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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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 derivates 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
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
available, several adult generations develop in the insect cadaver. When the nutrients are consumed, the offspring develop into DJ s, 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

Fig. 1. Life cycle of entomopathogenic nematodes in a scarabaeid beetle larva (Ehlers 2001)
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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 luminescenes, it is usually found during late stationary growth phase and
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

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*)
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
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.
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\(^{-1}\) 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, Alsaiyeh 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.
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 unstable 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 ml$^{-1}$ with $4 \times 10^3$ DJs 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 (Alsaiyah 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
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$^{-1}$ 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$^{-1}$, than in *S. feltiae*, at $106 \times 10^3$ ml$^{-1}$ 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 µm and of *S. feltiae* 849 µm (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
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\(^{-1}\) (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 ml$^{-1}$ in cultures containing $2 \times 10^3$ females ml$^{-1}$ (inoculum density $3-6 \times 10^3$ ml$^{-1}$). No significant density dependent effect of the inoculum on *S. feltiae* cultures was observed. Cultures with a parental female density of $2-4 \times 10^3$ ml$^{-1}$ (inoculum density $6-7 \times 10^3$ 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 < $2 \times 10^3$ 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$^{-1}$. 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$^{-1}$ for *S. carpocapsae* and $6-7 \times 10^3$ DJs ml$^{-1}$ 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 steinerenematids 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$^{-1}$ was obtained with an inoculum density of $8 \times 10^3$ DJs ml$^{-1}$ (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
to avoid the occurrence of higher F1 generation adult populations. Although the results indicate that a density above $10^9$ cells ml$^{-1}$ of *X. nematophila* is sufficient for *S. carpocapsae*, and $10^{10}$ cells 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.
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\(^{-1}\) 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
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% ± 2.2 in *S. carpocapsae*, 66.7% ± 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$^{-1}$ were recorded for *S. carpocapsae* and $106 \times 10^3$ DJs ml$^{-1}$ 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
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 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 ml\(^{-1}\) in order to obtain \(> 10^3\) parental females ml\(^{-1}\). However, this species shows a high batch to batch variability regarding the predisposition of the DJ 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 ml\(^{-1}\).

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

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 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 Hämolympserum der Lepidoptere Galleria mellonella in Entwicklung gingen, waren es bei S. feltiae nur 31%. Der
Eindringungsvorgang in das Insekten 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% ± 2,2 bei S. carpocapsae, 66,7% ± 2,6 bei S. feltiae), nicht aber in der F1 Generation.

Steinernematide Adulte reagieren auf sich verschlechternde Nährungsbedingungen 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*.


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⁻¹, um ca. 3,000 Weibchen ml⁻¹ 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⁻¹.

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

Schritte für eine zukünftige Prozessoptimierung definieren zu können.
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Life cycle and population development of entomopathogenic nematodes *Steinernema carpocapsae* and *S. feltiae*, (Nematoda, Rhabditida) in monoxenic liquid cultures

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In press in Nematology
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 10^2 cells ml^-1 and…”
  Replace “9” with “7”

- In Results, Page 4, 1st Paragraph, Line 1,
  “in Figure 1A”
  Erase “A”

- In Results, Page 4, 5th Paragraph, Line 5,
  “…This happened 1 day earlier than in S…”
  Replace “earlier” with “later”

- In Results, Page 5, 2nd Paragraph, Line 8,
  “…less volume (67.7 mm^3 ml^-1) than S. feltiae (107.6 mm^3…”
  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, Line 12-13,

“…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


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
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

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

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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 ± 2.2% in *S. carpocapsae*, 66.7 ± 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 × 10^6 DJ ml⁻¹ were recorded for *S. carpocapsae* and 106 × 10^6 DJ ml⁻¹ 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 *Photornhabdus*, 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 (Susurulk & Ehlers, 2008), can actively seek suitable insect hosts (Lewis, 2002; Torr et al., 2004) and invade the host insect's haemocoeel, 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; Hirao & 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...
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, offsprings develop further to reproductive adults for another reproductive cycle if food is abundant, or offsprings develop tendencies 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 lifecycle: 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 larvae. 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. carpocapsae 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⁻¹ NaCl, 5.0 g l⁻¹ yeast extract, 0.5 g l⁻¹ NH₄PO₄, 0.5 g l⁻¹ K₂HPO₄, 0.2 g l⁻¹ MgSO₄.7H₂O (Merck, Haar, Germany) at 180 rpm and 25°C. Bacterial cultures of 10⁶ cells ml⁻¹ 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⁻¹ yeast extract (Merck), 20.0 g l⁻¹ soy powder (Cargill, Hamburg, Germany), 4.0 g l⁻¹ NaCl, 0.35 g l⁻¹ KCl, 0.15 g l⁻¹ CaCl₂, 0.1 g l⁻¹ MgSO₄ (Merck), 6.0 g l⁻¹ lecithin and 40.0 g l⁻¹ 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 10⁹ cells ml⁻¹. The nematode culture medium was inoculated with 1% of a bacterial culture adjusted to 10⁶ cells ml⁻¹ and the bacteria were pre-cultured at 25°C for 36 h before inoculation of 3000 DJ ml⁻¹, 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 ml$^{-1}$ and protein inclusion bodies (Couche &Gregson, 1987) were detected in the cells by microscopic observation (Zeiss, Germany, interference contrast microscope at ×1000 magnification), 100 ml bacterial culture was transferred to sterile 500 ml Erlenmeyer flasks, inoculated with 4000-6000 DJ 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 ×600 magnification and nematodes were counted under an inverted microscope (Zeiss) at ×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 l$^{-1}$ HEPES (Carl Roth, Germany) and 11.7 g l$^{-1}$ NaCl (Merck)), 2.5% glutaraldehyde (v/v), pH 7.4 adjusted with 1 M NaOH, diluted with 4 ml of 1 M 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®, 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: $V (\text{in mm}^3) = (\pi \times \text{length} \times \text{width})/4$ for single nematodes (Poulin & Morand, 2000).

