, V16 and HDa

In the time series only 5% of the recovered B1 and 17% of HDa had dispersed from the first layer in the absence of an insect at 8 h; in the presence of *G. mellonella* these percentages were 14% and 23%, respectively (Figures 2.1B and 2.1C). Downward movement was slow (0.20 - 0.25 cm/h at 4 h) and the first nematodes reached the bottom layer at 4 h (B1) and 6 h (HDa). Migration of HDa seemed to increase at 6 h but this was not followed up at 8 h. At 8 h 1% and 7% of the recovered B1 and HDa, respectively, were found in the bottom ring. Test insects exposed to HDa at 6 and 8 h contained negligible numbers of hermaphrodites (≤ 7 per insect). Migration was not significantly increased by the presence of *G. mellonella* for either isolate ($P \geq 0.2$).

The nematodes of the other batch of B1, tested at 4 h, migrated better than those used in the time series; 2% and 10% of the recovered nematodes was found in the bottom layer at 4 h in cylinders without and with *G. mellonella*, respectively, resulting in higher distances migrated (Table 2.2). The mean distance migrated by NC1, V16 and HDa at 4 h did not differ from that of either batch of B1 at 4 h (Table 2.2). Two and 5% of the recovered nematodes was found in the bottom layer for NC1 and V16, respectively. Test insects either survived exposure to the nematodes in sand columns (HDa and V16) or they contained ≤ 1 hermaphrodite per insect (B1 and NC1).

Data of the four isolates tested at 48 h were less reliable than those at 4 h since percentage recovery from the sand was low (20 - 40%). In the absence of *G. mellonella* 10, 21, 2 and 16 % of the nematodes were found in the bottom layer for B1, NC1, V16 and HDa, respectively (mean distances migrated ($\bar{x} \pm$ S.E.); 2.0 ± 0.4 cm, 2.7 ± 0.2 cm, 1.2 ± 0.2 cm, and 2.5 ± 0.6 cm, respectively). Relatively high numbers of hermaphrodites were recovered from the insect cadavers ($\bar{x} \pm$ S.E.); 45 ± 6, 281 ± 49, 144 ± 25, and 184 ± 58 for B1, NC1, V16 and HDa, respectively, thus boosting the values for migration in these cylinders (2.8 ± 0.2 cm, 4.0 ± 0.4 cm, 3.4 ± 0.3 cm and 2.9 ± 0.5 cm, respectively).

HP88 and HI82

In the time series 53% and 68% of HP88 had left the top layer of sand at 8 h in the absence and presence of *G. mellonella*, respectively (Figure 2.1D). At the same time 4% and 33%, respectively, were found in the bottom layer. The nematodes moved slowly (0.3 - 0.5 cm/h at 4 h). Ten hermaphrodites were recovered from the one insect killed at 6 h and one and two hermaphrodites from the two insects killed at 8 h. The mean distance covered by HP88

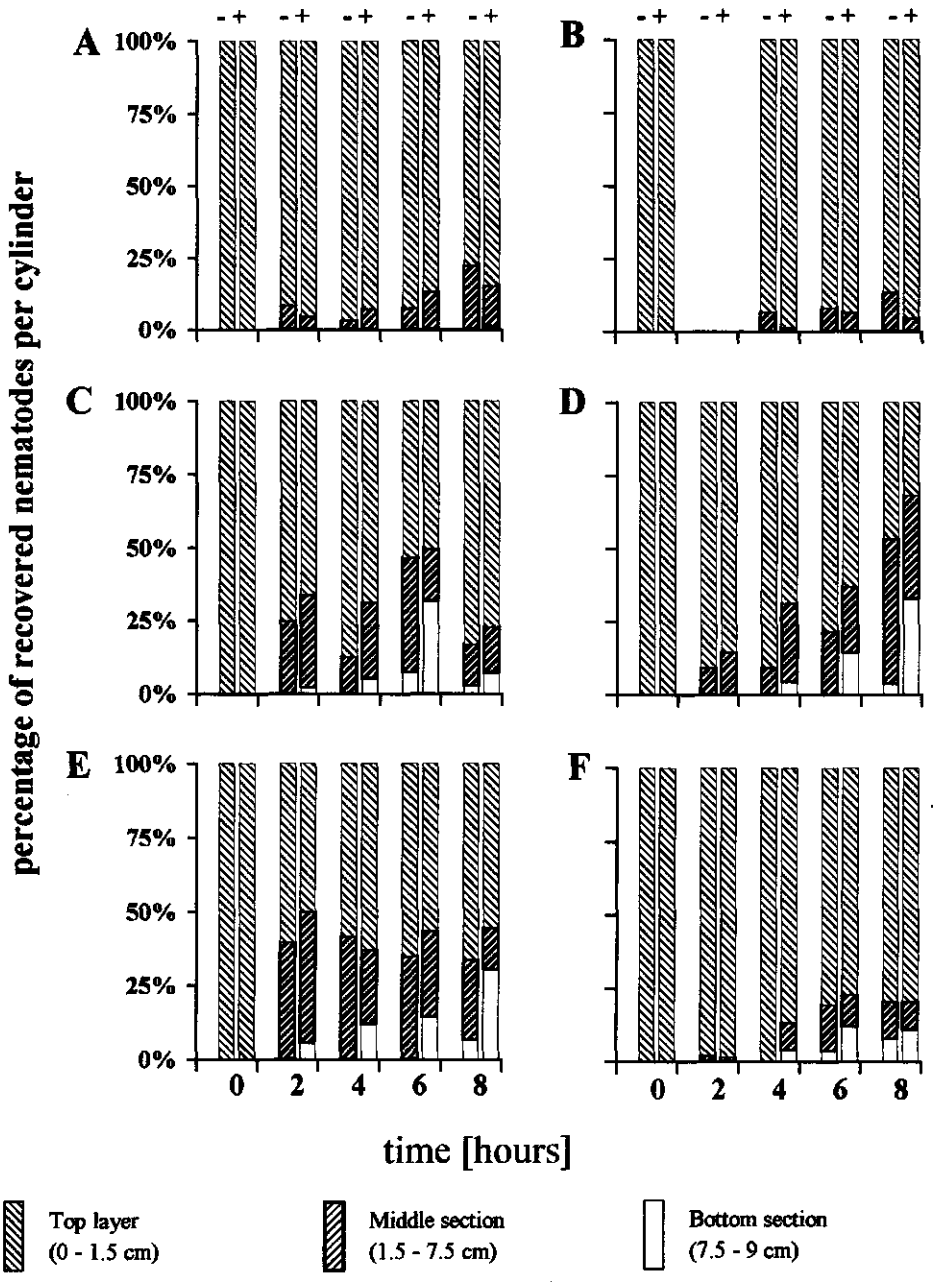

Figure 2.1. Percentage of recovered nematodes in the course of time in three sections of 9 cm high sand columns, in the absence (–) and presence (+) of a last instar of *G. mellonella* at the bottom of the cylinders, after application of approximately 2000 living heterorhabditid nematodes on top of the cylinders. A: *H. zealandica* NZH3; B: *H. bacteriophora* B1; C: *H. bacteriophora* HDa; D: HP88; E: K122; F, M145.

Table 2.2. Migration ($\bar{x}$; average distance covered [cm]), with standard error of the mean (S.E.), at 20 °C in 4 h of a number of (batches of) isolates of *Heterorhabditis* in a number of 9 cm sand columns (n) without (–) and with (+) a *G. mellonella* larva at the bottom. (For each isolate migration followed by different letters are significantly different from each other, $P \leq 0.05$).

	n	$\bar{x} \pm$ S.E. (–)	$\bar{x} \pm$ S.E. (+)
H. zealandica NZH3	2	0.9 ± 0.1 *a*	1.0 ± 0.0 *a* [t]
	3	1.1 ± 0.2 *a*	0.9 ± 0.1 *a* [q,d]
H. bacteriophora			
B1	4	1.0 ± 0.1 *a*	0.8 ± 0.0 *a* [t]
NC1	3	1.6 ± 0.2 *a*	2.5 ± 0.1 *a* [q,d]
V16	3	1.2 ± 0.1 *a*	1.9 ± 0.1 *a* [q,d]
Hda	3	1.0 ± 0.0 *a*	1.7 ± 0.2 *a* [q,d]
	3	1.2 ± 0.1 *a*	2.1 ± 0.1 *a* [q,t]
	3	1.4 ± 0.2 *a*	2.1 ± 0.2 *a* [q,d]
HP88	2	1.1 ± 0.2 *a*	2.1 ± 0.8 *a* [t]
HI82	3	2.0 ± 0.3 *a*	3.8 ± 0.7 *b* [q]
H. megidis HO1	4	2.8 ± 0.2 *a*	5.2 ± 0.5 *b* [t]
Heterorhabditis sp. (Irish group)			
K122	2	2.5 ± 0.1 *a*	2.6 ± 0.4 *a* [t]
M145	2	1.3 ± 0.0 *a*	1.4 ± 0.2 *a* [t]
M198	3	1.7 ± 0.2 *a*	4.2 ± 0.3 *b* [q]
	3	0.9 ± 0.0 *a*	1.3 ± 0.1 *a* [q]
Heterorhabditis sp. (NW European group)			
HW79	3	1.9 ± 0.1 *a*	7.5 ± 0.3 *b* [q]
HL81	4	3.0 ± 0.2 *a*	7.7 ± 0.1 *b* [t]
HF85	3	1.9 ± 0.3 *a*	6.7 ± 1.0 *b* [t]
HFr86	4	2.7 ± 0.1 *a*	6.7 ± 0.7 *b* [t]
HNb87	4	2.3 ± 0.2 *a*	7.0 ± 0.4 *b* [q,t]
HB1'87	3	2.3 ± 0.2 *a*	5.3 ± 0.6 *b* [q,t]
HE87	3	2.3 ± 0.1 *a*	5.2 ± 0.5 *b* [q,t]
HUK211	2	1.5 ± 0.2 *a*	2.8 ± 0.3 *a* [t]
HSH	3	3.1 ± 0.3 *a*	7.0 ± 0.1 *b* [q]
HKem	3	3.3 ± 0.1 *a*	6.3 ± 0.6 *b* [q]

[q] Batch subjected to evaluation of quality;
[t] data from time series, 4 h;
[d] batch also tested at 48 h (see text).

significantly increased in the presence of *G. mellonella* ($P \leq 0.02$) and in time ($P \leq 0.05$). Fewer nematodes of HI82 had left the top layer at 4 h (22 - 46%), but they moved at a higher rate (0.5 - 0.9 cm/h at 4 h), resulting in higher migration compared to HP88 (Table 2.2).

The Irish isolates M145, K122 and M198

In the time series only 34 - 45% of K122 and 20 - 21% of M145 had left the top layer at 8 h (Figure 2.1E and 2.1F), and nematodes moved slowly (0.6 cm/h and 0.3 cm/h at 4 h for K122 and M145, respectively). The percentage of recovered nematodes that remained in the top layer stabilized at 60% for K122 and 80% for M145. At 8 h, 30% of K122 reached the bottom layer, compared with 8 - 11% for M145. Test insect exposed to K122 were killed from 4 h onwards and contained 1.5, 10 and 11 hermaphrodites per insect at 4, 6 and 8 h, respectively. Those exposed to M145 contained 7 ± 3 ($\bar{x} \pm$ S.E.) hermaphrodites per insect at 6 and 8 h. In the additional experiments, more nematodes of K122 had left the top layer at 4 h (52 - 86%), resulting in higher distances covered (Table 2.3). Migration of K122 was enhanced by the presence of *G. mellonella*, but only in the additional experiments (Table 2.3). One batch of M198 dispersed better than the other two Irish isolates, but the other batch did not (Table 2.2).

H. megidis HO1

At 8 h, 59 and 68% of *H. megidis* HO1 had left the top layer in the absence and presence of *G. mellonella*, respectively (Figure 2.2A). In the presence of *G. mellonella*, 59% of the recovered nematodes was found in the bottom layer. These nematodes moved relatively fast (1.3 cm/h). From 4 h onwards, all test insects were killed and contained equal numbers of hermaphrodites per insect (35 ± 7 ($\bar{x} \pm$ S.E.)). In the cylinders without *G. mellonella*, a more or less stable situation was reached at 4 h with 45% in the top layer, 40% in the middle part and 15% in the bottom layer. HO1 responded significantly to the presence of *G. mellonella* and to time ($P \leq 0.002$).

The NW European isolates

Migration of the six Dutch isolates and the British HUK211 in the time series resembled that of *H. megidis* HO1, but the fractions of nematodes leaving the first layer of sand at 8 h were much greater, except for HUK211 (Figures 2.2 and 2.3). Sixteen and 55% of HUK211 had left the top layer at 8 h in the absence and presence of *G. mellonella*, respectively (Figure2.2A). In the presence of *G. mellonella* virtually all nematodes (95 - 99%) of HL81,

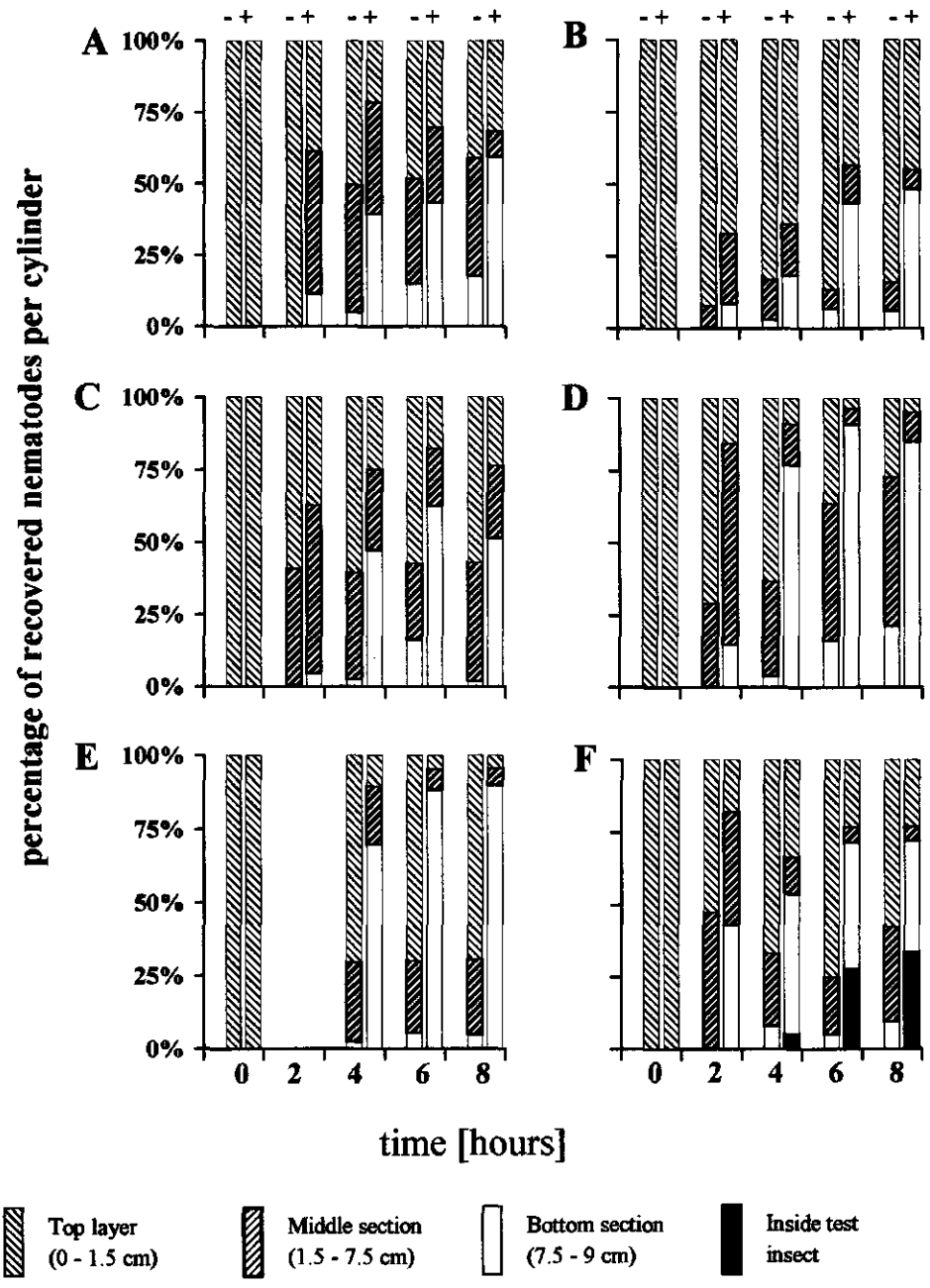

Figure 2.2. Percentage of recovered nematodes in the course of time in three sections of 9 cm high sand columns or inside *G. mellonella*, in the absence (–) and presence (+) of a last instar of *G. mellonella* at the bottom of the cylinders, after application of approximately 2000 living heterorhabditid nematodes on top of the cylinders. A: *H. megidis* HO1; B: HUK211; C: HB1'87; D: HNb87; E: HF85; F: HE87.

Table 2.3. Migration ($\bar{x}$; average distance covered [cm]), with standard error of the mean (S.E.), at 20 °C in 4 h of a number of batches of three isolates of *Heterorhabditis* in a number of 9 cm sand columns (n) without (–) and with (+) a *G. mellonella* larva at the bottom. (For each isolate migration data followed by different letters are significantly different from each other, $P \leq 0.05$).

	n	$\bar{x} \pm$ S.E. (–)	$\bar{x} \pm$ S.E. (+)
K122	3	3.4 ± 0.2 *a*	5.1 ± 0.2 *b* *[q]
HF85	3	2.5 ± 0.2 *a*	5.7 ± 0.1 *b* *[q]
	3	3.1 ± 0.1 *a*	5.9 ± 0.2 *b* *[q]
	3	3.7 ± 0.1 *a*	8.0 ± 0.1 *b* *[q]
	2	2.4 ± 0.2 *a*	6.4 ± 0.5 *b*
	2	3.3 ± 1.3 *a*	7.4 ± 0.1 *b*
	2	2.6 ± 0.5 *a*	7.5 ± 0.4 *b*
	2	3.7 ± 1.0 *a*	7.1 ± 0.1 *b*
	4	3.1 ± 0.2 *a*	6.6 ± 0.4 *b*
HUK211	3	2.1 ± 0.1 *a*	3.4 ± 0.2 *b* [q]
	3	2.8 ± 0.2 *a*	3.6 ± 0.1 *b* [q]

* Data obtained from K. Jung, Friesland College of Agriculture, Leeuwarden, The Netherlands
[q] Subjected to evaluation of quality (see text).

HF85, HFr86, and HNb87 left the first layer within 4 h and arrived in the bottom layer at 6 h. These nematodes moved fast (1.7 - 1.9 cm/h at 4 h). Since nematodes were already found in the bottom layer at 2 h, some individuals 'raced' through the cylinder at an average rate of 4.5 cm/h. Test insects exposed to HE87 in the time series contained 3 ± 3, 58 ± 19, 256 ± 21 and 337 ± 120 ($\bar{x} \pm$ S.E.) hermaphrodites per insect at 2, 4, 6, and 8 h, respectively. Unfortunately only test insects exposed to HE87 were dissected. The fraction of recovered nematodes leaving the top layer in cylinders without *G. mellonella* varied from 31% for HF85 to 77% for HL81 and HFr86. Additional results on other batches of HF85 at 4 h demonstrated a considerable variation in migration (Table 2.3). Migration of the German HSH, the Russian HKem and the Dutch HW79 at 4 h was comparable to that of the other NW European isolates (Table 2.2). In the time series all Dutch isolates and HUK211 responded significantly to the presence of the host insect ($P \leq 0.001$). In fact, all except HF85, responded more than proportionally to *G. mellonella* in the course of time (host × time interaction, $P \leq 0.05$).

Variation in migration between experiments was assessed for isolates that were tested frequently. Variance of mean migration between experiments was generally larger than within experiments; e.g. 2.12 versus 0.90 for HF85 ($n = 5$), 3.44 versus 0.12 for K122 ($n = 4$),

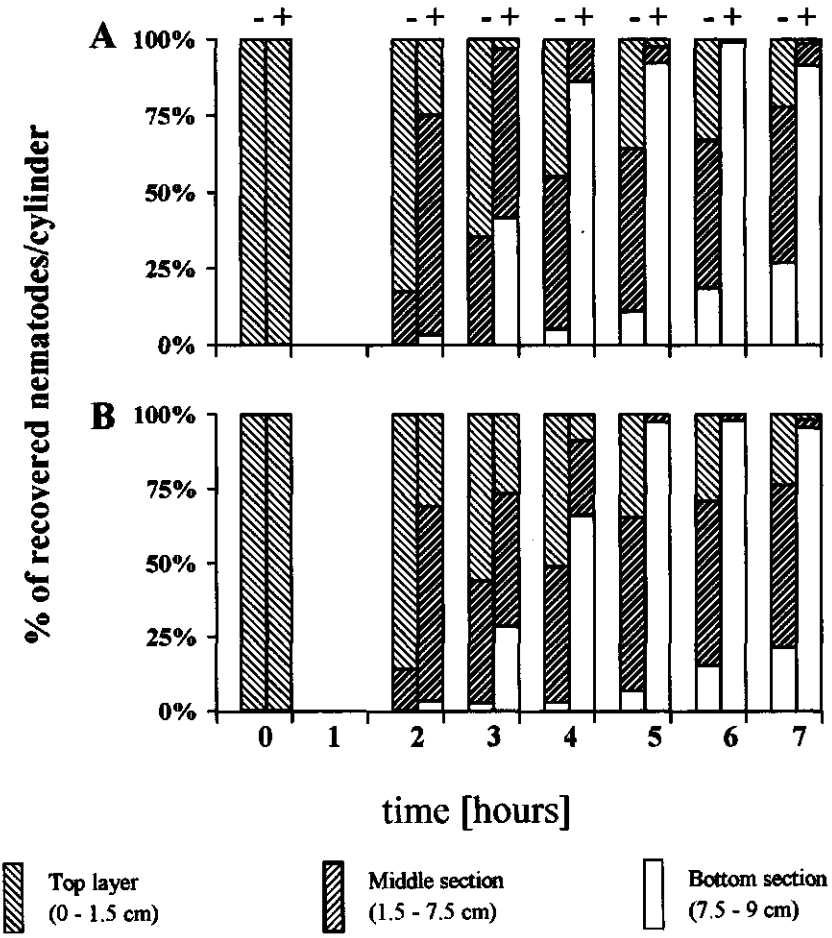

Figure 2.3. Percentage of recovered nematodes in the course of time in three sections of 9 cm high sand columns, in the absence (–) and presence (+) of a last instar of *G. mellonella* at the bottom of the cylinders, after application of approximately 2000 living heterorhabditid nematodes on top of the cylinders. A: HL81; B: HFr86.

and 1.34 versus 0.09 for HUK211 ($n = 3$). The isolates could be roughly ranked in order of descending migration at 4 h; NW European group (except HUK211) ≥ *H. megidis* > Irish group ≥ HP88, HI82, *H. bacteriophora* > *H. zealandica*.

Uniform dispersal would result in a 1 : 4 : 1 ratio in the number of nematodes in the three layers of a cylinder. Although this distribution was never found (χ^2 - test, $P \leq 0.05$), the distributions of HL81 (1 : 2.3 : 1.2), HFr86 (1.1 : 2.3 : 1) and HNb87 (1.3 : 2.5 : 1) in the absence of *G. mellonella* came very near. However, relatively large proportions of the

nematodes were consistently recovered from both ends of the cylinder for these most rapidly migrating isolates.

Discussion

In NW Europe, *O. sulcatus* is an increasingly serious pest of nursery stock, ornamentals and soft fruit such as strawberries. Hanula (1993) demonstrated that a biological control agent must penetrate to depths of 15 cm to be 95% effective against larvae of *O. sulcatus* on field grown yews. Although the actual vertical distribution of larvae may vary with plant species, the above data illustrate the need for actively migrating nematodes if insect parasitic nematodes were to be applied for effective black vine weevil control.

H. bacteriophora (syn. *H. heliothidis*; Poinar, 1990) and HP88, which are so far the only heterorhabditids included in studies on migration, generally outperformed steinernematids (e.g. Georgis and Poinar, 1983a; 1983b; 1983c; Alatorre-Rosas and Kaya, 1990). However, the present study showed that most heterorhabditids tested in this study, except *H. zealandica* NZH3, outperformed *H. bacteriophora* in the ability to migrate through a sand column at 20 °C, both in the rate of migration and in the proportion of actively migrating nematodes. In fact, the four *H. bacteriophora* isolates used here, including the type strains B1 and NC1, migrated better than previously reported for *H. bacteriophora* or *H. heliothidis* in sandy loam (20 - 25 °C, five days; Georgis and Poinar, 1983c). Soil type, isolate or quality of the batch used may all partially explain the observed differences.

Up to 99% of some of the heterorhabditids migrated when placed on a sand surface, contradicting Kaya's generalization (1990) that only a small proportion of steinernematid or heterorhabditid populations disperse when placed on the soil surface. Inactivity of a certain proportion of a population of nematodes is well documented among insect parasitic nematodes (Ishibashi and Kondo, 1990), and is assumed to be a survival strategy. When inactive, they can be stimulated to become active by various mechanical and chemical stimuli, such as CO_2 and other attractants from insects (Ishibashi and Kondo, 1990). In the present study, activation by substances released by *G. mellonella* may have contributed to the increase in the percentage actively migrating nematodes that most isolates exhibited when *G. mellonella* was present (chemo-orthokinesis).

