Comparison of Life History Table Data with Results from Liquid Culture of *Steinernema yirgalemense*

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Comparison of life history table data with results from liquid culture of *Steinernema yirgalemense*

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**Summary** – Using the hanging drop technique with nematode growth gelrite, the life history traits (LHT) of the entomopathogenic nematode *Steinernema yirgalemense* (strain Sy157-C) was investigated at a bacterial density of $10 \times 10^9$ cells ml$^{-1}$ at $25^\circ$C. With the same technique DJ recovery at three bacterial densities ($5 \times$, $10 \times$ and $20 \times 10^9$ cells ml$^{-1}$) was assessed. Results were compared with data from liquid culture. *S. yirgalemense* was cultivated in liquid culture at three temperatures, $25^\circ$C, $27^\circ$C and $30^\circ$C and DJ recovery, population dynamics, sex ratio and DJ yield were assessed. Yields of *S. yirgalemense* reached 322,520 DJs ml$^{-1}$ at $25^\circ$C. DJ recovery at three bacterial densities ($5 \times$, $10 \times$ and $20 \times 10^9$ cells ml$^{-1}$) in liquid culture was assessed at $25^\circ$C. The DJ survival at 4, 15 and $25^\circ$C was assessed within period of 35 days. The highest survival was recorded at $15^\circ$C (98.6%) followed by $25^\circ$C (96.6%) and much reduced survival at $4^\circ$C (10.3%).

**Keywords:** *endotokia matricida*, hanging drop, nematode growth gelrite, survival
Entomopathogenic nematodes (EPNs) are widely used to control economically important insect pests in different farming systems: from fruit orchards, cranberry bogs and turf grass to nurseries and greenhouses (Grewal et al., 2005; Georgis et al., 2006). The two most important nematode families which established their roles in biological control programs are Heterorhabditidae and Steinernematidae (Stock, 2005).

The most important requirement for successful and economically reasonable usage of EPNs in crop protection is large scale production at low cost within a short process time (Ehlers, 2001; Hirao & Ehlers, 2009a). This can only be achieved under well-defined liquid culture conditions and successful management of nematode population dynamics (Ehlers, 2001). Nowadays, EPNs are produced for commercial purposes by several companies in large liquid fermentation tanks which range from 7,500 up to 80,000 l (Ehlers et al., 1998; Grewal et al., 2005). Several EPN species such as *Heterorhabditis bacteriophora*, *H. indica*, *H. megidis*, *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, *S. riobrave* and *S. scapterisci*, have been mass produced (Smart, 1995; Kaya et al., 2006). However, one of the long term strategies in biological programs based on EPN utilization is exploring new nematode species and investigation of their potential as biocontrol agents.

The EPN *S. yirgalemense* (Rhabditida: Steinernematidae) was isolated for the first time in Yirgalem, Ethiopia and it is classified into the *bicornutum*–group, which is characterized by the presence of two horn-like structures in the labial region of the DJ stage (Nguyen et al., 2004). In several studies, this species showed biological control potential against false codling moth, *Thaumatotibia leucotreta* (Tortricidae) (Malan et al., 2011) and codling moth, *Cydia pomonella*
(Tortricidae) (De Waal et al., 2011). Moreover, it is assumed that *S. yirgalemense* may be used in future against enset root mealybug, *Cataenococcus ensete* - problematic pest of enset (*Ensete ventricosum*, Musaceae), which is traditionally cultured in Ethiopia (Azerefegne et al., 2009). Therefore, it is of crucial importance to collect more information on the biology of *S. yirgalemense* and its bacterial symbiont *Xenorhabdus* sp. Studying the life history table (LHTs), which characterizes age specific growth, reproduction and survival of organisms, allows understanding nematode behavior under different environmental conditions but also conditions of mass production (Roff, 1992; Stearns, 1992). Prior to launching large scale EPN production, it is necessary to perform investigations at small scale in order to obtain information on nematode population growth, offspring production and life span (Hirao et al., 2010). Many investigations have been conducted to assess population dynamics of nematodes and their symbiotic bacteria under liquid culture (de la Torre, 2003; Hirao & Ehlers, 2009a; Hirao & Ehlers, 2010; Hirao et al., 2010). However, these investigations were done at a population level but a study based on individual nematodes may provide a better insight into the fundamental reproductive biology of the worms.

The hanging drop technique using semi-solid nematode growth gelrite (NGG) has been applied to study both, free living nematodes (Muschiol & Traunspurger, 2007; Muschiol et al., 2009; Ayub et al., 2013a) and recently the EPN *S. riobrave* (Addis et al., 2014). The hanging drop method has many practical applications: it allows studying single individuals of the same age by culturing them under unlimited food supply leading to determination of the best bacterial concentration in liquid culture in order to obtain the highest dauer juvenile (DJ) yield. In addition, it allows studying culture conditions (e.g. temperature), DJ recovery, life span and mating
(Muschiol et al., 2009; Ayub et al., 2013b; Addis et al., 2014). Studying single individual
nematodes with a better accuracy allows generating reliable data about LHTs of an organism.

There is no published data on the biology, bacterial symbiont, population development
and reproduction of *S. yirgalemense* both in hanging drops and liquid culture. Therefore, the main
objectives of this study were to obtain information about fundamental LHTs of *S. yirgalemense*
using the hanging drop method and to assess DJ recovery both in hanging drops and in liquid
culture. In addition, the population dynamics of *S. yirgalemense* in monoxenic liquid cultures and
the influence of temperature on the survival during storage of *S. yirgalemense* were investigated.

