

**Life history traits of entomopathogenic nematodes of *Steinernema* spp. and  
*Heterorhabditis bacteriophora***

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## **Dedication**

This thesis is dedicated to my parents for their endless love, support and encouragement

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## 1. Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* together with their symbiotic bacteria *Xenorhabdus* and *Photorhabdus*, respectively (Poinar 1990) are lethal pathogens of insect pests. Currently, EPNs are commercially used for the management of important insect pests that attack above and below ground plant parts (Grewal et al. 2005; Lacey et al. 2015).

The formation of a durable infective stage, which can be stored for long periods, applied by conventional spraying methods and persist in the soil following application are the favourable characteristics of EPNs (Ehlers 2001). In addition, they are specific to insects, safe to non-target organisms including humans, other vertebrates and plants, and do not pollute the environment (Ehlers and Peters 1995), which has led EPNs to be exempted from pesticide registration in the United States and many nations of the European Union (Lacey et al. 2015).

EPN explorations have been conducted all over the world and a number of species have been reported. Campos-Herrera et al. (2012) have listed 63 species in the genus *Steinernema* and 17 described species in the genus *Heterorhabditis*. To date, the number of EPN species in the genera *Steinernema* and *Heterorhabditis* has increased to 90 and 20, respectively. However, important biological traits of EPNs such as virulence, reproductive potential, and response to different environmental stress are limited in the descriptions of new EPN species. For efficient uses of EPNs as biocontrol agents, efforts have been made to improve their beneficial traits through selection and breeding (Anbesse et al. 2013; Mukuka et al. 2010; Nimkingrat et al. 2013).

Among the described EPN species only few of them are currently mass produced for commercial purposes by four companies in large liquid bioreactor tanks, which reach up to 80,000 l (Ehlers 2003). *Heterorhabditis bacteriophora*, *S. carpocapsae*, *S. feltiae* and *S. kraussei* are currently mass produced, formulated and commercialised (Ehlers 2003; Grewal et al. 2005; Grewal and Peters 2005; Lacey et al. 2015). *Steinernema feltiae* and *H. bacteriophora* are the most widely spread EPN species reported all over the world and together with their symbiotic bacteria, *X. bovienii* and *P. luminescens*, respectively; they have been used to control a wide range of insect pests (Lacey et al. 2015). The symbiotic bacterium *X. bovienii* has created a symbiotic association with at least 9 different *Steinernema* spp., which is the highest number of symbiotic relations record among the described *Xenorhabdus*

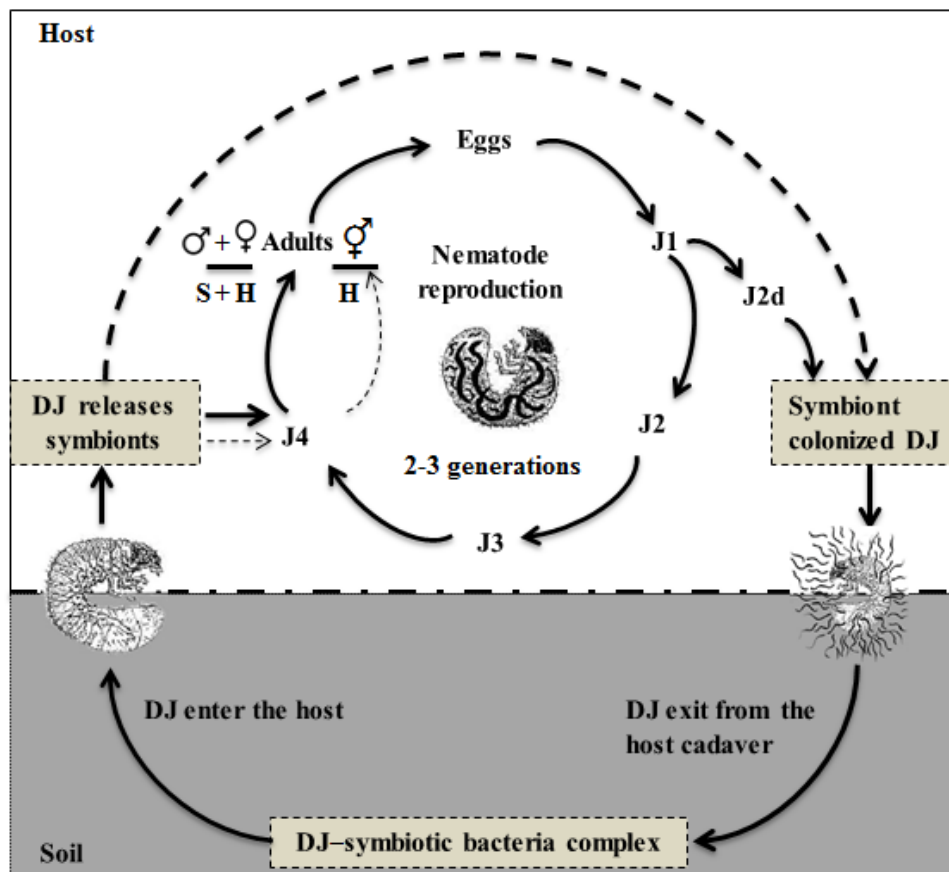
spp. Similarly, *P. luminescens* has shared the highest association (with more than 4 species) in the genus *Heterorhabditis* (Campos-Herrera et al. 2012).

*Steinernema riobrave* and *S. yirgalemense*, both belong to the ‘*bicornutum*-group’, were identified a decade ago (Nguyen et al. 2007). *Steinernema riobrave* was isolated in the lower Rio Grande Valley, Texas, USA (Cabanillas et al. 1994). Together with its symbiont *X. cabanillasii* (Tailliez et al. 2006), it has been found effective against different kinds of weevils, e.g. *Diaprepes abbreviatus* and termites (Cabanillas and Raulston 1996; Cabanillas 2003; Shapiro-Ilan et al. 2002; Stuart et al. 2004; Yu et al. 2010). Its ability to withstand both high soil temperature (Grewal et al. 1994) and cold temperatures (Shapiro-Ilan et al. 2014) and control a wide range of insect pests shows its promising potential as a biological control agent.

The EPN *S. yirgalemense* was first reported from Yirgalem, Ethiopia (Nguyen et al. 2004), later in Kenya (Mwaniki et al. 2008) and South Africa (Malan et al. 2011). Together with its symbiont *X. indica* (Ferreira et al. 2014) it has shown a control potential against different kinds of mealybugs, weevils, grubs and other economically important insect pests in Africa (Anbesse et al. 2008; De Waal et al. 2011; Le Vieux and Malan 2013; Malan et al. 2011; Mwaniki et al. 2009; Van Niekerk and Malan 2012).

The life cycle of EPNs and their associated symbiotic bacteria passes through a number of steps (Fig. 1). EPNs spend much of their life cycle feeding and reproducing in the host’s cadaver. Dauer juveniles (DJs) are the only free-living, infective stages of EPNs. Both, in *Steinernema* and *Heterorhabditis*, transmission from host to host is by means of DJs, which are capable of surviving in the soil and invade a new host through the insect’s natural openings such as anus, mouth, spiracles and in some cases through the cuticle (Dowds and Peters 2002; Koppenhöfer et al. 2007). These infective third stage juveniles contain 200–2,000 cells of their symbiont in their intestine (Chaston et al. 2013; Ciche et al. 2008). Upon entrance into the insect, the DJs respond to chemical cues, which are called food signals and release their symbiotic bacteria into the insect’s haemocoel through the mouth (*Heterorhabditis* spp.) or anus (*Steinernema* spp.) (Chaston et al. 2013; Ciche et al. 2006; Sugar et al. 2012). Proliferation of the symbionts in the haemolymph kills the host within 24–48 hours and creates a soup of bacteria and insect content, which the nematodes depend on for growth and reproduction.

The DJs exit from the developmentally arrested dauer stage due to food signals released from the insect haemolymph and resume feeding (Strauch and Ehlers 1998). This developmental process is called DJ recovery, a term first used in *C. elegans* (Golden and Riddle 1984) and the recovered DJ develops into a fourth stage juvenile (J4). In *Steinernema* spp. J4 develop into males and females, whereas in *Heterorhabditis* spp. the J4 derived from DJs always develop into hermaphrodites. The nematodes complete their development and reproduce two or more generations inside the host. At the time of food depletion and increased nematode population, the pre-dauer juveniles (J2ds) get colonized by their symbiotic bacteria and completely develop into DJs (Ciche et al. 2008; Hirao et al. 2010). Depending on the body size of the insect host few to hundreds of thousands of DJs emerge from the host cadaver and look for a new host (Griffin et al. 2005; Shapiro-Ilan et al. 2012).



**Fig. 1** *Steinernema*–*Xenorhabdus* spp. and *Heterorhabditis*–*Photorhabdus* spp. life cycle from infection of a scarabaeid beetle larva to emergence of dauer juveniles from the insect cadaver (modified from Ehlers 2001). J1–J4 = first to fourth juvenile stages; ♂ = male; ♀ = female; ♀ = hermaphrodite; J2d = pre-dauer juvenile; S = *Steinernema*; H = *Heterorhabditis*

After entry into the insect host or inoculation into artificial medium, the DJs of *Steinernema* and *Heterorhabditis* spp. pass through different developmental pathways (Fig. 2). These developmental pathways depend on the availability of food, nematode population density and nematode genera/species and other environmental factors (Hirao and Ehlers 2009c; Strauch et al. 1994). First the DJs recover and develop into the fourth stage juvenile (J4), which leads to the development of amphimictic parental nematodes in *Steinernema* spp. that reach sexual maturity approximately at the same time and automictic adults of *Heterorhabditis* spp. (developmental pathway 1).

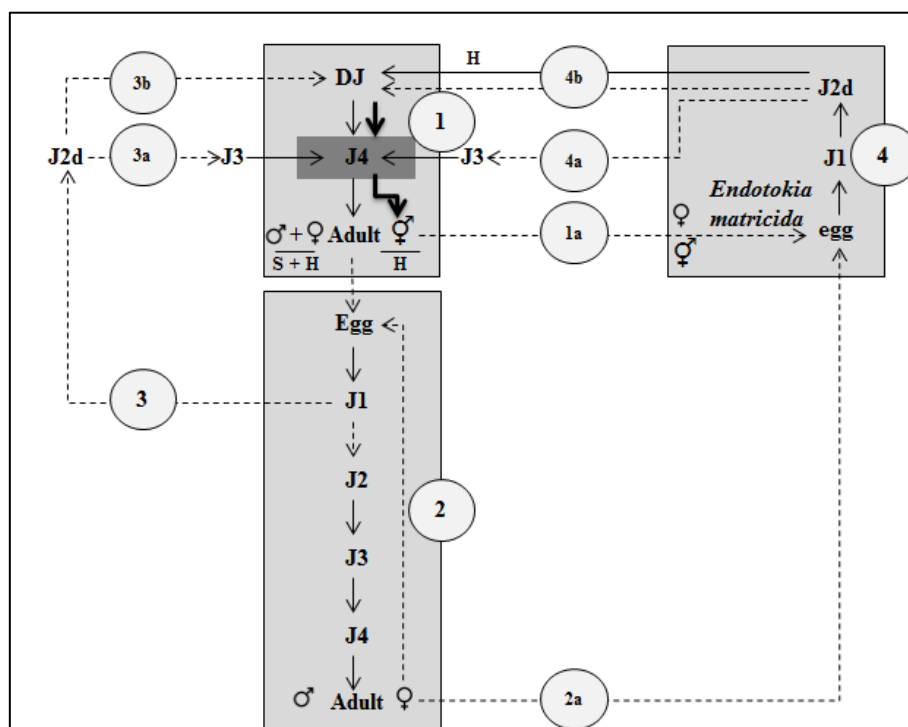
Under unfavourable environment conditions (e.g. low food), females of *Steinernema* spp. and hermaphrodites of *Heterorhabditis* spp. follow the developmental pathway 1a and secure the continuation of generation through *endotokia matricida*. *Endotokia matricida* is the process of egg retention and hatching of J1 inside the maternal uterus followed by a complete consumption of the maternal nematode's body in order to secure the development of well-fed DJs that guarantee the continuation of the next generation (Hirao et al. 2010; Johnigk and Ehlers 1999a). This step is very critical for EPN mass production in liquid culture, since successful fertilization of parental nematodes and build-up of offspring population accelerates the occurrence of *endotokia matricida* and later increases DJ development, which shortens the process time required for DJ harvesting.

Developmental pathway 2 occurs when the environmental conditions are conducive to support growth and development of filial generation adults. When the insect cadaver or artificial media is no longer supportive to nematode growth and reproduction, the females proceed to *endotokia matricida* (2a). The development of second and third generation adults in liquid culture occurs at low DJ inoculum density (Hirao and Ehlers 2009c; Johnigk et al. 2004; Johnigk and Ehlers 1999a) due to the presence of enough bacteria. In addition, it has been reported that at lower nematode density the level of nematode pheromones that induce DJ formation is minimal (Noguez et al. 2012; Popiel et al. 1989). In liquid culture amphimictic females of *Heterorhabditis* spp. do not produce offspring due to copulation problem (Strauch et al. 1994).

Developmental pathway 3 occurs when the food resource gets depleted as a result of nematode population build-up and increased production of nematode pheromones, which leads the J1s to develop in to J2d and later to DJs as indicated in the developmental pathway 3b. The fourth developmental pathway is the process in which the J1 hatches inside the uterus



of the parental and or filial generation females and hermaphrodites. At the expense of the maternal body, this step mainly leads to the development of DJs. Similar to developmental pathway 3a, any environmental change in the medium or increase in the density of the symbiotic bacteria in liquid culture as reported in *S. carpocapsae* and *S. feltiae* with low numbers of DJ inoculum density (Hirao and Ehlers 2009c) allows the J2ds to follow the developmental pathway 4a towards J3 instead of DJ development (developmental pathway 4b). The J2ds of *Steinernema* spp. obtained via *endotokia matricida* get released from the maternal carcass and any change in the medium or environmental condition may encourage their development towards J3s instead of DJs. However, in *Heterorhabditis* spp. J2ds obtained via *endotokia matricida* fully develop into DJs before leaving the maternal carcass and therefore development of J2ds to DJ is obligatory. J2ds of both nematode groups obtained from laid eggs have similar developmental pathway.



**Fig. 2** Life cycle and developmental pathways of the genera *Steinernema* and *Heterorhabditis*. Solid lines indicate obligatory developmental pathways. Dotted lines represent alternative developmental pathways. The numbers in circles represent the general sequences of the developmental steps starting from Dauer Juvenile (DJ). J1–J4 = first to fourth juvenile stages; ♂ = male; ♀ = female; ♀♂ = hermaphrodite; J2d = pre-dauer juvenile; S = *Steinernema*; H = *Heterorhabditis* (adapted from Hirao 2009)

For successful and economically reasonable usage, EPNs should be produced in large scale at a competitive cost and short process time (Ehlers 2001; Ehlers and Shapiro-Ilan 2005). Understanding the different developmental pathways and factors that are involved in the development of EPNs helps to improve liquid culture production. Mass production of EPNs in liquid culture, which starts from egg isolation, production of monoxenic cultures and pre-culturing of their symbiotic bacteria (Lunau et al. 1993). The monoxenic starter cultures are then scaled-up to liquid medium (Ehlers et al. 1998). In order to improve mass production in liquid culture a better knowledge of population dynamics of both, the nematode and its associated symbiotic bacteria, are necessary. In the past, many investigations have been conducted in liquid cultures to assess the population dynamics of *Steinernema–Xenorhabdus* spp. and *Heterorhabditis–Photorhabdus* spp. (Hirao and Ehlers 2009a, b, c; Hirao et al. 2010; Johnnig et al. 2004; Strauch and Ehlers 1998).

Previous population dynamics investigations in liquid culture were done at a population level and only allowed assessing the changes of nematode population and their symbiotic bacteria as a whole entity. In order to better understand liquid culture processes, it is necessary to study the life history traits (LHTs) of the nematode based on single or a pair of parental nematodes in a medium, which resembles liquid culture conditions and give an indication of the nematodes reproductive potential.

Life history of organisms encompasses the life of an individual from its birth or hatching to death, which describes the age-or stage-specific patterns of maturation, reproduction, survival and death (Braendle et al. 2011). Except *S. hermaphroditum*, which develops to self-fertile hermaphrodites (Griffin et al. 2001; Stock et al. 2004), all described *Steinernema* spp. are reproducing amphimictically. *Steinernema* spp. have a helix type of copulation and are capable of mating in liquid culture that allow them to have similar life cycle and developmental pathways both in solid and liquid media, while only automictic females of *Heterorhabditis* spp. are reproductive in liquid culture (Strauch et al. 1994).

LHT analysis is using a hanging drop method. The hanging drop technique uses a semi-solid Nematode Growth Gelrite medium (NGG) that was described by Muschiol and Traunspurger (2007) to assess the LHTs of free-living nematodes. Because of its advantages over liquid and solid culture medium, its application has received positive attention to study other free-living nematodes including the two model organisms *Caenorhabditis elegans* and *Pristionchus pacificus* and *Panagrolaimus* sp. (Ayub et al. 2013b; Gilarte et al. 2015;

Muschiol et al. 2009). This method allows studying single individual nematodes of similar age with a better accuracy to generate reliable data about the LHTs of nematodes. In addition, it allows studying culture conditions (e.g. food, temperature), DJ recovery and influence of mating (Ayub et al. 2013b; Muschiol et al. 2009). The problems associated with EPNs in liquid culture production could be alleviated with a better knowledge on their LHTs.

The hanging drop method allows knowing the nematodes reproductive potential through delivery of fresh bacteria every day, while liquid culture conditions prohibit following individual nematodes. Data generated based on the LHT assessment in hanging drops could be extrapolated in liquid culture production of nematodes (Ayub et al. 2013a). Knowledge on the optimum bacterial density and offspring production potential of a given species in hanging drops can be used as a basis for further improvement in liquid culture production. It could help to develop and parameterize a simple model to estimate DJ yield at the end of the process cycle and make decision measures at early stages. Moreover, it may contribute to analyse the gaps that hinder to reach the nematodes maximum DJ production potential.

The general purpose of this study was to investigate the LHTs of the EPNs, *S. feltiae*, *S. riobrave*, *S. yirgalemense* and *H. bacteriophora* in a semi-solid NGG in hanging drops in order to generate supportive information for further improvement of their liquid culture production. The study investigated the following:

- LHTs of *S. feltiae*, *S. riobrave*, *S. yirgalemense* and *H. bacteriophora*
- Effect of mating on offspring production and survival of *H. bacteriophora*
- Utilization of the LHT data as a basis for prediction of DJ yield in liquid culture at the end of the process cycle

## 2. Results

### 2.1. Influence of food and mating on the LHTs of EPNs

To produce basic information and improve liquid culture production of EPNs, the life history traits (LHTs) of *S. feltiae*, *S. riobrave*, *S. yirgalemense* and *H. bacteriophora* were assessed at 25°C by observing single pairs of male and female or individual hermaphrodites using a hanging drop technique. LHTs were assessed with a daily supply of the respective fresh symbiotic bacteria ranging from  $2.5 \times 10^9$  cells ml<sup>-1</sup> to  $20 \times 10^9$  cells ml<sup>-1</sup>.

A hanging drop method using semi-solid NGG medium allowed observation of growth and reproduction of amphimictic females of steinernematids and automictic females of heterorhabditids. Different life history parameters such as age of maternal nematodes at maturity, offspring production, population growth rates and survival were successfully assessed. In order to investigate recovery of DJs and development to adult nematodes and provide nematodes for hanging drop experiments, solid Nematode Growth Gelrite (NGG) plates were inoculated with  $20 \times 10^9$  cells ml<sup>-1</sup> and incubated over night before DJ inoculation. Culturing nematodes in NGG plates allows close observation of nematode development and helps to pick nematodes at the right time for both LHT study and mating experiments. The semi-solid NGG and solid NGG coated with symbiotic bacteria can also be used for selection and breeding experiments.

Males of *Heterorhabditis* spp. have a *bursa copulatrix* enabling them to attach and copulate with the female. Similar to liquid culture, *H. bacteriophora* males failed to copulate in hanging drops with hermaphrodites and females. On the other hand, they managed to copulate on a drop of 300 µl of solid NGG on the inner side of the lid of multi-well culture plates coated with 10 µl *P. luminescens*, which allowed assessing the influence of mating on offspring production and survival.

Assessing the LHT of EPNs, such as offspring production, population growth rate, and lifespan of nematodes at different bacterial densities and observation of mating helped to identify key factors and parameters to be considered for improvement of liquid culture production and development of a tool for estimation of DJ yields at an early stage of liquid culture production.

### 2.1.1. Offspring production

Increasing bacterial density had a significant positive increasing effect on offspring production. In addition, differences in the number of total offspring produced per maternal nematodes were observed among the EPN species. E.g., the maximum number of offspring per female or hermaphrodite recorded at the highest bacterial density ( $20 \times 10^9$  cells ml<sup>-1</sup> of the respective symbiotic bacteria) was 813, >1900 and 269, for *S. feltiae*, *S. riobrave* and *H. bacteriophora*, respectively (Table 1). The number of offspring per female of *S. yirgalemense* recorded at  $10 \times 10^9$  cells ml<sup>-1</sup> of *X. indica* was only 314 (Publication 3, Table 1).

The medium used for propagation of the symbiotic bacteria to be transferred to hanging drops was different for *S. yirgalemense* and might have exerted an effect on offspring production. In *S. feltiae* and *S. riobrave*, nutrient rich nematode liquid medium was used while symbionts of *S. yirgalemense* had been propagated in a nutrient poor YS broth. *Steinernema feltiae* and *S. riobrave* might have benefited through consumption of unutilized media components harvested together with their symbiotic bacteria or just by higher quality bacterial cells obtained from the medium with high nutrient concentration.

Offspring of EPNs originate either from externally laid eggs (extra-uterine) or from *endotokia matricida* (intra-uterine). At a given interval of time, maternal nematodes squeeze vulval muscles to deposit batches of eggs for the first 1–2 days. Starting from the second day of egg laying EPNs produce offspring via *endotokia matricida* regardless of bacterial density. Although bacterial density has a significant positive influence on the total offspring production, significant differences were not observed on the proportion of offspring produced from *endotokia matricida*. Nevertheless, the percentage of offspring produced via *endotokia matricida* decreased from 53 to 39%, 66 to 28% and 77 to 64% for *S. feltiae*, *S. riobrave* and *H. bacteriophora*, respectively with increasing cell densities of the symbiotic bacteria from  $5 \times 10^9$  cells ml<sup>-1</sup> to  $20 \times 10^9$  cells ml<sup>-1</sup> (Publication 1, Fig. 1B; Publication 2, Fig. 2A and B and Publication 4, Fig. 2B). The main reason for differences among the EPN species in the total offspring production is related to the body volume of maternal nematodes. With increasing bacterial density, the size of the ovary increases dramatically. For all nematode species investigated, the bacterial density was positively correlated to offspring production.

### 2.1.2. Nematode growth rate

Based on the assessment conducted in NGG plates coated with  $20 \times 10^9$  cells  $\text{ml}^{-1}$  it was observed that egg laying in *S. riobrave*, *S. yirgalemense* and *H. bacteriophora* started almost at similar age (4.5 days starting from J1), whereas females of *S. feltiae* started egg laying at day 4 (Publications 1–4). After twenty-four hours incubation, juveniles completely hatched from laid eggs.

Bacterial density did not play a significant role on the different mean generation times. The mean generation time ( $T_1$ ), which is the period of time necessary for a population growing at a constant rate  $r_m$  to increase by the factor  $R_0$  of *H. bacteriophora* was slightly longer ( $>6.16$  days) compared with steinernematids ( $< 5.92$  days) (Table 1). The intrinsic rate of natural increase  $r_m$  calculated as

$$\sum_{x=0}^d e^{-r_m x} l_x m_x = 1$$

has indicated a positive increase with increasing bacterial cell densities of the symbionts (Table 1). However, a significant difference in  $r_m$  was observed only between the lowest and the highest bacterial densities of *P. luminescens* for *H. bacteriophora*. *Heterorhabditis bacteriophora* has a relatively longer generation time and slow reproduction rate compared with steinernematids. As a result, the  $r_m$  of *H. bacteriophora*, e.g., at the highest bacterial density ( $0.89 \text{ day}^{-1}$ ) is lower compared with the steinernematids ( $> 1.19 \text{ day}^{-1}$ ) (Table 1). Due to its lower number of offspring per female and  $r_m$  ( $0.98 \text{ day}^{-1}$ ), *S. yirgalemense* has a higher population doubling time compared with *S. feltiae* and *S. riobrave* at the same bacterial density ( $10 \times 10^9$  cells  $\text{ml}^{-1}$ ).

### 2.1.3. Influence of mating on offspring production in *H. bacteriophora*

Offspring production of self-fertilizing and mated hermaphrodites of *H. bacteriophora* was not significantly different (Publication 4, Fig. 5). The highest number of offspring (349) was recorded from self-fertilizing hermaphrodites while amphimictic females produced on average only 52 individuals.

### 2.1.4. Average survival of maternal nematodes

The average survival of reproductive females of *Steinernema* spp. and hermaphrodites of *H. bacteriophora* was not significantly affected by bacterial density (Table 1). However, in *Steinernema* spp. the average survival of maternal nematodes slightly increased with increasing bacterial density. At the same bacterial density the average survival recorded for *H. bacteriophora* is higher compared with *Steinernema* spp. Both, amphimictic and automictic females of *Steinernema* spp. and *H. bacteriophora*, could not survive longer than 8.1 days, due to the occurrence of *endotokia matricida*, which has a significant advantage from evolutionary point of view.

The time required to reach the adult stage is almost similar for all assessed EPN species. Yet, a slightly longer lifespan of hermaphrodites of *H. bacteriophora* was recorded. The occurrence of *endotokia matricida* in *Steinernema* spp. was not significantly affected by bacterial density. Similarly, a detailed assessment of *H. bacteriophora* hermaphrodites indicated that mating and bacterial density has no significant effect on the beginning of *endotokia matricida*, hermaphrodite death and DJ release from the maternal carcass. Females of *H. bacteriophora* produced 98% of their offspring (52 per female) through *endotokia matricida* (Publication 4, Fig. 5). When juveniles of steinernematids develop in the maternal uterus, they start consuming the body contents of the female and are active enough to quickly damage the mother and they emigrate from the mother's body before they have readily developed to DJs. On the other hand, the juveniles of *H. bacteriophora* are less active and fully develop into DJs inside the maternal carcass.

**Table 1.** Life History Traits (LHTs) of *Steinernema feltiae* (SF), *S. riobrave* (SR), *S. yirgalemense* (SY) and *Heterorhabditis bacteriophora* (HB) assessed at 25°C in hanging drops at different bacterial densities of their respective symbiotic bacteria ( $\times 10^9$  cells  $\text{ml}^{-1}$ )

Life cycle parameters	2.5	5				10					20			
	HB	SF	SR HYB19	SR 7-12	HB	SF	SR HYB19	SR 7-12	SY	HB	SF	SR HYB19	SR 7-12	HB
$R_0$	50	359	792	683	100	589	1355	1332	314	204	813	1904	1904	269
$T_1$ (day)	6.97	5.35	5.92	5.35	6.16	5.50	5.18	5.46	5.87	6.53	5.56	5.74	5.41	6.25
$r_m$ ( $\text{day}^{-1}$ )	0.54	1.1	1.13	1.23	0.7	1.16	1.44	1.34	0.98	0.81	1.19	1.32	1.4	0.89
PDT (day)	1.39	0.64	0.62	0.57	1.01	0.6	0.5	0.52	0.71	0.85	0.59	0.53	0.5	0.78
Av. lifespan (day)	8.1	6.7	7.4	6.9	7.9	7	7.5	7.1	6.55	8	7.3	7.8	7.3	8
Remark	Pl. 4	Pl. 1	Pl. 2	Pl. 2	Pl. 4	Pl. 1	Pl. 2	Pl. 2	Pl. 3	Pl. 4	Pl. 1	Pl. 2	Pl. 2	Pl. 4

$R_0$  = net reproductive rate (offspring per female);  $T_1$  = alternative measures of generation time;  $r_m$  = intrinsic rate of natural increase; PDT = population doubling time, Pl = publication number in the present studies



## **2.2. Liquid culture production and DJ yield estimation**

Results obtained with the hanging drop method, which is designed to deliver fresh food at a given interval of time and follow individuals or pairs of nematodes, cannot be directly transferred to understand population development in liquid culture production. Nevertheless, parameters observed in hanging drops, which have a strong impact on female body volume and which is linked to offspring production, can be used for estimation of DJ yield in liquid culture. This hypothesis was tested through conducting a number of liquid culture experiments both in Erlenmeyer flasks and bioreactors. DJ recovery is an important parameter influencing success and failure of liquid culture production. Also this parameter was observed.

### **2.2.1. DJ recovery**

DJ recovery was assessed in hanging drops using *S. yirgalemense* at different bacterial densities of *X. indica*. DJ recovery was significantly positively associated with bacterial density. The maximum percentage of DJ recovery (55%) recorded in hanging drops at  $20 \times 10^9$  cells ml<sup>-1</sup> was found quite low compared with recovery recorded in liquid culture, which ranged from 63–75% at 72 h post DJ inoculation (Publication 3, Fig. 1 and Fig. 2A).

### **2.2.2. Female number**

In steinernematids the population of parental females depends on nematode species (sex-ratio) and percentage DJ recovery. On the other hand heterorhabditids parental nematode population solely depend on DJ recovery. The percentage of females in the adult nematode population in liquid culture at 72 h post DJ inoculation was not significantly influenced by the incubation temperature (Publication 3, Fig. 2B) and nematode batches (Publication 1). Still, the total number of females recorded per ml<sup>-1</sup> both in *S. yirgalemense* and *S. feltiae* differs depending on the percentage of DJ recovery although not significantly different. These differences in the total number of maternal nematodes in liquid culture of *S. feltiae* have shown a difference in the total nematode population at the end of the process cycle.

### 2.2.3. Female body volume

In hanging drops, food was delivered every day and the nematode's potential in body growth and its association towards offspring production was clearly observed. The beginning of *endotokia matricida* is the time at which the body volume of maternal nematodes reaches to the maximum (stop growing). In all three EPN species, *S. feltiae*, *S. riobrave* and *H. bacteriophora*, the body volume of parental nematodes increased with increasing bacterial density. The maximum body volume of maternal nematodes was recorded at the highest bacterial density ( $20 \times 10^9$  cells ml<sup>-1</sup>) for all EPN species. In turn, the body volume of maternal nematodes indicated a correlation of 0.89, 0.93, and 0.88 with the number of offspring recorded per female of *S. feltiae*, *S. riobrave* and *H. bacteriophora*, respectively (Publication 1, Fig. 3; Publication 2 and Publication 4, Fig. 4).

In liquid culture due to food limitation, a strong negative correlation between female number and DJ yield per female of *S. feltiae* ( $R = -0.9$ ) was observed and the contribution of female body volume towards DJ yield per female is reduced ( $R = 0.42$ ) (Publication 1, Fig. 5 and 6). The females in bioreactors with lower number of parental females, had a relatively larger body volume (0.033 mm<sup>3</sup>) compared with those from Erlenmeyer flasks (0.026 mm<sup>3</sup>) (Publication 1, Table 3). Based on the results of hanging drops and liquid culture, optimization of media components that encourage bacterial growth and process parameters, which favour DJ recovery followed by high nematode population growth and development is the central point towards improvement of nematode propagation.