Most heterorhabditid isolates tested here migrated at a higher rate than*H. bacteriophora* or *H. zealandica*: an 8 h period was generally sufficient to follow a large proportion of the population down to the bottom layer of the 9 cm sand column. The nematodes of most NW European isolates migrated at an incredible rate. The rapid downward

migration could not have resulted from passive movement caused by water drainage or gravity, because low water content (8%) in the sand did not allow water to drain. In fact, upward migration to a similar degree as downward migration has been obtained in identical cylinders when placed upside down (Westerman and Godthelp, 1991). Size of the nematodes in relation to particle size and the available pore space may also affect migration (Wallace, 1958). Indeed, isolates with long infective juveniles (NW European group, Irish group, *H. megidis*) generally migrated more rapidly than isolates with short infectives (*H. bacteriophora*, *H. zealandica*). However, of the related isolates HP88 and HI82, which are both approximately the same size, the former had the highest percentage nematodes leaving the top layer, but the nematodes of the latter isolate migrated more rapidly.

The rate of migration was doubled in the presence of *G. mellonella* for most isolates, which may have resulted from mechanisms such as chemotaxis and chemo-klinokinesis. Lei *et al.* (1992) observed that the nematodes of *H. zealandica* T327 moved randomly on agar dishes, but their movements became directed in the presence of puparia or larvae of the cabbage maggot, *Delia radicum*. Similarly, Gaugler *et al.* (1980) observed that on agar most individuals of a member of the related genus *Steinernema carpocapsae* oriented to a CO_2 gradient. It is likely that the nematodes in this study mainly oriented to a concentration gradient of volatile chemicals since the duration of the experiment was short for the formation of a gradient of water-soluble host cues, except in case of the 48 h experiments. The presence of *G. mellonella* corroborated the differences in migration between isolates without essentially altering the order. Lewis *et al.* (1992, 1993), who investigated the behaviour of steinernematids on an agar surface with respect to host searching strategies, predicted and found that species that moved in search of hosts, such as *S. glaseri*, would be more responsive to volatile host cues than species that adopted a 'sit-and-wait' strategy, such as *S. carpocapsae*. Heterorhabditid isolates of the NW European group were more active and responded much stronger to volatile cues of *G. mellonella* than *H. bacteriophora* or *H. zealandica*. *H. bacteriophora* and *H. zealandica*, although active searchers in comparison to most steinernematids, would be classified as 'sit-and-wait' strategists in comparison to isolates of the NW European group. Lewis *et al.* (1992) further suggested that these actively searching isolates may be better adapted for finding and parasitizing subterranean, sedentary insect larvae, such as *O. sulcatus*.

Mean migration at 4 h, which was chosen to compare migration among all batches tested, was not determined at a stable situation but, for most isolates, during the course of rapid movement. In this way, laborious dissecting of insect cadavers could be avoided.

However, migration defined in this manner is bound to be variable as it is sensitive to factors that influence the rate of migration or the proportion of actively migrating nematodes, such as subtle changes in the particle size composition, pH, moisture content, solute concentrations, condition, size and attractiveness of the test insects, or condition of the nematodes. Nevertheless, migration of HF85 (NW European group) proved unaffected by, for instance, particle size (range 105 - 425 mm) or pH (range 3 - 10) of the sand (Westerman, unpubl.). Probably variation in migration between replicates of one isolate can mainly be attributed to differences between batches of the nematodes, due to production and storage conditions (Kaya, 1990), as was demonstrated for heterorhabditids by Westerman and Stapel (1992). Moreover, migration will only give an indication of the ability of a batch to migrate at that time. For example, Westerman and Stapel (1992) demonstrated that migration decreased with storage time of the batch used and Lei *et al.* (1992) observed that newly collected nematodes did not orient to host cues, whereas one month old nematodes did.

Differences in mean migration were more or less congruent with putative taxonomic groups within the genus (Tables 2.2, 2.3). For example, isolates of the NW European group, with the exception of HUK211, exhibited almost identical courses of migration in time, distinct from those of the other groups. The behaviour of the related *H. megidis* and Irish isolates (Smits *et al.*, 1991; Dix *et al.*, 1992; Joyce *et al.*, 1994a; 1994b) had the nearest resemblance to that of the NW European group. The migration curves of the isolates of *H. bacteriophora* seem to differ from those of the taxonomically different, but closely related, isolate HP88 and *H. zealandica* (Dix *et al.*, 1992; Joyce *et al.*, 1994a; 1994b).

Since conditions may have been nearly optimal for migration, the results of this study only give an indication of the migration potential of the isolates examined. Migration and host searching in natural soils will depend on many other factors, such as the distribution and attractiveness of the target insect, soil texture, moisture and particle size composition, the presence and density of root systems and soil temperature. For example, larvae of *O. sulcatus* were not attractive to some NW European heterorhabditids (Westerman and Godthelp, 1991). Furthermore, migration of NW European heterorhabditids is seriously impaired in humous soils (Westerman, unpubl.). Shape and size of the root system of the plant will not only influence the distribution of target insects but may also influence migration and host finding of nematodes as was demonstrated by Choo *et al.* (1989) and Choo and Kaya (1991). Although dense roots reduced infectivity of *H. bacteriophora* NC1 in sandy soils, sparse roots increased nematode infectivity in humous soils. Twenty °C, as chosen in this study, might be close to optimal for application to indoor ornamentals. Outdoors, however, soil

temperatures would vary over a wide range and would be appreciable cooler during much of the year in NW Europe. Since temperature affects efficacy to a large extent, these experiments should be continued at lower temperatures for comparison with the available efficacy data (e.g. Simons and Van der Schaaf, 1986; Westerman and Van Zeeland, 1989).

Chapter 3

Comparative vertical migration of insect parasitic nematodes *Heterorhabditis* spp. and *Steinernema* spp. in sand at 9 °C

Insect parasitic nematodes of the genus *Heterorhabditis* Poinar can provide effective control of the black vine weevil, *Otiorhynchus sulcatus* F.; up to 100 % mortality of the larvae can be obtained in strawberries and ornamentals in the greenhouse. However, the nematodes are not very effective when soil temperatures are below 12 - 13 °C (e.g. Rutherford *et al.*, 1987), which limits the extension of the application of *Heterorhabditis* in open fields in regions of lower soil temperatures. Nematodes of the related genus, *Steinernema* Travassos, tend to be more effective at low temperatures than heterorhabditids (e.g. Molyneux, 1986); however, heterorhabditids are generally more pathogenic towards *O. sulcatus* and other soil dwelling coleopterans (e.g. Rutherford *et al.*, 1987; Hanula, 1993). Mobility is required for black vine weevil control, as larvae of *O. sulcatus* can be found at great depths, for instance, down to 15 cm in yew (Hanula, 1993). Species and isolates of *Heterorhabditis* differ in their migration at 20 °C (Chapter 2). Therefore, in the present study, mobility of sixteen isolates of *Heterorhabditis* and three of *Steinernema* was studied in sand columns at 9 °C.

Mean migration was assessed in 9 cm vertical sand columns at 9 °C with or without a larva of *G. mellonella* at the bottom as described in an earlier paper on migration at 20 °C (Chapter 2). Origin, culture and storage method of the nematode species and isolates were described in Chapter 2. The nematodes were left at the cold storage temperature (4 - 5 or 9 °C) until inoculation. Forty to 60%, and for HP88 70%, of the applied nematodes were recovered. The number of nematodes recovered from dead insects was so small (≤ 25, on average) that they were not included in the calculations. Isolates were either tested in time series of 16, 24, 32, 40, 48 and 56 hours or only at a 48 h incubation period.

Migration in the course of time is presented in the Figures 3.1 and 3.2 for eleven heterorhabditid and one steinernematid isolate. Mean migration data, analysed in a similar way as the 20 °C data (Chapter 2), significantly increased with time ($P \leq 0.05$) for all isolates tested in time series, except for HF85, HNb87 and HSH, and most isolates significantly responded to the presence of *G. mellonella*; HB1'87, HUK211 and *H. megidis* HO1

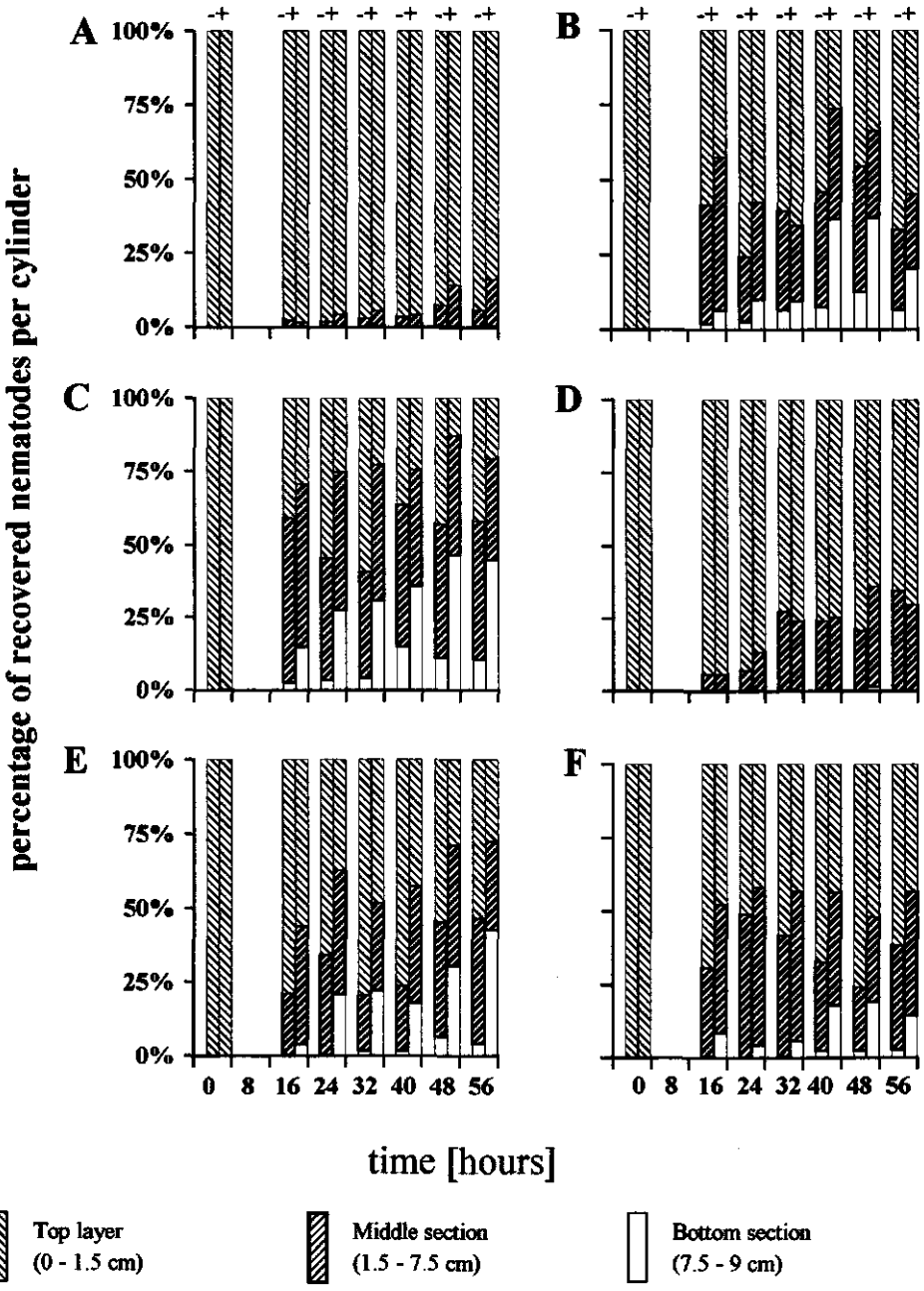

Figure 3.1. Percentage of recovered nematodes in the course of time in three sections of 9 cm high sand columns, in the absence (–) and presence (+) of a last instar of *Galleria mellonella* at the bottom of the cylinders, after application of approximately 2000 living heterorhabditid nematodes on top of the cylinders. A: HP88; B: *Heterorhabditis megidis* H01; C: K122; D: M145; E: HL81; F: HF85.

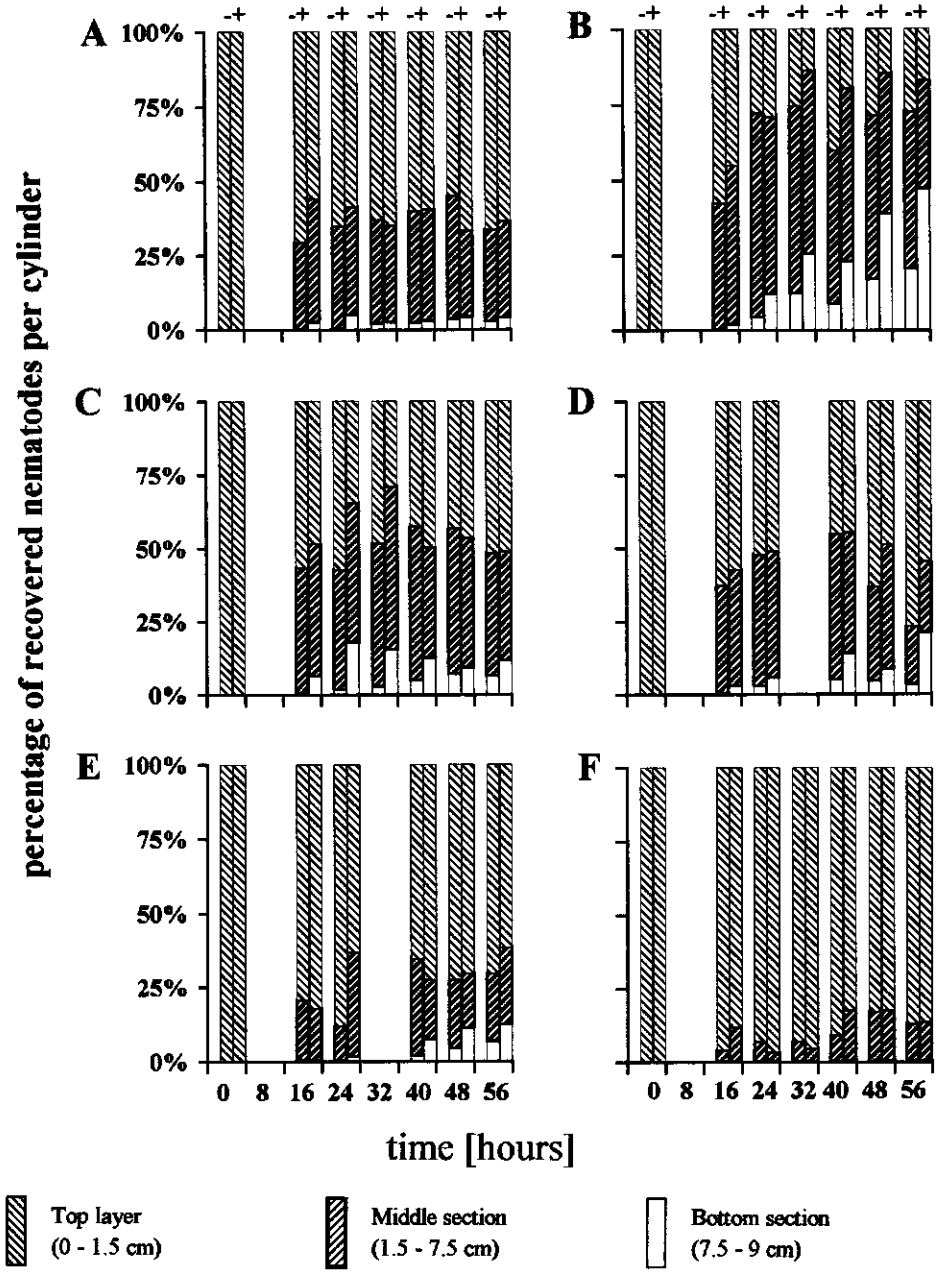

Figure 3.2. Percentage of recovered nematodes in the course of time in three sections of 9 cm high sand columns, in absence (–) and presence (+) of a last instar of *Galleria mellonella* at the bottom of the cylinders, after application of approximately 2000 living heterorhabditid or steinernematid nematodes on top of the cylinders. A: HNb87; B: HUK211; C: HB1'87; D: HSH; E: HKem; F: *Steinernema feltiae* OBSIII.

Table 3.1. Migration (mean distance covered [cm] ± S.E.) at 9 °C in 48 h of a number of *Heterorhabditis* and *Steinernema* species and isolates in a number (n) of 9 cm sand columns in presence or absence of a *Galleria mellonella* larva. (Migration data per isolate followed by the same letter are not significantly different from each other, Tukey's test, $P \leq 0.05$).

	n	$\bar{x}$ ± S.E. (−)	$\bar{x}$ ± S.E. (+)
H. zealandica	3	1.1 ± 0.0 *a*	1.0 ± 0.1 *a* [q]
H. bacteriophora	3	0.9 ± 0.0 *a*	0.9 ± 0.1 *a* [q]
HP88	3	1.0 ± 0.1 *a*	1.3 ± 0.1 *a* [q,t]
HI82	3	0.8 ± 0.0 *a*	0.9 ± 0.0 *a* [q]
H. megidis HO1	4	3.3 ± 0.3 *a*	4.6 ± 0.4 *a* [t]
Heterorhabditis sp. (Irish group)			
K122	4	3.3 ± 0.3 *a*	5.8 ± 0.3 *b* [t]
M145	4	1.6 ± 0.1 *a*	2.2 ± 0.3 *a* [t]
M198	3	1.2 ± 0.1 *a*	1.4 ± 0.1 *a* [q]
Heterorhabditis sp. (NW European group)			
HW79	3	1.4 ± 0.1 *a*	2.7 ± 0.2 *b* [q]
HL81	3	2.7 ± 0.0 *a*	4.6 ±0.2 *b* [q,t]
HF85	4	1.7 ± 0.2 *a*	3.3 ± 0.3 *b* [t]
HNb87	4	2.6 ± 0.1 *a*	2.2 ± 0.2 *a* [t]
HB1'87	4	3.1 ± 0.1 *a*	3.1 ± 0.2 *a* [t]
HUK211	4	4.1 ± 0.2 *a*	5.4 ± 0.2 *b* [t]
HSH	3	2.3 ± 0.2 *a*	3.0 ± 0.4 *a* [q,t]
Hkem	3	2.0 ± 0.4 *a*	2.3 ± 0.2 *a* [q,t]
Steinernema spp.			
S. feltiae OBSIII	3	1.4 ± 0.1 *a*	1.5 ± 0.0 *a* [q,t]
S. feltiae Mr	3	1.7 ± 0.4 *a*	1.6 ± 0.2 *a* [q]
S. carpocapsae UK	3	1.1 ± 0.3 *a*	0.9 ± 0.1 *a* [q]

[q] Batch subjected to evaluation of quality (see text)
[t] Data obtained from time series, 48 h.

responded more than proportionally to *G. mellonella* in the course of time (host × time interaction, P = 0.005, 0.03 and 0.001, respectively). Five isolates did not respond significantly to the presence of *G. mellonella*; HNb87, HSH, HKem, M145 and *S. feltiae* OBSIII. Often, 56 h were insufficient to follow the proportion mobile nematodes in the population down to the bottom layer. This was mainly due to rate differences, because generally only a small proportion of the nematodes was inactive and remained in the top

layer. Mean migration at 9 °C was higher in cylinders with than in cylinders without *G. mellonella* for most isolates, and the presence of *G. mellonella* corroborated differences in migration between isolates (Table 3.1).

In contrast to migration at 20 °C, differences at 9 °C do not seem to be related to putative taxonomic groups within *Heterorhabditis*. Isolates, identified here as being relatively mobile at 9 °C, belonged to different species and groups; e.g. the Irish K122, the NW European UK211 or *H. megidis* HO1. In addition, there were differences between isolates from the same locality (e.g. compare Dutch or Irish isolates), indicating that the ability to move in cold sand columns is not directly related to their geographic origin. However, not all species and groups were equally represented in this study.

The three steinernematids tested in this study, *S. carpocapsae* UK, *S. feltiae* Mr and *S. feltiae* OBSIII, were relatively immobile at 9 °C, especially when compared to the Irish and NW European heterorhabditids at 9 °C, or when compared to migration of *S. feltiae* Mr at 20 °C (3.2 ± 0.6 and 5.7 ± 0.1, in cylinders without and with *G. mellonella* at the bottom, respectively; unpublish.). However, steinernematids constitute a large group of species and isolates. Other isolates, e.g. the cold active isolate LIC (Morris *et al.*, 1990), may be more mobile at 9 °C.

The migration assay provides an indication of the migration potential in sand at 9 °C of the isolates (batches) examined. It is not possible to identify isolates that are mobile or relatively immobile at 9 °C solely on the basis of the present data, because the batches used may have differed in quality. However, isolates once qualified as relatively mobile at 9 °C are worth further testing. Whether migration in sand columns reflects the ability of the nematodes to move in natural soils, and whether this is relevant to efficacy in the field remains to be tested.

Chapter 4

Penetration of the insect parasitic nematode *Heterorhabditis* spp. into host insects at 9 and 20 °C

Abstract Biological control of black vine weevil, *Otiorhynchus sulcatus*, by means of the insect parasitic nematode, *Heterorhabditis* spp., is difficult at low temperatures. The influence of temperature on the penetration ability was investigated to identify whether this process is a bottleneck for biocontrol. Variation in penetration ability among and within *Heterorhabditis* was assessed. A negative exponential model was used to characterize penetration through time. The two parameters in this model, the proportion infectious nematodes and the relative penetration rate, were compared among different batches of the same nematode isolate (HF85). There was a considerable amount of variation among batches in the proportion infectious nematodes in HF85, but there was very little variation in the relative penetration rate. Among twelve other heterorhabditid isolates there was almost no variation in the proportion of nematodes infectious against *O. sulcatus* at 9 °C. Such variation did occur when two lepidopteran species, *Galleria mellonella* and *Spodoptera exigua*, were offered as hosts at 9 °C, or when hosts were presented at 20 °C. The relevance of variation in penetration parameters for control of *O. sulcatus* at low temperatures is discussed.

Introduction

In most temperate regions in the world, the black vine weevil, *Otiorhynchus sulcatus* F., is an increasingly serious pest of nursery stock, ornamentals and soft fruits, such as strawberries, grapes, and blackcurrants. Insect parasitic nematodes of the genus *Heterorhabditis* Poinar can provide effective control of the weevil; up to 100% mortality of the larvae can be obtained in strawberries and ornamentals in the greenhouse. However, the nematodes are little effective at low soil temperatures, i.e. below 12 - 13 °C (e.g. Rutherford *et al.*, 1987). These temperatures occur in The Netherlands and other NW European countries during spring and autumn when the larvae of the weevil should be controlled in nursery stock and ornamentals in open cultures.

Penetration into the haemocoel of the insect is required for insect death. Therefore, in this study the penetration ability of *Heterorhabditis* spp. was investigated. The influence of high (20 °C) and low (9 °C) temperature on the penetration ability was assessed. Penetration was compared between heterorhabditid isolates to see if isolates exist that have a better ability to penetrate at low temperatures. Because nematode behaviour can change as a result of duration and conditions of storage (e.g. Fan and Hominick, 1991b; Westerman, 1992), variation in penetration ability among batches of the same isolate was investigated.

Penetration into the lepidopterans *Galleria mellonella* (L.) and *Spodoptera exigua* (Hb.) was assessed to determine whether and in which way penetration into these highly susceptible host species differs from penetration into the less susceptible *O. sulcatus*.

To assess and compare the penetration ability, the penetration process had to be quantified. The increase in the number of penetrated nematodes in time can be described by a negative exponential curve when infectious nematodes penetrate at the same relative rate until all have penetrated (saturation curve). Results of other studies suggest a negative exponential increase (e.g. Mannion and Jansson, 1993). Therefore, a negative exponential model was adopted here, requiring two parameters to characterize the penetration process.

Materials and methods

Insect cultures, nematode cultures and storage

Origin of the heterorhabditids was documented in Chapter 2. In addition to isolates of *Heterorhabditis*, a few representatives of the related genus *Steinernema* Travassos were included for comparison. *S. feltiae* OBSIII was isolated in The Netherlands by Dr F. Galle (De Groene Vlieg Company, Nieuwe Tonge), *S. feltiae* Mr (Austria) and SF1 (Germany) were provided by Dr R.U. Ehlers (C.A. Univ., Kiel, Germany) and *S. carpocapsae* UK (UK) was provided by Dr N.G.M. Hague (Univ. Reading, UK). All nematodes were propagated on *G. mellonella* at 20 °C and stored in aerated water at 4 - 5 °C, as described in Chapter 2. Batches to be tested at 9 or 20 °C were transferred to this temperature three days before the beginning of the experiments to adjust. *G. mellonella* and *S. exigua* were reared according to standard procedures on artificial medium at 25 °C. *O. sulcatus* was reared on strawberry plants in a greenhouse at approximately 20 - 25 °C. Last instar larvae were collected from the pots and stored at 4 - 5 °C in potting soil with some strawberry roots, until use.