The different juvenile stages (from first-stage juvenile (J1) to fourth-stage (J4), pre-dauner 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 ×100 magnification. Pre-dauner 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 ml$^{-1}$ suspended in culture supernatant at the period for female collection. The females were observed every 3 h under an inverted microscope at ×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
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⁻¹ 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 ± 2.2% in S. carpocapsae, 66.7 ± 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
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**S. carpocapsae**
- Recovered DJ
- J4♂
- J4♀
- Parental ♂
- Parental ♀
- F1 J1
- F1 J2
- F1 J3
- F1 J4♂
- F1 J4♀
- F1 ♂
- F1 ♀
- F1 J2d
- F1 DJ

**S. feltiae**
- Recovered DJ
- J4♂
- J4♀
- Parental ♂
- Parental ♀
- F1 J1
- F1 J2
- F1 J3
- F1 J4♂
- F1 J4♀
- F1 ♂
- F1 ♀
- F1 J2d
- F1 DJ

**Days post DJ inoculation**

**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$^{-1}$, *S. feltiae* only $106 \times 10^3$ DJs ml$^{-1}$.

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 ($67.7$ mm$^3$ ml$^{-1}$) than *S. feltiae* ($107.6$ mm$^3$ 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 mono-xenic 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
Life cycle and population development of entomopathogenic nematodes

Fig. 3. Mean population dynamics ($n = 2$) 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 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 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

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Fig. 4. Mean volume of DJ yield (in mm$^3$ ml$^{-1}$) 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).

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Hatch in uterus
Uterus disruption
Maternal death
Gut lumen disruption
J2d emergence

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$^6$ cells ml$^{-1}$ 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* (Alsaiyah *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 carcass 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 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.

**Acknowledgements**

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**References**


Publication 1


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
DOI 10.1007/s00253-009-1961-4
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=Σ(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)

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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 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 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 (RT$_{50}$=17.1 h) than for Steinernema carpocapsae (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 DJ
investigated in this study as well as the influence of Steinernematids recover in insect blood serum was much reduced (Strauch and Ehlers 1998). Whether drops obtained by bleeding the insects, DJ recovery was unknown food signals in the hemolymph. In hemolymph after invasion of the insect Steinernema, which is believed to have evolved the symbiotic relation to entomopathogenic bacteria independently (Sudhaus 1993).

Materials and methods

Xenorhabdus 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 Synt 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−1) 5 NaCl, 5 yeast extract, 0.5 NH4PO4, 0.5 K2PO4, 0.2 MgSO4×7 H2O (Merck) at 180 rpm and 25°C. Bacterial cultures of 1010 cells ml−1 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−1) 15 yeast extract (Merck), 20 soy flour, 6 lecithin (Cargill), 40 vegetable oil (Raiffeisen), 4 NaCl, 0.35 KCl, 0.15 CaCl2, 0.1 MgSO4 (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 109 cells ml−1 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−1 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.
Establishment of secondary phase bacteria

The shift to secondary phase bacteria was induced by exposing the cultures to stress conditions according to Krasomil-Osterfeld (1995). X. nematophila was cultured for 20 days at 100 rpm and 25°C under osmotic stress in YS broth without NaCl. In order to identify secondary colonies, samples were streaked onto NBTA containing (g l⁻¹) 10 tryptase soy broth (TSB) (bioMerieux), 15 bacto-agar (Difco), 0.5 bromothymol blue (Sigma), supplemented after cooling to 35°C with 4 ml l⁻¹ of filter-sterilized 2,3,5-triphenyl-tetrazoliumchloride (Merck). Secondary phase bacteria produce red colonies on NBTA, whereas primary absorb bromothymol blue (Akhurst 1980). A single secondary colony was selected and sub-cultured again under osmotic stress. After four selection rounds, pure secondary phase bacteria were obtained. They were then once propagated in normal YS at 180 rpm and afterward checked for phase stability. Secondary phase of X. bovienii was obtained from 2-month-old monoxenic flask cultures of S. feltiae, which had been started with primary cells. The secondary phase was stable even without culture under osmotic stress. Storage was also done in 15% glycerol stocks at −20°C.

Phenotypic characterization of secondary phase of X. nematophila and X. bovienii

Further secondary phase characters were checked to support identification by dye absorption. Whereas primary bacteria are mobile, swarming on soft agar, and producing antibiotic activity against Bacillus cereus in overlay cultures, the secondary bacteria lose these characters (Boemare and Akhurst 1988). Swarming was checked after culturing primary and secondary in parallel culture plates for 72 h at 25°C on soft agar containing (g l⁻¹) 15 TSB and 8 agar (Difco). For testing the antibiotic activity, a YS broth culture of B. cereus was mixed at 5% into sterile TSB supplemented with 15 g l⁻¹ agar after cooling to 50°C. Primary and secondary cultures of X. nematophila and X. bovienii were incubated in YS broth until a cell density of 10¹⁰ cells ml⁻¹ was reached. Then, 1.5 ml samples were centrifuged at 14,000 rpm for 10 min, and the supernatant was filter-sterilized through a 0.22-µm pore size membrane (Milipore). Ten microliters of each filtrate was then inoculated onto the agar containing B. cereus. Growth inhibition indicates the presence of primary cells.

Effect of insect penetration on DJ recovery

In liquid culture production, DJs do not undergo host penetration prior to recovery. In order to determine whether host insect penetration affects recovery, DJs were either injected into an insect host or left to penetrate into the insect. Single last instars of the lepidopteran insect Galleria mellonella were exposed to 50 DJs of S. carpocapsae in 10 µl Ringer’s solution on a wet filter paper disc inside a cell well of a 24-cell well plate (Greiner). For S. feltiae invasion, G. mellonella larvae were buried into 10% moist sand in the well and also exposed to 50 DJs. At 6 h post-inoculation (hpi), the infected larvae were collected from cell wells and transferred into plastic boxes covered with wet filter paper and maintained at 25°C until recovery was assessed.

For DJ injection into the hemocoel of G. mellonella larvae, inoculum DJ density was adjusted at a density of 5,000 DJs ml⁻¹ in sterile Ringer’s solution. Final instar G. mellonella larvae were injected with 10 µl of the DJ suspension to achieve approximately 50 DJs per insect. Injection was performed with a microsyringe (ITO Corp, Japan). Recovery was observed at 6, 24, and 48 hpi. Twelve inoculated larvae were dissected in Petri dishes filled with Ringer’s solution, and the nematodes were collected into counting chambers (Cellstar, Germany) to check the recovery under an inverted microscope (Zeiss, Germany). Dauer juveniles were distinguished from recovered juveniles by morphological characters according to Strauch and Ehlers (1998). The head region of DJS is tapered, whereas recovered juveniles have a broad head region and the mouth is opened. The experiment was repeated twice.

