

Aus dem Institut für Phytopathologie  
der Christian-Albrechts-Universität zu Kiel

Life cycle and development of entomopathogenic nematodes  
*Steinernema carpocapsae* and *S. feltiae*  
in monoxenic liquid culture

Lebenszyklus und Entwicklung der entomopathogenen  
Nematoden *Steinernema carpocapsae* und *S. feltiae* in  
monoxenischer Flüssigkultur

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vorgelegt von

M.Sc. Ayako Hirao

aus Tokyo

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Dekan: Prof. Dr. Uwe Latacz-Lohmann

1. Berichterstatter: Prof. Dr. Ralf-Udo Ehlers

2. Berichterstatter: Prof. Dr. Hinrich Schulenburg

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**Appendix 1: Publication 1.** Life cycle and population development of entomopathogenic nematodes *Steinernema carpocapsae* and *S. feltiae*, (Nematoda, Rhabditida) in monoxenic liquid culture

**Appendix 2: Publication 2.** Influence of cell density and phase variation of bacterial symbionts (*Xenorhabdus* spp.) on dauer juvenile recovery and development of biocontrol nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida)

**Appendix 3: Publication 3.** Effect of temperature on the development of *Steinernema carpocapsae* and *Steinernema feltiae* (Nematoda: Rhabditida)

**Appendix 4: Publication 4.** Influence of inoculum density on population dynamics and dauer juvenile yields in liquid culture of biological control nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida)

**Danksagung**

# Introduction

## 1. Introduction

The soil living entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis* (Nematoda: Rhabditida) are used as biological control agents. Primarily the application of EPN as insecticides was developed for soil born pests (Grewal et al. 2005). The conditions in soil cause serve problems for the application of chemical pesticides. The chemicals need to percolate the soil in order to reach the insect pest larva, need to be highly toxic or persistent to be effective. These characters increase the risk for groundwater contaminations and non-target effects, contradicting the objectives of a modern regulation policy and consequently prevent the registration of new chemical insecticides against soil pests. For example, the highly effective parathion and its derivatives were banned from the market due to the high toxicity 2001 - 2002. The use of neonicotinoids, which can be effective against root feeding pests because of their systemic mode of action, is very much restricted. Furthermore, these substances are toxic against bees (European Food Safety Authority 2008). An actual case of bee intoxication was recorded in Germany due to seed treatment of maize with Clothianidin (Pistorius et al. 2009).

EPN are highly effective against soil born insects (Ehlers and Gerwien 1993, Toepfer et al. 2008). Since the EPN and bacteria recycle inside host insects, EPN can have long-term, sustainable effects on the control of pest populations (Peters 1996). Another advantage of EPN use is the safety for both, the user and the environment (Ehlers and Hokkanen 1996, Bathon 1996). They have little negative effects on non-target insect populations. Furthermore, neither the nematodes themselves nor the bacto-helminthic combination causes any harmful effects to vertebrates or plants (Bathon 1996, Powell and Webster 2004).

EPN can be stored for several months and are tolerant to moderate shear forces and can thus be applied with conventional spraying equipment. Even foliar applications for EPN had been successfully developed (e. g. against Diamond Back Moth: Schroer and Ehlers 2005). Today, EPNs are mainly used in environments where chemical pesticides cannot be delivered or if applied, would cause severe polluting effects, i.e. in the soil or in galleries of

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boring insects, or in cases where resistance to insecticides has developed (Ehlers 2001).

The life cycle of EPN is presented Fig. 1. A special developmental stage within the life cycle of most of rhabditid nematodes is the dauer juvenile (DJ). Dauer juveniles are morphologically distinct juveniles containing 200-2,000 cells of the symbiotic bacteria in their intestine (Endo and Nickle 1994). They are formed as a response to depleting food resources and adverse environmental conditions (Strauch et al. 1994). This stage is a free-living, infective and low metabolic stage that is well adapted for long-term survival in the soil (Lewis et al. 1995, Susurluk and Ehlers 2008). DJs can survive in the soil and wait for suitable host insect. The DJ invades a host through natural openings. In the hemolymph, the nematodes encounter optimal conditions for reproduction. As a response to chemical cues in the hemolymph, DJs molt and resume development. In accordance with the description of the life cycle of the closely related nematode, *Caenorhabdus elegans*, this developmental step is called „recovery“ and the chemical cues triggering recovery are called „food signal“ (Golden and Riddle 1982). During recovery, the DJ releases the symbiont cells into the insect's hemocoel. When EPN DJs invade an insect host, they receive a signal for recovery from the insect hemolymph. Ciche and Ensign (2003) investigated the release of bacterial cells via mouth using GFP-labeled *Photorhabdus luminescens* during *H. bacteriophora* DJ recovery following DJ inoculation in hemolymph of *Manduca sexta* larvae. They found that approximately 40% of DJs released the bacterial cells after 60 min. Snyder et al. (2007) investigated the bacterial cell of GFP-labeled *X. nematophila*. They were released from the intestine of *S. carpocapsae* DJs via the anus when inoculated in the hemolymph of *M. sexta* larvae. Twenty-five percent of inoculated *S. carpocapsae* DJs released the bacterial cells by 4 hours post inoculation. According to these results, DJ recovery offsets soon after DJ invasion into favorable condition. The bacteria produce toxins and other metabolites, which contribute to overcome the insect's defense mechanisms and kill the insect within approximately 48 hours after nematode invasion (Dowds and Peters 2002). The bacteria proliferate and produce favorable conditions for nematode reproduction. Feeding on the symbiotic bacterial cells, recovered juveniles develop into adults and produce offspring. When abundant resources are

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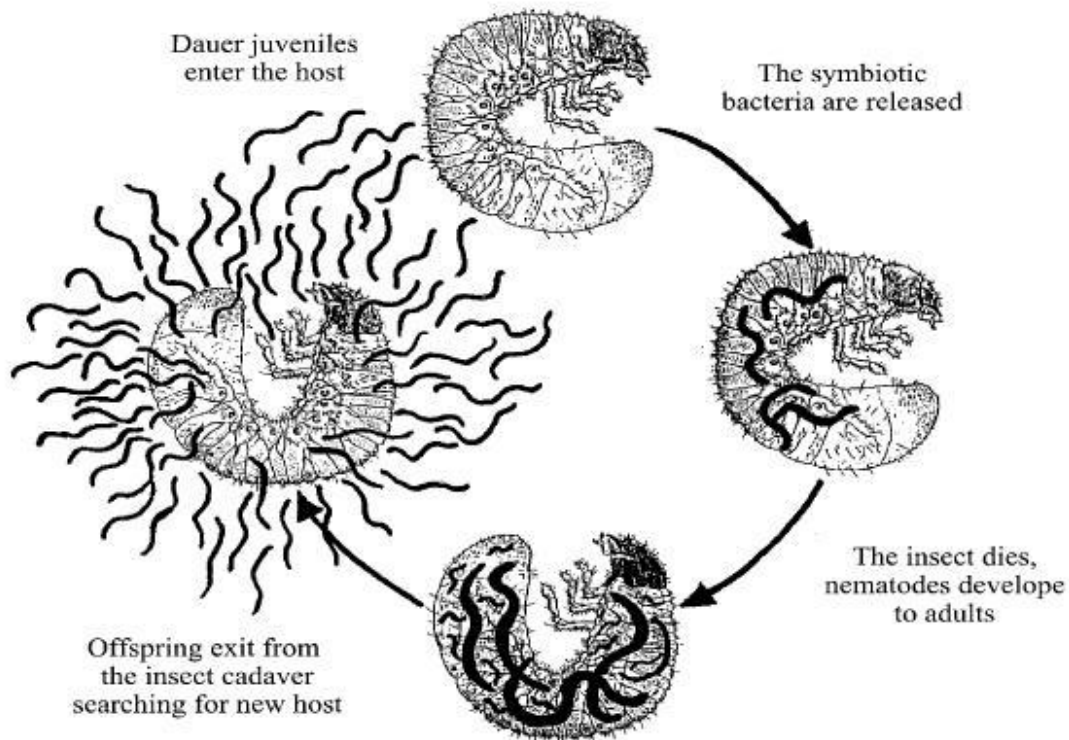


Fig. 1. Life cycle of entomopathogenic nematodes in a scarabaeid beetle larva (Ehlers 2001)

available, several adult generations develop in the insect cadaver. When the nutrients are consumed, the offspring develop into DJs, which retain the symbiotic bacteria in the intestine and leave the insect cadaver in a search for other hosts.

An economically efficient method for mass production is an important prerequisite for the development of any commercial biocontrol products. Today, eight species of EPNs, including *S. carpocapsae* and *S. feltiae*, are produced in liquid culture (Ehlers 2003). The technology for mass production in liquid culture has been established (Lunau et al. 1993, Ehlers et al. 1998). The mass production is carried out in monoxenic culture of species-specific symbiotic bacteria, which have been isolated from nematode infected insect larvae (Ehlers et al. 1990). Considering the development of both symbiotic bacteria and entomopathogenic nematodes, the typical medium for mass production includes a carbon source, a variety of proteins of animal and plant origin, yeast extract and lipids of animal or

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plant origin (Surrey and Davies 1996, Ehlers et al. 1998). Cultures are started with a bacterial pre-culture (24 to 36 hours) prior to DJ inoculation. The bacterial inoculum volume is between 0.5-1% of the total culture volume. After successful bacterial pre-culture, DJs, which are obtained from prior monoxenic cultures, are inoculated. The schematic life cycle of *Steinernema* nematode is represented in Fig. 2. Inoculated DJs receive chemical cues which are produced by their symbiotic bacteria. As a response to these secondary metabolites, DJs recover the development to reproductive stages. Recovered juveniles of *Heterorhabditis* spp. develop to hermaphrodite, which have both, oocytes and sperm, and reproduce offspring via automictic self-fertilization. In contrast, J4 juveniles of *Steinernema* spp. develop to males and females. They copulate and the females produce eggs. When the surrounding conditions provide sufficient food, the offspring develop into a further reproductive cycle. If the culture conditions are not suitable, newly hatched juveniles develop into DJs. Another pathway for offspring development, which is observed especially in the life cycle of rhabditid nematodes, is the *endotokia matricida*. Juveniles hatch and develop inside of maternal body and cause maternal death. Population development studies of *H. bacteriophora* and *H. indica* in liquid culture indicate that most of offspring DJs originated from *endotokia matricida*. These juveniles finally developed to DJs. This phenomenon has been reported from observations on the population development in cultures of *Steinernema* and *Heterorhabditis*, both in insect hosts and in liquid culture (Balliadi et al. 2001, Johnigk and Ehlers 1999b).

For commercial production, mean yields, process stability and process time are important factors. Many studies have been carried out for the optimization of DJ production of *Heterorhabditis* spp. For *H. bacteriophora*, DJ inoculation timing in bacterial pre-culture and optimal DJ inoculum density were determined (Johnigk et al. 2004, Ehlers 2001). For *H. megidis* aeration rate in bioreactor was determined (Strauch and Ehlers 2000). One of the factors, which cause process failure, is the bacterial phase variation. The symbiotic bacteria occur in at least two physiologically and morphologically distinct phases, named the primary and the secondary phase (Akhurst 1980). According to the description on the secondary phase of *Photorhabdus luminescens*, it is usually found during late stationary growth phase and

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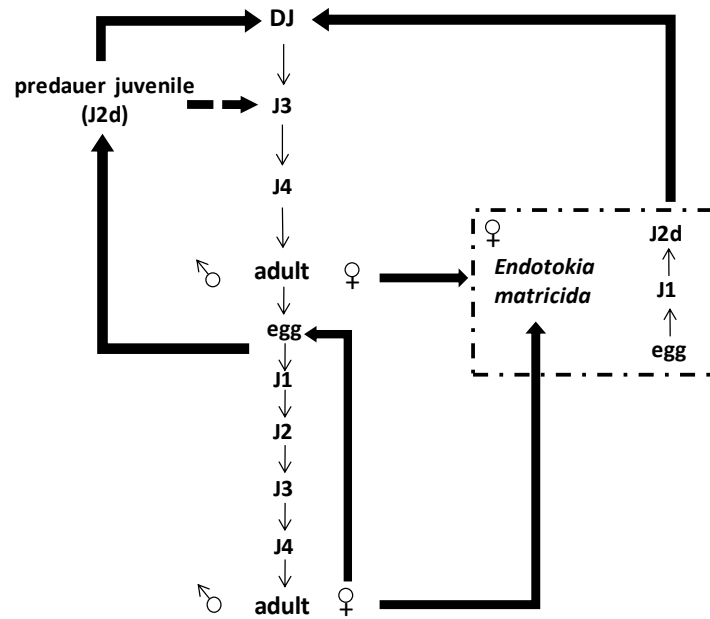


Fig. 2. Life-cycle of *Steinernema* nematodes. DJ; dauer juvenile, J1 – J4; each juvenile stage, ♂; male, ♀; female. J2d; pre-dauer juvenile. J1 juveniles hatch from laid eggs in medium and develop to pre-dauer juvenile (J2d) or hatch and develop to J2d inside the female body (*endotokia matricida*)

also can be induced by cultivating a primary form under osmotic stress conditions (Krasomil-Osterfeld 1995). The primary cells can absorb dye in agar plates containing bromothymol blue or neutral red (Boemare and Akhurst 1988). Primary cells carry inclusion proteins (Couche and Gregson 1987, Bowen and Ensign 2001), and they are one of the major nutritive resources for nematodes (You et al. 2006). The primary phase bacteria produce antibiotic substance, lipase, phospholipase, and protease. Secondary variants lose these characters (Boemare and Akhurst 1988). Nematodes grown on secondary phase yield significantly less DJs than if cultured on primary phase bacteria, particularly in liquid cultures (Han and Ehlers 2001).

DJ recovery is the first important developmental step, since it determines the number



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of adults in first reproduction cycle. When DJs of *C. elegans* are put into fresh food, they respond to cues and recover within 50-60 min. The food signal for *C. elegans* is a neutral, carbohydrate-like substance produced by *Escherichia coli* (Golden and Riddle 1984a). Components of artificial media, like yeast extract, can also induce recovery in *C. elegans* (Golden and Riddle 1984b). In monoxenic liquid culture, recovery is induced by signals produced by the symbiotic bacteria. Generally, DJ recovery and development are not well supported under absence of symbiotic bacteria (Han and Ehlers 2000). However, DJs of *H. bacteriophora* inoculated into bacterial cell free culture supernatants of their symbiotic bacterium react to cues, which are produced by their symbionts and recover up to 80% within 48 h (Strauch and Ehlers 1998). These bacterial secondary metabolites, which support DJ recovery, are called „bacterial food signal”. They are produced by primary phase bacterial cells during exponential growth before entry in the stationary phase of *P. luminescens* (Strauch and Ehlers 1998). Biochemical investigations on the *P. luminescens* food signal indicated that it is composed of negatively charged small molecules (< 50kDa) with different polarity (Aumann and Ehlers 2001). Only specific symbionts can induce DJ recovery of *Steinernema* spp., and non-specific symbionts of *Xenorhabdus* spp. cannot induce it (Sicard et al. 2006). The metabolites excreted by secondary phase cells of *P. luminescens* are not equally sufficient to induce recovery like the metabolites of primary phase cells (Han and Ehlers, 2001). Despite many studies about DJ recovery, the DJ recovery of *Steinernema* spp. in liquid culture has not yet been well described quantitatively, in particular the events during early recovery and the relation with their symbiotic bacteria deserve attention.

The biology of EPN is likely to be influenced by culture temperature. There are numerous studies on the influence of temperature on the infectivity of DJs. However, the temperature range for DJ infectivity does not necessarily overlap with the range for reproduction (Grewal et al, 1994). Generally, the free-living DJ is more tolerant to environmental extremes including temperature than the reproductive stages, which are sheltered in the infected insect. For instance, five isolates of *S. feltiae* infected *G. mellonella* larvae at temperatures ranging from 8 to 28°C, however, there was no DJ offspring observed

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at 28°C (Hazir et al. 2001). The infectivity of *S. carpocapsae* (All strain) and *H. megidis* (H90 strain) DJs to *G. mellonella* larva ranged from 12-16°C, while the highest reproduction rate was assessed at temperatures between 20-24°C. Lower culture temperature can affect the developmental period. For instance, the number of *S. feltiae* DJs was maximal at 15°C, but it took the longest duration until the emergence of DJs from insect cadavers among several culture temperatures (Sanders and Webster 1999). Culture temperature should be optimal not only for nematodes but also for symbiotic bacteria, since they are the major food resource for nematode development. It is often reported that the temperature, which allows greater bacterial production is higher than the optimal temperature for nematode development in liquid culture (Surrey and Davis 1996, Ehlers 2001). Culture temperature effects on *Steinernema* population development and the optimal temperature for DJ production in monoxenic liquid culture for *Steinernema* spp. have not yet been investigated.

The initial inoculum density of population is another important culture condition, which affects reproduction and DJ yields. Density-dependent differences in reproduction were observed in *H. megidis* infecting *G. mellonella* larvae (Ryder and Griffin 2002). Female fecundity was negatively correlated with density across generations. Additionally, high inoculum density induced more DJ offspring than offspring of other juvenile stages in the reproductive cycle. *Steinernema carpocapsae* prefer crowding condition for population development both in insects and in micro-assay liquid culture system (Popiel et al. 1989), indicating that a higher DJ yield could be obtained in cultures initiated with higher density of DJs. When the DJ formation can be induced by starving condition in J1, as it is reported in *H. bacteriophora* (Strauch et al. 1994), food abundance (mainly the presence of symbiotic bacteria) act antagonistically to DJ formation (Golden and Riddle 1984b). A maximum DJ density in the F1 generation therefore depends on the correct balance of nematode population density and nutrient availability at the peak of egg hatching. Another possible important factor is the presence of a crowding pheromone secreted by nematodes themselves, the Dauer Recover Inhibiting Factor (DRIF), firstly reported from populations of *C. elegans* (Golden and Riddle 1984a). This pheromone signals nematode density in the population.

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Density-dependent effects on DJ yield were reported in *H. bacteriophora* liquid culture (Ehlers 2001). Optimal density for the highest DJ yield was assessed in relation to the hermaphrodite density. Densities of 4,000 hermaphrodites ml<sup>-1</sup> resulted in maximum DJ yield. Lower densities induced offspring to develop to another reproductive cycle until they reach a population density inducing DJ formation.

Because the reproduction of *Heterorhabditis* is hermaphroditic, DJ yield estimation can be based on the number of hermaphrodites, which have developed from recovered juveniles. In the amphimictic genus *Steinernema*, however, the sex ratio should also be considered. Sex ratio of *Steinernema* is always female-biased (Grewal et al. 1993, Alsaiyah et al. 2009). Consequently, information on inoculum DJ density-dependent effects on DJ recovery, sex ratio, fecundity of female, nutrient availability on the time for F1 hatching should be necessary for the estimation of eventual DJ yields.

Many of important details of the life cycle in liquid culture have not yet been elucidated for *Steinernema* spp. and most of the descriptions on population development have been done in insect host (Wang and Bedding 1996). The purpose of this study was to analyze the biology of entomopathogenic nematodes, *Steinernema carpocapsae* and *S. feltiae*, with respect to an optimization of liquid culture.

The investigations were divided in four sections.

1. Description of life cycle and population dynamics in liquid culture

2. DJ recovery

DJ recovery was analyzed in relation to their symbiotic bacteria, *Xenorhabdus nematophila* and *X. bovienii*, whether the bacterial food signal and phase variation influence on DJ recovery.

3. Culture temperature effect

Four different culture temperatures were examined for each species in order to determine the influence on each population development, induction of DJ development and final DJ yield.

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### 4. Inoculum density effect

Influence of DJ inoculum density on population development of nematode and symbiotic bacteria was investigated to determine optimal inoculum density. Hence, the biological characteristics of *S. carpocapsae* and *S. feltiae* were compared.

### 2. Results and discussion

#### 2.1. DJ recovery

Dauer Juveniles (DJs) leave the enduring stage after receiving chemical cues, which indicate favorable environmental condition. The developmental step is called „recovery” (Golden and Riddle 1982, Strauch et al. 1994). In the case of entomopathogenic nematodes, following the invasion into a host insect, DJs receive cues from the insect’s hemolymph and recover. At 48 h post DJ invasion into the hemocoel, almost 100% of the DJ of *S. carpocapsae* and *S. feltiae* inoculated by injection or via host penetration in *G. mellonella* recovered, indicating that host penetration is not a pre-requisite for DJ recovery at 48 h (Publication 2, Table 1). In contrast, these two species responded differently to sterile purified serum of *G. mellonella* hemolymph (Publication 2, Table 2). The significantly lower DJ recovery of *S. feltiae* indicated that either the serum of this lepidopteran insect is less well suited for recovery, or *S. feltiae* responds to an instable component in the insect’s hemolymph, which is lost during the preparation of the serum.

In liquid culture, DJ recovery of *Heterorhabditis* spp. is not induced by culture medium, but induced by their symbiotic bacteria secondary metabolites, which are secreted during bacterial growth. These signals are called „bacterial food signals” (Strauch and Ehlers 1998, Aumann and Ehlers 2001). The influence of symbiotic bacteria (*Xenorhabdus* spp.) on DJ recovery of *Steinernema* spp. was investigated. DJ recovery of *S. carpocapsae* and *S. feltiae* is bacterial cell density dependent. Recovery recorded in these experiments usually was between 60 to 90% at higher bacterial density (Publication 2, Fig. 1). In contrast, Strauch and Ehlers (1998) reported that a mean recovery in their symbiont’s liquid culture for two *Heterorhabditis* species ranged between 0 to 81%. This high variability was not observed in the two steinernematid species.

DJ recovery was higher in primary bacterial cultures than in secondary phase cultures (Publication 2, Fig. 3), which might be one reason for culture failure beginning with

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the secondary phase bacterial pre-culture (Ehlers et al. 1990). The DJ recovery in bacterial cell-free culture supernatant indicated that *X. nematophila* and *X. bovienii* also excrete a bacterial food signal into the medium. However, the recovery was lower in supernatants than in bacterial cell suspensions, whereas in heterorhabditids, no difference was recorded between cell suspension and cell-free culture supernatant (Strauch and Ehlers 1998). These results indicate that either the *Xenorhabdus* food signal is instable or only living cells as such trigger recovery or the cells continue to produce food signal despite the presence of antibiotics.

Recovery of both species was reduced in secondary bacterial cells and also in the supernatant of the secondary phase (Publication 2, Fig. 3), confirming results of earlier studies on *S. carpocapsae* and *Heterorhabditis bacteriophora* (Han and Ehlers 2001).

The response of DJs in bacterial cell cultures differs between *S. carpocapsae* and *S. feltiae*. *S. carpocapsae* reacts to bacterial food signal faster than *S. feltiae* (Publication 2, Fig. 4). A delay in recovery can induce non-synchronous population development resulting in longer process duration with multiple generations (Ehlers et al. 1998).

Neither the culture temperature nor the initial DJ density had a significant influence on the recovery (Publication 3, Fig. 1) (Publication 4, Table 3). Only *S. carpocapsae* DJ recovery was reduced at the highest temperature (29°C) tested. In contrast, the recovery of *S. carpocapsae* was affected by DJ age (Publication 4, Table 3). Age-related DJ recovery is reported also in *C. elegans*, in which the response of DJs to environmental cues is highly age dependent, meaning that old DJs exhibit an increased tendency to recover (Golden and Riddle 1984a).

DJ recovery increases with increasing bacterial cell density, but is less or not at all affected by culture condition. Consequently, the DJ recovery is not a good indicator for any determination of optimal culture condition, particularly in *S. feltiae*, whose recovery surpasses 80% at any condition. In contrast, the DJ recovery of *Heterorhabditis* spp. DJs is one of the most important developmental steps, which defines final DJ yield (Strauch and Ehlers 1998). As heterorhabditid nematodes produce amphimictic adults as a response to

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low nematode density (as a result of low recovery), which are unable to copulate and produce offspring in liquid culture (Strauch et al. 1994), low recovery has a direct influence on the DJ yields. The steinernematid liquid culture, in contrast, is less vulnerable for low DJ recovery. Adults can copulate in liquid culture and thus the second generation is potentially able to contribute to the final DJ yield. Thus, any attempts to enhance DJ recovery of *Heterorhabditis* spp. liquid culture is resulting in improved process results, and in particular, the optimization of DJ inoculum timing in relation to the growth phase of the bacterial pre-culture has significantly increased process stability in *Heterorhabditis* spp. cultures (Johnigk et al. 2004). In steinernematid liquid culture this developmental step certainly needs less attention in *S. feltiae*, but might contribute to a better process stability in *S. carpocapsae*, which was variable in DJ recovery in liquid culture. However, this could be handled by assessing DJ recovery prior to inoculation of DJs and adaptation of the inoculum density.

### 2.2. Offspring production

#### 2.2.1. Developmental period in parental generation

The period of offspring hatching and subsequent offspring DJ occurrence depends on the developmental speed of the parental generation. The developmental period of *S. carpocapsae* and *S. feltiae* inoculated in bacterial pre-culture at the cell density of  $10^{10}$  cells  $\text{ml}^{-1}$  with  $4 \times 10^3$  DJs  $\text{ml}^{-1}$  at 25°C is shown in Publication 1, Fig. 1. The first offspring is observed on 4 – 4.5 days post DJ inoculation (dpi) in *S. carpocapsae*, while it took only 3 – 3.5 dpi in *S. feltiae* (see also Publication 3, Table 1). Data on the juvenile development recorded 12-60 h after DJ recovery in bacterial cell cultures of different densities indicated that a higher cell density of the symbiont *Xenorhabdus* spp. enhances juvenile development to adult (Publication 2, Fig. 2). Thus, high bacterial cell density in pre-cultures prior to DJ inoculation is necessary not only to induce high recovery but also trigger the following development to

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adults. Under the same culture condition as described above, the *S. feltiae* adult population peak was recorded on 2 dpi, while the parental female population of *S. carpocapsae* reached the peak only after 3 dpi, which is one day after the peak of the male population (Publication 1, Fig. 2, 3). It indicates that *S. feltiae* juveniles recovered from DJs develop faster than those of *S. carpocapsae*, although the recovery of *S. feltiae* takes 8 hours longer than of *S. carpocapsae* DJs.

Post recovery juvenile development can be affected by culture temperature (Publication 3, Table 1) as well. The development of the parental generation is fastest at 25°C for both *S. carpocapsae* and *S. feltiae*, whereas it is delayed at lower and higher temperatures. The negative influence of non-optimal temperature on nematode development can cause longer culture duration with non-synchronous DJ occurrence.

Developmental speed was not affected by initial DJ inoculum densities. When the culture conditions are comparable, the size of parental females is the same in both species.

### 2.2.2. Sex ratio in adult population

The female-biased sex ratio has been observed in all cultures, regardless of the culture temperature or DJ inoculum density, ranged between 53 – 81% in *S. carpocapsae* and between 64 – 79% in *S. feltiae*, with batch differences (Publication 4, Table 4). It is also reported in populations of other steinernematids and heterorhabditids in vivo and in vitro cultures (Alsayyah et al. 2009, Selvan et al. 1993). According to the report of Johnigk and Ehlers (1999a) for *Heterorhabditis* spp., female-biased adult populations were observed also in following generations, whereas it was observed only in parental generations in this studies of steinernematids.

### 2.2.3. Fecundity of parental female

In contrast to the conserved sex-ratio in parental adults, female fecundity (egg number in



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uterus) is apparently affected by culture conditions. With increasing temperature from 27° to 29°C, the fecundity of *S. carpocapsae* declined significantly. The maximum egg number was counted at 23° and 25°C in *S. feltiae* and a significant decrease of the fecundity was recorded at the lowest and the highest temperature (Publication 3, Fig. 2). This might be due to a lower insemination of the eggs as increasing temperature primarily affects the fertility of the male sperm.

The fertility of parental females decreased with increasing initial DJ densities in both species (Publication 4, Fig. 2). The reduction of offspring production per female due to high nematode density is also observed in *H. megidis* cultured in *G. mellonella* (Ryder and Griffin 2002). With an increasing female density (increased inoculum densities) and a constant bacterial cell density, females at higher density dispose of less nutritive resource. In other words, females at lower density with abundant resources are larger and produce more offspring, whereas females at higher density with less resource are smaller and also produce less offspring. Variable size of parental adults was also recorded by Johnigk and Ehlers (1999b) in heterorhabditid cultures. It represents a compensation mechanism, which enables the nematodes to react to changing nutritive environments. At low population density and abundant food, the parental females are large and produce many offspring, whereas at high female density they are shorter and less fertile. This is also the reason why the daily increase in DJ yield did not change with variable population density.

### 2.3. DJ yield

In the culture with an initial density of  $4 \times 10^3$  DJs ml<sup>-1</sup> incubated at 25°C, the first DJs are observed on 6 dpi in *S. feltiae* and 7 dpi in *S. carpocapsae* (Publication 1, Fig. 1). The mean DJ yield was always higher in *S. carpocapsae*, at  $158 \times 10^3$  ml<sup>-1</sup>, than in *S. feltiae*, at  $106 \times 10^3$  ml<sup>-1</sup> on 15 dpi. A continuous increase of DJ offspring is observed in *S. carpocapsae* from the first DJ occurrence until 15 dpi, in contrast, the DJ yield in *S. feltiae* reaches the maximum density around 10 dpi, indicating that most DJs in *S. feltiae* cultures are F1 offspring. The

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rapid increase of DJs in both species indicates that most DJs are the offspring of the parental generation.

