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Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis*

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Summary – *Heterorhabditis* spp. were cultured monoxenically in shaken liquid media inoculated with dauer juveniles (DJ). In all cultures some female phenotype adults produced offspring while others had only unfertilized eggs in their uteri. When *Heterorhabditis* spp. are grown on agar media they exhibited a " y " or " λ " type copulation behaviour. In contrast to *Steinernema* spp., heterorhabditid males were unable to attach to the vaginal region in liquid culture. Consequently, under liquid culture conditions only self-fertilizing hermaphrodites reproduce and the amphimictic part of the F1 generation can be identified by the presence of unfertilized eggs without shell in their uteri. During daily observations of the nematode development in liquid cultures containing approximately 20 F1 generation eggs, 30 % developed to amphimictic females, 38 % to males and 32 % to hermaphrodites. Single F1 generation first stage juveniles (J1) were either directly inoculated into *Photorhabdus luminescens* cultures or starved for 24 h in Ringer's solution before inoculation. Starvation significantly increased the automictic part in the F1 generation (53.3 % DJ, 40 % hermaphrodites and 6.6 % amphimictic adults). The ratio amphimictic : automictic adults was 1.84 when J1 were directly transferred to culture medium and 0.07 when they were starved prior to transfer. In all experiments males developed a day earlier than amphimictic females and females 2-3 days earlier than hermaphrodites. Obligatory for automictic reproduction is a yet undescribed second juvenile pre-dauer stage (J2d) distinguishable from amphimictic J2 by its corn-cob like structure of the cuticle in the head region. A preceding development from a J2d to a DJ is not obligatory for automictic reproduction. The F1 sex ratio males : amphimictic females was between 0.84 and 1.3. The observations have a significant impact on liquid culture population dynamics of *Heterorhabditis* species.

Résumé – ***Les conditions d'élevage définissent le caractère automictique ou amphimictique de la reproduction chez les nématodes Rhabditides entomopathogènes du genre* Heterohabditis** – Des *Heterohabditis* spp. sont maintenus en élevage monoxénique dans un milieu liquide inoculé à l'aide de « dauer » juvéniles (DJ). Dans chacun de ces élevages, quelques adultes de phénotype femelle produisent une descendance tandis que l'utérus des autres ne contient que des œufs non fécondés. Lorsque les *Heterohabditis* spp. sont élevés sur milieu gélosé, ils font montre d'un comportement copulatoire de type « y » ou « λ ». A l'inverse des *Steinernema* spp., les mâles d'Heterorhaditides sont, en milieu liquide, incapables de se fixer sur la région vaginale. En conséquence, dans les conditions d'élevage en milieu liquide, seuls les hermaphrodites autoféconds sont capables de se reproduire, les individus amphimictiques de la F1 pouvant être identifiés par la présence d'œufs non fécondés, sans coquille, dans leur utérus. L'observation journalière de nématodes comportant des œufs provenant d'environ 20 F1, montre que 30 % produisent des femelles amphimictiques, 38 % des mâles et 32 % des hermaphrodites. Des juvéniles de premier stade (J1) provenant d'une génération F1 sont soit inoculés, isolément, directement dans des cultures de *Photorhabdus luminescens*, soit laissés inanitiés pendant 24 heures dans une solution de Ringer avant inoculation. L'inanition augmente significativement la portion automictique de la F1 (DJ : 53, 3 %; adultes hermaphrodites : 40 %; adultes amphimictiques : 6,6 %). Le rapport entre les adultes amphimictiques et automictiques est de 1,84 lorsque les J1 sont transferrés directement à partir du milieu de culture et de 0,07 s'ils sont laissés inanitiés avant le transfert. Dans toutes les expériences, les mâles se développent un jour plus tôt que les femelles amphimictiques et les femelles deux à trois jours avant les individus hermaphrodites. Est obligatoire pour la reproduction automictique, un stade non encore décrit consistant en juvéniles de deuxième stade « pré-dauer » (J2d) se distinguant des J2 amphimictiques par la structure pavimenteuse de la cuticule dans la région céphalique. Un passage de J2d à DJ n'est pas obligatoire pour la reproduction automictique. L'indice andrique correspondant aux femelles amphimictiques de la F1 est de 0,84-1,3. Les présentes observations sont d'importance concernant la dynamique des populations des espèces d'*Heterohabditis* en milieu liquide.