Penetration model

The penetration model was based on the same assumptions regarding the penetration behaviour as those used by Bohan and Hominick (1995a). Mortality of nematodes during the experimental period is assumed negligible. The total nematode population (D) consists of a proportion infectious (C) and a proportion non-infectious ($1 - C$) nematodes. Only the infectious nematodes I ($= C \times D$) penetrate (Bohan and Hominick, 1996). The parameter C sets an upper limit to the number of nematodes that can actually penetrate the host, irrespective of the exposure time. When assuming an equal probability for all infectious

nematodes to penetrate, the penetration rate (dM/dt) is proportional to the number of infectious nematodes outside the insect:

$$dM/dt = B \times C \times D \qquad \text{[nem./h]} \qquad (4.1)$$

where M is the number of penetrated nematodes [nem.];
B is the relative penetration rate [1/h]

The number of infectious nematodes outside the host at time t, I_t, decreases with increasing numbers of penetrated nematodes, M_t:

$$I_t = I_0 - M_t \qquad \text{[nem.]} \qquad (4.2)$$

where I_0 is the number of infectious nematodes applied at time $t = 0$ ($= C \times D$) [nem.].

I_0 is the maximum number of nematodes that can penetrate. The rate of change in infectious nematodes outside the host is the same as the rate of penetration with reversed sign. Therefore, the decrease in the number of infectious nematode in time is described as:

$$I_t = I_0 \times e^{-B \times t} \qquad \text{[nem.]} \qquad (4.3)$$

Combining Equations (4.2) and (4.3), the increase in the number of penetrated nematodes in time is described as:

$$M_t = I_0 - I_t = C \times D \times (1 - e^{-B \times t}) \qquad \text{[nem.]} \qquad (4.4)$$

Parameter estimation

B and C can be estimated directly from Equation (4.4). A reliable estimate of B is obtained only when several observations are available for the initial part of the curve where the slope changes most. B cannot be estimated accurately if the plateau ($C \times D$) is (almost) reached at the time of the first sample. In this case it may still be possible to estimate B, by combining data from several penetration curves (same host, nematode isolate and temperature) with different numbers of applied nematodes (D) (Anderson, 1982). For each D, there is a relationship $M_t = C \times D \times (1 - e^{-B \times t})$. Data for a fixed sample time, t, and different D are combined. Pairs of data (D, M_t) for a range of initial nematode numbers are subjected to linear regression analysis through the origin:

$$M_t = A \times C \times D \qquad \text{[nem.]} \qquad (4.5)$$

with

$$A = 1 - e^{-B \times t} \tag{4.6}$$

After estimating A through linear regression using Equation (4.5), B is solved from Equation (4.6):

$$B = -1/t \times \ln(1 - A) \qquad [1/h] \tag{4.7}$$

This method requires that C is already known and that time t is chosen before curve (4.4) reaches its horizontal asymptote (i.e. before $M_t \rightarrow C \times D$).

Penetration assay

Single last instar larvae of *G. mellonella*, *O. sulcatus* or *S. exigua* were placed on the bottom of petri-dishes (25 ml, Ø 5 cm) and covered with 33 - 35 g moist (8% w/w) fine sterile sand (particle size: 93% between 180 - 425 µm). When insects had to be replaced during the experiment (see below), they were placed on top of the sand in a small indention, and larvae of *O. sulcatus* were enveloped in fine gauze to prevent them from moving through the petri-dish. All materials had been thermo-equilibrated overnight at either 9 or 20 °C. Nematodes in 0.5 ml water were applied evenly on the surface of the sand. Plates were closed, stacked in piles of five with a 40 - 50 g weight on top and placed in a plastic container lined with moist cloth, at the appropriate temperature. Mortality among control insects of *G. mellonella* and *S. exigua* was rare and, therefore, controls were included only occasionally for these two species (not mentioned). All insect larvae, dead or alive, were removed from the sand after the exposure period. Unless stated otherwise, the insects were washed under running water and dried in a towel to remove external nematodes. The insects were incubated at 20 °C for a total of 7 d to allow the nematodes to develop to last stage juveniles or adults. Dead insects were dissected or frozen to dissect at another time, and the numbers of developed larvae or hermaphrodites were counted. When no developed nematodes were found, the dissected insects were examined under the microscope for undeveloped larvae (J3). Dead larvae that did not show the characteristic signs (colour, constitution) associated with the bacterial symbionts of the nematodes, *Photorhabdus* Boemare, Akhurst and Mourant or *Xenorhabdus* Thomas and Poinar, and that did not contain nematodes were considered to have died from natural causes. Temperature during trials was recorded using a CSU-thermistor temperature probe connected to a 1202 Grant squirrel datalogger.

Because only dead insects were dissected, it is possible that some insects that were penetrated, as well as penetrated nematodes, were overlooked. Some surviving insects might have recovered from infection by removing invaded nematodes from the haemocoel before dissection. This would cause an error in the mean number of nematodes per insect and the proportion infected insects. The alternative, dissection immediately after exposure, would hardly be more accurate, because the small, undeveloped J3 juveniles are easily overlooked. Comparing the number of nematodes in infected (immediately after exposure) and killed insects (several days after exposure) in the same experiment indicated that the loss of penetrated nematodes in *G. mellonella* and *O. sulcatus* at 20 °C is negligible, except when the cadaver is contaminated with other bacteria (unpubl. data). However, the loss of penetrated nematodes may be larger at low temperatures (e.g. Thurston and Kaya, 1994).

The effect of host species (O. sulcatus vs G. mellonella) and temperature (9 vs 20 °C) on host penetration by four batches of the heterorhabditid HF85

The proportion infectious nematodes, C, was estimated by the mean cumulative number of nematodes that penetrated *G. mellonella* or *O. sulcatus*, at 9 or 20 °C, over four consecutive exposure periods of 3 d, or 7 d in case of *O. sulcatus* at 9 °C. C was assessed for four batches of HF85, which had been stored at low temperatures as described above for 6, 48, 118 and 132 d, respectively. For each combination of nematode batch, host and temperature, a group of 15 petri-dishes was prepared (see section on Penetration assay) and inoculated with 200 nematodes. After each period of 3 d or 7 d the larvae were replaced by new insects. Replacement was necessary because dissection had to take place before the nematodes started reproducing, i.e. within 7 - 8 days at 20 °C and within 4-5 weeks at 9 °C. After exposure the insects were not washed to prevent removal of nematodes from the system each time the insect is replaced. The number of penetrated nematodes was expressed as a percentage of the dose applied, D. The effect of nematode batch, temperature and insect host and the interaction between these factors was assessed using ANOVA on arcsin $\sqrt{}$ transformed (to stabilize the variance) proportion infectious nematodes. Means were separated according to pairwise L.s.d. with $\alpha = 0.05$.

The relative penetration rate, B, was estimated using Equations (4.5) and (4.6). For each combination of nematode batch, host and temperature, four groups of 15 petri-dishes were prepared and each was inoculated with either 50, 100, 150 or 200 nematodes. Insects were removed after 24 h or 7 d (*O. sulcatus*) at 9 °C (average temperatures 9.4 and 9.7 °C, respectively) or after 24 h at 20 °C (average temperature 20.6 °C). In the first instance, all

data were combined to test the effect of batches, host and temperature on the slope, A. In the second instance, the effect of batch alone was tested within each combination of temperature and host.

The effect of nematode isolate, temperature and insect species on the proportion infectious nematodes C

In four experiments the proportion infectious nematodes, C, of twelve isolates was estimated in the presence of *G. mellonella*, *O. sulcatus* or *S. exigua*, at 9 and 20 °C. For each combination of isolate, insect host and temperature a group of 20 (trial 1 and 2) or 25 petri-dishes (trial 3 and 4) was prepared and each petri-dish was inoculated with 300 nematodes. In trial 4 group size for *S. exigua* was 20; group size depended on the availability of *O. sulcatus* and *S. exigua*. Exposure time was 3 d at 20 °C (average temperature 20.3 and 19.7 °C for trial 3 and 4, respectively) and 5 d or 6 weeks (*O. sulcatus*) at 9 °C (average temperature 8.4, 7.7, 8.8 and 8.9 °C for trial 1 to 4, respectively). Data were analysed as described for the heterorhabditid HF85 (see above).

Results

The effect of host species (O. sulcatus vs G. mellonella) and temperature (9 vs 20 °C) on host penetration by four batches of the heterorhabditid HF85

The mean cumulative number of nematodes that entered the insect host in the course of time is presented in Figure 4.1. Estimates of C, expressed as a percentage of the dose applied, are summarised in Table 4.1. The proportion infectious nematodes C was higher for *G. mellonella* than for *O. sulcatus*, and higher at 20 than at 9 °C. The effect of an increase in temperature on C (over four batches) was larger in case of *O. sulcatus* (× 9.6) than in case of *G. mellonella* (× 4.4). Analysis showed that temperature, insect host, batch, two-way and three-way interactions significantly influenced C ($n = 240$; $P \leq 0.05$).

The slope A was similar for the four batches, but different for different insects and temperatures ($R^2_{adj} = 98\%$, $P < 0.001$) (Figure 4.2). If the regression was not forced through the origin, intercepts were not significantly different from zero ($P > 0.05$). They were therefore omitted. The relative penetration rates, B, estimated with Equation (4.7) are 1.18×10^{-2} and 7.85×10^{-2} [1/h] for *G. mellonella* 9 and 20 °C, respectively, and 0.07×10^{-2} and 1.83×10^{-2} [1/h] for *O. sulcatus* at 9 °C and 20 °C, respectively. Differences between batches were tested again per combination of host and temperature, using A ($= 1 - e^{-B \times t}$). In each

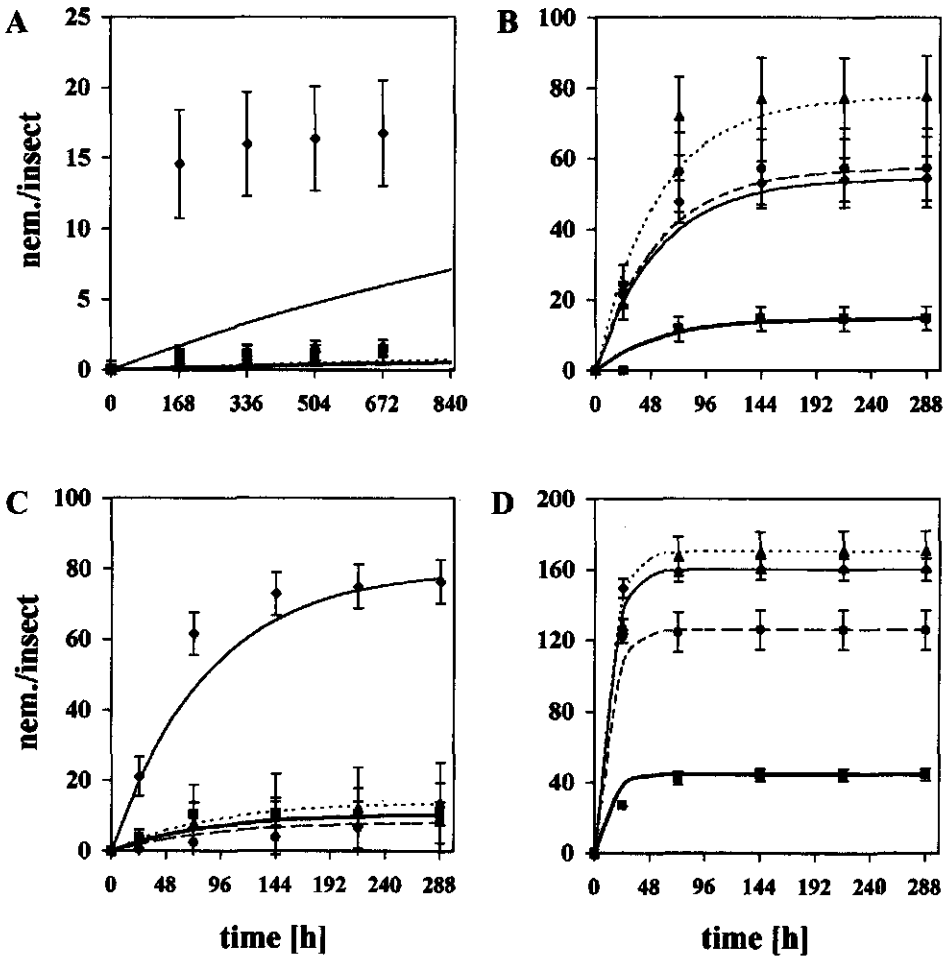

Figure 4.1. The mean cumulative number of nematodes that penetrated *O. sulcatus* (A, B) or *G. mellonella* (C, D) in petri-dishes filled with moist sand at 9 °C (A, C) or 20 °C (B, D) in the course of time, after exposure to one of four batches of the heterorhabditid HF85 (● —, batch 1; ▲ ····· , batch 2; ◆ ____ , batch 3; ■ ____ , batch 4).

combination, infectious nematodes of HF85 penetrated at the same relative rate, except in case of *O. sulcatus* at 20 °C. Nematodes of this batch did not penetrate ($A = 0$, $P > 0.05$; Figure 4.2B), while those of the other three batches did ($A > 0$, $P < 0.001$).

Curves of the increase in the number of penetrated nematodes in time, based on estimates of C and B, were compared to observations (Figure 4.1). Estimates of C are based on these observations; estimates of B are based on different data sets. In case of *O. sulcatus* at

Table 4.1. The proportion infectious nematodes, *C*, expressed as a percentage of the dose applied, that entered the insects, *G. mellonella* and *O. sulcatus* after repeated exposures at 9 and 20 °C. (Figures followed by different letters are significantly different from each other, L.s.d., $P \leq 0.05$).

	Temperature [°C]			
	9		20	
Batch No.	*G. mellonella*	*O. sulcatus*	*G. mellonella*	*O. sulcatus*
1	4.1 bc	0.7 a	63.0 f	28.7 d
2	6.9 c	0.9 ab	85.1 g	38.9 e
3	40.1 e	8.4 c	80.2 g	27.3 d
4	5.3 c	0.7 a	22.3 d	7.4 c

9 °C, the increase as depicted by the observations (points) is much faster than the increase as predicted by *B* (lines). The misfit of curve and observations can be explained by a very small difference in the experimental set-up used to estimate *C* and *B*, causing an overestimation of the speed of infection in the experiment to estimate *C* (points). Insects were washed after exposure to the nematodes in the experiment to estimate *B*, but washing was omitted in experiment to estimate *A*. Apparently, many nematodes adhered to the outside of *O. sulcatus* and penetrated after the official exposure period of 7 d at 9 °C, causing a much higher number of infections than would have been possible if external nematodes had been removed. *B* was estimated correctly (line). It seems that this problem only occurred for *O. sulcatus* at 9 °C. The above problem can be avoided by a single exposure of 6 - 7 d of *G. mellonella* at 9 or 20 °C and *O. sulcatus* at 20 °C, and a single exposure of 2 - 3 weeks of *O. sulcatus* at 9 °C. The experimental procedure for estimating *C* was, therefore, adapted in the next series of experiments.

The effect of nematode isolate, temperature and insect species on the proportion infectious nematodes, C

In this experiment, the proportion infectious nematodes in 12 heterorhabditid and four steinernematid was assessed by prolonged exposure of insects to a single dose of nematodes, such that maximum penetration was reached. Estimates of *C* for different isolates are summarized in Table 4.2. Estimated proportion infectious was higher at 20 than at 9 °C, and was always lower in *O. sulcatus* than in the other two hosts. Differences between isolates and between host species at 9 °C were small, except for high values for two steinernematids in the

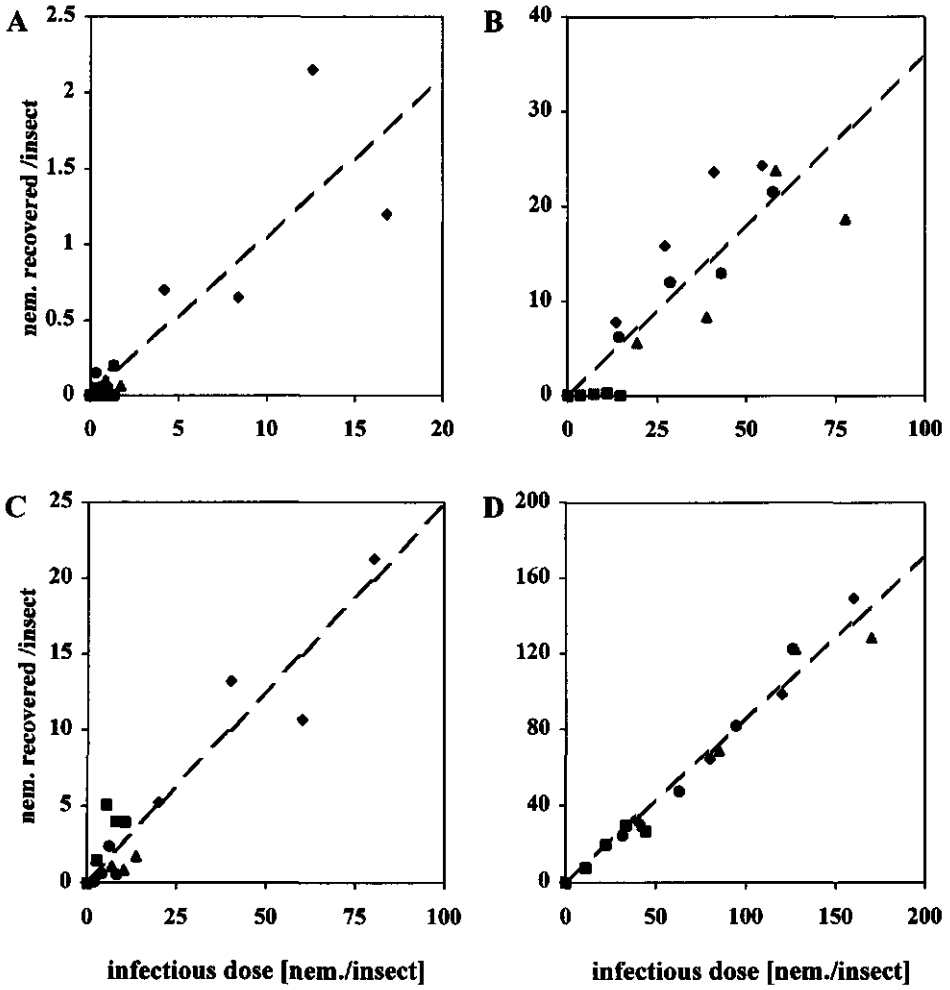

Figure 4.2. The mean number of nematodes recovered per insect (n = 15) and the estimated linear regression line as a function of the infectious dose applied [nem./insect], for single *O. sulcatus* (A, B) or *G. mellonella* (C, D) in petri-dishes filled with moist sand at 9 °C (A, C) or 20 °C (B, D), after exposure to one of four batches of the heterorhabditid HF85 (●, batch 1; ▲, batch 2; ◆, batch 3; ■, batch 4) for 24 h or 7 d (*O. sulcatus* at 9 °C).

presence of *G. mellonella* and *S. exigua*. There were no differences between isolates for *O. sulcatus* at 9 °C ($C \leq 1\%$; $P > 0.05$). Apart from two high values for the steinernematids, ranking of isolates was similar in the three hosts. The influences of temperature, insect host, nematodes isolate and the interactions between these factors on C were significant ($P \leq 0.001$) in all four trials.

Table 4.2. The proportion infectious nematodes, *C*, (%) estimated by the mean number of nematodes recovered from *G. mellonella* (*G.m.*), *S. exigua* (*S.e.*), or *O. sulcatus* (*O.s.*) larvae (n = 20 - 25), divided the dose applied [300 nem. per insect], after an exposure period of 3 d at 20 °C, and 5 d (*G. mellonella* and *S. exigua*) or 6 w (*O. sulcatus*) at 9 °C. Figures within a trial followed by different letters are significantly different from each other, L.s.d., $P \leq 0.05$.

		Mean number of nematodes recovered per host (% of dose applied)					
		9 °C			20 °C		
trial	Isolate	*G.m.*	*O.s.*	*S.e.*	*G.m.*	*O.s.*	*S.e.*
1	HF85[a]	0.7 a	0.0 a		87.6 f	43.2 d	
	HI82[b]	0.1 a	<0.1 a	n.t.	7.4 b	9.2 b	n.t.
	UK211[a]	0.3 a	0.1 a		55.8 e	28.0 c	
2	UK211[a]	0.2 ab	0.1 a	0.4 abc	46.8 i	22.0 g	45.8 i
	HSH[a]	4.3 d	0.2 ab	9.0 e	59.7 j	12.1 f	79.5 l
	HB1'87[a]	0.6 abc	1.0 abc	1.3 c	73.6 k	31.4 h	72.4 k
	HO1[c]	0.1 a	<0.1 a	0.1 a	22.5 g	10.1 e	33.6 h
	B1[b]	<0.1 a	0.0 a	<0.1 a	1.1 bc	0.4 ab	1.4 c
3	HF85[a]	1.0 bcd	0.0 a	1.0 bcd	33.1 gh	1.8 abc	68.7 j
	HP88[b]	3.1 de	0.4 ab	2.7 de	36.8 h	1.4 abc	77.2 k
	K122[d]	0.5 abc	0.0 a	0.6 abc	16.0 f	0.2 ab	47.4 i
	HSH[a]	0.3 abc	0.3 ab	0.4 abc	38.4 h	1.9 abc	73.5 jk
	M198[d]	0.4 abc	0.0 a	1.0 bcd	27.1 g	1.3 abc	73.0 jk
	HW79[a]	1.7 bcd	<0.1 a	1.2 cd	18.4 f	2.0 b	69.3 j
	HL81[a]	1.1 bcd	0.1 a	3.0 de	51.8 i	5.4 e	33.2 gh
4	Mr[e]	21.7 ef	0.3 a	18.5 e	65.7 j	0.5 a	40.8 i
	OBSIII[e]	27.8 fg	0.9 ab	26.5 f	66.9 j	1.9 ab	32.9 gh
	SF1[e]	5.6 c	0.5 a	7.8 c	43.5 i	3.0 b	36.9 h
	Uk[f]	0.3 ab	0.0 a	0.2 ab	38.3 hi	0.8 ab	13.2 d

n.t. = not tested
[a] NW European heterorhabditid
[b] *H. bacteriophora* or related group
[c] *H. megidis*
[d] Irish heterorhabditid
[e] *S. feltiae*
[f] *S. carpocapsae*

Discussion

The increase in the number of penetrated nematodes in insects in time was characterized by a negative exponential model (Figure 4.1). Two parameters, the proportion infectious nematodes and the relative penetration rate, successfully describe penetration in time. Bohan and Hominick (1995a) developed a more complicated model for penetration of steinernematid nematodes. This model was not used here because quantification of the four parameters in their model would require more elaborate data-sets, while the four parameters are less suitable for extensive comparison of nematode isolates and batches.

Temperature and insect species both influenced the proportion infectious nematodes. The results confirm that only a proportion of the nematodes is infectious, as observed by Fan and Hominick (1991a) and Bohan and Hominick (1996). However, this proportion is not a characteristic of a nematode species or isolate, but of the combination of nematode, host, and abiotic conditions. The effect of temperature on the proportion infectious nematodes was reported before (e.g. Fan and Hominick, 1991a; Schirocki and Hague, 1994; Bohan and Hominick, 1995a), but the effect of host species was unexpected. It is unknown what is causing the difference between infectious and non-infectious nematodes. Inactivity of nematodes is generally considered to be a survival strategy. Explanations from a biological or evolutionary perspective include the saving of energy, spreading of biotic and a-biotic risks, increasing the chances of matings between non-related individuals, avoiding over-exploitation of available hosts, and decreasing the chances of extinction of isolated populations (e.g. Ishibashi and Kondo, 1990; Bohan and Hominick, 1995b; De Leij, 1995). The present results show that nematodes can be stimulated to become infectious when presented with the right host or the right conditions.

There was little variation among the twelve heterorhabditid isolates in the proportion infectious nematodes in the presence of *O. sulcatus* at 9 °C (Table 4.2); it was always below 1%. Variation among isolates was (much) larger in the presence of lepidopteran hosts, *G. mellonella* and *S. exigua*, at 9 °C, and for all hosts at 20 °C. However, that is of little use when trying to find a more infectious nematode for control of the black vine weevil at low temperatures. The proportion infectious nematodes doesn't seem to be related to (putative) taxonomic groups within *Heterorhabditis*, although the two members of *H. bacteriophora* (B1 and HI82) were least infectious. Variation in the proportion infectious nematodes between batches of the same isolate was of the same order or larger than the variation among isolates. Storage temperature and the duration of storage are known to be involved (e.g. Fan

and Hominick, 1991b). When the production and storage factors responsible for this variation could be identified, it may be possible to optimise the marketed nematodes.

Steinernematid isolates seem to be less variable than heterorhabditid isolates in the proportion infectious nematodes. For example, usually 30 - 50% is infectious in the presence of *G. mellonella* at 15 - 20 °C (e.g. Table 4.2; Fan and Hominick, 1991a; Hague *et al.*, 1991; Schirocki and Hague, 1994; Bohan and Hominick, 1996). Under the same conditions, 1 - 90% is infectious among heterorhabditids (Tables 4.1, 4.2; Fan and Hominick, 1991a). In general, a higher proportion of the steinernematids is infectious at low temperatures compared to heterorhabditids (e.g. Table 4.2; Hague *et al.*, 1991; Bohan and Hominick, 1996; Mráček *et al.*, 1997). This could explain why steinernematids tend to be more effective at low temperatures than heterorhabditids (e.g. Molyneux, 1986). However, this will be of little use for control of the black vine weevil, because the proportion infectious nematodes in the presence of *O. sulcatus* at 9 °C was just as low for steinernematids as for heterorhabditids (Table 4.2). *Steinernema* is therefore not an alternative to *Heterorhabditis* for control of *O. sulcatus* at low temperatures.