### 2.2.4. DJ yield estimation

The information obtained from the hanging drop experiments indicated that the body volume of maternal nematodes at the beginning of *endotokia matricida* has a significant association with the total number of offspring recorded per female or hermaphrodite. In liquid culture it is not possible to assess individual nematodes. However, the total number of parental nematodes, body volume of parental nematodes and total adult nematode population can be assessed by taking samples.

With increasing density of maternal nematodes competition in liquid culture for limited resources is increasing. As a result the number of parental females at three days post DJ inoculation (beginning of *endotokia matricida*) showed a negative correlation ( $R = -0.9$ ) with DJ yield per female at the end of the process.

The relationship between the female body volume at the beginning of *endotokia matricida* and offspring production per female obtained in the hanging drops during the LHT assessment of the different EPN species was the motivation towards development of a tool for DJ yield estimation. However, the female body volume alone recorded at the beginning of *endotokia matricida* could not be used to predict DJ yield at the end of the process. Similarly, the correlation between the number of parental females  $\text{ml}^{-1}$  recorded at the beginning of *endotokia matricida* and the total DJ yield  $\text{ml}^{-1}$  at the end of the process cycle was low ( $R = 0.5$ ) (Publication 1, Fig. 6). Moreover, the variation in DJ yield estimation based on only parental females was high between process runs and type of culture equipment (Erlenmeyer flasks or bioreactors).

Combination of the two important parameters (female body volume and female number) provided reasonable results for prediction of nematode yields in liquid culture. This was then tested with results from both, Erlenmeyer flask and bioreactor culture, using *S. feltiae*. The total female body volume calculated as the average female body volume  $\times$  total number of parental females  $\text{ml}^{-1}$  at three days post DJ inoculation was found positively correlated ( $R = 0.72$ ) with DJ yields recorded at the end of the liquid culture process (Publication 1, Fig. 7). The total female body volume at process day three is a good indicator for estimation of nematode DJ yield at the end of the process (12–15 days) post DJ inoculation) in both Erlenmeyer flasks and bioreactors with acceptable error, which had a mean deviation of 9467 DJs  $\text{ml}^{-1}$ , that resembled about 5% of the final DJ yields.

### **3. Discussions**

Many aspects of the LHTs of an organism are influenced by its body size (Charnov 1993; Peters 1983; Stearns 1992). The body size of nematodes depends on its way of life (free-living or parasitic) and environmental conditions such as food and temperature. Therefore, assessing the response of body size of EPNs at different food densities has a great importance in LHT study. Under in vivo conditions, the various LHTs of EPNs such as offspring production, survival, body size, and time to and rate of DJ emergence from the insect cadaver have been linked with nematode population density (Ryder and Griffin 2002; Shapiro-Ilan et al. 2012). The role of bacterial cell density and DJ inoculum density on the final DJ yield of *S. feltiae* and *S. carpocapsae* in liquid culture were also assessed (Hirao and Ehlers 2009b, c).

#### **3.1. Influence of food on the LHTs of EPNs**

The life history theory of nematodes indicates that higher numbers of offspring production in animal-parasitic nematodes is related to larger body size of females compared to free-living nematodes, and size has evolved depending on the size of their associated hosts (Morand and Sorci 1998). Under in vitro conditions the LHTs of EPNs depends on the availability of bacterial cells in the culture and also culture conditions such as temperature.

##### **3.1.1. Offspring production**

Offspring production and the ultimate DJ yield of EPNs are influenced by both, genetic and environmental factors. Food and temperature are the two important environmental factors that play a significant role in the LHTs of organisms (Ayub et al. 2013b; Klass 1977; So et al. 2011). It has been reported that the body size of both free-living and parasitic nematodes, which is mainly governed by the availability of food, is positively associated with offspring production (Morand and Sorci 1998). The final body volume of adult nematodes is independent on their free-living stages as eggs or DJs or use of intermediate hosts rather depend on the nutritional conditions during inter-moult periods and adult stage (Morand 1996).

The most pronounced differences among the EPN species assessed in the hanging drops were in the number of offspring production per maternal nematodes. Increasing the cell density of the respective symbiotic bacteria has increased the number of offspring produced per parental

females. Similar positive effects of bacterial density on the fecundity of *C. elegans* and *C. briggsae* have been reported (Houthoofd and Vanfleteren 2006; Klass 1977; Schiemer 1982; Tain et al. 2008). These differences in offspring production arise from the differences in the body volume of maternal nematodes and the body size of their respective juveniles. The size of eggs might have also contributed to these differences.

Generally, the EPN species assessed in hanging drops have larger body volume and produced higher number of offspring compared with the free-living nematodes such as *Caenorhabditis elegans*, *Panagrolaimus* sp., *Poikilolaimus* sp. and *Pristionchus pacificus*, assessed with the same technique at similar bacterial density (Ayub et al. 2013b; Gilarte et al. 2015; Muschiol et al. 2009; Muschiol and Traunspurger 2007). In order to compensate losses of offspring during hazardous transmission from one host to the other, parasitic nematodes including EPNs have evolved towards extremely high fecundity (Morand 1996; Morand and Sorci 1998).

The number of offspring recorded per female of *S. yirgalemense* in hanging drops was lower compared with the other *Steinernema* spp. assessed. The possible reasons for this variation could be media differences used for propagation of the symbiotic bacteria for hanging drops. In *S. feltiae* and *S. riobrave* nutrient rich nematode liquid medium was used, while symbionts of *S. yirgalemense* were propagated in the nutrient poor YS medium. In addition to the density of bacterial cells, the nutritional quality of the bacteria for nematode growth and reproduction should be considered. Moreover, *S. feltiae* and *S. riobrave* might have benefited through consumption of unutilized media components, which were harvested together with their symbiotic bacteria.

The body volume of *S. yirgalemense* females was not assessed in hanging drops. But, based on measurements conducted with in vivo produced nematodes, with the assumption that all the assessments were done with the last instar of *Galleria mellonella*, the body volume of *S. yirgalemense* females is relatively smaller when compared with females of *S. riobrave* (Cabanillas et al. 1994; Nguyen et al. 2004) but greater than the body volume of *S. feltiae* females (Nguyen et al. 2007). On the other hand, the body volume of the DJs of *S. yirgalemense* is smaller when compared with the DJs of *S. feltiae* and *S. riobrave* (Cabanillas et al. 1994; Nguyen et al. 2004; Nguyen et al. 2007). The reason for this unexpected lower number of

offspring in *S. yirgalemense* with larger female body volume could be a result of media differences in the propagation of the symbiotic bacteria used for hanging drops. Assessment of the nematodes LHTs using symbiotic bacteria, cultured in different media may cause differences.

In liquid culture, in addition to maternal body volume, DJ body volume influences the final DJ yield. Particularly the J2ds of heterorhabditids obtained from *endotokia matricida* complete development into DJs inside the maternal carcass. E.g., the body volume of *H. bacteriophora* is higher than *H. indica* hermaphrodites and with a vice versa DJ body volume (Ehlers et al. 2000; Han 1996; Nguyen and Hunt 2007). However, the total DJ body volume of the two species was similar ( $54 \text{ mm}^3 \text{ ml}^{-1}$ , calculated based on Andrásy formula, Andrásy 1956) although there was a yield difference of 150,000 DJs  $\text{ml}^{-1}$ .

Adaptation of female nematodes to nutritional conditions by adjustment of their body size has also been observed in liquid culture production. Liquid culture is a batch system and does not allow provision of fresh food sequentially and the bacterial cells are limited and start declining following DJ recovery. The availability of bacterial density depends on the concentration of media compounds and the number of nematode population recovered from inoculated DJs. As a result the body volume of females of *S. feltiae* recorded in liquid culture at the beginning of *endotokia matricida* was much lower when compared with the hanging drops. Johnigk and Ehlers (1999a) reported that *H. bacteriophora* hermaphrodites cultured at higher bacterial density have an increased body length that was positively correlated with the production of higher numbers of offspring.

EPNs generate offspring from laid eggs and *endotokia matricida*. Increasing bacterial density results in an overall increase in the total number of offspring, while the percentage offspring produced via *endotokia matricida* is decreasing. Percentage of offspring production in parental hermaphrodites of *H. megidis* via *endotokia matricida* was positively associated with the density of DJs inoculated per insect (Ryder and Griffin 2002). In addition to food and DJ inoculum density, the proportion of offspring obtained from *endotokia matricida* could be affected by nematode species and generation.

A pronounced effect of *endotokia matricida* even at higher bacterial density was observed in *S. yirgalemense*, which 96% of the offspring originated from *endotokia matricida* (Publication

3). Based on the number of offspring recorded in hanging drops and percentage of offspring produced via *endotokia matricida*, it could be possible to suggest that the bacteria obtained from YS medium is not quality enough for nematode reproduction.

Under natural conditions, the availability of food at the time of female development is much lower than at the moment of hermaphrodite development. The last generation females of *H. bacteriophora* in *G. mellonella* produced 100% of their offspring via *endotokia matricida* (Baliadi et al. 2009). Still, in the presence of enough food, females of *H. bacteriophora* also produced 98% of their offspring (52 per female) through *endotokia matricida* (Publication 4, Fig. 5). Second generation females of *S. feltiae* taken from liquid culture and assessed in hanging drops with fresh bacteria also produced 97% their offspring via *endotokia matricida* (Addis unpublished data). Therefore, second generation females of EPNs might have evolved to produce higher proportions of their fewer offspring via *endotokia matricida* to withstand the harsh conditions that occur at a late stage of population development in an insect.

### **3.1.2. DJ development**

Food is one of the environmental factors that play a significant role in the LHTs of nematodes (Klass 1977). The developmental pathways of EPNs depend on two main factors, the food signal and DJ recovery inhibiting factor (DRIF) (Noguez et al. 2012; Strauch and Ehlers 1998; Strauch et al. 1994). The presence of a high food signal concentration and low level of DRIF encourages DJ recovery, whereas low levels of a food signal coupled with higher concentrations of DRIF encourages DJ formation.

Except the optimum time of DJ inoculation in liquid culture, the right amount of bacterial density required for high DJ yield production of EPNs is not well known. Utilizing the advantages of the hanging drops developed recently using semi-solid NGG (Muschiol and Traunspurger 2007), efforts have been made to investigate the LHTs of different EPN species or strains (Publication 1–4) including *endotokia matricida* and DJ development.

The presence of DJ recovery inhibition factors in *C. elegans* was reported three decades ago (Golden and Riddle 1984). Recently, the presence of pheromones in *H. bacteriophora* produced by adult nematodes cultured in agar plates, which inhibited DJ recovery upto 60% is reported (Noguez et al. 2012). Substances in spent *S. feltiae* and *H. bacteriophora* liquid culture

supernatant (9 days old), which are believed to be nematode pheromones, inhibited the recovery of DJs successfully (Addis, unpublished). The supernatants had also some effect on DJ formation. In the future, the chemical composition of the substance(s) needs to be analysed and further tested in order to utilize its role in liquid culture production of EPNs.

When the nutritional status of the host is no longer suitable for reproduction, *Steinernema* spp. form DJs. A similar scenario is also happening in monoxenic liquid culture production of these nematodes. It has been reported that high nematode population development encouraged the development of DJs, while the presence of food and soluble nutrients has negatively affected DJ formation (Popiel et al. 1989).

Differences were observed regarding pre-dauer juveniles (J2ds) originated via *endotokia matricida* between the three *Steinernema* spp. and *H. bacteriophora* in their development towards DJs. As previously reported, J2ds of *Heterorhabditis* spp. are motionless and fully develop into DJs inside the maternal carcass (Johnigk and Ehlers 1999a), whereas the J2ds of *S. feltiae*, *S. riobrave* and *S. yirgalemense* emigrated out of the maternal body into the surrounding environment before developing into DJs. Similarly, the J2ds of *S. carpocapsae* (Hirao et al. 2010) and *S. glaseri* (Kaya and Stock 1997) did not fully develop into DJs in the maternal carcass. However, the exact time required for the J2ds to completely develop in to DJs is not known. In hanging drops it is often observed that J2ds of *Steinernema* spp. quickly develop into J4 and later to adults. Whether the J2ds directly develop into DJs and recover quickly to proceed towards J4 or jump the process of complete DJ development and continue to the next developmental stage is not clear and needs further investigation.

### **3.1.3. Nematode growth rate**

The growth rate of organisms is governed by their genetic content and the environment (Fenchel 1974). Food and temperature are the two most important environmental factors that affect the growth rate of nematodes. The life cycle of nematodes, including EPNs comprises of egg, four different juvenile stages each accompanied with a periodic moulting and the adult stage. Significant growth of nematodes occurs between inter-moult and adult stages (Wharton 1986). Food availability during particular inter-moult periods and the adult stage determines the



nematodes body size and therefore has an implication on their LHTs and liquid culture production.

Increasing bacterial density in *S. feltiae*, *S. riobrave* and *H. bacteriophora* increases offspring production significantly without significant change on the lifespan of maternal nematodes. EPNs have a short mean generation time. The short developmental time in EPNs is beneficial to minimize the risks of mortality before reproduction and production of stress resistant DJs. EPNs have evolved to produce a higher number of offspring within a short period of time. As a result they have greater or equivalent population growth rate ( $r_m$ ) values compared with free-living nematodes such as *Panagrolaimus* sp., *Poikilolaimus* sp., *Panagrolaimus* sp. strain NFS 24-5, *C. elegans* and *P. pacificus*, which have an extended period of egg laying (Ayub et al. 2013b; Gilarte et al. 2015; Muschiol et al. 2009; Muschiol and Traunspurger 2007).

In addition to low food availability, lower incubation temperatures decrease the growth rate of nematodes, which was observed in the cold tolerant nematode, *S. kraussei* with longer generation time and a slow population growth rate (Addis unpublished data). Likewise, the population growth rate of *Panagrolaimus* sp. strain NFS 24-5 was significantly affected by culture temperature (Ayub et al. 2013b). An optimum incubation temperature in liquid culture production of *S. yirgalemense* has increased the rate and density of DJ development (Publication 3). This indicates that incubation temperature is one important parameter, which needs consideration in mass production of EPNs.

### **3.1.4. Influence of food and mating on the lifespan of nematodes**

Ciche et al. (2008) indicated that both in vivo and in vitro cultured nematodes of *H. bacteriophora* nematodes first produced eggs that develop into non-DJ stages followed by *endotokia matricida*. In agreement with the authors, all maternal nematodes of steinernematids and heterorhabditids laid eggs for the first few days followed by *endotokia matricida*. It has been observed that in the presence of enough bacteria in hanging drops, the majority of the J2ds of steinernematids and DJs of heterorhabditids that came out of the maternal carcass quickly recovered and developed into next nematode stages.

Preliminary investigation of both *Steinernema feltiae* and *H. bacteriophora* juveniles obtained from sterilized eggs using spent nematode supernatant indicated that DJs could also develop from laid eggs (Addis unpublished data). The amount of bacterial and nematode pheromone should be optimized in order to understand the role of food and pheromones on DJ recovery and DJ formation, respectively. Ciche et al. (2008) reasoned out that the small number of eggs were insufficient to account for the large numbers of DJs and concluded that DJ development is solely through *endotokia matricida*. In mating experiments, it was found that the contribution of automictic females was far lower compared to parental hermaphrodites, which produced seven-times more offspring per hermaphrodite (Publication 4, Fig. 5). Therefore, occurrence of *endotokia matricida* is an obligatory step in the LHT in EPNs but not the only way for DJ development. But, the quality of DJs obtained from eggs towards offspring production, survival and virulence needs further assessment.

*Endotokia matricida* was the main cause of mortality of reproductive females/hermaphrodites of all the assessed EPN species. Once *endotokia matricida* starts, the life span is defined with almost no variance in duration. As a consequence, female/hermaphrodites do not live much longer than one-week starting from the J1 stage. It has been reported that *endotokia matricida* is initiated when the J1 hatches inside the uterus mainly at the time of food depletion (Chen and Caswell-Chen 2003; Johnigk and Ehlers 1999a). Nevertheless, assessment of nematode lifespan with a daily delivery of fresh food in hanging drops using different levels of bacterial density did not show significant difference in their lifespan (Publication 1, Table 2; Publication 2, Table 1; Publication 4, Table 2). This would indicate that food supply has no influence on the beginning of the *endotokia matricida*. However, Johnigk and Ehlers (1999a) reported that maternal nematodes of heterorhabditids undergo *endotokia matricida* immediately when kept in a bacteria-free environment and did not lay any eggs. Hence, an influence of the food supply on the introduction of *endotokia matricida* is obvious and we can conclude that maternal nematodes undergo to *endotokia matricida* quickly when kept in bacteria-free solutions. In the present study, the amount of bacterial cell densities tested ( $\geq 2.5 \times 10^9$  bacterial cells ml<sup>-1</sup>) in hanging drops was not low enough to immediately induce *endotokia matricida*. Experiments indicating that food supply can influence the beginning of the *endotokia matricida* but probably need bacterial density much lower than  $2.5 \times 10^9$  cells ml<sup>-1</sup>.

In addition to food, a number of factors have been reported as a cause for the occurrence of *endotokia matricida*. In *C. elegans*, failure of the egg laying system due to ageing increases the incidence of *endotokia matricida*, which affects mainly mated hermaphrodites (Pickett and Kornfeld 2013). In liquid culture, incubation temperature had significant effect on the percentage of *S. yirgalemense* females, which entered into *endotokia matricida* at three days post DJ inoculation (Publication 3, Fig. 2C). Of females of *S. feltiae*, transferred to hanging drops from 8 days old liquid culture only 34% of the reproductive females' laid eggs before *endotokia matricida* started, while 100% of young reproductive females laid eggs before entering into *endotokia matricida* (Addis unpublished data). As a result, an increase in the number of fertilized eggs and probably deterioration of the egg laying system might be the reasons for the occurrence of *endotokia matricida* in EPNs and is part of their LHT.

It has been reported that mating increased both offspring production and incidence of *endotokia matricida* in *C. elegans* hermaphrodite populations. The incidence of *endotokia matricida* increased from 6% to 68% when hermaphrodites mate with males, which decreased the average lifespan from 16.7 to 9.8 days (Pickett and Kornfeld 2013). Unlike *C. elegans*, mating had no significant effect on offspring production and survival of *H. bacteriophora* hermaphrodites (Publication 4, Fig. 5 and 6). As mating does not increase the number of offspring, the hermaphrodites of *H. bacteriophora* obviously are not limited in sperm production like *C. elegans*. Looking at the life cycle and the occurrence of the different stages of *H. bacteriophora*, we can also see that males are usually matured two days after the start of *endotokia matricida* (Johnigk and Ehlers 1999b). Juveniles hatched inside the mother consume the sperm of the hermaphrodite and stop increase of offspring production. Hence, mating would not make sense at this stage of the development of the hermaphrodite.

In *S. feltiae*, duration of male exposure time and offspring production has a positive influence on offspring production, although no influence on the lifespan of females (Addis, unpublished data). Non-reproductive females of steinernematids survive longer compared with reproductive females. Single females of *S. feltiae* were kept in hanging drops in order to investigate whether an effect of food density on survival can be observed. Since the lifespan of reproductive females is always affected by *endotokia matricida*, regardless of bacterial density and mating duration, it was necessary to keep females without males. It was observed that 50%

of the non-reproductive females of *S. feltiae* survived 20 days at the optimum bacterial density; whereas reproductive females completed their lifespan within a week (Addis unpublished data). Ayub et al. (2013b) also indicated that non-reproductive females of *Panagrolaimus* sp. (strain NFS 24-5) survived on average 20 more days compared with reproductive females. Dietary restriction in *C. elegans* extended the lifespan of hermaphrodites by 57–62% (Klass 1977; Tain et al. 2008). Unlike *C. elegans*, lower food supply in *S. feltiae* did not extend the lifespan of non-reproductive nematodes. It is not expected to extend the lifespan of reproductive females with reduced food since mortality due to *endotokia matricida* is negatively correlated with food density.

### 3.2. Liquid culture production and DJ yield estimation

A synchronized and high proportion of DJ recovery in liquid culture leads to a synchronized nematode population development and higher number of parental adults, which in turn leads to higher numbers of DJ yields within a short process cycle (Aumann and Ehlers 2001; Ehlers 2001; Hirao and Ehlers 2009c; Strauch and Ehlers 1998). Of course, this rule is only true at optimal culture temperature as in *S. yirgalemense* the highest DJ recovery, recorded at incubation temperature of 30°C, did not support higher DJ yield (Publication 3). Following a successful DJ recovery, offspring production or the final DJ yield can still be affected by the body size of females/hermaphrodites, nematode species, the number of parental nematodes and the DJ body size (Hirao and Ehlers 2009a,b,c; Shapiro-Ilan et al. 2012; Strauch and Ehlers 1998; Strauch et al. 1994). In hanging drops, the body size of females of *S. feltiae*, *S. riobrave* and *H. bacteriophora* at the beginning of *endotokia matricida* was significantly associated with offspring production. The results of the LHT data mainly the body size of females was used as a basis for DJ yield estimation of *S. feltiae* at the end of the process cycle both in Erlenmeyer flasks and bioreactors.

In liquid culture, the number of parental females of *S. feltiae* at the beginning of *endotokia matricida* has a strong negative correlation with DJ yield per female ( $R = -0.9$ ) (Publication 1, Fig. 6). This ability to adapt to nutritional conditions has an implication to the final DJ yield in liquid culture. Hirao and Ehlers (2009c) indicated that DJ inoculum density has no significant influence on the final DJ yield of *S. carpocapsae* and *S. feltiae*, except cultures of *S. carpocapsae* at lower DJ inoculum densities. Consequently, nematodes adapt to low food

supply with a decrease in female body size and lower number of offspring per female and to high food supply at the same density of females with a higher number of offspring per female.

Liquid culture is a batch system. The bacterial density depends on the concentration of media compounds and declines as the nematodes feed on the bacteria. Hanging drops are fed batch cultures and the nematodes transferred every day to fresh bacteria, which is almost constant. Due to food limitation in liquid culture, the body volume of females recorded at the beginning of *endotokia matricida* was much lower when compared with hanging drops (Publication 1, Fig. 5 and Table 3). Likewise, the females in bioreactors with a lower number of parental females had a relatively larger body volume compared with those from Erlenmeyer flasks (Publication 1, Table 3). Therefore, it is expected that the number of offspring produced per female is higher in bioreactors. But the total DJ yield depends also on the total number of parental nematodes. When the number of parental females increased from 465 to 2,202 females'  $\text{ml}^{-1}$ , the DJ yield per female decreased from 302 to 86 (Publication 1, Fig. 6). Hira0 and Ehlers (2009c) also observed a negative association between number of parental females and offspring per female of *S. feltiae* and *S. carpocapsae*. This compensative adaptation of fecundity in response to number of parental nematodes minimizes the contribution of DJ inoculum density towards the final DJ yield.

The small difference observed between the number of offspring recorded at the lowest bacterial density in hanging drops and with the lowest number of parental females of *S. feltiae* in liquid culture indicates the positive role of food in offspring production. The average number of offspring produced per female at  $5 \times 10^9$  cells  $\text{ml}^{-1}$  in hanging drops (359 offspring per female) was much closer to the DJ yields per female (338 DJs  $\text{ml}^{-1}$ ) recorded in one of the bioreactors with the lowest number of parental females (Publication 1, Table 3). This indicates that improvement of the media components or designing a technique to use fed-batch approaches may contribute to increase the DJ yield produced in one process cycle.

Therefore, estimation of DJ yield at the end of the process cycle should consider the two most important factors, i.e. parental female number and female body volume. With this consideration, the total female body volume calculated as the average female body volume  $\times$  total number of parental females  $\text{ml}^{-1}$  at three days post DJ inoculation is positively correlated

( $R = 0.72$ ) with DJ yield and was a good indicator for final nematode yield of a liquid culture with acceptable deviation.

#### 4. Conclusions

In order to increase the role of EPNs in biocontrol, the cost of EPN based products should be cheaper. Much has been done to improve beneficial traits of EPNs such as virulence and tolerance to different environmental stresses. Although, knowledge on the reproductive biology and LHTs of EPNs has a practical advantage for breeding and ultimately for mass production, this area has received little attention.

Population dynamic studies in liquid culture starting from small Erlenmeyer flasks to big bioreactors needs a fundamental knowledge on both, the nematode and the respective symbiotic bacteria. Previous liquid culture investigations have been addressing the nematode and symbiotic bacteria relationship as a whole entity and did not allow observing single nematodes. The hanging drop method developed to assess the LHTs of free-living nematodes using semi-solid NGG was successfully implemented to investigate the LHTs of EPNs under different growing conditions and can be used to investigate other EPN species. Influence of mating on offspring production and survival of *H. bacteriophora* hermaphrodites was investigated successfully with a new method developed from 300  $\mu$ l solid NGG coated with 10  $\mu$ l of *P. luminescens* on the inner side of multi-well culture plates. The lid of multi-well plates was used as the base.

Investigations of the LHTs using semi-solid NGG in hanging drops in this study are the first approach for EPNs. These investigations have contributed to understand the LHTs and reproductive potentials of *S. feltiae*, *S. riobrave*, *S. yirgalemense* and *H. bacteriophora*. Results of the hanging drop investigation of *S. feltiae* have been successfully extrapolated for its DJ yield estimation at the end of the culture process both, in Erlenmeyer flasks and bioreactors.

In hanging drop investigations, an increased bacterial density has increased DJ recovery. However, production of *S. yirgalemense* in liquid culture indicated that high DJ recovery alone is not a guarantee for efficient EPN production, rather high DJ recovery followed by successful fertilization and nematode population build-up are the major phenomenon, which leads to higher number of DJ production. Similar to unsynchronized DJ recovery, which leads to the development of second and third adult generation, incubation temperatures and other process parameters that does not encourage fertilization and occurrence of *endotokia matricida* causes process failure.

Increasing the cell density of the respective symbiotic bacteria has increased offspring production per female by 2–3 folds. Variations in offspring production were also observed among the nematode species and genera tested, which ranges from 269–1904 offspring per maternal nematode. These variations are resulted mainly from the differences in the body volume of maternal nematodes. The lifespan of maternal nematodes is always limited to one week, which has evolved as an adaptation to produce stress resistant DJs quickly before the environment gets deteriorated.

The start of *endotokia matricida* in hanging drops has a slightly negative correlation with bacterial density. However, it affects all reproductive females of *S. feltiae*, *S. riobrave*, *S. yirgalemense* and *H. bacteriophora* hermaphrodites assessed regardless of bacterial density. The proportion of offspring obtained from *endotokia matricida* in second generation females of both *S. feltiae* and *H. bacteriophora* was significantly higher indicating that *endotokia matricida* has evolved as part of their LHT for successful transition and transmission. In liquid culture production of *S. yirgalemense*, early and increased percentage of *endotokia matricida* resulted in higher DJ yield and DJ proportion. Therefore, early and increased occurrence of *endotokia matricida* in liquid culture should be considered as a positive indicator for successful production. Higher number of females at the end of liquid culture process is the result of bad fertilization, which was explained by studying the lifespan of non-mated females of *S. feltiae* in hanging drops, which survived three times longer than reproductive females.

Bacterial density significantly increases the body volume of female nematodes, which is highly correlated with offspring production. The presence of a negative correlation between parental female number and DJ yield per female in liquid culture as a result of limited food reduces the contribution of parental nematodes towards the total DJ yield  $\text{ml}^{-1}$  at the end of the culture process. The hanging drop technique gives an idea about the reproductive potential of a given nematode species but cannot provide information to directly estimate DJ yield in liquid culture with limited food supply. On the other hand, the total female body volume [calculated as the average body volume of maternal nematodes  $\times$  the number of maternal nematodes  $\text{ml}^{-1}$  at the beginning of *endotokia matricida*] turned out to be a good indicator for DJ yield estimation at the end of the process cycle.



The condition in the medium at the time of juvenile development and emigration of J2ds from the maternal carcass may affect DJ recovery and DJ formation depending on availability of food and nematode density. In order to improve the model, the number of bacterial cells at the time of *endotokia matricida* and its role towards second generation adult development needs further investigation. In multi-well plates, it was observed that mortality of juveniles towards adult development is negligible. But, in liquid culture all the juveniles may not develop into DJs. Therefore, this estimation technique may fit better for EPNs with higher proportion of *endotokia matricida* and heterorhabditids, which the J2Ds completely develop in to DJs in the maternal carcass.

The two main parameters that showed significance contribution towards nematode population growth (bacterial density and incubation temperature) can be improved through selection of media components and optimum incubation temperatures for both the bacteria and nematode growth and development.

## 5. Summary

The entomopathogenic nematodes (EPNs), *Steinernema feltiae*, *S. riobrave*, *S. yirgalemense* and *Heterorhabditis bacteriophora* are symbiotically associated with the bacteria *Xenorhabdus bovienii*, *X. cabanillasii*, *X. indica* and *Photorhabdus luminescens*, respectively. *Steinernema feltiae* and *H. bacteriophora* are widely used in the biocontrol of economically important insect pests throughout the world. *Steinernema riobrave* and *S. yirgalemense* are also playing a role in controlling important insect pests in North America and Africa, respectively and have the potential to be used as a biocontrol agent in other regions, with similar agro ecology and insect pest problems.

Successful and economically reasonable usage of these EPN species in crop protection requires large scale production at low costs. This requirement can only be achieved under well-defined in vitro culture conditions and detailed knowledge on the population dynamics of both the nematodes and their associated symbiotic bacteria. In vitro culture of EPNs is carried out in liquid culture using artificial substrates with the symbiotic bacteria as a food source, which is essential for growth and reproduction. Therefore, investigations at small scale cultures to generate information on the fundamental reproductive parameters are necessary.

In this thesis, the life history traits (LHTs) of the EPNs *S. feltiae*, *S. riobrave*, *S. yirgalemense* and *H. bacteriophora* were investigated at different densities of their respective symbiotic bacteria. An adapted hanging drop method using a semi-solid Nematode Growth Gelrite (NGG) has allowed the assessment of the LHTs of steinernematids and hermaphrodites at defined bacterial densities. Males of *H. bacteriophora* failed to copulate in hanging drops both with amphimictic and automictic females. Drops of solid NGG on the inner side of the lid of multi-well culture plates coated with symbiotic bacteria allow males of *H. bacteriophora* to copulate. The influence of mating on offspring production and survival of *H. bacteriophora* was investigated. The results of the LHT analysis was used as a basis for prediction of *S. feltiae* DJ yield in monoxenic liquid culture. *Steinernema yirgalemense* was cultured at different incubation temperatures and the percentage DJ recovery recorded was compared with the results of hanging drops. Furthermore, the influence of the incubation temperature on the occurrence of *endotokia matricida* and DJ development was assessed.

Offspring production per maternal nematodes was found to be different among EPN species and bacterial densities. It has been observed that food has a significant role on offspring production through changing the body size of females. In addition to bacterial density, mating duration has a positive correlation with the total number of offspring produced by *S. feltiae* females, while mating has no effect on hermaphrodites of *H. bacteriophora*.