Although the DJ density is lower in *S. feltiae*, the total biomass of the *S. feltiae* DJ yield is larger than of *S. carpocapsae* cultures because of the difference in DJ size (Publication 1, Table 1 and Fig. 4). The mean size of *S. carpocapsae* DJs is 558  $\mu\text{m}$  and of *S. feltiae* 849  $\mu\text{m}$  (Adams and Nguyen 2002). Hence, the size of the DJ is conserved and not affected by the culture condition and nutrition.

### 2.3.1. *Endotokia matricida*

Comparing the huge number of pre-dauer juvenile (J2d) with the number of J1 in the F1 generation, it is assumed that most J2d originate from the juveniles, which hatched inside of the female rather than from J1, which hatched from eggs laid into the medium. Intra-uterus juvenile hatching, called *endotokia matricida*, is often an observed phenomenon in rhabditid nematodes. Detailed observation of *endotokia matricida* in *Heterorhabditis* spp. indicated that juveniles, which hatched inside the uterus, always develop to DJs (Johnigk and Ehlers 1999b). The progress of the *endotokia matricida* of *Steinernema* spp. in liquid culture has not yet been described.

The chronological development of the *endotokia matricida* is described in Publication 1, Fig. 5. No major difference in the development of the *endotokia matricida* was observed between *S. carpocapsae* and *S. feltiae*. But differences were recorded when comparing the development in *Heterorhabditis bacteriophora* and *H. indica* in liquid culture reported by Johnigk and Ehlers (1999b). J2ds of *Heterorhabditis* were motionless and never left the maternal carcass until molting to DJs. Baliadi et al. (2001) also reported a complete development of J2d to DJ inside of maternal carcass in *S. carpocapsae* and *H. bacteriophora* when reared in *G. mellonella* larvae. *S. carpocapsae* J1 hatching inside the uterus developed to DJs within 18 hours. In contrast, juveniles of both *S. carpocapsae* and *S. feltiae* in this study developed only until the J2d stage inside the uterus and the development lasted 12 – 21

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hours. The J2d completed their development to DJs outside the carcass of the dead female. Whether this is specific for in vitro liquid culture needs further confirmation. This observation has consequences for the identification of the harvest time as J2ds or DJs which have not readily developed can die during downstream processing of the liquid culture DJ yield.

### 2.3.2. Influence of culture condition on DJ yield

From the aspect of process improvement for mass production, the most considerable factor is DJ yield. In order to determine the most favorable culture condition, the influence of culture temperature and the inoculum DJ density were investigated. The DJ density was counted on 10 and 15 dpi.

The DJ yield was significantly different among different culture temperatures (Publication 3, Fig. 3). On 10 dpi, the highest DJ density was observed on 25°C in *S. carpocapsae* and it decreased significantly at 27°C. At 23 and 29°C, fewer DJs were recorded. The DJ density of *S. feltiae* was significantly higher at 23° and 25°C, than at 20° and 27°C. Comparing the non-DJ population in cultures at high and low non-optimal temperatures, most of nematodes were in the J2d stage at lower temperature, while they had developed to adults at higher temperature. DJ density of both species increased from 10 to 15 dpi. On 15 dpi, significant difference of DJ density was observed only in *S. carpocapsae* and not observed in *S. feltiae*. The low DJ density at higher temperature of both species is probably the result of reduced fecundity in the parental generation.

When comparing the variability of the DJ yields between the culture temperatures, the variability of the DJ proportion in *S. carpocapsae* deviates considerably from the mean only at 27°C. Such variability can have serious consequences for liquid culture production. A temperature shift of a few degrees can cause such high changes indicating that *S. carpocapsae* is much more sensible to variable temperature than *S. feltiae*.

The influence of initial inoculum density on the DJ yield was investigated in cultures at 25°C, beginning with a bacterial pre-culture adjusted to a cell density of  $10^{10}$  ml<sup>-1</sup> (only in

## Results and discussion

*X. nematophila*). Subsequent DJ yields were assessed on 10 and 15 dpi (Publication 4, Fig. 3). The data are presented in relation to the female density to avoid an influence of variable DJ recovery between batches. On average, the highest DJ density in *S. carpocapsae* on 15 dpi was  $175 \times 10^3$  DJs  $\text{ml}^{-1}$  in cultures containing  $2 \times 10^3$  females  $\text{ml}^{-1}$  (inoculum density  $3\text{-}6 \times 10^3$   $\text{ml}^{-1}$ ). No significant density dependent effect of the inoculum on *S. feltiae* cultures was observed. Cultures with a parental female density of  $2\text{-}4 \times 10^3$   $\text{ml}^{-1}$  (inoculum density  $6\text{-}7 \times 10^3$   $\text{ml}^{-1}$ ) produced the highest DJ yields.

### 2.3.3. Infectivity of offspring DJ

The infectivity of harvested DJs reared at different temperatures brought a new aspect. It has been reported that infectivity differs among different bioassay temperatures (Dunphy and Webster 1986, Grewal et al. 1994 and 1996, Wright 1992, Zervos et al. 1991), but results of this study indicate that the incubation temperature during DJ formation can also affect the DJ infectivity. The mean mortality recorded in this study was lower than 50% with both, *S. carpocapsae* and *S. feltiae*. DJs of both species reared at lower temperatures had the lowest infectivity (Publication 3, Fig. 5). Since all the bioassays were performed at 25°C, the difference of infectivity was not because of bioassay temperature, but because of the physiological difference of the DJs from cultures incubated at different process temperature. The population development of both species at the lowest temperature was the slowest, thus DJs of both species reared at the lowest temperature were physiologically younger than those that had been reared at higher temperatures. Hence the bacteria cells stored in the vesicle of DJs might not have multiplied yet inside the DJs and as consequence caused lower mortality among tested insects. Martens et al. (2003) reported that during the early phase of bacterial retention few cells are encountered in the DJ intestine of *S. carpocapsae* and that the cells grow and divide until filling up the DJ vesicle. The results of this study suggest that the DJ harvest period should be well timed in order to avoid impacts on DJ infectivity.

### 2.4. Population dynamics in filial generations

In the study on the life cycle (Publication 1) and also on the influence of the inoculation density on yields (Publication 1) the analysis of the population dynamics revealed a major difference between *S. feltiae* and *S. carpocapsae*. In *S. carpocapsae* cultures nearly all offspring of the parental adults developed to DJs, whereas in *S. feltiae* a significant second and third generation of adults was observed. Consequently, the DJ proportion after 15 days was 90.4% in *S. carpocapsae* and only 89.1% in *S. feltiae* cultures. The rest of the population consisted of J2d in *S. carpocapsae* and mainly of adults in *S. feltiae* cultures. It looks like second and third generation adults in *S. feltiae* cultures did not contribute to the DJ yield until day 15, as no increase of the J1, J2 and J2D population was recorded together with the increase of the F1 and F2 adult generations. Offspring production by filial *S. feltiae* generations seems to be negligible. This is particularly surprising when comparing their number with the number of parental females, which were recorded at much lower density.

In order to shorten the process time, the occurrence of a F1 adult population should be prevented in mass production. Another reason also favors the production within one generation. The presence of F1 adults in the DJ yields causes major problems during downstream processing. In the final product, DJs are packed at high density of 60 million in 50 g of formulation powder. Since all non-DJ stage nematodes rapidly die during mixing of the final formulation, their cadavers provide a source for the growth of contaminants, which spoil DJ products. Consequently, non-DJ stages need to be removed from the DJ suspension, which causes technical problems.

The production of the DJs within a one generation process is possible with *S. carpocapsae* providing the initial adult nematode density can be controlled by adjusting the DJ density (Publication 4, Fig. 4). Two generation processes are also possible in this nematode. In the culture with parental female density  $< 2 \times 10^3 \text{ ml}^{-1}$ , F1 female density surpassed the parental female density (Publication 4, Fig. 6A), but can be avoided by higher inoculation density.

## Results and discussion

However, the observations of the population dynamics of *S. feltiae* in several cultures indicate that the occurrence of a considerable number of F1 adults is the rule (Publication 1, Fig. 3), and cannot be controlled by the initial inoculum density (Publication 4, Fig. 6B). Thus it is difficult to reduce the process time. However, it would still be acceptable should the second generation (F1) adults contribute to the DJ yield. But since no increase of the J1, J2 and J2d density was observed with increase of F1 adult population, adults in following generation of *S. feltiae* seem to be unable to produce considerable number of DJ offspring. The reason for the occurrence of a second generation of adults in *S. feltiae* needs further consideration. The question is, why the J1 offspring of the parental generation do not readily develop to DJ and instead develop to a F1 adult population?

Generally, there are two antagonistic substances affecting DJ formation in nematode cultures, a „food signal“ which is known to trigger DJ recovery (Strauch and Ehlers 1998) and a „dauer recovery inhibiting factor (DRIF)“, which inhibits DJ recovery and induce DJ formation. The pheromone DRIF is described from *C. elegans* and is secreted by all developmental nematode stages (Golden and Riddle 1984b). The concentration of this pheromone thus is nematode density dependent. Fodor et al. (1990) reported of results that this pheromone is also produced by *S. carpocapsae*. The balance of bacterial food signal and the density of DRIF in the culture define DJ formation. Thus one would expect that the higher the nematode density, the higher is the DJ formation within the F1 generation. But this was not observed in *S. feltiae*. So, is this nematode deficient in the production of DRIF? Probably not, the reason for the high F1 adult population development is probably due to the high bacterial density resulting in a high concentration of food signal(s), which are antagonistic to DRIF. But the question remains, what caused the bacterial population to increase again although one would expect a typical growth curve with a stationary phase. Instead the population of *X. bovienii* even surpassed the density of the initial population at DJ inoculation. This late growth is probably the key for further process improvement in *S. feltiae*.

### 2.5. Optimal culture condition for *S. carpocapsae* and *S. feltiae*

## Results and discussion

The results of the investigations on influence of different factors on the DJ yield and the description of the life cycle of *S. carpocapsae* and *S. feltiae* in liquid culture under variable culture parameters can be useful to contribute to the further definition of optimal culture condition. High and stable recovery is induced by bacterial pre-culture containing high density bacterial populations. The cell density should surpass  $10^{10}$  cells ml<sup>-1</sup>. Another possible point for improvement is an investigation on the influence of DJ age on the recovery of *S. carpocapsae*. In order to avoid low recovery in mass production, the recovery of inoculum DJ should be checked prior to DJ inoculation. Optimal culture temperature for the development of all stage of nematodes is 25°C for both species. At this temperature, DJ population increases constantly without higher variability in DJ yields. Optimal DJ inoculum density was  $3-6 \times 10^3$  DJs ml<sup>-1</sup> for *S. carpocapsae* and  $6-7 \times 10^3$  DJs ml<sup>-1</sup> for *S. feltiae*. The culture started with this DJ inoculum density produced the highest DJ densities.

This study has elucidated the life cycle and population dynamics of two steinernematids and recommended the improved culture conditions. It can also be used to define objectives for further investigations. An improvement of the culture medium for *S. feltiae* and *X. bovienii* is recommended to increase the DJ density and avoid the high F1 adult populations. Culture medium improvement is also possible for *S. carpocapsae*. Increased bacterial cell density in pre-culture can provide higher DJ yield. However, it might require higher inoculum density, since the DJ yield of  $300 \times 10^3$  ml<sup>-1</sup> was obtained with an inoculum density of  $8 \times 10^3$  DJs ml<sup>-1</sup> (Han 1996). Thus, also in *S. carpocapsae* there is room for improvements. On the other hand, remarkable difference in the life cycle between the two species suggests that entomopathogenic nematodes might have evolved species-specific population development strategies. In order to optimize culture condition, detailed descriptions of the life cycle are required for each species.

# Conclusions

## 3. Conclusions

Mass production of entomopathogenic nematodes in liquid culture has three major objectives:

- Production of high DJ yields
- Production within a short process time
- High quality DJs

In order to further extend the use of entomopathogenic nematodes in biological control, nematode-based products should be cheap to be compatible with other control measures. Thus the production process is a major factor for achievement of these goals.

The key points for process instability are:

- Growth of secondary symbiont cells
- Low DJ recovery
- Low number of offspring of the parental adults
- High number of non-DJ stages at process end

This investigation contributed to the understanding of the life cycle of *S. carpocapsae* and *S. feltiae* and the role of bacterial density on nematode development. It also provided results to improve process conditions in terms of defining optimal process temperature and DJ inoculum density.

Although the technology for liquid culture mass production has been established, there still is room for improvements in order to reduce product costs even more and make available these nematodes also for low value agriculture crops. Process stability and yield variability still are problems which need to be solved.

Based on the presented results we can conclude that the first process step for further improvements is the investigation of the bacterial growth particularly of *X. bovienii* in order



## Conclusions

to avoid the occurrence of higher F1 generation adult populations. Although the results indicate that a density above  $10^9$  cells  $\text{ml}^{-1}$  of *X. nematophila* is sufficient for *S. carpocapsae*, and  $10^{10}$  cells  $\text{ml}^{-1}$  of *X. bovienii* for *S. feltiae*, reaching even higher cell densities should be a major focus of future activities. Only primary phase bacteria induced efficient DJ recovery. The preservation of the symbiont culture in the primary phase is another factor which will need attention and should be taken into consideration when improving growth of the bacterial pre-culture. However, the major question, which evolved from these studies is, why the culture of *X. bovienii* continues growth reaching an even higher density than the initial bacterial culture. The key to the reduction of the *S. feltiae* process time is in the prevention of this second population peak. In addition, it will provide easier conditions for downstream processing of the DJs.

DJ recovery is less of a problem in both species when compared with *Heterorhabditis* spp. *S. feltiae* DJ recovery is always higher than 80% regardless of the culture temperature and inoculum DJ density. In contrast, *S. carpocapsae* reacts more sensibly to culture condition and investigations on the DJ recovery might yield fruitful improvements. However, as parental females can compensate with increasing offspring production at lower density, the DJ recovery is not a major focus for the improvement of *Steinernema* liquid cultures.

Optimal culture temperature, at 25°C for both species, is of course just one process parameter. All investigations have been made in flask cultures. The improved conditions will need to be confirmed also in bioreactors and using these machines, which allow to change also other process parameters, additional improvements would be possible.

This contribution had a focus on culture conditions and less on DJ quality. But the few results obtained on DJ quality already indicate that this parameter will need attention during any kind of process improvement in the future.

# Summary

## 4. Summary

Entomopathogenic nematodes (EPN) *Steinernema carpocapsae* and *S. feltiae* (Rhabditida: Nematoda) are symbiotically associated with the bacteria *Xenorhabdus nematophila* and *X. bovienii*, respectively. These bacto-helminthic complexes are widely used in biological control of economically important pest insects. One problem restricting a wider use of EPN in plant protection is high production costs. Although the mass production technology in liquid culture has been established for commercial purposes more than 15 years ago, process instability still causes variability in yields. In this thesis, the critical developmental steps of *Steinernema carpocapsae* and *S. feltiae* in monoxenic liquid culture were investigated.

The infective stage of EPN is a so called dauer juvenile (DJ), which is a developmentally arrested third juvenile stage. Mass production of EPN in liquid culture media starts with a culture of their symbiotic bacteria prior to DJ inoculation. DJ recovery is the first developmental step to the fourth juvenile stage of the nematodes. Major reasons for process variability are reduced DJ recovery and variable nematode population development. In order to improve the culture process, this contribution investigated the life cycle of *S. carpocapsae* and *S. feltiae* in monoxenic liquid culture, recorded the bacterial growth and the influence of the bacterial symbionts on DJ recovery and population development and studied the influence of culture temperature and variable inoculation density on nematode development and DJ yields.

The liquid culture process can be subdivided into the following steps:

1. Pre-culture of the symbiotic bacteria (*Xenorhabdus* spp.)
2. Inoculation of DJs
3. Exit of the DJs from the dauer stage (so called recovery) and development to

males and females

4. Development of new DJs from the offspring of parental adults
5. Harvest of the new DJs after about 15 days post DJ inoculation

The recovery is induced by specific chemical cues, indicating a suitable environment for the development of EPN. These substances can be found in insects and cultures of the symbiotic bacteria, which are the essential food resource for the nematodes. Almost 100% of the DJs recovered in insects within 24 h. In liquid culture the synchronicity of the recovery process and the proportion of recovered DJ were dependent on the bacterial cell density. At  $10^{10}$  cells ml<sup>-1</sup> recovery of 80 % after 12 h was observed for both nematode species. Results on the nematode population dynamics indicated that bacterial cells at a higher density induce not only a higher DJ recovery, but also accelerate the development of recovered juveniles to adults.

The symbiotic bacteria often develop to so-called secondary phase cells, which are less supportive for nematode reproduction. In cell suspensions and supernatants of secondary cells the DJ recovery was reduced. The time at which 50% of the population had recovered after exposure to the bacteria was longer ( $RT_{50} = 17.1$  h) in *S. feltiae* than for *S. carpocapsae* ( $RT_{50} = 6.6$  h). Whereas > 90% *S. carpocapsae* DJs recovered in haemolymph serum of the lepidopteran insect *Galleria mellonella*, recovery of *S. feltiae* only reached 31%. Penetration into a host insect prior to exposure to the insect's food signal did not enhance DJ recovery.

The life cycle of the two nematode species in monoxenic liquid culture and the growth of their symbiotic bacteria were extensively investigated. To distinguish between the different juvenile and adult stages, their size was recorded. No differences were observed between the species in the size of the juvenile stages but significant differences were recorded in the length of the adults, pre-dauer (J2d) and dauer juvenile stages (DJ). On average, 90% of

## Summary

inoculated DJs of *S. feltiae* recovered and 77% of *S. carpocapsae*. In general, *S. feltiae* developed approximately one day faster than *S. carpocapsae*. The sex ratio was female-biased ( $59.2\% \pm 2.2$  in *S. carpocapsae*,  $66.7\% \pm 2.6$  in *S. feltiae*) in the parental population, but not in the F1 generations.

Steinernematid adults respond to depleting food resources with the cessation of egg-laying. Juveniles hatch inside the uterus and develop on cost of the maternal body content causing the death of the adult (*endotokia matricida*). In contrast to in vivo observations of steinernematids by other authors, who reported that readily developed DJs leave the carcass of the dead adult, in this study J2d emerged 12 hours after cessation of egg-laying.

The density of both bacterial cultures decreased due to feeding of the parental juveniles. However, other than *X. nematophila*, which continued at very low density, the density of *X. bovienii* increased again until 15 dpi. The vast majority of F1 offspring of *S. carpocapsae* developed to DJs whereas in *S. feltiae* a significant second and third generation of adults was observed, probably due to the increasing bacterial population. However, second and third generation adults in *S. feltiae* cultures did not significantly contribute to the DJ yield. Mean yields of  $158 \times 10^3$  DJs ml<sup>-1</sup> were recorded for *S. carpocapsae* and  $106 \times 10^3$  DJs ml<sup>-1</sup> for *S. feltiae*.

In order to determine optimal culture temperature for *S. carpocapsae* and *S. feltiae* the development of nematodes was observed at four different temperatures (*S. carpocapsae*: 23-29°C, *S. feltiae*: 20-27°C). No temperature effect was observed on DJ recovery of *S. feltiae*, while *S. carpocapsae* DJ recovery was significantly reduced at 29°C. The fecundity (assessed by the number of eggs in the uterus) in *S. carpocapsae* reached a maximum at 27°C and was significantly reduced at 29°C. The fecundity of *S. feltiae* was high at 23°C and 25°C and reduced at 20°C and 27°C. The offspring is observed earlier in cultures at 25°C in both

## Summary

species, indicating faster development of parental generation at this temperature. Also highest yields were observed at this temperature. The infectivity of *S. feltiae* was highest after production at 25°C and at 27°C for *S. carpocapsae*.

In commercial production of EPN a short process time is saving costs. The shortest possible process is a one-generation process. Inoculated DJs develop to adults and their offspring again to DJs. This development is induced during the J1 stage as a response to depleting food resources (low bacterial density). An alternative developmental pathway is the development to adults as a response to high food resources. The development is thus driven by the density of the symbiotic bacteria culture and the nematode population density. In order to determine the optimal inoculum density, the density-dependent effects on population development and DJ productivity were investigated. A major difference observed again between *S. feltiae* and *S. carpocapsae* cultures was that in the former, besides DJs, always produced second and third generations of adults. An influence of the DJ inoculum density was not recorded. In contrast, in *S. carpocapsae* cultures only at a very low inoculum density or low DJ recovery (low density of females  $< 2000 \text{ ml}^{-1}$ ) a second generation of adults developed. The difference between the species is possibly due to the different development of the symbiotic bacteria. The density of both bacteria decreased due to feeding of the nematodes after recovery. However, *X. bovienii* cell density increased again until day 15 post inoculation, whereas the *X. nematophila* cell density usually remained at low densities. The second growth, often surpassing the initial bacterial density, probably induces the development to a large number of adults in the second generation in *S. feltiae* – *X. bovienii* cultures.

A strong effect of inoculum density on the fertility of the parental females was recorded. Since the parental female density increased with increasing inoculum densities while the bacterial concentration remained constant, females disposed of less nutritional

## Summary

resources with increasing densities. Indeed, the bacterial cell density decreased more rapidly in cultures with higher populations. The egg number in the uterus per female was negatively correlated with the inoculum density. The number of offspring in a culture and in this way the DJ yield is determined by the initial number of females.

For *S. carpocapsae* the optimal DJ inoculum density should be between  $3$  and  $6 \times 10^3$  DJs  $\text{ml}^{-1}$  in order to obtain  $> 10^3$  parental females  $\text{ml}^{-1}$ . However, this species shows a high batch to batch variability regarding the predisposition of the DJs to recover from the dauer stage. Therefore, the DJ recovery in a batch to be used should be tested prior to inoculation. *S. feltiae* was less variable in DJ recovery usually reaching  $> 90\%$ . The recommended DJ inoculum density for this species is  $> 5 \times 10^3$  DJs  $\text{ml}^{-1}$ .

The results provide valuable information to define objectives for further investigations on the population dynamics and possible steps for future process improvements.

## 5. Zusammenfassung

Entomopathogene Nematoden (EPN) *Steinernema carpocapsae* und *S. feltiae* leben in einer engen Symbiose, jeweils mit *Xenorhabdus nematophila* oder *X. bovienii*. Diese Nematoden-Bakterien-Komplexe werden im biologischen Pflanzenschutz zur Bekämpfung ökonomisch bedeutender Schädlinge eingesetzt. Ein Problem, welches einen weiter verbreiteten Einsatz von EPN im Pflanzenschutz bisher verhindert, sind die hohen Produktionskosten. Obwohl die Technologie zur Massenproduktion in Flüssigkultur schon seit mehr als 15 Jahren Standard für die kommerzielle Herstellung ist, bestehen immer noch Probleme hinsichtlich der Prozessstabilität und der Variabilität der Erträge. In der vorliegenden Arbeit wurden die kritischen Entwicklungsschritte von *Steinernema carpocapsae* und *S. feltiae* in monoxenischer Flüssigkultur näher untersucht.

Das infektiöse Stadium der Nematoden ist die Dauerlarve (DL), ein drittes Juvenilstadium, das in diesem Entwicklungsstadium verharrt, bis es geeignete Bedingung für Wachstum und Vermehrung findet. Die Flüssigkultur beginnt mit der Kultivierung der symbiotischen Bakterien, bevor die DL inokuliert werden. Der erste Entwicklungsschritt in Flüssigkultur ist die so genannte „recovery“, d.h. die Aufhebung des Dauerstadiums und die Entwicklung über die J4 zu adulten Nematoden. Wichtige Gründe für die Instabilität des Produktionsprozesses sind eine reduzierte „recovery“ der inokulierten DL und eine variable Populationsentwicklung der Nematoden. Mit dem Ziel, die Kulturbedingungen zu verbessern, wurden im Rahmen der vorliegenden Arbeit der Lebenszyklus von *S. carpocapsae* und *S. feltiae* in Flüssigkultur untersucht, gleichzeitig das Bakterienwachstum beobachtet, der Einfluss der bakteriellen Symbionten auf die DL „recovery“ und die Populationsentwicklung untersucht und der Einfluss von Temperatur und unterschiedlicher Dichte des DL Inokulums

## Zusammenfassung

auf die DL Ernte beleuchtet.

Der Flüssigkulturprozess kann in folgende Schritte unterteilt werden:

1. Vorkultur der symbiotischen Bakterien (*Xenorhabdus* spp.)
2. Inokulation von Dauerlarven (DL)
3. Weiterentwicklung der DL zu Männchen und Weibchen
4. Entwicklung neuer DL aus den Nachkommen der inokulierten DL
5. Ernte der neuen DL 15 Tage nach der Inokulation

Die „recovery“ wird von chemischen Signalen induziert, die geeignete Umweltbedingungen für die Entwicklung von EPN anzeigen. Diese Substanzen wurden sowohl in Insekten als auch in Kulturen der bakteriellen Symbionten nachgewiesen, die die essentielle Nahrungsquelle für die Nematoden darstellen. In Insekten entwickeln sich nahezu 100% der DL innerhalb von 24 Stunden. In Flüssigkultur waren die Synchronität der „recovery“ und der Anteil sich weiterentwickelnder DL von der Zelldichte der Bakterienkultur abhängig. Bei  $10^{10}$  Bakterienzellen/ml konnte eine „recovery“ von annähernd 80% bei *S. feltiae* und über 80 % bei *S. carpocapsae* innerhalb von 12 Std. beobachtet werden. Die Versuche mit unterschiedlichen Bakterienzeldichten zeigten auch, dass eine hohe Bakterienzeldichte nicht nur die „recovery“ fördert, sondern auch die Entwicklung der Nematoden zu Adulten beschleunigt.

Die symbiotischen Bakterien entwickeln sich oft zu sogenannten Sekundärformen, die die Nematodenvermehrung nicht ausreichend unterstützen. In Zellsuspensionen und Überständen dieser Sekundärform war die DL „recovery“ reduziert. Die Zeit, zu der 50% der DL in Entwicklung gegangen waren, war länger bei *S. feltiae* ( $RT_{50}=17,1$  Std.) als bei *S. carpocapsae* ( $RT_{50}=6,6$  Std.). Während > 90% der DL in Hämolympenserum der Lepidoptere *Galleria mellonella* in Entwicklung gingen, waren es bei *S. feltiae* nur 31%. Der



## Zusammenfassung

Eindringungsvorgang in das Insekt steigert die „recovery“ der DL nicht.

Der Lebenszyklus der zwei Nematodenarten in monoxenischer Flüssigkultur und das Wachstum der symbiotischen Bakterien wurden eingehend beobachtet. Um die verschiedenen Stadien unterscheiden zu können, wurden Größenmessungen vorgenommen. Bei der Größe der Juvenilstadien wurde zwischen den Arten keine Unterschiede registriert, wohl aber bei der Länge der Adulten, Prä-Dauerlarven (J2d) und den DL. Im Durchschnitt entwickelten sich 90% der inokulierten DL nach Inokulation von *S. feltiae* aber nur 77% von *S. carpocapsae*. Im Allgemeinen ist die Entwicklung von *S. feltiae* einen Tag schneller als die von *S. carpocapsae*. Das Geschlechtsverhältnis in der Parentalgeneration ist zu Gunsten der Weibchen verzerrt ( $59,2\% \pm 2,2$  bei *S. carpocapsae*,  $66,7\% \pm 2,6$  bei *S. feltiae*), nicht aber in der F1 Generation.

Steinernematide Adulte reagieren auf sich verschlechternde Ernährungsbedingungen mit dem Einstellen der Eiablage. Die Juvenilen schlüpfen im Uterus aus dem Ei und entwickeln sich auf Kosten des Muttertiers, was letztendlich im Tod der Mutter endet (*endotokia matricida*). Im Gegensatz zu Beobachtungen anderer Autoren, die davon berichten, dass vollständig entwickelte DL die Hülle des toten Weibchens verlassen, wurde in dieser Untersuchung beobachtet, dass 12 Std. nach Beendigung der Eiablage Prä-Dauerlarven die Hülle verlassen.

Die Dichte der Bakterienkulturen ging aufgrund der Nahrungsaufnahme der Elternpopulation bei beiden Nematodenkulturen zurück. Anders als bei *X. nematophila*, die dann mit einer sehr geringen Zelldichte beobachtet wurden, stieg die Zelldichte bei *X. bovienii* bis zum 15. Tag wieder an. Der bei weitem größte Teil der F1 Nachkommenpopulation von *S. carpocapsae* entwickelte sich zu DL, während bei *S. feltiae* eine bedeutende zweite und dritte Adultgeneration festgestellt wurde, die sich wahrscheinlich

## Zusammenfassung

aufgrund der ansteigenden Bakterienkultur entwickelten. Allerdings trugen diese Generationen nicht wesentlich zum DL Ertrag bei. Die mittleren Erträge lagen  $158 \times 10^3$  DL/ml bei *S. carpocapsae* und bei  $106 \times 10^3$  DL/ml bei *S. feltiae*.

Um die optimale Temperatur für die Flüssigkultur zu bestimmen, wurden Versuche bei jeweils 4 verschiedenen Temperaturen durchgeführt (*S. carpocapsae*: 23-29°C, *S. feltiae*: 20-27°C). Die untersuchten Temperaturen hatten bei *S. feltiae* keinen Einfluss auf die „recovery“, während bei *S. carpocapsae* die „recovery“ bei 29°C signifikant zurückging. Die Fertilität (bestimmt anhand der Eier pro Weibchen) war bei *S. carpocapsae* bei 27°C am höchsten und bei 29°C am niedrigsten. Bei *S. feltiae* lag das Temperaturoptimum bei 23-25°C. Erste Nachkommen (J1) konnten bei beiden Nematodenarten am frühesten bei 25°C beobachtet werden. Auch der Dauerlarvenерtrag nach 15 Tagen war bei dieser Temperatur jeweils am höchsten. Die Infektiosität der DL war bei 25°C produzierten *S. feltiae* und bei 27°C produzierten *S. carpocapsae* am höchsten.