**Materials and methods**

**SYMBIOTIC BACTERIA AND NEMATODES**

The symbiotic bacteria *Xenorhabdus* sp. were isolated according to Ehlers et al. (1990)
from *Galleria mellonella* larvae (Lepidoptera: Pyralidae), previously infested with 100 DJs per
*G. mellonella* in Petri dish and kept at 25°C in the dark. After 24 h, the infected *G. mellonella*
was transferred into 70% ethanol for 10 seconds for surface sterilization. After sterilization, a
drop haemolymph was taken by piercing the leg of the larvae using a sterile needle. The drop was
streaked on NBTA agar plate (containing 10.0 g tryptic soya broth, 14 g bacto agar 0.025 g
bromothymolblue, and 4.0 ml of a 1% sterile filtrated 2,3,5 – triphenyl – tetracoliumchloride
solution (Akhurst, 1980). The plates were incubated at 25°C for 48 h in the dark.

A single dark blue colony of symbiotic bacteria was transferred from NBTA plate to 100
ml YS medium containing 5.0 g yeast extract, 5.0 g NaCl, 0.5 g NH₄H₂PO₄, 0.5 K₂HPO₄ and 0.2
g MgSO₄×7H₂O dissolved in 1 l of distilled water (Dye, 1968) and propagated at 25 °C on an
orbitary shaker at 180 rpm in the dark. After 24h, bacterial cultures were prepared for long term storage at \(-80^\circ\text{C}\), mixed with glycerol (15% v/v) and transferred to 2 ml sterile Eppendorf caps. *Steinernema yirgalemense* strain Sy 157-C used in this study was obtained by the courtesy of Dr Antoinette P. Malan from Stellenbosch University, South Africa. Monoxenic liquid cultures of *S. yirgalemense* were obtained through modification of Lunau *et al.* (1993): DJs obtained from *G. mellonella* were washed with sterile Ringer’s solution (9.0 g NaCl, 0.42 g KCl, 0.37 g CaCl$_2 \times$ 2H$_2$O, 0.2 g NaHCO$_3$ dissolved in 1 l of distilled water) through centrifugation at 4,500 rpm for two minutes. The supernatant was removed and DJs washed again with the same speed and time. After removing the supernatant \(\sim 4,000\) DJs were transferred to sterile nematode growth gelrite (NGG) plates (1.25 g peptone, 1.5 g NaCl, 1.5 g gelrite, 500 µl CaCl$_2 \times$ 2H$_2$O, 500 µl MgSO$_4 \times$ 7H$_2$O, 12.5 ml KH$_2$PO$_4$, 500 µl cholesterol dissolved with 486 ml of mineral water) pre-cultured with the symbiotic bacteria. Three days after inoculation of DJs, gravid females were collected in sterile Petri dish with sterile Ringer’s solution. The eggs were forced to come out from the female through mechanical damage using sharp scissors. The eggs were passed through 50 µm sieves and collected in 15µl centrifuge tube in order to separate eggs from remaining female body. The eggs were centrifuged for 2 minute at 3,000 rpm. The supernatant was removed and after adding sterile Ringer’s solution, centrifugation was repeated with the same speed and duration. The supernatant was removed and eggs were transferred into sterile Eppendorf caps and 1 ml of sterilization solution (0.5 ml 12% NaOCl, 1.5 ml 4 M NaOH in 10 ml distilled water) was added. The caps were gently shaken and then centrifuged for 2 min at 3,000 rpm. After discarding the supernatant, the caps were filled with sterile YS medium and centrifugation repeated. The eggs were transferred in to sterile multi-well plates with a volume of 250 µl YS medium and incubated

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for 72 hours at 25°C. After checking contamination the juveniles were transferred into Wouts agar plates (16.0 g Bacto Nutrient Broth, 12.0 g Bacto Agar, 5.0 g sun-flower oil, dissolved in 1 l of distilled water), which were previously inoculated with several drops of the symbiotic bacteria grown in YS broth. The plates were sealed in paraffin film and incubated at 25°C in the dark. When the Wouts agar plates were fully covered with DJs, the agar was cut into pieces and transferred in to 100 ml Erlenmeyer flasks containing 20 ml of nematode liquid medium (6.0 g of 1:1 mixture of lecithin and oil, 30.0 g rapeseed oil, 15.0 g yeast extract, 20.0 g soy flour, 4.0 g NaCl, 0.35 g KCl, 0.3 g CaCl$_2$ and 0.2 g MgSO$_4$·7H$_2$O in 1 l of tap water and pH adjusted to 6.7 according the Ehlers et al. (1998)), which had been pre-inoculated with symbiotic bacteria at 1% of the medium volume from 24 h YS broth cultures. Erlenmeyer flasks were placed on a shaker at 180 rpm and cultured for 2 weeks at 25°C in the dark. Afterwards, the DJs were kept at 19°C on a shaker for further experiments.

**ADJUSTMENT OF Xenorhabdus sp. DENSITY IN SEMI-SOLID NEMATODE GROWTH GELRITE (NGG)**

The symbiotic bacterium *Xenorhabdus* sp. was propagated in 100 ml YS for 48 h (500 ml Erlenmeyer flasks). The YS medium containing symbiotic bacteria was divided and poured into two 50 ml sterile centrifuge tubes under sterile conditions and centrifuged at 4,500 rpm for 10 minutes at 4°C. The supernatant was discarded and sterile K–medium (3.1 g NaCl, 2.4 g KCl per 1000 ml tap water) was added (3 times the volume of concentrated bacteria) and the bacteria were re-suspended by vortexing. The centrifugation was repeated for 5 minutes and the supernatant was removed. The centrifuge tubes were then filled with two times the volume of the bacteria with sterile semi-solid NGG (0.5 g casein peptone casein, 1.5 g NaCl, 0.75 g gelrite,
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500 µl CaCl₂·2H₂O, 500 µl MgSO₄·7H₂O, 12.5 ml KH₂PO₄ buffer solution and 500 µl cholesterol per 1 l mineral water). Afterwards a 100 µl bacterial suspension was diluted with 900 µl of 30% ethanol. Bacteria cell counting was done by the use of a Thoma chamber (0.01 mm depth; Poly Optic GmbH) under the phase contrast microscope (Carl Zeiss, Germany). After determining the bacterial cell density, the required bacterial cell densities were adjusted by making dilutions with semi-solid NGG.