At  $10 \times 10^9$  bacterial cells  $\text{ml}^{-1}$  the lowest percentage of offspring produced via *endotokia matricida* was recorded in *S. feltiae* (45%), while for *S. riobrave* it has been 54% and for *H. bacteriophora* 79%. About 95% of the offspring in *S. yirgalemense* originated from *endotokia matricida* at  $10 \times 10^9$  bacterial cells  $\text{ml}^{-1}$ . Second generation females of both *S. feltiae* and *H. bacteriophora* produced generally the majority of their offspring via *endotokia matricida*.

Unlike the total number of offspring production per female, significant differences in generation time (5.18–6.97 days) and average lifespan (6.55–8.1 days) was not observed between the species. Because of higher offspring numbers and lower generation times, the intrinsic growth rate of steinernematids was higher ( $>0.98 \text{ day}^{-1}$ ) compared to *H. bacteriophora* ( $<0.81 \text{ day}^{-1}$ ) at the same bacterial density.

All maternal nematodes of the assessed EPN species produce offspring for 1–2 days from laid eggs and afterwards through *endotokia matricida*. Reproductive females of all the studied species entered into *endotokia matricida* regardless of bacterial density after about one week and terminate their lifespan. In contrast, non-mated females of *S. feltiae* managed to survive almost three weeks, while automictic *H. bacteriophora* always get affected through self-fertilization. Mating frequency had no effect on the survival of both *S. feltiae* females and *H. bacteriophora* hermaphrodites. Unlike previous reports on *Caenorhabditis elegans* and other organisms, lower food supply did not extend the lifespan of the investigated nematode species for both reproductive and non-reproductive females.

It was observed by other authors that egg laying is ceased in *H. bacteriophora* hermaphrodites when they are transferred into a bacteria-free Ringer's solution, which finally results in hatching of the J1 inside the mother and consequently in the onset of *endotokia matricida*. However, the lack of food cannot be the reason for the onset of *endotokia matricida* in hanging drops, where the females or hermaphrodites were transferred every day into a fresh

bacterial suspension. It is not known yet what ceased the egg laying under that condition. It could be the age of the maternal nematodes.

Among the assessed EPN species, females of *S. riobrave* are the most fecund (>1900 offspring per female) followed by *S. feltiae* (813) recorded at the optimum bacterial density. The results of the hanging drop experiment indicated that in addition to species differences, increased bacterial density has a significant positive influence on offspring production. E.g., at  $5 \times 10^9$  bacterial cells  $\text{ml}^{-1}$  of the respective symbiotic bacteria, the number of offspring recorded per female of *S. feltiae*, *S. riobrave* strains and hermaphrodites of *H. bacteriophora* was 359, 680–792 and 50, respectively. With increasing bacterial density from  $5 \times 10^9$  to  $20 \times 10^9$  cells  $\text{ml}^{-1}$ , the studied EPN species offspring production increased by 2–3 folds.

In hanging drops the bacterial density and DJ recovery have also a positive association. The percentage of DJ recovery of *S. yirgalemense* was, however, lower when compared with recovery in liquid culture. The NGG used for hanging drops is relatively nutrient-poor that reduces the metabolism of the bacteria. Therefore, NGG may not be an appropriate method to test DJ recovery of EPNs in liquid culture.

Although the highest percentage of DJ recovery was recorded at 30°C, the DJ yield at 25°C (284,114 DJs  $\text{ml}^{-1}$ ) was more than ten times higher compared with DJ yields recorded at 30°C. This indicates that DJ recovery alone is not sufficient to ensure a high yield in liquid culture.

The beginning of *endotokia matricida* is considered to be the maximum point for maternal nematode body growth. In hanging drops, the body size of females of *S. feltiae*, *S. riobrave* and *H. bacteriophora* hermaphrodites at the beginning of *endotokia matricida* had a significant correlation of 0.89, 0.93 and 0.88, respectively with offspring production. This strong correlation was successfully used for the prediction of the DJ yield of *S. feltiae* liquid culture.

In liquid culture, food is a limiting factor since it is not possible in that batch process to feed in fresh bacteria continuously. In contrast to the hanging drop conditions, which was replaced daily, in liquid culture the nematodes compete for food. As a consequence of competition the number of parental females has a strong negative correlation with offspring

production per female. It could be demonstrated for *S. feltiae* that the total body volume of all females and the number of all females assessed at 3 days post DJ inoculation is a good parameter for the estimation of the DJ yield with an error of about 5% of the observed yield.

## 6. Zusammenfassung

Die entomopathogenen Nematoden (EPNs), *Steinernema feltiae*, *S. riobrave*, *S. yirgalemense* und *Heterorhabditis bacteriophora* sind jeweils symbiotisch assoziiert mit den Bakterien *Xenorhabdus bovienii*, *X. cabanillasii*, *X. indica* und *Photorhabdus luminescens*. *Steinernema feltiae* und *H. bacteriophora* werden in der ganzen Welt vielfältig im biologischen Pflanzenschutz von ökonomisch wichtigen Schadinsekten eingesetzt. *Steinernema riobrave* und *S. yirgalemense* spielen ebenfalls eine Rolle bei der Kontrolle von wichtigen Schadinsekten in Nordamerika, beziehungsweise Afrika und haben das Potential als biologischer Kontrollorganismus auch in anderen Regionen mit ähnlicher agrarökologischen Konditionen.

Der erfolgreiche und ökonomische sinnvolle Einsatz dieser EPN Arten im Pflanzenschutz setzt deren Massenproduktion bei geringen Kosten voraus. Diese Voraussetzung kann nur unter gut definierten *in vitro* Kulturbedingungen und detaillierter Kenntnisse der Populationsdynamiken der Nematoden als auch ihrer assoziierten symbiotischen Bakterien erreicht werden. Die *in vitro* Kultur der EPN wird in Flüssigkulturen durchgeführt unter der Verwendung von künstlichen Substraten mit ihren symbiotischen Bakterien als Nahrungsressource, die für Wachstum und Reproduktion essentiell sind. Aus diesem Grund sind Untersuchungen in klein-volumigen *in vitro* Kulturen bezüglich der zugrunde liegenden Parameter für die Vermehrung notwendig.

In dieser Arbeit wurden der Lebenszyklus (life history traits, LHTs) der EPNs *S. feltiae*, *S. riobrave*, *S. yirgalemense* und *H. bacteriophora* bei unterschiedlichen Dichten ihrer jeweiligen symbiotischen Bakterien untersucht. Eine adaptierte Methode in hängenden Tropfen mit einem semi-flüssigen Gelrite (NGG) erlaubte die Untersuchung von der LHTs von steinernematiden und heterorhabditiden bei definierten Bakteriendichten. Die Männchen von *H. bacteriophora* waren nicht in der Lage in den hängenden Tropfen mit den amphimiktischen und automiktischen Weibchen zu kopulieren. Tropfen aus festem NGG auf der inneren Seite des Deckels der Kulturschale, die mit einer Bakterien-schicht bedeckt wurden, ermöglichten es den Männchen die Kopulation. Der Einfluss der Paarung auf die Nachkommenproduktion und die Langlebigkeit von *H. bacteriophora* wurde untersucht. Die Resultate der LHT Analyse wurden als eine Basis für die Voraussage des DJ (Dauerjuvenile) Ertrages in monoxenischen *S. feltiae* Flüssigkulturen

verwendet. *Steinernema yirgalemense* wurde bei verschiedenen Temperaturen kultiviert und der Prozentsatz der DJ die sich weiterentwickelten verglichen mit den Daten aus den Versuchen in hängenden Tropfen. Des Weiteren wurde der Einfluss der Inkubationstemperatur auf das Einsetzen der *endotokia matricida* und der Entwicklung der DJ wurde untersucht.

Die Nachkommenproduktion der weiblichen Nematoden war unterschiedlich bei den EPN Arten und den Bakteriendichten. Es wurde beobachtet, dass die Nahrung eine signifikante Rolle bei der Nachkommenproduktion und der Größenentwicklung der Weibchen spielt. Zusätzlich zu der Bakteriendichte hatte die Dauer der verfügbaren Paarungszeit eine positive Korrelation mit der Gesamtzahl der Nachkommen von *S. feltiae* Weibchen, während die Kopulation keinen Effekt bei Hermaphroditen von *H. bacteriophora* hatte.

Bei  $10 \times 10^9$  Bakterienzellen  $\text{ml}^{-1}$  entwickelten sich am wenigsten Nachkommen über die *endotokia matricida* bei *S. feltiae* (45%), während es bei *S. riobrave* (54% und bei *H. bacteriophora* (79%) waren. Annähernd 95% der Nachkommen entwickelten sich via *endotokia matricida* bei  $10 \times 10^9$  Bakterienzellen  $\text{ml}^{-1}$ . Weibchen aus der zweiten Generation bei *S. feltiae* und *H. bacteriophora* produzierten generell den Großteil ihrer Nachkommen via *endotokia matricida*.

Im Gegensatz zu der Gesamtzahl der produzierten Nachkommen pro Weibchen konnten keine signifikanten Unterschiede hinsichtlich der Generationszeit (5.18–6.97 Tage) und der durchschnittlichen Lebensspanne (6.55–8.1 Tage) beobachtet werden. Aufgrund höherer Nachkommenszahlen und niedrigeren Generationszeiten war die intrinsische Wachstumsrate bei Steinernematiden höher ( $>0.98 \text{ Tage}^{-1}$ ) im Vergleich zu *H. bacteriophora* ( $<0.81 \text{ Tage}^{-1}$ ).

Sämtliche Weibchen der untersuchten EPN Arten produzierten 1-2 Tage Nachkommen über die Eiablage und anschließend via *endotokia matricida*. Bei den reproduktiven Weibchen aller untersuchten Arten begann die *endotokia matricida* unabhängig von der Bakteriendichte nach rund einer Woche und beendete so ihre Lebensspanne. Im Gegensatz dazu überlebten unbefruchtete Weibchen fast 3 Wochen, während die automiktischen *H. bacteriophora* immer durch die Selbstbefruchtung beeinträchtigt waren. Die Paarungsfrequenz hatte keinen Einfluss auf die Lebensdauer von *S. feltiae* Weibchen und *H. bacteriophora* Hermaphroditen. Im Gegensatz zu Veröffentlichungen zu *Caenorhabditis elegans* und anderen Organismen

verlängert ein geringeres Nahrungsangebot die Lebenspanne der hier untersuchten Nematodenarten bei befruchteten und unbefruchteten Weibchen nicht.

Von anderen Autoren wurde beobachtet, dass die Eiablage bei *H. bacteriophora* Hermaphroditen stoppte nachdem diese in bakterienfreie Ringersche Lösung überführt wurden, was schließlich dazu führte, dass die J1 im Muttertier schlüpften und so die *endotokia matricida* in Gang setzten. Allerdings kann Nahrungsmangel nicht der Grund sein für das Einsetzen der *endotokia matricida* in hängenden Tropfen, wo die Weibchen oder Hermaphroditen täglich in eine frische Bakteriensuspension überführt wurden. Es ist bisher nicht bekannt was die Eiablage unter diesen Bedingungen beendet. Möglicherweise ist es das Alter der Muttertiere.

Zwischen den untersuchten EPN Arten waren die Weibchen von *S. riobrave* die fruchtbarsten (>1900 Nachkommen pro Weibchen), gefolgt von *S. feltiae* (813) aufgezeichnet bei der jeweils optimalen Bakteriendichte. Die Resultate der hängenden Tropfen Methode zeigten, dass zusätzlich zu den Artunterschieden eine ansteigende Bakteriendichte sich positiv auf die Nachkommenproduktion auswirkt. Z. B. war bei  $5 \times 10^9$  Bakterienzellen  $\text{ml}^{-1}$  der jeweiligen symbiontischen Bakterien die Anzahl der Nachkommen pro Weibchen von *S. feltiae*, *S. riobrave* Stämmen und hermaphrodites of *H. bacteriophora* gleich 359, 680–792 beziehungsweise 50. Mit zunehmender Bakteriendichte von  $5 \times 10^9$  zu  $20 \times 10^9$  Zellen  $\text{ml}^{-1}$  stieg die Nachkommenproduktion der untersuchten EPN Arten um das 2–3 fache.

In hängenden Tropfen war die Bakteriendichte ebenfalls positiv assoziiert mit der Weiterentwicklung der DJ. Der Prozentsatz der DJ von *S. yirgalemense* war allerdings niedriger als der, der in Flüssigkulturen beobachtet wurde. Das NGG, welches für die hängenden Tropfen verwendet wurde ist relativ Nährstoffarm, was den Stoffwechsel der Bakterien einschränkt. Deshalb ist NGG wahrscheinlich ungeeignet um die Weiterentwicklung der DJ in Flüssigkultur zu untersuchen.

Obleich der höchste Anteil der DJ Weiterentwicklung bei 30°C beobachtet wurde, war der DJ Ertrag bei 25°C (284.114 DJs  $\text{ml}^{-1}$ ) mehr als 10 mal höher als bei 30°C. Das weist darauf hin, dass die Weiterentwicklung der DJ alleine nicht ausreicht, um einen hohen Ertrag in Flüssigkultur zu gewährleisten.



Der Beginn der *endotokia matricida* wird als der Zeitpunkt angenommen an dem das Körperwachstum der weiblichen Nematoden sein Maximum erreicht hat. In hängenden Tropfen hatte die Körpergröße der Weibchen von *S. feltiae*, *S. riobrave* und *H. bacteriophora* Hermaphroditen zu Beginn der *endotokia matricida* eine signifikante Korrelation von jeweils 0,89, 0,93 und 0,88 mit der Nachkommenproduktion. Diese starke Korrelation konnte erfolgreich genutzt werden, um die den DJ Ertrag von *S. feltiae* Kulturen zu prognostizieren.

In Flüssigkulturen ist die Nahrung der begrenzende Faktor, da es nicht möglich ist bei diesem Batch-Prozess frische Bakterien kontinuierlich nachzuliefern. Im Gegensatz zu den Bedingungen im hängenden Tropfen der täglich ausgetauscht wurde, konkurrieren die Nematoden in den Flüssigkulturen um Nahrung. Als Konsequenz dieser Konkurrenz war die Anzahl der Weibchen stark negativ korreliert mit der Nachkommenzahl. Für *S. feltiae* konnte gezeigt werden, dass das totale Körpervolumen aller Weibchen und die Anzahl der Weibchen 3 Tage nach der DJ Inokulation gute Parameter für die Abschätzung des DJ Ertrages sind, mit einer Abweichung von rund 5% von dem beobachteten Ertrag.

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**Annex 1: Publication 1**

**Life history trait analysis of the entomopathogenic nematode *Steinernema feltiae* provides the basis for prediction of dauer juvenile yields in monoxenic liquid culture**

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# Life history trait analysis of the entomopathogenic nematode *Steinernema feltiae* provides the basis for prediction of dauer juvenile yields in monoxenic liquid culture

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**Abstract** Entomopathogenic nematodes (*Steinernema* spp.) are used in integrated pest management to control insect pests in cryptic environments. The nematodes are mass produced in monoxenic liquid culture with their symbiotic bacteria *Xenorhabdus* spp. For a better understanding of nematode population dynamics, the life history traits (LHTs) of the entomopathogenic nematode *Steinernema feltiae* were assessed at 25 °C by observing single pairs of male and female nematodes using a hanging drop technique. To investigate the influence of different food supplies on nematode reproduction, the LHTs were assessed with a daily supply of  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  cells  $\text{ml}^{-1}$  of the nematode's bacterial symbiont *Xenorhabdus bovienii* in semi-solid nematode growth gelrite (NGG) medium. Increasing bacterial density had a significant positive influence on the average number of offspring produced, which ranged from 359 to 813 per female. The intrinsic rate of natural increase  $r_m$ , which ranges from 1.10 to 1.19  $\text{day}^{-1}$ , was neither influenced by the bacterial density, nor was the mean generation time  $T$  (5.12–5.25 days) and population doubling time (PDT) (0.64–0.59 days). The average lifespan of reproductive females, which ranged from 6.7 to 7.3 days, was positively correlated with bacterial density. A positive correlation between female body volume and

bacterial density was recorded ( $R = 0.67$ ) as well as a significant positive correlation between female body size and offspring production ( $R = 0.89$ ) in hanging drops. Whether these data can be used to predict nematode yields in liquid culture was tested. The total female body volume calculated as the average female body volume  $\times$  total number of parental females per millilitre 3 days after nematode inoculation was positively correlated ( $R = 0.72$ ) with nematode yields. The total female body volume on process day 3 is thus a good indicator for the estimation of nematode yield at the end of the process (12–15 days post dauer juvenile (DJ) inoculation) in both Erlenmeyer flasks and bioreactors. With a mean deviation of 9467 DJs  $\text{ml}^{-1}$ , the error resembles approximately 5 % of the final DJ yields.

**Keywords** Biological control · Dauer juvenile yield · *Endotokia matricida* · Generation time · Hanging drop

## Introduction

The entomopathogenic nematode–bacterium complex *Steinernema feltiae*–*Xenorhabdus bovienii* is one of the most widespread biological control agents used to control a wide range of insect pests (Lacey et al. 2015). A cost-effective commercial production of this nematode species in liquid culture demands maximum dauer juvenile (DJ) yields within a short process time (Ehlers 2001). A better knowledge of life history trait (LHT) data and population dynamics of both the nematode and its associated symbiotic bacterium, *X. bovienii*, can help to improve the efficacy of mass production.

The DJ is a third juvenile stage. It is the only free-living, infective stage of entomopathogenic nematodes (EPNs), which harbour few hundreds to thousands of symbiont bacterial cells in their intestines (Ciche et al. 2008). After invasion through the

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insect's natural openings and in some cases through the cuticle (Dowds and Peters 2002), the DJs respond to chemical cues, called food signals (Aumann and Ehlers 2002), and release their symbionts into the insect's haemocoel through regurgitation or defecation (Ciche et al. 2006; Sugar et al. 2012). The symbionts start multiplying in the insect's haemolymph and the DJs resume feeding. The exit from the developmentally arrested stage is called DJ recovery (Strauch and Ehlers 1998). Depending on the availability of food, the nematodes undergo one or two generations inside their host (Ehlers 2001).

Mass production of EPNs in liquid culture starts with the pre-culture of their symbiotic bacteria 24 h prior to DJ inoculation from monoxenic cultures (Lunau et al. 1993). Except one species, which consists of self-fertile hermaphrodites (Griffin et al. 2001), the remaining 79 described species of *Steinernema* are reproducing amphimictically. Unlike *Heterorhabditis* spp., which failed to mate in liquid culture, *Steinernema* spp. have a helix type of copulation that allows them to reproduce in liquid media (Strauch et al. 1994). After recovery, *Steinernema* spp. develop into fourth-stage juveniles and subsequently into male and female nematodes. Similar to in vivo conditions, females first lay eggs and with depletion of nutrients, they retain the eggs inside their uterus. The juveniles then hatch in the female body consuming its body content, a phenomenon called *endotokia matricida* (Johnigk and Ehlers 1999).

In the past, investigations targeting at optimization of DJ production were performed in monoxenic liquid cultures in flasks or bioreactors (e.g. Johnigk et al. 2004; Hirao et al. 2010). Culture temperature, time of DJ inoculation, DJ inoculum density and bacterial cell density at the time of DJ inoculation have an effect on the DJ yield of *S. feltiae* in liquid culture (Hirao and Ehlers 2009a, b, c; Hirao et al. 2010). It has also been reported that adults of second- and third-generation *S. feltiae* did not contribute towards total DJ yield after 8 days post DJ inoculation, although their number was sometimes higher than the density of the parental female population (Hirao et al. 2010). The presence of higher numbers of non-DJ stages at the end of the culture process creates a problem during downstream processing. The non-DJ stages rapidly die and provide suitable conditions for the growth of contaminants in the formulated nematode product, which is why they need to be removed prior to product formulation (Ehlers 2001). All these studies allowed assessing nematode and bacterial populations as a whole entity but did not allow observation of single parental individuals of nematodes for assessment of LHT.

The hanging drop method using semi-solid nematode growth gelrite (NGG) medium has many advantages over solid and liquid culture media (Muschiol and Traunspurger 2007) and has received positive attention both in biological and ecological studies (Ayub et al. 2013; Gilarte et al. 2015; Muschiol et al. 2009). In order to support further improvement of liquid culture technology of *S. feltiae*, the LHT analysis with single parents was performed. So far, EPNs were not assessed using

the hanging drop technique except a study conducted recently on *Steinernema riobrave* (Addis et al. 2014).

The hanging drop technique allows observation of single nematodes under controlled food supply and helps to define the culture conditions to obtain higher nematode yield. Offspring production and the ultimate DJ yield could be affected by both genetic and environmental factors, such as body size of females and mating behaviour, bacterial inoculum density and DJ recovery, nematode inoculum density, nematode species, and media components and process parameters (Han et al. 1992; Hirao et al. 2009a, b, c; Johnigk et al. 2004; Shapiro-Ilan et al. 2012; Strauch and Ehlers 1998; Strauch et al. 1994). Food is one of the environmental factors that play a significant role in the development and LHTs of organisms (Addis et al. 2014; Klass 1977). The objectives of this study were to investigate the LHT of *S. feltiae* and to understand the reproductive biology and influence of variable *X. bovienii* density on offspring production, growth and lifespan. In addition, it was tested whether results from hanging drop experiments can be used to develop a decision support system to estimate nematode yields in liquid culture at an early stage of the on-going production process.

## Materials and methods

### Monoxenic cultures

*S. feltiae* (strain EN02) and the symbiotic bacteria *X. bovienii*, which was isolated from *S. feltiae* (strain Sylt), were obtained from stock cultures of e-nema GmbH (Schwentinental, Germany). Monoxenic liquid cultures were established according to Lunau et al. (1993) and produced according to Ehlers et al. (1998) in 100 ml nematode liquid medium (NLM), containing (in g ml<sup>-1</sup>) 15 yeast extract, 20 soy flour, 6 lecithin + oil (1:1), 30 vegetable oil, 4 NaCl, 0.35 KCl, 0.3 CaCl<sub>2</sub> and 0.2 MgSO<sub>4</sub> × 7 H<sub>2</sub>O, adjusted to pH 6.7 in 500-ml Erlenmeyer flasks.

*X. bovienii* was propagated in YS broth in 100-ml Erlenmeyer flasks filled with 20 ml medium containing (in g l<sup>-1</sup>) 5 NaCl, 5 yeast extract, 0.5 NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.5 K<sub>2</sub>HPO<sub>4</sub> and 0.2 MgSO<sub>4</sub> × 7 H<sub>2</sub>O and incubated at 25 °C for 24 h. NLM was inoculated with 1 % (v/v) *X. bovienii* culture and propagated at 25 °C for 48 h before introduction of DJs from monoxenic cultures at a density of ~5000 DJs ml<sup>-1</sup>. Two weeks after DJ inoculation, the Erlenmeyer flasks with DJs were stored at 4 °C on a shaker at 70 rpm (rotation Ø 2 cm) to provide inoculum for later nematode cultures.

### Life history trait analysis

The LHTs of *S. feltiae* were studied by adopting the hanging drop method used for bacteria feeding nematodes (Ayub et al.

2013; Muschiol and Traunspurger 2007; Muschiol et al. 2009) and the EPN *S. riobrave* (Addis et al. 2014). The individual nematode development and offspring production of *S. feltiae* were assessed at food concentrations of  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  cells  $\text{ml}^{-1}$  of *X. bovienii*.

### Preparation of *X. bovienii* for solid and semi-solid nematode growth gelrite

Solid NGG and semi-solid NGG were prepared according to Addis et al. (2014). Semi-solid NGG was prepared from autoclaved stock solutions, mixed after cooling to 55 °C. The basis of the medium was (in  $\text{g l}^{-1}$ ) 1 peptone from casein, 3 NaCl and 1.5 gellan gum (Gelrite; SERVA, Heidelberg, Germany). To 1 l of this medium, 1 ml of  $147 \text{ g l}^{-1}$  suspension of  $\text{CaCl}_2 \times 2 \text{ H}_2\text{O}$ , 1 ml of  $246.6 \text{ g l}^{-1}$   $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$  and 25 ml of  $136 \text{ g l}^{-1}$   $\text{KH}_2\text{PO}_4$  buffer at pH of 6 were added. Finally, 1 ml of  $1 \text{ g l}^{-1}$  cholesterol suspended in ethanol (>99 %) was added. The symbiotic bacterium *X. bovienii* was propagated in NLM for 48 h, and the suspension was centrifuged at 3939g for 10 min at 4 °C. The supernatant was discarded, and the cells were washed with sterilized K-medium (containing in  $\text{g l}^{-1}$ : 3.1 NaCl and 2.4 KCl) and centrifuged again. The pellets were then dissolved in semi-solid NGG to obtain bacterial densities of  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  cells  $\text{ml}^{-1}$ . The cell densities were determined by counting the samples in a Thoma chamber (0.01 mm depth, Poly Optic). Semi-solid NGG bacterial suspensions were stored for a maximum of 3 days at 4 °C before use in hanging drops. Solid NGG was prepared as semi-solid NGG, except that the peptone concentration was increased to  $2.5 \text{ g l}^{-1}$  and gelrite to  $3 \text{ g l}^{-1}$  in the basic preparation.

### Life history trait assessment in hanging drops

The LHTs of *S. feltiae* were assessed according to Addis et al. (2014), which was adopted from Muschiol and Traunspurger (2007). Prior to set-up of the hanging drop experiments, 2 ml of *X. bovienii*-semi-solid NGG mixture was inoculated on NGG plates ( $100 \times 15$  mm filled with ~25 ml NGG) at a cell density of  $20 \times 10^9$  cells  $\text{ml}^{-1}$  and incubated overnight at 25 °C. After 24 h of incubation, ~4000 DJs per plate were introduced and incubated at 25 °C. About 36 h post DJ inoculation, single pre-adult female and male pairs were transferred from NGG plates into 10  $\mu\text{l}$  drops ( $\varnothing$  3.7 mm) of the *X. bovienii*-semi-solid NGG mixture kept on the inner side of the lids of multi-well plates with 12 wells and a diameter of 2.2 cm (Greiner 665102; Greiner Bio-One, Germany).

The bottom of each well was covered with ~0.14 g dry cellulose tissue paper that had been wetted with 650  $\mu\text{l}$  of tap water to keep the drops moist. After introduction of the nematodes, the multi-well plates were sealed with Parafilm and kept at 25 °C in airtight boxes. Every 24 h, the nematodes

were transferred into fresh drops and the plates containing old drops were kept for another 24 h for hatching of the offspring. Transfer of parental nematodes into a new drop was continued until the last female had died. When male nematodes died before the female, new males were introduced from NGG plates. The experiment was conducted four times with different batches of nematodes.

### Offspring production

The total offspring production of females was determined based on hatched juveniles. Counting of the offspring produced in each drop was done by adding 10  $\mu\text{l}$  of Rose Bengal solution ( $300 \mu\text{g ml}^{-1}$ , Sigma-Aldrich). The drops were covered with a circular 18-mm-diameter cover slip (Thermo Fisher Scientific) and examined under a dissecting microscope (Zeiss, Germany). Counting was facilitated by placing a  $2 \times 2$ -mm grid underneath the multi-well lid. The percentage of offspring originated from *endotokia matricida* (intra-uterine hatching of J1 and development to DJs) and from laid eggs (extra-uterine development) was determined by identifying and recording the females at the beginning of *endotokia matricida*, while transferring into new drops.

### Calculation of life cycle parameters

According to the results of the assessment made on the development of *S. feltiae* on NGG plates prior to the start of the study in hanging drops (data not shown), the age of *S. feltiae* females at egg laying in hanging drops was 4 days. The different formulae used to calculate the life cycle parameters of *S. feltiae* are described in Table 1. Age-specific survival probability ( $l_x$ ) was calculated for the number of maternal females at day  $x$  per total number of females used in the experiment for a given bacterial density and replication. Age-specific fecundity ( $m_x$ ) is the total number of offspring produced per number of females assessed at day  $x$ . The intrinsic rate of natural increase ( $r_m$ ) iteration was done by changing the values of  $r_m$  until  $\sum_{x=0}^d e^{-r_m x} l_x m_x$  becomes 1 (Addis et al. 2014).

### Correlation of female body size and offspring production

The effect of bacterial density on female body volume and its association with offspring production was studied in a parallel hanging drop experiment with  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  cells  $\text{ml}^{-1}$  of *X. bovienii* at 25 °C. Likewise, every day, the nematodes were transferred into fresh food. Instead of assessment of LHT parameters, the female body length and width were measured at the beginning of *endotokia matricida* (by this time, the females stop laying eggs) using a dissection

**Table 1** Formulae used to calculate the different life cycle parameters of *Steinernema feltiae* and their description. TFR,  $R_0$  and generation time were calculated on the basis of the life table calculated with  $l_x$  and  $m_x$  (data not shown), where  $x$  = time in days,  $l_x$  = age-specific survival probability and  $m_x$  = age-specific fecundity

Formulae	Description of the life cycle parameters
$TFR = \sum m_x$	Total fertility rate (TFR): the total number of offspring that would be produced by the female if they were able to survive until the end of their reproductive period
$R_0 = \sum l_x m_x$	Net reproductive rate ( $R_0$ ): the average number of offspring produced by a female during its lifetime, dependent on the age-specific survival probability and fecundity of the female
$T_0 = (1/R_0) \sum x l_x m_x$	Also known as the cohort generation time ( $T_c$ ): the mean age at reproduction of a cohort of females
$T_1 = (\ln R_0)/r_m$	The period of time necessary for a population growing at a constant rate $r_m$ to increase by the factor $R_0$
$T = \sum_{x=0}^d x e^{-r_m x} l_x m_x$	The mean age of the mother of newly hatched individuals in a population with a stable age distribution
$r_m$	Intrinsic rate of natural increase ( $r_m$ ): the growth rate of a population that has a stable age distribution and grows under an unlimited environment. It was calculated based on the Euler/Lotka equation (Vranken and Heip 1983)
	$\sum_{x=0}^d e^{-r_m x} l_x m_x = 1$
$\ln 2/r_m = PDT$	Population doubling time (PDT)

microscope and an attached camera. The images were analysed using the software analysis® (Soft Imaging Systems GmbH, Münster, Germany). The volume of females was calculated using the formula  $V = (L \times D^2) / 1.7$  (Andrássy 1956) where  $V$  = volume,  $L$  = body length and  $D$  = maximum body diameter. The body volume of a given female and its total number of offspring could not be assessed on the same individual since female individuals had to be heat killed at 65 °C prior to recording of the measurements. Therefore, for assessment of the correlation between female body volume and offspring production, the mean body volume of all 257 females assessed in one of the respective densities (~20 females per replication per bacterial density) was correlated with the mean number of offspring obtained from the LHT studies in hanging drops at an equal bacterial density.