Ziel bei der kommerziellen Produktion von EPN ist es den Dauerlarvenерtrag in möglichst kurzer Zeit zu produzieren, d.h. in einem Ein-Generationenprozess. Die geernteten DL sind dabei die direkten Nachkommen der inokulierten DL. Diese Entwicklung wird während des J1 Stadiums induziert und ist eine Reaktion auf schlechte Ernährungsbedingungen. Ein alternativer Entwicklungsweg ist die Bildung von Adulten aufgrund guter Ernährungsbedingungen. Die Entwicklung wird also von der Dichte der Bakterien und der Nematoden bestimmt. Es wurden Versuche mit unterschiedlicher Inokulumdichte durchgeführt, um dichteabhängige Effekte auf die Populationsentwicklung und die Nachkommenproduktion in Flüssigkultur zu untersuchen. In *S. feltiae* Kulturen wurden wiederum, unabhängig von der Inokulationsdichte, immer 2. und 3. Generationen von Adulten beobachten. Bei *S. carpocapsae* war dies nur bei niedrigen Inokulationsdichten zu

## Zusammenfassung

beobachten oder bei niedriger „recovery“, die eine Weibchendichte von weniger als 2000/ml in der ersten Generation zur Folge hatte. Dieser Unterschied zwischen den Arten liegt möglicherweise in dem unterschiedlichem Wachstum ihrer bakteriellen Symbionten begründet. Bei beiden Arten ging die Bakteriendichte wiederum durch die fressenden Nematoden zurück. Bei *X. bovienii* stieg die Zelldichte allerdings bis zu 15. Tag wieder an, während sie bei *X. nematophila* niedrig blieb. Die erneute Zunahme der Bakteriendichte, die sogar noch die Anfangsdichte überstieg, war wahrscheinlich der Grund für das Auftreten einer zweiten Generation adulter Tiere in *S. feltiae* – *X. bovienii* Kulturen.

Die Inokulumsdichte hatte einen starken Einfluss auf die Fertilität der Weibchen. Mit zunehmender Weibchendichte steigt die Nahrungskonkurrenz in den Kulturen und es konnte beobachtet werden, dass die Bakteriendichte dementsprechend schneller abnimmt. In Folge war die Anzahl der pro Weibchen negativ korreliert mit der Inokulumsdichte. Dies hatte wiederum zur Folge, dass der DL Ertrag trotz steigender Anzahl der Weibchen ab einer bestimmten Dichte nicht mehr zunahm.

Anhand dieser Beobachtungen konnte ein Bereich für die optimale Inokulationsdichte abgeleitet werden. Für *S. carpocapsae* liegt der optimale Bereich zwischen 3,000-6,000 DL ml<sup>-1</sup>, um ca. 3,000 Weibchen ml<sup>-1</sup> zu bekommen. Allerdings zeigte sich auch, dass es bei *S. carpocapsae* eine große Variabilität hinsichtlich der Prädisposition der DL für die „recovery“ geben kann. Um dadurch bedingte niedrige Weibchendichten zu Beginn der Kultur zu vermeiden, sollten die DL dieser Art vor der Inokulation hinsichtlich der „recovery“ getestet werden. *S. feliae* war weniger variabel in der „recovery“ mit meist >90%. Die empfohlene Inokulumsdichte liegt bei > 5,000 DL ml<sup>-1</sup>.

Die Ergebnisse stellen wertvolle Informationen zur Verfügung, die genutzt werden können, um weitere Forschungsziele über die Untersuchung der Populationodynamik und

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Schritte für eine zukünftige Prozessoptimierung definieren zu können.

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Publication 1

**Life cycle and population development of entomopathogenic nematodes *Steinernema carpocapsae* and *S. feltiae*, (Nematoda, Rhabditida) in monoxenic liquid cultures**

Ayako Hirao, Ralf -Udo Ehlers and Olaf Strauch

In press in Nematology

# Publication 1

Corrections to galley proof

- In Introduction, Page 2, 2nd Paragraph, Line 2,

“(e.g., Ehlers et al., 1998; Ehlers, 2000, 2001; Han &”

Replace “; Ehlers, 2000, 2001” with “, 2000; Ehlers, 2001”

- In Materials and methods, Page 2, 2nd Paragraph, Line 13,

“...1% of a bacterial culture adjusted to  $10^9$  cells ml<sup>-1</sup> and...”

Replace “9” with “7”

- In Results, Page 4, 1st Paragraph, Line 1,

“in Figure 1A”

Erase “A”

- In Results, Page 4, 5th Paragraph, Line5,

“...This happened 1 day earlier than in S....”

Replace “earlier” with “later”

- In Results, Page 5, 2nd Paragraph, Line8,

“... less volume (67.7 mm<sup>3</sup> ml<sup>-1</sup>) than *S. feltiae* (107.6 mm<sup>3</sup>...”

Replace “67.7” with “15.1” and also replace “107.6” with “24.8”

- In Figure 2 legend, Page 6, Line 3,

“... J1, J2, pre-dauer juvenile (J2d and DJ) densities are...”

Replace “(J2d and DJ)” with “(J2d) and DJ”

- In Figure 3 legend, Page 7, Line 3,

“... J1, J2, pre-dauer juvenile (J2d and DJ) densities are...”

Replace “(J2d and DJ)” with “(J2d) and DJ”

- In Results, Page 7, Line12-13,

“...continued to feed on bacterial cells in the medium until moulting to DJ.”

Erase “until moulting to DJ”

- In References, Page 9, between “CHAVARRIA-HERNANDEZ et al. (2007)” and

“EHLERS, R.-U. (2001)”

Insert one reference

“COUCHE, G.A. & GREGSON, R.P. (1987). Protein inclusions produced by the entomopathogenic bacterium *Xenorhabdus nematophila* subsp. *nematophilus*. *Journal of Bacteriology* 169, 5279-5288.”

- In References, Page 10, “GOLDEN, J.W. & RIDDLE, D.L. (1982). A pheromone influences larval development in the nematode *Caenorhabditis elegans*. *Nature* 218, 578-580.”

Replace “*Nature*” with “*Science*”

- In References, Page 10, “HIRAO, A. & EHLERS, R.-U. (2009c).”

Insert doi number after “in press”

DOI; 10.1007/s00253-009-2095-4

## Publication 1

- In Reference, Page 10, “JESSEN et al. (2000).”

Replace the part of underline “Carbon dioxide triggers dauer juvenile recovery of entomopathogenic nematodes (*Heterorhabditis* spp.)” with “Carbon dioxide triggers recovery from dauer juvenile stage in entomopathogenic nematodes (*Heterorhabditis* spp.)”

- In Reference, Page 10, “LUNAU et al. (1993).”

Replace “*Nematologica* 23, 97-102.” with “*Nematologica* 39, 385-399.”

- In Reference, Page 10, between “STRAUCH, O. & EHLERS, R.-U. (1998)” and “STRAUCH et al. (1994)”

Insert one reference

“STRAUCH, O. & EHLERS, R.-U. (2000). Influence of the aeration rate on the yields of the biocontrol nematode *Heterorhabditis megidis* in monoxenic liquid cultures. *Applied Microbiology and Biotechnology* 54, 9-13.”

## Life cycle and population development of entomopathogenic nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda, Rhabditida) in monoxenic liquid cultures

Ayako HIRAO, Ralf-Udo EHLERS\* and Olaf STRAUCH

*Institute for Phytopathology, Department for Biotechnology and Biological Control, Christian-Albrechts-University Kiel, Hermann-Rodewald Str. 9, 24118 Kiel, Germany*

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**Summary** – The life cycle and population dynamics of the entomopathogenic nematodes *Steinernema carpocapsae* and *S. feltiae* were studied in monoxenic liquid culture with their symbiotic bacteria *Xenorhabdus nematophila* and *X. bovienii*. To distinguish between the different juvenile and adult stages, their size was recorded. No differences were observed between the species in the size of the juvenile stages but significant differences were recorded in the length of the F1 adults, pre-dauer (J2d) and dauer juvenile stages (DJ). On average, 90% of inoculated DJ of *S. feltiae* recovered and 77% of *S. carpocapsae*. In general, *S. feltiae* developed from the inoculum DJ to the adult approximately 1 day faster than *S. carpocapsae*. The sex ratio was female-biased ( $59.2 \pm 2.2\%$  in *S. carpocapsae*,  $66.7 \pm 2.6\%$  in *S. feltiae*) in the parental population but not in the F1 generations. Steinernematid adults, like heterorhabditids, respond to depleting food resources with the cessation of egg laying. Juveniles hatch inside the uterus and develop at cost of the maternal body content causing the death of the adult (*endotokia matricida*). In contrast to *Heterorhabditis* spp. and *in vivo* observations of steinernematids by other authors, who reported that readily developed DJ leave the carcass of the dead adult, in this study J2d emerged 12 h after cessation of egg laying. The density of both bacterial cultures decreased due to the feeding of the parental juveniles. However, *X. nematophila* continued at very low density, whereas the density of *X. bovienii* increased again until 15 days post-inoculation. The vast majority of F1 *S. carpocapsae* offspring developed to DJ, whereas in *S. feltiae* a significant second and third generation of adults was observed, probably due to the increasing bacterial population. However, second and third generation adults in *S. feltiae* cultures did not contribute significantly to the DJ yield. Mean yields of  $158 \times 10^3$  DJ ml<sup>-1</sup> were recorded for *S. carpocapsae* and  $106 \times 10^3$  DJ ml<sup>-1</sup> for *S. feltiae*. The results provide valuable information for future process improvement.

**Keywords** – biological control, dauer juvenile recovery, *endotokia matricida*, *Xenorhabdus bovienii*, *X. nematophila*.

Entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis* (Nematoda: Rhabditida) are safe biological control agents (Ehlers, 2003) used to manage insect pests (Grewal *et al.*, 2005). They are symbiotically associated with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, members of the gamma subclass of the *Proteobacteria* (Ehlers *et al.*, 1988). Like other rhabditoid nematodes, they respond to depleting food resources and unfavourable environmental conditions with the formation of a developmentally-arrested, enduring third juvenile stage, the dauer juvenile (DJ). DJ are well adapted for long-term survival in the soil environment (Susurluk & Ehlers, 2008), can actively seek suitable insect hosts (Lewis, 2002; Torr *et al.*, 2004) and invade the host insect's haemocoel,

where they perceive food signals (Golden & Riddle, 1982) causing the DJ to recover from the arrested DJ stage and develop to J4 (Strauch *et al.*, 1998; Hiraio & Ehlers, 2009a). During DJ recovery, the symbiotic bacterial cells stored in the intestine of DJ are released, accelerating the killing of the host (Han & Ehlers, 2000). By feeding on bacterial cells and degraded host tissues, the recovered juveniles develop to adults. Under favourable nutritive conditions their offspring develop to another reproductive adult generation. When food resources are depleting they develop to DJ, which leave the insect cadaver (Strauch *et al.*, 1994).

For commercial purposes, EPN are mass produced in liquid culture bioreactors (Ehlers, 2001, 2007). Artificial liquid media are inoculated with symbiotic bacteria prior

\* Corresponding author, e-mail: ehlers@biotec.uni-kiel.de

to DJ inoculation. The bacterial inoculum volume is between 0.5 and 1.0% of the culture volume (Ehlers, 2001). Cultures begin with a 24–36 h bacterial pre-culture prior to DJ inoculation (Ehlers *et al.*, 1998). Inoculum DJ originate from monoxenic pre-cultures (Lunau *et al.*, 1993). In artificial media, DJ do not recover because of the lack of food signals (Strauch & Ehlers, 1998). Only the pre-culture of the symbiotic bacteria provides food signals triggering recovery (Aumann & Ehlers, 2001; Hirao & Ehlers, 2009a). Since the reproduction of *Steinernema* is amphimictic, males and females copulate and females produce eggs. Also, in liquid culture, offspring develop further to reproductive adults for another reproductive cycle if food is abundant, or offspring development tends towards DJ formation as a response to depleting food resources (Strauch *et al.*, 1994). Growth and reproduction of adult stages is also influenced by the nutritional conditions. With abundant food, hermaphrodites of *H. bacteriophora* grow larger and continue egg laying, whereas they stop laying eggs and the J1 hatch inside the uterus and feed on the maternal body as a response to food shortage. The juveniles develop to DJ causing the death of the hermaphrodite/female (*endotokia matricida*) and then leave the maternal carcass (Johnigk & Ehlers, 1999b). The majority of *H. bacteriophora* DJ harvested from liquid culture are juveniles originating from *endotokia matricida*. The density of the symbiotic bacteria cell is a good indicator of the availability of food resources and direct effects on nematode population development as DJ recovery and *endotokia matricida* are density-dependent (Hirao & Ehlers, 2009a; Johnigk & Ehlers, 1999b).

According to previous studies on *Heterorhabditis* spp. (*e.g.*, Ehlers *et al.*, 1998; Ehlers, 2000, 2001; Han & Ehlers, 2001; Johnigk *et al.*, 2002, 2004), an improvement of culture conditions should target at least three major developmental steps within the nematode life cycle: DJ recovery, fertility of adults and DJ formation. Investigations on the life cycle provided major fundamental knowledge to understand and improve the liquid culture production process (*e.g.*, Strauch *et al.*, 1994; Johnigk & Ehlers, 1999a, b). Information on the population development of *Steinernema* spp., however, is scarce. Most of the studies on population development of *Steinernema* spp. have been done in insect hosts. Wang and Bedding (1996) described the population development of *S. carpocapsae* and *H. bacteriophora* in *Galleria mellonella* larva. Only recently, the influence of the bacterial density, the process temperature and the DJ inoculum density on DJ recovery and population development in liquid cultures of *S. car-*

*pocapsae* and *S. feltiae* was investigated (Hirao & Ehlers, 2009a, b, c). However, a description of the life cycle of these two species is still missing. The objective of this study was to describe the life cycle of *Steinernema carpocapsae* and *S. feltiae* in monoxenic liquid culture.

## Materials and methods

### SYMBIOTIC BACTERIA AND NEMATODES

*Xenorhabdus nematophila* was isolated from insects infected with DJ of *S. carpocapsae* (All strain) and *X. bovienii* from *S. feltiae* (Sylt strain). Symbiotic bacteria were propagated in YS broth containing 5.0 g l<sup>-1</sup> NaCl, 5.0 g l<sup>-1</sup> yeast extract, 0.5 g l<sup>-1</sup> NH<sub>4</sub>PO<sub>4</sub>, 0.5 g l<sup>-1</sup> K<sub>2</sub>PO<sub>4</sub>, 0.2 g l<sup>-1</sup> MgSO<sub>4</sub> · 7H<sub>2</sub>O (Merck, Haar, Germany) at 180 rpm and 25°C. Bacterial cultures of 10<sup>10</sup> cells ml<sup>-1</sup> were transferred into 2 ml Eppendorf tubes and mixed with glycerol to obtain a 15% solution and stored at -20°C until use.

*Steinernema carpocapsae* (All strain) and *S. feltiae* (EN02 Hybrid strain) were used. The nematodes were propagated according to Ehlers *et al.* (1998) in liquid medium (LM) containing 15.0 g l<sup>-1</sup> yeast extract (Merck), 20.0 g l<sup>-1</sup> soy powder (Cargill, Hamburg, Germany), 4.0 g l<sup>-1</sup> NaCl, 0.35 g l<sup>-1</sup> KCl, 0.15 g l<sup>-1</sup> CaCl<sub>2</sub>, 0.1 g l<sup>-1</sup> MgSO<sub>4</sub> (Merck), 6.0 g l<sup>-1</sup> lecithin and 40.0 g l<sup>-1</sup> vegetable oil. Media were adjusted to pH 7.0 for *S. carpocapsae* and 6.5 for *S. feltiae* with 4 M KOH (Merck). Bacterial stocks were thawed and propagated at 25°C in YS broth until the cell density reached at least 10<sup>9</sup> cells ml<sup>-1</sup>. The nematode culture medium was inoculated with 1% of a bacterial culture adjusted to 10<sup>9</sup> cells ml<sup>-1</sup> and the bacteria were pre-cultured at 25°C for 36 h before inoculation of 3000 DJ ml<sup>-1</sup>, originating from monoxenic cultures. Incubation was at 25°C until harvest on day 15 post-inoculation (15 dpi) of bacteria. DJ were separated from culture debris by centrifugation (2500 rpm, 10 min), and washed in sterile Ringer's solution (Merck). These steps were repeated at least three times until the debris and bacteria were removed. Active DJ were then separated from inactive DJ and adults by suspending the DJ over a 30 µm sieve in Ringer's solution. DJ that had passed through the sieve after 4 h were collected in sterile Ringer's solution. DJ were stored at 4°C until use. All steps were performed under sterile conditions.



## MONOXENIC CULTURES

In order to provide a homogenous bacterial inoculum, *X. nematophila* and *X. bovienii* bacteria were produced each in one batch culture and then distributed over three Erlenmeyer flasks. The bacterial cultures were carried out according to Johnigk *et al.* (2004) in a bioreactor (Meredos, Goettingen, Germany) filled with 5 l liquid medium supplemented with 0.1% (v/v) anti-foam (Silfoam, SE2; Wacker Chemie, Munich, Germany). Process conditions were according to Hirao and Ehlers (2009b). When the bacterial pre-cultures had reached a cell density of  $10^{10}$  cells  $\text{ml}^{-1}$  and protein inclusion bodies (Couche & Gregson, 1987) were detected in the cells by microscopic observation (Zeiss, Germany, interference contrast microscope at  $\times 1000$  magnification), 100 ml bacterial culture was transferred to sterile 500 ml Erlenmeyer flasks, inoculated with 4000-6000 DJ  $\text{ml}^{-1}$  and incubated at 25°C. Three flask cultures were used for each species and the experiments were replicated twice. Flasks contaminated with foreign bacteria were discarded. Finally, six flask cultures were assessed for *S. carpocapsae* and seven flask cultures for *S. feltiae*.

## ASSESSMENT OF DEVELOPMENTAL STAGES AND BACTERIA

The nematode and bacterial population was recorded daily until 15 dpi. Samples containing at least 100 individuals were taken from the liquid cultures and diluted with Ringer's solution. Bacterial cell density was assessed by counting cells in a Thoma chamber under the microscope at  $\times 600$  magnification and nematodes were counted under an inverted microscope (Zeiss) at  $\times 100$  magnification. Measurements on the individual size of the nematodes were carried out on fixed specimens. Nematodes were transferred to a 55°C water bath and then into fixative (0.1 M HEPES buffer containing 23.8 g  $\text{l}^{-1}$  HEPES (Carl Roth, Germany) and 11.7 g  $\text{l}^{-1}$  NaCl (Merck), 2.5% glutaraldehyde (v/v), pH 7.4 adjusted with 1 M NaOH, diluted with 4 ml of 1 M  $\text{CaCl}_2$  (Merck)). Fixation was overnight at 4°C under slow agitation. Nematodes were then washed with HEPES buffer and the length and width of the nematodes were measured under the microscope using image analysis software (analysis<sup>®</sup>, Soft Imaging System, Germany). At least 12 individuals from each developmental stage were assessed. The volume of offspring DJ from populations of *S. carpocapsae* and *S. feltiae* cultures was estimated by the

formula:  $V \text{ (in mm}^3\text{)} = (\pi \times \text{length} \times \text{width})/4$  for single nematodes (Poulin & Morand, 2000).

The different juvenile stages (from first-stage juvenile (J1) to fourth-stage (J4), pre-dauer juveniles and DJ) and adults (males and females) were identified and counted separately. The vulva and uterus were used to distinguish between J4 females and adult females. The asymmetric gonad and spicules were used to identify males. The difference among J1, J2 and J3 was estimated by the relative size of each juvenile (Table 1), since the sexual primordium could not be determined with an inverted microscope at  $\times 100$  magnification. Pre-dauer juvenile (J2d) could be distinguished from normal J1 and J2; J2d have a thinner spindle shape with a longer pharyngeal region and a sharp symmetric tail. Due to the density of lipids in the gut cells, the J2d body is darker than that of other juvenile stages. Feeding behaviour of J2d was only observed during the early phase of this stage. When the size reached their maximum, J2d stopped feeding. Their gut lumen shrank and became invisible.

## DESCRIPTION OF *ENDOTOKIA MATRICIDA*

In order to describe the process of *endotokia matricida* in liquid culture, 40 *S. carpocapsae* females with fertilised eggs were collected from cultures at 81-87 h post DJ inoculation and at 69-72 h post DJ inoculation from *S. feltiae* cultures. The different time-scales were chosen because the two species developed differently. The females were transferred separately into wells of a 96-cell well plate (Greiner Bio-One, Dresden, Germany) filled with a bacterial cell suspension of  $10^7$  cells  $\text{ml}^{-1}$  suspended in culture supernatant at the period for female collection. The females were observed every 3 h under an inverted microscope at  $\times 100$  magnification.

## Results

The length and width of the parental female and F1 juvenile and adult stages are summarised in Table 1. No significant differences were recorded in the size of the juvenile stages of both species. Differences were observed between the stages in the length of the F1 adults, J2d and DJ. The length of parental females is almost doubled in *S. carpocapsae* and more than doubled in *S. feltiae* when compared with the F1 females.

A comparative overview of the population development for both species at 25°C in monoxenic liquid culture from inoculated DJ to the adults and the first filial

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**Table 1.** Mean body length and width (in  $\mu\text{m}$ ) of *Steinernema carpocapsae* (each stage  $n = 72$ ) and *S. feltiae* ( $n = 84$ ) juveniles and adults of parental and first filial generation obtained from monoxenic liquid cultures inoculated with 4000-6000 dauer juveniles (DJ) and incubated at 25°C. Data are expressed as Mean (min-max) body size in  $\mu\text{m}$ . Data marked with \* are significantly different between the same stages of the two species (t-test,  $P < 0.05$ ).

Generation	Stage	Day	Length		Width	
<i>S. carpocapsae</i>						
Parental	Female	4	2621.2*	(2399.4-2851.3)	145.0	(128.8-153.1)
F1	J1	4	225.1	(182.0-244.7)	14.3*	(12.3-16.5)
F1	J2	5	496.2	(394.9-505.7)	32.5*	(29.8-35.4)
F1	J3	5-6	763.1	(659.8-807.2)	37.7*	(35.9-40.3)
F1	J4M	6	788.8	(717.0-848.4)	38.7*	(35.9-46.1)
F1	J4F	6	979.4	(904.0-1048.3)	44.3*	(39.6-49.0)
F1	Male	7	913.0	(884.7-987.6)	44.1	(41.7-47.2)
F1	Female	7	1423.9*	(1205.2-1505.8)	72.5	(59.9-86.4)
F1	J2d	5	589.5*	(561.7-601.0)	27.7	(25.2-32.2)
F1	DJ	7	562.7*	(551.6-599.1)	22.3*	(20.1-25.7)
<i>S. feltiae</i>						
Parental	Female	3	2767.4*	(2633.1-2977.5)	154.4	(143.8-166.9)
F1	J1	3	223.0	(182.5-244.7)	16.4*	(12.7-16.4)
F1	J2	4	485.5	(390.8-507.1)	26.6*	(22.6-28.9)
F1	J3	5	770.2	(674.2-828.2)	31.4*	(29.5-32.9)
F1	J4M	6	796.8	(785.8-823.4)	33.7*	(32.0-37.2)
F1	J4F	6	976.6	(931.7-1098.3)	57.5*	(55.0-58.6)
F1	Male	7	816.4	(723.4-843.6)	41.6	(38.9-41.8)
F1	Female	7	1125.1*	(1025.3-1187.8)	70.9	(66.5-79.6)
F1	J2d	5	753.9*	(744.2-792.7)	30.7	(28.3-32.9)
F1	DJ	6	740.9*	(683.2-779.0)	28.4*	(25.2-30.7)

generation (F1) until 8 dpi is presented in Figure 1A; continuation until the F2 generation was not possible as individuals of the F1 and F2 generations cannot be distinguished. Detailed population dynamics of the complex *S. carpocapsae*-*X. nematophila* are presented in Figure 2 and of *S. feltiae*-*X. bovienii* in Figure 3.

The density of the bacterial symbionts (*X. nematophila* and *X. bovienii*) began to decrease on 2 dpi and reached their minimum density on the day when the J1 offspring density reached their maximum (5 dpi *X. nematophila*, 4 dpi *X. bovienii*). After reaching its minimum, the density of *X. bovienii* increased again until 15 dpi, in contrast to *X. nematophila*, which continued at very low density.

On average, 90% of the inoculated DJ recovered from the DJ in *S. feltiae* (2 dpi) and 77% in *S. carpocapsae* (3 dpi) cultures. However, the mean number of parental adult females was finally about 2000 ml<sup>-1</sup> for both species (Figs 2, 3). In general, *S. feltiae* developed faster in liquid culture than *S. carpocapsae*. Whereas the maximum adult female population of *S. carpocapsae* was recorded on

3 dpi (Fig. 2), the maximum number of both sexes of *S. feltiae* was observed already on 2 dpi (Fig. 3).

The sex of inoculated DJs of both species was assessed at the time of maximum adult density counted until 5 dpi and exhibited a female-biased sex ratio ( $59.2 \pm 2.2\%$  in *S. carpocapsae*,  $66.7 \pm 2.6\%$  in *S. feltiae*).

First offspring (J1) were observed on 4 dpi in *S. carpocapsae* and 3 dpi in *S. feltiae*. In general, the J1 developed to J2 or J2d in 1 day. In *S. carpocapsae* cultures, first DJ offspring occurred 7 dpi together with the first F1 adults. This happened 1 day earlier than in *S. feltiae* cultures.

The main difference between the population development of *S. feltiae* and *S. carpocapsae* was that nearly all the offspring of the latter species developed to DJ (Fig. 2), whereas in *S. feltiae* a significant second and third generation of adults was observed (Fig. 3). The DJ proportion after 15 days was 90.4% in *S. carpocapsae* and 89.1% in *S. feltiae* cultures. The rest of the population consisted of J2d in *S. carpocapsae* and mainly of adults in *S. feltiae* cultures. It appears that second and third generation

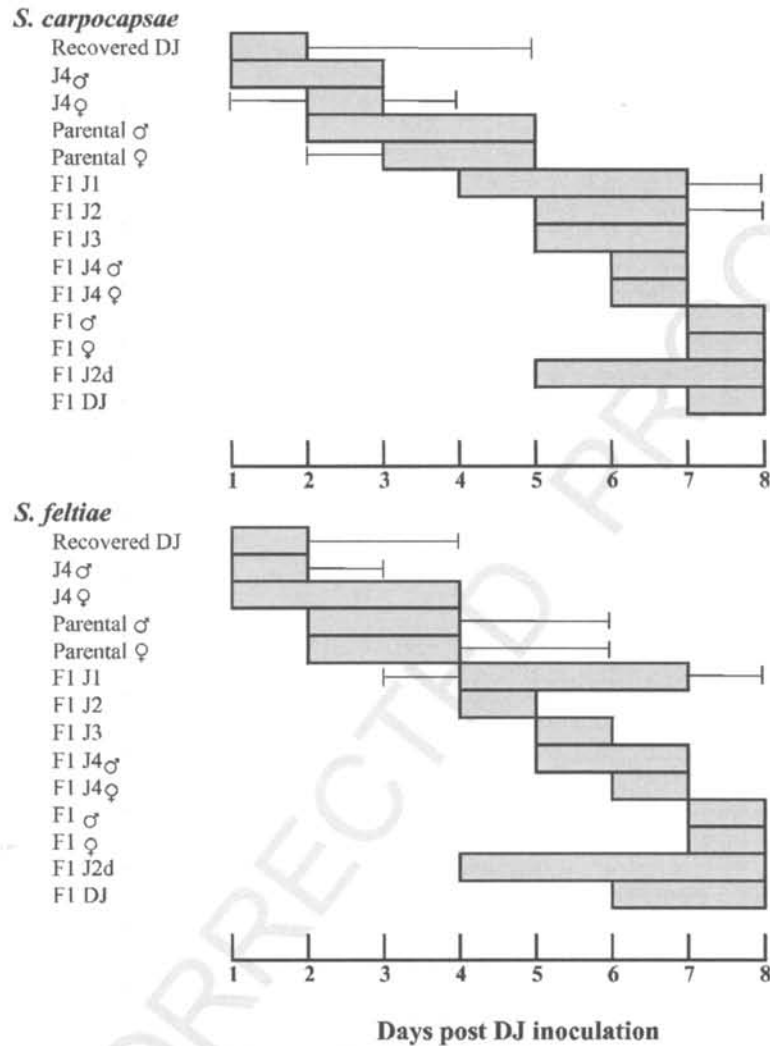


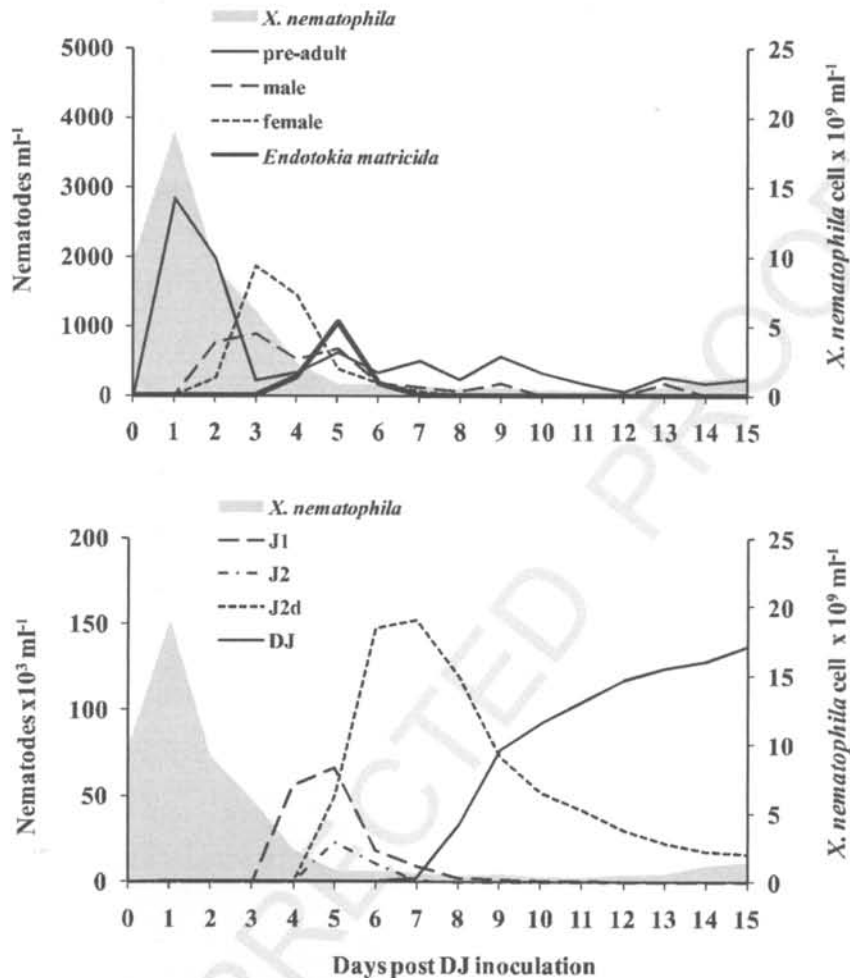
Fig. 1. Life span of parental and F1 juvenile and adults of *Steinernema carpocapsae* and *S. feltiae* in monoxenic liquid culture until 8 days post DJ inoculation at 25°C. Lines extending the bars indicate time span when the population of this stage was less than 10% of the maximum (DJ = dauer juvenile, J1-J4 = first- to fourth-stage juvenile, J2d = pre-dauer juvenile).

adults in *S. feltiae* cultures did not contribute to the DJ yield until day 15, as no increase of the J1, J2 and J2d population was recorded together with the increase of the F1 and F2 adult generations. Offspring production by filial *S. feltiae* generations seems to be negligible. This is particularly surprising when comparing their number with the number of parental females, which were recorded at much lower density.