Temperature and host species both influenced the relative penetration rate. The influence of temperature was expected. Usually, 9 °C is within the range of non-lethal temperatures at which nematode activity is inhibited (e.g. Wallace, 1963). The influence of host species can be explained when recognizing that the insect species differed in many aspects that could influence the ease with which the nematodes can penetrate, e.g. size, activity, size and shape of natural openings, thickness of the cuticle. So far, the relative penetration rate, *B*, was estimated for the NW European heterorhabditid HF85 only. There was no variation between batches of the same isolate in the relative penetration rate in spite of variation in the proportion infectious nematodes (Table 4.1). In this study, differences between isolates in the relative penetration rate were not investigated. The relative penetration rate from this study cannot be compared with infection rates from other studies (e.g. Bohan and Hominick, 1995a; Mráček *et al.*, 1997), because different models have been used. If isolates differ in the speed at which they penetrate, it may be possible to select an isolate with a higher relative penetration rate for penetration into *O. sulcatus* at low temperatures.

Chapter 5

Aggregation of insect parasitic nematodes, *Heterorhabditis* spp. and *Steinernema* spp., among host insects at 9 and 20 °C

Abstract The distribution of penetrated nematodes among target insects is an important factor for control success, because it determines which proportion of the insects is infected and which not. Aggregation of nematodes increases the infection chance of already infected insects and decreases the infection chance of uninfected insects. The negative binomial distribution (NBD) was used to describe the distribution of nematodes in *Otiorhynchus sulcatus*, *Galleria mellonella* and *Spodoptera exigua* at 9 and 20 °C. The NBD is characterized by the mean and the parameter k, which describes the degree of aggregation. A density dependent k was estimated. Nematode distribution was close to random in the lepidopteran larvae, *G. mellonella* and *S. exigua* at 20 °C, but aggregated in the black vine weevil, *O. sulcatus*, especially at 9 °C. Consequently, more nematodes will have to penetrate *O. sulcatus* at 9 °C than *G. mellonella* or *S. exigua* at 20 °C to cause the same proportion mortality at the same mean number of nematodes per host, assuming that a single penetrated nematode is sufficient to kill the insect. The relevance of aggregation of nematodes for control *O. sulcatus* is discussed.

Introduction

In most temperate regions in the world, the black vine weevil, *Otiorhynchus sulcatus* F., is an increasingly serious pest of nursery stock, ornamentals and soft fruit such as strawberries, grapes and blackcurrants. The larvae feed on the root system and collar of the plants. Control of the larvae by means of the insect parasitic nematode *Heterorhabditis* Poinar is excellent at high soil temperatures, but is problematic at lower temperatures, usually below 12 - 14 °C (e.g. Rutherford *et al.*, 1987). It is not clear why control fails in this situation.

To infect an insect, the nematodes have to move towards the target insect and penetrate into the haemocoel. Inside the haemocoel, they release insect pathogenic bacteria, *Photorhabdus luminescens* Boemare, Akhurst & Mourant, that are symbiotically associated with the nematodes. The nematodes themselves are virtually non-pathogenic (Han *et al.*, 1991; Gerritsen and Smits, 1993), and the killing is mainly done by the bacterial symbiont.

The distribution of invaded nematodes among the insects in a population is an important factor for success, because it determines which proportion of the insects is infected and which not. The distribution of invaded nematodes in the insect population depends on a number of factors, such as variability in insect susceptibility and attractiveness, and

penetration behaviour of the nematodes. For instance, when nematodes penetrate independently from each other, such that each has the same chance to penetrate, and when insects are equally susceptible and attractive, the insects will be infected randomly by the nematodes. On the other hand, when nematodes do not penetrate independently from each other, e.g. when penetration chances are higher after the first nematode has penetrated (e.g. Hay and Smits, 1995), or when insects differ in attractiveness and susceptibility, some will be infected more often than others. The resulting distribution of number of nematodes per insect will be aggregated, where many insects harbour a few or no nematodes and a few insects harbour many nematodes. Aggregation increases the chance of multiple infections and decreases the chance of new infections. Aggregation is thus associated with a lower proportion infected insects.

It is unknown how many nematodes have to penetrate to kill a *O. sulcatus* larva. It depends on the pathogenicity of the bacteria and the number of bacteria per nematode. One to six bacterial cells are sufficient to kill a larva of *Galleria mellonella* (L.) (Griffin *et al.*, 1989), but the LD_{50} for *O. sulcatus* is unknown and cannot be assessed experimentally. Because each nematode usually contains several hundreds of bacteria (Jung, 1996), it is assumed that a single invading nematode will be lethal to *O. sulcatus*. Consequently, infected insects will die and only non-infected insects will survive exposure.

The purpose of this study was to determine if aggregation is involved in the parasitization of *O. sulcatus* by insect parasitic nematodes of the genus *Heterorhabditis* and the related genus *Steinernema* Travassos. If so, it might (partly) explain the difficulty of controlling *O. sulcatus* with insect parasitic nematodes. The degree of aggregation of nematodes within the insect population and its influence on penetration chance is assessed and quantified. Because low temperature is an important constraint to successful black vine weevil control, the influence of high (20 °C) and low (9 °C) temperature on the degree of aggregation is assessed. The distribution of nematodes in *O. sulcatus* after exposure to the nematodes is compared to that in the lepidopterans *G. mellonella* and *Spodoptera exigua* (Hb.), to see if the distribution of nematodes is similar in highly susceptible and less susceptible insect populations.

The negative binomial distribution (NBD) was used to address the above questions, because this relatively simple model can describe a range of distributions from random (Poisson) to clustered. The NBD is characterized by the mean and the parameter k, which describes the degree of aggregation. The value of k is often density dependent, meaning that the distribution changes from random to aggregated with increasing mean number of

nematodes per insect. Therefore a variable k value was estimated for a large number of groups of insects exposed to nematodes. The NBD was used to describe the relationship between the proportion killed insects (incidence) and the mean number of nematodes per insect.

Materials and methods

Negative binomial distribution

The proportion of insects that will escape penetration is described by the probability that an insect contains no nematodes. For the negative binomial distribution this probability is described by:

$$P_0 = \left[1 + \mu/k\right]^{-k} \tag{5.1}$$

where P_0 is the probability that an insect contains no nematodes;
μ is the mean number of nematodes per insect [nem. per insect];
k is a measure of the degree of aggregation.

Because a penetrated insect can contain 1, 2, 3 or more nematodes, the proportion penetrated, I, is given by:

$$I = \sum_{x=1}^{\infty} P_x = 1 - P_0 = 1 - \left[1 + \mu/k\right]^{-k} \tag{5.2}$$

Aggregation decreases with increasing k. A characteristic of an aggregated distribution is that the variance (σ^2) exceeds the mean (μ). If $k \to \infty$, or $1/k \to 0$, the NBD becomes equivalent to the Poisson distribution. In that case, the variance equals the mean. If all insects are equally attractive and penetrable, and if the probability of a nematode penetrating is independent of penetrations by other nematodes, then a Poisson distribution of nematodes among hosts is expected. For a Poisson distribution the proportion penetrated insects is described by:

$$I = 1 - P_0 = 1 - e^{-\mu} \tag{5.3}$$

Often the degree of aggregation is density dependent, which means that the distribution changes from random at low infection levels to aggregated at high infection levels. The parameter k will vary with μ. The parameter k is related to the sample mean m as:

$$k = m^2 / (s^2 - m) \tag{5.4}$$

The relationship between m and the sample variance s^2 can be described by an empirical relationship, called Taylor's power law (TPL):

$$s^2 = a \times m^b \tag{5.5}$$

where a and b are constants (e.g. Southwood, 1978). The parameters a and b characterize the degree of aggregation. For $a \approx 1$ and $b \approx 1$ the number of nematodes per insect is a randomly distributed, for $b > 1$ it is aggregated at high values of m, and random at low values of m (depending also on the value of a). When substituting Equation (5.5) into Equation (5.4), a density dependent k is expressed in terms of a and b:

$$k = m / \left(a \times m^{b-1} - 1\right) \tag{5.6}$$

When Equation (5.6) is substituted in Equation (5.2) a density dependent NBD is created that depends on μ, a and b.

Experimental data

Groups of 10 to 30 larvae (usually 15, 20 or 25) of either *O. sulcatus*, *G. mellonella* or *S. exigua* were exposed to one of twelve heterorhabditid isolates or one of four isolates at 9 or 20 °C. Larvae of *S. exigua* and *G. mellonella* were reared on artificial medium at 25 °C. These insect populations had been maintained in the laboratory for years. Larvae of *O. sulcatus* were reared in the greenhouse on potted strawberries and this population was regularly replenished by adults collected in the wild. Nematodes had been propagated on *G. mellonella* at 20 °C and stored in aerated water at 4 - 5 °C according to standard procedures (Chapter 2). A list of the isolates used is given in Chapter 4. Nematodes were applied to individually confined insect larvae in small petri-dishes (25 ml, Ø 5 cm) filled with 33 - 35 g moist (8% w/w) fine sterile sand (particle size: 93% between 180 - 425 µm). Nematode dose and exposure time differed between groups of insects, but within a group each insect received approximately the same number of nematodes and was exposed to the nematodes for approximately the same period of time. Exposure time ranged from 1 to 3 d at 20 °C and from 1 to 7 d at 9 °C. Nematode dose ranged from 50 to 300 nematodes per insect. After exposure, the insects were incubated at 20 °C for a total of 7 d to allow the penetrated nematodes to develop to last stage larvae or adults. Dead insects were dissected to estimate the number of nematodes per insect. Dead insects that did not show the characteristic signs associated with the presence of the bacterial symbionts of the nematodes, *P. luminescens* or

Table 5.1. The number of groups of *G. mellonella*, *O. sulcatus* and *S. exigua*, exposed to the heterorhabditid HF85 or other heterorhabditid and steinernematid isolates, at 9 or 20 °C, each groups consisting of 10 to 30 insects (usually 15, 20 or 25 insects).

		Nematode isolates		
Insect species	Temp. [°C]	HF85	Other isolates	Total
G. mellonella	9	57	73	130
	20	75	39	114
O. sulcatus	9	29	14	43
	20	26	43	69
S. exigua	9	1	15	16
	20	2	21	23
				395

Xenorhabdus Thomas and Poinar (colour, constitution) were considered to have died from natural causes. In total 395 groups of insects were exposed to nematodes (Table 5.1), involving approximately 8000 insects.

Parameter estimation

For each group of insects, the mean number of nematodes recovered per insect, m, was assessed as an estimate of μ , the variation in number of nematodes per insect, s^2, was assessed as an estimate of σ^2, and the proportion of the insects killed by the nematodes, i, was assesses as an estimate of the proportion infected insects, I, assuming that a single invading nematode is lethal. The proportion insects that survived, or died from natural causes, was used as an estimate of the proportion uninfected insects, $1-I$. Group size (n) was known for each group. Consequently, 395 sets of data were available, each containing an estimate of μ, σ^2 and I.

The distribution of penetrated nematodes among insects was not assessed for each of the 395 groups of insects separately, but for a series of groups involving the same insect host, (*O. sulcatus*, *G. mellonella* or *S. exigua*), and the same temperature (9 or 20 °C). Nematodes used belonged to sixteen different isolates. Data on all isolates were pooled. Data for the NW European heterorhabditid HF85 were also treated separately, because half the data sets involved exposure to this isolate (Table 5.1). In case of *S. exigua*, data on all isolates were pooled. HF85 was not compared to the other isolates with respect to aggregation, because the category 'other' consisted of a variable set in both number of background of the nematodes.

The parameter k of the NBD was estimated for each of twelve series of groups (Table 5.1). Per series, the parameters a and b of Taylor's power law were assessed from the observed m and s^2, using Equation (5.5) after taking the natural logarithm:

$$\ln s^2 = \ln a + b \times \ln m \tag{5.7}$$

by linear regression of ln s^2 on ln m. The distribution of the number of nematodes per insect was assumed random, if a and especially b were not significantly different from 1 (t-test, $\alpha = 0.05$). Differences between the twelve series was tested using pairwise t-tests.

Test for goodness of fit of observed data to the Poisson or Negative binomial distribution

For each of the twelve series of data involving different treatments, the NBD with density dependent k, based on m and s^2, and the Poisson distribution, based on m, were used to predict the relationship between the proportion penetrated insects, I, and μ. The observed relationship between i and m were compared to the predicted relationships between I and μ (generalized linear regression analysis; binomial distribution; Genstat). The goodness of fit of the observations to the predicted relationships was evaluated on the basis of residual deviance (= log likelyhood ratio between fitted and satured model that explains all variation in the data, see Genstat 5 reference manual, 1988). Formally, the relationship between the observed i (estimate of I) and m (estimate of μ) cannot be tested for significance, because both i and m were estimated and thus subject to variation. For regression analysis the explanatory variable has to be fixed and known. Therefore, m was assumed fixed and known. When the deviance is small, i.e. $\leq \chi^2_{df}(0.05)$, with $df = n - 1$ for Poisson and $df = n - 2$ for NBD with density dependent k, the observations fit the probability model. In case of similar deviances for Poisson and NBD, the Poisson distribution is considered appropriate to describe the relationship between i and m. The above method to estimate the goodness of fit could not be used for *S. exigua* because infection was virtually always 100% ($i = 1$).

Results

The observed proportion penetrated insects, i, is plotted against the observed mean number of nematodes per insect, m, in Figure 5.1. The number of observations on *O. sulcatus* at 9 °C was low (43) in comparison to *G. mellonella* (130), and they were scattered (Figure 5.1D).

The estimated a and b of Taylor's power law for each treatment of isolate, host, and temperature are presented in Table 5.2. The relationship between the observed variance (ln s^2)

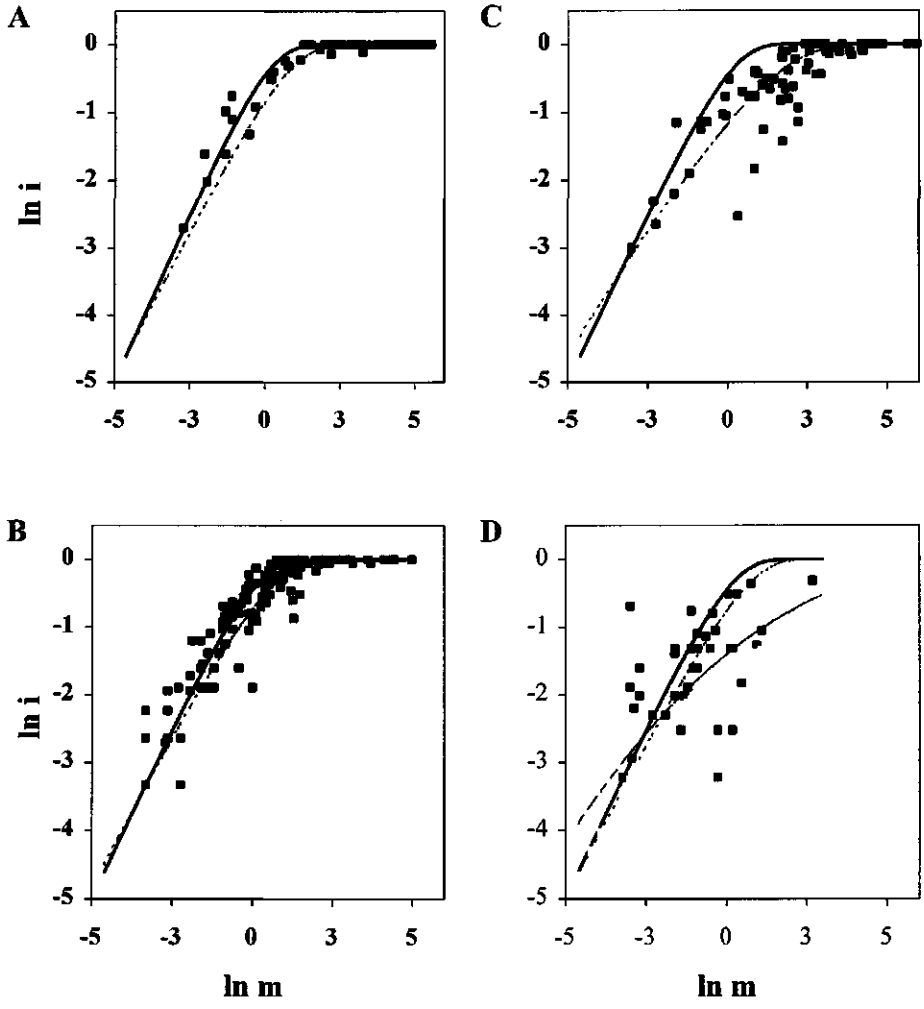

Figure 5.1. The relationship between the proportion killed insects, *i*, and the mean number of nematodes per larva, *m*, for nematodes applied to *G. mellonella* (A, B) and *O. sulcatus* (C, D), at 20 (A, C) and 9 °C (B, D). Observations are compared to the Poisson distribution (———), and the NBD with density dependent *k* (- - - -). For *O. sulcatus* at 9 °C two lines are presented for the NBD, one for HF85 (- - - -) and one for the other isolates (— — -).

and the observed mean number of nematodes per insect (ln *m*) and the estimated regression lines (Equation (5.7)) are illustrated in Figure 5.2 for *G. mellonella* and *O. sulcatus* at 20 °C. Regression was significant in all treatments (55% < R^2_{adj} < 98%; $P < 0.005$). Estimates of *a* were significantly different from 1 ($P \leq 0.05$) and were similar for all treatments, except for *O. sulcatus* at 20 °C, where *a* was higher than in the other treatments. Estimates of *b* were

Table 5.2. Estimates of *a* and *b* (± S.E.) of Taylor's power law for the number of nematodes of the heterorhabditid HF85 or other isolates that penetrated per larva of *G. mellonella*, *O. sulcatus*, and *S. exigua* at 9 or 20 °C.(Figures in a column followed by different letters are significantly different from each other, pairwise *t*-test, $\alpha = 0.05$).

Insect species	Temp. [°C]	Nematode isolate	estimate of *a* ± se	estimate of *b* ± se
G. mellonella	9	HF85	2.4 ± 0.29 *a*	1.4 ± 0.07 *b*
		Other	2.5 ± 0.36 *a*	1.1 ± 0.06 *a*
		All	2.3 ± 0.23 *a*	1.2 ± 0.05 *a*
	20	HF85	3.0 ± 0.72 *a*	1.3 ± 0.06 *ab*
		Other	2.7 ± 0.32 *a*	1.2 ± 0.03 *a*
		All	3.1 ± 0.36 *a*	1.2 ± 0.03 *a*
O. sulcatus	9	HF85	2.5 ± 0.53 *a*	1.2 ± 0.07 *a*
		Other	9.0 ± 1.65 *b*	1.8 ± 0.11 *c*
		All	3.7 ± 0.68 *ab*	1.3 ± 0.07 *ab*
	20	HF85	5.5 ± 1.07 *b*	1.7 ± 0.08 *c*
		Other	6.9 ± 1.73 *b*	1.4 ± 0.09 *b*
		All	5.9 ± 1.01 *b*	1.5 ± 0.06 *b*
S. exigua	9	All	2.1 ± 0.35 *a*	1.4 ± 0.07 *b*
	20	All	9.9 ± 8.79 *ab*	1.0 ± 0.19 *a*

Table 5.3. Goodness of fit, measured by residual deviance for various models (Poisson distribution and NBD with density dependent *k*) for nematodes (HF85, other isolates, and all isolates) applied to *G. mellonella* (A) and *O. sulcatus* (B).

A	9 °C			20 °C		
model	HF85	Other	All	HF85	Other	All
n	57	73	130	75	39	114
Poisson	2.75	7.52	10.26	0.27	7.44	7.72
NBD	2.31	2.86	5.03	0.77	0.20	1.08
B	9 °C			20 °C		
model	HF85	Other	All	HF85	Other	All
n	29	14	43	26	43	69
Poisson	10.68	4.50	15.18	3.20	6.00	9.21
NBD	3.18	0.76	5.51	1.02	2.71	3.64

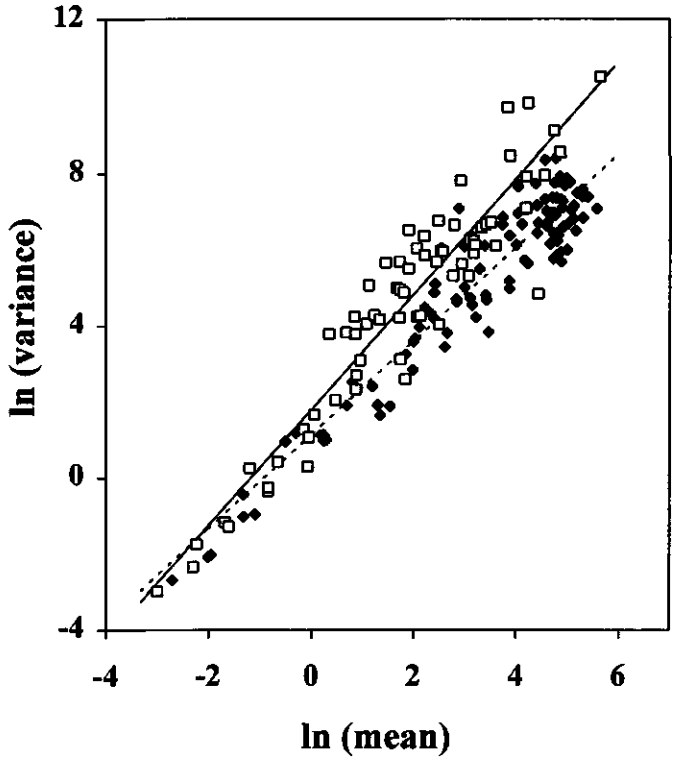

Figure 5.2. The relationship between the variance (ln s^2) and the mean number of nematodes per insect (ln m), and estimated regression line according to Taylor's power law for *G. mellonella* (◆, ...) and *O. sulcatus* (□, ———) at 20 °C.

significantly different from 1 ($P \leq 0.05$), except for *G. mellonella* at 9 °C (isolates other than HF85) and for *S. exigua* at 20 °C (all isolates). Estimates of b were higher for *O. sulcatus* (range 1.2 - 1.8) than for *G. mellonella* (range 1.1 - 1.4) and *S. exigua* (range 1.0 - 1.4), indicating that aggregation among *O. sulcatus* was greater than for the other two hosts.

The goodness of fit of the observations to the relationship between I and μ predicted by the probability distributions was high (low residual deviance), even for the data of *O. sulcatus* at 9 °C (Table 5.3). Usually the NBD with variable k gave the best description (lowest deviance). In case of HF85 applied to *G. mellonella* at 9 and 20 °C, deviances were equal for both distributions, in which case the simplest model, the Poisson distribution, is preferred. Predictions by the Poisson distribution and the NBD with variable k are included in Figure 5.1. In the case of *O. sulcatus* at 9 °C, theoretical curves were presented for HF85 and other isolates separately (Figure 5.1D).

Discussion

Available evidence (Tables 5.2, 5.3) indicates that insect parasitic nematodes are aggregated among insect hosts. The NBD with a density dependent *k* gave a good description of the observations. Aggregation was greater in *O. sulcatus* than in *G. mellonella* and S. *exigua*, and greater at low than at high temperatures. The effect of temperature was stronger for *O. sulcatus* than for the other two hosts. The number of nematodes per insect could be described by a Poisson distribution for *G. mellonella* exposed to the heterorhabditid isolate HF85 at both temperatures, indicating that *G. mellonella* was penetrated more or less randomly.

As a result of aggregation, many more nematodes will have to penetrate *O. sulcatus* at 9 °C than for instance *G. mellonella* or *S. exigua* at 20 °C to cause the same proportion infection. Because the NBD with variable *k* usually gave a comparable or better description of the relationship between *m* and *i* than the Poisson distribution, this distribution is used to estimate or extrapolate (in case of *O. sulcatus* at 9 °C) penetration chances; the mean number of invading nematodes required for 50 or 95% infection of the insect population. For *G. mellonella* these are 1 (50%) and 7 (95%) nematodes at 20 °C, and 1 (50%) and 6 (95%) nematodes at 9 °C. For *O. sulcatus* these are 3 (50%) and 28 (95%) nematodes at 20 °C, and between 1 (HF85) and 10 (other isolates) nematodes to cause 50% infection and between 6 (HF85) and 1190 (other isolates) to cause 95% infection at 9 °C. Aggregation would be less of a problem if all nematodes would penetrate. However, only a proportion of the nematodes is infectious (Chapter 4). In the presence of *G. mellonella* and *S. exigua* at 20 °C this proportion is usually high, i.e. 30 - 70%, however in the presence of *O. sulcatus* at 9 °C only about 1% of the infective stage nematodes is infectious. Consequently, between 600 to 119000 nematodes will have to be applied per *O. sulcatus* larva to obtain 95% mortality. This could explain the difficulties in practice with control of *O. sulcatus* at low temperatures.