**Observation of S. Yirgalemense Life Cycle In Solid NGG Plates**

The sterile solid NGG plates (prepared with 486 ml mineral water, 1.25 g peptone, 1.5 g NaCl, 1.5 g gelrite, 500 µl CaCl₂·2H₂O, 500 µl MgSO₄·7H₂O, 12.5 ml KH₂PO₄ and 500 µl cholesterol) were inoculated with 2 ml bacteria-semi-solid NGG drop mixture at 20 × 10⁹ cells ml⁻¹ and incubated at 25°C in dark. After 24 h, ~ 4,000 DJs were introduced to the NGG plate in order to observe recovery and development under the dissecting microscope. Several gravid females were taken out and washed in sterile Ringer’s prior to dissection. After dissection, eggs were collected and transferred into sterile multi-well plates containing 0.5 ml of YS broth in order to observe juvenile hatching and development. The multi-well plates were sealed and kept at 25°C in the dark and checked daily.
HANGING DROP TECHNIQUE AND ASSESSMENT OF LIFE HISTORY TRAITS

The NGG (nematode growth gelrite) agar plates were pre-inoculated with 2 ml of the symbiotic bacteria (20 × 10⁹ cells ml⁻¹) in semi-solid NGG and incubated at 25°C for 48h and were kept in the dark. After incubation, plates were inoculated with ~ 4,000 DJs and kept in the same condition for additional 36h until the first pre–adult individuals had developed. A single male and female nematode were taken from NGG agar plates and transferred into 10 µl bacteria-NGG mixture drops (10 × 10⁹ cells ml⁻¹) at the inner side of the lids of multi well plates (Fig. 1b). Every well was filled with ~ 14 g paper moisturized with 650 µl water in order to avoid the desiccation of the drops during the experiment (Fig. 1a). Plates were sealed with paraffin film and kept at 25°C in the dark. Every 24h nematodes were provided with fresh bacteria, by transferring them to new drops in new plates. The old plates were sealed as well and kept for 24 – 36 h to allow the juveniles to hatch from laid eggs prior to counting. The process of transferring females and males to fresh drops continued until endotokia matricida or the death of the female occurred. Prior to counting the offspring, every old drop was stained by adding the 10 µl Rose Bengal solution (300 µg ml⁻¹ Sigma-Aldrich). After covering the drops with circular 18 mm cover slips (Thermo Scientific), the offspring were counted under a dissecting microscope (Zeiss Stemi SV11). The offspring originating from laid eggs (extra–uterine offspring) was differentiated from offspring produced during endotokia matricida (intra–uterine offspring) and was recorded separately. For each of the three replicates 113 pairs of female and male nematodes originated from different nematode batches were subjected to examination as described above.
From the data obtained in hanging drop experiment the LHT analysis was launched and life cycle parameters (listed in Table 1) were calculated using Microsoft Excel. Total fertility rate (TFR), net reproductive rate $R_0$ and generation time ($T_0$, $T_1$, $T$) were calculated on the basis of the $l_i$ and $m_x$ (data not shown).

**Fig. 1.** Multi-well plate filled with moisturized tissue paper in the wells (a) and the multi-well plate lid inoculated with $10\mu l$ bacteria-semi-solid NGG ($10\times10^9$ cells ml$^{-1}$) and NGG drop mixture prepared for nematode transfer (b).
Table 1. Description of the life cycle parameters and formulas used for the calculation of each parameter.

<table>
<thead>
<tr>
<th>Life cycle parameter</th>
<th>Formulae</th>
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| **Intrinsic rate of natural increase** ($r_m$) also called the Euler equation (Vranken & Heip, 1983), represents the growth rate of a population that has a stable age distribution and grows under an unlimited environment (Muschiol et al., 2009). | $\sum_{x=0}^{d} e^{-r_m x} l_x m_x = 1$  
| $x =$ time (days), $l_x =$ age specific survival probability, $m_x =$ age-specific fecundity |
| **Population doubling time (PDT)**           | PDT = $\ln 2/r_m$                                                        |
| **Total fertility rate** (TFR): the total number of juveniles that would be produced by the females if they were able to survive until the end of the reproductive period. | TFR = $\sum m_x$                                                       |
| **Net reproductive rate** ($R_0$): The average number of juveniles produced by a female during its life time; dependent on the age specific survival probability and fecundity of the female. | $R_0 = \sum l_x m_x$                                                    |
| **The cohort generation time** ($T_0$) is the mean age at reproduction of a cohort of females. | $T_0 = (1/R_0) \sum x l_x m_x$                                           |
| **The mean generation time** ($T_1$): The period of time needed for a population growing at a constant rate $r_m$ to increase by the factor $R_0$. | $T_1 = (\ln R_0)/r_m$                                                   |
DJ RECOVERY IN HANGING DROPS

The influence of bacterial cell density on DJ recovery in hanging drops was assessed at 24 and 48h. The DJs, which were stored at 4°C and at 20°C were subjected to the experiment in order to compare the differences in their capacities for recovery. Approximately 20 DJs were transferred in to each out of four hanging NGG drops previously mixed with symbiotic bacteria. DJ recovery was assessed at three different bacterial cell densities (5 ×, 10 × and 20 × 10^9 cells ml⁻¹). The multi-well plates carrying the NGG drops were kept in darkness at 25°C and the numbers of recovered individuals were counted at 24 and 48 h by taking 2 drops at each assessment time point. This experiment was repeated 3 times with different batches of nematodes.