Until 15 dpi *S. carpocapsae* had produced a mean yield of  $158 \times 10^3$  DJ ml<sup>-1</sup>, *S. feltiae* only  $106 \times 10^3$  DJs ml<sup>-1</sup>.

The density of DJ remains more or less stable in *S. feltiae* cultures from 10 dpi onwards, whereas in *S. carpocapsae* cultures it continuously increased until 15 dpi. However, when estimating the volume of the DJ-yield (measured as DJ volume, Fig. 4), *S. carpocapsae* cultures produced less volume ( $67.7 \text{ mm}^3 \text{ ml}^{-1}$ ) than *S. feltiae* ( $107.6 \text{ mm}^3 \text{ ml}^{-1}$ ). This is caused by the lower body size of the DJ of *S. carpocapsae*.

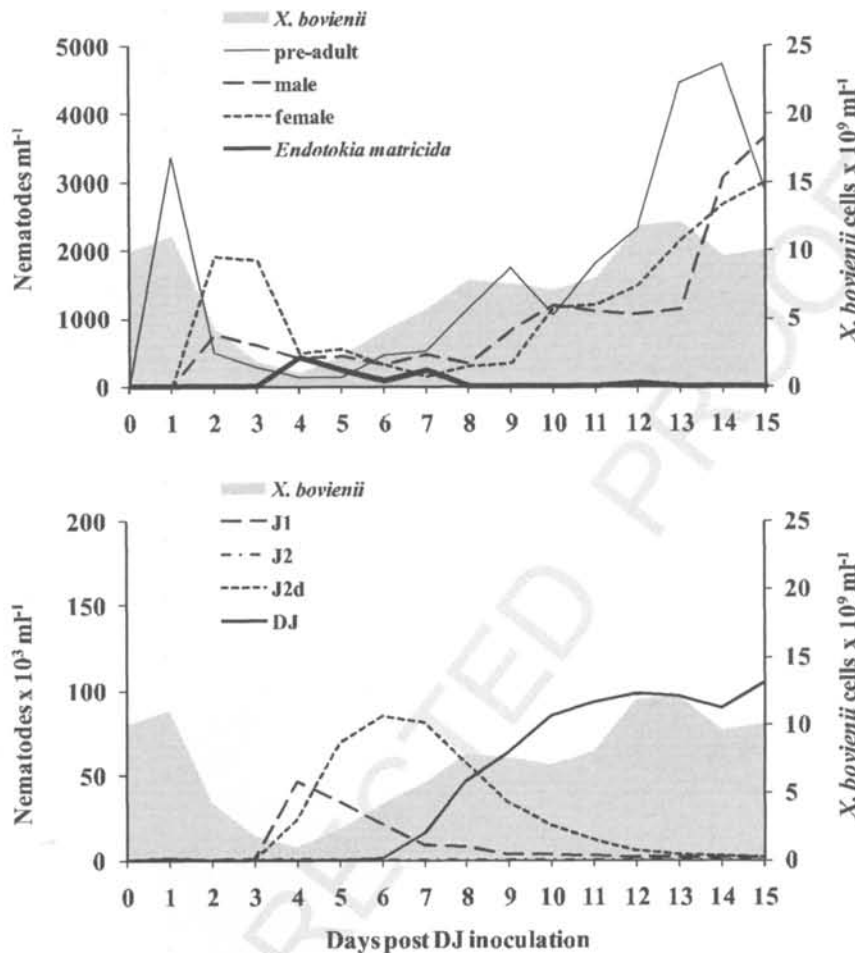
The *endotokia matricida* can be divided into five steps: i) J1 hatch inside the maternal uterus; ii) uterus



**Fig. 2.** Mean population dynamics ( $n = 6$ ) of the bacto-helminthic complex *Steinernema carpocapsae*-*Xenorhabdus nematophila* in monoxenic liquid culture at 25°C recorded from 0-15 days after DJ inoculation. Density of pre-adult stage juveniles (J3 and J4 stages), males, females and *endotokia matricida* females are presented in the upper graph; J1, J2, pre-dauer juvenile (J2d and DJ) densities are presented in the lower graph.

is disrupted by the juveniles; *iii*) maternal death; *iv*) maternal gut lumen disrupted by juveniles; and *v*) pre-dauer juveniles emerge from the maternal cadaver. The vast majority of the pre-dauer juveniles developed inside the females bodies (*endotokia matricida*). The chronology of the *endotokia matricida* is presented in Figure 5. *Endotokia matricida* begins with the hatching of J1 from eggs inside the mother, which was observed on 4 dpi for *S. carpocapsae* and *S. feltiae*. In *S. carpocapsae* cultures it was observed at same time when J1 hatched from eggs laid into the medium and in *S. feltiae* it was 1 day later. After initiation of *endotokia matricida*, oviposition

ceases but the females continue feeding. Juveniles fed on fluid in the uterus. Early hatched juveniles tended to swim to the edge of the uterus toward the anterior part of the maternal body. Feeding of J1 seems to cause rupture of the uterus in the anterior part and juveniles get access to the body lumen. Hatching of juveniles continued after uterus rupture. Soon after juvenile invasion into the head part of the female, the head region collapsed and maternal feeding ceased. Then, movement of the maternal gut content could no longer be observed, indicating maternal death. Even though the juveniles entered the maternal body cavity, the intestine and pharynx remained



**Fig. 3.** Mean population dynamics ( $n = 7$ ) of the bacto-helminthic complex *Steinernema feltiae*-*Xenorhabdus bovienii* in monoxenic liquid culture at 25°C recorded from 0-15 days after DJ inoculation. Density of pre-adult stage juveniles (J3 and J4 stages), males, females and endotokia matricida females are presented in the upper graph; J1, J2, pre-dauer juvenile (J2d and DJ) densities are presented in the lower graph.

intact. Three hours after maternal death, forceful feeding movements of the juveniles resulted in rupture of the maternal gut tissue and the gut content was distributed over the body cavity. Juveniles fed on the gut content, consisting of intact and digested symbiont cells. By this time most of the juveniles had developed to the J2d stage. Thus, the juveniles had no access to the bacterial symbiont cells before they had developed to J2d stages. Twelve hours after the first juveniles had hatched in the uterus, J2d stages emerged from the maternal cadaver through the anterior part of the female carcass. The J2d stages continued to feed on bacterial cells in the medium until

moulting to DJ. No DJ were observed inside the maternal carcass.

### Discussion

Liquid culture production is state of the art in commercial EPN production (Ehlers, 2007). Consequently, many publications have dealt with improvement of process conditions (e.g., Friedman, 1990; Ehlers *et al.*, 1998; Strauch & Ehlers, 2000; Jessen *et al.*, 2000; Johnigk *et al.*, 2004; Chavarria-Hernandez *et al.*, 2006, 2007; Hirao & Ehlers, 2009b, c). However, detailed descriptions on the life cy-

cle of *Steinernema* in liquid culture are scarce. In order to monitor and improve culture conditions for DJ production in liquid culture, basic knowledge of the life cycle for each species is required. The data on the life span, density dynamics, body size and subsequent DJ density obtained

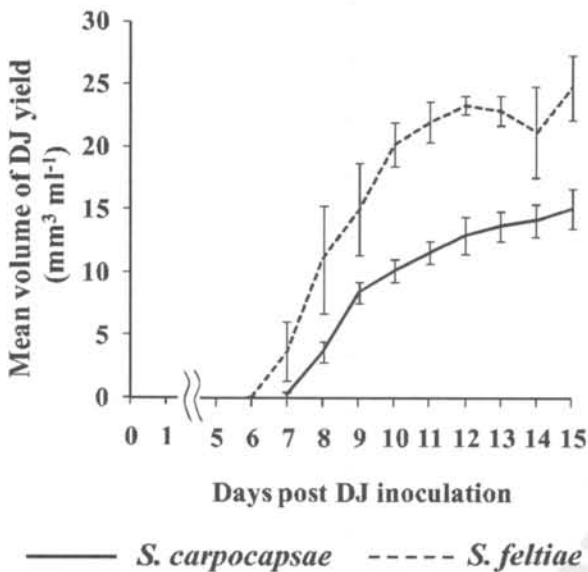


Fig. 4. Mean volume of DJ yield (in mm<sup>3</sup> ml<sup>-1</sup>) recorded from monoxenic liquid cultures of *Steinernema carpocapsae* (n = 6) and *S. feltiae* (n = 7) incubated at 25°C assessed from population density (Figs 2 and 3) and DJ size (Table 1).

in this investigation can be used to estimate/predict population development and yields in commercial production and for further improvements of culture conditions.

The rapid increase of the DJ density in both species indicates that most DJ are F1 offspring. The much higher density of J2d than J1 suggests that most J2d originated from *endotokia matricida* rather than from J1, which hatched from eggs laid into the medium. The number of juveniles that can develop inside females is limited by the size of the parental female and offspring juveniles. Although the density of parental females was almost equal between the two species, higher numbers of *S. carpocapsae* J2d developed per female than in *S. feltiae*. The reason is probably the smaller DJ size in *S. carpocapsae* (Table 1).

Differences in the population development between *S. carpocapsae* and *S. feltiae* were observed in the adult population dynamics. Whereas almost all offspring of *S. carpocapsae* developed to J2d and DJ by 15 dpi, a large number of offspring developed to adults in *S. feltiae* populations, which may cause major problems during downstream processing. All non-DJ stages rapidly die when DJ are formulated and then provide a medium for the growth of contaminants, which spoil the product. Thus, these stages must be removed from the DJ suspension prior to product formulation. The results confirm observations on the population dynamics made during investigations on the influence of the DJ inoculum density on DJ yields and population development. Thus, the occurrence

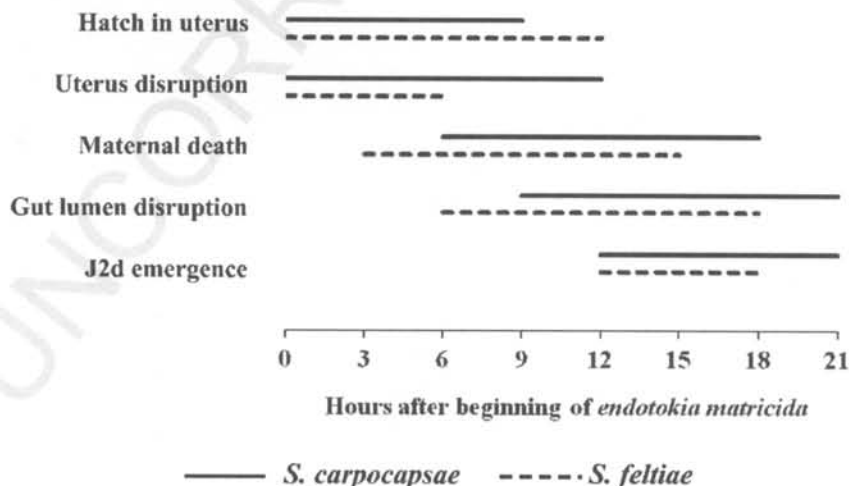


Fig. 5. Development of *endotokia matricida* of *Steinernema carpocapsae* and *S. feltiae* in monoxenic liquid cultures incubated at 25°C recorded from 0-21 h after hatching of the first juvenile inside the uterus. Mean from 40 single females with fertilised eggs observed in cell wells inoculated with 10<sup>7</sup> cells ml<sup>-1</sup> with the species-specific symbiont cells.

of F1 adults seems to be the rule rather than an exception and should be the focus of future process improvement in order to try to avoid the development of a second generation adult population. This increase is accompanied by an increase in the bacterial density, which is probably responsible for the switch to adult development instead of DJ formation. *Xenorhabdus bovienii* cell density continued at a high level from 8 dpi onwards and reached two maxima, the first at 1 dpi and the second at 12 dpi. This indicates that during the first exponential growth phase *X. bovienii* is not efficiently exploiting the resources of the culture medium.

The female-biased sex ratio is reported from *in vivo* and *in vitro* cultures of three other species of *Steinernema* (Alsayyah *et al.*, 2009). However, in this study it was only observed in the parental but not in the F1 and the F2 generations in *S. feltiae*. Future investigations on the sex determination should take this observation into consideration.

Although it is reported that the recovery of *S. feltiae* in bacterial cultures takes 8 h longer than DJ recovery of *S. carpocapsae* (Hirao & Ehlers, 2009a), the peak of female density is observed 1 day earlier in *S. feltiae* than in *S. carpocapsae* cultures and results in a 1 day earlier occurrence of offspring and DJ.

No major differences were observed in *endotokia matricida* between the two species. However, differences exist when comparing with the *endotokia matricida* of *H. bacteriophora* and *H. indica* in liquid culture. Johnigk and Ehlers (1999b) reported that J2d developed inside the maternal carcass and were motionless before they moulted to DJ. The complete development to DJ inside the maternal cadaver was also observed in *S. carpocapsae* and *H. bacteriophora* reared in *G. mellonella* larva (Baliadi *et al.*, 2001). However, in this study juveniles did not complete their development to DJ inside the maternal body but escaped from the carcass by their strong movements. Baliadi *et al.* (2001) also reported that juveniles already developed in the maternal body 18 h after the start of *endotokia matricida* when nematodes were reared in *G. mellonella*. In this study juveniles of *endotokia matricida* develop only until J2d stages in 12-21 h. The final formation of the DJ outside the female body makes juveniles more vulnerable to stress. In *Heterorhabditis* spp. the DJ that developed from *endotokia matricida* were of superior quality than those that developed outside the body of the female. Whether this is the case in *Steinernema* spp. needs further investigation.

In conclusion, the description of the life cycle of these two steinernematids in liquid culture has revealed some differences to the life cycle *in vivo* and new information on the population dynamics, which will be useful for future investigation focusing on further process improvement.

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Publication 2

**Influence of cell density and phase variants of bacterial symbionts (*Xenorhabdus* spp.) on dauer juvenile recovery and development of biocontrol nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida)**

A. Hirao and R.-U. Ehlers

Applied Microbiology and Biotechnology

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## Publication 2

Corrections to galley proof

Line 39: erase "are"

Line 89: .. (Boemare and Akhurst 1988).

Line 120: erase: ". In hemolymph drops obtained by bleeding the insects, DJ recovery was  
much reduced"

Line 136-137: "...isolated from *Steinernema carpocapsae* (ALL strain) and .."

Line 147: "*S. carpocapsae* (ALL strain) and ...

Line 263: erase the complete sentence: "DJ recovery in hemolymph was compared with the  
DJ recovery in living *G. mellonella* insect inoculated without penetration."

Line 273: "cell densities of ..."

Line 277: erase "at"

Line 326: "... to cell wells containing.."

Line 334: ...percent. Pairwise comparisons were done with the ..."

Line 341: " $A = \sum(t...$ " Comment: the sign for "sum" is missing.

Figure 1. Please replace with the new Figure we have attached.

Line 441: "Whereas the percentage..."

Line 527: supernatant

Line 578: erase the complete reference Boemare 2002

# Influence of cell density and phase variants of bacterial symbionts (*Xenorhabdus* spp.) on dauer juvenile recovery and development of biocontrol nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida)

A. Hirao & R.-U. Ehlers

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**Abstract** The rhabditid nematodes *Steinernema carpocapsae* and *Steinernema feltiae* are used in biological control of insect pests. Mass production is done in liquid culture media pre-incubated with their bacterial symbionts *Xenorhabdus nematophila* and *Xenorhabdus bovienii*, respectively, before nematode dauer juveniles (DJs) are inoculated. As a response to food signals produced by the bacterial symbionts, the DJs exit from the developmentally arrested dauer stage (they recover development) and grow to adults, which produce DJ offspring. Variable DJ recovery after inoculation often causes process failure due to non-synchronous population development and low numbers of adult nematodes. This contribution investigated the influence of the bacterial cell density on DJ recovery and development to adults. At higher density of  $10^{10}$  bacterial cells  $\text{ml}^{-1}$ , a higher percentage of DJ recovery was induced, and adults occurred earlier in both *Steinernema* spp. than at lower density of  $10^9$  and  $10^8$  cells  $\text{ml}^{-1}$ . *Xenorhabdus* symbionts produce phase variants. Recovery in bacteria-free supernatants was lower than in supernatants containing bacterial cells for both primary and secondary phase *Xenorhabdus* spp. and lower in secondary than in primary phase supernatants or cell suspensions. In general, recovery was lower for *Steinernema feltiae* and the time at which 50% of the population had recovered after exposure to the food signal was longer ( $\text{RT}_{50}=17.1$  h) than for *Steinernema carpocapsae* ( $\text{RT}_{50}=6.6$  h). Whereas >90% *S. carpocapsae* DJs are recovered in hemolymph serum of the lepidopteran insect *Galleria*

*mellonella*, recovery of *S. feltiae* only reached 31%. Penetration into a host insect prior to exposure to the insect's food signal did not enhance DJ recovery. Consequences for liquid culture mass production of the nematodes and differences between species of the genera *Steinernema* and *Heterorhabditis* are discussed.

**Keywords** Biocontrol nematodes · *Steinernema carpocapsae* · *S. feltiae* · Dauer juvenile recovery · *Xenorhabdus nematophila* · *X. bovienii* · Liquid culture

## Introduction

Entomopathogenic nematodes of the genus *Steinernema* (Nematoda: Rhabditida) are symbiotically associated with enterobacteria of the genus *Xenorhabdus*, member of the gamma subclass of the *Proteobacteria* (Ehlers et al. 1988). The bacto-helminthic complexes are safe biocontrol agents (Ehlers 2003) used to manage several insect pests in ornamentals and food crops (e.g., Grewal et al. 2005; Toepfer et al. 2003). Like other rhabditid nematodes, *Steinernema* spp. have a developmentally arrested third juvenile stage, called dauer juvenile (DJ), which is the only free-living stage. The term dauer (German for enduring) describes a morphologically distinct stage, formed as a response to depleting food sources and adverse environmental conditions. DJs are well adapted for long-term survival in the soil environment (Susurluk and Ehlers 2008). Each DJ carries some hundred cells of its specific symbiont in a pocket in the anterior part of its intestine (Bird and Akhurst 1983). The DJ is actively searching for suitable insect hosts (Rasmann et al. 2005) and penetrates into the insect's hemocoel. Inside the insect, the complex encounters suitable conditions for reproduction and the DJs leave the

A. Hirao · R.-U. Ehlers (✉)

Institute for Phytopathology, Department for Biotechnology and Biological Control, Christian-Albrechts-University Kiel, Hermann-Rodewald-Str. 9, 24118 Kiel, Germany  
e-mail: ehlers@biotec.uni-kiel.de

72 stage (Han and Ehlers 2000). In accordance to the rhabditid  
 73 nematode *Caenorhabditis elegans*, the exit from the DJ stage  
 74 is called “recovery” (Golden and Riddle 1982; Strauch et al.  
 75 1994). Recovery is a response to food signals in the  
 76 hemolymph. During recovery, the bacteria are released,  
 77 which accelerates the killing of the host (Han and Ehlers  
 78 2000). The nematodes feed on the symbiont cells, grow to  
 79 adults, and produce DJ offspring, which leave the insect’s  
 80 cadaver on the search for new hosts (Ciche et al. 2006).

81 *Xenorhabdus* spp. produce phase variants with different  
 82 phenotypes (Akhurst 1980). Secondary forms are detected  
 83 during the stationary growth phase in cultures, which have  
 84 been inoculated with primary phase. Primary cells absorb  
 85 bromothymol blue from NBTA agar plates. Secondary  
 86 colonies cannot absorb the dyes from agar media (Akhurst  
 87 1980). Primary cells carry inclusion proteins and produce  
 88 antibiotic substances, lipase, phospholipase, and protease.  
 89 Secondary variants lose these characters (Boemare 2002).  
 90 Nematodes grown on secondary phase yield significantly  
 91 less DJs than if cultured on primary phase bacteria,  
 92 particularly in liquid cultures (Han and Ehlers 2001).

93 For biocontrol purposes, nematodes are produced in  
 94 industry scale bioreactors. Much information on liquid  
 95 culture production of the related biocontrol nematode  
 96 *Heterorhabditis bacteriophora* Poinar 1979 has been  
 97 published (Ehlers 2001). Artificial liquid media are pre-  
 98 incubated with the symbiotic bacteria for approximately  
 99 1 day before DJs are inoculated. In vitro production is  
 100 possible due to pre-culture of the symbiotic bacterium  
 101 *Photorhabdus luminescens*, which excretes food signals  
 102 into the medium and trigger DJ recovery (Aumann and  
 103 Ehlers 2001). Artificial growth media lack any kind of food  
 104 signal. When Strauch and Ehlers (1998) transferred DJs  
 105 into culture filtrates of *P. luminescens*, the highest recovery  
 106 was recorded in samples taken during the late logarithmic  
 107 growth phase. Ehlers et al. (1998) reported that low DJ  
 108 recovery leads to a non-synchronous population develop-  
 109 ment of *H. bacteriophora*, prolonged culture duration, and  
 110 unstable yields. In order to reach the necessary density also  
 111 at low DJ recovery, higher numbers of DJs must be  
 112 inoculated, which makes necessary additional scale-up  
 113 steps, thus increasing the production costs (Ehlers et al.  
 114 1998). Consequently, the key to a successful and cost-  
 115 effective liquid culture process is through the management  
 116 of DJ recovery. These conditions have not yet been  
 117 investigated in species of the genus *Steinernema*.

118 Under natural conditions, *Heterorhabditis* DJs recover  
 119 after invasion of the insect’s hemocoel as a response to yet  
 120 unknown food signals in the hemolymph. In hemolymph  
 121 drops obtained by bleeding the insects, DJ recovery was  
 122 much reduced (Strauch and Ehlers 1998). Whether  
 123 steinernematids recover in insect blood serum was  
 124 investigated in this study as well as the influence of

host penetration on DJ recovery, and the influence of  
*Xenorhabdus* cell density and their phase variants on  
 nematode DJ recovery were evaluated. This study also was  
 performed to compare results obtained with *Heterorhabditis*  
 DJ recovery with those from the genus *Steinernema*, which  
 is believed to have evolved the symbiotic relation to  
 entomopathogenic bacteria independently (Sudhaus 1993).

## Materials and methods

### Symbiotic bacteria

*Xenorhabditis* spp. were isolated from nematode-infested  
 insect larvae of *Galleria mellonella* according to Ehlers et  
 al. (1990). *Xenorhabdus nematophila* was isolated from all  
 the strains of *Steinernema carpocapsae* and *Xenorhabdus*  
*bovienii* from the Sylt strain of *Steinernema feltiae*.  
 Symbiotic bacteria were then propagated in YS broth in  
 100-ml Erlenmeyer flasks filled with 30 ml medium  
 containing (in g l<sup>-1</sup>) 5 NaCl, 5 yeast extract, 0.5 NH<sub>4</sub>PO<sub>4</sub>,  
 0.5 K<sub>2</sub>PO<sub>4</sub>, 0.2 MgSO<sub>4</sub> × 7 H<sub>2</sub>O (Merck) at 180 rpm and  
 25°C. Bacterial cultures of 10<sup>10</sup> cells ml<sup>-1</sup> were transferred  
 into 2-ml Eppendorf tubes and mixed with glycerol to  
 obtain a 15% solution and stored at -20°C until use.

### Nematodes

*S. carpocapsae* (all strains) and *S. feltiae* (EN02 hybrid  
 strain) were used. Monoxenic cultures were established  
 according to Lunau et al. (1993). The DJ inoculum was  
 produced according to Ehlers et al. (1998) in liquid medium  
 (LM) containing (in g l<sup>-1</sup>) 15 yeast extract (Merck), 20 soy  
 flour, 6 lecithin (Cargill), 40 vegetable oil (Raiffeisen), 4  
 NaCl, 0.35 KCl, 0.15 CaCl<sub>2</sub>, 0.1 MgSO<sub>4</sub> (Merck). Media  
 were adjusted to pH 7.0 for *S. carpocapsae* and 6.5 for *S.*  
*feltiae* with 4 M KOH (Merck). LM was inoculated with  
 the symbiotic bacteria of the specific nematode. Therefore,  
 bacterial stocks were thawed and propagated at 25°C in YS  
 broth until a cell density of 10<sup>9</sup> cells ml<sup>-1</sup> was reached. LM  
 was inoculated at 1% with the YS bacterial culture and  
 incubated for 36 h at 25°C. Then, DJs from monoxenic  
 cultures were inoculated at 3,000 DJs ml<sup>-1</sup> and incubated at  
 25°C until harvest at 15 days after inoculation of the  
 symbiotic bacteria.

Nematodes were collected by centrifugation (2,500 rpm,  
 10 min). To remove culture debris and bacteria, DJs were  
 washed in Ringer’s solution (Merck) and centrifuged again.  
 Then, active DJs were separated from inactive ones by  
 suspending the DJs over a 30-µm sieve in water and  
 collecting DJs, which had passed the sieve after 4 h in  
 sterile Ringer’s solution. DJs were stored at 4°C until use.  
 All steps were performed under sterile conditions.