Effect of insect serum from G. mellonella larvae on DJ recovery

G. mellonella larvae were collected and surface-sterilized by washing them in 70% ethanol. The hemolymph was collected from a proleg into a 2.0 ml cap, which contained 0.5% phenylthiourea-acetone (Sigma) at 1/10 of the total volume to avoid melanization of the hemolymph (Arakawa 1995). Collected hemolymph was stored at −20°C until use. Before the experiment, the stock was thawed and purified by centrifugation and filtration with a 0.2 µm filter. Then, ampicillin and streptomycin sulfate (Merck) were added each at 0.1% (w/v) to avoid any activity of symbiotic bacteria, and the sterile serum was incubated for 24 h. All handling was carried out under sterile condition. Hemolymph (500 µl) was added to each well, and 500 DJs per well were inoculated. The cultures were then incubated on a shaker at 180 rpm and 25°C. Recovery was assessed from at least 50 juveniles at 24 and 48 hpi. DJ recovery in hemolymph was compared with the DJ recovery in living insect inoculated without penetration. Galleria mellonella insect inoculated without penetration.

Influence of bacterial cell density on DJ recovery

Bacterial symbionts were cultured in LM in shaken flasks for 36 h at 180 rpm and 25°C. Cell density was counted...
In order to investigate how long the DJs need to be exposed to the bacterial food signal for induction of the recovery process, the same experiment was repeated, but only at a cell density of $10^{10}$ cells ml$^{-1}$. Then, 50 DJs were removed from the suspension at 0, 0.5, 2, 4, 6, 8, 10, 12, 24, and 36 h after inoculation, washed in sterile Ringer’s solution, and then transferred to cell well containing 500 µl Ringer’s solution supplemented with the antibiotics as described above. Recovery of all 50 individuals was assessed at 48 hpi. The experiment was also done in three replicates and repeated twice. The time period, at which 50% of the inoculated DJs had recovered ($RT_{50}$), was calculated by Probit analysis (Finney 1971).

Statistic analysis

Recovery data are presented in percent. Two-pair comparison was analyzed by the Chi-squares test. Recovery data among multiple variants were converted to arcsin values, and then mean for different variants were analyzed for significant differences by ANOVA and the Tukey HSD test. The recovery data over time were analyzed and statistically compared using the trapezoidal rule $A = (t_{n+1} - t_n)(r_n + r_{n+1})/2$ ($t$ is the time after DJ inoculation, $n$ is the sample no., $r_n$ is the recovery after $t_n$), estimating the integral of the curve. The higher the area under the curve, the stronger is the effect of the variant on the recovery induction. Means for different variants were analyzed for significant differences by ANOVA and the Tukey HSD test. For evaluation of the DJ synchronization index, the regression of the recovery data over time was calculated, and the indices (slopes of graphs) were compared for significant differences by ANOVA and the Tukey HSD test.

Results

Effect of insect penetration on DJ recovery

Recovery of DJs in insect hemocoel is presented in Table 1. At 6 and 24 hpi, recovery of injected $S.\ carpocapsae$ DJs was higher than the recovery of the DJ, which had undergone host searching and penetration. At 48 hpi, the recovery of $S.\ carpocapsae$ and $S.\ feltiae$ DJs reached approximately 100% regardless which way they reached the hemocoel. The delay in recovery of DJs, which naturally penetrated the insect, is due to the time needed to reach the host insect and advance to the hemocoel through penetration of the integument, the wall of the
Influence of host penetration on dauer juvenile (DJ) recovery

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

<table>
<thead>
<tr>
<th>HPI</th>
<th>Injection (N=3,120)</th>
<th>Penetration (N=2,160)</th>
<th>Injection (N=2,316)</th>
<th>Penetration (N=695)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>41.4a 21.1b</td>
<td>63.2A 13.1B</td>
<td>99.5a 81.6b</td>
<td>99.8A 93.8A</td>
</tr>
<tr>
<td>24</td>
<td>99.9a 81.6b</td>
<td>99.8A 93.8A</td>
<td>99.9a 100.0a</td>
<td>99.8A 100.0A</td>
</tr>
<tr>
<td>48</td>
<td>99.9a 100.0a</td>
<td>99.9a 100.0a</td>
<td>63.2A 13.1B</td>
<td>63.2A 13.1B</td>
</tr>
</tbody>
</table>

DJs had reached the hemolymph 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, \( P \leq 0.05 \)).

Influence of host penetration on DJ recovery

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)

<table>
<thead>
<tr>
<th>HPI</th>
<th><em>S. carpocapsae</em> N=645</th>
<th><em>S. feltiae</em> N=712</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>88.6a</td>
<td>22.4b</td>
</tr>
<tr>
<td>48</td>
<td>96.9a</td>
<td>31.0b</td>
</tr>
</tbody>
</table>

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 on nematode development after recovery

As recorded in the previous experiments, the recovery of both nematode species was again highest at the highest bacterial density, and recovery of S. carpocapsae was less affected by the decreasing cell density than S. feltiae (Fig. 2). In both species, the recovery increased until 24 hpi and then was constant in S. carpocapsae and at 10^9 cells ml^{-1} in S. feltiae, but continued to increase from 31% at 12 hpi to 77% at 60 hpi at 10^9 cell ml^{-1} and from 20% to 57% at 10^8 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 cells ml^{-1} (Fig. 2a, b), the adult population was observed already at 36 hpi, while in 10^8 cells ml^{-1}, the first adults occurred at 48 hpi (Fig. 2c).

S. feltiae adult population was first observed at 24 hpi at 10^10 cells ml^{-1} (Fig. 2d), 36 hpi in 10^9 cells ml^{-1} (Fig. 2e), and 48 hpi in 10^8 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 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 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; 468

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

<table>
<thead>
<tr>
<th>Nematode species</th>
<th>Bacterial cell density</th>
<th>Log 10</th>
<th>Log 9</th>
<th>Log 8</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>r²</td>
<td>Slope</td>
<td>r²</td>
<td>Slope</td>
</tr>
<tr>
<td>S. c.</td>
<td>14.1a</td>
<td>0.99</td>
<td>12.3ab</td>
<td>0.99</td>
<td>9.0bc</td>
</tr>
<tr>
<td>S. f.</td>
<td>7.5a</td>
<td>0.99</td>
<td>5.3ab</td>
<td>0.96</td>
<td>2.3b</td>
</tr>
</tbody>
</table>

Slope and regression coefficient (r²) 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≤0.05)

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31% at 12 hpi to 77% at 60 hpi at 10^9 cell ml^{−1} (432

24 hpi and then was constant in S. carpocapsae and at 10^9 cells ml^{−1} in S. feltiae, but continued to increase from 31% at 12 hpi to 77% at 60 hpi at 10^9 cell ml^{−1} and from 20% to 57% at 10^8 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} cells ml^{−1} (Fig. 2a, b), the adult population was observed already at 36 hpi, while in 10^{8} cells ml^{−1}, the first adults occurred at 48 hpi (Fig. 2c).