Key-words : *Heterorhabditis* spp., amphimixis, automixis, sex ratio, reproduction, *in vitro* culture conditions, nematodes.

Rhabditid nematodes of the genera *Heterorhabditis* and *Steinernema* are effective agents for biological control of soil-borne insect pests (Ehlers & Peters, 1994). The infective third stage dauer juvenile (DJ) of these nematodes seeks and invades larvae of a variety of soil-dwelling insect species, including many economically important pests (e.g. Klein, 1990; Georgis, 1992). The DJ is developmentally arrested and morphologically and

physiologically adapted for long-term survival under detrimental environmental conditions in the soil. Particularly the heterorhabditid DJs are ensheathed in the second stage cuticle (Campbell & Gaugler, 1991 *a, b*) which has a corn-cob like structure in the head region (Mráček & Bednarek, 1992). DJs of both genera carry cells of a symbiotic bacterium of the genera *Xenorhabdus* or *Photorhabdus* in their intestinal lumen (Bird & Akhurst, 1983; Endo & Nickle, 1991). Every nematode species is associated with its specific bacterial species (Pütz *et al.*, 1990). After penetration into the insect the DJs release their bacteria into the haemolymph. Providing the insect's humoral and cellular defence mechanisms do not succeed in eliminating the nematode-bacterium complex, the host dies within 3 days after infection (Peters & Ehlers, 1994). The bacteria proliferate, the nematodes feed on the bacteria cells and the host tissue and develop into adults. Adults of *Steinernema* spp. are always amphimictic females and males. In contrast, heterorhabditid DJs develop into protandric hermaphrodites (Poinar, 1975), a character they share with the rhabditid *Caenorhabditis elegans* (Wood, 1988). In contrast to the latter species, the subsequent second generation of *Heterorhabditis* spp. consists of amphimictic male and female adults (Poinar, 1990). The females or hermaphrodites lay eggs from which the first stage juveniles (J1) hatch within about 2 days (Lunau *et al.*, 1993). The life cycle of both steinernematid and heterorhabditid species consists of four juvenile stages (J1 to J4). Under permissive nutritional conditions two or three generations can develop inside the dead host. When the nematode population has reached a certain density threshold and nutritional conditions worsen, an alternative developmental pathway leads to the formation of third-stage DJs which leave the insect corpse and search for new hosts (Poinar, 1990).

For use in biocontrol an *in vivo* production of the nematodes is much too labour intensive and consequently impracticable. Liquid culture techniques have been developed to produce *S. carpocapsae* in large-scale bioreactors (Friedman, 1990). However, heterorhabditid nematodes often perform better against certain insects than steinernematids (e.g. Georgis & Gaugler, 1991), but stable conditions for the production of *Heterorhabditis* spp. have not yet been achieved. Problems also arise with the sensitivity of the nematodes to environmental extremes, e.g. high temperature, solar radiation, and desiccation (Ehlers, 1992), thus preventing exploitation of their maximal potential as control agents under field conditions. Genetic improvement has been suggested as a means of increasing their tolerance to environmental hardships (Gaugler, 1986, 1987). For genetic studies and improvement *Heterorhabditis* spp. are well suited candidates. Because of their autotokous reproduction, mutations become homozygous automatically, pure lines can be maintained, single gene mutants can easily be isolated and the frequently occurring males allow the transfer of genetic markers for crossbreeding (Glazer *et al.*, 1991). However, a comprehensive knowledge of the life cycle and mode of reproduction is needed as a prerequisite for genetic studies aimed at improving the efficacy of the nematodes and also for successful propagation under liquid culture conditions.

The life cycle of *H. bacteriophora* (HP88) *in vitro* and *in vivo* was described by Zioni *et al.* (1992 *a*). However, there are conflicting data on the mode of reproduction : Poinar (1975) claimed that adults of the first generation of this nematode reproduce automictically, whereas the second generation progeny is amphimictic. Likewise, Dix *et al.* (1992) have demonstrated that the initial female progeny produced by first generation hermaphrodites are exclusively amphimictic. Early second generation females, when selected with immature gonads and injected into insect corpses, failed to produce offspring. In contrast, Glazer *et al.* (1991) propagated *H. bacteriophora* HP88 in every generation from single virgin juveniles to generate homozygous inbred lines and suggested that *H. bacteriophora* propagates exclusively through self-fertilization. Likewise Zioni *et al.* (1992 *b*) produced a homozygous inbred line of a dumpy mutant by fifteen successive generations initiated from single virgin hermaphrodites. Describing the life cycle, Zioni *et al.* (1992 *a*) reported the presence of only hermaphrodites and males in the second generation.