NEMATODE LIQUID CULTURES

In order to establish an identical bacterial inoculum for each assessed nematode batches, bacteria were pre-cultured in YS medium (20 ml in 100 ml Erlenmeyer flasks) for 48h (at 25°C and 180 rpm) and 1 % (v/v) of the cultured bacteria was transferred into sterile 40 or 100 ml medium depending on the size of Erlenmeyer flasks. After 48h post bacteria inoculation, 5,000 DJs ml⁻¹ of medium were added. DJ recovery, DJ yield and nematode population dynamics were assessed at 25°C, 27°C and 30°C. For nematode population monitoring, the samples were taken

\[ T = \sum_{x=0}^{d} xe^{-r_{m}x} l_{x} m_{x} \]
on the 4\textsuperscript{th}, 5\textsuperscript{th}, 8\textsuperscript{th}, 11\textsuperscript{th} and 15\textsuperscript{th} days and the presence of different developmental stages was counted and recorded using an inverted microscope (Axiovert 25, Carl Zeiss, Germany). The number of females, males, DJs and other stages (J1 – J4) were distinguished by the presence of reproductive structures or gastrointestinal system, respectively. For DJ recovery, samples were taken on the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} days post DJ inoculation. In order to estimate the proportion of DJs at the end of culturing process, samples were taken on 15\textsuperscript{th} day post DJ inoculation and DJs yield was counted.

\textit{Estimation of bacteria cell density in liquid cultures}

Samples were taken on the day of nematode inoculation (day 0) and on 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd}, 4\textsuperscript{th}, 5\textsuperscript{th}, 8\textsuperscript{th} and 11\textsuperscript{th} days post DJ inoculation into Erlenmeyer flasks. The bacteria cell density was counted using a Thoma chamber (0.01 mm depth, Poly Optic GmbH).

\textit{DJ recovery in nematode liquid cultures}

The DJ recovery in liquid culture was assessed by taking samples for 3 days starting from the day of DJ inoculation. The ratio between recovered and non-recovered DJs was recorded and presented in percentage. Additionally, the influence of three different bacterial densities (5 ×, 10 × and 20 × 10\textsuperscript{9} cells ml\textsuperscript{-1}) in 20 ml liquid culture (100 ml Erlenmeyer flasks) was assessed at 24h and 48 h post DJ inoculation by taking samples with minimum of 100 nematodes.
ASSessment of DJ Survival at Different Storage Temperatures

The DJs subjected to storage experiment were obtained from liquid cultures after washing with sterile Ringer’s solution and sieving through a mesh size of 10 μm using the vacuum suction instruments. The 50 ml plastic culture flasks (SARSDET, INC) filled up to 7 ml volume with Ringer’s solution containing 10,000 DJs/ml were stored for 5 weeks at 4°C, 15°C and 25°C. Every 7 days sub-samples containing a minimum of 100 DJs were counted under inverted microscope and dead and live DJs were recorded. For each temperature the average survival of DJs was calculated. The evaluation of the influence of the 3 different storage temperatures to DJ’s survival was based on 3 replicates conducted using different batch of nematodes.

Statistical Analysis

Before conducting ANOVA, percentage values for DJ recovery were arcsine transformed. Effects of different bacterial densities and incubation temperatures on DJ recovery, sex ratio and DJ yield were analyzed using the analysis of variance (ANOVA) and Tukey’s honestly significant difference test (HSD) Data for LHT analysis were processed using the formulas listed in table 1.
Results

LIFE HISTORY TRAIT ANALYSIS

Life cycle of S. yirgalemense in solid NGG plates

The occurrence of DJ recovery and development of recovered nematodes to adult stages was observed under dissecting microscope in solid NGG plates while juvenile hatching and further nematode development was observed in multi-well plates.

DJ recovery was observed within 24 h post DJ inoculation. Although, there was an increase in recovery until 36 h, the majority of the DJs had not recovered. Between 48–60h adults of both males and females had developed and started mating. Gravid females were observed between 60-72h post DJ inoculation and eggs were also released to NGG plates. After about 72h, the J1 started hatching from deposited eggs. At the same time in only few females, intra-uterine offspring development (*endotokia matricida*) was initiated. However, 96h post inoculation the number of females in *endotokia matricida* prevailed. Once females enter into *endotokia matricida*, they continue to feed, although offspring hatch and develop to DJs remaining inside the maternal body. Within 5-10 h from *endotokia matricida* initiation, the majority of females were completely degraded.

Within 24 h from transferring eggs to YS broth in the multi-well plates, juveniles in the first and second stage were observed. During the following 24h they developed to J3 and J4 stages. According to observation, it can be concluded that *S. yirgalemense* requires about 4 days to complete its life cycle – from eggs to eggs at 25°C under conditions of continuous

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development. In other words, it takes 3 days for a female to reach maturity and this data were used for the calculation of life history table.

**Offspring production in hanging drops**

In order to obtain information about the average offspring production, life span and other life history parameters, female and male pairs were kept in hanging drops at 25°C with $10 \times 10^9$ bacterial cells ml$^{-1}$. The average number of offspring per female was 314 and about 96% of the individuals originated from intra-uterine development, also known as *endotokia matricida* (Fig. 2). Apparently, in this nematode species, the period of egg deposition was very short and *endotokia matricida* starts immediately after fertilization and ends within 24h. Intra-uterine developed juveniles remained inside the mother’s cadaver until the cadaver degraded completely and the juveniles were migrating into the medium.