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df = 7, 70; P < 0.0001), the first significant increase in recovery is after exposure of 8 to 12 h (Tukey HSD, P = 0.0003) and again from 12 to 24 h (Tukey HSD, P = 0.003). This difference between the two species is obvious also when the RT50 is compared with 6.6 h exposure for S. carpocapsae and 17.1 h for S. feltiae.

Discussion

In liquid culture mass production, the recovery of inoculated DJ is the first step in the nematode’s life cycle determining success or failure of the production process (Ehlers 2001). Low DJ recovery of heterorhabditid nematodes results in low adult density at the moment of offspring production. As a consequence, many of the offspring do not develop to DJs, 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
Like the symbionts *Photorhabdus* spp. of the nematode *Heterorhabditis* spp. (Strauch and Ehlers 1998), *X. nematophila* and *X. bovienii* also excrete a food signal into the medium; however, the recovery is reduced in the superantant, whereas in *heterorhabditids*, no difference was recorded between cell suspension and cell-free culture supernatant (Strauch and Ehlers 1998). Either the *Xenorhabdus* food signal is instable or living cells as such trigger recovery or the cells continue to produce food signal despite the presence of antibiotics. An effect of the antibiotics on the recovery was excluded as was the influence of the penetration process on the DJ recovery. Like in *Heterorhabditis*, the recovery of the two steinernematids inside the insects was always 100%. But other than recorded for *heterorhabditid* species, *S. carpocapsae* also recovered well in sterile insect serum produced from the hemolymph of *G. mellonella*, whereas the response of *S. feltiae* was much reduced (Table 2). Possibly, *heterorhabditids* and *S. feltiae* DJs respond to an instable component in the insect’s hemolymph, which is lost during preparation of the serum.

Other reason why high DJ recovery is a pre-requisite for successful liquid culture production of *heterorhabditid*, less influenced by the bacterial cell density than observed for *S. feltiae*. For the latter nematode, a high cell density is necessary before DJ inoculation in order to obtain high DJ recovery.

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

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 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, $P \leq 0.05$).

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 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, $P \leq 0.05$).
Dauer juvenile recovery of both *Steinernema* spp. was higher in primary phase bacterial cells than in secondary cells, which might be one reason why cultures that started with secondary phase symbionts most often fail (Ehlers et al. 1990; Han and Ehlers 2001). The observation that recovery was reduced in culture supernatants was confirmed also for the secondary symbiont.

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

Acknowledgments The scholarship to the first author by the German Academic Exchange Service (www.daad.de) is gratefully acknowledged. Thanks are also due to Olaf Strauch for help with data evaluation and to Friederike Bernsdorff for technical support.

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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-µm 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

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 nematophilus* 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 sensitive 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.

Keywords Biological control · Entomopathogenic nematodes · Process temperature · Dauer juvenile density · Reproduction · Liquid culture

Introduction 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). DJ 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.
Cost efficiency in nematode mass production is driven by the ratio of DJ output to the DJs inoculated and by the process time. Incubation temperature is an important factor influencing nematode development. In this study, the influence of culture temperature on nematode population development in liquid culture was investigated to determine the optimal temperature for the development of *S. carpocapsae* and *S. feltiae*.

### Materials and methods

#### Symbiotic bacteria

*Xenorhabdus* spp. were isolated from nematode-infested insect larvae of *Galleria mellonella* according to Ehlers et al. (1990). *Xenorhabdus nematophila* was isolated from *S. carpocapsae* (ALL strain) and *Xenorhabdus bovienii* from *S. feltiae* (SYLT strain). Symbiotic bacteria were then propagated in 100-ml Erlenmeyer flasks containing 30 ml YS broth containing (in g l\(^{-1}\)) 5 NaCl, 5 yeast extract, 0.5 KPO\(_4\), and 0.2 MgSO\(_4\) *×* 7 H\(_2\)O (Merck).

Bacteria were cultured at 180 rpm and 25°C. When cell density had reached 10\(^{10}\) cells ml\(^{-1}\), the suspension was transferred into 2-ml Eppendorf tubes, mixed with glycerol at 15% (v/v), and stored at −20°C until use.

#### Nematodes

*S. carpocapsae* (ALL strain) and *S. feltiae* (EN02 hybrid strain; SYLT strain was one strain used to produce this hybrid) 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 containing (in g l\(^{-1}\)) 15 yeast extract (Merk), 20 soy flour (Cargill), 4 NaCl, 0.35 KCl, 0.15 CaCl\(_2\), 0.1 MgSO\(_4\) (Merk), 6 lecithin, and 40 vegetable oil. Media were adjusted to pH 7.0 for *S. carpocapsae* and 6.5 for *S. feltiae*, with 4 M KOH (Merk). LM was inoculated with the symbiotic bacteria of the specific nematode. Briefly, bacterial stock was thawed and propagated at 25°C in YS broth until a cell density of 10\(^{6}\) cells ml\(^{-1}\) was reached.

This YS bacterial culture was then inoculated into LM at 1% volume of the final culture volume and incubated for 36 h at 25°C. Then, DJs from monoxenic cultures were inoculated at 3,000 DJs ml\(^{-1}\) and incubated at 25°C until harvest at 15 days after DJ inoculation.

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

Pre-culture of symbiotic bacteria

In order to provide a homogenous bacterial inoculum for the nematode culture, the bacteria were produced in one batch culture and then distributed over several Erlemeyer flasks inoculated with nematodes. The bacterial cultures were carried out according to Johnigk et al. (2004) in a bioreactor (Belach, Sweden) filled with 10 l LM, supplemented with 0.1% (v/v) anti-foam (Silifom, SE2, Wacker Chemie), and steam-sterilized at 120°C for 20 min. After sterilization, the temperature was kept at 25°C with agitation at 500 rpm until DJ inoculation into the bioreactor. Prior to inoculation, bacterial stocks were propagated in YS broth. Inoculation was at 1% bioreactor volume with cultures of a cell density of 10\(^9\) cells ml\(^{-1}\). During incubation, the temperature was controlled at 25°C, the pressure at 0.1 bar, and the airflow was 1.5 l min\(^{-1}\). The pH and O\(_2\) saturation were recorded but not controlled. Agitation speed was controlled between 300 and 800 rpm to keep the O\(_2\) saturation at a minimum of 20%.