It is unknown what causes aggregation of the nematodes in the insects. The NBD can be derived from a number of hypothetical models (e.g. Southwood, 1978; Pielou, 1969). Anderson *et al.* (1978) demonstrated that small fluctuations in hosts susceptibility could generated aggregation of parasites among hosts. Here, possible sources of variation are related to how nematodes penetrate. For example, variability in susceptibility and attractiveness of the insects may be caused by differences in cuticle thickness and hardiness (e.g. just moulted, close to moulting, small injuries), if nematodes predominantly penetrate through the cuticle (e.g. Peters and Ehlers, 1994). Differences in the size of the spiracles or in the frequency of CO_2 bursts (see Gaugler, 1988) may be involved, if nematodes penetrate

through the tracheal system. Differences in the chewing activity of the mandibles or defecation rate (e.g. Forschler and Gardner, 1991) may be involved if nematodes enter predominantly through the mouth and the intestinal wall. Similarly, differences in behaviour, size, proportion infectious nematodes, or the degree of activity of the nematodes can induce variability in the penetration behaviour of the nematodes. More information regarding the route of penetration, and insect and nematode behaviour is required to identify the causes.

Low temperature enhanced the level of aggregation, indicating that variability in host susceptibility or nematode behaviour increased. Maybe low temperature reduced the frequency of CO_2 bursts from the spiracles, as a result of lower metabolic rate, or it slowed down the hardening of the cuticle after moulting. This variability increasing effect of low temperature suggests that other factors in the soil, such as host plant, soil type, humidity, insect population density, or other insect pathogens, could similarly increase or decrease the level of aggregation, depending on their influence on insects and nematodes. Hence, more research is required to investigate if the estimated parameters are constants or variable depending on the outcome of biological interactions.

Differences in the degree of aggregation between insect species may be related to culturing conditions (see Materials and methods) and natural habitat. Larvae of *O. sulcatus* live and pupate in the soil, where they can naturally contact nematodes. It is likely that this insect has developed some resistance or avoidance to penetration and that it will be more variable in susceptibility than *G. mellonella* and *S. exigua* that do not naturally contact nematodes. The first species lives in beehives, the second lives on above ground parts of the plant.

Not included in the degree of aggregation measured in this study are the effects of nematode preference for certain individual insects in a population and the uneven spatial distribution of insects and nematodes. Here, a known number of nematodes was applied to individually confined insects. In the field, nematode preference for certain insects and spatial variability could result in additional aggregation. Aggregation, as estimated in this study is therefore a minimum.

Chapter 6

Exploring the scope for improving biocontrol of black vine weevil, *Otiorhynchus sulcatus*, with *Heterorhabditis* spp. at low temperatures; a simulation study

Abstract Control success with heterorhabditid nematodes varies with nematode species, isolate, production and storage conditions, and environmental conditions after application. These factors affect nematode behaviour. A simulation model was developed to relate control success to the underlying behavioural processes. The model simulates movement of a nematode population in space and time from the moment of application on a sand column until penetration into a host. It was used to identify, (1) which nematode traits can best be used for improvement, and (2) what is the most promising strategy of improvement: screening species or isolates, breeding, improving production and storage conditions, or controlling environmental conditions after application. The questions were addressed by quantifying the sensitivity of simulated control success to changes in nematode behavioural parameters, and relating this sensitivity to genetic or environmental variation found in nematodes. There is scope for improvement if there is both sensitivity and variability. The study was carried out for *Heterorhabditis* spp. against larvae of the black vine weevil *Otiorhynchus sulcatus* at low temperatures.

Parameters characterizing nematode movement had little influence on simulated control success. Parameters characterizing aggregation and arrestment had a large effect on control, but there is no genetic or environmental variation in *Heterorhabditis* for these traits. Parameters characterizing penetration had a moderate effect on control. The most promising option to enhance control by *Heterorhabditis* in this system would be to raise the proportion infectious nematodes of an isolate up to its genetic maximum, by improving production and storage conditions. An additional advantage of this approach would be that variation in biocontrol would be reduced, resulting in a more reliable product.

Introduction

The control of soil-inhabiting pests by insect parasitic nematodes, *Heterorhabditis* Poinar, and *Steinernema* Travassos, can be very effective. To infect an insect, the nematodes have to move towards the target insect (movement) and penetrate into the haemocoel (penetration). Inside the haemocoel, they release a symbiotically associated bacterium from the intestine. These bacteria are insect pathogenic and are mainly responsible for killing the insect host (virulence). The success of a soil application with nematodes is influenced by a combination of factors, including pest species, environmental factors (mainly temperature and moisture),

soil factors (e.g. type and soil structure), and nematode isolate (Georgis and Gaugler, 1991) and pre-treatment of the nematodes (production and storage).

The black vine weevil, *Otiorhynchus sulcatus* F., is a major pest in nursery stock and ornamentals. The larvae feed on the root system and collar of the plants. Control of the larvae with *Heterorhabditis* is excellent at moderate to high soil temperatures (15 - 20 °C) , but may fail at low soil temperatures below 12 - 14 °C, which are common in Dutch autumns when treatments may be required. It is not completely clear what behavioural bottleneck(s) (e.g. movement, penetration) hamper(s) control in this situation. The purpose of this study is to clarify this issue and to help design control options and identify strategies for screening or artificial selection of isolates that are better suited to control *O. sulcatus* at low temperatures.

Modelling is used to integrate the available knowledge of the processes underlying the nematode-host-soil system and simulate the control success. These processes include nematode migration, immobilization and remobilization, arrestment near hosts, penetration into hosts, and aggregation of nematodes among hosts (a characteristic of the frequency distribution of the number of nematodes per host). The model simulates the spatio-temporal dynamics of a nematode population from the moment of application on a sand column until penetration into the host. The model is used for a feasibility study into options for enhancing biological control, i.e. (1) to identify which nematode traits can be best used for improvement, and (2) which strategy of improvement is most promising. Potential improvement strategies are screening species or isolates, breeding, improving production and storage conditions, or controlling environmental conditions after application. The questions are addressed by quantifying the sensitivity of simulated control success to parameters characterizing nematode behavioural traits, and relating this sensitivity to available genetic or environmental variation in these parameters. There is scope for improvement if there is both sensitivity and variability. To compare favourable and unfavourable control situations, the model was parameterized for *O. sulcatus* at 9 and 20 °C and for the lepidopteran *Galleria mellonella* (L.) at 9 and 20 °C.

Materials and methods

The conceptual basis of the model is described as well as the methods used to determine the structure and process parameter values that gave the best correspondence between simulations and observations (calibration). Genetic and environmental variation in parameter values was assessed or summarized from the literature. The optimized model was used for sensitivity analysis and scenario studies.

Table 6.1. Parameters (capitals) and variables (italic) used in Figure 6.1.

Immobilization		
	immobile	number of immobile nematodes in layer 1
	mobile x	number of mobile nematodes in layer x
	I	relative immobilization rate
	EI	relative rate at which immobile nematodes resume movement
Versions for movement and host localization		
Fig. 6.1A	M_x	layer-dependent relative dispersal rates
Fig. 6.1B	*M*	relative dispersal rate
	arrested	number of nematodes arrested in layer 6
	A	relative arrestment rate
	EA	relative rate at which arrested nematodes escape arrestment
Fig. 6.1C	*DM*	relative rate for directed movement
Penetration of infectious nematodes into an insect host (Figure 6.1D)		
	penetrated	number of nematodes that penetrated the insect
	B	relative penetration rate
	C	proportion infectious nematodes in the population

Modelling concepts

Three basic processes were accounted for in the model:

(1) immobilization near the soil surface, where nematodes are initially applied;

(2) movement in soil, potentially resulting in accumulation near hosts;

(3) penetration into hosts.

Mortality of nematodes in the soil or in the insect was not considered.

The model represents a system consisting of a 9 cm high sand column with (optional) an insect host (*O. sulcatus* or *G. mellonella*) at the bottom. Nematodes are applied at the top of the cylinder. The numbers of infectious nematodes in six soil layers (1.5 cm) are used as state variables characterizing the change of the system in time. Non-infectious nematodes are accounted for in a separate array of state variables (see below). There are state variables for temporarily immobilized nematodes in the top layer and for arrested nematodes in the layer containing a host insect (Figure 6.1). An explanation of the symbols is listed in Table 6.1. The model was programmed in the simulation language FST (Rappoldt and Van Kraalingen, 1996).

Immobilization. Immobilization in the top layer (state variable IMMOBILE) was governed by a relative rate of immobilization, *I*. Immobility seems to be a temporary state and can be influenced by external factors. It was, therefore, assumed reversible. Remobilization was

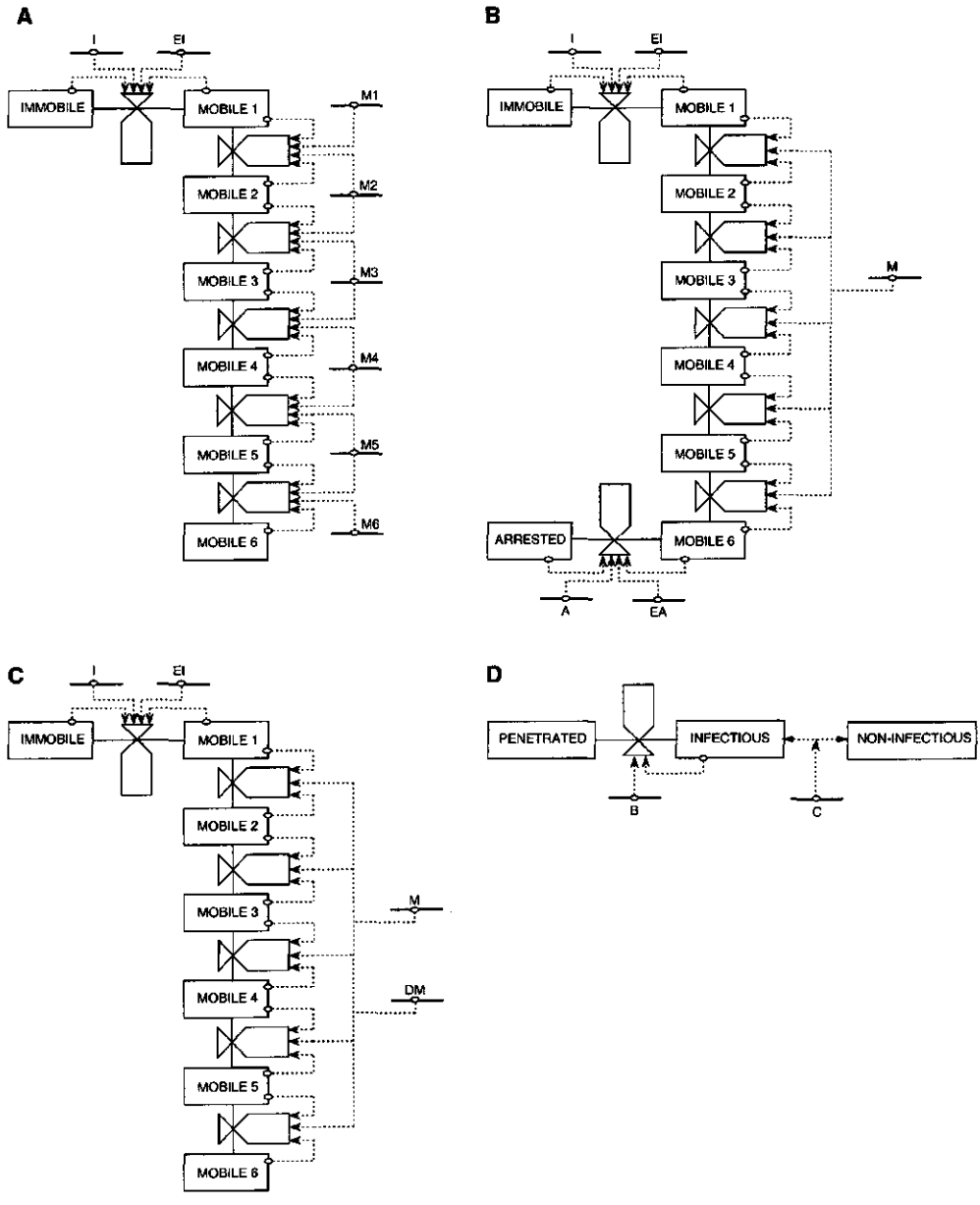

Figure 6.1. Flow diagrams for models for immobilization, movement and host localization of heterorhabditid nematodes to an attractive host (A to C), and for penetration into an insect host (D). For explanation see text and Table 6.1.

determined by a relative rate of remobilization, *EI*. The number of nematodes immobilized per unit time equals the product of *I* and the number of nematodes in the layer. The same applies *mutatis mutandis* to the number of nematodes that were remobilized per unit time. The interpretation of the rate parameters *I* and *EI* is facilitated by recognizing that in a single layered system, the proportion immobilized nematodes would converge to an equilibrium value of $I/(I + EI)$. The parameters *I* and *EI* were determined by calibration (see below).

Nematode movement. Mobile nematodes (MOBILE 1 to 6) disperse in the sand column. Dispersal was characterized by the proportion of nematodes in a sand layer dispersing to an adjacent layer per unit time. This proportion was called the relative rate of dispersal, *M*, and equals half the inverse of the average residence time per sand layer. (This representation is equivalent to diffusion with a diffusion coefficient of $M \times L^2$, where *L* equals the layer thickness of 1.5 cm). The magnitude of *M* with respect to *EI* and *I* determines the importance of movement in relation to immobilization.

Approaches to modelling host localization. Above concept for dispersal is incomplete when there are hosts in the system. The presence of an attractive host (*G. mellonella* or the lepidopteran *Agrotis segetum* Schiff.) stimulates movement, causing a lower proportion of immobile nematodes. Moreover, it causes nematodes to accumulate near hosts in due course (e.g. Chapters 2, 3). Stimulation and arrestment are related to host cue-induced changes in the speed of movement ((chemo-)orthokinese) and the rate of turning of the nematodes ((chemo)-klinokinese) (Kennedy, 1978; Hunneke *et al.*, 1994). Directed movement of individuals towards chemical cues excreted by the host (chemotaxis) can also result in an increased rate of host localization (e.g. Lei *et al.*, 1992; Lewis *et al.*, 1993). Because it is not clear which of these behavioural phenomena best describe the actual movement behaviour in the present system, three versions of the model were compared. In the first model (Figure 6.1A), a layer-dependent mobility rate M_x was used to allow a gradual slowing down of movement near the insect, either with or without a separate state variable for immobile nematodes. In the second model version (Figure 6.1B), a state variable (ARRESTED) was introduced to represent nematodes arrested in the direct neighbourhood of hosts, due to increased turning rate or complete stopping of movement. The parameters *A* and *EA* controlled the rates at which nematodes were arrested or escaped from arrestment. When $A = 0$, there is no arrestment, when $EA = 0$, arrestment is complete. The value of the ratio $A/(A + EA)$ characterizes the equilibrium fraction of arrested nematodes. In the third model version (Figure 6.1C), directed movement was simulated by introducing an extra parameter for directed movement, *DM*, that accounted for an extra downward flow of nematodes caused by the host or by gravity, in

addition to the downward component of random movement. The value of *DM* was determined by calibration (see below). Since *O. sulcatus* is not attractive (Westerman and Godthelp, 1990), movement in the presence of this host was assumed identical to movement in columns without an insect. For all three versions of the model, the values of *M* were determined by calibration (see below).

Penetration into hosts. Penetration is controlled by two parameters: the relative penetration rate, *B*, and the proportion infectious nematodes in the population, *C* (Chapter 4). The rate of penetration is the product of *B* and the number of infectious nematodes in a layer. The proportion infectious, i.e. those nematodes that are able to infect (Bohan and Hominick, 1996), can change over a period of months (Fan and Hominick, 1991b), but such changes were ignored in the model. Here, *C* is a constant. Once inside a host, the nematodes become parasitic and do not return to the sand environment. The insects act as a sink (Figure 6.1D). The parameters *B* and *C* were estimated in petri-dish experiments (Chapter 4). Non-infectious individuals were assumed to behave in exactly the same way as infectious nematodes, except that they do not penetrate. They were included in the model because in experiments no distinction can be made between infectious and non-infectious nematodes in the soil. Immobilization, movement and arrestment of infectious and non-infectious nematodes were modelled in parallel. State variables for mobile, immobile and arrested nematodes were represented in duplo for this purpose, and all processes were calculated for both groups separately.

Model choice, calibration and parameter estimation

Parameter values and model structure with respect to immobilization, movement and host localization were determined by calibration on data from experiments. In a set of experiments, movement of nematodes in 9 cm high sand columns (4.5 cm diam.) at 20 °C was measured with insect hosts (*G. mellonella* or *A. segetum*) at the bottom. Approximately 2000 nematodes were applied on top of the sand column and the percentage recovered nematodes in each of six, 1.5 cm high rings was assessed at four time intervals after application (45 - 360 min.), as described in Chapter 2. Two sand columns were evaluated at each time; one with and one without a host. Four time series were done with the NW European heterorhabditid HNh2'87 and four others with HNb87. The nematodes were used within 35 d of extraction. Culture and storage method of the nematodes are described in Chapter 2.

Optimization of model structure. Optimization of model structure for movement in the presence of an attractive host was done simultaneously with parameter calibration using the

simulation and calibration software package SENECA (see below). The criteria for accepting a model version and parameter values were goodness of fit of the model, the number of parameters, and stability of parameter estimates in different calibrations using the same data. In case of comparable goodness of fit, the model version with the fewest parameters was chosen.

Parameter calibration. The model was calibrated for nematode movement in sand columns with and without an attractive host. Calibration of parameters was done using the software package SENECA 2.0 (De Hoop *et al.*, 1992). SENECA uses Controlled Random Search (Price, 1979) for finding best parameter sets. For each parameter, 50 values were randomly drawn from an uniform distribution within a plausible range. Minimum values in this range were set to zero while the largest estimates found in the literature were used as maxima. The highest value for M at 20 °C was roughly estimated at 0.013 [1/min.], based on the fact that the fastest nematodes in Chapter 2 moved through 6 sand layers in 4 hours (M = 0.5 × 1/residence time per layer). A wider range (usually 0 - 0.5 or 0 - 1.0) was used for calibration of M_1 to M_6, M and DM. Because values for I and EI and for A and EA are interdependent and dependent on the values for the dispersal rate, initial maximum ranges were arbitrarily set at 0.5, and adapted when this seemed necessary, e.g. when one of the parameters approached the maximum of the range. Similarly, the maximum value for M at 9 °C, was roughly estimated at 0.18 [1/h.], but a wider range (usually 0 - 0.5 or 0 - 1.0) was used for calibration. Goodness of fit for the number of nematodes in a layer (GOF) was here defined as the average of the residuals between model result and observed data (see De Hoop *et al.*, 1992). Here, nematode counts in six soil layers at four time intervals in each experimental series was used as GOF criteria. For GOF = 0, the model results fit perfectly to the observed data. The Euler integration method with adaptive step size was used. Time step was defined in minutes for the model at 20 °C and in hours for the model at 9 °C. As a rule of thumb, the maximum time step (Δt) was chosen as one-tenth of the smallest residence time, i.e. 0.1 [min] for the model at 20 and 0.1 [h] for the model at 9 °C. The maximum absolute value of the relative change in a state variable during one time step (e.g. d(MOBILE1)/MOBILE1 × Δt) was set at 5%.

Genetic and environmental variation in parameters characterizing immobilization, movement and host localization. Because pre-treatment of the above batches had been different, causing different movement patterns, the eight above series were calibrated separately and used to estimate environmental variation. Data from previous studies (Chapters 2, 3) on movement in time of a number of heterorhabditid isolates, with pooled data for layer 2 to 5, were used to estimate genetic variation in immobilization, movement and host localization at 9 and 20 °C.

Sensitivity analysis

The sensitivity of the model was tested for changes of 50% in the parameters controlling immobilization, movement, and penetration into *G. mellonella* or *O. sulcatus* at 9 °C, starting from parameter values for a single isolate (isolate HF85, batch 1; Chapter 4). In addition, the effect of arrestment was tested. In case of *G. mellonella* the proportion arrested nematodes ($A/(EA + A)$) was changed from approximately 1 (arrestment complete) to 0.66 ($A = 2 \times EA$), in case of *O. sulcatus* it was changed from an assumed absence of arrestment $A/(EA + A) \rightarrow 0$ to 0.66. The effect of the changes on the number of penetrated nematodes was evaluated after a simulation period of 6 weeks.

Scenario studies

Scenario studies were carried out to assess 1) the effect of host location and sand volume on the number of penetrating nematodes in the course of time, and 2) the effect of differences in behaviour of nematode isolates on the dose that has to be applied on top of a sand column to kill a single insect at the bottom.

The effect of host location and sand volume. Penetration in the course of time in a sand column (six layer system) was compared to that in a small sand petri-dish (single layer system). The effect of the position of the insect in the sand column (layer 1 to layer 6) on penetration success were examined. The effects were tested in case of *G. mellonella* and *O. sulcatus* as a host, at 9 and 20 °C. Parameter values for the heterorhabditid HF85 (batch 1; Chapter 4) were used in these simulations.

Effect of genetic and environmental variation. Four isolates, representing two taxonomic groups (HF85 and UK211, NW European group; B1 and HP88, *H. bacteriophora*), were selected for this scenario study because they exhibit different movement behaviour, response to attractive hosts and penetration. Parameter values and variation in these values, in as far as known, are summarized in Table 6.2. Parameter values for mobility of HP88 at 9 °C were also used for B1, for which estimates were not available. Parameter ranges were used in case of the proportion infectious C for UK211 and HF85, because multiple estimates were available, two for UK211 and six for HF85 (Table 6.2). Parameter values for the relative penetration rate B were available for HF85 only, therefore, these were used for all four isolates.

The penetration success was assessed after a simulation period of 3 weeks at 20 °C and 6 weeks at 9 °C. The mean number of nematodes per insect was translated into the

Table 6.2. Parameter estimates for the NW European *Heterorhabditis* sp. HF85 and UK211, and for *H. bacteriophora* HP88 and B1, in the presence of *G. mellonella* (*G.m.*) or *O. sulcatus* (*O.s.*) at 9 or 20 °C, used to calculate the dose that has to be applied to kill an insect at the bottom of a sand column (see Scenario studies).

isolate	insect	temp. [°C]	immobilization $\frac{I}{EI+I}$	movement $M \times 10^{-3}$ [1/min]	arrestment $\frac{A}{EA+A}$	penetration $B \times 10^{-5}$ [1/min]	% infectious C
HF85	*G.m.*	20	0.14	96.4	1	131.0	22.3 - 87.6*
	G.m.	9	0.45	2.2	1	19.7	0.7 - 40.1*
	O.s.	20	0.91	89.0	0	31.0	1.8 - 43.2*
	O.s.	9	0.64	1.1	0	1.2	0.0 - 8.4*
UK211	*G.m.*	20	0.84	63.8	1	n.a.	46.8 - 55.8$^{\nabla}$
	G.m.	9	0.07	3.2	1	n.a.	0.2 - 0.3$^{\nabla}$
	O.s.	20	0.96	98.4	0	n.a.	22.0 - 28.0$^{\nabla}$
	O.s.	9	0.36	3.2	0	n.a.	0.1 - 0.1$^{\nabla}$
HP88	*G.m.*	20	0.80	25.3	1	n.a.	36.8
	G.m.	9	0.91	0.4	1	n.a.	3.1
	O.s.	20	0.78	5.9	0	n.a.	1.4
	O.s.	9	0.99	0.5	0	n.a.	0.4
B1	*G.m.*	20	0.95	5.1	1	n.a.	1.1
	G.m.	9	n.a.	n.a.	1	n.a.	0.1
	O.s.	20	0.87	3.7	0	n.a.	0.4
	O.s.	9	n.a.	n.a.	0	n.a.	0.0

n.a. no estimate available
* range of six estimates
$^{\nabla}$ range of two estimates

proportion infected insects using knowledge of the frequency distributions of number of nematodes per host (Chapter 5). The frequency distributions of nematodes per host, after nematodes have been in direct contact with the host, were described by negative binomial distributions. The distribution is characterized by a parameter k which is a measure of the degree of aggregation of nematodes among hosts, and which appeared to be density dependent. It could be described by the stable parameters a and b (Chapter 5). Rough estimates of LD_{50} and LD_{95}, the average number of nematodes that have to penetrate per host to cause a lethal infection in 50 and 95% of the insect population, were deduced from these distributions. The dose that has to be applied on top of the sand column to kill an insect at the bottom was calculated from the penetration success and these LD_{50} and LD_{95} estimates. The lethal dose was assumed similar among isolates and, in the presence of *G. mellonella*, similar

at the two temperatures. LD_{50} and LD_{95} values used were 1 and 7 nem. per insect for *G. mellonella*, and 3 and 28 nem. per insect for *O. sulcatus*. The value of 28 nem. per insect (LD_{95} for *O. sulcatus* at 20 °C), as LD_{95} for *O. sulcatus* at 9 °C, was used for the sake of simplicity. Estimates of the LD_{95} at 9 °C in the study in Chapter 5 ranged from 6 to 1190 nem. per insect.

Results

Model structure and parameter values

Immobilization and nematode movement. The observed and simulated distribution of nematodes in a sand column in the course of time is illustrated for the heterorhabditid HNb87 in Figure 6.2A. Goodness of fit was high (GOF ≤ 0.6) for all series. There was some variation in parameter values when calibration was repeated.