The aim in this paper is to resolve these apparently conflicting observations and to establish the mode of reproduction of *Heterorhabditis* spp. in the F1 generation. Liquid culture techniques were applied to elucidate the mode of reproduction and to determine conditions influencing nematode development.

Material and methods

Nematode strains

Heterorhabditis sp., strain HD01, was received from H. Bathon (BBA, Darmstadt, Germany) and *H. megidis* (HE) from P. Smits (IPO, Wageningen, The Netherlands). *H. megidis* (HSH1) was isolated in Schleswig-Holstein, Germany (Ehlers, 1989). The HP88 strain of *H. bacteriophora* was obtained from Biosys (Palo Alto, CA, USA). *Steinernema anomali* was obtained from Kozodoi (Kozodoi, 1984).

Nematode development in liquid culture

All monoxenic *in vitro* cultures were prepared according to Lunau *et al.* (1993). One day prior to nematode inoculation media were inoculated with strain-specific *Xenorhabdus* or *Photorhabdus* sp., isolated and propagated according to Akhurst (1980). Culturing was done at 25 °C in the dark.

For observations of nematode development in liquid culture monoxenic DJ of the *Heterorhabditis* strains HSH1, HD01 and HE were inoculated into Liquid Lipid Medium (LLM : Lunau *et al.*, 1993) or other media,

appropriately adjusted for liquid culturing of the corresponding strains. Samples were taken every 2-3 days and the nematode density and population dynamics were recorded. Bacterial density was assessed by counting cells in a Thoma chamber under the microscope (Normaski optic, 400x).

MATING BEHAVIOUR

The strains HP88, HD01 and HSH1 were cultured in Petri dishes on Lipid Agar (LA : Wouts, 1981) and inoculated with dauer juveniles from monoxenic cultures. When the first preadult juveniles (early J4) of the F1 generation had developed, 20 Lipid Agar plates/strain were inoculated with single female J4s. Additional ten plates per strain were inoculated with one J4 female and one male nematode. Copulation and development of eggs and offspring were observed with an inverted microscope. HSH1 and *S. anomali* were also cultured in 6 cm diameter Petri dishes in LLM.

SEX RATIO IN THE F1 GENERATION (HSH1)

P. luminescens was grown in Lipid Salt Medium (LSM in g/l H_2O : 0.5 beef extract, 0.5 yeast extract, 1.7 peptone, 0.1 glucose, 5.2 NaCl, 0.1 K_2H-PO_4 and 3.0 vegetable oil) and the bacterial density was assessed after 40 h. The LSM medium is low in nutrient concentration thus allowing observation of the culture development under the inverted microscope. Twenty four cell culture wells of 16 mm diameter were filled with 200 µl bacterial suspension adjusted to 10^9 cells/ml by dilution with cell-free culture supernatant. Approximately 50 nematode eggs obtained from gravid hermaphrodites isolated from infested *G. mellonella* were inoculated per well (Lunau *et al.,* 1993). The development of the hatched nematodes was observed daily over a period of 8 days.

INFLUENCE OF CULTURE CONDITIONS ON THE SEX RATIO (HSH1)

Cell culture wells of 6.4 mm diameter were filled with 40 µl of a 24 h *P. luminescens* culture in LSM adjusted to 10^9 cells/ml by dilution with sterile Ringer's solution. Single J1 were transferred into the wells from a 5-day-old monoxenic liquid culture, which had been inoculated with DJ. The development of a total of 192 individuals was observed daily over a period of 9 days. 96 of the J1 were kept in bacteria free Ringer's solution one day before they were transferred to the bacteria suspension.