Bioreactor parameters were recorded by the software Phantom (Belach, Sweden).

Nematode flask cultures

When the bacterial pre-culture had reached a cell density of 10\(^{10}\) cells ml\(^{-1}\) and protein inclusion bodies could be observed in all cells, 100 ml of the culture was transferred into sterile 500-ml Erlenmeyer flasks inoculated with 4,000 DJs ml\(^{-1}\) (ranging from 3,166 to 4,666 DJs ml\(^{-1}\)) and incubated at four different temperatures (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 influence of the different culture temperatures on DJ recovery, egg production, and DJ proportion of the total nematode population were recorded daily. DJ recovery (in percent) was assessed according to Hirao and Ehlers (2009) 1 day before the first progeny hatched from eggs (before progeny DJs are occurring). The number of eggs in the uterus (fecundity) of females, which had developed from inoculated DJs, was assessed on the day on which the number of females with egg reached its maximum. Twelve females were collected from one flask culture; dissected and fertilized eggs were counted. For monitoring of the nematode and DJ populations, nematode samples were taken every day, and the developmental stage of at least 100 nematodes was assessed under an inverted microscope (Zeiss, Germany). Offspring DJs were distinguished from inoculum DJs by their darker appearance due to the higher content of lipid droplets, which is consumed in older (inoculated) DJs. Another parameter was the presence of the cuticle of the prior juvenile stage, the pre-dauer J2d.
stage, which was still present in offspring DJs but lost by inoculated DJs. The progeny DJ density was assessed on 10 and 15 days post-DJ inoculation (dpi). Each temperature was tested with three flask cultures, and the experiments were replicated four times.

Infectivity test

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

Statistical analysis

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

Results

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

<table>
<thead>
<tr>
<th>Culture temperature (°C)</th>
<th>20</th>
<th>23</th>
<th>25</th>
<th>27</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. carpocapsae</td>
<td>NT</td>
<td>5.8</td>
<td>4.5</td>
<td>5.0</td>
<td>6.8</td>
</tr>
<tr>
<td>S. feltiae</td>
<td>4.6</td>
<td>3.5</td>
<td>3.5</td>
<td>4.0</td>
<td>NT</td>
</tr>
</tbody>
</table>

At least 100 nematodes per flask were sampled every day and the day on which newly hatched progeny was recorded.

NT not tested.

S. 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 (103.1×10^3 DJs ml^-1). At 27°C, the DJ density was significantly reduced to 54.2×10^3 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 50×10^3 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 200×10^3 DJs ml^-1 at 25°C, followed by 100 and 120×10^3 DJs ml^-1 at 23°C and 27°C, respectively. The density at 29°C remained low at 20×10^3 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.
(ANOVA, $F=6.449, df=3, 29, P=0.002$) and for *S. feltiae* (ANOVA, $F=11.274, df=3, 28, P<0.0001$). The infectivity of *S. carpocapsae* DJ varied between 22.2% and 37.5%, with the highest infectivity observed for DJs which had been cultured at 27°C (Tukey’s HSD, $P=0.046$). The infectivity of *S. feltiae* DJs cultured at 20°C was significantly lower (16.6%, $P=0.0005$).

**Discussion**

This study determined the optimal temperature for DJ production in liquid culture of the entomopathogenic nematodes *S. carpocapsae* and *S. feltiae*. The results indicate that the optimal culture temperature for both nematodes is 25°C, when taking into consideration the DJ yields and the proportion of DJs of the total nematode population at the end of the process time. Both factors are the most important parameters defining the success of the liquid culture process. The DJ is the developmental stage used in biological insect control. The proportion of DJs at process end has an influence on the success of the following downstream process. A high proportion of non-DJ stages makes necessary additional cleaning steps. Adults and juveniles other than DJs cannot survive in the formulated nematode product and would spoil it due to the high content of dead organic matter, which promotes growth of contaminants and reduces the shelf life of the DJs in the final product. The elimination of the non-DJ stages during downstream processing is therefore a basic necessi-

![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.](image)

**Q5**

*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.
ty, and the lower the proportion of non-DJ stages in liquid culture, the easier is the harvesting and subsequent cleaning (Ehlers 2001). The temperature at which the lowest proportion of non-DJ stages of the total nematode population was recorded correlated with the temperature supporting maximum DJ yields.

An important developmental step deciding on success and failure of the liquid culture process is the recovery of inoculated DJs of the entomopathogenic nematodes *Heterorhabditis* spp. (Strauch and Ehlers 1998). However, for the two steinernematids tested, the recovery was not a good indicator for final DJ density. Particularly for *S. feltiae*, DJ recovery was surpassing 90% at any culture temperature. The influence of the culture temperature seems to be of minor significance for the DJ recovery in both steinernematids tested. Only at the highest temperature of 29°C, the recovery of *S. carpocapsae* declined.

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

As one could expect, the temperature had an influence on the time scale of the nematode development. The development was delayed at lower temperatures. The delay of DJ occurrence at higher temperature might indicate that the temperature is closed to the maximally tolerated high temperature for reproduction. Grewal et al. (1994) examined the influence of temperature on infectivity, establishment, and reproduction for several *Steinernema* and *Heterorhabditis* species in *G. mellonella* larvae at different temperatures, ranging from 8°C to 39°C. It resulted that the temperature range for establishment and reproduction was relatively narrow compared to the temperature range for infectivity. The lowest temperature at which the nematode could reproduce in *G. mellonella* larvae was 20°C for *S. carpocapsae* (ALL strain) and 10°C for *S. feltiae* (SN and Argentina strain); whereas, the highest temperature for reproduction in *G. mellonella* larvae was 30°C for *S. carpocapsae* and 25°C for *S. feltiae*. Not only the delayed development but also the reduction of fecundity caused a low final DJ density at higher temperatures. Fecundity of both *S. carpocapsae* and *S. feltiae* was low at the highest temperature tested (Fig. cxx) and resulted in low DJ yield. The data on DJ yields and DJ proportion indicate that DJ yield on 10 dpi can be predicted by the number of eggs and the developmental speed from inoculated DJ to adult. In contrast, DJ yield on 15 dpi cannot be predicted from these parental factors anymore.