Host localization. The first model version to describe host localization in columns with an attractive host at the bottom using a layer-specific mobility rate, M_1 to M_6, yielded unstable estimates, indicating that too many parameters were fitted for the available data. The second model, using a separate state variable for arrested nematodes and the parameters A and EA to control the rates at which nematodes are arrested or escape from arrestment in the layer containing the host insect, gave a good description of the process. Parameter estimates were stable and goodness of fit was high (GOF ≤ 0.5). The third model, adding an extra parameter DM for directed movement towards the host, gave a slightly better fit to the data (GOF ≤ 0.4), but estimates tended to be more variable than in the second version and the improvement in GOF was too small to warrant inclusion of this extra parameter in the model. Therefore, the second model version was chosen to describe the process of host localization. Observed and simulated movement according to the second model in the presence of an attractive host in the course of time is illustrated for the heterorhabditid HNb87 in Figure 6.2B. Arrestment was complete; the estimated parameter A was so high in comparison to EA that virtually no nematodes escaped from the bottom layer once they were in ($A/(EA + A) > 0.995$).

Genetic and environmental variation in parameters characterizing immobilization, movement and host localization. In the absence of a host, estimates of M ranged from 0.00274 to 0.00795 [1/min] for the heterorhabditid HNh2'86 and from 0.00465 to 0.00999 [1/min] for the heterorhabditid HNb87. The proportion immobilized nematodes, $I/(EI + I)$, for HNh2'86 ranged from 0.28 to 0.74. The proportion immobilized nematodes for HNb87 ranged from 0.21 to 0.73. In the presence of *G. mellonella,* estimates of M were a factor 10 higher than in

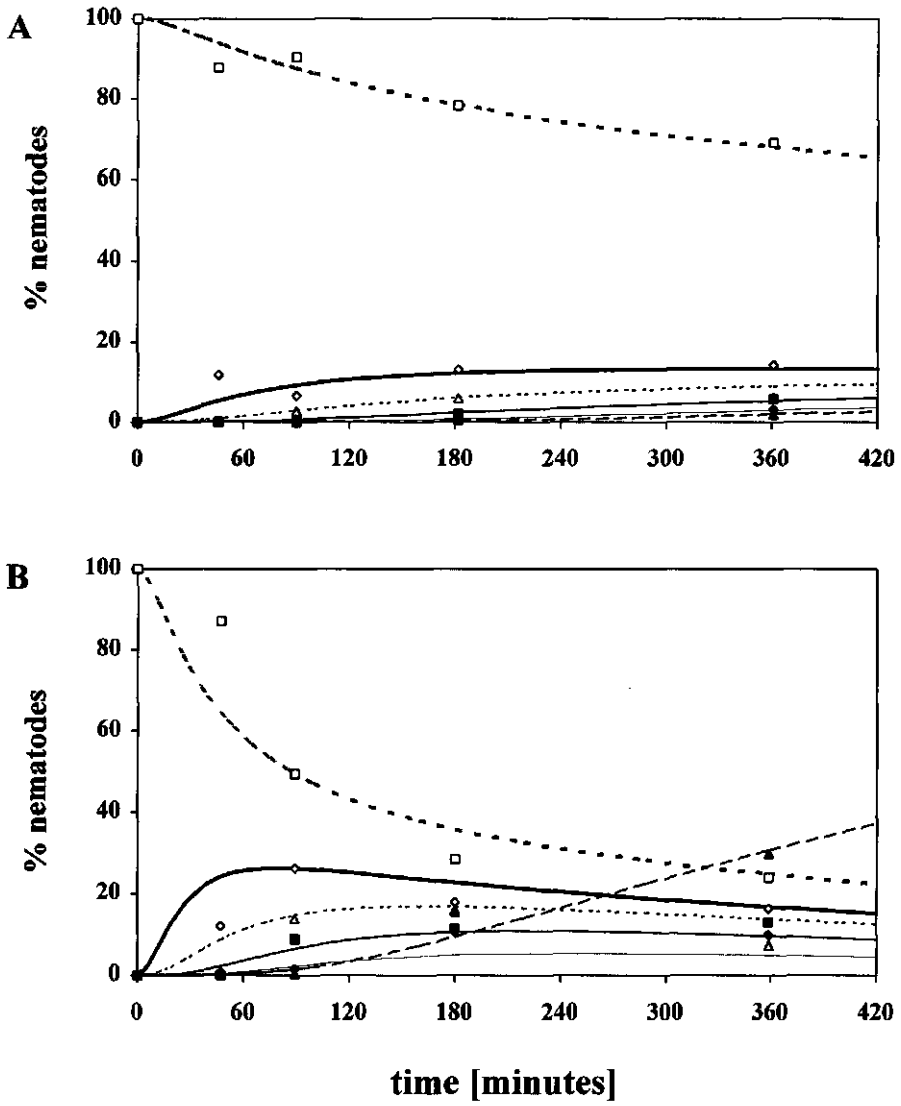

Figure 6.2. Observed (points) and simulated (lines) distribution of heterorhabditid nematodes (HNb87) over six, 1.5 cm thick sand layers in a 9 cm long vertical sand column in the course of time, in the absence (A) or presence (B) of an attractive host, after application of 100 nematodes on top of the column at time $t = 0$. GOF = 0.2 and 0.3, respectively. Layer 1 (□, - - -); layer 2 (◊, ———); layer 3 (Δ, ·····); layer 4 (■, ———); layer 5 (◆, ———) and layer 6 (▲, — — •).

the absence of this host and ranged from 0.0145 to 0.0427 [1/min] for HNh2'87 and from 0.0227 to 0.0939 [1/min] for HNb87. The proportion immobilized nematodes, $I/(EI + I)$, ranged from 0.44 to 0.65 for HNh2'86 and from 0.15 to 0.25 for HNb87. Parameter estimates

Table 6.3. Estimated relative dispersal rate, M ($\times 10^{-3}$ [1/min]), and proportion immobilized nematodes $I/(EI + I)$, in cylinders with or without a *G. mellonella* at the bottom, for 14 heterorhabditid and one steinernematid isolate at 20 °C and for ten heterorhabditid nematodes at 9 °C.

	20 °C				9 °C			
	without host		*G. mellonella*		without host		*G. mellonella*	
isolate	M	I / $EI+I$	M	I / $EI+I$	M	I / $EI+I$	M	I / $EI+I$
NZH3[a]	4.1	0.80	4.2	0.85	n.a.	n.a.	n.a.	n.a.
B1[b]	3.7	0.87	5.1	0.95	n.a.	n.a.	n.a.	n.a.
Hda[b]	15.0	0.88	28.5	0.83	n.a.	n.a.	n.a.	n.a.
HP88[b]	5.9	0.78	25.3	0.80	0.5	0.99	0.4	0.91
HO1[c]	28.2	0.66	39.7	0.37	n.a.	n.a.	n.a.	n.a.
K122[d]	8.9	0.89	32.0	0.80	3.5	0.65	3.9	0.16
M145[d]	51.2	0.95	31.5	0.94	0.5	0.67	0.6	0.64
HF85[e]	89.0	0.91	96.4	0.14	1.1	0.64	2.2	0.45
HL81[e]	25.4	0.28	95.4	0.07	1.3	0.64	3.8	0.56
HNb87[e]	35.4	0.58	95.9	0.06	1.3	0.64	1.0	0.57
HB1'87[e]	16.2	0.70	54.0	0.45	1.7	0.46	2.1	0.48
HE87[e]	37.7	0.84	101.8	0.78	n.a.	n.a.	n.a.	n.a.
HFr86[e]	18.4	0.52	91.8	0.11	n.a.	n.a.	n.a.	n.a.
UK211[e]	98.4	0.96	63.8	0.84	3.2	0.36	3.2	0.07
HSH[e]	n.a.	n.a.	n.a.	n.a.	2.6	0.74	1.9	0.54
Hkem[e]	n.a.	n.a.	n.a.	n.a.	5.0	0.62	2.1	0.76
OBSIII[f]	7.1	0.77	5.7	0.64	n.a.	n.a.	n.a.	n.a.

n.a. estimate not available
[a] *H. zealandica*; [b] *H. bacteriophora*; [c] *H. megidis*; [d] *Heterorhabditis* sp. (Irish group); [e] *Heterorhabditis* sp. (NW European group); [f] *S. feltiae*

for 14 heterorhabditid and one steinernematid isolate at 20 °C and ten heterorhabditid isolates at 9 °C are presented in Table 6.3 (GOF ≤ 0.5 and GOF ≤ 0.9 for 20 and 9 °C, respectively). The proportion arrested nematodes, $A/(EA + A)$, is not shown, because arrestment was complete for all 15 isolates.

Sensitivity analysis

Sensitivity analysis (Table 6.4) revealed that the model for *G. mellonella* at 9 °C was especially sensitive to changes in the proportion infectious nematodes C and a reduction in arrestment ($A/(EA + A)$: $1 \rightarrow 0.66$). It was less sensitive to changes in the relative penetration rate B, and almost insensitive to changes in parameters characterizing immobilization and dispersal. The model for *O. sulcatus* at 9 °C was sensitive to changes in all parameters: arrestment ($A/(EA + A)$: $0 \rightarrow 0.66$), the penetration parameters B and C, and, to a lesser

Table 6.4. Percentage change in the number of penetrated nematodes after 6 weeks, after a 50% change in parameter values for the heterorhabditid HF85 at 9 °C, starting from 4.10 nem. per *G. mellonella* and 0.05 nem. per *O. sulcatus* per 100 nematodes applied, at standard parameter values.

		G. mellonella			*O. sulcatus*		
input	dimension	standard value	+50%	−50%	standard value	+50%	−50%
I	[1/h.]	3.9	−0.1	0.1	5.0	−11.2	14.5
EI	[1/h.]	4.8	0.1	−0.3	2.7	9.1	−20.4
M	[1/h.]	12.9×10^{-2}	0.1	−3.1	6.7×10^{-2}	4.9	−14.3
A/(EA+A)	[h./h.]	1	i	−3.7*	0	86.6*	i
		1	i	−22.2**	0	24.0**	i
B	[1/h.]	1.2×10^{-2}	0.1	−0.9	0.1×10^{-2}	45.7	−48.5
C	[infect. nem./ total nem.]	4.1×10^{-2}	49.9	−49.9	0.7×10^{-2}	50.1	−50.1

i irrelevant
* $A = 1 \times 10^{-2}$[1/h.]; $EA = 0.5 \times 10^{-2}$[1/h.]
** $A = 1 \times 10^{-3}$[1/h.]; $EA = 0.5 \times 10^{-3}$[1/h.]

extent, the parameters characterizing immobilization and movement. If the degree of aggregation among hosts had been included in the model, the model would have been sensitive to changes in the parameters governing the degree of aggregation.

Scenario studies

The effect of sand volume and host location. The results of this first scenario study illustrated and explained the importance of the above behavioural traits, arrestment, the relative penetration rate and the proportion infectious nematodes, for control of *O. sulcatus* at 9 °C in comparison to *G. mellonella* at 20 °C. The proportion infectious nematodes, *C*, determines the final level of infection; arrestment and the relative penetration rate influence the speed of infection. Figure 6.3 shows the increase in the number of nematodes that penetrated the host in time, after application of 100 nematodes (at $t = 0$) on top of a single layer system (petri-dish) or a six layer system (sand column). The proportion infectious nematodes, *C*, determined the maximum level of infection, which was the same for the single and the six layer system. The period of time chosen for simulation was sufficient to reach the maximum level in the single layer system, but insufficient in the six layer system except for *G. mellonella* at 20 °C (Figure 6.3D). The figure clearly shows that infection was delayed in the larger volume of six layer system compared to the single layer, and that the delay was much larger in case of *O. sulcatus* than for *G. mellonella*. Arrestment in the presence of

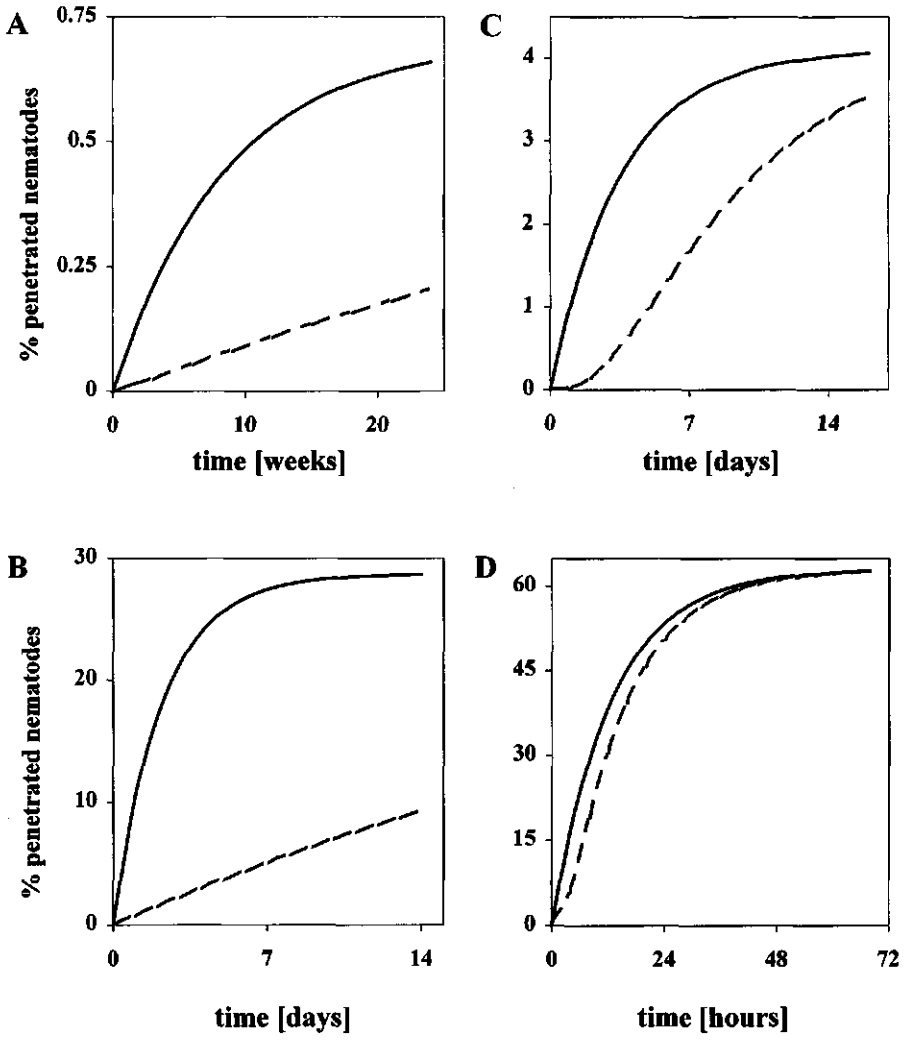

Figure 6.3. Simulated numbers of nematodes that entered the host, *O. sulcatus* (A, B) or *G. mellonella* (C, D) in the course of time at 9 °C (A, C) or 20 °C (B, D), after application of 100 nematodes of the heterorhabditid HF85 to a single (———) or a six layer system (- - -) with the insect at the bottom.

G. mellonella caused a concentration of (mobile) nematodes in the host layer, because nematodes remained there once they had entered it. Nematodes in the host layer were -as it were- queuing up to penetrate the host. A larger sand volume (six versus one layer system) caused a delay in infection, because the nematodes needed more time to reach the host layer.

Changing the position of *G. mellonella* from layer 6 to 1 of the higher sand layers again reduced the time necessary to reach the host layer, to the level of the single layer system. Arrestment does not operate in the presence of *O. sulcatus*. Mobile nematodes continuously dispersed into and out of the host layer. Penetration removed a part of these transients, and this explains the importance of the value of the relative penetration rate, *B*, in case of *O. sulcatus*. The larger sand volume of the six layer system caused that the nematodes were more 'diluted' and fewer transients were present in the host layer. Host position had no effect on the number of nematodes in the host layer, and thus no effect on the speed of infection, unless *O. sulcatus* was placed in the top layer, where there is a reservoir of immobilized nematodes. *O. sulcatus* does not stimulate nematodes to become mobile and, therefore, most nematodes reside in the top layer. A proportion *C* of these were capable of penetration. With *O. sulcatus* in the top layer, the course of infection resembled that of a single layer system, but was not identical to it.

The effect of genetic and environmental variation. The results of this second scenario study illustrate the effect of variation among and within nematode isolates on control success, as measured by the dose necessary to kill a single insect at the bottom of a sand column. The estimated nematode dose (per insect) that has to be applied on top of a sand column to kill 50 or 95% of the insect population is summarized in Table 6.5. In case of *G. mellonella* as a host, the value of *C*, the proportion infectious nematodes, is most important (see sensitivity analysis) and the volume of the sand column causes only a small delay in infection (see above scenario study). The values of *C* were high for HF85, UK211 and HP88 at 20 °C, and for HF85 and UK211 at 9 °C. All infectious nematodes of these isolates will enter *G. mellonella* in a sand column within the simulation period. In case of *O. sulcatus* as a host, all parameter values are important (see sensitivity analysis), although the penetration parameters, *C* and *B*, are most important, and the sand column causes a serious delay in infection (see above scenario study). Values for *C* were high for HF85 and HUK211 at 20 °C. The influence of *B* could not be illustrated, because values for *B* were assumed similar for all isolates. Only in one instance a low dose was required to kill *O. sulcatus* at 9 °C; 480 nem. per insect for killing 50 % and 4400 nem. per insect for killing 95% of the *O. sulcatus* larvae. It was caused by an exceptionally high 8.4% infectious nematodes for HF85 under these conditions. It illustrates the influence of environmental variation in the proportion infectious nematodes on model outcome.

Table 6.5. Estimated dose (nem. per insect) of four heterorhabditid isolates that has to be applied on top of a sand column to kill 50 (A) or 95% (B) of the host population.

A	Dose [nem. per insect] required to kill 50% of			
host	*G. mellonella*		*O. sulcatus*	
temp. [°C]	20	9	20	9
HF85†	2 - 4	3 - 140	16 - 380	480 - 4×10^5
UK211‡	2	330 - 500	41 - 53	3.1×10^4
HP88	3	120	380	4.0×10^4
B1	120	3900	1800	3.8×10^6
B	Dose [nem. per insect] required to kill 95% of			
host	*G. mellonella*		*O. sulcatus*	
temp. [°C]	20	9	20	9
HF85†	8 - 31	18 - 1000	150 - 3500	4400 - 3.7×10^6
UK211‡	12 - 15	2300 - 3500	380 - 490	2.9×10^5
HP88	19	870	3500	3.8×10^5
B1	780	2.7×10^4	1.6×10^4	3.5×10^7

† range caused by six estimates for C ; see Table 6.2
‡ range caused by two estimates for C ; see Table 6.2

Discussion

A model was developed that simulates the dynamics of a heterorhabditid nematode population in space and time from the moment of application on a sand column until penetration into the host. It is based on the available knowledge of the behavioural processes underlyingbiocontrol, i.e. movement, immobilization and remobilization, arrestment near the host, and penetration into the host. In order to be able to incorporate the processes of immobilization, movement and host localization in the model, these processes had to be quantified first. Calibration was used to estimate parameter values and to find the model that gives the best description of the observations based upon available assays. Because all relevant processes of biocontrol were integrated in one model, it was possible to study the biocontrol process quantitatively as a whole. This would not have been possible otherwise. In addition to the quantitative information, the model provided a conceptual framework to improve understanding of the insect-nematode-soil system, and to identify bottlenecks in biocontrol under particular circumstances. The model does not explain how or why the nematodes behaved the way they behaved, e.g. immobilized after application, accumulated near an attractive host, or were non-infectious. This would require modelling of nematode

behaviour at individual or lower levels.

Here, the model was used to explore the scope for improvement of control of *O. sulcatus* by *Heterorhabditis* spp. at low temperatures. Parameter values characterizing the *O. sulcatus-Heterorhabditis*-sand system were used as input in the model to understand and explain in quantitative sense why heterorhabditids fail to control *O. sulcatus* at low temperatures and to identify behavioural bottlenecks. The question remains in which way these bottlenecks can best be alleviated. This question will be addressed by relating sensitivity in model parameters to available 'nature' and 'nurture' based variation. The summary of available information on sensitivity and variation in Table 6.6 shows that three categories of behavioural traits could be distinguished:

1. Traits with influential but invariable parameters. The model was most sensitive to changes in parameters characterizing the degree of aggregation of nematodes among hosts and the presence or absence of arrestment. Aggregation and arrestment do not seem to vary among or within *Heterorhabditis* spp. but among insect hosts. For a given pest situation, aggregation and arrestment are given facts that set bounds to control possibilities by heterorhabditid nematodes. They can only be manipulated by choosing other insect species or by influencing health and condition of the insects.
2. Traits with influential and variable parameters. The next most influential parameters were those characterizing penetration, i.e. the proportion infectious nematodes and the relative penetration rate. The model was only sensitive to the relative penetration rate in case of *O. sulcatus* (where there is no arrestment). It is unknown if there is variation between isolates in the relative penetration rate. In case of *O. sulcatus* at low temperatures, the proportion infectious nematodes was variable within nematode isolates.
3. Traits with variable parameters that have little influence on model outcome. This category includes movement in the soil and the process of immobilization and remobilization of nematodes after application to the soils surface. Parameters characterizing these processes were variable between and within isolates, but had little influence on model outcome.

Behavioural traits of category 1 and 2 have a large influence on model outcome, as pointed out by sensitivity analysis, and are, therefore, most important to control success. Of these, only the traits of category 2 show variation in parameter values and are, therefore, eligible to improvement. Behavioural traits of category 3 show variation in parameter values, however, improving these traits would have little influence on control success.

Table 6.6. Summary of available information on sensitivity of behavioural traits, the level of genetic and environmental variation in these traits, and the anticipated effect of genetic selection and optimization of production and storage methods on the improvement of the biological control of *O. sulcatus* by *Heterorhabditis* spp. at low temperatures.

			Source of variation				Effect of	
behaviour	para-meters	sensitivity to 50% change	genetic	reference	environmen-tal	reference	selective breeding	production and sto-rage
% immobile	*EI/I*	low	intermediate-high	Table 6.3	intermediate	Results 20 °C	low	low
movement	*M*	low	high	Table 6.2	intermediate	Results 20 °C; Chapter 3	low	low
% arrested	*EA/A*	low-high	none	Table 6.2	none	Results 20 °C	low	none
penetration	*B*	intermediate	unknown	---	none	Chapter 4	unknown	none
% infectious	*C*	intermediate	low	Chapter 4	high	Chapter 4	low	high
aggregation	*a, b*	high	none	Chapter 5	none	Chapter 5	none	none

high ≈ 70-100%
intermediate ≈ 35-70%
low ≈ 0-35%

Traits with the largest potential for improvement of control of *O. sulcatus* at 9 °C are the proportion infectious nematodes, *C*, which shows environmentally induced variation, and the relative penetration rate, *B*, which shows genetic variation. The relative penetration rate may be improved by field collection of new isolates, selection and breeding, if there would be genetic variation for this trait (which is unknown). Heritability of *B* would be high, because so far no environmentally induced variation was found for this trait.

The non-genetic variation in *C* is probably induced during pre-treatment of the nematodes, so during production and storage. Production and storage (conditions, duration, methods) have a large influence on many aspects of the behaviour (e.g. Westerman, 1992; Fan and Hominick, 1991b). At this stage, it is unknown how these should be changed such that the proportion infectious nematodes is enhanced up to its genetic maximum. An additional advantage of controlling production and storage conditions would be that variation in the performance of the nematodes would be reduced, resulting in a more reliable product.

Selective breeding to enhance movement of the nematodes is not an option for improvement of control of *O. sulcatus* by *Heterorhabditis* at low temperatures. Gaugler and co-workers reached the same conclusions with respect to control of the scarabs *Popillia japonica* and *Maladera matrida* by the insect parasitic nematode *Steinernema carpocapsae.* They enhanced the response of *S. carpocapsae* to scarab beetle larvae on an agar surface by breeding (Gaugler and Campbell, 1991). It is not clear what they exactly selected for, a higher proportion mobile nematodes, enhanced speed of movement, enhanced arrestment, or a combination of these. However, the improved response on agar did not result in better control results against the scarabs in field and pot trials (Gaugler *et al.*, 1994).

In summary, a clear-cut solution for the poor control of *O. sulcatus* by *Heterorhabditis* spp. at low temperatures cannot be given. The best option is to optimize production and storage conditions to raise the proportion infectious nematodes of an isolate up to its genetic maximum. Hence, future research efforts to improve control of *O. sulcatus* by *Heterorhabditis* spp. at low temperatures should primarily focus on this strategy.

Chapter 7

General discussion

In this study, the possibilities to improve the inundative biological control of the black vine weevil, *Otiorhynchus sulcatus*, by means of insect parasitic nematodes, *Heterorhabditis* spp., at low temperatures were investigated. A systems analytical approach was followed, comprising:

1. experiments;
2. modelling, using the experimental data;
3. a feasibility study to explore options for improvement, using the model.

Problems and choices during experimentation

Experimentation fills the larger part of this thesis. Experiments were performed to complement existing information on nematode behaviour relevant to biocontrol, with the purpose to understand and characterize these processes quantitatively and dynamically. Information on nematode behaviour is usually fragmentary, qualitative, and static, and most information does not relate to heterorhabditid nematodes but to nematodes of the genus *Steinernema.* The behavioural processes that were studied are: movement and host attraction, penetration, and aggregation of penetrated nematodes among hosts. After a series of preliminary experiments it was decided not to study two processes; 1) the actual killing of the insect by the symbiotic bacterium *Photorhabdus luminescens* and 2) mortality of the nematodes.