Results

DEVELOPMENT IN LIQUID CULTURE

Nematode development was recorded of 75 flask liquid cultures of HE, 56 of HD01 and several hundred HSH1. Population dynamics were similar in all cultures with differences between strains in culture time to reach maximum yields and reproduction potential. An example for a typical population development in monoxenic liquid culture of strain HSH1 is given in Fig. 1. Bacterial density decreased after 4 days due to nematodes feeding on bacteria cells. Some of the inoculated DJ developed to hermaphrodites, which reproduced and J1 offspring occurred on day 4. They developed either to dauer juveniles (increase in DJ density from day 5 onwards) or further to adult nematodes (first F1 adults recorded on day 5) with males occurring about 1 day earlier than female phenotype adults. In cultures of all strains female phenotype nematodes were observed carrying unfertilized eggs (as shown in Fig. 2A). They died without producing offspring. These nematodes were counted as females. Nematodes with female phenotype but with fertilized eggs (like those shown in Fig. 2B) or juveniles in the uterus were counted as hermaphrodites although it could not be assessed whether these so-called hermaphrodites of the F1 generation reproduced by self- or by cross-fertilisation. It could also not be resolved why a certain part of the female phenotype nematodes did not reproduce. Copulation plugs were never observed on female phenotype nematodes in liquid media. Maximum DJ concentration (56 000/ml) was reached on day 15.

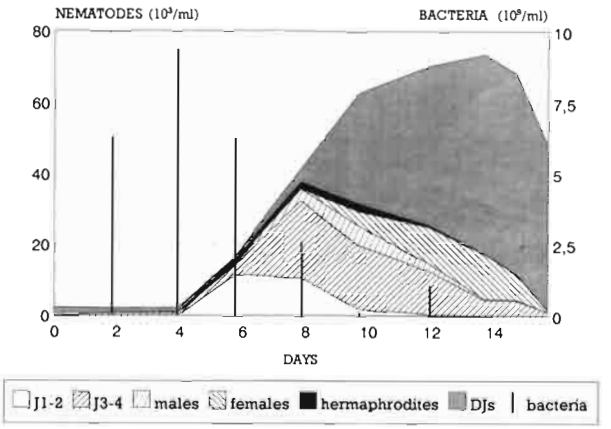

Fig. 1. *Population dynamic of* Heterorhabditis megidis *(strain HSH1) and cell counts of* Photorhabdus luminescens *(strain XSH) in shaken liquid culture, inoculated with 3000 dauer juveniles/ml.*

MATING BEHAVIOUR OF *HETERORHABDITIS* AND *STEINERNEMA*

Males of *Heterorhabditis* spp. possess a bursa copulatrix (e.g. Poinar *et al.,* 1987). On LA the heterorhabditid males were observed gliding along the female body and then attaching with their tail to the vaginal region of the female (" y " or " λ " type copulation). Next the spicula were penetrated through the vulva lips (Fig. 2C). At the end of the copulation phase the males secreted a jelly-like substance formed into a copulation plug closing the vagina of the female (Fig. 2B). Thus the presence of a copulation plug in the female vaginal region is evidence of a successful copulation. This typical