The association between female body volume and DJ yield in monoxenic liquid cultures was estimated. The symbiotic bacteria were cultured for 48 h in yeast-salt (YS) medium in 500-ml Erlenmeyer flask at 180 rpm and 25 °C and inoculated at 1 % (v/v) into NLM and again cultured for 48 h at the same temperature and shaker speed before inoculation of ~5000 DJs ml<sup>-1</sup>. The experiment was repeated four times with three different batches of nematodes. The numbers of parental females were counted at 72 h post DJ inoculation. A total of 36 Erlenmeyer flasks were assessed. The body volume of 958 females and 2448 DJs was measured using the Andrassy formula (Andrássy 1956). The body size of females was measured at the beginning of *endotokia matricida* (~72 h post DJ inoculation) and DJ yield and DJ volume at the time of harvest (15 days post DJ inoculation). In addition, for the purpose of further comparison, similar data were collected from two bio-reactor runs filled with 20,000 l nematode liquid medium.

## Statistical analysis

The influence of bacterial density on the LHTs of *S. feltiae* was analysed using analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test at  $P \leq 0.05$ . The relationship of bacterial density with female body volume, total number of offspring per female, intra-uterine-originated offspring per female and different LHT parameters was assessed using the Pearson correlation test at  $P \leq 0.05$ . Similarly, the correlation of female body volume with offspring production in hanging drops and with DJ yield per female and total DJ yield per millilitre in liquid culture and the female number with DJ yield per female and total DJ yield per millilitre in liquid culture was performed using the Pearson correlation test at  $P \leq 0.05$ . Prior to ANOVA, the percentage data were arcsine transformed.

## Results

### Offspring production

A significantly positive correlation was recorded between bacterial density and offspring production per female of *S. feltiae* ( $R = 0.53$ ;  $P < 0.0001$ ) (Fig. 1a). The lowest average offspring production per female (359) was recorded at  $5 \times 10^9$  and the highest (813) at  $20 \times 10^9$  cells ml<sup>-1</sup> of *X. bovienii*. The contribution of offspring production per female resulting from extra- or intra-uterine development was assessed at different bacterial densities. The percentage of offspring originating from intra-uterine development (*endotokia matricida*) was negatively correlated with bacterial density ( $R = -0.18$ ;

$P < 0.0001$ ) (Fig. 1b). The highest percentage of offspring (53 %) originating from *endotokia matricida* was observed at  $5 \times 10^9$  cells ml<sup>-1</sup> and decreased towards 39 % at the highest bacterial density (Fig. 1b).

### Population growth parameters

Offspring production increased with increasing bacterial density (Fig. 1a), and the total fertility rate (TFR,  $R = 0.85$ ;  $P = 0.0004$ ) and net reproductive rate ( $R_0$ ,  $R = 0.82$ ;  $P = 0.001$ ) were positively correlated with the bacterial density (Table 2). Significant correlations between bacterial density and the different mean generation times ( $T_0$ ,  $T_1$  and  $T$ ), population doubling time (PDT) and the intrinsic rate of natural increase ( $r_m$ ) were not observed. All other LHT parameters did not differ significantly between the different bacterial densities. At the lowest bacterial density, the lowest cohort generation time  $T_0 = 5.6$  was recorded, as well as the lowest  $T_1 = 5.4$  days (period of time necessary for a population growing at a constant rate  $r_m$  to increase by the factor  $R_0$ ) and lowest  $T = 5.1$  days (mean age of the mother of newly hatched individuals in a population with a stable age distribution). The highest  $T_0 = 5.9$ ,  $T_1 = 5.6$ ,  $T = 5.3$  days and intrinsic rate of natural increase  $r_m = 1.2$  day<sup>-1</sup> were recorded at the highest bacterial density. At the lowest bacterial density, the lowest  $r_m = 1.1$  day<sup>-1</sup> was recorded. The PDT slightly decreased with increasing bacterial density; however, there was no significant difference recorded (Table 2).

The average lifespan of female nematodes ranged from 6.7 to 7.3 days starting from the J1 hatching from the egg and was positively correlated with the bacterial density ( $R = 0.27$ ;  $P < 0.0001$ ) (Table 2). With the beginning of the *endotokia matricida*, the time until death of the female is defined since hatched J1s inside the uterus start destroying the internal organs of the mother.

### Correlation between female body size and offspring production

In hanging drops, the female body volume was positively correlated with the bacterial density ( $R = 0.67$ ;  $P < 0.0001$ ) (Fig. 2). The body volume of females ranged from 0.040 to 0.045, 0.045 to 0.064 and 0.077 to 0.093 mm<sup>3</sup> at  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  cells ml<sup>-1</sup>, respectively, resulting in a mean body volume of 0.043, 0.058 and 0.085 mm<sup>3</sup>, respectively (Fig. 3). The correlation between mean female body volume at the beginning of *endotokia matricida* and the mean total number of offspring per female was significantly positive ( $R = 0.89$ ;  $P < 0.00001$ ) (Fig. 3).

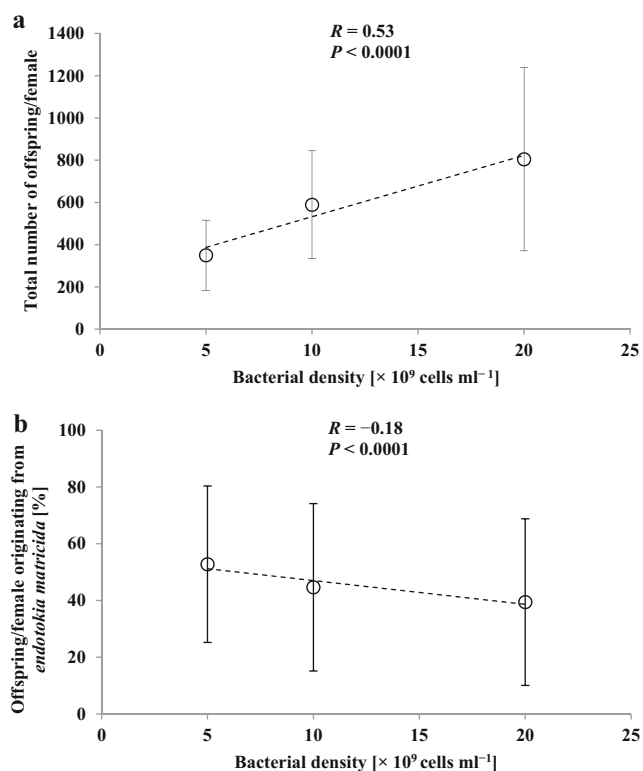
In liquid culture in Erlenmeyer flasks, a negative correlation was recorded between the number of parental females recorded 3 days post DJ inoculation and the body volume of parental females measured at the same time ( $R = -0.33$ ;  $P = 0.048$ ) (Fig. 4). Due to limited food availability in liquid (batch culture) compared to hanging drops (adults transferred to fresh food daily), the body volume of female nematodes was lower, ranging from 0.021 to 0.033 mm<sup>3</sup> (Fig. 5). The

**Table 2** Correlation between bacterial density and mean values of the different life cycle parameters of *Steinernema feltiae* assessed at  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  cells ml<sup>-1</sup> of *Xenorhabdus bovienii* in hanging drops at 25 °C

	Bacterial density (cells ml <sup>-1</sup> )			Correlation coefficient	P value
	$5 \times 10^9$	$10 \times 10^9$	$20 \times 10^9$		
<i>N</i>	144	151	188		
TFR	493 ± 125 <sup>a</sup>	761 ± 95 <sup>ab</sup>	1032 ± 196 <sup>b</sup>	0.85	0.0004
$R_0$	359 ± 112 <sup>a</sup>	589 ± 87 <sup>ab</sup>	813 ± 197 <sup>b</sup>	0.82	0.001
$T_0$ (days)	5.61 ± 0.21 <sup>a</sup>	5.78 ± 0.20 <sup>a</sup>	5.89 ± 0.24 <sup>a</sup>	0.45	0.14
$T_1$ (days)	5.35 ± 0.21 <sup>a</sup>	5.50 ± 0.19 <sup>a</sup>	5.56 ± 0.23 <sup>a</sup>	0.40	0.203
$T$ (days)	5.12 ± 0.18 <sup>a</sup>	5.24 ± 0.15 <sup>a</sup>	5.25 ± 0.17 <sup>a</sup>	0.31	0.33
$r_m$ (day <sup>-1</sup> )	1.10 ± 0.09 <sup>a</sup>	1.16 ± 0.06 <sup>a</sup>	1.19 ± 0.08 <sup>a</sup>	0.45	0.14
PDT (days)	0.64 ± 0.05 <sup>a</sup>	0.60 ± 0.04 <sup>a</sup>	0.59 ± 0.04 <sup>a</sup>	-0.46	0.134
Average lifespan (days)	6.7 ± 0.3 <sup>a</sup>	7 ± 0.3 <sup>a</sup>	7.3 ± 0.4 <sup>a</sup>	0.27	<0.0001

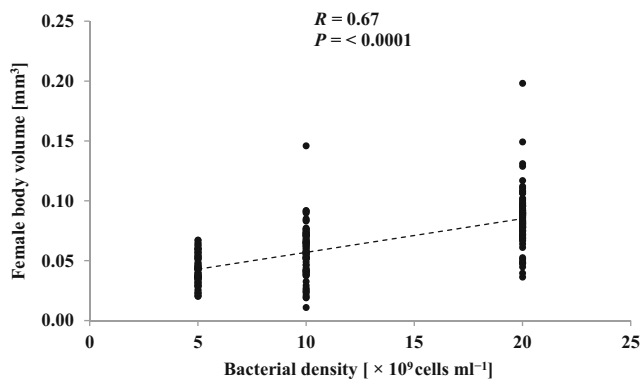
Values represent means of four replicates ± SD. Different superscript letters indicate significant differences between densities within rows (post hoc Tukey's HSD test,  $P \leq 0.05$ ). For the correlation between offspring production and bacterial density, individual values obtained from each female (*N*) were used whereas for  $R_0$  values in each replication obtained from the fecundity schedule of all the assessed females for a given bacterial density was applied for ANOVA. The correlation was analysed by the Pearson correlation test

*N* number of females observed, *TFR* total fertility rate,  $R_0$  net reproductive rate,  $T_0$  mean age at reproduction of a cohort of females,  $T_1$  period of time necessary for a population growing at a constant rate  $r_m$  to increase by the factor  $R_0$ ,  $T$  mean age of the mothers of a set of newly hatched individuals in a population with a stable age distribution,  $r_m$  intrinsic rate of natural increase, *PDT* population doubling time

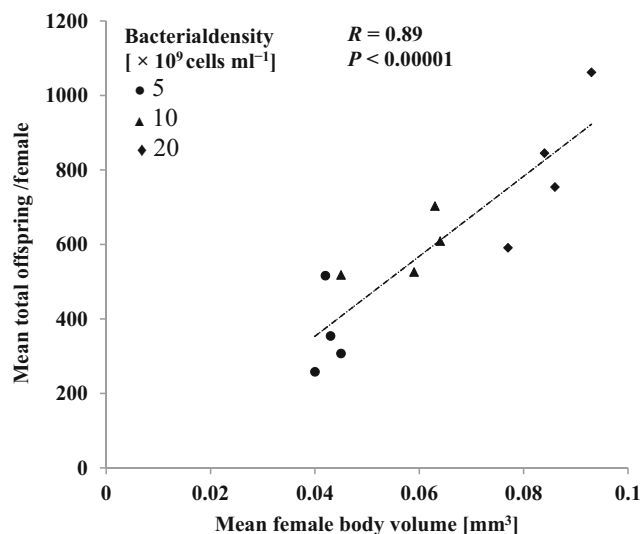


**Fig. 1** Correlation of *Xenorhabdus bovienii* cell density with total number of offspring per female (a) and percentage of offspring per female originating from *endotokia matricida* (intra-uterine development) (b) of *Steinernema feltiae* assessed in hanging drops at 25 °C. The calculation of correlation was carried out with all investigated females ( $n = 483$ ). Open circles are mean values of four replicates and bars represent the standard deviation

correlation between mean female body volume and DJ yield per female was significant ( $R = 0.42$ ;  $P = 0.011$ ) (Fig. 5). However, the relationship was not strong like that recorded in hanging drops. The influence of the mean female body volume towards total DJ yield per millilitre at the end of the process cycle was not significant ( $R = 0.11$ ;  $P = 0.54$ ) (Fig. 5).



**Fig. 2** Correlation between *X. bovienii* cell density and body volume of *S. feltiae* females assessed at the beginning of *endotokia matricida* in hanging drops at 25 °C ( $n = 257$  females)



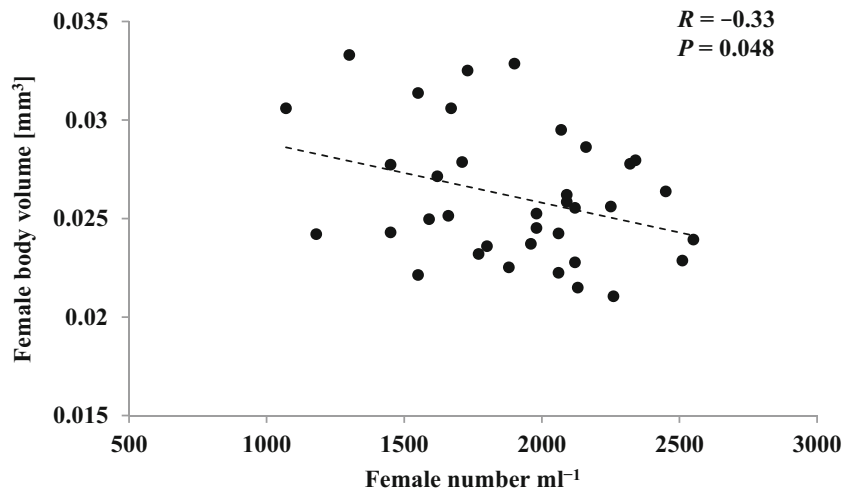
**Fig. 3** Correlation between mean female body volume measured at the beginning of *endotokia matricida* and mean total offspring per female of *S. feltiae* assessed in hanging drops at  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  cells  $\text{ml}^{-1}$  of *X. bovienii* at 25 °C. Data points represent the mean body volume obtained from at least 20 females and mean total offspring per female (minimum 21 and maximum 60 females) from a parallel LHT experiment assessed at the same bacterial density

In Erlenmeyer flasks, the number of parental females ranged from 1070 to 2550  $\text{ml}^{-1}$  and 400 to 530  $\text{ml}^{-1}$  in the two bioreactor processes (Table 3). The correlation between the number of parental females at the beginning of *endotokia matricida* and the total DJ yield per millilitre at 15 days post DJ inoculation was significant ( $R = 0.5$ ;  $P = 0.002$ ) (Fig. 6). A significant negative correlation was recorded for the number of parental females and the DJ yield per female ( $R = -0.9$ ;  $P < 0.00001$ ) (Fig. 6).

The number of parental nematodes in bioreactor runs (400–530  $\text{ml}^{-1}$ ) was on average four times lower than the number of parental females recorded in Erlenmeyer flasks (1899  $\text{ml}^{-1}$ ). In flasks, the average number of DJs obtained per female was 96, which was three times less when compared with the mean number of DJs per female (302) recorded in bioreactors. The body volume of DJs obtained from bioreactors was in the mean range of DJ volume recorded from flasks. In flasks, the DJ yields ranged from 153,184 to 209,679 DJs  $\text{ml}^{-1}$ , while the DJ yield recorded in bioreactors was relatively lower (135,000–140,333 DJs  $\text{ml}^{-1}$ ) (Table 3 and Fig. 7). The total female body volume obtained by multiplying the average female body volume by the mean number of parental nematodes per millilitre recorded at 3 days post DJ inoculation was found significantly associated with the total DJ yield per millilitre ( $R = 0.72$ ;  $P < 0.0001$ ) (Fig. 7). From the results obtained from female body volume and the number of parental numbers, it was possible to predict the final DJ yield with a mean deviation of 9467 and a maximum deviation of 23,686 DJs  $\text{ml}^{-1}$  for both Erlenmeyer flasks and bioreactors (Fig. 7).



**Fig. 4** Correlation (line) between parental female number per millilitre and average female body volume of at least 20 females per Erlenmeyer flask (black circles) assessed at 3 days post DJ inoculation in monoxenic liquid culture in Erlenmeyer flasks ( $n = 36$ ) at 25 °C

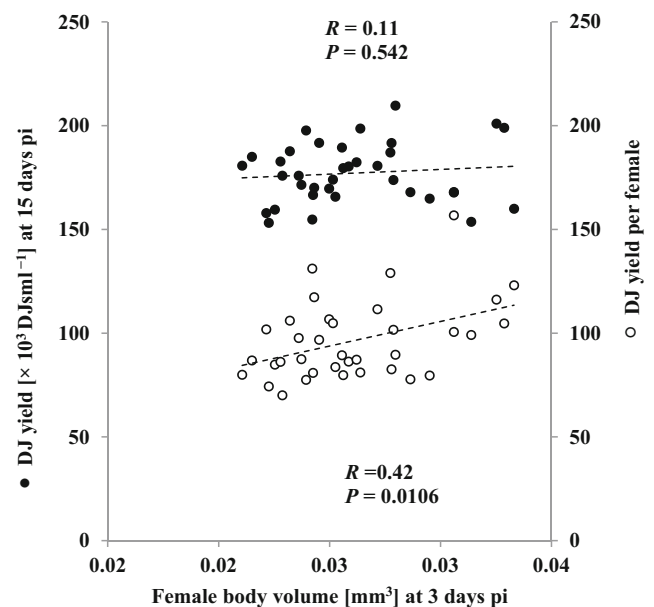


## Discussion

In addition to the genetic background, food and temperature are the two important factors that affect the population growth rate of organisms (Fenchel 1974). The LHT analysis revealed a strong correlation between availability of food, namely bacterial density, and the number of offspring a female nematode produces. A strong positive correlation between bacterial density and TFR and net reproductive rate ( $R_0$ ) of *S. feltiae* was recorded. An increase in *X. bovienii* from  $5$  to  $20 \times 10^9$  cells  $\text{ml}^{-1}$  increased offspring production in *S. feltiae* females by 56 %. The same has been reported for other nematode species (Hirao et al. 2010; Johnigk and Ehlers 1999; Klass 1977; So et al. 2011). At a similar increase of bacterial density, *S. riobrave* offspring production increased by >60 % (Addis et al. 2014). Schiemer (1982) reported a 91 % increase in the number of eggs produced by *Caenorhabditis briggsae* females when the density of *Escherichia coli* increased from  $2 \times 10^8$  to  $5 \times 10^{10}$  cells  $\text{ml}^{-1}$ . The results of the LHT analysis of *S. feltiae* also indicate an increase in body volume of the females with increasing bacterial density and a strong correlation of female body volume with offspring production per female ( $R = 0.89$ ). This indicates that the nematodes can adapt to variable food supply by alteration of the female body volume, which is correlated with offspring production.

Another way of adaptation to environmental conditions and/or food supply is the phenomenon of *endotokia matricida*. Johnigk and Ehlers (1999) described in detail the intra-uterine hatching of the first-stage juveniles and subsequent development to DJs in *Heterorhabditis* spp. During this process, the female body content is consumed by the offspring and as a consequence, the mother dies. Johnigk and Ehlers (1999) assumed that *endotokia matricida* is an adaptation to potential collapse of the food supply in a dead insect due to growth of contaminating microorganisms or feeding of scavengers on the insect cadaver. Thus, the

*endotokia matricida* secures the development of DJs with enough fat reserves allowing them to survive longer under unfavourable environmental conditions. Chen and Caswell-Chen (2004) also indicated that under extreme starvation, intra-uterine-originated offspring of *Caenorhabditis elegans* managed to develop into DJs, while extra-uterine-originated offspring were unable to develop into DJs under similar conditions. Therefore, information on offspring numbers resulting from extra- or intra-uterine development is important for mass production in liquid culture. Offspring outside of the female can be exposed to reduced bacterial concentrations, resulting in DJs with less fat reserves than those which have developed inside the female by *endotokia matricida*.



**Fig. 5** Correlation of female body volume of *S. feltiae* measured at 3 days post DJ inoculation (pi) with DJ yield per millilitre (filled circles) and DJ yield per female (open circles) assessed at 15 days pi in monoxenic liquid culture in Erlenmeyer flasks at 25 °C ( $n = 36$ )

**Table 3** Number of parental females and female body volume at 72 h post DJ inoculation and DJ yield per millilitre, DJ yield per female and DJ body volume at 15 days post DJ inoculation of *Steinernema feltiae*

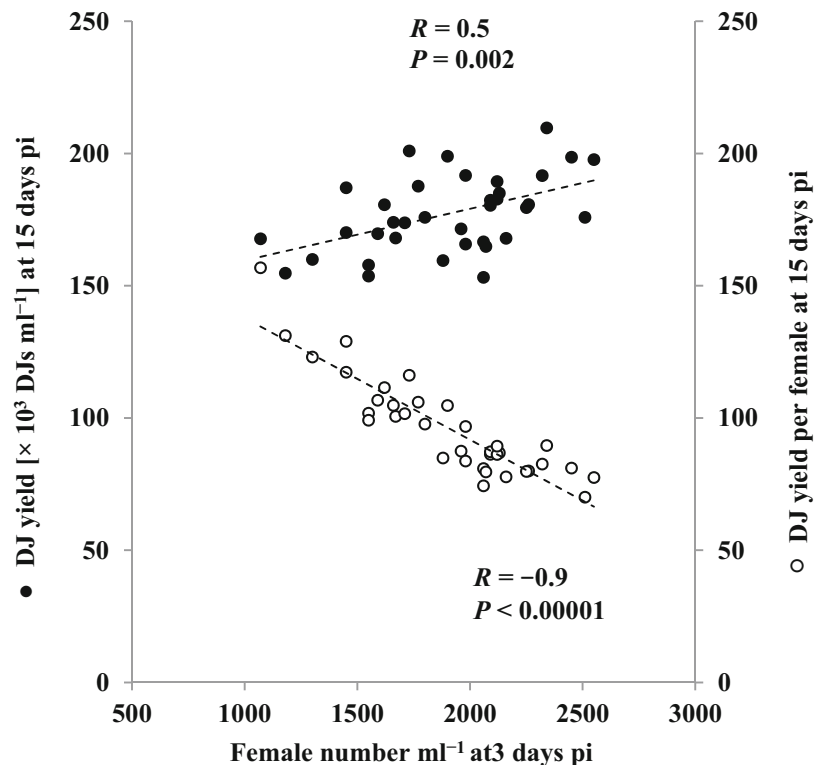
Type of culture	Number	Number of parental females per millilitre	Female body volume (mm <sup>3</sup> )	DJ yield per millilitre	DJ yield per female	DJ body volume (mm <sup>3</sup> )
Erlenmeyer flasks	36	1899 (1070–2550)	0.026 (0.021–0.033)	177,109 (153,184–209,679)	96 (70–157)	0.0003 (0.00025–0.0004)
Bioreactors	2	465 (400–530)	0.033 (0.032–0.033)	137,666 (135,000–140,333)	302 (265–338)	0.0003 (0.0002–0.0003)

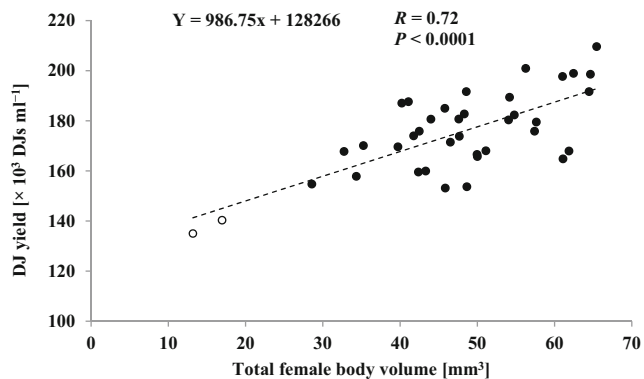
Individual data obtained from all 483 females indicate a weak but significantly negative correlation between bacterial density and intra-uterine offspring production in *S. feltiae*. The highest number of offspring from *endotokia matricida* (53 %) was recorded at the lowest bacterial density, indicating that the egg laying (extra-uterine development of offspring) is continuing with an abundant food supply. Similar results were recorded by Johnigk and Ehlers (1999) for *Heterorhabditis* spp. A relatively higher percentage of offspring from *endotokia matricida* was also recorded in *S. riobrave* reaching 66 % at lower bacterial density (Addis et al. 2014). However, unlike pre-dauer juveniles (J2ds) of *Heterorhabditis* spp., which are motionless and fully develop into DJs in the maternal carcass (Johnigk and Ehlers 1999), *S. feltiae* J2ds were active and emigrated out of the female body into the surrounding environment before developing into DJs. Similarly, J2ds of *Steinernema carpocapsae* (Hirao et al. 2010), *Steinernema glaseri* (Kaya and Stock 1997) and *S. riobrave* (Addis et al. 2014) did not fully develop into DJs in the maternal carcass. As J2ds are non-feeding already, like the

DJs, alimentionation outside the female body is not of concern anymore. However, harvest should be timed until DJs have readily developed, because J2ds are still vulnerable to stress conditions during downstream processing and storage.

A number of studies report that dietary restriction increases the longevity of *C. elegans* hermaphrodites (Klass 1977; Tain et al. 2008). The hypothesis that restricted alimentionation can prolong lifetime can be abandoned taking into consideration results obtained with *S. feltiae*. Investigations of the different bacterial densities at  $0.01 \times$ ,  $0.1 \times$ ,  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  were tested, and higher bacterial densities resulted in an increase of the average lifespan of females to 20 days (Addis, unpublished data). The optimum bacterial density for *S. feltiae* might be higher than  $20 \times 10^9$  cells ml<sup>-1</sup>; however, experimental conditions at higher bacterial densities were not possible. The average lifespan of *S. feltiae* females increased from 6.7 to 7.3 days when bacterial density increased from 5 to  $20 \times 10^9$  cells ml<sup>-1</sup> with a significant but weak correlation ( $R = 0.27$ ). However, once *endotokia matricida* starts, the

**Fig. 6** Correlation of parental female number per millilitre counted at 3 days post DJ inoculation (pi) with DJ yield per millilitre (filled circles) and DJ yield per female (open circles) of *S. feltiae* assessed at 15 days pi in monoxenic liquid culture in Erlenmeyer flasks at 25 °C ( $n = 36$ )





**Fig. 7** Correlation between total female body volume (average female body volume  $\times$  total number of female number assessed at 3 days post DJ inoculation) and DJ yield per millilitre of *S. feltiae* at 15 days post DJ inoculation in monoxenic liquid culture in Erlenmeyer flasks (filled circles) and bioreactors (open circles) at 25 °C

death of the females is observed at the latest 4 days. Lifespan of *S. riobrave* females also ended due to *endotokia matricida* regardless of bacterial density (Addis et al. 2014). Thus, prolonged lifespan of females due to favourable nutritional conditions can only be observed in the absence of males. Hermaphrodites of *Heterorhabditis bacteriophora* enter into *endotokia matricida* even in the presence of enough food (Ciche et al. 2008), which indicates that *endotokia matricida* is part of the LHTs of EPNs.

*S. feltiae* has a short mean generation time and produced a higher number of offspring within a short period of time, resulting in high  $r_m$  values when compared to those free-living nematodes with longer generation time and lower number of offspring. The mean generation time ( $T$ ) recorded for *S. feltiae* ranged from 5.12 to 5.25 days, which is lower than the mean generation time of *Panagrolaimus* sp., *Poikilolaimus* sp. (Muschiol and Traunspurger 2007) and *Panagrolaimus* sp. strain NFS 24-5 (Ayub et al. 2013), but higher than the mean generation time values of *C. elegans* (Muschiol et al. 2009). Depending on the bacterial density, the population of *S. feltiae* increased at a rate of 1.10–1.19  $\text{day}^{-1}$ , which is similar compared to *S. riobrave* with  $r_m$  values of 1.13–1.44  $\text{day}^{-1}$  (Addis et al. 2014). Due to the higher  $R_0$ , the  $r_m$  recorded for *S. feltiae* and *S. riobrave* is quite high when compared to those for *Panagrolaimus* sp., *Poikilolaimus* sp. and *Panagrolaimus* sp. strain NFS 24-5 with  $r_m$  values of 0.309, 0.165 and 0.81  $\text{day}^{-1}$ , respectively (Ayub et al. 2013; Muschiol and Traunspurger 2007). On the other hand the  $r_m$  of *S. feltiae* was lower than that reported from *C. elegans*, which has a lower generation time although with lower  $R_0$  of 291 and 289 for the strains N2 and MY6, respectively (Muschiol et al. 2009). In addition, the population growth rate of *S. feltiae* was compared to that of *Pristionchus pacificus* and was found to be similar with a net reproductive rate  $r_m$  of 1.125  $\text{day}^{-1}$  (Gilarte et al. 2015). As observed in free-living nematodes, preference to different bacterial densities may present between different EPN species. Increasing the cell density of *E. coli* from  $5 \times 10^8$  to  $10^{10}$

has increased the population growth rate of *Panagrolaimus* sp. to 0.262  $\text{day}^{-1}$  while *Poikilolaimus* sp. performed best at  $5 \times 10^8$  (Schroeder et al. 2010). In the free-living nematode *Panagrolaimus* sp. strain NFS 24-5, the optimum culture temperature increased the  $r_m$  values from 0.53 to 0.93  $\text{day}^{-1}$  (Ayub et al. 2013). Therefore, in addition to bacterial density, incubation temperature needs attention in mass production of EPNs.

Adaptation to nutritional conditions has also been observed in liquid culture. In bioreactors, females produced a higher number of DJs per female when compared with results from Erlenmeyer flasks, which was due to a lower number of females per millilitre. The highest DJ yield per female (302 DJs) was obtained from the lowest number of parental females in bioreactors (average of 465 females  $\text{ml}^{-1}$ ). It decreased with increasing number of parental females and reached a minimum (86 DJs per female) at the highest number of parental females (2202 females  $\text{ml}^{-1}$ ) recorded in Erlenmeyer flasks. The average number of offspring produced per female at  $5 \times 10^9$  cells  $\text{ml}^{-1}$  in hanging drops (359 offspring per female) is much closer to the DJ yield per female (338 DJs  $\text{ml}^{-1}$ ) recorded in one of the bioreactors with the lowest parental females. The females in bioreactors had a relatively larger body volume (0.033  $\text{mm}^3$ ) than those in Erlenmeyer flasks (0.026  $\text{mm}^3$ ) but a similar volume of DJs (0.0003  $\text{mm}^3$ ), and this has contributed to the higher number of DJs per female recorded in bioreactors.

In hanging drops, a significantly positive correlation between female body size and offspring production was recorded as a result of bacterial density, which showed a strong correlation with female body volume ( $R = 0.67$ ). In liquid culture due to food limitation, a strong negative correlation between female number and DJ yield per female ( $R = -0.9$ ) was observed, and the contribution of female body volume towards DJ yield was reduced ( $R = 0.42$ ). However, the correlation of the total female body volume in a culture obtained by multiplying the average female body volume with the total number of parental females per millilitre was high ( $R = 0.72$ ) and can thus be used as an indicator to estimate the final DJ yield of a culture both in Erlenmeyer flasks and bioreactors.

In conclusion, assessment of the life history traits in the hanging drop technique provides important baseline information that helps to better understand and improve the liquid culture production process. Results have been successfully extrapolated for the estimation of DJ yields at the end of the culture process.

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**Compliance with ethical standards** This article does not contain any studies with human participants or vertebrate animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no competing interests.