172	Establishment of secondary phase bacteria	220
173	The shift to secondary phase bacteria was induced by	222
174	exposing the cultures to stress conditions according to	223
175	Krasomil-Osterfeld (1995). <i>X. nematophila</i> was cultured for	224
176	20 days at 100 rpm and 25°C under osmotic stress in YS	225
177	broth without NaCl. In order to identify secondary colonies,	226
178	samples were streaked onto NBTA containing (in g l <sup>-1</sup> ) 10	227
179	tryptic soy broth (TSB) (bioMerieux), 15 bacto-agar	228
180	(Difco), 0.5 bromothymol blue (Sigma), supplemented after	229
181	cooling to 35°C with 4 ml l <sup>-1</sup> of filter-sterilized 2,3,5-	230
182	triphenyl-tetrazoliumchloride (Merck). Secondary phase	231
183	bacteria produce red colonies on NBTA, whereas primary	232
184	absorb bromothymol blue (Akhurst 1980). A single second-	233
185	ary colony was selected and sub-cultured again under osmotic	234
186	stress. After four selection rounds, pure secondary phase	235
187	bacteria were obtained. They were then once propagated in	236
188	normal YS at 180 rpm and afterward checked for phase	237
189	stability. Secondary phase of <i>X. bovienii</i> was obtained from	238
190	2-month-old monoxenic flask cultures of <i>S. feltiae</i> , which	239
191	had been started with primary cells. The secondary phase	240
192	was stable even without culture under osmotic stress. Storage	241
193	was also done in 15% glycerol stocks at -20°C.	242
194	Phenotypic characterization of secondary phase	243
195	of <i>X. nematophila</i> and <i>X. bovienii</i>	244
196	Further secondary phase characters were checked to support	245
197	identification by dye absorption. Whereas primary bacteria	246
198	are mobile, swarming on soft agar, and producing antibiotic	247
199	activity against <i>Bacillus cereus</i> in overlay cultures, the	248
200	secondary bacteria lose these characters (Boemare and	249
201	Akhurst 1988). Swarming was checked after culturing	250
202	primary and secondary in parallel culture plates for 72 h	251
203	at 25°C on soft agar containing (in g l <sup>-1</sup> ) 15 TSB and 8 agar	252
204	(Difco). For testing the antibiotic activity, a YS broth	253
205	culture of <i>B. cereus</i> was mixed at 5% into sterile TSB	254
206	supplemented with 15 g l <sup>-1</sup> agar after cooling to 50°C.	255
207	Primary and secondary cultures of <i>X. nematophila</i> and <i>X.</i>	256
208	<i>bovienii</i> were incubated in YS broth until a cell density of	257
209	10 <sup>10</sup> cells ml <sup>-1</sup> was reached. Then, 1.5 ml samples were	258
210	centrifuged at 14,000 rpm for 10 min, and the supernatant	259
211	was filter-sterilized through a 0.22-µm pore size membrane	260
212	(Milipore). Ten microliters of each filtrate was then	261
213	inoculated onto the agar containing <i>B. cereus</i> . Growth	262
214	inhibition indicates the presence of primary cells.	263
215	Effect of insect penetration on DJ recovery	264
216	In liquid culture production, DJs do not undergo host	265
217	penetration prior to recovery. In order to determine whether	266
218	host insect penetration affects recovery, DJs were either	267
219	injected into an insect host or left to penetrate into the	268
	insect. Single last instars of the lepidopteran insect <i>Galleria</i>	220
	<i>mellonella</i> were exposed to 50 DJs of <i>S. carpocapsae</i> in	221
	10 µl Ringer's solution on a wet filter paper disc inside a	222
	cell well of a 24-cell well plate (Greiner). For <i>S. feltiae</i>	223
	invasion, <i>G. mellonella</i> larvae were buried into 10% moist	224
	sand in the well and also exposed to 50 DJs. At 6 h post-	225
	inoculation (hpi), the infected larvae were collected from	226
	cell wells and transferred into plastic boxes covered with	227
	wet filter paper and maintained at 25°C until recovery was	228
	assessed.	229
	For DJ injection into the hemocoel of <i>G. mellonella</i>	230
	larvae, inoculum DJ density was adjusted at a density of	231
	5,000 DJs ml <sup>-1</sup> in sterile Ringer's solution. Final instar <i>G.</i>	232
	<i>mellonella</i> larvae were injected with 10 µl of the DJ	233
	suspension to achieve approximately 50 DJs per insect.	234
	Injection was performed with a microsyringe (ITO Corp,	235
	Japan). Recovery was observed at 6, 24, and 48 hpi. Twelve	236
	inoculated larvae were dissected in Petri dishes filled with	237
	Ringer's solution, and the nematodes were collected into	238
	counting chambers (Cellstar, Germany) to check the recovery	239
	under an inverted microscope (Zeiss, Germany). Dauer	240
	juveniles were distinguished from recovered juveniles by	241
	morphological characters according to Strauch and Ehlers	242
	(1998). The head region of DJs is tapered, whereas recovered	243
	juveniles have a broad head region and the mouth is opened.	244
	The experiment was repeated twice.	245
	Effect of insect serum from <i>G. mellonella</i> larvae on DJ	246
	recovery	247
	<i>G. mellonella</i> larvae were collected and surface-sterilized	248
	by washing them in 70% ethanol. The hemolymph was	249
	collected from a proleg into a 2.0 ml cap, which contained	250
	0.5% phenylthiourea-acetone (Sigma) at 1/10 of the total	251
	volume to avoid melanization of the hemolymph (Arakawa	252
	1995). Collected hemolymph was stored at -20°C until use.	253
	Before the experiment, the stock was thawed and purified	254
	by centrifugation and filtration with a 0.2 µm filter. Then,	255
	ampicillin and streptomycin sulfate (Merck) were added	256
	each at 0.1% (w/v) to avoid any activity of symbiotic	257
	bacteria, and the sterile serum was incubated for 24 h. All	258
	handling was carried out under sterile condition. Hemo-	259
	lymph (500 µl) was added to each well, and 500 DJs per	260
	well were inoculated. The cultures were then incubated on	261
	the shaker at 180 rpm and 25°C. Recovery was assessed	262
	from at least 50 juveniles at 24 and 48 hpi. DJ recovery in	263
	hemolymph was compared with the DJ recovery in living	264
	<i>G. mellonella</i> insect inoculated without penetration.	265
	Influence of bacterial cell density on DJ recovery	266
	Bacterial symbionts were cultured in LM in shaken flasks	267
	for 36 h at 180 rpm and 25°C. Cell density was counted	268

269	using a Thoma chamber. Then, the bacterial suspension was	DJ recovery after different exposure periods in bacterial cell	318
270	centrifuged at 14,000 rpm for 30 min at 4°C and the	suspension	319
271	supernatant was filter-sterilized (0.2 μm, Millipore).		
272	Dilutions were produced by adding sterile supernatant to	In order to investigate how long the DJs need to be exposed	320
273	the bacterial suspension to obtain cell density of 10 <sup>8</sup> , 10 <sup>9</sup> ,	to the bacterial food signal for induction of the recovery	321
274	and 10 <sup>10</sup> cells ml <sup>-1</sup> . To stop bacterial growth and avoid	process, the same experiment was repeated, but only at a	322
275	contamination, ampicillin and streptomycin sulfate were	cell density of 10 <sup>10</sup> cells ml <sup>-1</sup> . Then, 50 DJs were removed	323
276	added as described above to the bacterial suspensions,	from the suspension at 0, 0.5, 2, 4, 6, 8, 10, 12, 24, and	324
277	supernatant, or Ringer's solution at 24 h prior to DJ	36 h after inoculation, washed in sterile Ringer's solution,	325
278	inoculation. Cell wells of 24 well plates (Greiner) were	and then transferred to cell well containing 500 μl Ringer's	326
279	each filled with 500 μl bacterial suspension, culture	solution supplemented with the antibiotics as described	327
280	supernatant, or Ringer's solution and 500 DJs from	above. Recovery of all 50 individuals was assessed at 48	328
281	monoxenic culture per cell well. The plates were then	hpi. The experiment was also done in three replicates and	329
282	incubated on a shaker at 180 rpm and 25°C. Every 2 h, at	repeated twice. The time period, at which 50% of the	330
283	least 50 DJs were removed, and the recovery was assessed	inoculated DJs had recovered (RT <sub>50</sub> ), was calculated by	331
284	according to Strauch and Ehlers (1998). Each bacterial	Probit analysis (Finney 1971).	332
285	density, supernatant, and control in Ringer's solution		
286	was tested in three replicates (cell wells), and the	Statistic analysis	333
287	experiment was repeated twice with different nematode		
288	batches.		
289	Influence of bacterial cell density on development after	Recovery data are presented in percent. Two-pair	334
290	recovery	comparison was analyzed by the Chi-squares test.	335
		Recovery data among multiple variants were converted	336
291	The same experiment was repeated without testing	to arcsin values, and then mean for different variants	337
292	supernatant and Ringer's solution. In order to test the	were analyzed for significant differences by ANOVA and	338
293	influence of the bacterial density on recovery and	the Tukey HSD test. The recovery data over time were	339
294	nematode development, the number of DJs, recovered	analyzed and statistically compared using the trapezoidal	340
295	DJs, and adults were assessed at 12, 24, 36, 48, and 60 h	rule $A = (t_{n+1} - t_n)(r_n + r_{n+1})/2$ ( $t$ is the time after DJ	341
296	after nematode inoculation. Each time, a minimum of 50	inoculation, $n$ is the sample no., $r_n$ is the recovery after $t_n$ ),	342
297	individuals was observed in each variant. Female	estimating the integral of the curve. The higher the area	343
298	nematodes were identified based on the presence of a	under the curve, the stronger is the effect of the variant on	344
299	vulva and males on the presence of the spicula. Each	the recovery induction. Means for different variants were	345
300	bacterial density was tested in three replicates, and the	analyzed for significant differences by ANOVA and the	346
301	experiment was repeated twice with different nematode	Tukey HSD test. For evaluation of the DJ synchronization	347
302	batches.	index, the regression of the recovery data over time was	348
		calculated, and the indices (slopes of graphs) were	349
		compared for significant differences by ANOVA and the	350
303	Influence of bacterial phase variant on DJ recovery	Tukey HSD test.	351
304	Primary and secondary phase bacteria were propagated	<b>Results</b>	352
305	in YS broth for 36 h. Bacterial cell-free supernatant was		
306	prepared from each phase variant, and suspensions were	Effect of insect penetration on DJ recovery	353
307	adjusted to a density of 10 <sup>10</sup> cells ml <sup>-1</sup> with the		
308	supernatant supplemented with the antibiotics. Then,	Recovery of DJs in insect hemocoel is presented in Table 1.	354
309	cell wells were filled with the suspension and nematodes	At 6 and 24 hpi, recovery of injected <i>S. carpocapsae</i> DJs	355
310	as described above. In these experiments, the same	was higher than the recovery of the DJ, which had	356
311	amount of wells was filled with the bacterial suspension	undergone host searching and penetration. At 48 hpi, the	357
312	without the addition of antibiotics in order to investigate	recovery of <i>S. carpocapsae</i> and <i>S. feltiae</i> DJs reached	358
313	the influence of the antibiotics on DJ recovery. Recovery	approximately 100% regardless which way they reached	359
314	was observed 48 h after nematode inoculation. A	the hemocoel. The delay in recovery of DJs, which	360
315	minimum of 50 nematodes was observed per variant.	naturally penetrated the insect, is due to the time needed	361
316	The experiments were also done in three replicates and	to reach the host insect and advance to the hemocoel	362
317	repeated twice.	through penetration of the integument, the wall of the	363

**Table 1** Influence of host penetration on dauer juvenile (DJ) recovery of *Steinernema carpocapsae* and *S. feltiae* in the hemolymph of *Galleria mellonella*

		Recovery of dauer juvenile (%)			
		<i>S. carpocapsae</i>		<i>S. feltiae</i>	
hpi		Injection (N=3,120)	Penetration (N=2,160)	Injection (N=2,316)	Penetration (N=695)
t1.5	6	41.4a	21.1b	63.2A	13.1B
t1.6	24	99.5a	81.6b	99.8A	93.8A
t1.7	48	99.9a	100.0a	99.8A	100.0A

DJs had reached the hemocoel either after natural host penetration or through direct injection into the hemocoel. Recovery was recorded at different hours after injection or host insect exposure to DJs (hpi). Percentages indicated with different letters (small letter, *S. carpocapsae*; capital letter, *S. feltiae*) are significantly different at the same assessment time between recovery of DJs which had been injected or which had naturally penetrated the insect (Chi-square test,  $P \leq 0.05$ )

tracheal system of the intestine. Thus, host penetration is not a pre-requisite for DJ recovery nor is the penetration process resulting in a higher recovery of the DJs.

**Influence of antibiotics on DJ recovery**

An influence of the antibiotics ampicillin or streptomycin sulfate on DJ recovery can be excluded since no significant difference of DJ recovery was recorded 48 hpi in wells with or without antibiotic treatment (data not shown).

**Recovery in serum of *G. mellonella***

Of *S. carpocapsae* DJs, 88.6% and 96.9% recovered in the sterile serum at 24 and 48 hpi, respectively. In contrast, only 22.4% and 31.0% of *S. feltiae* DJs recovered at 24 and 48 hpi, respectively (Table 2), indicating that serum of this lepidopteran insect is less well suited for recovery of *S. feltiae*.

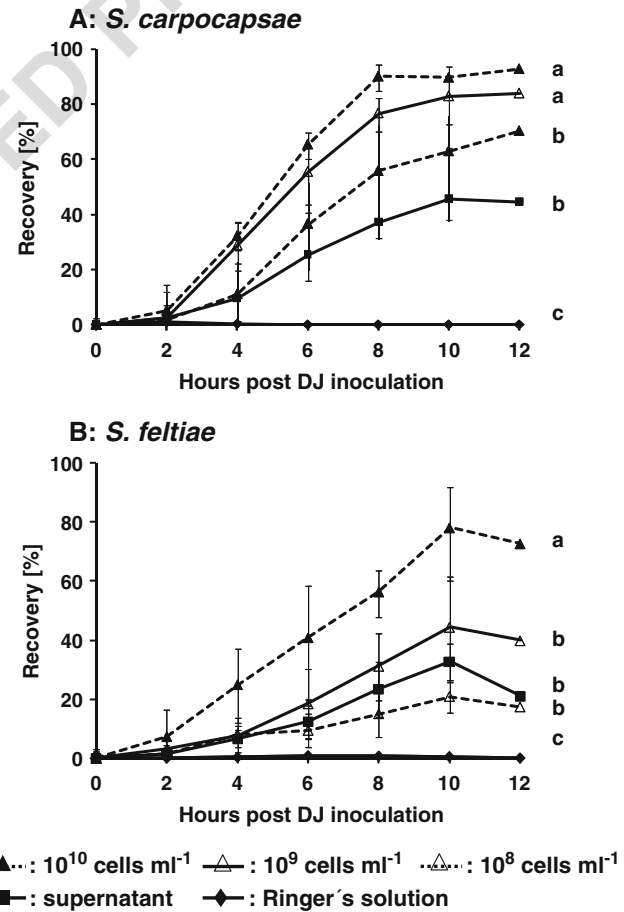
**Table 2** Recovery of *Steinernema carpocapsae* and *S. feltiae* dauer juveniles in purified sterile serum of the insect *Galleria mellonella* 24 and 48 h post-inoculation (hpi)

hpi	<i>S. carpocapsae</i> N=645	<i>S. feltiae</i> N=712	
t2.3	24	88.6a	22.4b
t2.4	48	96.9a	31.0b

Percentages indicated with different letters indicate significant difference at the same assessment time between the different nematode species (Chi-square test,  $P \leq 0.05$ )

**Influence of bacterial cell density and bacterial culture supernatant**

The results on the influence of the cell density of the nematode's specific bacterial symbionts are presented in Fig. 1. Significant differences were recorded for both *S. carpocapsae* (ANOVA,  $F=60.6$ ;  $df=4, 41$ ;  $P < 0.0001$ ) and *S. feltiae* (ANOVA,  $F=40.7$ ;  $df=4, 34$ ;  $P < 0.0001$ ). Ringer's solution obviously lacks any kind of food signal as no recovery was recorded for both nematode species even after 12 hpi. Both species recovered in bacterial culture supernatant, indicating that both *X. nematophila* and *X. bovienii* excrete food signals into the culture medium. *S. carpocapsae* reached 44% recovery in supernatant, which was lower than in any of the bacterial cell suspensions. *S. feltiae* reached 20% DJ recovery at 12 hpi in supernatant with no significant difference to the cell densities  $10^8$



**Fig. 1** Dauer juvenile recovery (%) of *S. carpocapsae* (a) and *S. feltiae* (b) recorded 2 to 12 h after inoculation into Ringer's solution, culture supernatant, or different cell densities of *X. nematophila* (a) or *X. bovienii* (b) at 25°C. Bars on graphs give upper and lower 95% confidence interval. Different letters at graphs indicate significant differences between treatments evaluated by comparison of the area under each curve (Tukey HSD test,  $P \leq 0.05$ )

395 (Tukey HSD,  $P=0.804$ ) and  $10^9 \text{ ml}^{-1}$  ( $P=0.5$ ). Maximum  
 396 recovery was recorded at cell densities of  $10^{10} \text{ ml}^{-1}$  for both  
 397 species. The reduction of the cell density to  $10^9 \text{ ml}^{-1}$  did not  
 398 result in a significant drop in DJ recovery in *S. carpocapsae*  
 399 ( $P=0.384$ ). Only the reduction to a density of  $10^8 \text{ ml}^{-1}$   
 400 caused a significant reduction in recovery to 70% at 12 hpi  
 401 ( $P=0.026$ ). In contrast, the reduction of the cell density of  
 402 *X. bovienii* to  $10^9$  and  $10^8 \text{ ml}^{-1}$  caused a significant  
 403 reduction of the recovery in *S. feltiae* to 39% and 18%  
 404 (both  $P<0.0001$ ) at 12 hpi, respectively. The response to  
 405 the bacterial food signal is more rapid in *S. carpocapsae*  
 406 than in *S. feltiae* (Fig. 1). The DJ synchronization index  
 407 was calculated by regression of the recovery data over time  
 408 until 8 h after inoculation of the DJs into the bacterial  
 409 suspension and supernatant (Table 3). The better the  
 410 synchronization of the DJ recovery, the higher is the index.

411 The faster progress of the recovery in higher cell  
 412 densities was confirmed by observations on exsheathment  
 413 and feeding of *S. carpocapsae*. The step introducing the  
 414 recovery process in DJs is the exsheathment from the  
 415 cuticle of the prior juvenile stage, the pre-dauer J2d stage.  
 416 In *X. nematophila* cell suspension of  $10^{10} \text{ cells ml}^{-1}$ , the  
 417 first *S. carpocapsae* juveniles shed the outer cuticle  
 418 at 8 hpi, but no exsheathment was observed in  $10^9$  and  
 419  $10^8 \text{ cells ml}^{-1}$  suspensions until 12 hpi. In  $10^{10} \text{ cells ml}^{-1}$ ,  
 420 the pharyngeal pumping, indicating the start of the feeding,  
 421 was observed at 8 hpi, while no feeding was observed in  
 422  $10^9$  and  $10^8 \text{ cells ml}^{-1}$  bacterial cell suspension until 12 hpi.

423 Influence of bacterial cell density on nematode  
 424 development after recovery

425 As recorded in the previous experiments, the recovery of  
 426 both nematode species was again highest at the highest  
 427 bacterial density, and recovery of *S. carpocapsae* was less  
 428 affected by the decreasing cell density than *S. feltiae*  
 429 (Fig. 2). In both species, the recovery increased until  
 430 24 hpi and then was constant in *S. carpocapsae* and at  
 431  $10^{10} \text{ cells ml}^{-1}$  in *S. feltiae*, but continued to increase from  
 432 31% at 12 hpi to 77% at 60 hpi at  $10^9 \text{ cell ml}^{-1}$  and from

20% to 57% at  $10^8 \text{ cell ml}^{-1}$  in *S. feltiae*. The bacterial cell  
 density also affected the development of recovered juveniles  
 to adults. In the population of *S. carpocapsae* incubated at a  
 bacterial cell density of  $10^{10}$  and  $10^9 \text{ cells ml}^{-1}$  (Fig. 2a, b),  
 the adult population was observed already at 36 hpi, while in  
 $10^8 \text{ cells ml}^{-1}$ , the first adults occurred at 48 hpi (Fig. 2c).  
 The *S. feltiae* adult population was first observed at 24 hpi at  
 $10^{10} \text{ cells ml}^{-1}$  (Fig. 2d), 36 hpi in  $10^9 \text{ cells ml}^{-1}$  (Fig. 2e),  
 and 48 hpi in  $10^8 \text{ cells ml}^{-1}$  (Fig. 2f). Where the percentage  
 of adults in *S. carpocapsae* reached 86.4%, 52.4%, and  
 11.6% at  $10^{10}$ ,  $10^9$ , and  $10^8 \text{ cells ml}^{-1}$  at 60 hpi, respectively,  
 the percentage reached only 71.2%, 19.4%, and 8.5% in  
*S. feltiae*, respectively. There was no major difference in  
 sex ratio between different bacterial cell densities in *S.*  
*carpocapsae*, whereas in *S. feltiae*, more females than males  
 were recorded at higher cell density.

Influence of bacterial phase variation on DJ recovery

Recovery of both species, *S. carpocapsae* and *S. feltiae*,  
 was reduced in secondary cells and also in supernatant of  
 secondary cells (Fig. 3a, b). Other than in Ringer's solution,  
 in which no recovery was recorded (Fig. 1), in medium YS  
 broth, a low recovery of approximately 10% was observed.  
 Recovery in supernatants was always lower than in cell  
 suspensions, confirming the results obtained in the first  
 experiments (Fig. 1).

Time scale of DJ recovery

When DJs were exposed to  $10^{10} \text{ cells ml}^{-1}$  over a period of  
 2 to 36 h and then transferred to Ringer's solution, the  
 response of *S. carpocapsae* DJs to the food signal was  
 almost immediate with a significant increase over time  
 (ANOVA,  $F=43.726$ ;  $df=7, 70$ ;  $P<0.0001$ ). Significant  
 steps were recorded between 2 and 4 h (Tukey HSD,  $P=$   
 $0.001$ ) and 4 and 6 h (Tukey HSD,  $P=0.004$ ) of exposure,  
 followed by a continuous, but small increase to 84%  
 recovery of DJs exposed for 36 h to the bacterial  
 suspension (Fig. 4a). In *S. feltiae* (ANOVA,  $F=27.248$ ;

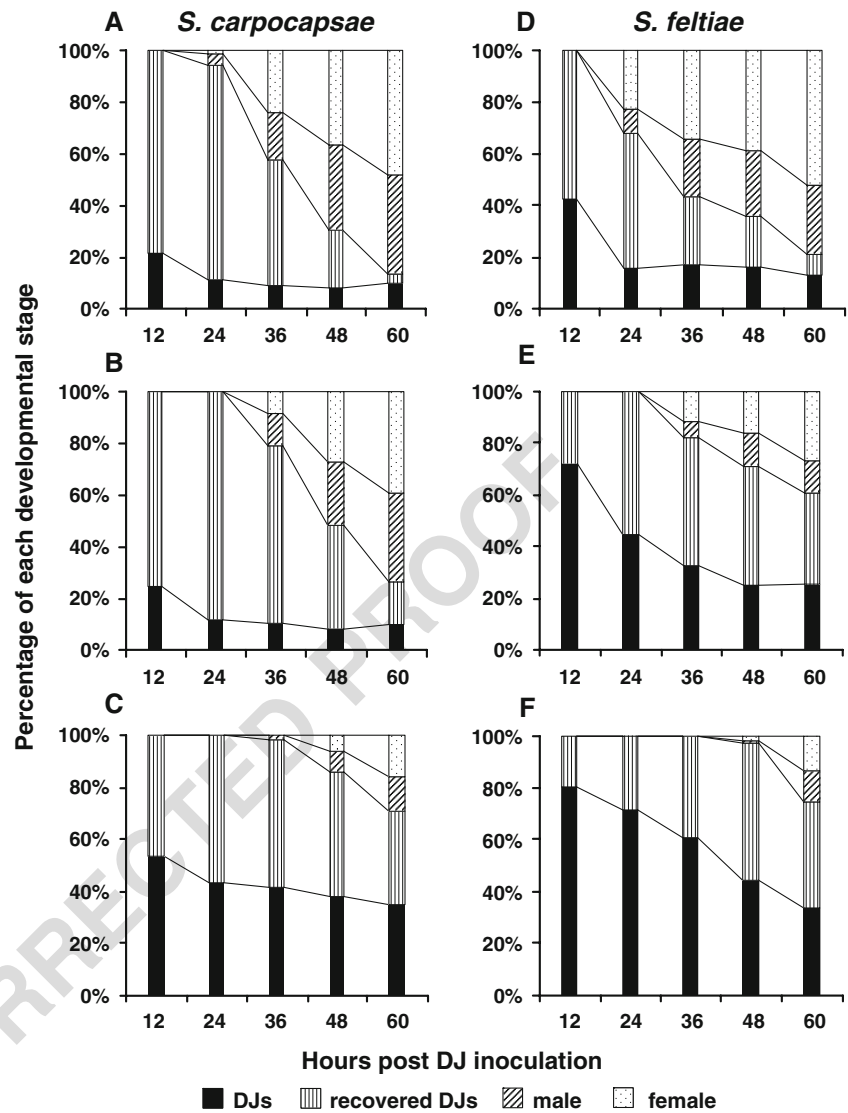
t3.1 **Table 3** Index for synchronization of dauer juvenile recovery in *Steinernema carpocapsae* (S.c.) and *S. feltiae* (S.f.)

t3.2	Nematode species	Bacterial cell density							
		Log 10		Log 9		Log 8		Supernatant	
		Slope	$r^2$	Slope	$r^2$	Slope	$r^2$	Slope	$r^2$
t3.5	S. c.	14.1a	0.99	12.3ab	0.99	9.0bc	0.97	5.8c	0.98
t3.6	S. f.	7.5a	0.99	5.3ab	0.96	2.3b	0.96	4.1ab	0.94

Slope and regression coefficient ( $r^2$ ) of regressions calculated from data on DJ recovery over time until 8 h post-inoculation. Numbers in one row followed by the same letter are not significantly different (Tukey HSD test,  $P\leq 0.05$ )



**Fig. 2** Influence of bacterial cell density on recovery and nematode development to adult males and females. Nematode population is given in percentage of the different developmental stages. Recovery in percent is the percentage of all stages except dauer juveniles (DJs). Third and fourth stage juveniles were added to recovered DJs. DJs were exposed to bacterial suspensions containing  $10^{10}$  (a and d),  $10^9$  (b and e), and  $10^8$  (c and f) cells  $ml^{-1}$ . *S. carpocapsae* (a–c) received *X. nematophila* and *S. feltiae* (d–f) *X. bovienii* bacteria

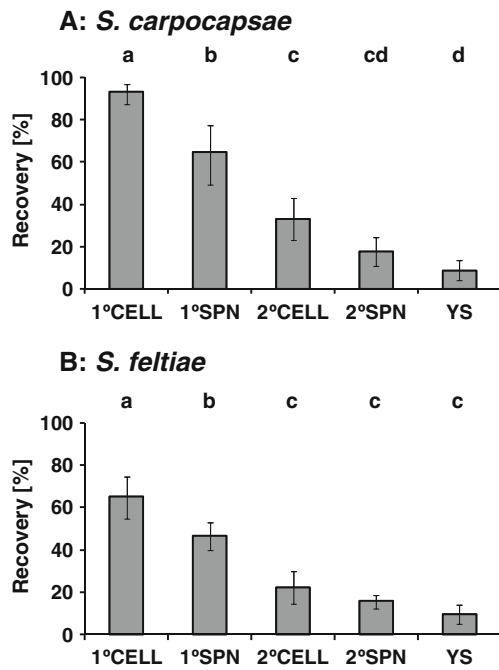


469  $df=7, 70; P<0.0001$ ), the first significant increase in  
 470 recovery is after exposure of 8 to 12 h (Tukey HSD,  $P=$   
 471  $0.0003$ ) and again from 12 to 24 h (Tukey HSD,  $P=0.003$ ).  
 472 This difference between the two species is obvious also  
 473 when the  $RT_{50}$  is compared with 6.6 h exposure for *S.*  
 474 *carpocapsae* and 17.1 h for *S. feltiae*.

475 **Discussion**

476 In liquid culture mass production, the recovery of inoculated  
 477 DJs is the first step in the nematode's life cycle determining  
 478 success or failure of the production process (Ehlers 2001).  
 479 Low DJ recovery of heterorhabditid nematodes results in low  
 480 adult density at the moment of offspring production. As a  
 481 consequence, many of the offspring do not develop to DJs,  
 482 instead, more individuals develop to adults (Strauch et al.

1994) resulting in two-generation processes and a longer  
 process duration (Ehlers et al. 1998). A non-synchronous  
 population development due to a delay in recovery has the  
 same consequences. In heterorhabditid nematodes, Strauch  
 and Ehlers (1998) reported a mean recovery in their  
 symbiont's liquid culture for two *Heterorhabditis megidis*  
 strains at 23% and 45% and for *H. bacteriophora* at 38%,  
 ranging between 0% and 81%. This high variability was  
 not observed in the two steinernematid species. Recovery  
 recorded in these experiments usually was between 60%  
 and 90% at higher bacterial density. Thus, variable  
 recovery in steinernematids is less of a problem compared  
 with heterorhabditids. Compared to heterorhabditid  
 nematodes, which recover in liquid culture over a period  
 of several days (Strauch and Ehlers 1998; Johnigk et al.  
 2004), DJs of the two steinernematid species recover  
 within 24 h. In *S. carpocapsae*, the DJ recovery is even



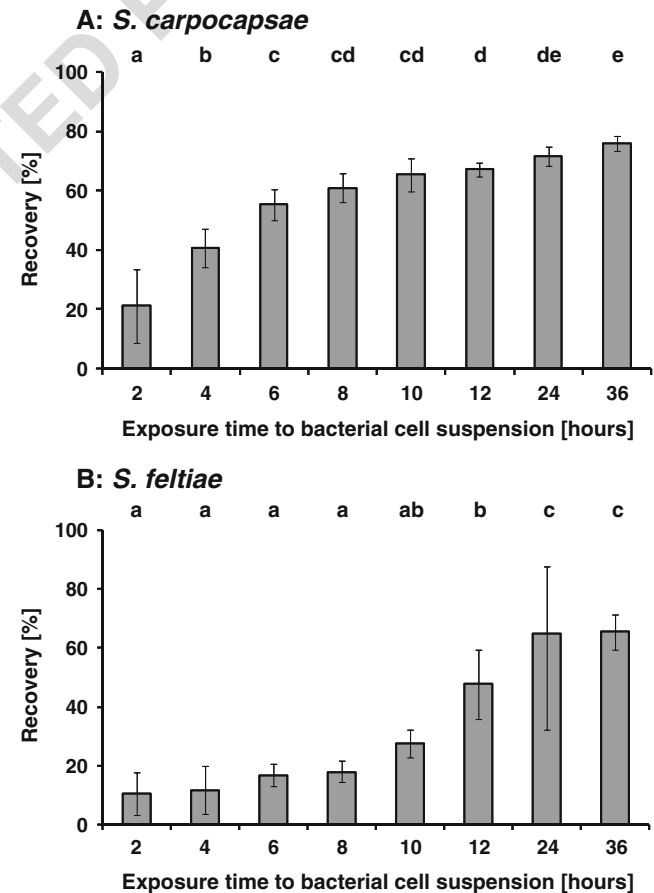
**Fig. 3** Dauer juvenile recovery (%) of *S. carpocapsae* (a) and *S. feltiae* (b) recorded after 48 h exposure at 25°C to YS broth, cell suspensions of  $10^{10}$  cells  $\text{ml}^{-1}$  of primary (1°) or secondary (2°) phase cells suspensions or culture supernatants (SPN) of *X. nematophila* (a) or *X. bovienii* (b). Lines above bars give upper and lower 95% confidence interval. Different letters on bars indicate significant differences between treatments (Tukey HSD test,  $P \leq 0.05$ )

500 less influenced by the bacterial cell density than observed  
 501 for *S. feltiae*. For the latter nematode, a high cell density is  
 502 necessary before DJ inoculation in order to obtain high DJ  
 503 recovery.