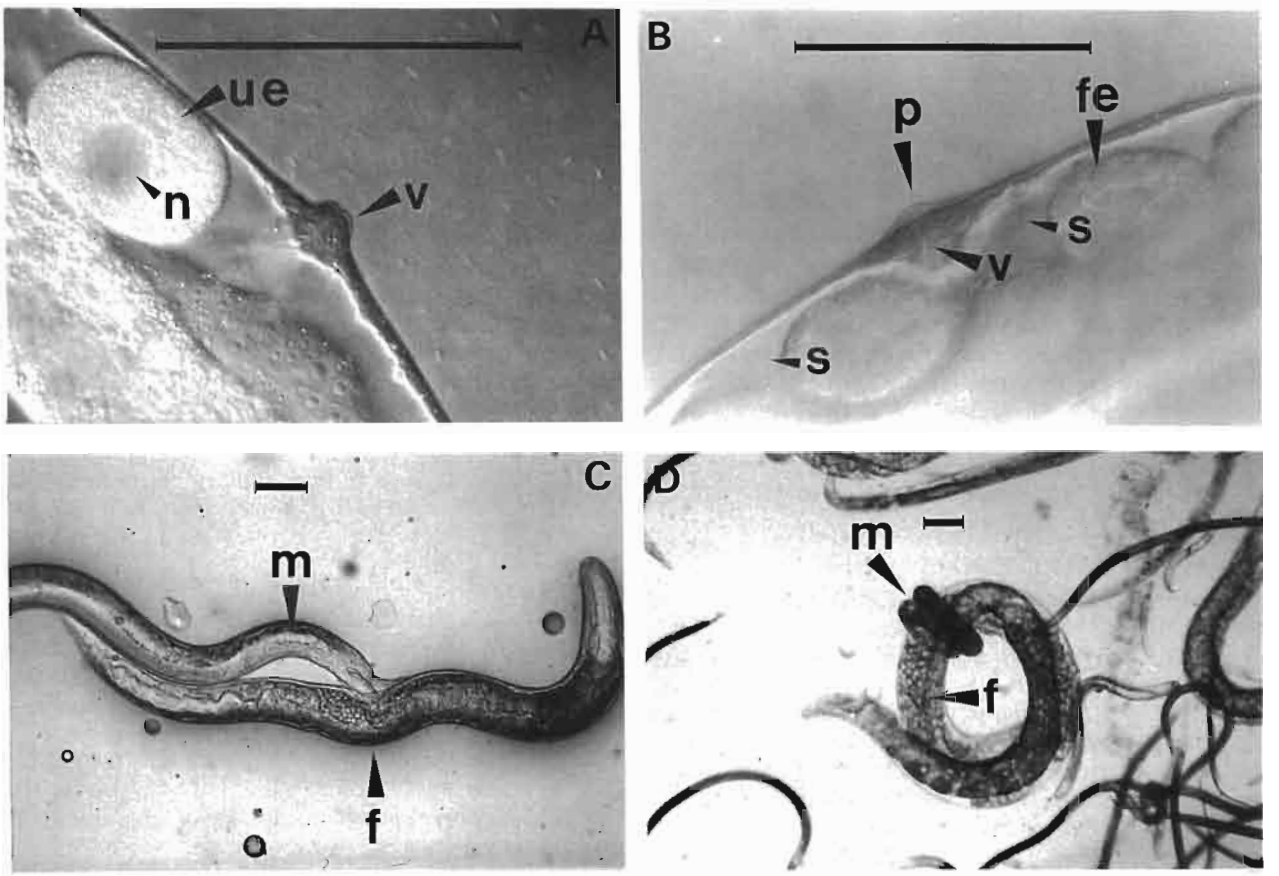

Fig. 2. *A : F1 generation amphimictic female of* Heterorhabditis megidis *(strain HSH1) with protruding vulval lips* (v). *Female isolated from liquid culture containing also F1 generation males. Unfertilized egg* (ue) *recognizable by lack of cell cleavage and egg shell and centred nucleus* (n); *B : F1 generation amphimictic female of* Heterorhabditis megidis *(strain HSH1) with copulation plug* (p) *closing the vulval opening* (v). *Female isolated from agar surface culture containing also F1 generation males. Fertilized egg* (fe) *recognizable by presence of egg shell* (s) *and cell cleavage; C : " λ " type copulation of female* (f) *and male* (m) Heterorhabditis megidis *(strain HSH1) on Agar surface; D : Helix type copulation of female* (f) *and male* (m) Steinernema anomali *in liquid culture.* (Bars = 100 *μm).*

copulation behaviour was observed only on the agar surfaces, never in liquid culture media. Other indicators of a successful mating are the cell cleavages of fertilized eggs and the occurrence of progeny. The nucleus in the centre of unfertilized eggs is relatively large and transparent and the egg has no shell (Fig. 2A). Fertilized eggs develop a shell and cell-cleavage starts (Fig. 2B). Females in liquid media always carried unfertilized eggs even when male nematodes were present and no copulation plug was observed. Copulation behaviour of steinernematids is different as they lack a bursa copulatrix (e. g. Poinar, 1990). Male *S. anomali* coiled around the female body (helix type copulation) which was observed on agar and in liquid media (Fig. 2D) also for other steinernematid species (*S. carpocapsae, S. feltiae, S. scapterisci, S. glaseri*).