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**Annex 2: Publication 2**

**Life history trait analysis of the entomopathogenic nematode *Steinernema riobrave***

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# Life history trait analysis of the entomopathogenic nematode *Steinernema riobrave*

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**Summary** – Life history traits (LHT) of *Steinernema riobrave* strain Sr 7-12 and Sr HYB19 were assessed at 25°C in monoxenic culture using a hanging drop technique. The LHT were studied with  $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells ml<sup>-1</sup> of *Xenorhabdus cabanillasii* in semi-solid Nematode Growth Gelrite. Increased *X. cabanillasii* densities had a significant positive influence on offspring production on both Sr 7-12 and the hybrid Sr HYB19. At the higher bacterial food density, the total fertility rate (TFR) per female of Sr 7-12 was 2022 offspring and the net reproductive rate ( $R_0$ ) reached 1904 offspring. Similarly, for Sr HYB19, the TFR per female was 2434 and  $R_0$  was 1903. The percentage offspring produced *via* intra-uterine development (*endotokia matricida*) was relatively higher at  $5 \times 10^9$  bacterial cells ml<sup>-1</sup> than at higher bacterial density, with 64% and 66% of the total offspring produced by Sr 7-12 and Sr HYB19, respectively. A positive correlation ( $R = 0.648$ ) was recorded for offspring production and bacterial food density and for female body volume with bacterial density ( $R = 0.610$ ). Female nematodes survived longer at higher bacterial food density; however, females of both strains could not survive longer than 7.8 days, beginning from hatching and always ending 1 day after initiation of *endotokia matricida*. Based on LHT results, there is a potential to maximise yield of dauer juveniles in liquid culture through increasing bacterial food density.

**Keywords** – bacterial density, *endotokia matricida*, life span, offspring,  $R_0$ , TFR.

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae are important biocontrol agents of insect pests. Together with their symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, respectively, they are lethal pathogens used for the management of many important insect pests (Grewal *et al.*, 2005).

*Steinernema riobrave* Cabanillas, Poinar & Raulston was recovered from corn earworms *Helicoverpa zea* in the Lower Rio Grande Valley, Texas, USA (Cabanillas *et al.*, 1994). It belongs to the ‘*bicornutum*-group’ which includes *S. abbasi*, *S. bicornutum*, *S. ceratophorum*, *S. pakistanense* and *S. yirgalemense* (Nguyen *et al.*, 2007). This nematode has potential for control of the corn earworm, *H. zea* (Cabanillas & Raulston, 1995, 1996), the citrus root weevil, *Diaprepes abbreviatus* (Shapiro-Ilan & McCoy, 2000a, b; Stuart *et al.*, 2004), the plum curculio, *Conotrachelus nenuphar* (Shapiro-Ilan *et al.*, 2002), the cotton boll weevil, *Anthonomus grandis*

(Cabanillas, 2003) and different species of subterranean termites (Yu *et al.*, 2010). Moreover, it is able to withstand high temperature, enabling its use in hot climates (Grewal *et al.*, 1994; Yu *et al.*, 2006). Thus, *S. riobrave* has a promising potential as a biocontrol agent for different insect pests, and improvement of commercial production technology could help to make it available at competitive costs.

Understanding the symbiosis between EPN and their associated bacteria was the first step in the utilisation of EPN as commercial biocontrol agents (Poinar & Grewal, 2012). Success in liquid culture depends on the ability to accurately manage nematode population dynamics (Ehlers, 2001; Hirao *et al.*, 2010). Many nematode species such as *Heterorhabditis bacteriophora*, *S. carpocapsae* or *S. feltiae* have been effectively produced in 7500–80 000 l bioreactors at yields as high as 250 000 dauer juveniles (DJ) ml<sup>-1</sup>, depending on the nematode species (Ehlers *et*

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*al.*, 1998; Strauch & Ehlers, 1998). A cost-effective EPN production process in liquid culture demands maximum DJ yield within a short process time. The success depends on synchronous DJ recovery, female fertility and high proportion of DJ formation (Ehlers, 2001). In order to understand the complex interactions between the EPN and their symbiotic bacteria during commercial production, it is fundamental to study the reproductive biology of the helminthic-bacterium complex. In the past, this was done by observing the development of complete populations in liquid culture (*e.g.*, Johnigk & Ehlers, 1999a; Hirao *et al.*, 2010). However, precise data on the life history traits (LHT) of EPN species are still lacking.

Muschiol & Traunspurger (2007) developed a hanging drop method using semi-solid Nematode Growth Gelrite (NGG), which allows the observation of single individuals at similar age with reliable accuracy (Muschiol *et al.*, 2009). The technique can be used to assess LHT, providing detailed information on, for example, nematode age at sexual maturity, lifespan, net reproductive rate, total fertility rate, generation time, intrinsic rate of natural increase, population doubling time and somatic growth rate. The objective of this study was to use the hanging drop method to study the fundamental reproductive biology and LHT of strain Sr 7-12 and a hybrid strain of *S. riobrave*, and to assess the influence of variable bacterial food density on offspring production, growth and life span of these strains.

## Materials and methods

### NEMATODES

Strains of the EPN *S. riobrave* (Sr 3-2, Sr 3-7, Sr 3-8a, Sr 3-8b, Sr 7-12, Sr 8-14, Sr NF and Sr TP) were obtained from Dr David I. Shapiro Ilan (USDA-ARS, SAA, SE Fruit and Tree Nut Research Unit, Byron, GA, USA) and strains Sr BTW and Sr LIN4 were delivered by Dr Larry Duncan (University of Florida, Gainesville, FL, USA). *Steinernema riobrave* designated as Sr HYB19 was developed through round-robin mating of all ten strains according to Mukuka *et al.* (2010). Strain Sr 7-12 was selected for the study because it had the highest reproductive potential of all strains with an average of 260 125 DJ in *Galleria mellonella* and the second lowest average lethal concentration (LC<sub>50</sub>) of 10 DJ in *Tenebrio molitor* (unpubl. data).

### MONOXENIC CULTURES

The symbiotic bacterium *X. cabanillasii* (Cabanillas *et al.*, 1994) was isolated from larvae of *G. mellonella* infected with *S. riobrave* (strain Sr 7-12) according to Ehlers *et al.* (1990). *Xenorhabdus cabanillasii* was inoculated into nematode liquid medium (NLM) (containing 15 g l<sup>-1</sup> yeast extract, 20 g l<sup>-1</sup> soy flour, 6 g l<sup>-1</sup> lecithin + oil (1:1), 30 g l<sup>-1</sup> vegetable oil, 4 g l<sup>-1</sup> NaCl, 0.35 g l<sup>-1</sup> KCl, 0.3 g l<sup>-1</sup> CaCl<sub>2</sub>, 0.2 g l<sup>-1</sup> MgSO<sub>4</sub> · 7 H<sub>2</sub>O and adjusted to pH 6.7) in 500-ml Erlenmeyer flasks filled with 100 ml medium. The flasks were then incubated at 180 rpm (rotor diam. 4 cm) and 25°C for 2 days. Monoxenic cultures were produced according to Ehlers *et al.* (1998) in NLM. Nematode liquid medium was inoculated with *X. cabanillasii* and propagated at 25°C for 48 h before introduction of DJ from monoxenic cultures at a density of 5000 DJ (ml medium)<sup>-1</sup>. After 15 days, cultures were stored at 4°C on a shaker at 70 rpm (rotor diam. 2 cm) before use in LHT experiments.

### LIFE HISTORY TRAIT ANALYSIS

Life history traits of both Sr 7-12 and Sr HYB19 were studied by adopting the hanging drop method used by Muschiol & Traunspurger (2007), and nematode development and offspring production were assessed at 5 × 10<sup>9</sup>, 10 × 10<sup>9</sup> and 20 × 10<sup>9</sup> *X. cabanillasii* cells ml<sup>-1</sup>.

#### *Preparation of X. cabanillasii in solid and semi-solid Nematode Growth Gelrite*

Solid NGG was prepared to produce adult inoculum for hanging drops and semi-solid NGG was used for the hanging drops for LHT analysis. Semi-solid NGG was prepared by mixing different components, which had been autoclaved separately and mixed after cooling to 55°C. The basis of the medium was 1 g l<sup>-1</sup> peptone from casein, 3 g l<sup>-1</sup> NaCl and 1.5 g l<sup>-1</sup> gellan gum (Gelrite; SERVA). To 1 l of this medium 1 ml of a 14.7 g l<sup>-1</sup> suspension of CaCl<sub>2</sub> · 2 H<sub>2</sub>O, 1 ml of 24.66 g l<sup>-1</sup> MgSO<sub>4</sub> · 7 H<sub>2</sub>O and 25 ml of 13.6 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> buffer were added. Finally 1 ml of 1 g l<sup>-1</sup> cholesterol suspended in ethanol (>99%) was added. The symbiotic bacterium *X. cabanillasii* was propagated in NLM for 48 h and the suspension centrifuged at 3939 g for 10 min. at 4°C. The supernatant was discarded and cells washed with sterilised K-medium (containing 3.1 g l<sup>-1</sup> NaCl and 2.4 g l<sup>-1</sup> KCl) and again centrifuged. The pellets were then dissolved in semi-solid NGG to obtain bacterial densities of 5 × 10<sup>9</sup>, 10 × 10<sup>9</sup> and 20 × 10<sup>9</sup> bacterial cells ml<sup>-1</sup>.

Cell densities were determined by counting aliquots in a Thoma chamber (0.01 mm depth; Poly Optic). Semi-solid NGG bacterial suspensions were stored for no longer than 3 days at 4°C before use in hanging drops.

Solid NGG was prepared as semi-solid NGG, except that the peptone concentration was increased to 2.5 g l<sup>-1</sup> and Gelrite to 3 g l<sup>-1</sup> in the basic preparation.

#### *LHT assessment on hanging drop*

Adult nematode inoculum for hanging drop experiments were taken from Petri dishes (diam. 56 mm) filled with solid NGG. The plates had been inoculated with 2 ml of 20 × 10<sup>9</sup> ml<sup>-1</sup> *X. cabanillasii* in semi-solid NGG and incubated overnight at 25°C. After 24 h, 4000 DJ were introduced to each plate from monoxenic cultures and incubated at 25°C. Single pre-adult male and female were then transferred from NGG plates approx. 36 h post DJ inoculation into 10 μl *X. cabanillasii* – semi-solid NGG mixture drops. Drops were kept on the inner side of lids of multi-well plates with 12 wells of 2.2 cm diam. (Greiner 665102; Greiner Bio-One). The bottom of each well was covered with approx. 140 mg dry cellulose tissue paper that had been wetted with 650 μl of tap water to keep the drops moist. After introduction of the nematodes, the multi-well plates were sealed with paraffin film and kept at 25°C in air tight boxes. Every 24 h the pair of female and male nematodes were transferred into a fresh 10 μl bacterial drop and the plates containing old drops were kept for another 24 h for hatching of offspring. Transfer of these parental nematodes into a new drop was continued until the last female had died. When male nematodes died before the female, new males were introduced from NGG plates. The experiment was conducted three times with different batches of nematodes and different cultures of symbiotic bacteria.

#### *Assessment of offspring production*

Total offspring production of females was determined based on hatched juveniles. Counting of the offspring produced in each drop was done by adding 10 μl of Rose Bengal solution (300 μg ml<sup>-1</sup>; Sigma-Aldrich). Then drops were covered with a circular 18-mm diam. cover slip (Thermo Scientific) and examined under a dissecting microscope (Zeiss Stemi SV11). Counting was facilitated by placing a grid underneath the multi-well lid. In drops in which the total number of offspring were too high for counting, drops were diluted in 2 ml water and counting was done by taking subsamples.

The percentage of offspring developed inside the uterus during *endotokia matricida* (Johnigk & Ehlers, 1999a)

or from laid eggs (intra- and extra-uterine development, respectively) was determined.

#### *Female body size*

Effect of bacterial food density on female body size was studied in a separate experiment by transferring single male and female pairs of Sr HYB19 into hanging drops with 5 × 10<sup>9</sup>, 10 × 10<sup>9</sup> and 20 × 10<sup>9</sup> cells ml<sup>-1</sup> of *X. cabanillasii* at 25°C. For each bacterial food density 20 male and female pairs of Sr HYB19 were used.

Every day the nematodes were transferred into new bacterial food and measurement of total body length and width was taken at the beginning of *endotokia matricida* using a dissection microscope and an attached camera. The image were analysed using analySIS® (Soft Imaging Systems) image analysis software. The influence of bacterial food density on female body size was analysed using a one-way ANOVA and a *post hoc* Tukey's honestly significant differences (HSD) test from the mean values obtained from the three replications.

#### *Calculation of life cycle parameters and statistical analysis*

Life cycle parameters such as the intrinsic rate of natural increase ( $r_m$ ), population doubling time (PDT), total fertility rate (TFR), net reproductive rate ( $R_0$ ), alternative measures of generation time ( $T_0$ ,  $T_1$ ,  $T$ ) and average life span of the females were calculated. The intrinsic rate of natural increase ( $r_m$ ) was calculated based on the Euler/Lotka equation (Vranken & Heip, 1983) given by:

$$\sum_{x=0}^d e^{-r_m x} l_x m_x = 1,$$

where  $r_m$  = intrinsic rate of natural increase,  $x$  = time (days),  $l_x$  = age specific survival probability and  $m_x$  = age-specific fecundity.

The intrinsic rate of natural increase ( $r_m$ ) iteration was done by goal seek in 'What if Analysis' using Microsoft Excel for Windows by changing the values of  $r_m$  until the sum of the values derived from the Euler equation reached one. Population doubling times (PDT) were calculated using the formula: PDT = ln 2/ $r_m$ . TFR,  $R_0$  and generation time was calculated on the basis of the life table constructed with  $l_x$  and  $m_x$ . TFR is the total number of offspring (intra- and extra-uterine) that would be produced by the females if they were able to survive until the end of their reproductive period and given by: TFR =  $\sum m_x$ . Net reproductive rate ( $R_0$ ) is defined as the



average number of offspring that a female in a population produced during its lifetime:  $R_0 = \sum l_x m_x$ . It depends on the age-specific survival probability and fecundity of the female.

Alternative generation time was measured using the following equations (as described by Vranken & Heip, 1983).

The mean generation time ( $T_1$ ) refers to that period of time necessary for a population growing at a constant rate  $r_m$  to increase by the factor  $R_0$ :

$$T_1 = (\ln R_0)/r_m; \quad (1)$$

the mean age of mothers in a cohort at hatching of female offspring ( $T_0$ , also referred to as  $T_c$ , the cohort generation time):

$$T_0/T_c = (1/R_0) \sum x l_x m_x; \quad (2)$$

the age of the mother of an average newly hatched in an exponentially growing population ( $T$ ):

$$T = \sum_{x=0}^d x e^{-r_m x} l_x m_x. \quad (3)$$

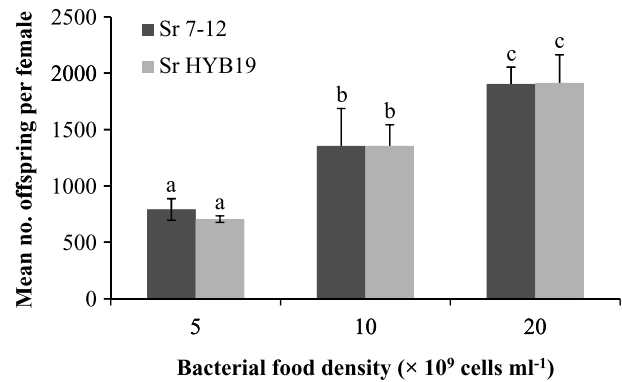
Prior to analysis of the percentage of extra- and intra-uterine produced offspring, data were arcsine transformed. The influence of bacterial food density on offspring production and the life cycle parameter of *S. riobrave* was analysed using a two-way ANOVA and a *post hoc* Tukey's honestly significant differences (HSD) test from the mean values obtained from the three replications. The relationship between bacterial food density and offspring production, intra- and extra-uterine offspring production and female body size was performed using the Pearson Correlation test.

## Results

### LIFE HISTORY TRAIT ANALYSIS

#### Offspring production

Offspring production was highly influenced by the bacterial food density ( $F = 50.153$ ;  $df = 2, 12$ ;  $P < 0.001$ ). The highest offspring production was recorded at bacterial food densities of  $20 \times 10^9$  cells  $ml^{-1}$  with an average per female of 1904 offspring for Sr 7-12 and 1913 for Sr HYB19 (Fig. 1). However, significant difference was not observed between the two strains at all bacterial food densities tested ( $F = 0.0721$ ;  $df = 1, 12$ ;  $P = 0.793$ ). A highly significant positive correlation



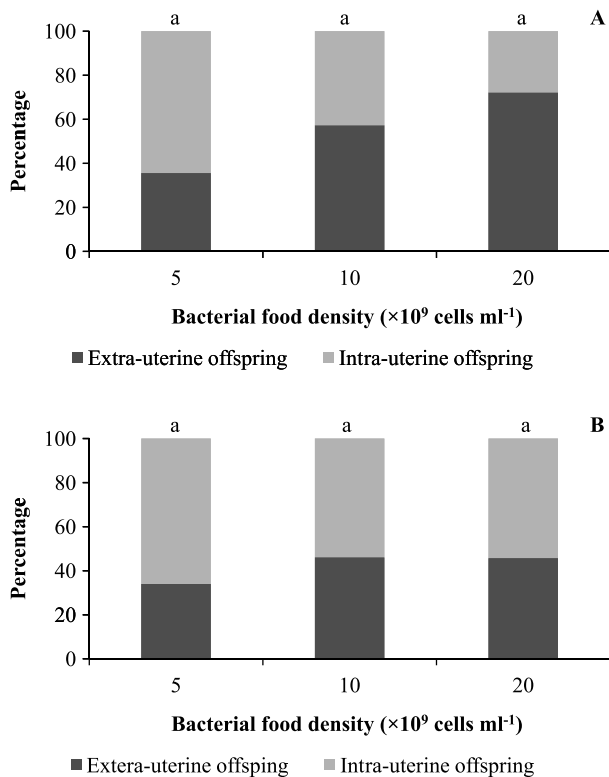
**Fig. 1.** Mean number of offspring produced per female of *Steinernema riobrave* strains Sr 7-12 and Sr HYB19 at 25°C and bacterial food densities of  $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells  $ml^{-1}$ . Different lower case letters above error bars indicate statistically significant differences between bacterial food densities for a given strain (Tukey's HSD test; at  $P \leq 0.05$ ).

( $R = 0.648$ ;  $P < 0.0001$ ) was obtained between bacterial food density and total offspring production.

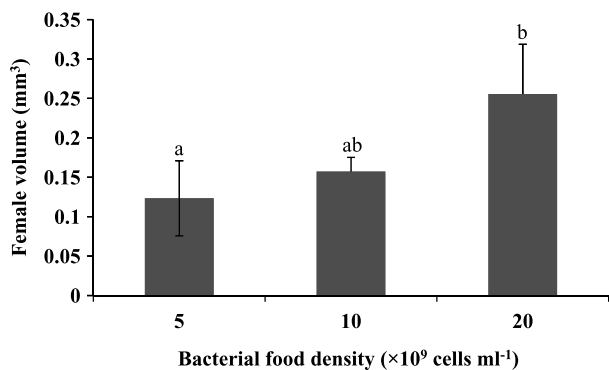
The contribution of extra- and intra-uterine offspring production at different food densities was also assessed. The percentage of extra- and intra-uterine offspring production was not significantly different among the bacterial food densities (intra-uterine:  $F = 1.011$ ;  $df = 2, 12$ ;  $P = 0.393$ ). The proportion of extra-uterine offspring production was higher at higher bacterial food densities for both, Sr 7-12 and Sr HYB19. The percentage offspring produced *via* intra-uterine development was relatively higher at the density of  $5 \times 10^9$  cells  $ml^{-1}$  compared to higher density and accounted for 64% and 66% of the total offspring produced in Sr 7-12 and Sr HYB19, respectively (Fig. 2A, B). Statistically significant differences were not detected between the two strains both in extra-uterine and intra-uterine offspring production ( $F = 0.197$ ;  $df = 1, 12$ ;  $P \leq 0.665$ ). Bacterial food density had a significantly negative association with intra-uterine offspring production ( $R = -0.309$ ;  $P < 0.0001$ ).

#### Female body size

The influence of *X. cabanillasii* densities on the volume of Sr HYB19 females was significant ( $F = 6.455$ ;  $df = 2, 6$ ;  $P = 0.032$ ). The mean body volume of females recorded was 0.123, 0.157 and 0.256  $mm^3$  at bacterial food densities of  $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells  $ml^{-1}$ , respectively (Fig. 3).



**Fig. 2.** Contribution of extra- and intra-uterine offspring production per female of *Steinernema riobrave* strains Sr 7-12 (A) and Sr HYB19 (B) at 25°C in hanging drops at bacterial food densities of  $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells ml $^{-1}$ . Different letters above bars indicate significant differences among bacterial food densities (Tukey's HSD test at  $P \leq 0.05$ ). Statistically significant differences between the two strains were not found at all bacterial food densities ( $P \leq 0.05$ ).



**Fig. 3.** Female body volume of *Steinernema riobrave* strain Sr HYB19 at three bacterial food densities ( $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells ml $^{-1}$ ) at 25°C. Different letters above bars indicate significant differences among bacterial food densities (Tukey's HSD test at  $P \leq 0.05$ ).

### Population growth parameters

For calculation of  $m_x$  and  $l_x$ , the age of Sr 7-12 and Sr HYB19 females at egg laying in hanging drops was defined at 4.5 days based on the assessment of developmental stages on NGG plates prior to the start of the study in hanging drops (data not shown). Offspring production (TFR,  $R_0$ ), generation time ( $T$ ,  $T_0$ ,  $T_1$ ) and intrinsic rate of population growth ( $r_m$ ) and population doubling time (PDT) were calculated according to the life table constructed with  $l_x$  and  $m_x$  (data not shown). With increasing food densities a significant increase in TFR was recorded (*post hoc* Tukey's test ( $F = 10.726$ ;  $df = 2, 12$ ;  $P = 0.002$ ) (Table 1)). At the higher bacterial food density Sr 7-12 and Sr HYB19 had the maximum TFR per female of 2022 and 2434 offspring, respectively (Table 1).

Significant differences were detected for  $R_0$  among the different bacterial food densities for both strains ( $F = 49.321$ ;  $df = 2, 12$ ;  $P = 0.001$ ). Similar to TFR Sr 7-12 and Sr HYB19 had the maximum  $R_0$  per female of 1904 and 1903, respectively, at the higher bacterial density (Table 1). For other population growth parameters, *i.e.*,  $r_m$ , PDT,  $T_0$ ,  $T_1$  and  $T$ , the differences observed between the bacterial food densities were not statistically significantly different for both, Sr 7-12 and Sr HYB19 ( $P \leq 0.05$ ; Table 1). No significant differences were observed between the two strains in all the population growth parameters ( $P \leq 0.05$ ).

The average life span of female nematodes was recorded at  $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells ml $^{-1}$  of *X. cabanillasii*. The result indicated that bacterial food densities had no significant influence on the life span of the females ( $F = 0.789$ ,  $df = 1, 12$ ;  $P = 0.476$ ) (Table 1). Although Sr HYB19 had a slightly lower lifespan, it was not significantly different from Sr 7-12 ( $F = 4.748$ ,  $df = 1, 12$ ;  $P = 0.056$ ). *Endotokia matricida* was not significantly affected by bacterial food densities. The beginning of *endotokia matricida* defined the death of the female approximately 1 day after start of hatching of J1 inside the uterus.

### Discussion

This contribution, for the first time, reports LHT of an EPN. The investigation provided fundamental details on the reproductive biology of the nematode *S. riobrave*, which might be used to improve commercial production in the future and possibly help one day to model nematode population development and reproductive and developmental parameters in monoxenic liquid culture. Success

**Table 1.** Life cycle parameters of *Steinernema riobrave* strains Sr 7-12 and Sr HYB19 assessed at different bacterial food densities in hanging drops at 25°C.

Life cycle parameters	Bacterial food density (cells ml <sup>-1</sup> )					
	5 × 10 <sup>9</sup>		10 × 10 <sup>9</sup>		20 × 10 <sup>9</sup>	
	Sr 7-12	Sr HYB19	Sr 7-12	Sr HYB19	Sr 7-12	Sr HYB19
<i>N</i>	96	66	81	66	79	57
TFR	975 ± 293 <sup>a</sup>	822 ± 190 <sup>a</sup>	1413 ± 316 <sup>ab</sup>	1440 ± 352 <sup>ab</sup>	2022 ± 239 <sup>b</sup>	2434 ± 1050 <sup>b</sup>
<i>R</i> <sub>0</sub>	792 ± 96 <sup>a</sup>	683 ± 74 <sup>a</sup>	1355 ± 333 <sup>b</sup>	1332 ± 203 <sup>b</sup>	1904 ± 150 <sup>c</sup>	1904 ± 244 <sup>c</sup>
<i>T</i> <sub>0</sub> (days)	4.05 ± 1.49 <sup>a</sup>	5.47 ± 0.45 <sup>a</sup>	4.94 ± 1.59 <sup>a</sup>	4.82 ± 1.5 <sup>a</sup>	5.88 ± 0.21 <sup>a</sup>	5.62 ± 0.37 <sup>a</sup>
<i>T</i> <sub>1</sub> (days)	5.92 ± 0.21 <sup>a</sup>	5.35 ± 0.48 <sup>a</sup>	5.18 ± 1.19 <sup>a</sup>	5.46 ± 0.25 <sup>a</sup>	5.74 ± 0.16 <sup>a</sup>	5.41 ± 0.37 <sup>a</sup>
<i>T</i> (days)	4.80 ± 1.70 <sup>a</sup>	5.25 ± 0.50 <sup>a</sup>	3.73 ± 0.88 <sup>a</sup>	4.85 ± 0.74 <sup>a</sup>	5.62 ± 0.11 <sup>a</sup>	5.22 ± 0.38 <sup>a</sup>
<i>r</i> <sub>m</sub> (day <sup>-1</sup> )	1.13 ± 0.06 <sup>a</sup>	1.23 ± 0.13 <sup>a</sup>	1.44 ± 0.33 <sup>a</sup>	1.34 ± 0.09 <sup>a</sup>	1.32 ± 0.03 <sup>a</sup>	1.397 ± 0.07 <sup>a</sup>
PDT (days)	0.62 ± 0.03 <sup>a</sup>	0.57 ± 0.06 <sup>a</sup>	0.496 ± 0.10 <sup>a</sup>	0.52 ± 0.04 <sup>a</sup>	0.53 ± 0.11 <sup>a</sup>	0.496 ± 0.03 <sup>a</sup>
Average life span (days)	7.4 ± 0.3 <sup>a</sup>	6.9 ± 0.7 <sup>a</sup>	7.5 ± 0.2 <sup>a</sup>	7.1 ± 0.7 <sup>a</sup>	7.8 ± 0.3 <sup>a</sup>	7.3 ± 0.7 <sup>a</sup>

Values represent means from the replicates ± SD. Different superscript letters indicate significant differences between values within rows (*post hoc* Tukey's HSD test,  $P \leq 0.05$ ). *N* = total number of females observed during three replicates; TFR = total fertility rate; *R*<sub>0</sub> = net reproductive rate, *T*<sub>0</sub> = mean age at reproduction of a cohort of females (also referred to as *T*<sub>c</sub>, the cohort generation time); *T*<sub>1</sub> = period of time necessary for a population growing at a constant rate *r*<sub>m</sub> to increase by the factor *R*<sub>0</sub>; *T* = mean age of the mothers of a set of new newly-hatched individuals in a population with a stable age distribution; *r*<sub>m</sub> = intrinsic rate of natural increase; PDT = population doubling time.

in liquid culture depends on the ability to manage nematode population dynamics accurately (Ehlers & Shapiro-Ilan, 2005). Many investigations have observed whole populations in liquid culture (*e.g.*, Ehlers *et al.*, 1998; Johnigk & Ehlers, 1999a, b; Hirao *et al.*, 2010) without the possibility of assessing fundamental growth parameters, hence making a precise interpretation of reproductive parameters impossible. The hanging drop technique now allows precise experiments using single adult nematodes to investigate the influence of food (in this case density of *X. cabanillasii*) or food quality (symbiont strains or phase variants), but also physical growth parameters (*e.g.*, temperature, media compounds). Parameters such as offspring production and growth have been assessed during this study, but DJ recovery, mating behaviour or survival can also be studied in detail.

Offspring production of *S. riobrave* was increased with increasing bacterial food density. Offspring in steinernematids are the result of extra- and intra-uterine offspring production. Intra-uterine development or *endotokia matricida* is initiated when the first-stage juvenile hatches from the egg inside the uterus. The assumption that higher food density would delay the start of *endotokia matricida*, as reported by Johnigk & Ehlers (1999b) for *H. bacteriophora* and *H. indica*, could not be confirmed for *S. riobrave*. Although a higher number of offspring was counted at high food density, this did not influence the be-

ginning of *endotokia matricida*. Increasing food density increases the volume of the female and longer females produced more eggs. However, the life span is obviously not influenced by increasing availability of food and, consequently, only the rate of egg production seems to be enhanced by a higher bacterial density.

Johnigk & Ehlers (1999b) reported that, under conditions of nutritive depletion, intra-uterine offspring production is a favourable and efficient way to raise well-fed DJ. Development outside of the adult can possibly even result in failure to reach the DJ stage due to lack of food resources. In this study, a higher percentage of intra-uterine offspring production was also observed at the lower bacterial food density.

Body size of nematodes is generally controlled by genetic and environmental factors such as food. An increased bacterial food densities had contributed an increased somatic growth of Sr HYB19 females, which in turn contributed to the higher number of offspring production. According to Johnigk & Ehlers (1999b), the body size of hermaphrodites of *Heterorhabditis* spp. was greatly influenced by the availability of their symbiotic bacteria.

Both strains of *S. riobrave* have comparably low *r*<sub>m</sub> and much higher PDT values when compared with free-living nematodes. The *r*<sub>m</sub> of *S. riobrave* on average is four- and six-fold lower compared to the free-living

nematodes *Panagrolaimus* sp. and *Poikilolaimus* sp. with  $r_m$  values of 0.309 and 0.165 (Muschiol & Traunspurger, 2007). Similarly, 75% and 87% increase in the PDT were observed when compared with *Panagrolaimus* sp. and *Poikilolaimus* sp., which took 2.24 and 4.21 days, respectively (Muschiol & Traunspurger, 2007). Recently, Ayub *et al.* (2013) recorded relatively closer  $r_m$  (0.81) and PDT (0.9) days for *Panagrolaimus* sp. (strain NFS 24-5) at 25°C.

An influence of the bacterial food density on the generation time and population growth rate was not recorded. Life span is defined by the start of *endotokia matricida*. Death of maternal nematodes due to intra-uterine birth is a common phenomenon in rhabditid nematodes (Johnigk & Ehlers, 1999b; Chen & Caswell-Chen, 2004; Hirao *et al.*, 2010). *Endotokia matricida* was found the cause of early death of female nematodes in both strains. Few non-fertile females that failed to produce offspring in hanging drops, although males were present, survived more than three weeks, indicating that an extension of the life span of females is possible.