504 Another reason why high DJ recovery is a pre-requisite  
 505 for successful liquid culture production of heterorhabditid,  
 506 but less so for steinernematids, is related to the different  
 507 copulation behavior of nematodes of the two genera. Adult  
 508 steinernematid offspring in the F1 generation can copulate  
 509 and multiply in liquid culture, whereas males of the F1  
 510 generation in heterorhabditids cannot attach to the female.  
 511 Fertilization is impossible, and amphimictic adults thus  
 512 cannot multiply (Strauch et al. 1994). Steinernematids can  
 513 compensate for low DJ recovery and immediately utilize  
 514 the remaining resources by development of a second adult  
 515 generation. Second-generation adults of heterorhabditids  
 516 produce no offspring. Instead, reproductive second  
 517 generation is developing from few F1 DJs, which recover  
 518 at low nematode density (result of low recovery) and  
 519 reproduce as self-fertilizing hermaphrodites (Johnigk and  
 520 Ehlers 1999a, b). This developmental process takes longer  
 521 and is often related to losses in the F1 DJ populations  
 522 during the development of the F2 DJs.

523 Like the symbionts *Photorhabdus* spp. of the nematode  
 524 *Heterorhabditis* spp. (Strauch and Ehlers 1998), *X.*

*nematophila* and *X. bovienii* also excrete a food signal 525  
 into the medium; however, the recovery is reduced in the 526  
 superantant, whereas in heterorhabditids, no difference 527  
 was recorded between cell suspension and cell-free culture 528  
 supernatant (Strauch and Ehlers 1998). Either the 529  
*Xenorhabdus* food signal is instable or living cells as such 530  
 trigger recovery or the cells continue to produce food 531  
 signal despite the presence of antibiotics. An effect of the 532  
 antibiotics on the recovery was excluded as was the influence 533  
 of the penetration process on the DJ recovery. Like in 534  
*Heterorhabditis*, the recovery of the two steinernematids 535  
 inside the insects was always 100%. But other than recorded 536  
 for heterorhabditid species, *S. carpocapsae* also recovered 537  
 well in sterile insect serum produced from the hemolymph 538  
 of *G. mellonella*, whereas the response of *S. feltiae* was 539  
 much reduced (Table 2). Possibly, heterorhabditids and *S.* 540  
*feltiae* DJs respond to an instable component in the insect's 541  
 hemolymph, which is lost during preparation of the serum. 542



**Fig. 4** Dauer juvenile recovery (%) of *S. carpocapsae* exposed to *X. nematophila* (a) and *S. feltiae* exposed to *X. bovienii* (b) after different exposure time to bacterial cell densities of  $10^{10}$  cells  $\text{ml}^{-1}$  at 25°C. Recovery was assessed 48 h after the beginning of the exposure period. Lines above bars give standard deviation. Different letters on bars indicate significant differences between treatments (Tukey HSD test,  $P \leq 0.05$ )

543 Dauer juvenile recovery of both *Steinernema* spp. was  
 544 higher in primary phase bacterial cells than in secondary  
 545 cells, which might be one reason why cultures that started  
 546 with secondary phase symbionts most often fail (Ehlers  
 547 et al. 1990; Han and Ehlers 2001). The observation that  
 548 recovery was reduced in culture supernatants was confirmed  
 549 also for the secondary supernatant.

550 Another important observation resulting from these  
 551 experiments was that not only the recovery is positively  
 552 related to the cell density of the bacterial symbiont  
 553 *Xenorhabdus* spp. but also the time necessary for develop-  
 554 ment to adults, which is retarded in lower cell densities in  
 555 both nematode species tested. The dependence on the speed  
 556 of the nematode development implies the need for high-  
 557 density bacterial precultures prior to nematode inoculation.

558

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Publication 3

**Effect of temperature on the development of *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida) in liquid culture**

A. Hirao and R.-U. Ehlers

Applied Microbiology and Biotechnology

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Corrections to galley proof

Line 7: Point missing after "R": R.-U.

Line 89: behind "broth" put: (Akhurst 1980)

Line 132: Instead of "DJ" put "bacterial": "...until bacterial inoculation..."

Line 158: egg put "s": "..eggs.."

Figure 3. Text first line: After "dauer juvenile" put "(DJ)". "...temperature on dauer juvenile (DJ) density.."

Line 273: Instead of "remained" put "were": "...non-DJ stages were in the.."

Line 286: Instead of "remained" put "were": "...progeny were in the ..."

Line 287: After "they" insert "had": "...,while they had developed to.."

Figure 4. Text second line: After "dauer juvenile" put "(DJ)": " ...of the dauer juvenile (DJ) progeny...."

Line 292: put "s" after "DJ": " ..of *S. carpocapsae* DJs varied..."

Line 293: put ", " before "which": "..for DJs, which ..."

Figure 5. Text second line: After "dauer juvenile" put "(DJ)": " ...bars) dauer juvenile (DJ) at 25°C...."

Line 368: After "Fig." put "2" and erase "cxx": "...tested (Fig. 2) and ..."

Line 385: erase "-" after "800": "...approximately 800- $\mu$ m long;..."

Line 385: erase ", " after "wheras": "...; whereas DJS of ..."

Order of two References is not correct:

1. Ehlers-Lunau before Ehlers-Stoessel
2. Grewal-Gaugler before Grewal-Selvan

# Effect of temperature on the development of *Steinernema carpocapsae* and *Steinernema feltiae* (Nematoda: Rhabditida) in liquid culture

A. Hirao & R.-U. Ehlers

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**Abstract** For commercial use of the entomopathogenic nematodes *Steinernema carpocapsae* and *Steinernema feltiae* in biological control of insect pests, they are produced in liquid culture on artificial media pre-incubated with their symbiotic bacteria *Xenorhabdus nematophila* and *Xenorhabdus bovienii*, respectively. After 1 day of the bacterial culture, nematode dauer juveniles (DJs) are inoculated, which recover development. The adult nematodes produce DJ offspring, which are harvested and can be sprayed. This study determined optimal temperatures to obtain high DJ progeny within a short process time. Temperatures assessed were 23°C, 25°C, 27°C, and 29°C for *S. carpocapsae* and 20°C, 23°C, 25°C, and 27°C for *S. feltiae*. The recovery of inoculated DJs was hardly affected and was reduced only in *S. carpocapsae* at 29°C. The fecundity (eggs in uterus) in *S. carpocapsae* reached a maximum at 27°C; whereas, maximum yields were recorded at 25°C. For both *Steinernema* spp., highest DJ densities were obtained after 15 days incubation at 25°C. Optimal culture temperature for both nematode species is 25°C. *S. carpocapsae* was more sensible to suboptimal temperature than *S. feltiae*. Results on total DJ density and DJ proportion of the total nematode population were more variable at non-optimal temperature condition for *S. carpocapsae* than for *S. feltiae*. Suboptimal culture temperature also reduced DJ infectivity.

36 **Keywords** Biological control · Entomopathogenic  
 37 nematodes · Process temperature · Dauer juvenile density ·  
 38 Reproduction · Liquid culture

A. Hirao · R.-U. Ehlers (✉)  
 Institute for Phytopathology, Department for Biotechnology  
 and Biological Control, Christian-Albrechts-University Kiel,  
 Hermann-Rodewald-Str. 9,  
 24118 Kiel, Germany  
 e-mail: ehlers@biotec.uni-kiel.de

## Introduction 39

For biological control of pest insects, the entomopathogenic nematodes *Steinernema carpocapsae* and *Steinernema feltiae* (Nematoda: Rhabditida) are produced in large-scale liquid culture (Ehlers 2001). These soil-inhabiting nematodes have a specific third stage juvenile, which is adapted to long-term survival and therefore called “dauer juvenile” (DJ). It carries cells of its specific enteric bacterial symbiont of the genus *Xenorhabdus* spp. in a pouch in the anterior part of the intestine (Ciche et al. 2006). DJs actively search for insects, invade the insect body via natural openings or less sclerotic parts of the cuticle, and release the bacteria. Approximately 2 days after invasion, the insects die of septicemia (Dowds and Peters 2002). Inside the insect, the DJ is exposed to components of the hemolymph (so called “food signals”) that trigger the exit of the DJ stage. This developmental step is called “recovery” (Strauch and Ehlers 1998). During recovery, the DJ releases its symbiotic bacteria. They proliferate after the insect is dead, providing the major food source for the nematode. The DJs develop to amphimictic males and females, which copulate and produce offspring. The nematodes hatching from the eggs develop either to reproductive adult stages producing further adult generations or to DJs. In the closely related nematode *Heterorhabditis bacteriophora*, DJ formation is a response to poor nutritional concentration (Strauch et al. 1994). Newly developed DJs leave the cadaver searching for new insect host.

The life cycle of *Steinernema* spp. in liquid culture mass production is similar to the life cycle in insects. The main difference is that the symbiotic bacteria are pre-cultured in artificial media prior to DJ inoculation in order to produce secondary metabolites (bacterial food signals), which also induce DJ recovery.

73	Cost efficiency in nematode mass production is driven	collected in sterile Ringer's solution. DJs were stored at 4°C	120
74	by the ratio of DJ output to the DJs inoculated and by the	until use. All steps were performed under sterile conditions.	121
75	process time. Incubation temperature is an important factor		
76	influencing nematode development. In this study, the	Pre-culture of symbiotic bacteria	122
77	influence of culture temperature on nematode population		
78	development in liquid culture was investigated to determine	In order to provide a homogenous bacterial inoculum for the	123
79	the optimal temperature for the development of <i>S. carpocapsae</i>	nematode culture, the bacteria were produced in one batch	124
80	and <i>S. feltiae</i> .	culture and then distributed over several Erlenmeyer flasks	125
		inoculated with nematodes. The bacterial cultures were	126
81	<b>Materials and methods</b>	carried out according to Johnigk et al. (2004) in a bioreactor	127
82	Symbiotic bacteria	(Belach, Sweden) filled with 10 l LM, supplemented with	128
		0.1% (v/v) anti-foam (Silfoam, SE2, Wacker Chemie), and	129
83	<i>Xenorhabdus</i> spp. were isolated from nematode-infested	steam-sterilized at 120°C for 20 min. After sterilization, the	130
84	insect larvae of <i>Galleria mellonella</i> according to Ehlers et	temperature was kept at 25°C with agitation at 500 rpm until	131
85	al. (1990). <i>Xenorhabdus nematophila</i> was isolated from <i>S.</i>	DJ inoculation into the bioreactor. Prior to inoculation,	132
86	<i>carpocapsae</i> (ALL strain) and <i>Xenorhabdus bovienii</i> from	bacterial stocks were propagated in YS broth. Inoculation	133
87	<i>S. feltiae</i> (SYLT strain). Symbiotic bacteria were then	was at 1% bioreactor volume with cultures of a cell density	134
88	propagated in 100-ml Erlenmeyer flasks containing 30 ml	of 10 <sup>9</sup> cells ml <sup>-1</sup> . During incubation, the temperature was	135
89	YS broth containing (in g l <sup>-1</sup> ) 5 NaCl, 5 yeast extract, 0.5	controlled at 25°C, the pressure at 0.1 bar, and the airflow	136
90	NH <sub>4</sub> PO <sub>4</sub> , 0.5 K <sub>2</sub> PO <sub>4</sub> , and 0.2 MgSO <sub>4</sub> ×7 H <sub>2</sub> O (Merck).	was 1.5 l min <sup>-1</sup> . The pH and O <sub>2</sub> saturation were recorded but	137
91	Bacteria were cultured at 180 rpm and 25°C. When cell	not controlled. Agitation speed was controlled between 300	138
92	density had reached 10 <sup>10</sup> cells ml <sup>-1</sup> , the suspension was	and 800 rpm to keep the O <sub>2</sub> saturation at a minimum of 20%.	139
93	transferred into 2-ml Eppendorf tubes, mixed with glycerol	Bioreactor parameters were recorded by the software	140
94	at 15% (v/v), and stored at -20°C until use.	Phantom (Belach, Sweden).	141
		Nematode flask cultures	142
95	Nematodes		
96	<i>S. carpocapsae</i> (ALL strain) and <i>S. feltiae</i> (EN02 hybrid	When the bacterial pre-culture had reached a cell density of	143
97	strain; SYLT strain was one strain used to produce this	10 <sup>10</sup> cells ml <sup>-1</sup> and protein inclusion bodies could be	144
98	hybrid) were used. Monoxenic cultures were established	observed in all cells, 100 ml of the culture was transferred	145
99	according to Lunau et al. (1993). The DJ inoculum was	into sterile 500-ml Erlenmeyer flasks inoculated with	146
100	produced according to Ehlers et al. (1998) in liquid medium	4,000 DJs ml <sup>-1</sup> (ranging from 3,166 to 4,666 DJs ml <sup>-1</sup> )	147
101	(LM) containing (in g l <sup>-1</sup> ) 15 yeast extract (Merck), 20 soy	and incubated at four different temperatures (23°C, 25°C,	148
102	flour (Cargill), 4 NaCl, 0.35 KCl, 0.15 CaCl <sub>2</sub> , 0.1 MgSO <sub>4</sub>	27°C, and 29°C for <i>S. carpocapsae</i> and 20°C, 23°C, 25°C,	149
103	(Merck), 6 lecithin, and 40 vegetable oil. Media were	and 27°C for <i>S. feltiae</i> ). The influence of the different culture	150
104	adjusted to pH 7.0 for <i>S. carpocapsae</i> and 6.5 for <i>S. feltiae</i> ,	temperatures on DJ recovery, egg production, and DJ	151
105	with 4 M KOH (Merck). LM was inoculated with the	proportion of the total nematode population were recorded	152
106	symbiotic bacteria of the specific nematode. Briefly,	daily. DJ recovery (in percent) was assessed according to	153
107	bacterial stock were thawed and propagated at 25°C in	Hirao and Ehlers (2009) 1 day before the first progeny	154
108	YS broth until a cell density of 10 <sup>9</sup> cells ml <sup>-1</sup> was reached.	hatched from eggs (before progeny DJs are occurring). The	155
109	This YS bacterial culture was then inoculated into LM at	number of eggs in the uterus (fecundity) of females, which	156
110	1% volume of the final culture volume and incubated for	had developed from inoculated DJs, was assessed on the day	157
111	36 h at 25°C. Then, DJs from monoxenic cultures were	on which the number of females with egg reached its	158
112	inoculated at 3,000 DJs ml <sup>-1</sup> and incubated at 25°C until	maximum. Twelve females were collected from one flask	159
113	harvest at 15 days after DJ inoculation.	culture; dissected and fertilized eggs were counted. For	160
114	Nematodes were collected by centrifugation (2,500 rpm,	monitoring of the nematode and DJ populations, nematode	161
115	10 min). To remove culture debris and bacteria, DJs were	samples were taken every day, and the developmental stage	162
116	washed in Ringer's solution (Merck) and centrifuged again.	of at least 100 nematodes was assessed under an inverted	163
117	Then, active DJs were separated from inactive DJs and	microscope (Zeiss, Germany). Offspring DJs were distin-	164
118	adults by suspending the DJs over a 30-µm sieve in water,	guished from inoculum DJs by their darker appearance due	165
119	and DJs, which had passed through the sieve after 4 h, were	to the higher content of lipid droplets, which is consumed in	166
		older (inoculated) DJs. Another parameter was the presence	167
		of the cuticle of the prior juvenile stage, the pre-dauer J2d	168

169 stage, which was still present in offspring DJs but lost by  
 170 inoculated DJs. The progeny DJ density was assessed on 10  
 171 and 15 days post-DJ inoculation (dpi). Each temperature was  
 172 tested with three flask cultures, and the experiments were  
 173 replicated four times.

174 **Infectivity test**

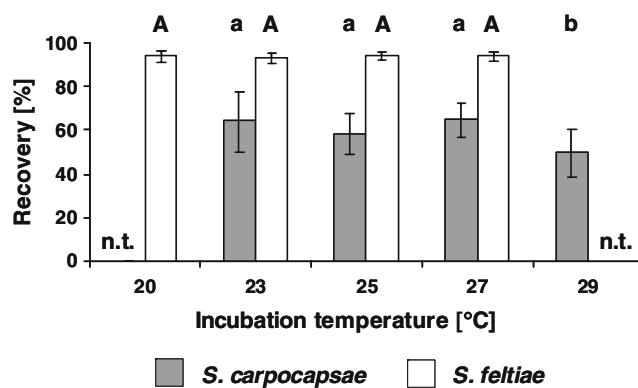
175 The infectivity of DJ offspring was tested with DJs  
 176 harvested on 10 dpi. The infectivity of *S. carpocapsae*  
 177 DJs was tested with final instar *G. mellonella* larvae  
 178 (Lepidoptera) by the one-on-one assay (Conserve and  
 179 Miller 1999). Briefly, single DJs of *S. carpocapsae* were  
 180 placed next to a single *G. mellonella* larva on wet filter  
 181 paper in wells of 24-cell well plates (Greiner). Larval  
 182 mortality was checked on 5 days post nematode applica-  
 183 tion. *Tenebrio molitor* larvae (Coleoptera) were used for  
 184 infectivity test with *S. feltiae*. Forty larvae of *T. molitor*  
 185 were buried in 200 g sand of 10% moisture, and 200 DJs in  
 186 1-ml Ringer's solution were applied on the surface of the  
 187 sand. The same volume of Ringer's solution without  
 188 nematodes was inoculated to insects in the untreated  
 189 control. Larval mortality was checked on 7 dpi. Each  
 190 bioassay had three replicates, and the experiment was  
 191 repeated twice. Insect mortality data were corrected for  
 192 control mortality using Abbott's formula (Abbott 1925).

193 **Statistical analysis**

194 Percentage values for DJ recovery and DJ infectivity were  
 195 arcsine transformed. Effects of incubation temperature on  
 196 recovery, fecundity, progeny DJ density and proportion in  
 197 nematode population, and infectivity were analyzed using  
 198 analysis of variance (ANOVA) and the Tukey's honestly  
 199 significant difference (HSD) test.

200 **Results**

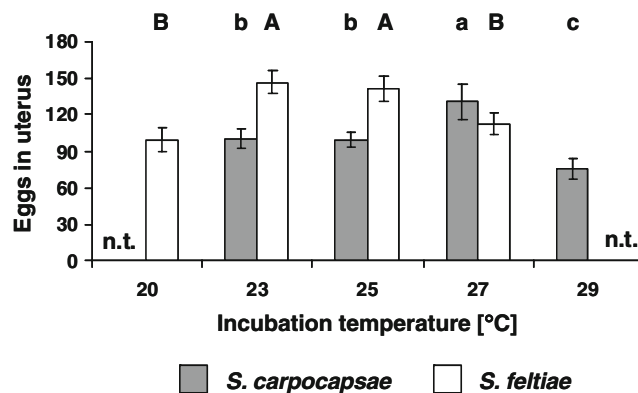
201 No significant difference of the DJ inoculum density among  
 202 the four different temperatures tested for *S. carpocapsae*  
 203 (ANOVA,  $F=1.040$ ,  $df=3, 35$ ,  $P>0.387$ ) and *S. feltiae*  
 204 (ANOVA,  $F=0.266$ ,  $df=3, 36$ ,  $P>0.849$ ) was recorded.  
 205 Thus, variable inoculum density was within limits, which  
 206 should not have had a major influence on the results.  
 207 Significant differences of DJ recovery at different culture  
 208 temperatures were observed only for *S. carpocapsae*  
 209 (ANOVA,  $F=4.745$ ,  $df=3, 35$ ,  $P=0.007$ ), but not for *S.*  
 210 *feltiae* (ANOVA,  $F=0.476$ ,  $df=3, 36$ ,  $P=0.701$ ; Fig. 1). The  
 211 mean DJ recovery of *S. carpocapsae* ranged between 56%  
 212 and 66% and was significantly reduced only at 29°C  
 213 (Tukey's HSD,  $P=0.002$ ). DJ recovery of *S. feltiae* was  
 214 over 90% in all cultures.



**Fig. 1** Dauer juvenile recovery (in percent) of *S. carpocapsae* (gray bars) and *S. feltiae* (white bars) at different temperatures. DJ recovery for *S. carpocapsae* was assessed on 4.8, 3.5, 4, and 5.8 days post inoculation (dpi) at 23°C, 25°C, 27°C, and 29°C, respectively, and DJ recovery for *S. feltiae* on 3.6, 2.5, 2.5, and 3 dpi at 20°C, 23°C, 25°C, and 27°C, respectively (NT not tested). Lines on bars indicate the 95% confidence interval. Different letters on bars indicate significant differences between treatments (Tukey's HSD test,  $P\leq 0.05$ )

215 The fecundity of *S. carpocapsae* females was signifi- 215  
 216 cantly different among temperatures (ANOVA,  $F=18.334$ , 216  
 217  $df=3, 172$ ,  $P<0.0001$ ). The highest fecundity for *S.* 217  
 218 *carpocapsae* with 130.8 eggs per female was observed at 218  
 219 27°C (Tukey's HSD,  $P<0.0001$ ), while the fecundity was 219  
 220 reduced at 29°C (75.3 eggs per female). The egg numbers 220  
 221 at 23°C and 25°C was approximately 100 eggs, and there 221  
 222 was no significant difference ( $P=0.99$ ). The fecundity of 222  
 223 *S. feltiae* incubated at 23°C and 25°C were over 140 eggs 223  
 224 per female, and it was significantly higher than the 224  
 225 fecundity at 20°C and 27°C (ANOVA,  $F=21.063$ ,  $df=3$ , 225  
 226 224,  $P<0.0001$ ; Fig. 2).

227 Culture temperature also had an influence on the 227  
 228 occurrence of the first progeny juveniles (Table 1). At 29° 228  
 229 C, the longest duration until first progeny occurrence of *S.* 229



**Fig. 2** Influence of culture temperature on fecundity of female developed from inoculum dauer juveniles presented as number of eggs per female of *S. carpocapsae* (gray bars) and *S. feltiae* (white bars; NT not tested). Lines on bars indicate the 95% confidence interval. Different letters on bars indicate significant differences between treatments (Tukey's HSD test,  $P\leq 0.05$ )



**Table 1** Influence of culture temperature on the occurrence of progeny

	Culture temperature (°C)				
	20	23	25	27	29
<i>S. carpocapsae</i>	NT	5.8	4.5	5.0	6.8
<i>S. feltiae</i>	4.6	3.5	3.5	4.0	NT

At least 100 nematodes per flask were sampled every day and the day on which newly hatched progeny was recorded  
NT not tested

*carpocapsae* was recorded (6.8 dpi). The shortest period was recorded for both nematodes at 25°C, with 4.5 dpi for *S. carpocapsae* and 3.5 dpi for *S. feltiae*.

The total DJ progeny was assessed on 10 and 15 dpi (Fig. 3a, b). The DJ density on 10 dpi was significantly different between the temperatures for *S. carpocapsae* (ANOVA,  $F=40.262$ ,  $df=3$ ,  $34$ ,  $P<0.0001$ ). The highest

density of *S. carpocapsae* DJs on 10 dpi was observed at 25°C ( $103.1 \times 10^3$  DJs  $ml^{-1}$ ). At 27°C, the DJ density was significantly reduced to  $54.2 \times 10^3$  DJs  $ml^{-1}$  (Tukey's HSD,  $P=0.0002$ ); whereas, at 23°C and 29°C, only few DJs were recorded. The temperature effect on the DJ density on 10 dpi for *S. feltiae* was also significant (ANOVA,  $F=19.016$ ,  $df=3$ ,  $35$ ,  $P<0.001$ ). The DJ density was significantly higher at 23°C and 25°C (approximately  $50 \times 10^3$  DJs  $ml^{-1}$ ) than at 20°C and 27°C ( $P=0.004$ ).

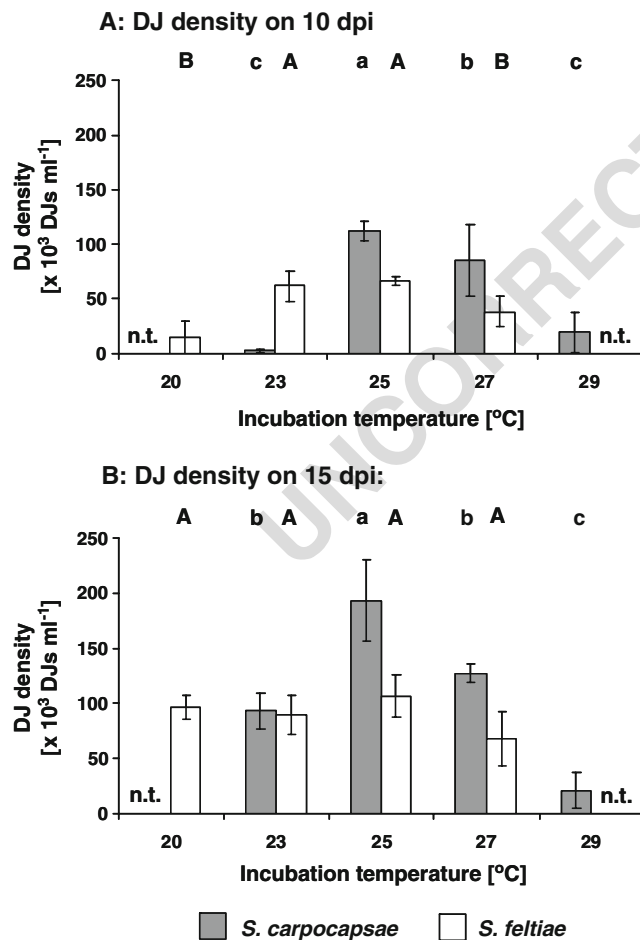
The DJ density for both nematodes increased from 10 to 15 dpi. The temperature effect on DJ density on 15 dpi was significant for *S. carpocapsae* (ANOVA,  $F=40.235$ ,  $df=3$ ,  $23$ ,  $P<0.0001$ ), but not for *S. feltiae* (ANOVA,  $F=3.217$ ,  $df=3$ ,  $20$ ,  $P=0.045$ ). The highest DJ density for *S. carpocapsae* reached almost  $200 \times 10^3$  DJs  $ml^{-1}$  observed at 25°C, followed by 100 and  $120 \times 10^3$  DJs  $ml^{-1}$  at 23°C and 27°C, respectively. The density at 29°C remained low at  $20 \times 10^3$  DJs  $ml^{-1}$  and was significantly lower than at all other temperatures tested ( $P=0.002$ ).

The highest DJ density of *S. feltiae* was also recorded at 25°C, but there was no significant difference among the four temperatures tested (ANOVA,  $F=3.217$ ,  $df=3$ ,  $20$ ,  $P>0.14$ ).

The proportion of progeny DJs of the total nematode population until 15 dpi is presented in Fig. 4. At 23°C, the occurrence of the first *S. carpocapsae* DJs is retarded (11 dpi), and the total proportion did not surpass 50%. At 25°C and 27°C, F1 DJs were first recorded at 7 dpi; however, major differences between these culture temperatures were observed. Whereas the proportion of DJs surpassed 85% at 25°C, it reached less than 65% at 27°C, and the variability between the flasks was much more pronounced than at 25°C. The DJ proportion at 29°C hardly reached 30%, and the first occurrence was not until 9 dpi. At all tested culture temperatures, the DJ proportion was steadily increasing until 15 dpi. At 29°C, all non-DJ stages had developed to adults on 15 dpi; whereas, at 23°C, non-DJ stages remained in the pre-dauer stage.