![Graph showing offspring production per female](image)

**Fig. 2.** The mean total number of offspring produced per *S. yirgalemense* female and average offspring number from intra-uterine and extra-uterine offspring production in hanging drops with $10 \times 10^9$ cells ml$^{-1}$ bacterial cell density at 25°C.
**Life history trait analysis and life span of females**

Based on data from hanging drop experiment conducted at 25°C and using $10 \times 10^9$ cells ml$^{-1}$ bacterial cell density, LHT analysis was performed. The life cycle parameters such as TFR (total fertility rate), $R_0$ (net reproductive rate), $r_m$ (intrinsic rate of natural increase), $T_0$ (cohort generation time), $T_1$ (generation time), $T$ (the mean age of the mothers of a set of newly-hatched individuals in a population with a stable age distribution) and PDT (population doubling time) were calculated.

*Steinernema yirgalemense* has a TFR and $R_0$ of 487 and 314 at $10 \times 10^9$ cells per ml, respectively (Table 2). The intrinsic rate of natural increase $r_m$ of $0.19\pm 0.11$ and PDT of $0.59 \pm 0.06$ were calculated. Generation time parameters were $4.98 \pm 0.49$, $4.87 \pm 0.49$ and $4.72 \pm 0.56$ days for $T_0$, $T_1$ and $T$, respectively (Table 2). The average life span of *S. yirgalemense* females was assessed at $5.55 \pm 0.67$ days (Table 2).
Table 2. Life cycle parameters (Mean ± SD of the three replicates): TFR (total fertility rate), $R_0$ (net reproductive rate), $r_m$ (intrinsic rate of natural increase), $T_0$ (cohort generation time), $T_1$ (generation time), $T$ (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), $d$ (days) and average life span;

<table>
<thead>
<tr>
<th>n</th>
<th>TFR (d)</th>
<th>$R_0$ (d)</th>
<th>$r_m$ (d-1)</th>
<th>$T_0$ (d)</th>
<th>$T_1$ (d)</th>
<th>$T$ (d)</th>
<th>PDT (d)</th>
<th>Average life span (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>113</td>
<td>488 ± 65</td>
<td>314 ± 15</td>
<td>1.19 ± 0.11</td>
<td>4.98 ± 0.49</td>
<td>4.87 ± 0.49</td>
<td>4.72 ± 0.56</td>
<td>0.59 ± 0.06</td>
<td>5.55 ± 0.67</td>
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</table>

**DJ recovery in hanging drops**

DJ recovery in hanging drops was assessed at 3 different bacterial densities of $5 \times 10^9$, $10 \times 10^9$ and $20 \times 10^9$ cells ml$^{-1}$ at 24 and 48h at 25°C using two nematode batches, which had been kept at 4°C and 20°C. Significant difference were not observed in DJ recovery between the different bacterial densities tested for nematodes from 4°C both after 24 h (Tukey’s HSD test: $F = 1.166$; DF = 2, 15; $p<0.338$) and 48 h (Tukey’s HSD test: $F = 1.560$; DF = 2, 15; $p<0.242$) (Fig. 2). After 24h the highest recovery was recorded at $20 \times 10^9$ cells ml$^{-1}$ bacterial cell density (39%) while the lowest recovery was at $5 \times 10^9$ cells ml$^{-1}$ (27%). The same percent recovery was recorded after 48h for the same bacterial density.
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Significant differences in DJ recovery were observed between the different bacterial densities tested on nematodes, which had been stored at 20°C prior to inoculation, both at 24h (Tukey’s HSD test: F = 5.985; DF = 2, 15; p < 0.012) and 48 h (Tukey’s HSD test: F = 6.810; DF = 2, 15; p < 0.008) (Fig. 3). After 24h the highest DJ recovery was observed at 20 × 10⁹ cells ml⁻¹ bacterial cell density (43%) and the lowest at 5 × 10⁹ cells ml⁻¹ (18%). After 48 h at 20 × 10⁹ cells ml⁻¹ bacterial cell density recovery increased to 55% and to 29% at 5 × 10⁹ cells ml⁻¹ bacterial density.

Fig. 3. Recovery (%) 24 and 48 h after inoculation of DJs which had been stored at 4°C prior to inoculation into hanging drops with three different bacterial densities (5 ×, 10 × and 20 × 10⁹ cells ml⁻¹); different letters above error bars indicate significant differences between bacterial densities (p > 0.05).
The influence of three different culturing temperatures (25°C, 27°C and 30°C) on DJ yield, DJ recovery and population dynamics were examined.

Fig. 4. Recovery (%) of DJs kept at 20°C prior to experiment in hanging drops at 24h and 48h with three different bacterial densities (5 ×, 10 × and 20 × 10⁹ cells ml⁻¹); different letters above error bars indicate significant differences between the different bacterial densities (p < 0.05).

Nematode Liquid Cultures

The influence of three different culturing temperatures (25°C, 27°C and 30°C) on DJ yield, DJ recovery and population dynamics were examined.
DJ recovery in nematode liquid cultures was assessed 24h, 48h and 72 h after DJ inoculation using 3 different culturing temperatures (25°C, 27°C and 30°C). Significant differences in DJ recovery between the different culturing temperatures were not recorded at any time points; i.e. 24 h, 48 and 72 hours; at 24h (Tukey’s HSD test: F = 0.959; DF = 2, 6; p < 0.042), 48h (Tukey’s HSD test: F = 0.959; DF = 2, 6; p < 0.042) and 72h (Tukey’s HSD test: F = 0.0.082; DF = 2, 3; p < 0.923) (Fig. 4A, B, C). However, at the end of the assessment, the highest percent of DJ recovery was observed at 27°C (Fig. 4C).
Fig. 5. DJ recovery (%) for three culturing temperatures 25°C, 27°C and 30°C at 24h (A), 48h (B) and 72h (C); Small case letters above error bars indicate significant between assessed temperatures ($p < 0.923$).
The sex ratio