In *Caenorhabditis elegans* occurrence of *endotokia matricida* was reported due to depletion of food (Chen & Caswell-Chen, 2004). On the other hand, it had been observed that dietary restriction reduces fecundity and growth but increases longevity (Tain *et al.*, 2008). Life span of *S. riobrave* is obviously not increased through decreasing the amount of available food or the bacterial food density in hanging drops was still too high to measure any effects. The presented results led one to suspect that, other than in *H. bacteriophora*, life span is not prolonged by increasing food supply but is bound to end within a defined time once eggs have been fertilised.

Extrapolation from results of hanging drop experiments to optimal cell density in *in vitro* liquid culture appears unrealistic. Whether similar yields per female recorded during this investigation will be reached in liquid batch cultures as well needs further experiments with artificially increased bacterial density. In conclusion, LHT analysis can provide valuable information on the reproductive biology and optimal growing conditions for EPN and support interpretation of population development in liquid culture to further improve and optimise mass production of *S. riobrave* in liquid culture.

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**Annex 3: Publication 3**

**Life history traits, liquid culture production and storage temperatures of *Steinernema yirgalemense***

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# Life history traits, liquid culture production and storage temperatures of *Steinernema yirgalemense*

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**Summary** – Using the hanging drop technique with nematode growth gelrite medium, life history traits of *Steinernema yirgalemense* (strain Sy 157-C) were investigated at a bacterial density of  $10 \times 10^9$  cells ml<sup>-1</sup> of *Xenorhabdus indica* at 25°C. With the same technique, the exit of dauer juveniles (DJ) from the arrested stage (recovery) was assessed at  $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells ml<sup>-1</sup> of *X. indica*. Additionally, *S. yirgalemense* was incubated in nematode liquid medium at 25, 27 and 30°C. At each culture temperature, DJ recovery, sex ratio at 3 days post DJ inoculation and DJ yield and DJ as a percentage of non-DJ stages at 15 days post DJ inoculation were assessed. DJ survival in Ringer's solution stored at 4, 15 and 25°C was assessed for 66 days. *Steinernema yirgalemense* has a total fertility rate and net reproductive rate of 487 and 314 offspring per female, respectively. The intrinsic rate of natural increase  $r_m$  was 0.98 day<sup>-1</sup>, population doubling time PDT = 0.71 days and mean generation time  $T = 5.72$  days. The average lifespan of *S. yirgalemense* females starting from first-stage juveniles was 6.55 days. In liquid culture, DJ recovery ranged from 63-75% at 72 h post DJ inoculation and was not significantly different between the incubation temperatures. Parental male to female ratio was not influenced by incubation temperature and usually was at a ratio of 1:2. The percentage of females that entered into *endotokia matricida* at 72 h post DJ inoculation was 61% at 25°C, whereas at 27 and 30°C it was 24% and 0.5%, respectively. The highest DJ yield was recorded at 25°C (284 114 DJ ml<sup>-1</sup>) followed by 27°C (176 932 DJ ml<sup>-1</sup>) and the lowest at 30°C with 26 298 DJ ml<sup>-1</sup>. At a storage temperature of 4°C, DJ survival did not exceed 42 days, whereas at 15 and 25°C more than 95% of the DJ survived 66 days. Although *S. yirgalemense* DJ survived for long periods at both 15 and 25°C in liquid storage, their survival in formulated product and virulence after storage needs further investigation.

**Keywords** – dauer juvenile yield, *endotokia matricida*, hanging drop, *Xenorhabdus indica*.

Entomopathogenic nematodes (EPN) in the families Heterorhabditidae and Steinernematidae are widely used to control economically important insect pests in different farming systems (Grewal *et al.*, 2005; Georgis *et al.*, 2006; Koppenhöfer, 2007; Lacey & Georgis, 2012). EPN explorations have been conducted all over the world and a number of species have been described. Campos-Herrera *et al.* (2012) have listed 17 described species in the genus *Heterorhabditis* and 63 species in the genus *Steinernema*. To date, the total number of described species in both genera has reached more than 90 (Lacey *et al.*, 2015).

The most important requirement for successful and economically reasonable use of EPN in crop protection is large-scale production at low cost within a short process

time (Ehlers, 2001; Ehlers & Shapiro-Ilan, 2005; Hirao & Ehlers, 2009a; Shapiro-Ilan *et al.*, 2012). This can only be achieved under well-defined liquid culture conditions and successful management of nematode population dynamics (Ehlers, 2001). Nowadays, four companies, using large liquid bioreactor tanks of up to 80 000 l, produce EPN for commercial purposes. Species such as *Heterorhabditis bacteriophora*, *H. indica*, *H. megidis*, *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, *S. riobrave* and *S. scapterisci* have been mass produced, formulated and commercialised (Ehlers *et al.*, 1998; Grewal *et al.*, 2005; Grewal & Peters, 2005; Lacey *et al.*, 2015).

The EPN *S. yirgalemense* was isolated for the first time in Yirgalem, Ethiopia (Nguyen *et al.*, 2004), and later in

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Kenya (Mwaniki *et al.*, 2008) and South Africa (Malan *et al.*, 2011). It has a promising biological control potential against the barley chafer grub, *Copegnatus curtipennis* (Anbesse *et al.*, 2008), the sweet potato weevil, *Cylas puncticollis* (Mwaniki *et al.*, 2009), the false codling moth, *Thaumatotibia leucotreta* (Malan *et al.*, 2011), the codling moth, *Cydia pomonella* (De Waal *et al.*, 2011), the citrus mealybug, *Planococcus citri* (Van Niekerk & Malan, 2012) and the vine mealybug, *Planococcus ficus* (Le Vieux & Malan, 2013). Preliminary investigations showed that *S. yirgalemense* can also infect and reproduce in enset root mealybug, *Cataenococcus ensete*, which is an economically important pest of enset (*Ensete ventricosum*, Musaceae) (Addis *et al.*, 2010). Prior to commercial exploitation of its biocontrol potential, it is important to collect information on the biology and reproduction potential of *S. yirgalemense* and its bacterial symbiont *Xenorhabdus indica*.

For large-scale EPN production, it is necessary to perform investigations on a small scale in order to obtain information on fundamental reproductive parameters, scale-up potential and adaptability to downstream processing technology (Ehlers, 2001; Hirao *et al.*, 2010). Many investigations have been conducted to assess population dynamics of *Steinernema* and *Heterorhabditis* spp. and their symbiotic bacteria under liquid culture (Strauch & Ehlers, 1998; Johnigk *et al.*, 2004; Hirao & Ehlers, 2009a, b; Hirao *et al.*, 2010). These investigations were done at population level, but a study based on individual nematodes may provide a better insight into the fundamental reproductive biology of EPN species.

The hanging drop technique using a semi-solid nematode growth gelrite medium (NGG) has been applied to study both free-living nematodes and EPN (Muschiol & Traunspurger, 2007; Muschiol *et al.*, 2009; Ayub *et al.*, 2013; Addis *et al.*, 2014). This method allows the study of single individuals of similar age. In addition, it allows the study of culture conditions (*e.g.*, temperature), lifespan and mating (Muschiol *et al.*, 2009; Ayub *et al.*, 2013; Addis *et al.*, 2014) or dauer juvenile (DJ) recovery. Recovery is the developmental step of leaving the arrested dauer stage as a reaction to chemical cues from food, insect haemolymph or symbiotic bacteria, which is known as food signal (Golden & Riddle, 1982; Strauch & Ehlers, 1998; Hirao & Ehlers, 2009a). Studying individual nematodes with a better accuracy allows the generation of reliable data about life history traits (LHT) of an organism.

Recently, the mass production potential of *S. yirgalemense* in liquid culture was assessed at 25°C (Ferreira *et*

*al.*, 2015). The authors reported a yield of 75 000 DJ ml<sup>-1</sup>, 15 days post DJ inoculation, which needs further improvement to utilise its potential. However, there is no available information on the LHT and the effects of incubation temperature on its development and DJ yield in liquid culture production. Exposure of mass-produced DJ to unfavorable storage and transportation temperatures affects their performance in the field against target pests. Therefore, the objectives of this study were to generate information about the fundamental LHT and DJ recovery of *S. yirgalemense* using the hanging drop method. In addition, the influence of incubation temperature on DJ recovery and DJ yield in monoxenic liquid culture was investigated. Finally, DJ survival at different storage temperatures was investigated.

## Materials and methods

### SYMBIOTIC BACTERIA AND NEMATODES

*Steinernema yirgalemense* strain Sy 157-C was obtained from Dr Antoinette P. Malan from Stellenbosch University, South Africa. Attempts to re-isolate the type specimen at the type locality were not successful. The symbiotic bacterium, *X. indica* (Ferreira *et al.*, 2014), was isolated according to Ehlers *et al.* (1990).

Monoxenic liquid cultures of *S. yirgalemense* were established according to Lunau *et al.* (1993). Further scaling-up of DJ grown on Wouts agar plates was done in nematode liquid medium (NLM) prepared according to Ehlers *et al.* (1998). NLM contains (in g l<sup>-1</sup>) 6.0 of a 1:1 mixture of lecithin and oil, 30.0 rapeseed oil, 15.0 yeast extract, 20.0 soy flour, 4.0 NaCl, 0.35 KCl, 0.3 CaCl<sub>2</sub> and 0.2 MgSO<sub>4</sub>·7H<sub>2</sub>O and pH adjusted to 6.7. The medium was inoculated with symbiotic bacteria at 1% (v/v) from 24 h yeast extract-salt (YS) broth cultures (in g l<sup>-1</sup>: 5.0 NaCl, 5.0 yeast extract, 0.5 NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.5 K<sub>2</sub>HPO<sub>4</sub> and 0.2 MgSO<sub>4</sub>·7H<sub>2</sub>O) 1 day prior to nematode inoculation. Erlenmeyer flasks were incubated on a shaker at 180 rpm (rotation diam. 4 cm) at 25°C for 15 days. Afterwards, the DJ were kept at 19°C on a shaker for further experiments.

### XENORHABDUS INDICA PREPARATION FOR HANGING DROPS

Solid and semi-solid nematode growth gelrite (NGG) were prepared according to Muschiol & Traunspurger (2007). In order to prepare semi-solid NGG, different components were autoclaved separately and mixed after



cooling to 55°C. The basis of the medium is (in g l<sup>-1</sup>): 1.0 peptone from casein, 3.0 NaCl and 1.5 gellan gum (Gelrite, SERVA). To 1 l of this medium 1 ml of a 147 g l<sup>-1</sup> suspension of CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 ml of 246.6 g l<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O and 25 ml of 136 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> buffer was added. At the end, 1.0 ml of 1.0 g l<sup>-1</sup> cholesterol suspended in >99% ethanol was added.

*Xenorhabdus indica* was propagated in 100 ml YS medium in Erlenmeyer flasks for 48 h, at 25°C to allow the bacteria to reach the stationary phase (Ferreira *et al.*, 2015). The suspension was centrifuged at 3939 g for 10 min at 4°C. The supernatant was discarded and sterile K-medium (containing in g l<sup>-1</sup>: 3.1 NaCl and 2.4 KCl) was added to wash the cells and centrifugation was repeated for 5 min. In order to get the required bacterial cell numbers for production of pre-adult nematodes and assessment of LHT, pellets were re-suspended in semi-solid NGG and cell densities were determined using a Thoma chamber (0.01 mm depth; Poly Optic). After adjusting the required bacterial cell densities, semi-solid NGG-bacterial suspensions were stored at 4°C and used for a maximum of 3 days.

#### ASSESSMENT OF LHT

Assessment of the LHT of *S. yirgalemense* was done according to Addis *et al.* (2014). For production of adult nematode inoculums, NGG plates were prepared as semi-solid NGG with an increased concentration of peptone to 2.5 g l<sup>-1</sup> and Gelrite to 3 g l<sup>-1</sup> in the basic preparation. The NGG plates (100 × 15 mm filled with approximately 25 ml NGG) were inoculated with 2 ml of *X. indica* at a density of 20 × 10<sup>9</sup> cells ml<sup>-1</sup>. After 24 h incubation at 25°C, ca 4000 DJ were introduced and kept under the same conditions for an additional 36 h until pre-adult individuals had developed. Single female and male pairs were transferred into 10 µl hanging drops (diam. 3.7 mm) with 10 × 10<sup>9</sup> cells ml<sup>-1</sup>. The drops were positioned at the inner side of the lids of multi-well plates with 12 wells of 2.2 cm diam. (Greiner 665102; Greiner Bio-One). The bottom of each well was filled with ca 0.14 g cellulose tissue paper that had been moistened with 650 µl water in order to avoid desiccation of drops. NGG plates were sealed with parafilm and kept at 25°C. Every 24 h nematodes were transferred to fresh bacteria in new drops. The old plates were sealed and kept for 24 h to allow the juveniles to hatch prior to counting. The process of transferring females and males to fresh drops continued until the death of the last female had occurred. Drops were stained by adding 10 µl Rose Bengal solution

(300 µg ml<sup>-1</sup>; Sigma-Aldrich) and covered with circular 18 mm cover slips (Thermo Scientific). Counting was facilitated by using a 2 × 2 mm grid underneath the lids. Counting was done using a dissecting microscope (Zeiss). Offspring that had developed from laid eggs (extra-uterine) or from intra-uterine offspring (*endotokia matricida*) were separately recorded for the 113 females assessed.

#### DJ RECOVERY IN HANGING DROPS

The influence of bacterial cell density on DJ recovery in hanging drops was assessed at 5 × 10<sup>9</sup>, 10 × 10<sup>9</sup> and 20 × 10<sup>9</sup> cells ml<sup>-1</sup>. For each bacterial density, ~20 DJ were transferred to four drops previously mixed with *X. indica*-semi-solid NGG mixture. The multi-well plates with the NGG drops were kept at 25°C and the numbers of recovered individuals were counted 24 and 48 h post DJ inoculation. At each time of assessment, two drops (ca 40 DJ) were assessed for each bacterial density and the experiment was repeated three times with different batches of nematodes.

#### NEMATODE LIQUID CULTURES

Symbiotic bacteria were pre-cultured in YS medium for 48 h at 25°C and 1 ml of the cultured bacteria was transferred into 100 ml sterile NLM in 500 ml size Erlenmeyer flasks. After 48 h incubation at 25, 27 and 30°C, approximately 5000 DJ ml<sup>-1</sup> medium were inoculated. DJ recovery and sex ratio were determined 72 h post DJ inoculation, whereas DJ yield and non-DJ stages were counted at 15 days post DJ inoculation. For each incubation temperature, three Erlenmeyer flasks were used and the experiment was repeated three times with different batches of nematodes.

#### DJ SURVIVAL AT DIFFERENT STORAGE TEMPERATURES

The DJ subjected to different storage temperatures were obtained from monoxenic liquid cultures. They were washed with sterile Ringer's solution and sieved through a mesh size of 10 µm using a vacuum suction instrument. Fifty ml culture flasks (Sarstedt) received 7 ml Ringer's solution containing 10 000 DJ ml<sup>-1</sup> and stored for more than 9 weeks at 4, 15 and 25°C. Every 7 days subsamples with a minimum of 100 DJ were taken and dead and alive DJ were counted using an inverted microscope. For each temperature the average survival of DJ was

calculated. One flask per replicate per storage temperature was assessed and the experiment was replicated three times with different batches of nematodes (in total nine culture flasks were used). Analysis of variance (one-way ANOVA) was conducted using survival values recorded only at day 66.

#### STATISTICAL ANALYSIS

Before conducting ANOVA, percentage values for DJ recovery, adult females and females with *endotokia matricida*, DJ density and DJ survival were arcsine-transformed. Effects of different bacterial food densities and incubation temperatures on DJ recovery, sex ratio, DJ yield and storage temperature on DJ survival were analysed using ANOVA and Tukey's HSD at  $P \leq 0.05$ . The relationship between bacterial density and DJ recovery in hanging drops was performed using the Pearson correlation test. The different life cycle parameters were calculated based on the Euler/Lotka equation (Vranken & Heip, 1983) given by:

$$\sum_{x=0}^d e^{-r_m x} l_x m_x = 1,$$

where  $r_m$  = intrinsic rate of natural increase,  $x$  = time (days),  $l_x$  = age-specific survival probability, and  $m_x$  = age-specific fecundity.

The intrinsic rate of natural increase ( $r_m$ ) iteration was done according to Addis *et al.* (2014). Population doubling time (PDT) was calculated using the formula:  $PDT = \ln 2/r_m$ . Total fertility rate (TFR), net reproductive rate ( $R_0$ ) and generation time were calculated on the basis of the life table constructed with  $l_x$  and  $m_x$  after defining the time of egg laying in hanging drops at 4.5 days based on the assessment of developmental stages in NGG plates (data not shown). TFR is the total number of offspring that would be produced by the females if they were able to survive until the end of their reproductive period and given by:  $TFR = \sum m_x$ .  $R_0$ , which depends on the age-specific survival probability and fecundity, is the average number of offspring that a female in a population produces during its life time:  $R_0 = \sum l_x m_x$ . Alternative generation time was calculated using the equations described by Vranken & Heip (1983). The mean generation time ( $T_1$ ) refers to that period of time necessary for a population growing at a constant rate  $r_m$  to increase by the factor  $R_0$ :  $T_1 = (\ln R_0)/r_m$ . The mean age of mothers in a cohort at hatching of female offspring is  $T_0$  (also known as  $T_c$ , the cohort generation time):

$T_0/T_c = (1/R_0) \sum x l_x m_x$ . The mean age of the mother of newly-hatched individuals in a population with a stable age distribution ( $T$ ):

$$T = \sum_{x=0}^d x e^{-r_m x} l_x m_x.$$

## Results

### LIFE HISTORY TRAIT ASSESSMENT IN HANGING DROPS

#### Offspring production

The average number of offspring per female was 314 and about 96% of the individuals originated from intra-uterine development (*endotokia matricida*). Apparently, in this nematode species, the period of egg deposition is very short and *endotokia matricida* starts immediately after fertilisation and ends the life of the female nematode within the next 24 h. Intra-uterine developed juveniles were migrating into the medium before they had fully developed into DJ inside the maternal carcass.

#### Analysis of life history trait parameters

*Steinernema yirgalemense* has a TFR and  $R_0$  of 487 and 314 offspring per female, respectively (Table 1). The intrinsic rate of natural increase  $r_m = 0.98$  and  $PDT = 0.71$ . Generation time parameters were 5.98, 5.87 and 5.72 days for  $T_0$ ,  $T_1$  and  $T$ , respectively (Table 1). The average lifespan of *S. yirgalemense* females starting from first-stage juvenile was 6.55 days (Table 1).

#### DJ recovery

DJ recovery in hanging drops was assessed at  $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells  $\text{ml}^{-1}$  at 25°C. A moderate positive correlation between bacterial food density and DJ recovery was observed both at 24 h ( $R = 0.67$ ;  $P = 0.0026$ ) and 48 h ( $R = 0.65$ ;  $P = 0.0035$ ) post DJ inoculation (Fig. 1). Percent DJ recovery ranged from 5% to 77%.

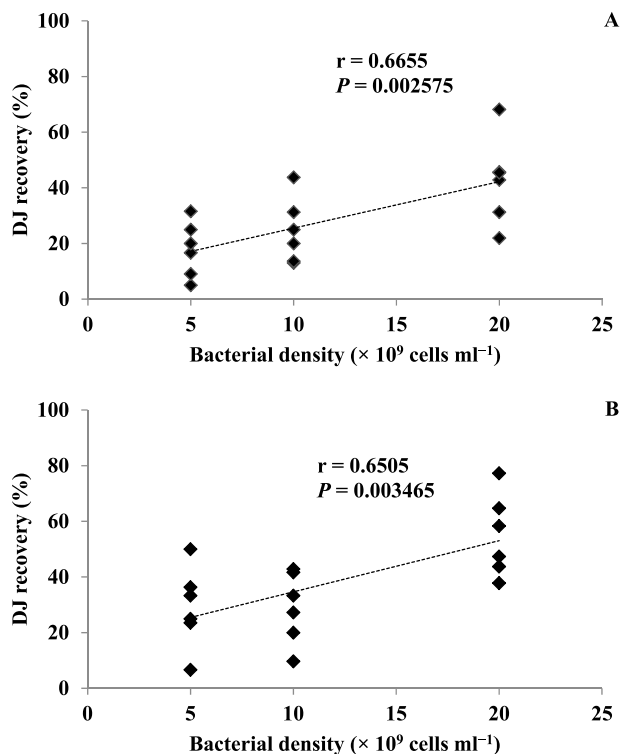
### LIQUID CULTURE PRODUCTION OF *S. YIRGALEMENSE*

In liquid culture, no significant differences in DJ recovery between the different incubation temperatures were recorded at 72 h post DJ inoculation ( $F = 1.146$ ;  $df = 2, 6$ ;  $P = 0.379$ ) (Fig. 2A). It ranged from 63 to 75% at 72 h post DJ inoculation. Similarly, the total number of

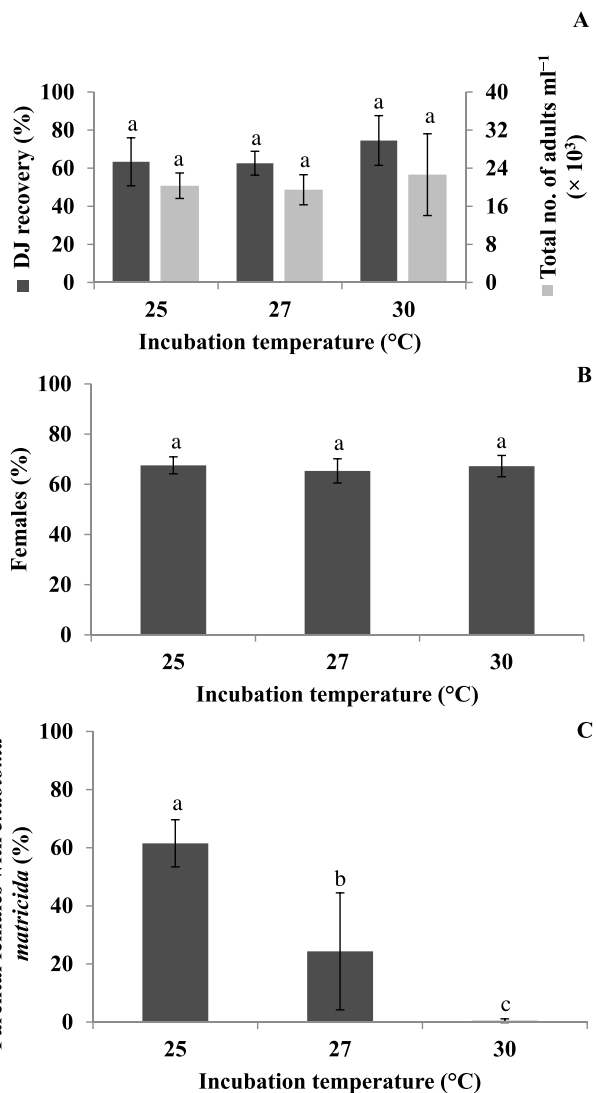
**Table 1.** Life history trait parameters for *Steinernema yirgalemense* strain Sy 157-C assessed at  $10 \times 10^9$  cells  $\text{ml}^{-1}$  of *Xenorhabdus indica* in hanging drops at 25°C.

Life cycle parameter	Mean $\pm$ SD
n	113
TFR (offspring/female)	488 $\pm$ 65
$R_0$ (offspring/female)	314 $\pm$ 15
$T_0$ (days)	5.98 $\pm$ 0.49
$T_1$ (days)	5.87 $\pm$ 0.49
$T$ (days)	5.72 $\pm$ 0.56
$r_m$ ( $\text{day}^{-1}$ )	0.98 $\pm$ 0.08
PDT (days)	0.71 $\pm$ 0.06
Average lifespan (days)	6.55 $\pm$ 0.67

TFR = total fertility rate;  $R_0$  = net reproductive rate;  $r_m$  = intrinsic rate of natural increase;  $T_0$  = cohort generation time;  $T_1$  = period of time necessary for a population growing at a constant rate  $r_m$  to increase by the factor  $R_0$ ;  $T$  = the mean age of the mothers of a set of newly-hatched individuals in a population with a stable age distribution; PDT = population doubling time; n = total number of female nematodes.



**Fig. 1.** Correlation of *Steinernema yirgalemense* percent DJ recovery and bacterial density assessed in hanging drops at 24 h (A) and 48 h (B) at  $5 \times 10^9$ ,  $10 \times 10^9$  and  $20 \times 10^9$  cells  $\text{ml}^{-1}$  of *Xenorhabdus indica* (Pearson's correlation at  $P \leq 0.05$ ).



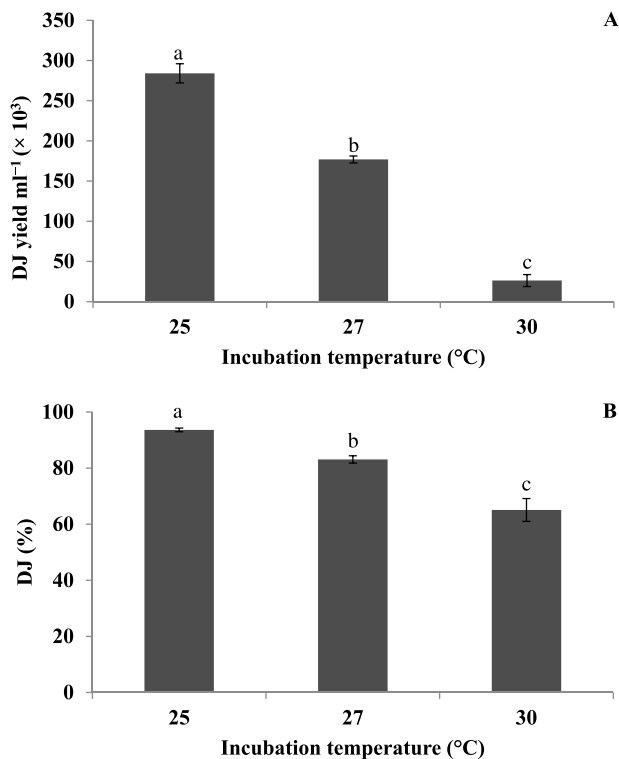
**Fig. 2.** Percentage of DJ recovery and total adults (A), percentage of females (B) and percentage of females with *endotokia matricida* (C) at 72 h post DJ inoculation of *Steinernema yirgalemense* assessed at 25, 27 and 30°C in monoxenic liquid culture (n = 9 Erlenmeyer flasks for each culture temperature). Lower case letters above error bars indicate significant differences between incubation temperatures (Tukey's HSD test at  $P \leq 0.05$ ).

parental nematodes was not significantly different ( $F = 0.268$ ;  $\text{df} = 2, 6$ ;  $P = 0.773$ ) (Fig. 2A).

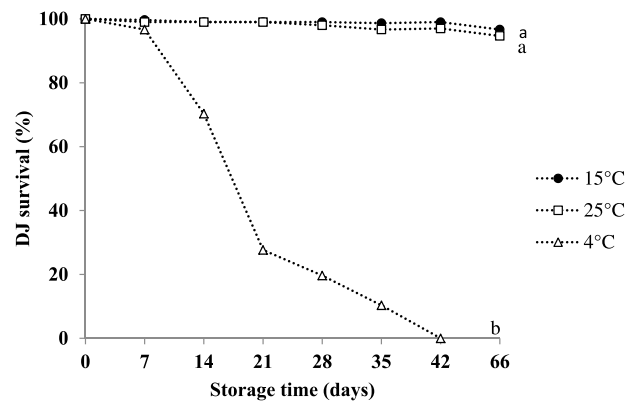
The percentage of females in the adult nematode population at 72 h post DJ inoculation was not significantly influenced by the incubation temperature ( $F = 0.272$ ;  $\text{df} = 2, 6$ ;  $P = 0.771$ ) (Fig. 2B). *Steinernema yirgalemense* sex ratio is female-biased with a male to female ratio of

around 1:2 (Fig. 2B). A significant difference in the percentage of females with *endotokia matricida* at 72 h post DJ inoculation was observed between the different incubation temperatures ( $F = 17.846$ ;  $df = 2, 6$ ;  $P = 0.003$ ) (Fig. 2C). The percentage of females that entered into *endotokia matricida* at 72 h post DJ inoculation at 25°C was 61%, whereas at 27 and 30°C it was only 24 and 0.5%, respectively (Fig. 2C).

The number of DJ developed at 15 days post DJ inoculation in liquid culture was significantly higher at 25°C (284 114 DJ ml<sup>-1</sup>) compared to the higher temperatures ( $F = 698.189$ ;  $df = 2, 6$ ;  $P < 0.0001$ ) (Fig. 3A). The DJ yield recorded at 27°C was 176 932 DJ ml<sup>-1</sup> and at 30°C it was only 26 298 DJ ml<sup>-1</sup>. Incubation temperature significantly affected the percentage of DJ of the total nematode population at 15 days post DJ inoculation ( $F = 148.037$ ;  $df = 2, 6$ ;  $P < 0.0001$ ) (Fig. 3B). At 25°C, a significantly higher percentage of DJ (94%) was



**Fig. 3.** Mean DJ yield (A) and DJ as percentage of non-DJ stages (B) of *Steinernema yirgalemense*, 15 days post DJ inoculation at 25, 27 and 30°C incubation temperatures. Lower case letters above error bars indicate significant differences between incubation temperatures (Tukey's HSD test at  $P = 0.021$ ).



**Fig. 4.** Percentage survival of *Steinernema yirgalemense* DJ in Ringers at storage temperatures of 4, 15 and 25°C assessed during a 9-week period at a density of 10 000 DJ ml<sup>-1</sup> in culture flasks. Different lower case letters indicate significant differences between temperatures tested at day 66 (Tukey's HSD test at  $P < 0.001$ ).

recorded, whereas at 30°C the lowest percentage of DJ (64%) was found (Fig. 3B).

#### DJ SURVIVAL AT THREE DIFFERENT STORAGE TEMPERATURES

Survival of *S. yirgalemense* DJ recorded at day 66 was significantly different between the different storage temperatures and the lowest survival was observed at 4°C ( $F = 942.188$ ;  $df = 2, 6$ ;  $P < 0.0001$ ) (Fig. 4). At 4°C, 100% of the DJ were dead within 42 days, whereas at storage temperatures of 15°C and 25°C the survival recorded at day 66 was 97% and 95%, respectively (Fig. 4).

## Discussion

The hanging drop technique provides the opportunity to study single nematode or nematode pairs under unlimited food supply and defined environmental conditions with a more detailed analysis (Muschiol *et al.*, 2009). As a result, it has been important in studies of the LHT of both free-living nematodes (Muschiol & Traunspurger, 2007; Muschiol *et al.*, 2009; Ayub *et al.*, 2013; Gilarte *et al.*, 2015) and EPN (Addis *et al.*, 2014, 2015).