Ad 1. After penetration of the insect by the nematodes, the insect is killed by septicaemia caused by the symbiotic bacterium *Photorhabdus luminescens.* Nematodes of *Heterorhabditis* spp. are virtually non-pathogenic (Han *et al.*, 1991; Gerritsen and Smits, 1993). It is unknown how many bacteria are required to kill an *O. sulcatus* larva, because it cannot be determined experimentally, as *O. sulcatus* does not survive injection. The LD_{50} for *G. mellonella* is low (LD_{50} = 1 - 6 bacteria per insect; Griffin *et al.*, 1989) and a single nematode usually contains hundreds of bacteria (Jung, 1996). It was, therefore, assumed that a single invading nematode contains sufficient bacteria to kill a *G. mellonella* or a *O. sulcatus* larva. It may not be enough to kill larvae of more resistant pest species, such as scarabs or craneflies (Wulff *et al.*, 1994). Bacterial growth rate is directly related to the speed of killing. Low temperature affects the growth rate of the bacteria, but growth is not stopped at

temperatures as low as 9 °C (unpubl. data; Clarke and Dowds, 1994). Low temperature has, therefore, no effect on the ability of the bacteria to kill the host.

Ad 2. The possibility that nematode mortality could be one of the main factors responsible for failure or success of a nematode application was put aside in this study. Nematode mortality is higher at high temperatures than at low temperatures (e.g. Jung, 1991), yet control of *O. sulcatus* is successful at high temperatures and unsuccessful at low temperatures. Mortality of nematodes was not considered to be an important factor influencing the experimental results. Experiments did not last long enough to induce substantial mortality, i.e. up to 12 days at 20 °C and up to 28 days at 9 °C (e.g. Jung, 1991). In preliminary experiments and in other studies (e.g. Epsky and Capinera, 1993; Peters and Ehlers, 1994) nematode mortality inside the host was checked indirectly by comparing the number of nematodes in the insect immediately after exposure with the number of nematodes after insect death, two to three days later. At 20 °C, virtually all nematodes that penetrate are recovered later on in a range of host species. Nematode mortality did occur when the insect cadaver was contaminated with bacteria other than the symbiont (unpubl data). In *O. sulcatus*, mortality of nematodes is usually more variable, but negligible on average. Nematode mortality inside the host may have been higher at 9 °C (e.g. Thurston and Kaya, 1994). In comparison to other factors and processes that clearly influence control success, the effect of nematode mortality was relatively unimportant and therefore ignored.

While searching for genetic variation in behavioural traits of the nematodes among isolates, variation was encountered that did not have a genetic basis. Nematodes of similar genetic constitution varied in behaviour from experiment to experiment. All aspects of behaviour were found to be variable, except the relative penetration rate. The occurrence of such environmentally induced variation was completely unexpected at first. The unstability and unpredictability of nematode behaviour complicated and slowed down research progress in this study. Much effort was put into standardising experimental and storage conditions to minimize these sources of variation. Inconsistencies in control results with heterorhabditid and steinernematid nematodes had been observed in many studies (e.g. Georgis and Gaugler, 1991) and were now related to variability in behaviour of the nematodes. 'Visual' characteristics of nematode populations were identified that were empirically related to nematode performance (Westerman and Stapel, 1992). These were used to exclude nematode batches with expected poor quality prior to experimentation. Because substantial non-genetic variation remained despite these precautions, replicates of isolates and experiments were included deliberately to assess the degree of variation. It turned out to be a valuable decision,

because the environmentally induced variation in *Heterorhabditis* provided cues to the best option for improvement of control of *O. sulcatus* at low temperatures; i.e. standardization of nematode quality.

Bioassays and modelling

The usual approach to address problems as handled in this thesis, i.e. how to improve the performance of insect parasitic nematodes for control of a soil pest, is by comparing the potential of nematode isolates in bioassays to identify more promising isolates on the basis of LD_{50} estimates, the dose applied per insect necessary to kill 50% of the insect population (e.g. Woodring and Kaya, 1988; Westerman, 1994). Bioassays originate from the testing of chemical and microbial pesticides and conclusions are based on the statistical analysis of dose-response curves. Thanks to its quantitative nature, the model was suitable to compare nematode isolates as in bioassays, but it was capable of doing more than that. Because the model was based on mechanistic integration of behavioural processes of nematodes, it was possible 1) to identify low proportion infectious nematodes as the main factor responsible for failure of control of *O. sulcatus* at low temperatures, and 2) to identify environmental variation as the only source of variation in the proportion infectious nematodes, indicating that controlling this variation is the best way to improve control of *O. sulcatus*. In laboratory bioassays, all relevant behavioural processes (e.g. movement, host localization, penetration) are usually included in the set-up, but only a resultant is measured and it is not possible to separate and quantify the contributions of each to the final result. The dose response relationships are descriptive and not suitable to identify the behavioural causes or optimal strategies. The model is, therefore, complementary to the bioassay, it provides additional insight and allows the evaluation of options for improvement.

The model developed in this study proved useful in identifying nematode behavioural traits and strategies for improvement of *Heterorhabditis* for control of *O. sulcatus* at low temperatures. In other nematode-insect-soil systems, additional factors and other behavioural traits may be bottlenecks in control success. For example, Georgis and Gaugler (1991) were able to identify factors that were most important for inundative biocontrol of the Japanese beetle, *Popillia japonica* in turfgrass, after analysing a large number of field trials. Apart from (low) temperature and choice of nematode isolate, moisture content, soil type and thickness of the thatch-layer were the main factors influencing control success. To understand how these factors influence the various behavioural traits of the nematodes, the model may be adapted or extended to identify behavioural bottlenecks and strategies for improvement in

situations other than for *O. sulcatus* at low temperatures.

Feasibility of inundative biological control with heterorhabditid nematodes

The results of the model showed that the possibilities to control insects by means of *Heterorhabditis* are best for those insect-nematode-soil systems where 1) aggregation of nematodes among hosts is low and where 2) arrestment, 3) the proportion infectious nematodes and 4) the relative penetration rate are high. In this study, these nematode behavioural characteristics were most important to model outcome in all four situations of host (*O. sulcatus* and *G. mellonella*) and temperature (9 and 20 °C). The model was initiated with parameter values that were estimated under laboratory conditions that excluded the effect of many factors that could adversely influence control results (e.g. low moisture content, other soil type). Field results may compare unfavourable with model results. The results of the model provide maxima of what can be achieved, i.e. a best case scenario.

The model illustrates why it is much easier to use *Heterorhabditis* to control highly susceptible 'pests' such as *G. mellonella* than to control more resistant pests such as *O. sulcatus*. Host species largely influence the aggregation of nematodes in hosts and (the absence or presence of) arrestment. Aggregation was low and arrestment high in the presence of *G. mellonella*, while the opposite was true in the presence of *O. sulcatus*. Because there is no variation in *Heterorhabditis* for these traits, aggregation and arrestment could be used as a first screen to assess control possibilities of potential target pests. However, arrestment and the degree of aggregation are not easily measured. Host species also influence the proportion infectious nematodes and the relative penetration rate, and maybe one of these traits could be used for a first screen instead. This study also showed that host species influence possibilities to improve control by *Heterorhabditis*. Variation in the proportion infectious nematodes in the presence of *G. mellonella* was larger than in the presence of *O. sulcatus*. Because variation in nematode behaviour is necessary for improvement, the possibilities to improve control by *Heterorhabditis* are best for those insects that induce large variation.

Possibilities to control insects by means of *Heterorhabditis* at low temperatures are poor. As expected (Griffin, 1993), low temperature affects almost every aspects of the behaviour of the nematodes; fewer nematodes are mobile and infectious, and the nematodes disperse and penetrate at a lower rate. The effects of low temperature on the degree of aggregation, the proportion infectious nematodes and the relative penetration rate are such that only few nematodes will be able to penetrate the host. Possibilities to improve control at low temperatures are slim, but will depend on target species at which control is aimed. In this

study, low temperature reduced variation in nematode behaviour, thus reducing the possibilities to improve performance at low temperatures. Control may be improved for those insect host species that induce some variation at low temperatures.

Conclusions reached in this study may apply to most of the genus *Heterorhabditis*. The isolates of *Heterorhabditis* that were used in this study show genetic variation that may be representative to other isolates within the genus; they represent different (putative) taxonomic groups and represent different geographic and climatic origin.

Prospects to improve control of *O. sulcatus* by heterorhabditid nematodes at low temperatures and directions for further research

O. sulcatus at low temperatures is on the very edge of what can be controlled by heterorhabditids, both with respect to insect species and with respect to temperature. The best option is to use the environmental variation in the proportion infectious nematodes. This variation is induced during pre-treatment of the nematodes, so during production and storage. At this stage, it is unknown how production and storage conditions should be changed such that the proportion infectious nematodes is enhanced and stabilized. The knowledge of the physiological and developmental processes that take place during production and storage are poorly understood. Circumstances during storage have only occasionally been related to nematode behaviour after storage (e.g. Fan and Hominick, 1991b; Jung, 1996; Griffin, 1997). As long as the biological mechanisms that regulate the proportion infectious nematodes in a population are not understood, the effects of changed production and storage conditions on the proportion infectious nematodes can only be evaluated empirically, so by trial and error. This is not an encouraging prospect in view of the many factors that are involved in storage and production. Future research should, therefore, be directed at finding out why nematodes are non-infectious and how this is regulated.

Now, production and storage conditions are largely controlled by commercial producers. It is possible that the optimization of production and storage aimed at enhancing the proportion infectious nematodes is not fully compatible with their aims, i.e. maximising yields and reducing losses during storage. However, if they want to extend the market for insect parasitic nematodes, for instance to outdoor crops, they will have to invest in quality control. Now, they are selling a product of which only about 1% is infectious against *O. sulcatus* at low temperatures. This may be raised to 8% as indicated in Chapter 4. An additional advantage of such quality control would be that the producers would be able to sell a more reliable product. Now, control results in the field vary as a result of the variable proportion

infectious nematodes in the product (Chapter 6). Controlling the percentage infectious nematodes would eliminate this source of variation.

An alternative to using *Heterorhabditis* under low temperatures conditions, is applying nematodes earlier in the season against younger larvae, so during late summer or early autumn, when the risks of low soil temperatures are smaller. The nematodes would have to be effective against young larvae and they would have to be very persistent, e.g. effective up to 10 weeks, to ensure control of late appearing larvae. Insect parasitic nematodes usually meet neither of these requirements (e.g. Georgis and Poinar, 1984; Klingler, 1988; Kaya, 1990; Curran, 1993). To implement this strategy, research would have to focus on improving efficacy against early instars and improving persistence. Because there is a large amount of genetic variation and probably also environmental variation in persistence in *Heterorhabditis* and *Steinernema* (e.g. Rovesti *et al.*, 1991; Curran, 1993), there are certainly possibilities to improve *Heterorhabditis* for use in this control strategy.

Samenvatting

De taxuskever, *Otiorhynchus sulcatus*, is een belangrijke plaag in sier- en boomteelt gewassen in Nederland. De larven van de kever vreten aan de wortels en wortelhals van de plant. Biologische bestrijding van de larven met behulp van insecten-parasitaire nematoden, *Heterorhabditis*, werkt uitstekend bij bodemtemperaturen boven de 12 à 13 °C. In vollegronds teelten komt de temperatuur vaak beneden deze waarden in de perioden dat bestrijding van de oudere larven plaatsvind, namelijk in het najaar (september - november) en vroege voorjaar (maart - mei). De toepassing van insecten-parasitaire nematoden voor de bestrijding van taxuskever blijft daardoor vaak beperkt tot kas- en container teelten, waar lage temperaturen geen belemmering vormen. Het gebruik van insecten-parasitaire nematoden kan een bijdrage leveren aan het terugdringen van het gebruik van chemische pesticiden, mits de werkzaamheid bij lage bodemtemperaturen (9 - 12 °C) verbeterd kan worden.

In dit proefschrift zijn de mogelijkheden tot verbetering van de biologische bestrijding met insecten-parasitaire nematoden onderzocht met behulp van een systeem analytische benadering. Deze bestond uit drie onderdelen:

1) Experimenteel onderzoek van zoekgedrag van nematoden in grondkolommen en doding van de gastheer.
2) Ontwikkeling van een model dat de biologische bestrijding simuleert op basis van integratie van onderliggende processen, met gebruikmaking van de experimentele resultaten.
3) Verkenning van de mogelijkheden om de bestrijding te verbeteren, met behulp van het model.

Gedragseigenschappen die een grote invloed hebben op de uitkomst van het model (gevoeligheidsanalyse) en waarvoor variatie voorhanden is binnen nematodentaxa, kunnen dienen voor het verbeteren van de bestrijding. Daarom zijn de gedragingen van verschillende isolaten van *Heterorhabditis* vergeleken. De invloed van temperatuur (9 en 20 °C) en gastheer (*O. sulcatus* en *Galleria mellonella*) op het gedrag van de nematoden is onderzocht om na te gaan hoe belangrijk ze zijn voor het bestrijdingsresultaat. In tegenstelling tot *O. sulcatus* is *G. mellonella*, de wasmot, zeer vatbaar en attractief voor insecten-parasitaire nematoden.

In Hoofdstuk 2 is beschreven hoe de nematoden hun gastheer vinden in zandkolommen bij 20 °C. Er zijn grote genetische verschillen in mobiliteit en gastheerzoekgedrag tussen isolaten. Deze verschillen vallen samen met de soortsindeling

binnen *Heterorhabditis*. Er is tevens niet-genetische variatie in mobiliteit en zoekgedrag binnen isolaten, veroorzaakt door condities tijdens productie en opslag. De aanwezigheid van *O. sulcatus* in de zandkolom heeft geen enkele invloed op het gedrag van de nematoden in grondkolommen terwijl *G. mellonella* een grote aantrekkingskracht uitoefent; meer nematoden bewegen naar beneden, ze bewegen sneller en ze aggregeren inde buurt van de larve (arrestment). Lage temperatuur (Hoofdstuk 3) verlaagt de mobiliteit van de nematoden ten opzichte van 20 °C. Verschillen bij 9 °C vallen niet samen met taxonomische verschillen.

In Hoofdstuk 4 is het penetratie-gedrag van de nematoden beschreven. Slechts een gedeelte van de nematoden is daadwerkelijk in staat om het insect binnen te dringen (infectieus). Er is genetische en niet-genetische variatie in het percentage infectieuze nematoden. Temperatuur en gastheer hebben beide invloed op het percentage infectieuze nematoden. In aanwezigheid van *O. sulcatus* bij 9 °C is slechts een heel klein gedeelte van de nematoden infectieus (≤ 1%), ongeacht het isolaat. De snelheid waarmee de infectieuze nematoden binnen dringen (relatieve penetratie-snelheid) blijkt stabiel te zijn voor het isolaat HF85 van *Heterorhabditis* sp. In deze studie is niet onderzocht of de relatieve penetratie-snelheid varieert tussen isolaten. Temperatuur en gastheer hebben invloed op de relatieve penetratie-snelheid. Deze is het kleinst voor *O. sulcatus* bij 9 °C.

In Hoofdstuk 5 wordt de variabiliteit van penetratie in gastheerinsecten gekwantificeerd. Wanneer enkele insecten veel nematoden bevatten en de meeste insecten weinig of geen, dan is de verdeling geclusterd. Dit clusteringsverschijnsel is bij *O. sulcatus* veel sterker dan bij *G. mellonella*. Clustering is bij lage temperatuur sterker dan bij hoge temperatuur. De grote mate van clustering bij *O. sulcatus* bij 9 °C heeft tot gevolg dat pas bij een hoog gemiddeld aantal nematoden per gastheer een adequate fractie doding wordt bereikt.

In Hoofdstuk 6 zijn de experimentele resultaten van Hoofdstuk 2 tot en met 5 gebruikt om een model te construeren dat de verdeling van nematoden simuleert in ruimte en in tijd tussen het moment van toedienen aan de zandkolom tot en met het binnendringen in de gastheer. De gedragingen ten aanzien van mobiliteit en zoekgedrag zijn vertaald in termen van percentage beweeglijke nematoden, relatieve bewegingssnelheid en accumulatie van nematoden bij de gastheer (arrestment). Dit is gedaan met behulp van model calibratie. Model beschrijvingen van mobiliteit en zoekgedrag werden geleidelijk verbeterd door veronderstellingen ten aanzien van mobiliteit en zoekgedrag te veranderen, parameters te calibreren en het resultaat te beoordelen. Gevoeligheidsanalyse van het model voor *O. sulcatus* bij 9 °C toont aan dat de mate van clustering en het al of niet aggregeren van nematoden in de buurt van het insect (arrestment) een grote invloed hebben op de

bestrijdingsresultaten. Er zijn in beide eigenschappen geen verschillen tussen of binnen isolaten van *Heterorhabditis* die benut zouden kunnen worden om de nematoden te verbeteren. Ook de penetratie-parameters, het percentage infectieuze nematoden en de penetratie-snelheid, blijken invloed te hebben op de uitkomst van het model. Er zijn echter geen genetische verschillen tussen isolaten van *Heterorhabditis* ten aanzien van het percentage infectieuse nematoden (in geval van *O. sulcatus* bij 9 °C). Wel is er niet-genetische variatie, die veroorzaakt wordt door productie en opslag omstandigheden. Deze studie toont aan dat de beste mogelijkheden om het bestrijdingssucces te verbeteren liggen in het zodanig optimaliseren van productie en opslag van nematoden, dat het percentage infectieuse nematoden verhoogd wordt tot het genetische maximum.

In Hoofdstuk 7 wordt ingegaan op obstakels die zijn genomen en keuzes die gemaakt zijn tijdens deze studie, en op de gebruikte onderzoeksmethoden. Er wordt dieper ingegaan op de betekenis van de onderzoeksresultaten voor de bestrijdingsmogelijkheden van bodemplagen met behulp van *Heterorhabditis* en op de vooruitzichten om de bestrijding van *O. sulcatus* ook daadwerkelijk te verbeteren.

Summary

The black vine weevil, *Otiorhynchus sulcatus*, is an important pest in ornamentals and nursery stock in the Netherlands. The larvae of the weevil feed on the root system and base of the plant. Biological control of the larvae by means of insect parasitic nematodes, *Heterorhabditis*, is excellent at soil temperatures above approximately 12 - 13 °C. In open cultures, temperatures frequently drop below these values during the periods that control of the older larvae takes place, namely in autumn (September - November) and spring (March - May). The use of nematodes is restricted mainly to greenhouse and container crops, where low temperature is no limitation. The use of nematodes can contribute to a decrease in the use of chemical pesticides, if performance can be improved at low soil temperatures (9 - 12 °C). In this thesis possibilities to improve the biocontrol by means of insect parasitic nematodes are explored using a systems analytical approach, comprising three components:

1) Experimental research of the host finding behaviour of the nematodes in soil columns and killing of the host.
2) Development of a model that simulates the biological control on the basis of integration of process knowledge, using the experimental data.
3) Exploring the possibilities to improve control, using the model.

Nematode behavioural traits that have a large influence on model outcome (sensitivity analysis) and for which variation is present in the nematode taxa are eligible to improvement. Therefore, the behaviour of nematodes was compared between various isolates of *Heterorhabditis*. The influence of temperature (9 and 20 °C) and host species (*O. sulcatus* and *Galleria mellonella*) on nematode behaviour was assessed to determine the contribution of these factors to control success. In contrast to *O. sulcatus, G. mellonella*, the wax moth, is highly susceptible and attractive to the insect parasitic nematodes.

In Chapter 2, host finding behaviour of the nematodes in sand column at 20 °C is described. There are significant differences in movement and host finding between isolates. These differences are congruent with putative taxonomic groups in the genus *Heterorhabditis*. There is also non-genetic variation in movement and host-finding, caused by storage and production conditions. The presence of *O. sulcatus* in the sand column has no influence on nematode behaviour, while *G. mellonella* is highly attractive; more nematodes are mobile, they move faster and they accumulate near the host (arrestment). Low temperature (Chapter 3) slowed down movement in comparison to 20 °C. Differences between isolates at 9 °C are not congruent with taxonomic groups.

In Chapter 4, penetration behaviour of the nematodes into insect hosts is described. Only part of the nematodes is capable of penetration (infectious). There is genetic and non-genetic variation in the proportion infectious nematodes. Temperature and host species both influence the proportion infectious nematodes. In the presence of *O. sulcatus* at 9 °C a tiny proportion (≤ 1%) is infectious, irrespective of the isolate. The speed at which the nematode penetrate (relative penetration rate) appears to be stable within the isolate HF85 of *Heterorhabditis* sp. In this study, differences in relative penetration rate between isolates were not investigated. Temperature and host species both influence the relative penetration rate. Both are smallest for *O. sulcatus* at 9 °C.

In Chapter 5, variability in penetration of host insects is quantified. When a few insects contain many nematodes and many insects contain a few or no nematodes, the distribution is aggregated. In *O. sulcatus* the degree of aggregation is much higher than in *G. mellonella*. Aggregation is stronger at low than at high temperature. The high degree of aggregation for *O. sulcatus* at 9 °C implies that only at high average numbers of penetrated nematodes per host an adequate proportion mortality is obtained.

In Chapter 6, the experimental results of Chapters 2 to 5 are used to construct a model that simulates the distribution of nematodes in time and space from the moment of application to a sand column until penetration into the host. Behaviour with respect to movement and host finding were translated in terms of percentage mobile nematodes, relative dispersal rate, and arrested nematodes. This was done by model calibration. Model descriptions of movement and host finding were gradually improved by changing the assumptions with respect to movement and host finding, calibrating the parameters, and evaluating the result of the model. Sensitivity analysis of the model for *O. sulcatus* at 9 °C shows that the degree of aggregation and the absence or presence of arrestment induced by the insect host have a large influence on control results. There are no differences in either of these traits between or within isolates of *Heterorhabditis* that could be used to improve control. The penetration parameters, the percentage infectious nematodes and the relative penetration rate, also influence model outcome. There are no genetic differences between isolates in the proportion infectious nematodes (in case of *O. sulcatus* at 9 °C), but there is non-genetic variation in this respect, caused by production and storage conditions. This study indicates that the best options to improve control should be sought in the optimization of production and storage conditions, such that the percentage infectious nematodes is increased to its genetic maximum.

In Chapter 7, obstacles encountered and choices made during this study were

discussed, as well as the research approach. The relevance of the results for options to control soil pests by means of *Heterorhabditis*, and the prospects to actually improve control of *O. sulcatus* are further discussed.

Gearfetting

De takstuorre, *Otiorhynchus sulcatus*, is in wichtiche pleach yn sier- en beamoanfok gewaaks yn Nederlân. De larven fan de tuorre frette de woartels en woartelhals fan de plant oan. Biologyske bestriding fan de larven mei help fan parasitêre nematoaden foar ynsekten, *Heterorhabditis*, wurket treflik by grûntemperatueren boppe de 12 á 13 °C. Yn oanfok op folle grûn komt de temperatuer faak ûnder dizze wearden yn perioaden dat bestriding fan âldere larven plakfynt, nammentlik yn'e hjerst (septimber - novimber) en maaitiid (maart - maaie). De tapassing fan ynsekten parasitêre nematoaden foar de bestriding fan de takstuorre bliuwt dêrtroch faaks beheind ta kas- en kontainer oanfok, der't lege temperatueren gjin behindering foarmje. It gebrûk fan ynsekten parasitêre nematoaden kin in bydrage leverje oan it werombringen fan it gebrûk fan gemyske pestisiden, mits de wurksumheid by lege boaiemtemperatueren (9 - 12 °C) ferbettere wurde kin.

Yn dit proefskrift binne de mooglikheden ta ferbettering fan de biologyske bestriding mei parasitêre nematoaden foar ynsekten ûndersocht mei help fan in systeem analytyske oanpak. Dizze bestie út trije ûnderdielen:

1) Eksperimenteel ûndersyk fan sykgedrach fan nematoaden yn grûnkolommen en deadzjen fan de gasthear.
2) Ûntwikkeling fan in model dat de biologyske bestriding simulearret op basis fan yntegraasje fan ûnderlizzende prosessen mei gebrûk fan de eksperimentele resultaten.
3) Ferkenning fan de mooglikheden om de bestriding te ferbetterjen, mei help fan it model.

Gedrachseigenskippen dy't in grutte ynfloed ha op de útkomst fan it model (gefoelichheidsanalyze) en der't fariaasje foarhannen is binnen de nematoadentaksen, kinne foar it ferbetterjen fan de bestriding tsjinje. Dêrom is der in fergelyk makke fan ferskillende isolaten fan *Heterorhabditis*. De ynfloed fan temperatuer (9 en 20 °C) en gasthear (*O. sulcatus en Galleria mellonella*) op it gedrach fan de nematoaden is ûndersocht om nei te gean hoe wichtich se binne foar it bestridingsresultaat. Yn tsjinstelling ta *O. sulcatus* is *G. mellonella*, de grutte waaksmot, hiel fetber en attraktyf foar ynsekten parasitêre nematoaden.

Yn Haadstik 2 is beskreaun hoe't de nematoaden har gasthear fine yn sânkolommen by 20 °C. Der binne grutte genetyske ferskillen yn mobiliteit en gasthearsykgedrach tusken isolaten. Dizze ferskillen falle gear mei de soartsyndieling binnen *Heterohabditis*. Der is ek net-genetyske fariaasje yn mobiliteit en sykgedrach binnen isolaten, feroarsake troch kondysjes wilens produksje en opslach. De oanwêzichheid fan *O. sulcatus* yn de sânkolom hat gjin inkele

ynfloed op it gedrach fan de nematoaden yn grûnkolommen wylst *G. mellonella* in grutte krêft hat dy't nei mekoar ta lûkt; mear nematoaden bewege nei ûnderen ta, se bewege hurder en se aggregearje yn de buert fan de larve (arrestment). Lege temperatuer (Haadstik 3) ferleget de mobiliteit fan de nematoaden wat 20 °C oanbelanget. Ferskillen by 9 °C falle net gear mei taksonomyske ferskillen.