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

The aspect of infectivity of harvested DJs needs particular attention. It has been reported that infectivity differs among different bioassay temperatures (Dunphy and Webster 1986; Grewal et al. 1994, 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. Both bioassay methods have been designed for infectivity in commercial production, and the expected mortality for good quality nematodes in these bioassay methods is 50%. The mean mortality recorded in this study was lower than 50% with both *S. carpocapsae* and *S. feltiae*. *S. carpocapsae* and *S. feltiae* DJs reared at lower temperatures had the lowest infectivity. Since all the bioassays were performed at 25°C, the difference of infectivity might have been caused by the number of symbiotic bacteria retained in the DJs. In other words, the DJs at lower temperature were physiologically younger than those of reared at higher temperatures; hence, the

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![Fig. 5](image-url)  
**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, $P \leq 0.05$).
bacteria stored in the vesicle of the DJ's had not multiplied yet inside the DJ's, and, as a consequence, fewer insects were killed. Martens et al. (2003) reported that at the early phase of bacterial retention, there were fewer cells in the DJ intestine and that the cells grow and divide until filling the complete DJ vesicle in the intestine. Harvest of DJ's should therefore be well timed to avoid deficiencies in infectivity.

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References

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

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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: "105 × 10³ 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"

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

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 10×10³ DJs per milliliter for *S. carpocapsae* and 1 to 8×10³ 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 <2×10³ 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 3–6×10³ DJs per milliliter in order to obtain >10³ 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 >5×10³ DJs per milliliter to reach >2×10³ parental females per milliliter. The mean yield recorded for *S. carpocapsae* was 135×10³ and 105×10³ 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
insects (Ciche et al. 2006). In the insect's hemolymph, DJs encounter yet unknown signals (Strauch and Ehlers 1998), which induce the exit from the developmentally arrested DJ stage. Analogous to the closely related nematode Caenorhabditis elegans, this developmental step is called "recovery" (Strauch and Ehlers 1998) and the recovery inducing signal food signal (Golden and Riddle 1984). During recovery, the DJs release the bacterial symbionts into the insect's haemocoel. They proliferate and produce suitable conditions for nematode reproduction. EPN cannot reproduce well without the presence of their symbionts (Han and Ehlers 2000). The nematodes develop through a J4 stage to adults. In steinernematids, these adults are amphimictic, whereas heterorhabditid nematodes develop to hermaphrodites, and the offspring of these self-fertilizing adults can develop to amphimictic stages or to DJs. In Heterorhabditis spp., this alternative pathway is induced during the J1 stage (Johnigk and Ehlers 1999a). A high bacterial cell density induces the development of another adult generation. Low food supply induces the development to DJs (Strauch et al. 1994). As long as food is abundant, the nematodes develop additional adult generations. With degrading food supply (insect cadaver consumed) DJ formation is induced. Another result of depleting nutrients and bacteria is the cessation of egg-laying by the adults. Instead, J1 hatch from eggs inside the uterus and develop to DJs on cost of the maternal body, a phenomenon called endotokia matricida (Johnigk and Ehlers 1999b). DJ offspring exit from the insect cadaver approximately 2 weeks after infestation (Ciche et al. 2006).

Extensive use of EPN in biocontrol depends on their cost-effective mass production, which is done in monoxenic liquid culture (Ehlers 2001). The life cycle in liquid culture resembles that in vivo for species of both genera (Johnigk and Ehlers 1999a; Wang and Bedding 1996; Hirao and Ehlers 2000c), with the major difference that in vitro, artificial media are pre-incubated with the symbiotic bacteria prior to DJ inoculation. DJ recovery then is a response to bacterial food signals (Strauch and Ehlers 1998; Aumann and Ehlers 2001; Hirao and Ehlers 2009a) rather than to signals in the hemocoel of a newly infected insect. Of critical importance for successful commercial mass production are high DJ yields within a short process time and an acceptable activity and infectivity (quality) of the resulting DJs (Ehlers 2001). Hence, a process that is producing a maximum DJ concentration within one nematode generation is superior to a two-generation process (Ehlers et al. 1998). Maximum DJ yields depend on high food resources. On the other hand, DJ formation is a response to depleting food resources. Thus, the actual availability of food in these batch cultures is important. An initial optimum nutrition supplies abundant food for bacterial growth and offspring production by the parental (inoculated) nematode population. Subsequently, starvation conditions should induce the development to DJs. In theory, a high initial nematode population should be well supplied to produce large numbers of offspring and consume most of the food, thus initiating DJ-inducing conditions. Hence, it should be possible to define an optimal inoculum density for a given liquid culture medium. In fact, Ehlers (2001) recorded a maximum final yield of Heterorhabditis bacteriophora at an initial hermaphrodite density between 3,000 and 5,000 recorded on the third day after nematode inoculation. The aim of the present study was to define the optimum inoculation density for the biocontrol nematodes Steinernema carpocapsae and Steinernema feltiae to obtain a maximum DJ yield within a short process time. Population development of symbiotic bacteria and the different nematode stages was monitored, including DJ recovery and sex ratio.

Materials and methods

Monoxenic culture of bacto-helminthic complex

Xenorhabditis spp. were isolated from nematode-infested insect larvae of Galleria mellonella, cultured and stored according to Hirao and Ehlers (2009b). Xenorhabdus nematophilae was isolated from the All strain of S. carpocapsae, Xenorhabdus bovienii from the Sylt strain of S. feltiae, S. carpocapsae (All strain) and S. feltiae (EN02 hybrid strain, includes Sylt 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⁻¹) 15 yeast extract (Merek), 20 soy powder (Cargill), 4 NaCl, 0.35 KCl, 0.15 CaCl₂, 0.1 MgSO₄ (Merek), 6 lecithin diluted with same volume of vegetable oil and 40 vegetable oil. Media were adjusted to pH7.0 for S. carpocapsae and 6.5 for S. feltiae with 4 M KOH (Merek). For nematode inoculum production, LM was inoculated with the symbiotic bacteria and when cell density had reached 10⁵ cells per milliliter inoculated with 3,000 DJs per milliliter and incubated at 25°C until harvest at 15 days after inoculation of the symbiotic bacteria. The DJs from monoxenic cultures were washed and stored at 4°C until use (Hirao and Ehlers 2009b).