DJ recovery is one of the most important steps for successful liquid culture establishment in EPN production. Prolonged and low DJ recovery leads to a non-synchronous nematode population development that increases the process time due to development of further

generations, which do not contribute significantly to the final DJ yield (Ehlers, 2001; Hirao *et al.*, 2010). In liquid culture *S. yirgalemense* DJ recovery ranged from 63 to 75% at 72 h post DJ inoculation and was not significantly affected by incubation temperatures. The percentage DJ recovery recorded at 25°C (63%) is comparable to that of *S. carpocapsae* (66%) and significantly lower than that of *S. feltiae* (90%) observed at a similar incubation temperature (Hirao & Ehlers, 2010). Remarkably, higher DJ recovery in liquid culture was found when compared with results obtained in hanging drops. Hanging drops are loaded with washed and concentrated cell suspensions in poor NGG medium. Washing of bacterial pellets with K-medium removes food signals excreted by the bacteria into the supernatant. In the relatively nutrient-poor NGG medium bacterial metabolism is reduced, which is probably the reason for lower DJ recovery compared to recovery in rich nematode liquid medium. The hanging drop technique may not be a meaningful method to test recovery of DJ in liquid culture. Ferreira *et al.* (2015) reported a 67% DJ recovery of *S. yirgalemense* within 48 h post DJ inoculation at 25°C, which is similar to the results of this study obtained at the same culture temperature. In order to get the optimal parental nematode density, factors that affect DJ recovery, *e.g.*, time of DJ inoculation, nematode batches and bacterial isolates, should be further investigated at the optimal culture temperature (25°C).

The percentage of parental females observed 3 days post DJ inoculation ranged from 65 to 68% and was not significantly affected by the incubation temperatures. Similar to the present study, many studies indicated that *Steinernema* spp. are female-biased (Grewal *et al.*, 1993; Alsaihan *et al.*, 2009; Hirao & Ehlers, 2009c). Ferreira *et al.* (2015) also recorded *S. yirgalemense* as female-biased at the beginning of liquid culture production, but there was a switch to male bias between 5-9 and 12-15 days post DJ inoculation. From our observations made in NGG plates, hanging drops and liquid culture experiments, the switch towards male bias is the result of *endotokia matricida*, which affects the females, while the males remain alive.

EPN generate offspring *via* intra-uterine (laid eggs) and extra-uterine (*endotokia matricida*) production. Among the total of 314 offspring per female of *S. yirgalemense* recorded in hanging drops, 96% of them reproduced *via endotokia matricida*. *Endotokia matricida* is a common phenomenon in rhabditid nematodes that occurs when juveniles start hatching inside the maternal uterus and plays an important role in nematode reproduction (Johnigk & Ehlers, 1999; Chen & Caswell-Chen, 2004; Ciche *et al.*,

2008). It secures the development of DJ with enough fat reserves allowing them to survive for long periods under unfavourable environmental conditions. Chen & Caswell-Chen (2004) indicated that under extreme starvation intra-uterine originated offspring of *C. elegans* managed to develop into DJ, whilst extra-uterine originated offspring were unable to develop into DJ under similar conditions.

*Endotokia matricida* was believed to be caused by depletion of food (Johnigk & Ehlers, 1999). However, provision of fresh food every day in hanging drops did not extend the lifespan of *S. yirgalemense*. Similar to the two strains of *S. riobrave* (Addis *et al.*, 2014) and many other *Steinernema* spp. and *H. bacteriophora*, all assessed in hanging drops (Addis *et al.*, 2015), the life of all reproductive females of *S. yirgalemense* ended due to *endotokia matricida*, irrespective of bacterial density, which fixes their lifespan to about 1 week. Ciche *et al.* (2008) reported that both hermaphrodites and females of *H. bacteriophora* always produce a certain proportion of their offspring *via endotokia matricida*. Although DJ are not produced only *via endotokia matricida*, entrance of females and hermaphrodites into *endotokia matricida* seems to be part of their LHT.

Results of the liquid culture production indicate that the percentage of females that entered into *endotokia matricida* at 72 h post DJ inoculation differs according to the culture temperature. At the higher incubation temperatures the percentage of females with *endotokia matricida* was lower than at the optimum temperature (25°C). Lower percentage of *endotokia matricida* at higher incubation temperatures might be associated with male sterility or some behavioural changes that may prevent successful mating.

Offspring production and the final DJ yield depend on the body size of nematodes (both maternal nematodes and DJ), DJ inoculum density, DJ recovery, nematode species, media components and process parameters (Shapiro-Ilan *et al.*, 2012). Based on the body volume of the females and DJ of *S. yirgalemense*, one can expect to obtain comparable offspring numbers with *S. riobrave* and higher numbers of offspring than with *S. feltiae* (Cabanillas *et al.*, 1994; Nguyen *et al.*, 2004). However, the number of offspring per female of *S. yirgalemense* recorded in hanging drops is considerably lower when compared with the two strains of *S. riobrave* and *S. feltiae* with 1355 and 589 offspring per female, respectively (Addis *et al.*, 2014, 2015). As a result of lower offspring numbers and a relatively higher generation time, the intrinsic rate of natural increase ( $r_m$ ) obtained in hanging

drops of *S. yirgalemense* (0.89 days) is lower compared with *S. riobrave* and *S. feltiae* (Addis et al., 2014, 2015).

*Steinernema yirgalemense* can reach quite high DJ yields (284 114 DJ ml<sup>-1</sup>) at 25°C within the same process time and incubation temperature when compared to *S. carpocapsae* (135 000 ml<sup>-1</sup>) and *S. feltiae* (105 000 ml<sup>-1</sup>) (Hirao & Ehlers, 2009c) and comparable results with *S. riobrave*, which gave more than 300 000 DJ ml<sup>-1</sup> (Addis, unpubl. results). Ferreira et al. (2015) reported remarkably low DJ yield (75 000 DJ ml<sup>-1</sup>) in monoxenic liquid culture at similar incubation temperature and process time with a slight difference in the amount of some components of NLM. In this study, incubation of pre-culture bacteria was done at the respective temperatures, whereas Ferreira et al. (2015) pre-cultured the symbiotic bacteria at 30°C for 48 h and after introduction of DJ the incubation temperature was reduced to 25°C. Pre-culturing the bacteria at 30°C may have an effect on the subsequent growth and development of this nematode and could be one of the reasons for the difference in DJ yield. On the other hand, continuous production of *S. yirgalemense* and *S. riobrave* in liquid culture has increased the DJ yield over the reproduction cycles, which might be due to adaptation and selection (Addis, pers. obs.).

The presence of a low number of non-DJ stages at the end of the culture process at 25°C makes further steps during downstream processing easier. Hirao & Ehlers (2009a) also reported that the temperature that supported a maximum DJ yield of *S. carpocapsae* and *S. feltiae* had the lowest proportion of non-DJ stages. Non-reproductive females survive for long periods, which increases the population of females at the end of the culture process. In hanging drops it was observed that ca 5% of females of *S. yirgalemense* that had failed to produce any offspring survived about 2 weeks (data not shown). Similar observations were reported both in *S. riobrave* (Addis et al., 2014) and *Panagrolaimus* sp. (Ayub et al., 2013). Non-DJ stages cannot survive for a long time in formulated products and when dead they create a favourable environment for the growth of contaminants that reduce the shelf life of nematode biocontrol products. Therefore, the presence of a higher proportion of non-DJ stages at the time of harvesting negatively affects the quality and the cost of a final product due to harvesting and subsequent cleaning steps (Ehlers, 2001; Shapiro-Ilan et al., 2012).

Both storage temperature and duration impact the performance of EPN in the field by directly affecting the DJ and their associated symbiotic bacteria. At 4°C none of

the DJ survived longer than 42 days, whereas nearly no mortality was observed at 15°C and 25°C until 66 days. Preliminary assessments with DJ in clay formulated products, which were kept at room temperature for 1 month, recorded 88% survival (data not shown), which needs further quantification using controlled storage temperatures. *Steinernema yirgalemense* was first collected and identified from the tropics (Nguyen et al., 2004) and, as expected, the DJ cannot tolerate low temperatures, although some nematodes have a specific thermal niche breadth regardless of their geographical origin, e.g., *S. riobrave*, reported from the tropics, tolerates lower temperatures (Grewal & Peters, 2005; Shapiro-Ilan et al., 2014). Recently Hill et al. (2015) reported that 50% of *S. yirgalemense* DJ survived 40.8°C for 1 h. Survival and virulence of *S. yirgalemense* DJ in formulated products need further investigation in order to optimise storage temperature.

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**Annex 4: Publication 4**

**Life History Traits of the Entomopathogenic Nematode *Heterorhabditis bacteriophora***

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# Life History Traits of the Entomopathogenic Nematode *Heterorhabditis bacteriophora*

--Manuscript Draft--

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<b>Article Type:</b>	Research Article
<b>Full Title:</b>	Life History Traits of the Entomopathogenic Nematode <i>Heterorhabditis bacteriophora</i>
<b>Short Title:</b>	Life History Traits of <i>Heterorhabditis bacteriophora</i>
<b>Corresponding Author:</b>	Temesgen Addis Christian-Albrechts University Kiel Kiel, GERMANY
<b>Keywords:</b>	amphimictic females, dauer juvenile, endotokia matricida, hanging drop, hermaphrodites, lifespan, mating, offspring production
<b>Abstract:</b>	<p>Entomopathogenic nematodes (EPNs) <i>Heterorhabditis</i> and <i>Steinernema</i> spp. are highly effective biological control agents used against insect pests in cryptic environments. They are associated with symbiotic bacteria of the genera <i>Photorhabdus</i> and <i>Xenorhabdus</i>, respectively. The bacteria, embedded in the third stage dauer juvenile (DJ), are released into the haemocoel of host insects after invasion and provide the food for the nematode. DJs develop into self-fertilizing hermaphrodites, which lay eggs until juveniles hatch inside the uterus and feed on the body content of the mother (endotokia matricida). The Life History Traits (LHTs) of <i>H. bacteriophora</i> were studied at <math>2.5 \times 10^9</math>, <math>5 \times 10^9</math> and <math>20 \times 10^9</math> cells per ml of <i>P. luminescens</i> at 25°C in semi-solid nematode growth gelrite (NGG) medium using a hanging drop technique, which allows assessing individual nematodes. The number of offspring produced per hermaphrodite increased from 50 at <math>2.5 \times 10^9</math> cells per ml to 269 at <math>20 \times 10^9</math> cells per ml of <i>P. luminescens</i>. This increase resulted from a strong positive correlation (<math>R = 0.88</math>) between an increasing hermaphrodite body volume and offspring production. The bacterial density did not influence the beginning of endotokia matricida, hermaphrodite death, DJ release from the maternal carcass and the percentage of juveniles obtained through endotokia matricida. On average, hermaphrodites survived about 8 days starting from the J1 stage, regardless of the bacterial density. The life span was limited due to the start of endotokia matricida. The adulthood of non-reproductive females lasted significantly longer (6.3 days) compared to reproductive females (4.2 days) and self-fertilizing hermaphrodites (4.4 days). Unlike in <i>Caenorhabditis elegans</i>, mating of automictic hermaphrodites of <i>H. bacteriophora</i> could not increase offspring production and survival. Amphimictic females of <i>H. bacteriophora</i> produce lower numbers of offspring (52 individuals per female) and a higher percentage of the offspring (98%) are obtained via endotokia matricida, compared to hermaphrodites (50%). Endotokia matricida is an obligatory developmental step in <i>Heterorhabditis</i> spp. whereas it is facultative in <i>C. elegans</i>.</p>
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1 **Life History Traits of the Entomopathogenic Nematode**  
2 ***Heterorhabditis bacteriophora***

3

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12

13 **Abstract**

14 Entomopathogenic nematodes (EPNs) *Heterorhabditis* and *Steinernema* spp. are highly  
15 effective biological control agents used against insect pests in cryptic environments.  
16 They are associated with symbiotic bacteria of the genera *Photorhabdus* and  
17 *Xenorhabdus*, respectively. The bacteria, embedded in the third stage dauer juvenile (DJ),  
18 are released into the haemocoel of host insects after invasion and provide the food for  
19 the nematode. DJs develop into self-fertilizing hermaphrodites, which lay eggs until  
20 juveniles hatch inside the uterus and feed on the body content of the mother (*endotokia*  
21 *matricida*). The Life History Traits (LHTs) of *H. bacteriophora* were studied at  $2.5 \times$ ,  $5 \times$ ,  
22  $10 \times$  and  $20 \times 10^9$  cells ml<sup>-1</sup> of *P. luminescens* at 25°C in semi-solid nematode growth  
23 gelrite (NGG) medium using a hanging drop technique, which allows assessing  
24 individual nematodes. The number of offspring produced per hermaphrodite increased  
25 from 50 at  $2.5 \times 10^9$  cells ml<sup>-1</sup> to 269 at  $20 \times 10^9$  cells ml<sup>-1</sup> of *P. luminescens*. This  
26 increase resulted from a strong positive correlation ( $R = 0.88$ ) between an increasing  
27 hermaphrodite body volume and offspring production. The bacterial density did not  
28 influence the beginning of *endotokia matricida*, hermaphrodite death, DJ release from  
29 the maternal carcass and the percentage of juveniles obtained through *endotokia*  
30 *matricida*. On average, hermaphrodites survived about 8 days starting from the J1 stage,  
31 regardless of the bacterial density. The life span was limited due to the start of *endotokia*  
32 *matricida*. The adulthood of non-reproductive females lasted significantly longer (6.3  
33 days) compared to reproductive females (4.2 days) and self-fertilizing hermaphrodites  
34 (4.4 days). Unlike in *Caenorhabditis elegans*, mating of automictic hermaphrodites of *H.*  
35 *bacteriophora* could not increase offspring production and survival. Amphimictic  
36 females of *H. bacteriophora* produce lower numbers of offspring (52 individuals per  
37 female) and a higher percentage of the offspring (98%) are obtained *via endotokia*

38 *matricida*, compared to hermaphrodites (50%). *Endotokia matricida* is an obligatory  
39 developmental step in *Heterorhabditis* spp. whereas it is facultative in *C. elegans*.

40

## 41 **Introduction**

42 Entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema*  
43 are commercially produced to control insect pests in cryptic environments [1,2]. These  
44 biocontrol agents are engaged in a relationship with bacterial symbionts in the genera  
45 *Photorhabdus* and *Xenorhabdus*, respectively [3]. EPNs attract attention globally because  
46 of health and environmental concerns over the use of synthetic pesticides [2]. Until  
47 today, the number of described EPN species in the genera *Heterorhabditis* and  
48 *Steinernema* has increased to 20 and 90, respectively. Important beneficial traits of EPNs  
49 such as virulence, reproductive potential and response to different environmental  
50 stresses are limited to few species/strains. For more efficient uses of EPNs, efforts are  
51 made to select EPN species, strains and inbred lines in order to improve some of their  
52 beneficial traits through selection and breeding [4-6].

53 *Heterorhabditis bacteriophora* is among the few EPN species currently produced  
54 at industrial scale [7]. The developmentally arrested third stage dauer juveniles (DJs)  
55 are used in biocontrol and can be applied with conventional spraying equipment. The  
56 DJs carry cells of their symbiont in the intestine, which they release into the haemocoel  
57 of infested insects. The bacteria kill the host within 1-2 days and the nematodes feed on  
58 the bacteria and reproduce. When the insect cadaver is consumed, DJs develop, which  
59 leave the cadaver on search for other host insects [1,2]

60           The life cycle, mode of reproduction, the influence of food on the developmental  
61 pathways and the phenomenon of the *endotokia matricida* (development of juveniles  
62 inside the body of the mother leading to maternal death) of *H. bacteriophora* have been  
63 studied in liquid culture [8-10]. However, these studies focused on a population level.  
64 For the assessment of life history traits (LHTs) of *H. bacteriophora* we observed single or  
65 pairs of parental nematodes in semi-solid hanging media drops supplemented with  
66 bacterial cells. The method allows observing the nematode's reproductive potential  
67 through delivery of fresh food every day. Data generated based on the LHT assessment  
68 in hanging drops can be extrapolated to liquid culture and used as a basic reference for  
69 further improvement and management of commercial liquid culture [11,12]. It can also  
70 help to develop and parameterize a simple model to estimate DJ yield at the end of the  
71 process cycle. Such decision support systems help to improve organization of mass  
72 production.

73           *Heterorhabditis bacteriophora* produces many of its offspring via *endotokia*  
74 *matricida*. Juveniles hatch in the uterus, feed on the content of the maternal body and  
75 develop to DJs. During this process, the symbiotic bacteria are transferred to the DJ  
76 progeny [10]. It has been reported that low bacterial density enhances the beginning of  
77 *endotokia matricida* in *Heterorhabditis* spp. [8]. However, recent studies on *Steinernema*  
78 spp. report that *endotokia matricida* occurrence is not only related to food availability  
79 [12-14]. In order to clearly investigate the role of food on *endotokia matricida* in *H.*  
80 *bacteriophora*, individual hermaphrodites were observed at variable bacterial densities.

81           In insect hosts and in artificial media, DJs of *H. bacteriophora* always develop in to  
82 hermaphrodites. The offspring obtained from laid eggs of hermaphrodites can develop  
83 into males and females, which undergo cross-fertilization and produce offspring. Due to

84 the Y-type copulation behavior of heterorhabditids, attachment of the male to the female  
85 vulva is necessary. In liquid media males fail to attach to the female and thus fertilization  
86 is not possible. Hence, only self-fertilized hermaphrodites reproduce in liquid media [9].  
87 Although mating is not an important parameter for *H. bacteriophora* liquid culture  
88 production, knowledge of its effect on offspring production and survival helps to  
89 generate valuable scientific information in the area of breeding and genetic studies.  
90 Therefore, the objectives of this study also were to investigate the effect of mating of the  
91 automictic hermaphrodite and the amphimictic female on offspring production. Data  
92 were compared with LHT results obtained for *Steinernema* spp.

93

## 94 **Materials and Methods**

### 95 Nematode and symbiotic bacterium

96 The commercial strain of *H. bacteriophora* (strain EN01) was used and its symbiotic  
97 bacterium *P. luminescens* isolated from infected insects.

### 98 Monoxenic cultures

99 Monoxenic cultures were produced from sterilized eggs [15]. Mass production in shaken  
100 flasks was done in nematode liquid medium (NLM) [16]. Prior to inoculation with  
101 ~4,000 DJs ml<sup>-1</sup>, *P. luminescens* had been incubated at 180 rpm (rotation diam. 4 cm) at  
102 25°C for 24 h. After 15 days post DJ inoculation, the cultures were stored at 4°C on a  
103 shaker at 70 rpm (rotation diam. 2 cm) for further experiments.

### 104 Life history trait assessment

105

106 Life history traits (LHTs) of *H. bacteriophora* were studied using semi-solid nematode  
107 growth gelrite (NGG) medium in hanging drops on inverted lids of multi-well plates [17].  
108 The basic medium comprises of (in g l<sup>-1</sup>): 1 peptone from casein, 3 NaCl and 1.5 Gellan  
109 Gum (Gelrite; SERVA, Heidelberg, Germany). After the autoclaved medium had cooled  
110 down to 55°C, autoclaved solutions at 1 ml of a 147 g l<sup>-1</sup> of CaCl<sub>2</sub>×2 H<sub>2</sub>O, 1 ml of 246.6 g  
111 l<sup>-1</sup> MgSO<sub>4</sub>×7 H<sub>2</sub>O and 25 ml of 136 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> buffer at a pH of 6 were added. Finally, 1  
112 ml of filter-sterilized 1 g l<sup>-1</sup> cholesterol suspended in ethanol (> 99%) was added. Solid  
113 NGG was prepared similarly except that the concentration of peptone were increased to  
114 2.5 g l<sup>-1</sup> and gelrite to 3 g l<sup>-1</sup> in the basic preparation of solid NGG [17]. Symbiotic  
115 bacteria propagation, harvesting and cell density quantification was done using the  
116 procedure developed for *S. riobrave* [13]. *Photorhabdus luminescens* was propagated in  
117 NLM for 24 h and the suspension was centrifuged at 3,939 *g* for 10 min. at 4°C. The  
118 supernatant was discarded and bacterial pellets were re-suspended and washed with  
119 sterilized K-medium (containing 3.1 g l<sup>-1</sup> NaCl and 2.4 g l<sup>-1</sup> KCl). Centrifugation was  
120 repeated at the same speed for 5 min. Bacterial pellets were re-suspended using semi-  
121 solid NGG. Cell densities were determined by counting samples in a Thoma chamber  
122 (0.01 mm depth; Poly Optic) and the required bacterial densities of 2.5 ×, 5 ×, 10 × and  
123 20 × 10<sup>9</sup> cells ml<sup>-1</sup> were obtained through serial dilution with semi-solid NGG. Semi-  
124 solid NGG-bacterial suspensions were stored at 4°C for no longer than 3 days before use.

125 To produce nematodes for transfer into hanging drops they were grown on NGG  
126 Petri dishes (100 × 15 mm, filled with ~25 ml medium) seeded with 2 ml of 20 × 10<sup>9</sup>  
127 cells ml<sup>-1</sup> of *P. luminescens* and incubated at 25°C for 24 h. Thereafter, ~4,000 DJs per  
128 plate were inoculated from monoxenic cultures and incubated at 25°C. About 40 h post  
129 DJ inoculation, young adult hermaphrodites were transferred individually from NGG



130 plates into 10  $\mu$ l drops of *P. luminescens*–semi-solid NGG mixture. Drops were kept on  
131 the inner side of the lids of multi-well plates with 12 wells of 2.2 cm diam. (Greiner  
132 665102; Greiner Bio-One). The bottom of each well had been covered with approx. 140  
133 mg dry cellulose tissue paper that was wetted with 650  $\mu$ l of water in order to keep the  
134 relative humidity in the multi-well plates and prevent evaporation from the hanging  
135 drops. After introduction of the nematodes, the multi-well plates were sealed with  
136 parafilm and kept at 25°C in airtight boxes. Every 24 h, hermaphrodites were  
137 transferred into fresh bacterial drops and the plates containing old drops were kept for  
138 another 24 h for hatching of juveniles. Transfer of hermaphrodites into new drops was  
139 continued until the final hermaphrodite had died. The experiment was conducted three  
140 times with different batches of nematodes.

141 Offspring production of each hermaphrodite was determined based on hatched  
142 juveniles from laid eggs and offspring, which had developed *via endotokia matricida*.  
143 Counting of the offspring produced in each drop was done by adding 10  $\mu$ l of Rose  
144 Bengal solution (300  $\mu$ g ml<sup>-1</sup>; Sigma-Aldrich). Then, drops were covered with a circular  
145 18-mm diam. cover slip (Thermo Scientific) and examined under a dissecting  
146 microscope (Zeiss Stemi SV11). Counting was facilitated by putting a grid underneath of  
147 the multi-well lid. Percentage of offspring developed *via endotokia matricida* (intra-  
148 uterine) and extra-uterine was determined.

#### 149 Hermaphrodite body size

150 Effect of bacterial density on hermaphrodite body size was assessed by transferring a  
151 pre-adult hermaphrodites obtained from NGG plates into hanging drops with 2.5  $\times$ , 5  $\times$ ,  
152 10  $\times$  and 20  $\times$  10<sup>9</sup> cells ml<sup>-1</sup> of *P. luminescens* and incubated at 25°C. Every day, the

153 nematodes were transferred into fresh bacterial food drops and the total body length  
154 and width were measured at the beginning of *endotokia matricida* using a dissection  
155 microscope and an attached camera. The measurements were facilitated by use of the  
156 image analysis software analySIS® (Soft Imaging Systems) and the body volume of  
157 hermaphrodite was calculated using the formula  $V = (L \times D^2)/1.7$  [18], where, V =  
158 volume, L = body length and D = maximum body diameter.

#### 159 Influence of mating on offspring production and survival

160 As mating of *Heterorhabditis* spp. is impossible in semi-solid NGG or any other liquid  
161 medium[9] a special medium had to be prepared to enable copulation of males with  
162 hermaphrodites and females. A drop of 300  $\mu\text{l}$  of solid NGG on the inner side of lid of  
163 multi-well plates was topped with 10  $\mu\text{l}$  of a  $20 \times 10^9$  cell  $\text{ml}^{-1}$  suspension of semi-solid  
164 NGG- *P. luminescens* (Fig. 1). This solid arena with a thin layer of liquid bacterial  
165 suspension provided a favorable environment for mating. One pre-adult hermaphrodite  
166 from NGG plates about 36 h post DJ inoculation was then combined with one male  
167 nematode also obtained from NGG dishes. To assess the reproductive potential of  
168 amphimictic adults, pairs of single female and male in a drop were observed. For  
169 comparison, single hermaphrodites and single females were introduced into the drops  
170 individually.

171 After introduction of the nematodes, the multi-well plates were sealed with  
172 parafilm and kept at 25°C in airtight boxes. Daily transfer of nematodes to fresh drops  
173 was continued until all the maternal nematodes had died. Upon death of male  
174 nematodes, new males were introduced at the time of the daily transfer. Offspring  
175 production and survival of hermaphrodites and females was assessed by counting

176 hatched J1 juveniles, which had been transferred into counting chambers under an  
177 inverted microscope (Zeiss). This experiment was repeated three times with different  
178 batches of nematodes.

#### 179 Statistical analysis

180 From the data obtained in hanging drop experiments the LHT analysis of *H.*  
181 *bacteriophora* was calculated based on the Euler or Lotka equation [19] (Table 1) and  $r_m$   
182 was calculated using Microsoft Excel [13]. Total fertility rate (TFR), net reproductive  
183 rate ( $R_0$ ) and different mean generation times ( $T_0$ ,  $T_1$ ,  $T$ ) were calculated on the basis of  
184 the  $l_x$  and  $m_x$  values generated from the raw data (data not shown). For calculation of  $m_x$   
185 and  $l_x$ , the age of *H. bacteriophora* hermaphrodites at egg laying in hanging drops was  
186 assessed on NGG plates prior to the start of hanging drop study. Some surface sterilized  
187 eggs were introduced into NGG Petri plates coated with 2 ml of  $20 \times 10^9$  cells ml<sup>-1</sup> of *P.*  
188 *luminescens* and incubated at 25°C until the hatched J1 developed into an egg-laying  
189 hermaphrodite. Egg laying started when hermaphrodites were 4.5 days old on average.

190 Prior to analysis of variance (ANOVA), percentages were arcsine transformed.  
191 The influence of bacterial density on offspring production and the different life cycle  
192 parameters were analyzed using a one way ANOVA and a post hoc Tukey's HSD test at  $P$   
193  $\leq 0.05$ . Similarly, ANOVA was implemented to analyze the influence of mating on  
194 offspring production and survival. The relationship of bacterial density with female body  
195 volume was assessed using the Pearson correlation test at  $P \leq 0.05$ .

196

197 **Results**

198 Influence of bacterial density on the LHTs of *H. bacteriophora*

199 Increasing bacterial density significantly influenced the average number of offspring  
 200 produced per hermaphrodite ( $F = 12.163$ ;  $df = 3, 8$ ;  $p = 0.002$ ) (Fig. 2A). The lowest  
 201 offspring production with an average of 50 offspring per hermaphrodite was recorded at  
 202  $2.5 \times 10^9$  cells ml<sup>-1</sup> and the highest with 269 at  $20 \times 10^9$  cells ml<sup>-1</sup> (Fig. 2A).

203 Although the bacterial density had a positive influence on the total number of  
 204 offspring produced per hermaphrodites, the percentage of offspring originating from  
 205 *endotokia matricida* was not significantly different among bacterial densities ( $F = 1.273$ ;  
 206  $df = 3, 8$ ;  $p = 0.347$ ) (Fig. 2B). However, the highest percentage of intra-uterine offspring  
 207 production (82%) was found at  $2.5 \times 10^9$  cells ml<sup>-1</sup> and it decreased towards the highest  
 208 bacterial density (64%). As indicated in Fig. 2A, the bacterial density had a significant  
 209 positive effect on the Total Fertility Rate (TFR) ( $F = 13.44$ ;  $df = 3, 8$ ;  $p = 0.002$ ) and Net  
 210 Reproductive Rate ( $R_0$ ) ( $F = 12.14$ ;  $df = 3, 8$ ;  $p = 0.002$ ) (Table 2).

211 Similar to offspring production, the population growth rate ( $r_m$ ) was significantly  
 212 affected by bacterial density ( $F = 6.558$ ;  $df = 3, 8$ ;  $p = 0.015$ ) (Table 2). At  $2.5 \times 10^9$  cells  
 213 ml<sup>-1</sup> the  $r_m$  was 0.54 day<sup>-1</sup> and increased to 0.89 day<sup>-1</sup> at the highest bacterial density.  
 214 The different mean generation times ( $T_0$ ,  $T_1$  and  $T$ ) were not significantly affected by the  
 215 bacterial density ( $p \leq 0.05$ ). However, the population doubling time (PDT) decreased  
 216 with an increasing bacterial density from 1.39 days to 0.78 days.

217 The lifespan of all hermaphrodites of *H. bacteriophora* ended due to *endotokia*  
 218 *matricida*. It was not significantly different between bacterial densities ( $F = 0.163$ ,  $df = 3$ ,

219 8;  $p = 0.919$ ) (Table 2). On average, hermaphrodites survived about 8 days starting from  
220 the J1 stage, regardless of the bacterial density.

221 Bacterial density had no effect on the start of the *endotokia matricida* ( $F = 0.254$ ;  
222  $df = 3, 8$ ;  $p = 0.856$ ), time of the parental hermaphrodite death ( $F = 0.254$ ;  $df = 3, 8$ ;  $p =$   
223  $0.857$ ) and time of DJ release from the carcass of the hermaphrodite ( $F = 0.695$ ;  $df = 3, 8$ ;  
224  $p = 0.580$ ) (Fig. 3). The life of all hermaphrodites ended due to *endotokia matricida* after  
225 4 days of adulthood. DJ emigration from the maternal carcass started on average two  
226 days after the beginning of the *endotokia matricida* and was similar in all bacterial  
227 densities tested.

#### 228 Hermaphrodite body size

229 A strong positive correlation ( $R = 0.88$ ;  $p = 0.0002$ ) between hermaphrodite body  
230 volume and offspring production was observed (Fig. 4). The mean body volume of *H.*  
231 *bacteriophora* hermaphrodites, which was significantly influenced by the bacterial  
232 density ( $F = 14.702$ ;  $df = 3, 8$ ;  $p = 0.001$ ) was on average 0.004, 0.008, 0.013 and 0.019  
233  $\text{mm}^3$  at  $2.5 \times$ ,  $5 \times$ ,  $10 \times$  and  $20 \times 10^9$  cells  $\text{ml}^{-1}$ , respectively (Fig. 4).

#### 234 Effect of mating on offspring production and survival

235 Offspring production between self-fertilizing and mated hermaphrodites was not  
236 significantly different (Fig. 5). However, significantly lower numbers of offspring were  
237 produced by amphimictic second generation females ( $F = 70.363$ ;  $df = 2, 9$ ;  $p = 0.0001$ )  
238 (Fig. 5). The highest number of offspring (349) was recorded from self-fertilizing  
239 hermaphrodites while amphimictic females produced on average only 52 individuals.

240 The percentage of offspring produced through *endotokia matricida* was not  
241 significantly different between self-fertilized and mated hermaphrodites (Fig. 5). In the  
242 two groups of hermaphrodites, less than 50% of the offspring originated from *endotokia*  
243 *matricida*, while females of *H. bacteriophora* produced significantly higher (98%) of  
244 their offspring via *endotokia matricida* (*endotokia matricida*:  $F = 195.857$ ;  $df = 2, 9$ ;  $p =$   
245  $0.0001$ ) (Fig. 5).