The number of males and females on 5\textsuperscript{th} day post DJ inoculation of liquid culture is considered as good indicator for prediction of the population development. In liquid culture of \textit{S. yirgalemense} sex ratio was recorded already on the 4\textsuperscript{th} day, because the recovered DJs rapidly transformed to adults and started copulating. Therefore, the number of males and females counted on day 4 post inoculation was taken for the estimation of the sex ratio of the first generation adults. The average number of females per ml was highest at 30°C (739) and the lowest at 25°C (636). Overall, a very low average number of males was recorded at all culturing temperatures: 50, 73 and 113 DJs ml\textsuperscript{-1} at 25°C, 27°C and 30°C respectively.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{The average number of females and males per ml, recorded on day 4 post DJ inoculation assessed at different culture temperatures (25°C, 27°C and 30°C); different letters above error bars indicate significant differences between assessed temperatures (p >0.05).}
\end{figure}
No significant difference in number of females and males due to the different culturing temperatures was observed ($p > 0.05$) (Fig. 6).

**DJ yield**

The estimation of DJ yield in liquid culture was done on the 15\textsuperscript{th} day post DJ inoculation. The highest DJ yield was recorded at 25°C (322,520 DJs ml\textsuperscript{-1}) while the yield was significantly lower at 30 °C (32,978 DJs ml\textsuperscript{-1}) (Tukey’s HSD test DF: 2, 6; $F = 7.900; p = 0.021$) (Fig. 7).

**Fig. 7.** The average DJ yield on day 15 post DJ inoculation at different culture temperatures (25°C, 27°C and 30°C). The small letters above bars indicate significant differences between temperatures ($p = 0.021$).
**POPULATION DYNAMICS**

In order to observe the dynamics of different stages in nematode liquid culture under different culturing temperatures of 25°C, 27°C and 30°C, samples were taken on the 4th, 5th, 8th, 11th and 15th day post DJ inoculation. Starting from the day of bacteria inoculation, the density of symbiotic bacteria was recorded.

In liquid culture incubated at 25°C, starting from first day 0, the exponential growth of bacteria was recorded (Fig. 8 A). The peak was reached on the second day post DJ inoculation ($18 \times 10^9$ cells ml$^{-1}$). The descending trend started from the 3rd day until the day 5 (to $8 \times 10^9$ cells ml$^{-1}$), which was due to feeding of the recovered nematodes. Very similar patterns in bacterial growth were recorded at 27°C and 30°C. The maximum increase of bacterial density at 27°C was recorded on 2nd day ($14 \times 10^9$ cells ml$^{-1}$) and on the same day for the 30°C ($15 \times 10^9$ cells ml$^{-1}$). The drop in bacterial growth started from the 3rd day and lasted until the 4th day at 27°C (to $5 \times 10^9$ cells ml$^{-1}$) and until the 5th day at 30°C incubation temperature ($5 \times 10^9$ cells ml$^{-1}$). From the 4th to the 5th day, an increase in bacterial density (to $14 \times 10^9$ cells ml$^{-1}$) was recorded at 25°C followed by slow descending tendency until the end of culturing process (Fig. 8A). At 27° and 30°C the bacteria increased until the 8th day (to $13 \times 10^9$ cells ml$^{-1}$ and $8 \times 10^9$ cells ml$^{-1}$, respectively) (Fig. 8B and C). In accordance to results from DJ recovery in liquid culture (see the section on DJ recovery in liquid culture), the females and males recorded on 4th day at three assessed culture temperatures, originate from recovered DJs (Fig. 8 A-C). Already on the 4th day, mating was observed as well as fertilized females and first released eggs. Therefore, the occurrence of first offspring (J1 stages) was recorded at 25°C and at 27°C on the 4th 5th (Fig. 8 A,
B). On the other hand, very low number of recovered males and females were recorded at 30°C (Fig. 8 C). The period of egg deposition lasted for a very short time, because in many females initiation of endotokia matricida was observed on the 5th day (at 25°C and 27°C). The vast majority of offspring which arose from endotokia matricida, after migrating from maternal body continued to feed and to develop to adults. That possibly explains the high number of females and males recorded on the 8th day at three culturing temperatures: 80,840 females and 2,881 males per ml at 25°C; 10,305 females and 3,277 males per ml at 27°C; However, the population of females from 27°C continued to grow and it reached its maximum on the 15th day (13,220 females per ml) even though it consisted of mostly old, small and non-fertilized individuals. On the other hand, the maximum number of females and males at 30°C was reached on the 11th day but in general, they did not look healthy and sexually active.

The increase in numbers during 15 days culturing process was recorded for DJs at three culture temperatures but also with remarkable differences in average numbers on the final day: the 384,481 DJs ml⁻¹ was recorded at 25°C, 305,333 at 27°C and 69,462 at 30°C (Fig. 8 A-C). Even though, there were no possibilities for their differentiation, according to the observation it is most probable that the majority of the DJs originated from second generation adults. Due to the nutritive depletion offspring of the 2nd generation females transformed to non-feeding dauer juvenile stage, instead of continuing development to adults.
DJ recovery at different bacterial inoculum densities in nematode liquid culture

DJ recovery in nematode liquid culture was assessed at three different bacterial densities 5×, 10× and 2 × 10⁹ cells ml⁻¹ at 25°C and in a control, which was not supplied with concentrated bacteria but treated in a regular way (the estimated bacterial density was 3×10⁹ cells ml⁻¹). A significant difference between the control and the treatment with the highest bacterial density...
both at 24 h (Tukey’s HSD test DF: 3.8; F = 6.161; p = 0.018), but also at 48h (Tukey’s HSD test DF: 3.8; F = 4.538; p = 0.039) post DJ inoculation was recorded (Fig. 9).