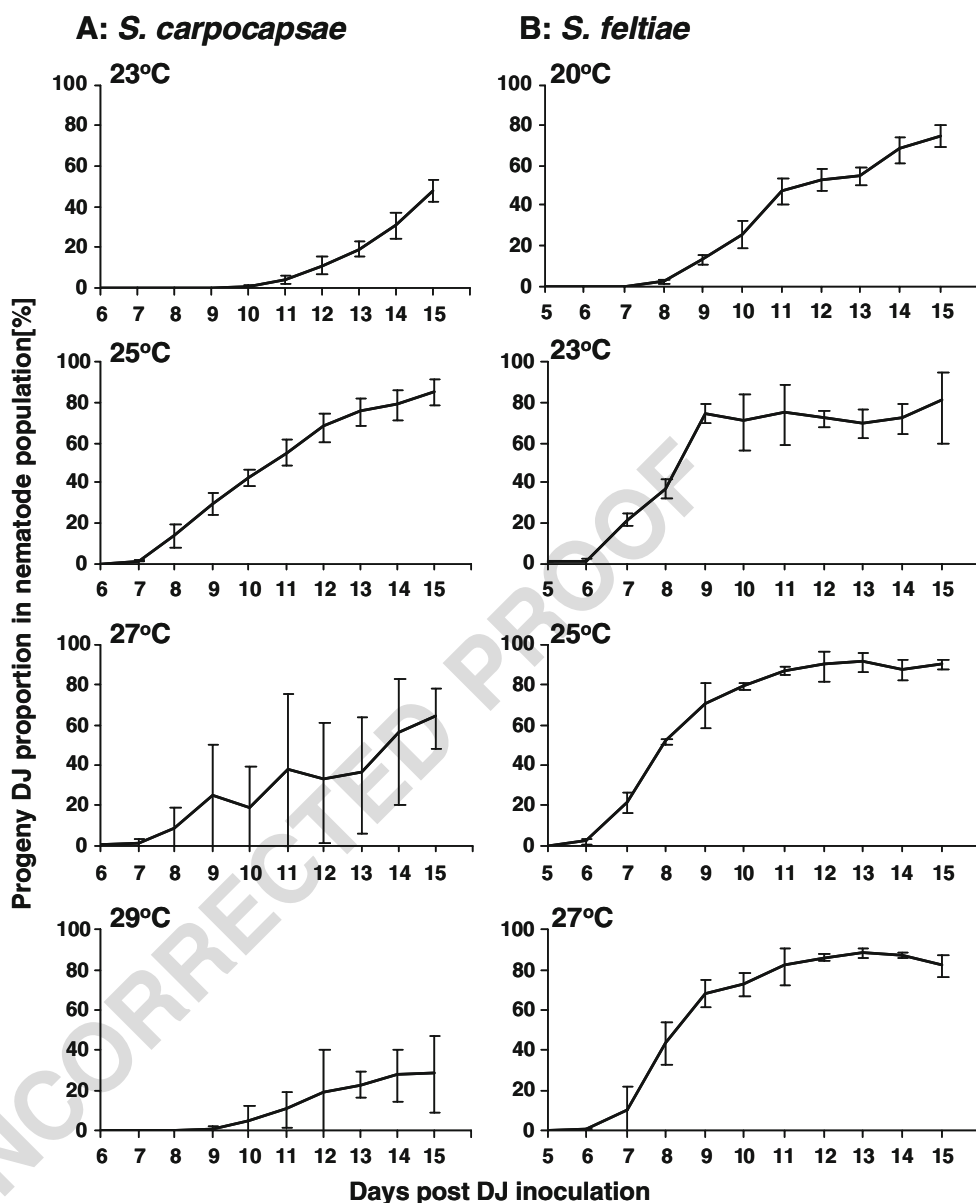
In cultures of *S. feltiae*, the development of F1 generation DJs was earlier than in *S. carpocapsae*. At 23°C and 25°C, first F1 DJs were observed on 6 dpi. At 20°C, the occurrence was retarded by 2 days and at 27°C by 1 day. Except for the DJ proportion at 20°C, which increased until 15 dpi, the maximum DJ proportion at the other temperatures was reached well before 15 dpi. At 23°C, a very high proportion was already recorded on 9 dpi. After reaching the high DJ proportion, the records were with little deviation and remained stable until 15 dpi. The maxima were reached on 12 dpi with 90% at 25°C, 88% at 27°C, and 75% at 23°C. Maximum DJ proportion at 20°C was 47.6%. Most of the non-DJ progeny remained in the pre-DJ stage at 20°C, 23°C, and 25°C, while they developed to adults at 27°C.

Significant effects of the culture temperature on the infectivity of harvested DJs were recorded for *S. carpocapsae*



**Fig. 3** Influence of culture temperature on dauer juvenile density on (a) 10 and (b) 15 days post-DJ inoculation (dpi) of *S. carpocapsae* (gray bars) and *S. feltiae* (white bars; NT not tested). Lines on bars indicate the 95% confidence interval. Different letters on bars indicate significant differences between treatments (Tukey's HSD test,  $P \leq 0.05$ )

**Fig. 4** Proportion of the dauer juvenile progeny of the total nematode population (in percent) of *S. carpocapsae* (left) and *S. feltiae* (right) recorded from 6 days post inoculation (dpi) for *S. carpocapsae* and from 5 dpi for *S. feltiae*. Lines on bars indicate the 95% confidence interval

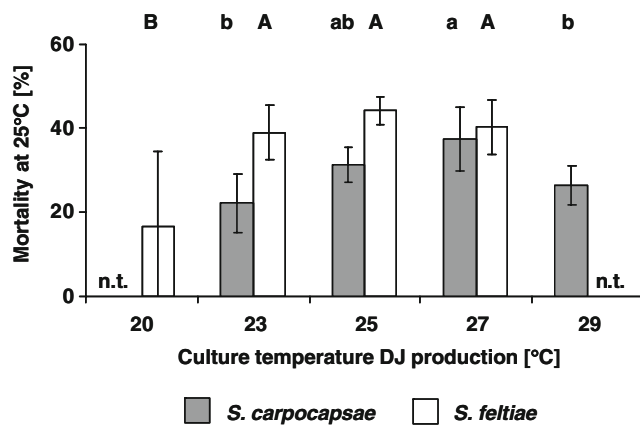


290 (ANOVA,  $F=6.449$ ,  $df=3$ ,  $29$ ,  $P=0.002$ ) and for *S. feltiae*  
 291 (ANOVA,  $F=11.274$ ,  $df=3$ ,  $28$ ,  $P<0.0001$ ). The infectivity  
 292 of *S. carpocapsae* DJ varied between 22.2% and 37.5%,  
 293 with the highest infectivity observed for DJs which had been  
 294 cultured at 27°C (Tukey's HSD,  $P=0.046$ ). The infectivity of  
 295 *S. feltiae* cultured at 23°C, 25°C, and 27°C ranged between  
 296 40.3% and 45.8%, and there was no significant difference  
 297 (Fig. 5). The infectivity of *S. feltiae* DJs cultured at 20°C  
 298 was significantly lower (16.6%,  $P=0.0005$ ).

299 **Discussion**

300 This study determined the optimal temperature for DJ  
 301 production in liquid culture of the entomopathogenic  
 302 nematodes *S. carpocapsae* and *S. feltiae*. The results

indicate that the optimal culture temperature for both 303  
 nematodes is 25°C, when taking into consideration the DJ 304  
 yields and the proportion of DJs of the total nematode 305  
 population at the end of the process time. Both factors are 306  
 the most important parameters defining the success of the 307  
 liquid culture process. The DJ is the developmental stage 308  
 used in biological insect control. The proportion of DJs at 309  
 process end has an influence on the success of the 310  
 following downstream process. A high proportion of non- 311  
 DJ stages make necessary additional cleaning steps. Adults 312  
 and juveniles other than DJs cannot survive in the 313  
 formulated nematode product and would spoil it due to 314  
 the high content of dead organic matter, which promotes 315  
 growth of contaminants and reduces the shelf life of the DJs 316  
 in the final product. The elimination of the non-DJ stages 317  
 during downstream processing is therefore a basic necessi- 318



**Fig. 5** Infectivity of *S. carpocapsae* (gray bars) and *S. feltiae* (white bars) dauer juveniles at 25°C produced at variable culture temperature (NT not tested). Lines on bars indicate the 95% confidence interval. Different letters on bars indicate significant differences between treatments (Tukey's HSD test,  $P < 0.05$ )

319 ty, and the lower the proportion of non-DJ stages in liquid  
320 culture, the easier is the harvesting and subsequent cleaning  
321 (Ehlers 2001). The temperature at which the lowest  
322 proportion of non-DJ stages of the total nematode popula-  
323 tion was recorded correlated with the temperature support-  
324 ing maximum DJ yields.

325 An important developmental step deciding on success  
326 and failure of the liquid culture process is the recovery of  
327 inoculated DJs of the entomopathogenic nematodes *Hetero-*  
328 *rorhabditis* spp. (Strauch and Ehlers 1998). However, for the  
329 two steinernematids tested, the recovery was not a good  
330 indicator for final DJ density. Particularly for *S. feltiae*, DJ  
331 recovery was surpassing 90% at any culture temperature.  
332 The influence of the culture temperature seems to be of  
333 minor significance for the DJ recovery in both steinernema-  
334 tids tested. Only at the highest temperature of 29°C, the  
335 recovery of *S. carpocapsae* declined.

336 The fecundity was highest at 27°C for *S. carpocapsae*,  
337 although maximum yields were recorded at 25°C. This might  
338 be due to a lower insemination of the eggs, as increasing  
339 temperature primarily affects fertility of the male sperm.  
340 With increasing temperature (29°C), the fecundity declined  
341 significantly. In *S. feltiae*, the maximum number of eggs was  
342 recorded at 23°C and 25°C, and a decrease was recorded at  
343 the lowest and the highest temperature. A temperature of  
344 29°C was not assessed for *S. feltiae*, as this nematode is  
345 known to fail in production of offspring under in vivo con-  
346 ditions at a temperature surpassing 28°C (Hazir et al. 2001).

347 As one could expect, the temperature had an influence  
348 on the time scale of the nematode development. The  
349 development was delayed at lower temperatures. The delay  
350 of DJ occurrence at higher temperature might indicate that  
351 the temperature is closed to the maximally tolerated high  
352 temperature for reproduction. Grewal et al. (1994) exam-  
353 ined the influence of temperature on infectivity, establish-

ment, and reproduction for several *Steinernema* and 354  
*Heterorhabditis* species in *G. mellonella* larvae at different 355  
temperatures, ranging from 8°C to 39°C. It resulted that the 356  
temperature range for establishment and reproduction was 357  
relatively narrow compared to the temperature range for 358  
infectivity. The lowest temperature at which the nematode 359  
could reproduce in *G. mellonella* larvae was 20°C for *S.* 360  
*carpocapsae* (ALL strain) and 10°C for *S. feltiae* (SN and 361  
Argentina strain); whereas, the highest temperature for 362  
reproduction in *G. mellonella* larvae was 30°C for *S.* 363  
*carpocapsae* and 25°C for *S. feltiae*. Not only the delayed 364  
development but also the reduction of fecundity caused a 365  
low final DJ density at higher temperatures. Fecundity of 366  
both *S. carpocapsae* and *S. feltiae* was low at the highest 367  
temperature tested (Fig. cxx) and resulted in low DJ yield. 368  
The data on DJ yields and DJ proportion indicate that DJ 369  
yield on 10 dpi can be predicted by the number of eggs and 370  
the developmental speed from inoculated DJ to adult. In 371  
contrast, DJ yield on 15 dpi cannot be predicted from these 372  
parental factors anymore. 373

When comparing the data of both nematodes, one major 374  
difference is observed in the reaction to changing culture 375  
temperature between *S. carpocapsae* and *S. feltiae*. The 376  
nematode *S. feltiae* is less sensible to changing temperature 377  
in DJ yields and proportion of DJs of the total nematode 378  
population. The results in yields are less variable at different 379  
temperatures. In contrast, the increase in temperature from 380  
25°C to 27°C caused a significant variability in proportion of 381  
the DJs in *S. carpocapsae*, and also, the total DJ density is 382  
more variable than recorded for *S. feltiae*. The lower total DJ 383  
density obtained with *S. feltiae*, however, is not surprising, as 384  
DJs are approximately 800-µm long; whereas, DJs of *S.* 385  
*carpocapsae*, in average, reach only 500 µm. Hence, the 386  
biomass in cultures of these two species is probably 387  
comparable despite the different numbers. 388

The aspect of infectivity of harvested DJs needs 389  
particular attention. It has been reported that infectivity 390  
differs among different bioassay temperatures (Dunphy 391  
and Webster 1986; Grewal et al. 1994, 1996; Wright 1992; 392  
Zervos et al. 1991), but results of this study indicate that 393  
the incubation temperature during DJ formation can also 394  
affect the DJ infectivity. Both bioassay methods have been 395  
designed for infectivity in commercial production, and the 396  
expected mortality for good quality nematodes in these 397  
bioassay methods is 50%. The mean mortality recorded in 398  
this study was lower than 50% with both *S. carpocapsae* 399  
and *S. feltiae*. *S. carpocapsae* and *S. feltiae* DJs reared at 400  
lower temperatures had the lowest infectivity. Since all the 401  
bioassays were performed at 25°C, the difference of 402  
infectivity might have been caused by the number of 403  
symbiotic bacteria retained in the DJs. In other words, the 404  
DJs at lower temperature were physiologically younger 405  
than those of reared at higher temperatures; hence, the 406

407 bacteria stored in the vesicle of the DJs had not multiplied  
 408 yet inside the DJs, and, as a consequence, fewer insects  
 409 were killed. Martens et al. (2003) reported that at the early  
 410 phase of bacterial retention, there were fewer cells in the  
 411 DJ intestine and that the cells grow and divide until filling  
 412 the complete DJ vesicle in the intestine. Harvest of DJs  
 413 should therefore be well timed to avoid deficiencies in  
 414 infectivity.

415  
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Publication 4

**Influence of inoculum density on population dynamics and  
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Rhabditida)**

A. Hirao and R.-U. Ehlers

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## Publication 4

Correction to galley proof

Line 8: We have one name (Hirao) with abbreviation of the first name and the other with the full name (Ehlers). Either "Ayako Hirao . Ralf-Udo Ehlers" or "A. Hirao . R.-U. Ehlers"

Line 15: "nematophila"

Line 53: " $10^5 \times 10^3$  per mililiter"

Line 83: "amphimictic"

Line 104-105: Instead of "Hirao and Ehlers 2009c" put "Hirao et al. 2009"

Line 428: Insert a free space between "preculture" and "(Table 1)"

Line 484: Insert a free space between "steinernematids" and "(Hirao"

Line 491: Erase "and Olaf Strauch"

Line 526: "*Caenorhabditis elegans*" in italics

Line 517: Erase free space after "Kluwer"

Line 535: "bacteriophora"

Line 545: Instead of "Hirao A, Ehlers R.-U (2009c)" put "Hirao A, Ehlers R-U, Strauch  
O (2009)"

# Influence of inoculum density on population dynamics and dauer juvenile yields in liquid culture of biocontrol nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida)

A. Hirao & Ralf-Udo Ehlers

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**Abstract** For improvement of mass production of the rhabditid biocontrol nematodes *Steinernema carpocapsae* and *Steinernema feltiae* in monoxenic liquid culture with their bacterial symbionts *Xenorhabdus nematopila* and *Xenorhabdus bovienii*, respectively, the effect of the initial nematode inoculum density on population development and final concentration of dauer juveniles (DJs) was investigated. Symbiotic bacterial cultures are pre-incubated for 1 day prior to inoculation of DJs. DJs are developmentally arrested and recover development as a reaction to food signals provided by their symbionts. After development to adults, the nematodes produce DJ offspring. Inoculum density ranged from 1 to  $10 \times 10^3$  DJ per milliliter for *S. carpocapsae* and 1 to  $8 \times 10^3$  DJs per milliliter for *S. feltiae*. No significant influence of the inoculum density on the final DJ yields in both nematode species was recorded, except for *S. carpocapsae* cultures with a parental female density  $< 2 \times 10^3$  DJs per milliliter, in which the yields increased with increasing inoculation density. A strong negative response of the parental female fecundity to increasing DJ inoculum densities was recorded for both species with a maximum offspring number per female of  $> 300$  for *S. carpocapsae* and almost 200 for *S. feltiae*. The compensative adaptation of fecundity to nematode population density is responsible for the lack of an inoculum (or parental female) density effect on DJ yields. At optimal inoculation density of *S. carpocapsae*, offspring were produced by the parental female population, whereas *S.*

*feltiae* always developed a F1 female population, which contributed to the DJ yields and was the reason for a more scattered distribution of the yields. The F1 female generation was accompanied by a second peak in *X. bovienii* density. The optimal DJ inoculum density for *S. carpocapsae* is  $3-6 \times 10^3$  DJs per milliliter in order to obtain  $> 10^3$  parental females per milliliter. Density-dependent effects were neither observed on the DJ recovery nor on the sex ratio in the parental adult generation. As recovery varied between different batches, assessment of the recovery of inoculum DJ batches is recommended. *S. feltiae* was less variable in DJ recovery usually reaching  $> 90\%$ . The recommended DJ inoculum density is  $> 5 \times 10^3$  DJs per milliliter to reach  $> 2 \times 10^3$  parental females per milliliter. The mean yield recorded for *S. carpocapsae* was  $135 \times 10^3$  and  $105 \times 10^3$  for *S. feltiae*.

**Keywords** Biocontrol nematodes · Liquid culture · Inoculum density · *Steinernema* · *Xenorhabdus* · Dauer juvenile yields

## Introduction

Entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis* (Nematoda: Rhabditida) are safe biocontrol agents (Ehlers 2003) widely used to manage insect pests in plant protection (Grewal et al. 2005). They are symbiotically associated with enterobacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, members of the gamma subclass of the *Proteobacteria* (Ehlers et al. 1988). Developmentally arrested, third-stage dauer juveniles (DJs) of the nematodes occur free-living in the soil searching for host insects. They carry several hundred cells of their specific symbiont in the anterior part of the intestine. The DJs seek and invade suitable host

A. Hirao · R.-U. Ehlers (✉)  
Institute for Phytopathology,  
Department for Biotechnology and Biological Control,  
Christian-Albrechts-University Kiel,  
Hermann-Rodewald-Str. 9,  
24118 Kiel, Germany  
e-mail: ehlers@biotec.uni-kiel.de

70 insects (Ciche et al. 2006). In the insect's hemolymph, DJs  
 71 encounter yet unknown signals (Strauch and Ehlers 1998),  
 72 which induce the exit from the developmentally arrested  
 73 DJ stage. Analogous to the closely related nematode  
 74 *Caenorhabditis elegans*, this developmental step is called  
 75 "recovery" (Strauch and Ehlers 1998) and the recovery  
 76 inducing signal food signal (Golden and Riddle 1984).  
 77 During recovery, the DJs release the bacterial symbionts  
 78 into the insect's haemocoel. They proliferate and produce  
 79 suitable conditions for nematode reproduction. EPN cannot  
 80 reproduce well without the presence of their symbionts  
 81 (Han and Ehlers 2000). The nematodes develop through a  
 82 J4 stage to adults. In steinernematids, these adults are  
 83 amphimictic, whereas heterorhabditid nematodes develop  
 84 to hermaphrodites, and the offspring of these self-fertilizing  
 85 adults can develop to amphimictic stages or to DJs. In  
 86 *Heterorhabditis* spp., this alternative pathway is induced  
 87 during the J1 stage (Johnigk and Ehlers 1999a). A high  
 88 bacterial cell density induces the development of another  
 89 adult generation. Low food supply induces the development  
 90 to DJs (Strauch et al. 1994). As long as food is abundant,  
 91 the nematodes develop additional adult generations. With  
 92 degrading food supply (insect cadaver consumed) DJ  
 93 formation is induced. Another result of depleting nutrients  
 94 and bacteria is the cessation of egg-laying by the adults.  
 95 Instead, J1 hatch from eggs inside the uterus and develop  
 96 to DJs on cost of the maternal body, a phenomenon called  
 97 endotokia matricida (Johnigk and Ehlers 1999b). DJ  
 98 offspring exit from the insect cadaver approximately  
 99 2 weeks after infestation (Ciche et al. 2006).

100 Extensive use of EPN in biocontrol depends on their  
 101 cost-effective mass production, which is done in monoxenic  
 102 liquid culture (Ehlers 2001). The life cycle in liquid culture  
 103 resembles that in vivo for species of both genera (Johnigk  
 104 and Ehlers 1999a; Wang and Bedding 1996; Hirao and  
 105 Ehlers 2009c), with the major difference that in vitro,  
 106 artificial media are pre-incubated with the symbiotic  
 107 bacteria prior to DJ inoculation. DJ recovery then is a  
 108 response to bacterial food signals (Strauch and Ehlers 1998;  
 109 Aumann and Ehlers 2001; Hirao and Ehlers 2009a) rather  
 110 than to signals in the hemocoel of a newly infected insect.  
 111 Of critical importance for successful commercial mass  
 112 production are high DJ yields within a short process time  
 113 and an acceptable activity and infectivity (quality) of the  
 114 resulting DJs (Ehlers 2001). Hence, a process that is  
 115 producing a maximum DJ concentration within one  
 116 nematode generation is superior to a two-generation process  
 117 (Ehlers et al. 1998). Maximum DJ yields depend on high  
 118 food resources. On the other hand, DJ formation is a  
 119 response to depleting food resources. Thus, the actual  
 120 availability of food in these batch cultures is important. An  
 121 initial optimum nutrition supplies abundant food for  
 122 bacterial growth and offspring production by the parental

(inoculated) nematode population. Subsequently, starvation 123  
 conditions should induce the development to DJs. In theory, 124  
 a high initial nematode population should be well supplied 125  
 to produce large numbers of offspring and consume most 126  
 of the food, thus initiating DJ-inducing conditions. Hence, 127  
 it should be possible to define an optimal inoculum 128  
 density for a given liquid culture medium. In fact, Ehlers 129  
 (2001) recorded a maximum final yield of *Heterorhabditis* 130  
*bacteriophora* at an initial hermaphrodite density between 131  
 3,000 and 5,000 recorded on the third day after nematode 132  
 inoculation. The aim of the present study was to define the 133  
 optimum inoculation density for the biocontrol nematodes 134  
*Steinernema carpocapsae* and *Steinernema feltiae* to 135  
 obtain a maximum DJ yield within a short process time. 136  
 Population development of symbiotic bacteria and the 137  
 different nematode stages was monitored, including DJ 138  
 recovery and sex ratio. 139

## 140 Materials and methods

### 141 Monoxenic culture of bacto-helminthic complex

142 *Xenorhabditis* spp. were isolated from nematode-infested 142  
 insect larvae of *Galleria mellonella*, cultured and stored 143  
 according to Hirao and Ehlers (2009b). *Xenorhabditis* 144  
*nematophila* was isolated from the All strain of *S.* 145  
*carpocapsae*, *Xenorhabditis bovienii* from the Sylt strain 146  
 of *S. feltiae*. *S. carpocapsae* (All strain) and *S. feltiae* 147  
 (EN02 hybrid strain, includes Sylt strain) were used. 148  
 Monoxenic cultures were established according to Lunau 149  
 et al. (1993). The DJ inoculum was produced according 150  
 to Ehlers et al. (1998) in liquid medium (LM) containing 151  
 (in g l<sup>-1</sup>) 15 yeast extract (Merck), 20 soy powder 152  
 (Cargill), 4 NaCl, 0.35 KCl, 0.15 CaCl<sub>2</sub>, 0.1 MgSO<sub>4</sub> 153  
 (Merck), 6 lecithin diluted with same volume of vegetable 154  
 oil and 40 vegetable oil. Media were adjusted to pH7.0 for 155  
*S. carpocapsae* and 6.5 for *S. feltiae* with 4 M KOH 156  
 (Merck). For nematode inoculum production, LM was 157  
 inoculated with the symbiotic bacteria and when cell 158  
 density had reached 10<sup>9</sup> cells per milliliter inoculated with 159  
 3,000 DJs per milliliter and incubated at 25°C until harvest 160  
 at 15 days after inoculation of the symbiotic bacteria. The 161  
 DJs from monoxenic cultures were washed and stored at 162  
 4°C until use (Hirao and Ehlers 2009b). 163

### 164 Influence of nematode inoculum density on population 165 dynamics and yields

166 The influence of the nematode inoculum density was tested 166  
 in monoxenic flask cultures. In order to provide a homoge- 167  
 nous symbiont inoculum, the bacterial culture was done in 168  
 bioreactors (5 l Meredos, Germany, for *S. carpocapsae*, 10 l 169



170 Belach, Sweden, for *S. feltiae*) according to Johnigk et al.  
 171 (2004). LM was supplemented with 0.1% (v/v) anti-foam  
 172 (Silfoam SE2, Wacker Chemie, Munich, Germany). Process  
 173 conditions were according to Hirao and Ehlers (2009b). The  
 174 cell density of each pre-culture and the time of harvest are  
 175 presented in Table 1. Bacterial cell density was assessed by  
 176 counting cells in a Thoma chamber under the microscope at  
 177 600-fold magnification. The cultures were used when a cell  
 178 density >10<sup>10</sup> cells per milliliter had been reached and all  
 179 cells contained protein inclusion bodies (Couche and  
 180 Gregson 1987). *X. nematophila* pre-culture was adjusted to  
 181 a cell density at 10<sup>10</sup> cells per milliliter by dilution with  
 182 culture supernatant. Cells were removed by centrifugation at  
 183 14,000 rpm from the same bacterial culture and the  
 184 supernatant used for dilution. *X. bovienii* pre-cultures were  
 185 used without dilution since their density was close to 10<sup>10</sup>  
 186 cells per milliliter. Each sterile 500-ml wide-necked  
 187 Erlenmeyer flask received 100 ml bacterial culture broth.

188 The flasks were then inoculated with 1,000–10,000 DJs  
 189 per milliliter of *S. carpocapsae* DJ or with 1,000–8,000 DJs  
 190 per milliliter of *S. feltiae* and incubated at 180 rpm, at 25°C.  
 191 Samples were taken daily, and at least 100 nematodes were  
 192 assessed under an inverted microscope (Zeiss, Germany)  
 193 and the bacterial density recorded as described. The  
 194 experiments we performed three times with three different  
 195 nematode inoculum batches. The inoculated nematode  
 196 density in each flask was determined 1 day after DJ  
 197 inoculation. These initial mean DJ densities and minimum  
 198 and maximum are presented in Table 2. Each DJ density  
 199 was tested in three flasks.

200 DJ recovery was assessed according to Hirao and Ehlers  
 201 (2009a) and represents the percentage of the sum of J3, J4,  
 202 and adults of the nematode population 1 day before first  
 203 progeny hatched from eggs. Female proportion in parental  
 204 adult populations was assessed when the maximal density  
 205 of males and females was observed the day before DJ  
 206 offspring were recorded. Fertility of the parental females  
 207 was assessed until 7 days post-DJ inoculation (dpi) on  
 208 which the offspring number per female had reached its  
 209 maximum density. DJ yields were assessed on 10 and

t1.1 **Table 1** Cell density in bioreactor of pre-culture *X. nematophila* and  
 t1.2 *X. bovienii* and process time at which the bacteria were transferred to  
 t1.3 monoxenic flask cultures

Batch	<i>X. nematophila</i>		<i>X. bovienii</i>	
	Cell density (×10 <sup>9</sup> cells per milliliter)	Process time (hours)	Cell density (×10 <sup>9</sup> cells per milliliter)	Process time (hours)
t1.4 1	24.0	26	9.5	30
t1.5 2	32.0	27	11.2	29
t1.6 3	22.0	24	10.8	26

**Table 2** Mean (N=3) DJ inoculum density (minimum and maximum  
 in parenthesis) of three different batches of *Steinernema carpocapsae*  
 and *S. feltiae* counted 1 day after DJ inoculation

Mean (min–max) DJ density ×10 <sup>3</sup> ml <sup>-1</sup>					
Batch 1	Batch 2		Batch 3		
<i>S. carpocapsae</i>					
1.3	(1.2–1.4)	1.7	(1.7–1.8)	1.0	(0.9–1.0)
2.6	(2.5–2.6)	4.0	(3.9–4.0)	1.9	(1.8–2.0)
3.6	(3.3–3.8)	5.2	(5.0–5.5)	2.9	(2.8–2.9)
4.8	(4.8–4.9)	6.9	(6.8–6.9)	3.8	(3.7–4.0)
5.2	(5.1–5.3)	8.4	(8.1–8.6)	4.9	(4.6–5.2)
7.6	(6.6–8.3)	10.7	(10.3–11.2)	6.0	(5.8–6.1)
8.4	(8.1–8.6)			7.3	(7.1–7.5)
				8.3	(7.9–8.4)
<i>S. feltiae</i>					
1.6	(1.5–1.6)	0.9	(0.8–0.9)	1.6	(1.4–1.8)
3.8	(3.6–4.0)	3.3	(3.1–3.4)	4.8	(4.2–5.2)
		5.6	(5.2–6.0)	7.2	(6.9–7.7)
		7.8	(7.4–8.2)		

15 dpi. Offspring DJs were distinguished from old,  
 inoculated DJs by their darker appearance due to more  
 densely packed lipid droplets and the presence of the cuticle  
 of the pre-dauer stage (J2d) in offspring DJs.

Samples of each culture were streaked on NBTA agar  
 (Akhurst 1980) to check for the presence of contaminants  
 on 7 and 14 dpi. Whenever a flask was contaminated, all  
 three replicates were discarded.

**Statistical analysis**

DJ recovery and the female proportion in parental adult  
 generation are presented in percentage. After arcsin  
 transformation, data were assessed by analysis of variance  
 (ANOVA) and Tukey honestly significant difference (HSD)  
 test. The correlation between inoculum DJ age and DJ  
 recovery was assessed by two-paired comparison.