246 The adulthood of non-reproductive females lasted significantly longer (6.3 days)  
247 compared to reproductive females, which had a significantly shorter lifespan ( $F =$   
248  $25.760$ ;  $df = 3, 12$ ;  $p = 0.0001$ ) (Fig. 6). Mated hermaphrodites had the shortest mean  
249 adulthood lifespan (3.9 days) followed by mated females (4.2 days) and self-fertilizing  
250 hermaphrodites (4.4 days) (Fig. 6).

251

## 252 Discussion

253 This contribution provides a detailed analysis of the influence of food and mating  
254 on the LHTs of *H. bacteriophora*. For the first time, LHTs of *H. bacteriophora* are reported  
255 and provide an insight into the reproductive biology of this nematode.

256 The number of offspring produced per hermaphrodite of *H. bacteriophora*  
257 increased from 50 to 269 with increasing *P. luminescens* cell density from  $2.5 \times 10^9$  to  $20 \times$   
258  $10^9$  cells  $\text{ml}^{-1}$ . Comparing the maximum number with the maximum offspring production  
259 of *S. feltiae* (maximum 813) and *S. riobrave* (>1,900 individuals per female) [12,13], the  
260 number of offspring per hermaphrodite of *H. bacteriophora* is much lower. These  
261 differences in offspring production at species level are a result of differences in the body

262 volume of maternal nematodes and their respective DJs [12,13,20,21]. For the body  
263 volume of *H. bacteriophora* hermaphrodites a positive correlation with offspring  
264 production was assessed, similar to *S. feltiae* and *S. riobrave* [12,13]. However, the body  
265 volume of *H. bacteriophora* hermaphrodites is lower compared with the female body  
266 volume of *S. feltiae* and *S. riobrave* [12,13] and consequently resulted in lower numbers  
267 of offspring at the same bacterial densities. An even lower number of offspring per  
268 female was recorded for LHTs of *S. yirgalemense* [14]. The lower number should,  
269 however, not be compared with results obtained with *H. bacteriophora* as the bacterial  
270 symbiont *X. indica* of *S. yirgalemense* had been propagated on a medium of  
271 comparatively much lower nutrient concentration (yeast extract only carbon and  
272 nitrogen source), which might have affected the quality of the bacteria and their  
273 potential to support nematode growth and reproduction.

274         Offspring of EPNs originate either from laid eggs or from *endotokia matricida*.  
275 *Heterorhabditis* and *Steinernema* spp. always produce a certain proportion of their  
276 offspring via *endotokia matricida*. In contrast, in *C. elegans* *endotokia matricida* is a  
277 facultative process [22], also induced by the presence of males. *Endotokia matricida* is a  
278 common phenomenon in rhabditid nematodes. It starts when egg laying ceases and  
279 juveniles hatch inside the maternal uterus. They first feed on sperm and then destroy  
280 the uterus tissue. Later the intestine cells lyse providing nutrients to the juveniles.  
281 *Endotokia matricida* leads to the death of the mother and to complete consumption of  
282 the body content by her offspring. It plays a significant role in nematode population  
283 maintenance and survival [8,10,22]. In EPN, *endotokia matricida* can secure offspring  
284 production when scavengers attack the insect cadaver, which would severely endanger

285 development of offspring in the cadaver, while, protected inside the mother's body, they  
286 can terminate development to DJs.

287 During *endotokia matricida*, *P. luminescens* is transmitted to DJs. Cells of the  
288 symbiont are deposited in the rectal gland cells during the fourth juvenile stage from  
289 where they are transported into the maternal body cavity. After juveniles have  
290 destroyed the uterus tissue they can load themselves with the bacterial symbiont cells  
291 during development to the DJ [10].

292 In this study, a significant influence of the bacterial density on the percentage of  
293 offspring produced *via endotokia matricida* was not observed. At  $2.5 \times 10^9$  cells ml<sup>-1</sup> of *P.*  
294 *luminescens*, 82% of *H. bacteriophora* offspring originated from *endotokia matricida* and  
295 the number decreased with increasing bacterial density. However, Johnigk and Ehlers  
296 [8] observed that egg laying ceased immediately when hermaphrodites were transferred  
297 into suspensions without any bacteria, indicating that an influence of the bacterial  
298 density exists. Obviously, the density of  $2.5 \times 10^9$  cells ml<sup>-1</sup> is not low enough to observe  
299 significant effects. A similar decreasing trend was also observed in *S. feltiae* and *S.*  
300 *riobrave* [12,13], although also not significant. In addition, the beginning of *endotokia*  
301 *matricida* and the lifespan of *H. bacteriophora* hermaphrodites was not influenced by  
302 bacterial density, as observed for *Steinernema* spp. [12,13]. Again, the amount of  
303 bacterial cells in hanging drops might have been above the threshold to immediately  
304 induce the *endotokia matricida*, unlike when *H. bacteriophora* hermaphrodites are kept  
305 in bacteria-free solution [8].

306 Regardless of bacterial density, juveniles of *H. bacteriophora* produced through  
307 *endotokia matricida* stay inside the body of the mother for 2 days until they have readily



308 developed into DJs and then exit the maternal carcass. In *S. feltiae*, *S. riobrave* and *S.*  
309 *yirgalemense* pre-dauer juveniles (J2d) leave their maternal carcass before they have  
310 terminated their development to DJs [12-14]. Similar observations have been reported  
311 for other *Steinernema* spp. cultured *in vivo* [24] or *in vitro* [25]. The DJs of *Steinernema*  
312 spp. mature outside of the maternal body, whereas DJs of *H. bacteriophora* can be  
313 observed motionless and straight inside the maternal cuticle.

314 At similar bacterial density the range of  $r_m$  values recorded for *H. bacteriophora*  
315 was between 0.7–0.89 day<sup>-1</sup> and is lower compared with *S. feltiae* and *S. riobrave*, which  
316 grew at a rate of greater than 1.1 day<sup>-1</sup> [12,13]. The growth rate of organisms depends  
317 on their genetic background and environment conditions, particularly food supply and  
318 temperature [26,27]. *Heterorhabditis bacteriophora* have a relatively longer generation  
319 time and slow reproduction rate compared to *S. feltiae* and *S. riobrave* and as a result  
320 have relatively lower  $r_m$  values. In addition to food availability, lower temperature  
321 decreases the growth rate of nematodes as observed in the cold tolerant EPN *S. kraussei*  
322 (Addis, unpublished data). Similarly, the population growth rate of *Panagrolaimus* sp.  
323 (strain NFS-24) was significantly influenced by culture temperature [28]. Therefore, an  
324 optimal incubation temperature in liquid culture increases the growth rate of  
325 nematodes and hence shortens the process time.

326 Amphimictic adults of *Heterorhabditis* spp. have a *bursa copulatrix* enabling them  
327 to attach to the female at the vulva region. The position of the male in relation to the  
328 female during copulation forms a lambda or “y”. *Steinernema* spp. do not possess a *bursa*  
329 and have a helix type of copulation, curling around the female body. Whereas  
330 heterorhabditids are unable to mate in liquid media, steinernematids can inseminate  
331 female also in a liquid environment [9]. Similarly, males of *H. bacteriophora* failed to

332 copulate with both, hermaphrodites and females, in hanging drops. In order to assess  
333 the influence of mating on offspring production and survival on females and  
334 hermaphrodites a new method was developed. A drop of 300  $\mu$ l of solid NGG on the  
335 inner side of the lid of multi-well culture plates was topped with a suspension of semi-  
336 solid NGG- *P. luminescens*. This provided conditions that allowed successful mating in  
337 autotictic and amphimictic females. This method can also be used to conduct crossing  
338 experiments with amphimictic nematodes.

339         Unlike in *C. elegans*, mating had no significant effect on offspring production of *H.*  
340 *bacteriophora* hermaphrodites. In *C. elegans*, self-fertile hermaphrodites are limited in  
341 sperm and offspring production ceases within a maximum of 6 days, while in mated  
342 hermaphrodites offspring production continues up to 12 days [29]. Obviously, the  
343 hermaphrodites of *Heterorhabditis* spp. produce enough sperm to inseminate the eggs  
344 produced during its life cycle. The occurrence of *Endotokia matricida* increases in *C.*  
345 *elegans* due to mating of hermaphrodites and reaches 68% in the hermaphrodite  
346 population [29], whereas in *H. bacteriophora* all reproductive female phenotypes  
347 terminate offspring production *via endotokia matricida*. In *C. elegans* mating  
348 significantly decreases the lifespan of hermaphrodites through induction of *endotokia*  
349 *matricida*, mechanical damage and behavioral changes [29,31,32]. The mean lifespan of  
350 mated and self-fertilizing *C. elegans* hermaphrodite was about a week while those  
351 escaping *endotokia matricida* survived more than two weeks [29]. The adulthood of *H.*  
352 *bacteriophora* hermaphrodites and females lasts less than 4.5 days. The short lifespan of  
353 *H. bacteriophora* hermaphrodites is due to the start of *endotokia matricida*.  
354 *Heterorhabditis bacteriophora* males occur two days after the start of *endotokia*

355 *matricida* in hermaphrodites [30], thus insemination would anyhow not result in high  
356 number of offspring as juveniles would consume the sperm.

357 In conclusion, mating does not increase offspring production as observed in *C.*  
358 *elegans* and *endotokia matricida* is an obligatory developmental step in heterorhabditis  
359 whereas it is mostly facultative in *C. elegans*.

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364

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463 **Table1.** Formulae used to calculate the different life cycle parameters of *Heterorhabditis*  
 464 *bacteriophora* and their description. The Total Fertility Rate (TFR) and Net Reproductive Rate  
 465 ( $R_0$ ) were calculated on the basis of the life table calculated with  $l_x$  and  $m_x$  (data not shown)  
 466 where,  $x$  = time in days,  $l_x$  = age specific survival probability and  $m_x$  = age-specific fecundity

Formulae	Description of the life cycle parameters
$TFR = \sum m_x$	Total fertility rate (TFR): The total number of offspring that would be produced by the hermaphrodite if they were able to survive until the end of their reproductive period.
$R_0 = \sum l_x m_x$	Net reproductive rate ( $R_0$ ): The average number of offspring produced by a hermaphrodite during its life time, dependent on the age specific survival probability and fecundity of the female.
$T_0 = (1/R_0) \sum x l_x m_x$	Also known as the cohort generation time ( $T_c$ ): The mean age at reproduction of a cohort of hermaphrodites.
$T_1 = (\ln R_0) / r_m$	The period of time necessary for a population growing at a constant rate $r_m$ to increase by the factor $R_0$ .
$T = \sum_{x=0}^d x e^{-r_m x} l_x m_x$	The mean age of the mother of newly-hatched individuals in a population with a stable age distribution.
$r_m$	Intrinsic rate of natural increase ( $r_m$ ): The growth rate of a population that has a stable age distribution and grows under an unlimited environment. It was calculated based on the Euler/Lotka equation [19]  $\sum_{x=0}^d e^{-r_m x} l_x m_x = 1$
$PDT = \ln 2 / r_m$	Population doubling time (PDT)

467

468



469 **Table 2.** Life history parameters of *Heterorhabditis bacteriophora* assessed at  $2.5 \times$ ,  $5 \times$ ,  
 470  $10 \times$  and  $20 \times 10^9$  cells ml<sup>-1</sup> of *Photorhabdus luminescens* in hanging drops at 25°C.

Life cycle parameters	Bacterial density (cells ml <sup>-1</sup> )			
	$2.5 \times 10^9$	$5 \times 10^9$	$10 \times 10^9$	$20 \times 10^9$
N	48	47	56	52
TFR	75 ± 50 <sup>a</sup>	146 ± 57 <sup>ab</sup>	280 ± 47 <sup>bc</sup>	336 ± 70 <sup>c</sup>
$R_0$	50 ± 45 <sup>a</sup>	100 ± 40 <sup>ab</sup>	204 ± 37 <sup>bc</sup>	269 ± 69 <sup>c</sup>
$T_0$ (days)	7.43 ± 1.07 <sup>a</sup>	6.66 ± 0.39 <sup>a</sup>	7.37 ± 0.65 <sup>a</sup>	6.96 ± 0.50 <sup>a</sup>
$T_1$ (days)	6.97 ± 1.18 <sup>a</sup>	6.16 ± 0.43 <sup>a</sup>	6.53 ± 0.21 <sup>a</sup>	6.25 ± 0.51 <sup>a</sup>
T (days)	6.7 ± 1.25 <sup>a</sup>	5.58 ± 0.53 <sup>a</sup>	5.69 ± 0.12 <sup>a</sup>	6.32 ± 1.76 <sup>a</sup>
$r_m$ (day <sup>-1</sup> )	0.54 ± 0.18 <sup>a</sup>	0.70 ± 0.09 <sup>ab</sup>	0.81 ± 0.02 <sup>ab</sup>	0.89 ± 0.03 <sup>b</sup>
PDT (days)	1.39 ± 0.48 <sup>a</sup>	1.01 ± 0.13 <sup>ab</sup>	0.85 ± 0.02 <sup>b</sup>	0.78 ± 0.03 <sup>b</sup>
Average lifespan (days)	8.1 ± 0.31 <sup>a</sup>	7.9 ± 0.31 <sup>a</sup>	8 ± 0.21 <sup>a</sup>	8 ± 0.21 <sup>a</sup>

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472 Values are means of three replicates ± standard deviation. Means followed by different  
 473 superscript letters within the same row indicate significant differences (Tukey's HSD  
 474 test,  $P \leq 0.05$ ). N = total number of hermaphrodite assessed; TFR = total fertility rate;  $R_0$   
 475 = net reproductive rate;  $T_0/T_1/T$  = alternative measures of generation time;  $r_m$  = rate of  
 476 natural increase; PDT = population doubling time.

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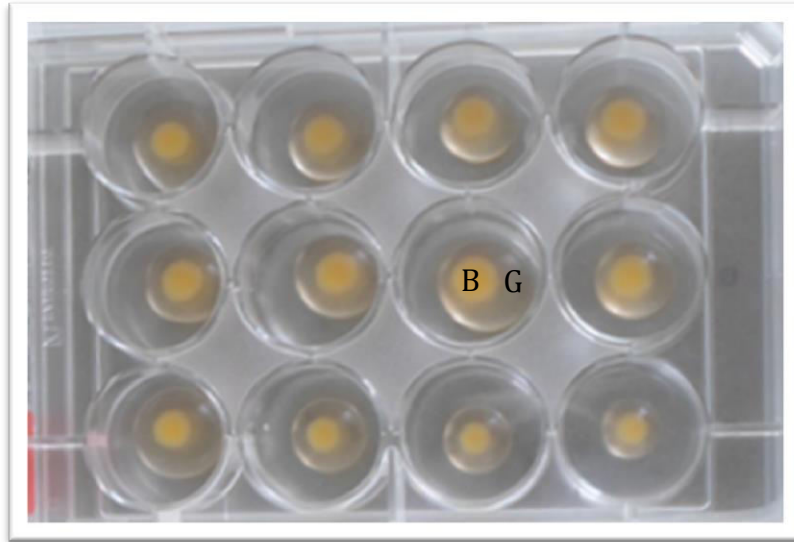
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484 **Fig. 1.** Multi-well plates with solidified 300  $\mu\text{l}$  nematode growth gelrite (G) coated with

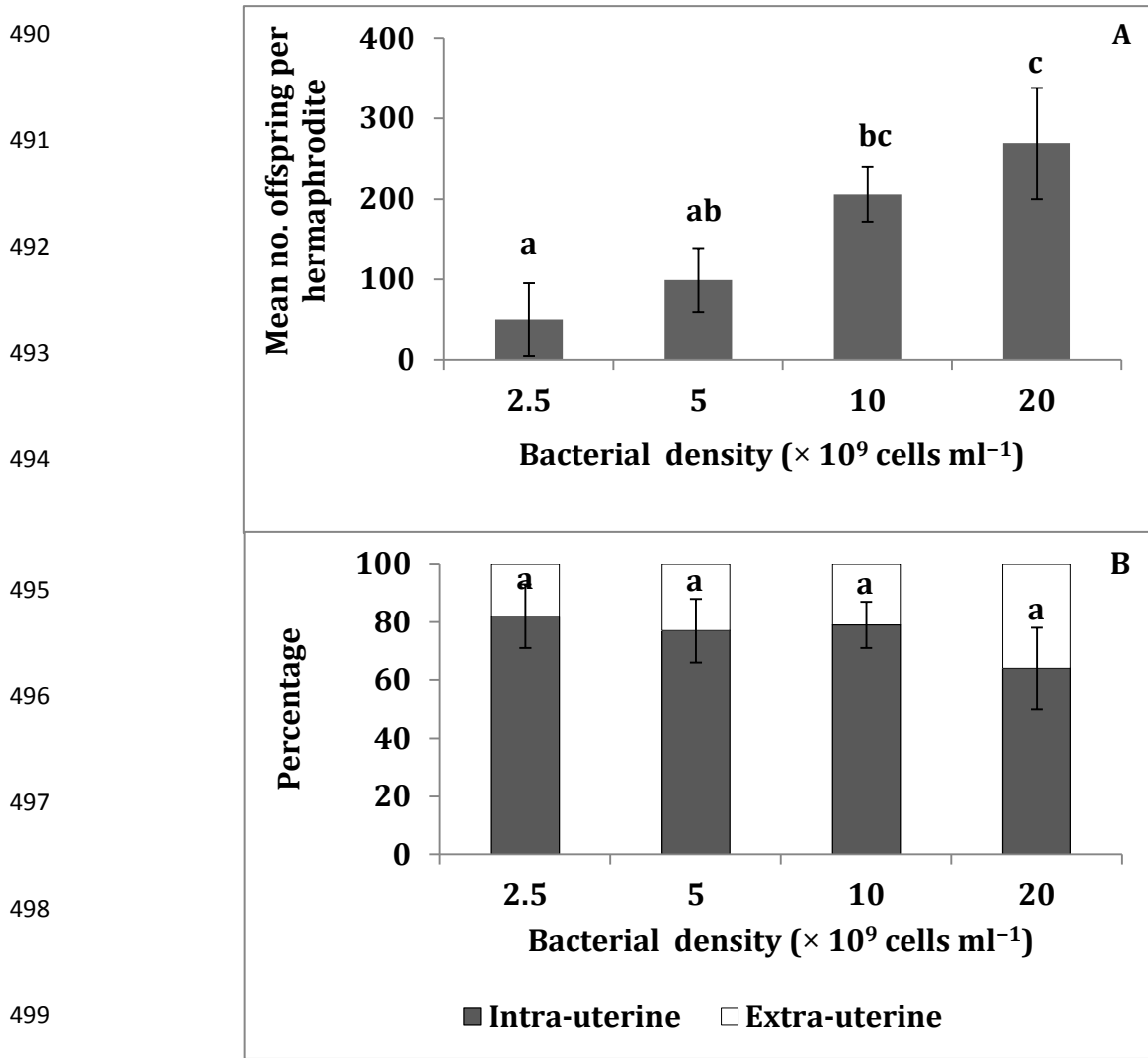
485 10  $\mu\text{l}$  *Photorhabdus luminescens*-semi-solid nematode growth gelrite (B) at a density of

486  $20 \times 10^9$  cell  $\text{ml}^{-1}$  used for assessment of the influence of mating on offspring production

487 and survival of *Heterorhabditis bacteriophora* hermaphrodites and females at 25°C. The

488 drops and the bacteria are on the inner side of the lid.

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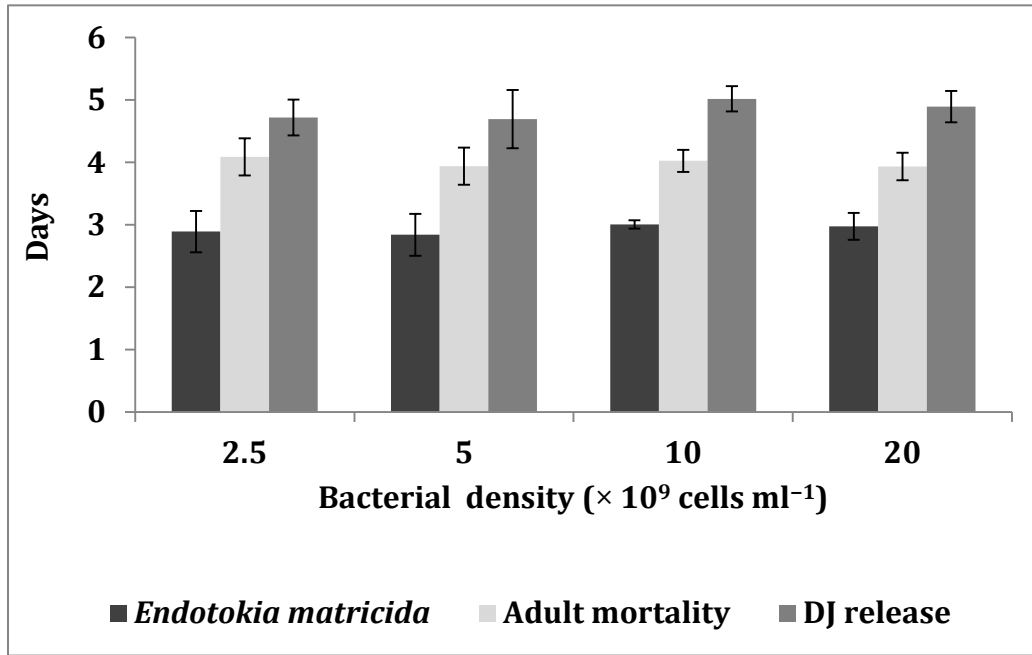
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501 **Fig. 2.** Mean number of offspring (A) and percentage of offspring production (B) per  
 502 hermaphrodite of *Heterorhabditis bacteriophora* originating from laid eggs (extra-  
 503 uterine) and from *endotokia matricida* (intra-uterine) at 25°C in hanging drops at 2.5 ×,  
 504 5 ×, 10 × and 20 × 10<sup>9</sup> cells ml<sup>-1</sup> of *Photorhabdus luminescens*. Different lower case  
 505 letters above error bars indicate significant differences among bacterial densities ( $P \leq$   
 506 0.05).

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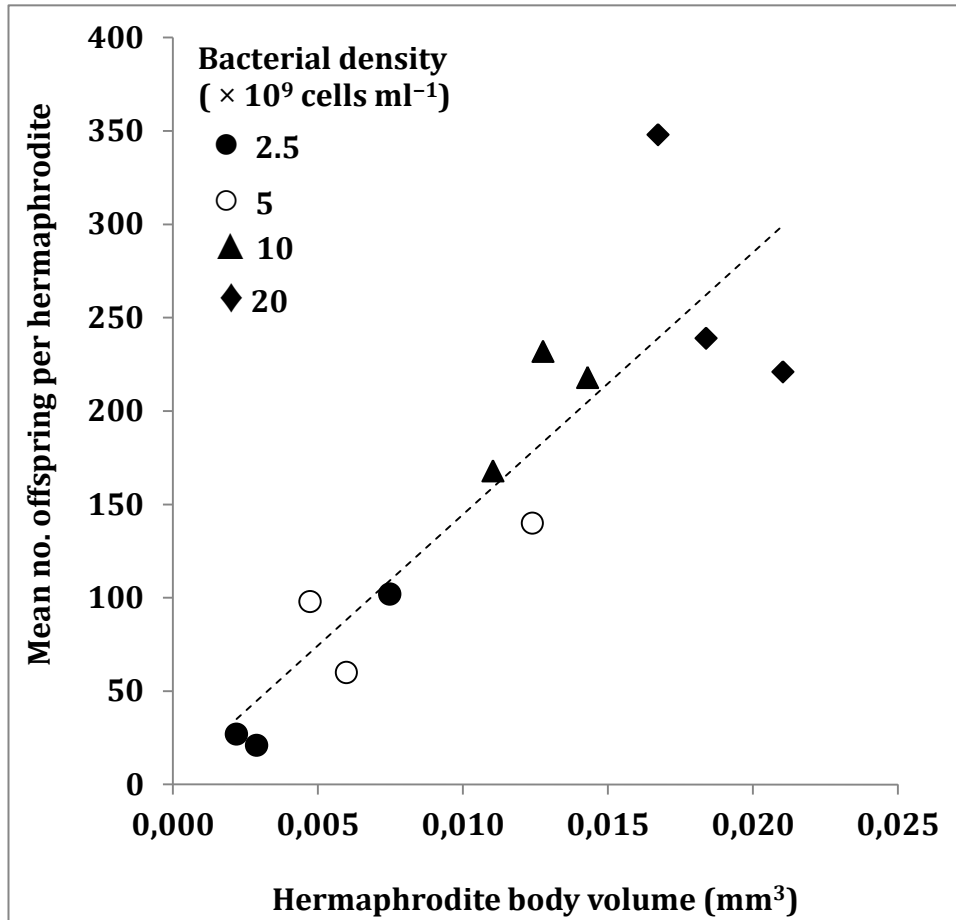
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520 **Fig. 3.** Influence of bacterial density on the occurrence of *endotokia matricida*, adult  
521 mortality and DJ release from maternal carcass of *Heterorhabditis bacteriophora*  
522 hermaphrodites assessed in hanging drops at 2.5  $\times$ , 5  $\times$ , 10  $\times$  and 20  $\times 10^9$  cells ml $^{-1}$  of  
523 *Photorhabdus luminescens* at 25°C. Significant differences were not observed between  
524 bacterial densities ( $P \leq 0.05$ ).

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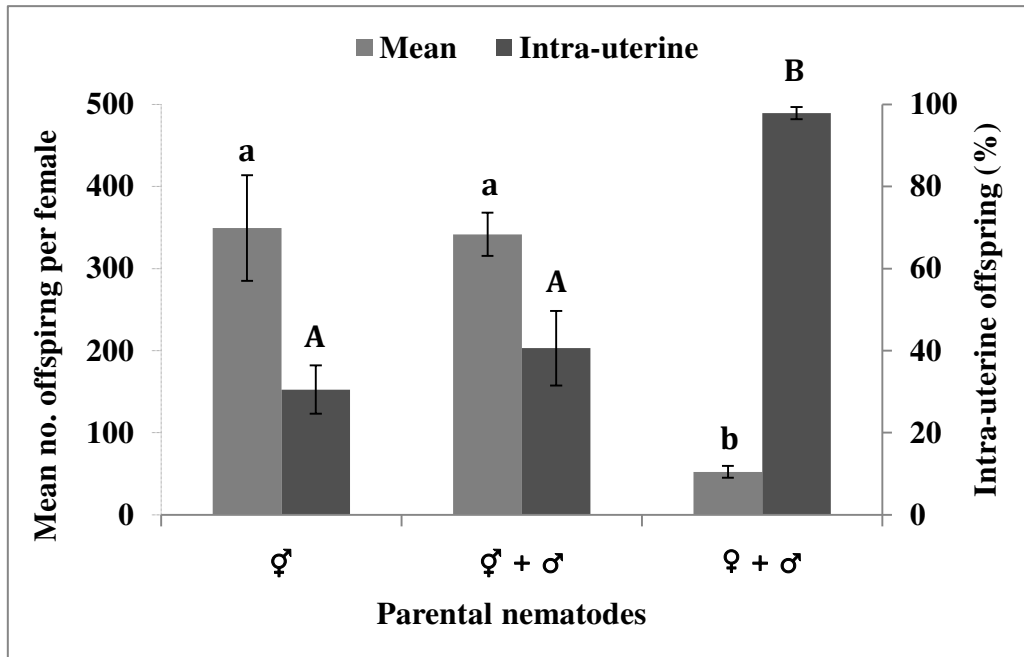


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535 **Fig. 4.** Hermaphrodite body volume of *Heterorhabditis bacteriophora* assessed at 2.5 ×, 5  
536 ×, 10 × and 20 × 10<sup>9</sup> cells ml<sup>-1</sup> of *Photorhabdus luminescens* at 25°C in hanging drops. A  
537 positive correlation between hermaphrodite body volume and offspring production was  
538 observed ( $R = 0.88$ ;  $p = 0.0002$ ).

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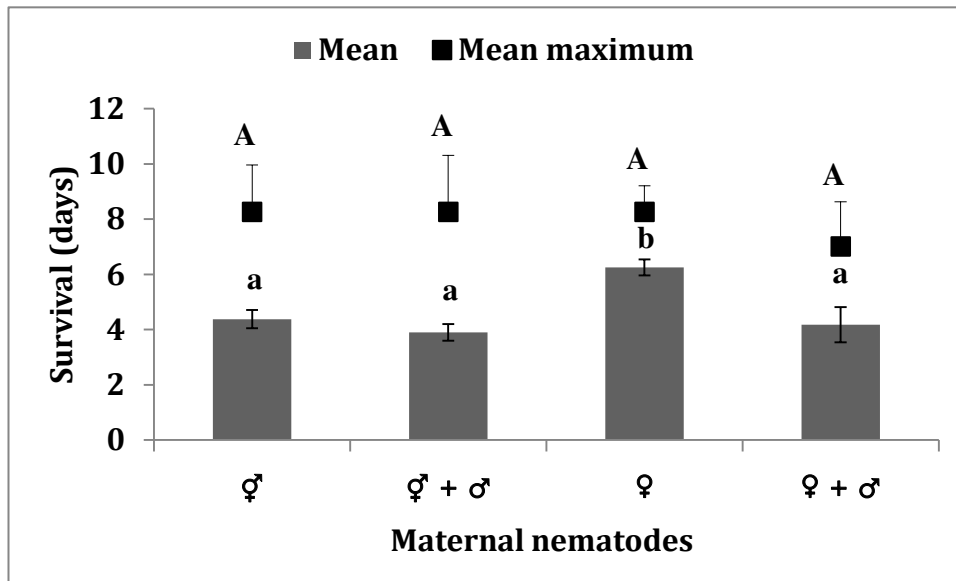
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555 **Fig. 5.** Mean number and percentage of offspring originating from *endotokia matricida*  
556 (intra-uterine) per self-fertilizing hermaphrodite (♀), mated hermaphrodite (♀ × ♂) and  
557 mated female (♀ × ♂) of *Heterorhabditis bacteriophora* strain EN01 assessed in 'egg-  
558 yolk' at 25°C at  $20 \times 10^9$  cells ml<sup>-1</sup> of *Photorhabdus luminescens*. Different lower and  
559 upper case letters above error bars indicate significant differences among parental  
560 nematodes in mean total offspring and those originating from *endotokia matricida*,  
561 respectively (Tukey's HSD test at  $P \leq 0.05$ ).

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570 **Fig. 6.** Mean and mean maximum adult survival of self-fertilizing hermaphrodite (♀),  
571 mated hermaphrodites (♀ × ♂), female (♀) and mated female (♀ × ♂) of *Heterorhabditis*  
572 *bacteriophora* at 25°C assessed on lids of multi-well plates with 300 µl solid NGG  
573 medium coated with 10 µl *Photorhabdus luminescens* at  $20 \times 10^9$  cell ml<sup>-1</sup>. Different  
574 lower case letters above error bars indicate significant differences in survival among  
575 parental nematodes (Tukey's HSD test at  $P \leq 0.0001$ ).

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 Curriculum Vitae
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