Yn Haadstik 4 is it penetraasje gedrach fan de nematoaden beskreaun. Inkeld in diel fan de nematoaden is feitlik by steat om it ynsekt der yn te kringen (ynfeksieus). Der is genetyske en net-genetyske fariaasje yn it persintaazje ynfeksieuze nematoaden. Temperatuer en gasthear ha beide ynfloed op it persintaazje ynfeksieuze nematoaden. Yn oanwêzichheid fan *O. sulcatus* by 9 °C is mar in hiel lyts diel fan de nematoaden ynfeksieus (≤ 1%), nettsjinsteande it isolaat. De gong, der't de ynfeksieuze nematoaden der mei yn kringe (relative penetraasje gong) blykt stabyl te wêzen foar it isolaat HF85 fan *Heterorhabditis* sp. Yn dizze stúdzje is net ûndersocht oft de relative penetraasje gong farieart tusken isolaten.Temperatuer en gasthear ha ynfloed op de relative penetraasje gong. Dizze is it lytst foar *O. sulcatus* by 9 °C.

Yn Haadstik 5 wurdt de fariabiliteit fan penetraasje yn gasthearynsekten kwantifisearre. Wannear't inkele ynsekten in protte nematoaden befetsje en de measte ynsekten in bytsje of gjin nematoaden dan is de ferdieling klustere. Dit klusteringferskynsel is by *O. sulcatus* folle sterker as by *G. mellonella*. Klustering is by lege temperatuer sterker as by hege temperatuer. De grutte mjitte fan klustering by *O. sulcatus* by 9 °C hat ta gefolch dat krekt by in trochsneed heech oantal nematoaden per gasthear in adekwate fraksje deading berikt wurdt.

Yn Haadstik 6 binne de eksperimentele resultaten fan haadstik 2 oant en mei 5 brûkt om in model te konstruearjen dat de ferdieling fan nematoaden simulearret yn romte en yn tiid tusken it momint fan tatsjinning oan de sânkolom oant en mei it ynkringen yn de gasthear. De gedragingen oangeande de mobiliteit en sykgedrach binne oerset yn termen fan persintaazje beweechlike nematoaden, relative bewegingsgong en akkumulaasje fan nematoaden by de gasthear (arrestment). Dit is dien mei help fan modelkalibraasje. Modelbeskriuwingen fan mobiliteit en sykgedrach wurde stadichwei ferbettere troch ûnderstellingen oangeande mobiliteit en sykgedrach te feroarjen, parameters te kalibrearjen en it resultaat te beoardieljen. Gefoelichheidsanalyze fan it model foar *O. sulcatus* by 9 °C lit sjen dat de mjitte fan klustering en it al of net aggregearjen fan nematoaden yn de buert fan it ynsekt (arrestment) in grutte ynfloed ha op de bestridingsresultaten. Der binne yn beide eigenskippen gjin ferskillen tusken of yn isolaten fan *Heterorhabditis* dy't brûkt wurde kinne soenen om de nematoaden te

ferbetterjen. Ek de penetraasjeparameters, it persintaazje ynfeksieuze nematoaden en de penetraasje gong, hawwe ynfloed op de útkomst fan it model. Der binne lykwols gjin genetyske ferkillen tusken isolaten fan *Heterorhabditis* oangeande it persintaazje ynfeksieuze nematoaden (yn gefal fan *O. sulcatus* by 9 °C). Wol is der net-genetyske fariaasje dy't feroarsake wurdt troch produksje en opslachomstannichheden. Dizze studzje lit sjen dat de beste mooglikheden om it bestridingssukses te ferbetterjen, lizze yn it op sa'n wize optimalisearjen fan produksje en opslach fan nematoaden, dat it persintaazje ynfeksieuze nematoaden ferhege wurdt ta it genetysk maksimum.

Yn Haadstik 7 wurdt yngien op tûkelteammen dy't west hawwe en de kar dy't makke is yn'e tiid fan dizze stúdzje, en op de brûkte ûndersyksmethoaden. Der wurdt djipper yngien op de betsjutting fan de ûndersyksresultaten foar de bestridingsmooglikheden fan boaiempleagen mei help fan *Heterorhabditis* op it foarútsjoch om de bestriding fan *O. sulcatus* ek feitlik te ferbetterjen.

References

Alatorre-Rosas, R. and Kaya, H.K. 1990. Interspecific competition between entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* for an insect host in sand. *Journal of Invertebrate Pathology* **55** : 179-188.

Anderson, R.M. 1982. Epidemiology. In: Cox, F.E.G.(Ed.), *Modern Parasitology*. Blackwell Scientific Publications, Oxford. p. 204-251.

Anderson, R.M., Whitfield, P.J., and Dobson, A.P. 1978. Experimental studies of infection dynamics: infection of the definitive host by the cercariae of *Transversotrema patialense*. *Parasitology* **77** : 189-200.

Bedding, R.A., Molyneux, A.S and Akhurst, R.J. 1983. *Heterorhabditis* spp., *Neoaplectana* spp., and *Steinernema kraussei*: interspecific and intraspecific differences in infectivity for insects. *Experimental Parasitology* **55** : 249-257.

Bohan, D.A. and Hominick, W.M. 1995a. Examination of the *Steinernema feltiae* (site 76 strain) infection interaction with the *Galleria mellonella* host, using an infection model. *Parasitology* **111** : 617-625.

Bohan, D.A. and Hominick, W.M. 1995b. Intra-population infectious structure and temporal variation in *Steinernema feltiae*. In: Griffin, C.T., Gwynn, R.L. and Masson, J.P. (Eds.), *Biotechnology, ecology and transmission strategies of entomopathogenic nematodes*. COST 819, CSC-EC EAEC, Brussels, Luxembourg. p. 83-94.

Bohan, D.A. and Hominick, W.M. 1996. Investigations on the presence of an infectious proportion amongst populations of *Steinernema feltiae* (Site 76 strain) infective stages. *Parasitology* **112** : 113-118.

Choo, H.Y. and Kaya, H.K. 1991. Influence of soil texture and presence of roots on host finding by *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology* **58** : 279-280.

Choo, H.Y., Kaya, H.K., Burlando, T.M. and Gaugler, R. 1989. Entomopathogenic nematodes: host-finding ability in the presence of plant roots. *Environmental Entomology* **18** : 1136-1140.

Clarke, D.J. and Dowds, B.C.A. 1994. Cold adaptation in Photorhabdus spp. In: Burnell, A.M., Ehlers, R.U. and Masson, J.P. (Eds.), *Biotechnology, genetics of entomopathogenic nematode-bacterium complexes*. COST 812 ECSC-EC EAEC, Brussels, Luxembourg. p. 170-177.

Curran, J. 1993. Post-application biology of entomopathogenic nematodes in soil. In: Bedding, R., Akhurst, R, and Kaya, H. (Eds.), *Nematodes and the biological control of insect pests*. CSIRO, East Melbourne, p. 67-78.

Dix, I., Burnell, A.M., Griffin, C.T., Joyce, S.A., Nugent, M.J. and Downes, M.J. 1992. The identification of biological species in the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) by cross-breeding second-generation amphimictic adults. *Parasitology* **104** : 509-518.

Doutt, R.L. and DeBach, P. 1964. Some biological control concepts and questions. In: DeBach, P. (Ed.), *Biological control of insect pests and weeds*. Reinhold, New York. p. 118-142.

Epsky, N.D. and Capinera, J.L. 1993. Quantification of invasion of two strains of *Steinernema carpocapsae* (Weiser) into three lepidopteran larvae. *Journal of Nematology* **25** : 173-180.

Evenhuis, H.H. 1978. Bionomics and control of the black vine weevil, *Otiorhynchus sulcatus*. *Mededelingen van de Faculteit der Landbouwwetenschappen, Rijksuniversiteit Gent* **43** : 607-611.

Fan, X. and Hominick, W.M. 1991a. Efficiency of the *Galleria* (wax moth) baiting technique for recovering infective stages of entomopathogenic rhabditids (Steinernematidae and Heterorhabditidae) from sand and soil. *Revue de Nématologie* **14** : 381-387.

Fan, X. and Hominick, W.M. 1991b. Effects of low storage temperature on survival and infectivity of two *Steinernema* species (Nematoda: Steinernematidae). *Revue de Nématologie* **14** : 407-412.

Forschler, B.T. and Gardner, W.A. 1991. Parasitism of *Phyllophaga hirticula* (Coleoptera: Scarabaeidae) by *Heterorhabditis heliothidis* and *Steinernam carpocapsae*. *Journal of Invertebrate Pathology* **58** : 396-407.

Gaugler, R. 1988. Ecological considerations in the biological control of soil-inhabiting insects with entomopathogenic nematodes. *Agriculture, Ecosystems and Environment* **24** : 351-360.

Gaugler, R. and Campbell, J.F. 1991. Selection for enhanced host-finding of scarab larvae (Coleoptera: Scarabaeidae) in an entomopathogenic nematode. *Environmental Entomology* **20** : 700-706.

Gaugler, R. LeBeck, L., Nakagaki, B. and Boush, G.M. 1980. Orientation of the entomogenous nematode *Neoaplectana carpocapsae* to carbon dioxide. *Environmental Entomology* **9** : 649-652.

Gaugler, R., Glazer, I., Campbell, J.F. and Liran, N. 1994. Laboratory and field evaluation of an entomopathogenic nematode genetically selected for improved host-finding. *Journal of Invertebrate Pathology* **63** : 68-73.

Genstat 5 Reference manual, 1988. Clarendon Press, Oxford. 749 pp.

Georgis, R. 1990. Formulation and application technology. In : Gaugler, R. and Kaya, H.K. (Eds.), *Entomopathogenic nematodes in biological control*. CRC Press, Boca Raton, Florida. p. 173-191.

Georgis, R. and Gaugler, R. 1991. Predictability in biological control using entomopathogenic nematodes. *Journal of Economic Entomology* **34** : 713-720.

Georgis, R. and Poinar, G.O. 1983a. Effect of soil texture on the distribution and infectivity of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae). *Journal of Nematology* **15** : 308-311.

Georgis, R. and Poinar, G.O. 1983b. Effect of soil texture on the distribution and infectivity of *Neoaplectana glaseri* (Nematoda: Steinernematidae). *Journal of Nematology* **15** : 329-332.

Georgis, R. and Poinar, G.O. 1983c. Vertical migration of *Heterorhabditis bacteriophora* and *H. heliothidis* (Nematoda: Heterorhabditidae) in sandy loam soil. *Journal of Nematology* **15** : 652-654.

Georgis, R. and Poinar, G.O. 1984. Greenhouse control of the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) by heterorhabditid and steinernematid nematodes. *Environmental Entomology* **13** : 1138-1140.

Gerritsen, L.J.M. and Smits, P.H. 1993. Variation in pathogenicity of recombinations of *Heterorhabditis* and *Xenorhabdus luminescens* strains. *Fundamental and applied Nematology* **16** : 367-373.

Griffin, C.T. 1993. Temperature responses of entomopathogenic nematodes: Implications for the success of biological control programmes. In: Bedding, R., Akhurst, R. and Kaya, H. (Eds.), *Nematodes and the biological control of insect pests*. CSIRO, East Melbourne, p. 115-126.

Griffin, C.T. 1997. Effects of prior storage conditions on the infectivity of *Heterorhabditis* sp. (Nematoda: Heterorhabditidae) *Fundamental and applied Nematology* (in press).

Griffin, C.T., Simons, W.R. and Smits, P.H. 1989. Activity and infectivity of four isolates of Heterorhabditis spp. *Journal of Invertebrate Pathology* **53** : 107-112.

Hague, N.G.M., Haukeland, S. and Otto, A.A. 1991. Effect of temperature on the efficacy of steinernematid nematodes. *Mededelingen van de Faculteit der*

Landbouwwetenschappen, Rijksuniversiteit Gent **56** : 1245-1251.

Han, R., Wouts, W.M. and Li, L. 1991. Development and virulence of *Heterorhabditis* spp. strains associated with different *Xenorhabdus luminescens* isolates. *Journal of Invertebrate Pathology* **58** : 27-32.

Hanula, J.L. 1993. Vertical distribution of black vine weevil (Coleoptera: Curculionidae) immatures and infection by entomogenous nematodes in soil columns and field soil. *Journal of Economic Entomology* **86** : 340-347.

Hay, D.B. and Smits, P.H. 1995. A model of host-infection behaviour for the entomopathogenic nematode *Heterorhabditis megidis* : evidence for primary and secondary infection strategies. In: Griffin, C.T., Gwynn, R.L. and Masson, J.P. (Eds.), *Ecology and transmission strategies of entomopathogenic nematodes.* COST 819, ECSC-EC EAEC, Brussels, Luxembourg. p. 77-82.

Hoop B.J. de, Herman, P.M.J., Scholten, H. and Soetaart, K. 1992. SENECA 2.0, A simulation environment for ecological applications. Netherland Institute of Ecology, Centre for Estruarine and Costal Ecology, Yerseke, The Netherlands. 224 pp.

Hunneke, K., Peters, A and Ehlers, R.U. 1994. Movement patterns of dauer juveniles in response to host cues. *IOBC/WPRS Bulletin* **17** : 112-115.

Ishibashi, N. and Kondo, E. 1990. Behavior of infective juveniles. In : Gaugler, R. and Kaya, H.K. (Eds.), *Entomopathogenic nematodes in biological control.* CRC Press, Boca Raton, Florida. p. 139-150

Joyce, S.A., Griffin, C.T. and Burnell, A.M. 1994a. The use of isoelectric focusing and the polyamide gel electrophoresis of soluble proteins in the taxonomy of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae). *Nematologica* **40** : 601-612.

Joyce, S.A., Burnell, A.M. and Powers, O. 1994b. Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA genes. *Journal of Nematology* **26** : 260-270.

Jung, K. 1991. Observations on the infective juveniles of the insect parasitic nematode, *Heterorhabditis* sp., at two storage temperatures. *Mededelingen van de Faculteit der Landbouwwetenschappen, Rijksuniversiteit Gent* **56** : 1305-1312.

Jung, K. 1996. Storage of entomopathogenic nematodes of the genus Heterorhabditis at two temperature: effect on infectivity, energy reserve and number of bacteria. *IOBC/WPRS Bulletin* **19** :103-106.

Kaya, H.K. 1985. Entomogenous nematodes for insect control in IPM systems. In: Hoy, M.A. and Herzog, D.C. (Eds.), *Biological control in agricultural IPM Systems.* Academic

Press, New York, p. 283-302.

Kaya, H.K. 1990. Soil ecology. In: Gaugler, R. and Kaya, H.K. (Eds.), *Entomopathogenic nematodes in biological control*. CRC Press, Boca Raton, Florida, p. 93-116.

Kennedy, J.S. 1978. The concepts of olfactory 'arrestment' and 'attraction'. *Physiological Entomology* **3** : 91-98.

Klingler, J. 1959. Biologische Beobachtungen über den Gefurchten Dichmaulrüssler (*Otiorhynchus sulcatus* Fabr.) während seines Massenauftretens der letzten Jahre auf Reben der deutschen Schweiz. *Landwirtschaftlichen Jahrbuch der Schweiz* **73** : 409-438.

Klingler, J. 1988. Investigations on the parasitism of *Otiorhynchus salicicola* and *O. sulcatus* (Col.: Curculionidae) by *Heterorhabditis* sp. (Nematoda). *Entomophaga* **33** : 325-331.

Lei, Z., Rutherford, T.A. and Webster, J.M. 1992. Heterorhabditid behavior in the presence of the cabbage maggot, *Delia radicum*, and its host plants. *Journal of Nematology* **24** : 9-15.

Leij, F.A.A.M. de. 1995. Survival of entomopathogenic nematodes in soil. In: Griffin, C.T., Gwynn, R.L. and Masson, J.P. (Eds.), *Biotechnology, ecology and transmission strategies of entomopathogenic nematodes*,' COST 819, CSC-EC EAEC, Brussels, Luxembourg. p. 1-6.

Lewis, E.E., Gaugler, R. and Harrison, R. 1992. Entomopathogenic host finding: response to host contact cues by cruise and ambush foragers. *Parasitology* **105** : 309-315.

Lewis, E.E., Gaugler, R. and Harrison, R. 1993. Response of cruiser and ambusher entomopathogenic nematodes (Steinernematidae) to host volatile cues. *Canadian Journal of Zoology* **71** : 765-769.

Mannion, C.M. and Jansson, R.K. 1993. Infectivity of five entomopathogenic nematodes to the sweetpotato weevil, *Cyclas formicarius* (F.), (Coleoptera: Apionidae) in three experimental arenas. *Journal of Invertebrate Pathology* **62** : 29-36.

Ministry of Agriculture, Nature Management and Fishery. 1990. Rapportage Werkgroep boomteelt, Achtergronddocument Meerjaren-plan Gewasbescherming. (in Dutch) De Haag. 112 pp.

Molyneux, A.S. 1986. *Heterorhabditis* spp. and *Steinernema* (= *Neoaplectana*) spp: temperature, and aspects of behavior and infectivity. *Experimental Parasitology* **62** : 169-180.

Moorhouse, E.R., Charnley, A.K. and Gillespie, A.T. 1992. A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Annals*

of applied Biology **121** : 431-454.

Morris, O.N., Converse, V. and Harding, J. 1990. Virulence of entomopathogenic nematode-bacteria complexes for larvae of noctuids, a geometrid, and a pyralid. *The Canadian Entomologist* **122** : 309-319.

Mráček, Z., Bečvár, S., Řezáč, P., Kindlmann, P. and Webster, J.M. 1997. Canadian steinernematid (Nematoda) isolates and their infectivity, under cold conditions, to greater wax moth (*Galleria mellonella*) larvae. *Biological control* **8** : 160-164.

Peters, A. and Ehlers, R.U. 1994. Susceptibility of leatherjackets (*Tipula paludosa* and *Tipula oleracea*; Tipulidae; Nematocera) to the entomopathogenic nematode *Steinernema feltiae*. *Journal of Invertebrate Pathology* **63** : 163-171.

Pielou, E.C. 1969. *An introduction to mathematical ecology*. New York: Wiley-Interscience. 286 pp.

Poinar, G.O. 1975. *Entomogenous nematodes*. Brill, Leiden, 317 pp.

Poinar, G.O. 1990. Biology and taxonomy. In: Gaugler, R. and Kaya, H.K. (Eds.), *Entomopathogenic nematodes in biological control*. CRC Press, Boca Raton, Florida. p. 23-62.

Price, W.L. 1979. A controlled random search procedure for global optimization. *The Computer Journal* **20** : 113-133.

Rabbinge, R., Ward, S.A. and Laar, H.H. van. (Eds.) 1989. Simulation and system management in crop protection. Simulation Monographs, Pudoc, Wageningen. 420 pp.

Rappoldt, C. and Kraalingen, D.W.G. van. 1996. The Fortran Simulation Translator, FST version 2.0. Introduction and Reference Manual. *Quantitative Approaches in Systems Analysis* **No. 5**, 178 pp.

Reibnitz C. von, Lange, K. and Backhaus, G.F. 1993. Uberall Dickmaulrüssler. *Deutsche Baumschule* **20** : 72-74.

Rovesti, L., Heinzpeter, E.W. and Deseö, K.V. 1991. Distribution and persistence of *Steinernema* spp. and *Heterorhabditis* spp. (Nematoda) under field conditions. *Anzeiger für Schädlingskunde, Pflanzenschutz und Umweltschutz* **64** :18-22.

Rutherford, T.A., Trotter, D. and Webster, J.M. 1987. The potential of heterorhabditid nematodes as control agents of root weevils. *The Canadian Entomologist* **119** : 67-73.

Schirocki, A. and Hague, N.G.M. 1994. The effect of temperature on the susceptibility of the black vine weevil, *Otiorhynchus sulcatus*, to different isolates of *Steinernema* and *Heterorhabditis*. *IOBC/WPRS bulletin* **17** : 61-64.

Simons, W.R. and Schaaf, D.A. van der. 1986. Infectivity of three *Heterorhabditis* isolates for

Otiorhynchus sulcatus at different temperatures. In: Samson, R.A., Vlak, J.M. and Peters, D. (Eds.), *Fundamental and applied aspects of invertebrate pathology.* Proceedings 4th ICIP, Veldhoven, The Netherlands, 1986. p. 285-289.

Smits, P.H., Groenen, J.T.M. and Raay, G. de. 1991. Characterization of *Heterorhabditis* isolates using DNA restriction fragment length polymorphism. *Revue Nématology* **14** : 445-453.

Southwood, T.R.E. 1978. *Ecological methods with particular reference to the study of insect populations* (2nd ed.). Chapman and Hall, London, 391 pp.

Thurston, G.S. and Kaya, H.K. 1994. Physical stressors affecting interactions of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and an entomopathogenic nematode. *The Canadian Entomologist* **126** : 261-267.

Tol, R.W.H.M. van. 1993. Efficacy of control of the black vine weevil (*Otiorhynchus sulcatus*) with strains of *Heterorhabditis* sp., *Steinernema* sp. and the fungus *Metarhizium anisophilae* in nursery stock. *Mededelingen van de Faculteit der Landbouwwetenschappen, Rijksuniversiteit Gent* **58 :** 461-467.

Wallace, H.R. 1958. Movement of eelworms. II. A comparative study of the movement in soil of *Heterodera schachtii* Schmidt and of *Ditylenchus dipsaci* (Kuhn) Filipjev. *Annals of applied Biology* **46** : 86-94.

Wallace, H.R.1963. *The biology of plant parasitic nematodes.* Edward Arnold Ltd. London. 280 pp.

Westerman, P.R. 1992. The influence of time of storage on performance of the insect parasitic nematode, *Heterorhabditis* sp. *Fundamental and applied Nematology* **15** : 407-412.

Westerman, P.R.1994. An essay on assays. In: A.M. Burnell, R.U. Ehlers & J.P. Masson (Eds.) *Biotechnology, genetics of entomopathogenic nematode-bacterium complexes.* COST 812 ECSC-EC EAEC, Brussels, Luxembourg. p. 129-142.

Westerman, P.R. and Zeeland, M.G van. 1989. Comparison of *Heterorhabditis* isolates for control of *Otiorhynchus sulcatus* at low temperatures. *Mededelingen van de Faculteit der Landbouwwetenschappen, Rijksuniversiteit Gent* **54** : 1115-1124.

Westerman, P.R. and Godthelp, J.H. 1990. The host-searching ability of the insect parasitic nematode *Heterorhabditis* sp. in sand columns. *Mededelingen van de Faculteit der Landbouwwetenschappen, Rijksuniversiteit Gent* **55** : 691-698.

Westerman, P.R. and Godthelp, J.H. 1991. Natural and specific migration of the insect parasitic nematode *Heterorhabditis* sp., towards various insect hosts. *IOBC/WPRS Bulletin* **14** : 63.

parasitic nematode *Heterorhabditis* sp., towards various insect hosts. *IOBC/WPRS Bulletin* **14** : 63.

Westerman, P.R. and Stapel, M. 1992. Linear regression models describing the performance of the insect parasitic nematode, *Heterorhabditis* sp. during storage. *Fundamental and applied Nematology* **15**: 525-530.

Woodring, J.L. and Kaya, H.K. 1988. Steinernematid and heterorhabditid nematodes: a handbook of techniques. Southern Cooperative Series Bulletin 331, Arkansas Agricultural Experimental Station, Fayetteville, U.S.A. 30 pp.

Wright, P.J. and Jackson, T.A. 1988. Low temperature activity and infectivity of a parasitic nematode against porina and grass grub larvae. Proceedings 41st New Zealand Weed and Pest Control Conference, p. 138-140.

Wulff, A., Peters, A. and Ehlers, R.U. 1994. Pathogenicity of the *Steinernema feltiae-Xenorhabdus bovienii* complex to *Tipula oleracea*. *IOBC/WPRS Bulletin* **17** : 99-102.

Zimmerman, G. and Simons, W.R. 1986. Experiences with biological control of the black vine weevil, *Otiorhynchus sulcatus* (F.). In: Samson, R.A, Vlak, J.M. and Peters, D. (Eds.), *Fundamental and applied aspects of invertebrate pathology*. Proceedings 4th ICIP, Veldhoven, The Netherlands, 1986. p. 529-533.

Curriculum vitae

Paula Westerman werd op 2 oktober 1961 geboren in Doetinchem. In 1980 behaalde zij het Atheneum-β diploma aan het Willem van Oranje College te Waalwijk. Daarna begon zij met een studie planteziektenkunde aan de Landbouwuniversiteit teWageningen. In 1984 werd het kandidaatsdiploma behaald met lof en in 1987 werd het doctoraalexamen behaald. De hoofdvakken waren entomologie en plantenverdeling en het bijvak virologie. In 1987 werd zij aangesteld als onderzoeksmedewerkster nematologie op de toenmalige Bijzondere Hogere Landbouwschool te Leeuwarden, tegenwoordig het Van Hall Instituut. Zij was belast met het onderzoek naar de biologische bestrijding van de taxuskever met behulp van insecten parasitaire nematoden. Deze onderzoekswerkzaamheden hebben geleid tot het huidige proefschrift.