Influence of nematode inoculum density on population dynamics and yields

The influence of the nematode inoculum density was tested in monoxenic flask cultures. In order to provide a homogeneous symbiont inoculum, the bacterial culture was done in bioreactors (5 l Meredos, Germany, for S. carpocapsae, 10 l
Belach, Sweden, for *S. feltiae* according to Johnigk et al. (2004). LM was supplemented with 0.1% (v/v) anti-foam  
(Silfoam SE2, Wacker Chemie, Munich, Germany). Process  
conditions were according to Hirao and Ehlers (2009b). The  
cell density of each pre-culture and the time of harvest were  
presented in Table 1. Bacterial cell density was assessed by  
counting cells in a Thoma chamber under the microscope at  
600-fold magnification. The cultures were used when a cell  
density >10^10 cells per milliliter had been reached and all  
cells contained protein inclusion bodies (Couche and  
Gregson 1987). *X. nematophila* pre-culture was adjusted to  
a cell density at 10^10 cells per milliliter by dilution with  
culture supernatant. Cells were removed by centrifugation at  
14,000 rpm from the same bacterial culture and the  
supernatant used for dilution. *X. bovienii* pre-cultures were  
used without dilution since their density was close to 10^10  
cells per milliliter. Each sterile 500-ml wide-necked  
Erlenmeyer flask received 100 ml bacterial culture broth.  
The flasks were then inoculated with 1,000–10,000 DJs  
per milliliter of *S. carpocapsae* DJ or with 1,000–8,000 DJs  
per milliliter of *S. feltiae* and incubated at 180 rpm, at 25°C.  
Samples were taken daily, and at least 100 nematodes were  
assessed under an inverted microscope (Zeiss, Germany)  
and the bacterial density recorded as described. The  
experiments were performed three times with three different  
nematode inoculum batches. The inoculated nematode  
density in each flask was determined 1 day after DJ  
inoculation. These initial mean DJ densities and minimum  
and maximum are presented in Table 2. Each DJ density  
was tested in three flasks.

DJ recovery was assessed according to Hirao and Ehlers  
(2009a) and represents the percentage of the sum of J3, J4,  
and adults of the nematode population 1 day before first  
progeny hatched from eggs. Female proportion in parental  
adult populations was assessed when the maximal density  
of males and females was observed the day before DJ  
inoculation. The offspring number per female had reached its  
maximum density. DJ yields were assessed on 10 and  
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.

### Table 1

<table>
<thead>
<tr>
<th>Batch</th>
<th><em>X. nematophila</em></th>
<th><em>X. bovienii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell density (×10^9 cells per milliliter)</td>
<td>Process time (hours)</td>
</tr>
<tr>
<td>t1.1</td>
<td>1</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>22.0</td>
</tr>
</tbody>
</table>

### 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 = 24, P < 0.001), recovery reached at least 80% and usually
surpassed 90%. There was no difference in the day at which offspring J1 were first recorded between the different inoculum densities; hence, there is no indication for a delayed recovery or delayed development of the parental generation. Evaluation of the recovery data indicated no density-dependent differences for both species. A significant correlation between the recovery and the age of the DJ inoculum (Table 3) was detected for S. carpocapsae ($R^2 = 0.98, P<0.0001$), whereas the recovery of S. feltiae was not affected by the DJ age and was equally high in all cultures.

Parental female density, sex ratio, and fertility The parental female density increased at a constant value in relation to the DJ inoculum (Fig. 1). The proportion of the females in the sex ratio is strongly biased toward the female (Table 4).

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

<table>
<thead>
<tr>
<th>Batch</th>
<th>S. carpocapsae</th>
<th>S. feltiae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>Age (days)</td>
</tr>
<tr>
<td>1</td>
<td>70.6b (67.3–73.1)</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>82.9c (69.9–86.3)</td>
<td>105</td>
</tr>
<tr>
<td>3</td>
<td>55.6a (52.0–57.5)</td>
<td>41</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Batch</th>
<th>Females (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. carpocapsae</td>
</tr>
<tr>
<td>1</td>
<td>70.7b (56.1–76.4)</td>
</tr>
<tr>
<td>2</td>
<td>63.9a (57.9–77.5)</td>
</tr>
<tr>
<td>3</td>
<td>72.8b (52.9–80.9)</td>
</tr>
</tbody>
</table>

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

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

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

### Influence of DJ inoculum density on DJ yield

The mean yield recorded for *S. carpocapsae* was 135×10³ with a variability (standard error) of 1–17×10³ DJs per milliliter between the three flasks and for *S. feltiae* 105×10³ with a variability of 5–10×10³ DJ per milliliter (two cultures surpassed a variability of 25×10³ DJ per milliliter). DJ yields generally increased from 10 to 15 dpi in cultures of both steinernematids, although this was more pronounced in *S. carpocapsae*. No correlation was recorded between the variance in yields of the three replicate cultures and the DJ inoculum density or the total yields in both nematode species.

In *S. carpocapsae*, the highest final DJ density (175×10³ DJs ml⁻¹) was counted on 15 dpi in cultures started with 4×10³ DJs per milliliter in batch 2. On average, among the three batches, inoculum densities between 3 and 6×10³ DJs per milliliter (parental female density, 1–3×10³ ml⁻¹) produced high final DJ numbers (approximately 150×10³ DJs per milliliter) with daily increases between 15 and 20×10³ DJs per milliliter. When the parental female population density surpassed 3×10³ per milliliter, a decline in yields was observed in *S. carpocapsae*. In *S. feltiae*, this decline was not observed, and no significant differences were recorded.

### To interpret density-dependent effects, the DJ yields were related to the parental female population instead to the DJ inoculum density. Due to variable DJ recovery the relation to the DJ inoculum density would be misleading. Yields recorded on 10 and 15 dpi are presented in Fig. 3. In general, there was no significant influence of the parental female density on the final DJ yields in both nematode species, except for *S. carpocapsae* cultures with a parental female density <2×10³ DJs per milliliter. This is particularly obvious for batch 3 cultures of *S. carpocapsae* when analyzing the results recorded on day 15 post-inoculation. These cultures received very low DJ numbers, and the total yields were the lowest with the lowest daily increase (between 5 and 10 DJs×10³ ml⁻¹ day⁻¹). The yields in batch 3 increased with increasing DJ inoculum density and particularly between 10 and 15 dpi (Fig. 3a).