![Diagram showing DJ recovery (%) at three different bacterial densities 5 ×, 10 × and 20 × 10^9 cells ml⁻¹ and control (3 × 10^9 cells ml⁻¹) observed at 24h and 48 h; different letters indicate significant difference between assessed bacterial densities within data recorded after 24h or 48h (p ≤ 0.05).](image_url)

**Fig. 9.** DJ recovery (%) at three different bacterial densities 5 ×, 10 × and 20 × 10^9 cells ml⁻¹ and control (3 × 10^9 cells ml⁻¹) observed at 24h and 48 h; different letters indicate significant difference between assessed bacterial densities within data recorded after 24h or 48h (p ≤ 0.05).
DJ SURVIVAL AT THREE DIFFERENT STORAGE TEMPERATURES

The DJs kept in Ringer’s solution were exposed for 5 weeks to storage temperatures of 4°C, 15°C and 25°C in order to assess their survival capability. Survival of *S. yirgalemense* was significantly different during the 35 days assessment and the lowest survival was observed at 4°C (Tukey’s HSD test: F = 708.88; DF=2, 6 ; p < 0.0001) (Fig. 10). More than 50% of the DJs at 4°C storage temperature were dead within three weeks, whereas, after 35 days survival of DJs remained more than 90% at 15 and 25°C (Fig. 10).

![Graph showing DJ survival at three different storage temperatures](image)

**Fig. 10.** Percentage survival of *S. yirgalemense* DJs at three different storage temperatures of 4°C, 15°C and 25°C assessed during a five weeks period. The different small case letters indicate significant differences between temperatures tested (p < 0.001).
DISCUSSION

LIFE-HISTORY TRAIT ANALYSIS

Studying the LHTs of EPN has an important role for the improvement of their production under liquid culture condition. So far, LHTs of the EPN *S. yirgalemense* has not been conducted before. The hanging drop technique provides the opportunity to study single nematode or nematode pairs under unlimited food supply and defined environmental conditions with more detailed insight (Muschiol et al., 2009). This allows generating data about life cycle parameters of subjected nematode species, to study its life span or offspring production. Furthermore, it may in the future allow to accurately model or to predict the entire population development under the same or similar environmental conditions. This is of great importance, because the success of nematode liquid culture and large scale production is very much dependent on population dynamics management (Grewal et al., 2005).

*Offspring production in hanging drop experiment*

Conducted hanging drop experiment showed that the females of *S. yirgalemense* strain Sy 157-C in average produces 314 juveniles per female, which is much lower when compared with *S. riobrave* with nearly 1,500 individuals per females under identical conditions (Teshome, 2013). The symbiotic bacteria were found difficult to handle in hanging drops and were therefore cultured in YS medium instead of NLM used for *S. riobrave* strains and this might have influence on the total number of offspring produced.
In *S. yirgalemense* less than 5% of the offspring originate from deposited eggs whilst in *S. riobrave* it ranged from 45% to 58% in strains Sr 7-12 and HYB19, respectively (Teshome, 2013). It has been shown that the *endotokia matricida* occurs readily under conditions of depleted food in *H. bacteriophora* and *H. indica* (Johnigk & Ehlers, 1999). On the other hand, the higher food density did not postponed the beginning of *endotokia matricida* in *S. riobrave* (Sr 7-12 and hybrid strain HYB19) (Addis *et al.*, 2014). In order to compare the influence of different food concentration on intra-uterine offspring production in *S. yirgalemense*, further investigations are needed.

The information on offspring production obtained from the hanging drop experiment can be used to predict the DJ yield production in liquid culture. For instance, assuming the inoculated 5,000 DJ ml\(^{-1}\) liquid medium recover to females and males at equal sex ratio (1:1), the value of cumulative offspring production for each day in hanging drop can be multiplied by 2,500 females. If the reproductive period of *S. yirgalemense* female takes 4 days and in that time period female produce 407 individuals (mean cumulative offspring production), the predicted yield ml\(^{-1}\) of liquid culture could be 1,016,749 DJs ml\(^{-1}\) after 6.5 days post DJ inoculation. However, this value can be considered as theoretical since conditions for nematode growth and development in hanging drops and in liquid culture are significantly different.

*Life cycle parameters and life span of females*

The population growth parameters *r*\(_m\) and PDT were 1.19 and 0.59 days, respectively. In a study on LHTs of *S. riobrave* by Teshome (2013), the values for intrinsic rate of natural increase were slightly higher for strain Sr 7-12 (1.44) and Sr HYB19 (1.34). On the other hand
the values for the population doubling time (PDT) in *S. riobrave* (Sr 7–12: 0.496 days; HYB19: 0.52) were very similar to *S. yirgalemense*.

*S. yirgalemense* has more or less a similar generation time compared to *S. riobrave* strains. The most pronounced difference between *S. yirgalemense* and *S. riobrave* was certainly in parameters related to offspring production (TFR and $R_0$). The TFR in *S. yirgalemense* was found three times lower when compared with both strains Sr 7-12 (1,413) and Sr HYB19 (1,439). Similarly, $R_0$ values of *S. yirgalemense* were found much lower when compared with the two strains of *S. riobrave* (Addis *et al.*, 2014).

The average life span of *S. yirgalemense* of 5.55 days was remarkably lower than that of *S. riobrave* strains that extended to 7.5 days (Addis *et al.*, 2014). The life of all reproductive females ended due to the occurrence of *endotokia matricida*. On the other hand, those females which failed to produce offspring were able to survive for more than a week. The proportion of these females was accounted to 8% and may not create process failure in liquid cultures.