**Result**

Effect of inoculum density and DJ age on DJ recovery

Results on median and minimum and maximum DJ  
 recovery are presented in Table 3. *S. carpocapsae* DJ  
 recovery, ranging from 56% to 83%, differed significantly  
 between nematode batches (ANOVA, F=644.075; df=2,  
 62; P<0.0001). Although there was a significant batch to  
 batch difference in *S. feltiae* (ANOVA, F=8.896, df=2, 24,  
 P<0.001), recovery reached at least 80% and usually

t3.1 **Table 3** Median (minimum and maximum in parenthesis) of *Steinernema carpocapsae* and *S. feltiae* dauer juvenile recovery

Batch	<i>S. carpocapsae</i>		<i>S. feltiae</i>	
	Recovery (%)	Age (days)	Recovery (%)	Age (days)
t3.4 1	70.6b (67.3–73.1)	75	83.6a (72.1–93.9)	128
t3.5 2	82.9c (69.9–86.3)	105	93.3b (89.3–97.5)	65
t3.6 3	55.6a (52.0–57.5)	41	94.5b (89.7–99.3)	15

Percentages indicated with different letters within one column are significantly different (Tukey HSD test,  $P \leq 0.05$ )

234 surpassed 90%. There was no difference in the day at which  
 235 offspring J1 were first recorded between the different  
 236 inoculum densities; hence, there is no indication for a  
 237 delayed recovery or delayed development of the parental  
 238 generation. Evaluation of the recovery data indicated no  
 239 density-dependent differences for both species. A signifi-  
 240 cant correlation between the recovery and the age of the DJ  
 241 inoculum (Table 3) was detected for *S. carpocapsae* ( $R =$

t4.1 **Table 4** Median (minimum and maximum in parenthesis) of female  
 proportion in parental adult population of *Steinernema carpocapsae*  
 and *S. feltiae* in three batches

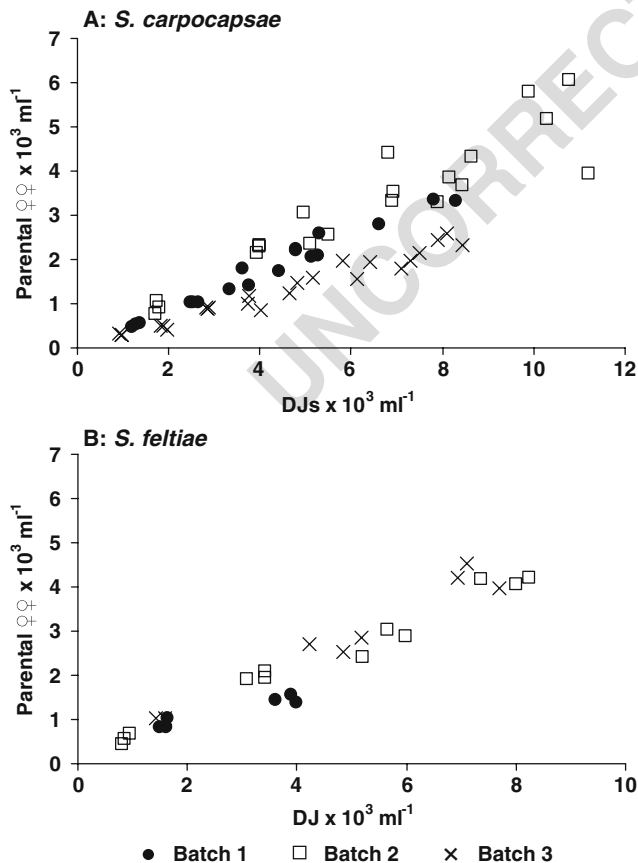
Batch	Females (%)			
	<i>S. carpocapsae</i>		<i>S. feltiae</i>	
t4.4 1	70.7b	(56.1–76.4)	67.9a	(64.3–69.4)
t4.5 2	63.9a	(57.9–77.5)	73.4b	(68.2–79.4)
t4.6 3	72.8b	(52.9–80.9)	73.5b	(67.8–75.2)

Percentages within one column indicated with different letters are significantly different (Tukey HSD test,  $P \leq 0.05$ )

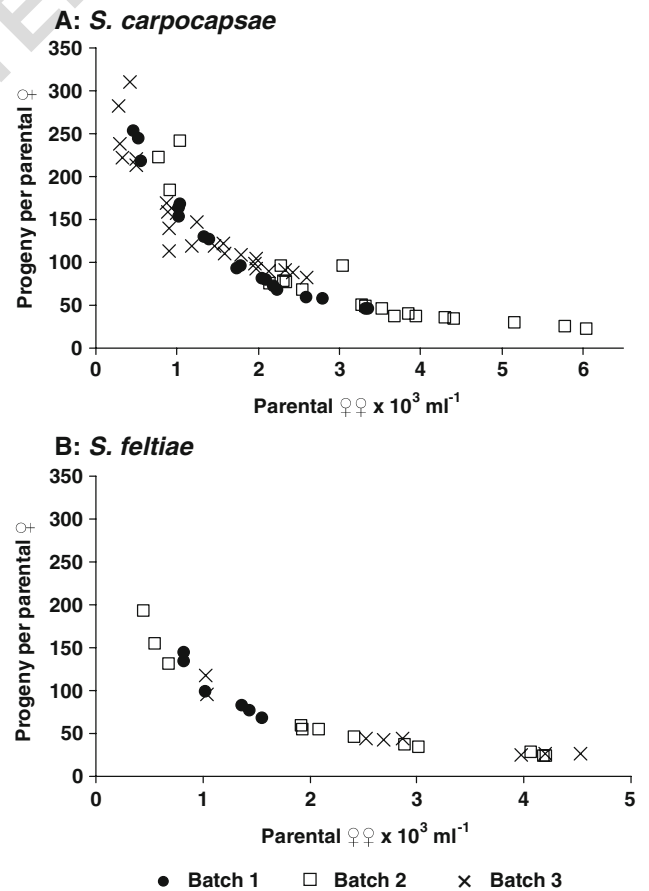
0.98,  $P < 0.0001$ ), whereas the recovery of *S. feltiae* was not  
 242 affected by the DJ age and was equally high in all cultures. 243

Parental female density, sex ratio, and fertility 244

The parental female density increased at a constant value in  
 245 relation to the DJ inoculum (Fig. 1). The proportion of the  
 246 females in the sex ratio is strongly biased toward the female  
 247



**Fig. 1** Influence of dauer juvenile (DJ) inoculum density on number of parental females of *Steinernema carpocapsae* (a) and *S. feltiae* (b)



**Fig. 2** Influence of parental female density on the number of progeny per female of *Steinernema carpocapsae* (a) and *S. feltiae*. Fertility was assessed until 7 days post-DJ inoculation

248 sex. No density-dependent effect on the proportion of the  
 249 parental female population within one batch was recorded,  
 250 but significant differences existed between the batches  
 251 (Table 4). The female proportion in batch 2 of *S.*  
 252 *carpocapsae* ( $F=7.709$ ,  $df=2$ ,  $59$ ,  $P<0.001$ ) and batch 1  
 253 of *S. feltiae* ( $F=10.652$ ,  $df=2$ ,  $23$ ,  $P<0.001$ ) were  
 254 significantly lower than in the other batches of the  
 255 respective species.

256 The fertility of parental females decreased with increas-  
 257 ing initial DJ densities in both species, with a maximum  
 258 offspring number per female of  $>300$  for *S. carpocapsae*  
 259 (Fig. 2a) and almost 200 for *S. feltiae* (Fig. 2b) The  
 260 fecundity was not different between batches.

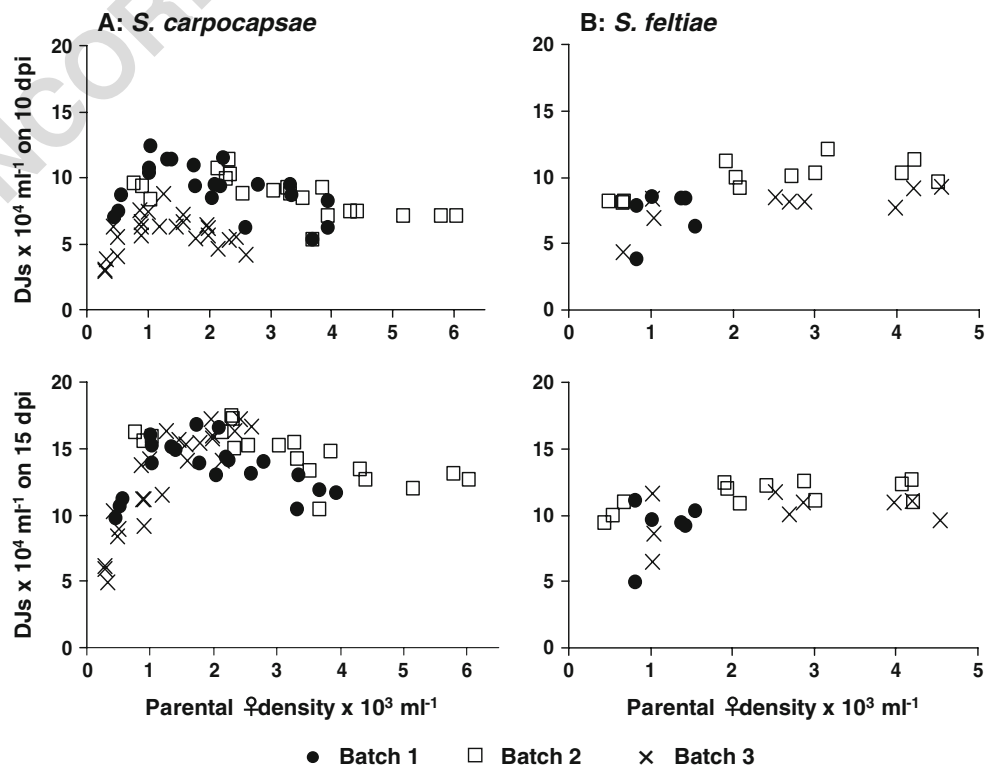
261 Influence of DJ inoculum density on DJ yield

262 The mean yield recorded for *S. carpocapsae* was  $135 \times 10^3$   
 263 with a variability (standard error) of  $1-17 \times 10^3$  DJs per  
 264 milliliter between the three flasks and for *S. feltiae*  $105 \times 10^3$   
 265 with a variability of  $5-10 \times 10^3$  DJ per milliliter (two  
 266 cultures surpassed a variability of  $25 \times 10^3$  DJ per milliliter).  
 267 DJ yields generally increased from 10 to 15 dpi in cultures  
 268 of both steinernematids, although this was more pro-  
 269 nounced in *S. carpocapsae*. No correlation was recorded  
 270 between the variance in yields of the three replicate cultures  
 271 and the DJ inoculum density or the total yields in both  
 272 nematode species.

273 To interpret density-dependent effects, the DJ yields  
 274 were related to the parental female population instead to the  
 275 DJ inoculum density. Due to variable DJ recovery the  
 276 relation to the DJ inoculum density would be misleading.  
 277 Yields recorded on 10 and 15 dpi are presented in Fig. 3. In  
 278 general, there was no significant influence of the parental  
 279 female density on the final DJ yields in both nematode  
 280 species, except for *S. carpocapsae* cultures with a parental  
 281 female density  $<2 \times 10^3$  DJs per milliliter. This is particu-  
 282 larly obvious for batch 3 cultures of *S. carpocapsae* when  
 283 analyzing the results recorded on day 15 post-inoculation.  
 284 These cultures received very low DJ numbers, and the total  
 285 yields were the lowest with the lowest daily increase  
 286 (between 5 and  $10 \text{ DJs} \times 10^3 \text{ ml}^{-1} \text{ day}^{-1}$ ). The yields in batch  
 287 3 increased with increasing DJ inoculum density and  
 288 particularly between 10 and 15 dpi (Fig. 3a).

289 In *S. carpocapsae*, the highest final DJ density ( $175 \times 10^3$   
 290  $\text{DJs ml}^{-1}$ ) was counted on 15 dpi in cultures started with  
 291  $4 \times 10^3$  DJs per milliliter in batch 2. On average, among the  
 292 three batches, inoculum densities between 3 and  $6 \times 10^3$  DJs  
 293 per milliliter (parental female density,  $1-3 \times 10^3 \text{ ml}^{-1}$ )  
 294 produced high final DJ numbers (approximately  $150 \times 10^3$   
 295  $\text{DJs per milliliter}$ ) with daily increases between 15 and  $20 \times$   
 296  $10^3$  DJs per milliliter. When the parental female population  
 297 density surpassed  $3 \times 10^3$  per milliliter, a decline in yields  
 298 was observed in *S. carpocapsae*. In *S. feltiae*, this decline  
 299 was not observed, and no significant differences were

Fig. 3 Influence of parental female density on dauer juvenile (DJ) density of *Steinernema carpocapsae* (a) and *S. feltiae* (b) at 10 and 15 days post-DJ inoculation



300 recorded in DJ yields. Furthermore, no influence of the  
 301 parental female (respectively inoculation) density on yields  
 302 was recorded. In *S. feltiae* cultures (Fig. 3b), the highest DJ  
 303 yield was  $125 \times 10^3$  DJs per milliliter in cultures started with  
 304  $6-7 \times 10^3$  DJs per milliliter ( $4 \times 10^3$  parental females per  
 305 milliliter) in batch 2 on 15 dpi. In cultures with parental  
 306 female densities  $< 2.5 \times 10^3$  nematodes per milliliter, the  
 307 yields dropped. The daily increase in DJ density was  
 308 between 10 and  $14 \times 10^3$  DJs per milliliter.

309 Bacterial and nematode population dynamics

310 A total of 90 flasks were examined daily for the  
 311 development of the population dynamics, however, here,  
 312 only two examples per species, one with a low and another  
 313 with a high DJ inoculation density, are presented, which  
 314 best reflect typical developments of the population dynam-  
 315 ics. Figure 4 presents the development recorded for *S.*  
 316 *carpocapsae*. At a low inoculum density (Fig. 4a), the  
 317 bacterial cell density of *X. nematophila* further increased

after DJ inoculation from  $10^{10}$  to  $2.5 \times 10^{10}$  cells per 318  
 milliliter on 2 dpi. Offspring DJs increase from 6 dpi 319  
 onwards. Part of the parental offspring further developed to 320  
 a F1 adult generation, and F1 females reached a maximum 321  
 density of  $> 10 \times 10^3$  on 11 dpi. In the culture, which 322  
 received a high DJ inoculum (Fig. 4b), the bacterial density 323  
 increased only to  $1.5 \times 10^{10}$  cells per milliliter on 1 dpi and 324  
 then was consumed by the parental adult generation. F1 325  
 generation adults remained  $< 300$  nematodes per milliliter, 326  
 and the DJ offspring density increased beginning on day 7 327  
 post-inoculation. 328

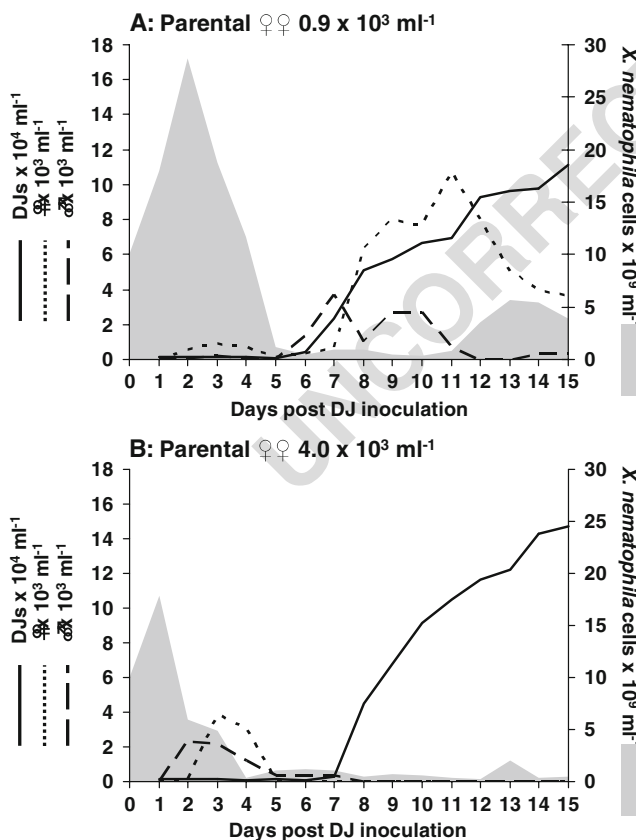
The population dynamics in the *S. feltiae* culture differed 329  
 significantly. In general, *X. boyvienii* produces less cells per 330  
 milliliter than *X. nematophila*, observed also in the pre- 331  
 cultures. In all cultures, an F1 adult generation was 332  
 recorded, whether the flask received a high or low DJ 333  
 inoculum. In almost all cases, the second peak in the cell 334  
 density of *X. boyvienii* surpassed that of the initial density 335  
 (Table 1). The population dynamics of the adult population 336  
 is not much different between cultures with a high or a low 337  
 inoculum. 338

The development of an F1 generation adult population 339  
 in *S. carpocapsae* cultures depends on an inoculum density 340  
 of  $< 2 \times 10^3$  DJs per milliliter, and this population much 341  
 surpasses the density of the parental population (Fig. 6a). 342  
 However, in *S. feltiae*, the occurrence of a second 343  
 generation adult population is the rule, independent from 344  
 the inoculum density. 345

Discussion 346

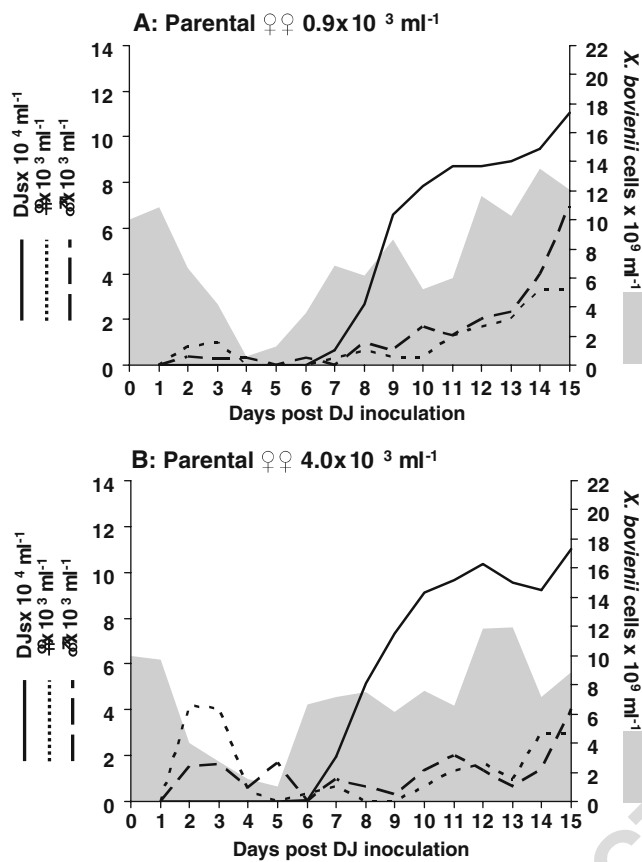
This contribution aimed at elucidating the influence of the 347  
 DJ inoculum density on DJ recovery, parental female 348  
 fertility, and DJ yields in liquid cultures of *S. carpocapsae* 349  
 and *S. feltiae*. According to the results, an optimal parental 350  
 female density can be defined for both nematode species, 351  
 $1-3 \times 10^3$  females per milliliter for *S. carpocapsae* and  $> 2 \times$  352  
 $10^3$  females per milliliter for *S. feltiae* (Fig. 3). Considering 353  
 that half of the inoculated population are males, the number 354  
 could just be doubled to get the DJ inoculum density. 355  
 However, the sex ratio is female-biased (Table 4), and 356  
 double DJ density guarantees an even high proportion of 357  
 females. 358

However, DJ recovery must be taken into consideration. 359  
 As recovery can vary in *S. carpocapsae* (Table 3), the 360  
 optimum inoculation density should be increased. For *S.* 361  
*carpocapsae*, the optimal DJ inoculum density should be 362  
 between  $3$  and  $6 \times 10^3$  DJs per milliliter in order to obtain 363  
 $> 10^3$  parental females per milliliter (Fig. 3a). To prevent 364  
 low yields due to low numbers of parental females, the DJ 365  
 recovery in a batch to be used as inoculum should be tested 366  
 prior to DJ transfer. *S. feltiae* was less variable in DJ 367



**Fig. 4** Population development of dauer juvenile (DJ) yield, adult nematodes, and bacterial cell density in two monoxenic *Steinerema carpocapsae*-*Xenorhabdus nematophila* liquid cultures inoculated with  $10^{10}$  bacterial cells per milliliter and with a low (a) or high (b) parental female density, incubated at 25°C over a period of 15 days after dauer juvenile (DJ) inoculation

Q1



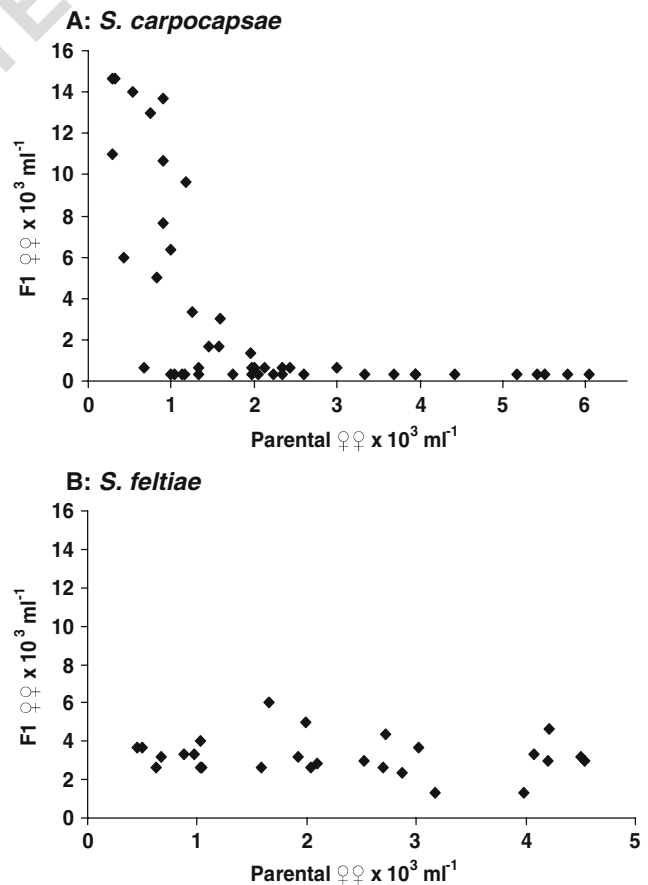
**Fig. 5** Population dynamics of dauer juvenile (DJ) yield, adult nematodes, and bacterial cell density in two monoxenic *Steinernema feltiae*-*Xenorhabdus bovienii* liquid cultures inoculated with  $10^{10}$  bacterial cells per milliliter and with a low (a) or high (b) parental female density, incubated at 25°C over a period of 15 days after dauer juvenile (DJ) inoculation

368 recovery usually reaching >90%. The recommended DJ  
369 inoculum density is  $>5 \times 10^3$  DJs per milliliter (Fig. 3b).

370 DJ recovery is the first key factor for population  
371 development, since it determines the number of adults in  
372 the parental generation, which subsequently affects the size  
373 of the F1 generation. Non-synchronous recovery leads to a  
374 second generation development and, particularly in hetero-  
375 rhabditid cultures, can cause total loss of the process due to  
376 the inability of F1 amphimictic adults to copulate in liquid  
377 culture (Strauch et al. 1994). An improvement of inoculum  
378 timing significantly increased process stability of *Hetero-*  
379 *rhabditis* spp. cultures (Johnigk et al. 2004). In contrast,  
380 liquid cultures of *Steinernema* spp. seem to be less  
381 vulnerable to low DJ recovery, as adults can copulate under  
382 liquid culture conditions (Strauch et al. 1994) and *S. feltiae*  
383 DJs seem to recover well as a response to the bacterial food  
384 signal as recorded by Hirao and Ehlers (2009b). Neverthe-  
385 less, recovery of *S. carpocapsae* provides an opportunity  
386 for improvement and should be further investigated; in  
387 particular, the influence of the DJ age on recovery needs  
388 further confirmation.

The results indicate that the DJ inoculum density has no  
effect on DJ recovery. Since the bacterial cell density was  
adjusted at  $10^{10}$  cells per milliliter, the availability of food  
per nematode was lower in cultures with a higher inoculum  
density, i.e., the cell density per nematode varied nearly ten  
times between the lowest and the highest inoculum density  
in both species at the start of the cultures. The constant DJ  
recovery recorded in all cultures of one batch indicates that  
bacterial cell density was high enough to trigger DJ  
recovery. Hirao and Ehlers (2009a) reported that DJ  
recovery of *S. carpocapsae* did not vary between  $10^{10}$   
and  $10^9$  cells per milliliter, whereas DJ of *S. feltiae*  
responded with lower recovery to decreasing bacterial cell  
density in cell well studies supplemented with antibiotics to  
fix the bacterial cell density. The lack of antibiotics in this  
study caused the bacteria to continue growth, and this was  
probably the reason for a constant DJ recovery at different  
inoculum densities.

Definition of the DJ inoculum to better synchronize  
population development and prevent a second adult F1  
generation is a feasible approach for the mass production of



**Fig. 6** Influence of the parental female density on the F1 female density (second generation) in *Steinernema carpocapsae* (a) and *S. feltiae* (b)

410 *S. carpocapsae* (Fig. 6a). This is not possible in *S. feltiae*.  
 411 The results clearly indicate that the occurrence of second  
 412 generation adults is the rule in *S. feltiae* (Fig. 5) and cannot  
 413 be influenced by the DJ inoculum and the subsequent  
 414 parental female population (Fig. 3b). The majority of the DJ  
 415 yield is provided by the F1 adult females. Still, a one  
 416 generation process would be superior also for *S. feltiae*, as  
 417 the definition of the harvest time is difficult when DJs  
 418 formation is scattered over a larger period of time. Thus,  
 419 liquid culture production of *S. feltiae* needs further  
 420 investigation concerning the management of the population  
 421 dynamics in order to produce DJs within one generation.  
 422 Analyzing the bacterial growth, it is obvious that nutrients  
 423 provided by the medium are not well exploited by *X.*  
 424 *bovienii*. Possibly, the presence of the nematodes provide  
 425 certain factors, which trigger growth of *X. bovienii* and lead  
 426 to the development of the second bacterial population peak,  
 427 which was even surpassing the density of the initial density  
 428 in the bioreactor pre-culture (Table 1). An improvement of  
 429 the culture medium for *X. bovienii* coupled with an  
 430 increasing DJ inoculum might be a possible approach for  
 431 improvement of the *S. feltiae* culture. However, the  
 432 nutritional quality of bacteria growing during the second  
 433 growth phase might be inferior to the original population,  
 434 and its significance for nematode nutrition should not be  
 435 over-estimated. During the late growth phase, cultures of  
 436 *Xenorhabdus* often consist of many small cells instead of  
 437 the standard size observed during the exponential growth  
 438 phase (Hulbert et al. 1989). An increasing number of these  
 439 small cells have also been observed in this study during the  
 440 second peak of the bacterial growth (Fig. 6).

441 Han (1996) investigated the effect of the inoculum  
 442 density on yields of *S. carpocapsae* in liquid culture and  
 443 obtained  $>300 \times 10^3$  DJ ml<sup>-1</sup> after 16 days. Comparing the  
 444 results obtained in this study with a maximum of  $175 \times 10^3$   
 445 DJ per milliliter, there is also room for improvement of the  
 446 *S. carpocapsae* process. However, one must consider that,  
 447 in the presented study, the bacterial cell density was  
 448 reduced to 10<sup>10</sup> cell per milliliter. Starting cultures at a  
 449 higher cell density will provide higher yields, but then  
 450 higher inoculum numbers should be used, as Han (1996)  
 451 obtained his highest yields with an inoculum density of  $8 \times$   
 452  $10^3$  DJs per milliliter.

453 The sex ratio was constantly biased toward the females  
 454 but was not influenced by the inoculum density. This  
 455 female-biased sex ratio has been reported before also from  
 456 other *Steinernema* spp. (Selvan et al. 1993; Alsaiyah et al.  
 457 2009). Whether the female-biased sex ratio in liquid culture  
 458 is caused by a lower recovery of males compared to  
 459 females needs to be tested.

460 A strong effect of inoculum density on the fertility of the  
 461 parental females was recorded. With the bacterial concen-  
 462 tration kept constant, less nutritional resources were

available for females with increasing densities. Indeed, the  
 bacterial cell density decreased more rapidly in cultures  
 with higher populations (Figs. 4 and 5). The egg number in  
 the uterus per female was negatively correlated with the  
 inoculum density. The reproduction can be predicted by the  
 parental female density and the fecundity, at least in *S.*  
*carpocapsae* cultures without or with a negligible F1  
 female population. Variable size of parental adults was also  
 recorded by Johnigk and Ehlers (1999b) in heterorhabditid  
 cultures. It represents a compensation mechanism, which  
 enables the nematodes to react to changing nutritive  
 environments. At low population density and abundant  
 food, the parental females are large and produce many  
 offspring, whereas at high female density, they are shorter  
 and less fertile (Fig. 1). This is also the reason why the  
 daily increase in DJ yield did not change with variable  
 population density.

In conclusion, this study provided valuable information  
 on the influence of the inoculation density on the DJ yields  
 and population dynamics of *S. carpocapsae* and *S. feltiae* in  
 liquid culture and confirmed earlier observations on DJ  
 recovery of steinernematids (Hirao and Ehlers, 2009a,b).  
 The results can certainly not be generalized and need to be  
 adapted to changing culture media and nematode species/  
 strain used in mass production.

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