In *S. carpocapsae*, this decline was not observed, and no significant differences were recorded. In *S. feltiae*, this decline was not observed, and no significant differences were recorded.
recorded in DJ yields. Furthermore, no influence of the parental female (respectively inoculation) density on yields was recorded. In *S. feltiae* cultures (Fig. 3b), the highest DJ yield was 125 × 10^3 DJs per milliliter in cultures started with 6–7 × 10^3 DJs per milliliter (4 × 10^3 parental females per milliliter) in batch 2 on 15 dpi. In cultures with parental female densities <2.5 × 10^3 nematodes per milliliter, the yields dropped. The daily increase in DJ density was between 10 and 14 × 10^3 DJs per milliliter. A total of 90 flasks were examined daily for the bacterial and nematode population dynamics between 10 and 14 × 10^3 DJs per milliliter. For *S. feltiae* cultures (Fig. 3), the highest DJ yield was 125 × 10^3 DJs per milliliter in cultures started with 6–7 × 10^3 DJs per milliliter (4 × 10^3 parental females per milliliter) in batch 2 on 15 dpi. In cultures with parental female densities <2.5 × 10^3 nematodes per milliliter, the yields dropped. The daily increase in DJ density was between 10 and 14 × 10^3 DJs per milliliter. A total of 90 flasks were examined daily for the bacterial and nematode population dynamics between 10 and 14 × 10^3 DJs per milliliter. For *S. feltiae* cultures (Fig. 3b), the highest DJ yield was 125 × 10^3 DJs per milliliter in cultures started with 6–7 × 10^3 DJs per milliliter (4 × 10^3 parental females per milliliter) in batch 2 on 15 dpi. In cultures with parental female densities <2.5 × 10^3 nematodes per milliliter, the yields dropped. The daily increase in DJ density was between 10 and 14 × 10^3 DJs per milliliter. A total of 90 flasks were examined daily for the bacterial and nematode population dynamics between 10 and 14 × 10^3 DJs per milliliter.

**Bacterial and nematode population dynamics**

At a low inoculum density (Fig. 4a), the bacterial cell density of *X. nematophila* further increased after DJ inoculation from 10^10 to 2.5 × 10^10 cells per milliliter on 2 dpi. Offspring DJs increase from 6 dpi onwards. Part of the parental offspring further developed to a F1 adult generation, and F1 females reached a maximum density of >10 × 10^3 on 11 dpi. In the culture, which received a high DJ inoculum (Fig. 4b), the bacterial density increased only to 1.5 × 10^10 cells per milliliter on 1 dpi and then was consumed by the parental adult generation. F1 generation adults remained <300 nematodes per milliliter, and the DJ offspring density increased beginning on day 7 post-inoculation.

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

**Discussion**

This contribution aimed at elucidating the influence of the DJ inoculum density on DJ recovery, parental female fertility, and DJ yields in liquid cultures of *S. carpocapsae* and *S. feltiae*. According to the results, an optimal parental female density can be defined for both nematode species, 1–3 × 10^5 females per milliliter for *S. carpocapsae* and >2 × 10^5 females per milliliter for *S. feltiae* (Fig. 3). Considering that half of the inoculated population are males, the number could just be doubled to get the DJ inoculum density. However, the sex ratio is female-biased (Table 4), and double DJ density guarantees an even high proportion of females.

However, DJ recovery must be taken into consideration. As recovery can vary in *S. carpocapsae* (Table 3), the optimum inoculation density should be increased. For *S. carpocapsae*, the optimal DJ inoculum density should be between 3 and 6 × 10^3 DJs per milliliter in order to obtain >10^5 parental females per milliliter (Fig. 3a). To prevent low yields due to low numbers of parental females, the DJ recovery in a batch to be used as inoculum should be tested prior to DJ transfer. *S. feltiae* was less variable in DJ

![Fig. 4 Population development of dauer juvenile (DJ) yield, adult nematodes, and bacterial cell density in two monoxenic *Steinernema 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](image-url)
recovery usually reaching >90%. The recommended DJ inoculum density is >5 × 10^3 DJs per milliliter (Fig. 3b).

DJ recovery is the first key factor for population development, since it determines the number of adults in the parental generation, which subsequently affects the size of the F1 generation. Non-synchronous recovery leads to a second generation development and, particularly in heterorhabditid cultures, can cause total loss of the process due to the inability of F1 amphimictic adults to copulate in liquid culture (Strauch et al. 1994). An improvement of inoculum timing significantly increased process stability of Heterorhabditis spp. cultures (Johnigk et al. 2004). In contrast, liquid cultures of Steinernema spp. seem to be less vulnerable to low DJ recovery, as adults can copulate under liquid culture conditions (Strauch et al. 1994) and S. feltiae DJs seem to recover well as a response to the bacterial food signal as recorded by Hirao and Ehlers (2009b). Nevertheless, recovery of S. carpocapsae provides an opportunity for improvement and should be further investigated; in particular, the influence of the DJ age on recovery needs 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...
S. carpocapsae (Fig. 6a). This is not possible in S. feltiae. The results clearly indicate that the occurrence of second generation adults is the rule in S. feltiae (Fig. 5) and cannot be influenced by the DJ inoculum and the subsequent parental female population (Fig. 3b). The majority of the DJ yield is provided by the F1 adult females. Still, a one generation process would be superior also for S. feltiae, as the definition of the harvest time is difficult when Djs formation is scattered over a larger period of time. Thus, liquid culture production of S. feltiae needs further investigation concerning the management of the population dynamics in order to produce Djs within one generation.

Analyzing the bacterial growth, it is obvious that nutrients provided by the medium are not well exploited by X. bovienii. Possibly, the presence of the nematodes provide certain factors, which trigger growth of X. bovienii and lead to the development of the second bacterial population peak, which was even surpassing the density of the initial density in the bioreactor pre-culture (Table 1). An improvement of the culture medium for X. bovienii coupled with an increasing DJ inoculum might be a possible approach for improvement of the S. feltiae culture. However, the nutritional quality of bacteria growing during the second growth phase might be inferior to the original population, and its significance for nematode nutrition should not be over-estimated. During the late growth phase, cultures of Xenorhabdus often consist of many small cells instead of the standard size observed during the exponential growth phase (Hulbert et al. 1989). An increasing number of these small cells have also been observed in this study during the second peak of the bacterial growth (Fig. 6).

Han (1996) investigated the effect of the inoculum density on yields of S. carpocapsae in liquid culture and obtained >300×10^3 DJ ml^-1 after 16 days. Comparing the results obtained in this study with a maximum of 175×10^3 DJ per milliliter, there is also room for improvement of the S. carpocapsae process. However, one must consider that, in the presented study, the bacterial cell density was reduced to 10^10 cell per milliliter. Starting cultures at a higher cell density will provide higher yields, but then higher inoculum numbers should be used, as Han (1996) obtained his highest yields with an inoculum density of 8×10^3 DJS per milliliter.

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

A strong effect of inoculum density on the fertility of the parental females was recorded. With the bacterial concentration 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 offsprings, 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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References


Hirao A, Ehlers RU (2009c) Life cycle and population development of the entomopathogenic nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda, Rhabditida) in monoxenic liquid cultures. Nematology (in press)


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