**DJ recovery in hanging drops**

DJ recovery is one of the most important steps for successful liquid culture establishment in EPN production. It can be of big constraint due to its high variability (Strauch & Ehlers, 1998). A prolonged recovery period may negatively affect process time and also decrease the final yield due to lack of synchronization (Ehlers *et al.*, 1998). Prior to starting the liquid culture experiment, DJ recovery in hanging drops under defined bacterial density $5 \times 10^9$, $10 \times 10^9$ and $20 \times 10^9$ cells ml$^{-1}$ at 25°C was assessed. DJs priory stored at 20°C showed betted DJ recovery than those from 4°C. The reason was later assessed by conducting storage temperature and 4°C was found lethal for
nematodes. The remarkably higher DJ recovery in hanging drops at 24h and 48h in comparison to DJ recovery in liquid culture could be due to the higher concentration of bacteria.

NEMATODE LIQUID CULTURES

DJ recovery in liquid cultures

*Steinernema yirgalemense* had showed a very low DJ recovery at all the three culture temperatures, 25°C, 27°C and 30°C. This nematode species had close to 20% DJ recovery within 72 hours which is found to be much lower when compared with *S. carpocapsae* (83%) and *S. feltiae* (90%) (Hirao & Ehlers, 2010).

DJ recovery was variable between experiments, which might be due to responds of the symbiotic bacteria and also the difference in nematode (Strauch & Ehlers, 1998; Aumann & Ehlers, 2001). The role of both batch differences and bacteria cultures should be further investigated at optimal culture temperature of 25°C. Nevertheless, the DJs itself can be responsible for great variability in recovery among replicates or nematode batches.

*Steinernema yirgalemense* seems to be different from other *Steinernema* spp. in male: female ratio. It has been observed that the number of males is more than three-times lower than the number of females observed 4 days after DJ inoculation. In *S. carpocapsae* 69% of adults is consisted for females and in *S. feltiae* 72% are females (Hirao & Ehlers, 2010).

*Steinernema yirgalemense* can reach quite high DJ yields at 25°C within the same process time recorded for *S. feltiae* and *S. carpocapsae*. The investigated nematode gave 322,520 DJs ml\(^{-1}\), which is almost two to three times higher when compared with *S. feltiae* and *S. carpocapsae,*
which gave 110,000 DJs ml\(^{-1}\) and 200,000 DJs ml\(^{-1}\), respectively (Hirao & Ehlers, 2009). However, a high variability between experiments was observed and further optimization of the bacteria and nematode cultures are needed. High variability in commercial production is one of the reasons for process failure (Ehlers, 2001). Hence, more detailed studies, especially on nematode – symbiotic bacteria interaction in liquid culture remain to be done. Despite very high number of DJs observed on day 15 post inoculation, the number of other stages, especially females (see the section on population dynamics), was observed. The presence of other stages on the day of harvesting is not desired because it may have negative effect on the quality of the final product because they represent ideal substrates for many microorganisms (Hirao & Ehlers, 2009). Therefore, the study on optimization of processing time should be considered.

**Population dynamics**

DJ recovery and further development was affected by culture temperature. At 25°C the nematodes performed best, both in DJ recovery and yield, whereas the highest culture temperature resulted in almost complete failure of the reproduction. Nematodes have their own specific requirement for both infectivity and reproduction (Grewal et al., 1994; Hirao & Ehlers, 2009).

The symbiotic bacteria showed the best performance at 25°C which led to better performance of the development of the nematode. The numbers of DJs recovered were lower when compared with other *Steinernema* spp. (Hirao & Ehlers, 2010). However, the females laid only a small proportion of eggs and the majority of the offspring obtained were from *endotokia matricida*, which is the source of well-fed DJ (Johnigk & Ehlers, 1999). It had been observed that
cultures of this nematode go through two generations within two weeks’ time. This could cause serious problem in commercial production because the presence of other stages at the time of harvesting increases cost of production because of necessary additional steps in cleaning DJ suspensions and can this reduce the quality of the final product (Hirao & Ehlers, 2009). However, this population dynamics observation should be further investigated at the best temperature that allowed higher DJ production with different DJ inoculums density.

**DJ recovery at different bacterial inoculums densities in nematode liquid culture**

The bacterial inoculums density has an important role in triggering DJ recovery. Occurrence of high recovery at the highest bacterial inoculum density could be related to high food signal production released to the medium. However, *S. yirgalemense* had showed maximum average recovery of 27%, which was far lower when compared with other steinernematid nematodes (Hirao & Ehlers, 2010). The shift from primary to the secondary phase of bacteria (Akhurst, 1980) may occur under conditions of osmotic stress and this shift may have negative effect on DJ recovery that leads to process failure (Han & Ehlers, 2001). However, in this study, although low DJ recovery was assessed, the final DJ yield was severely affected; the influence of the recovery on the final yield seems to be low. This could be explained with quick reproduction of the nematodes after recovery, and probably an increased volume of females which in turn produce higher numbers of DJ per female (Hirao & Ehlers, 2009a).
DJ SURVIVAL AT THREE DIFFERENT STORAGE TEMPERATURES

The results of this study gave the first insight into the *S. yirgalemense* survival at different storage temperatures. This nematode was first collected and identified from the tropics (Ethiopia) and it is not uncommon to respond negatively to lower storage temperatures. Good storage conditions should provide maximum survival of DJs and prolonged conservation of their infectivity (Strauch *et al.*, 2000). The temperatures of 15°C provided better survival of DJs. However, further decreasing temperatures should be tested in order to get the optimum temperature for both storage and transport of nematode products.

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