Sex ratio in the F1 generation (HSH1)

Of the approximately 1200 inoculated eggs 40 % developed into adults, with an average of 20 individuals in each well (SD = 6). Observations had shown that copulation of *Heterorhabditis* sp. does not occur under liquid culture conditions. Therefore amphimictic females and automictic hermaphrodites could be distinguished by the presence of unfertilized *vs* fertilized eggs in their uteri. The number and percentage of different adult stages are given in Fig. 3. In the F1 generation deriving from eggs isolated from parent hermaphrodites out of *G. mellonella* all three sexes were recorded : females (30 %), males (38 %) and hermaphrodites (32 %). The ratio amphimictic males : females was 1.3. The ratio amphimictic : automictic adults was 2.1. Males devel-

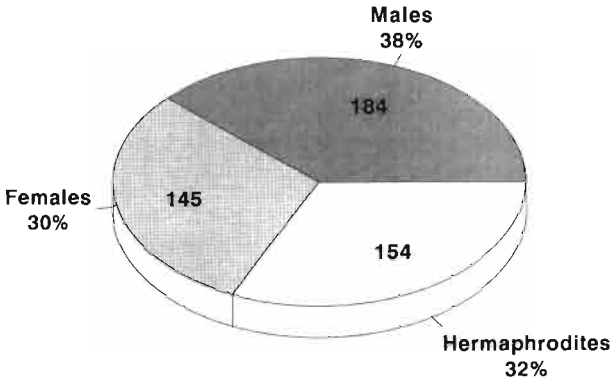

Fig. 3. *Total nematode counts from 24 cell well cultures and percentage of automictic (hermaphroditic) and amphimictic (male and female) adults in the F1-generation of* Heterorhabditis megidis *(strain HSH1) 8 days after inoculation of approximately 50 sterile eggs per well isolated from* in vivo (Galleria mellonella) *cultured hermaphrodites. Each well contained 200 µl of a 1 day old* Photorhabdus luminescens *culture adjusted to 10⁹ cells/ml by dilution with cell free bacterial culture supernatant (Medium : LSM, see text).*

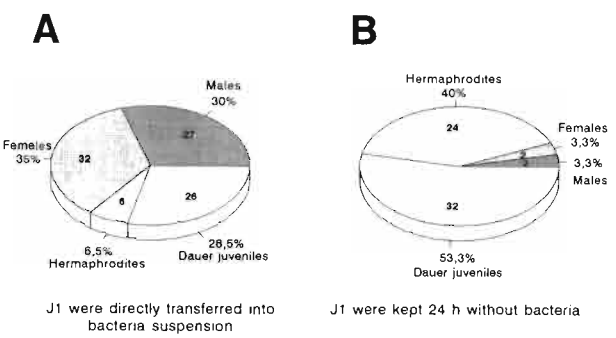

Fig. 4. *A : Total nematode counts from 96 cell well cultures and percentage of automictic (hermaphroditic) and amphimictic (male and female) adults in the F1-generation of* Heterorhabditis megidis *(strain HSH1) 9 days after inoculation of single first-stage juveniles per well isolated from a 5-day-old monoxenic* in vitro *liquid culture inoculated with dauer juveniles. Each well contained 40 µl of a 1-day-old* Photorhabdus luminescens *culture adjusted to 10⁹ cells/ml by dilution with Ringer's solution (Medium : LSM, see text); B : Same culture conditions like A but first stage juveniles kept in Ringer's solution for 24 h prior to inoculation into cell wells.*

oped a day earlier than amphimictic females and females 2-3 days earlier than hermaphrodites. None of the hermaphrodites was observed to pass through the DJ stage.

INFLUENCE OF CULTURE CONDITIONS ON THE SEX RATIO (HSH1)

Culture conditions had a severe impact on the ratio amphimictic : automictic adults. Whereas only 35 % of the J1s immediately transferred into the bacteria suspension developed to automictic DJ and hermaphrodites (Fig. 4A) nearly all J1s developed to automictics (93 %) when they were kept without bacteria for 24 h prior to the transfer into the bacteria culture (Fig. 4B). Since DJs exclusively develop into hermaphrodites, for the calculation of the ratio DJs were counted as automictic. The ratio amphimictics : automictics was 1.84 when J1 were transferred into bacteria suspension directly after isolation and, with a ratio of 0.07, was significantly lower ($\alpha < 0.01$, χ^2 test with Yates correction) when J1s were starved for 24 h prior to inoculation into the culture broth. The ratio male : female was 0.84 and 1, respectively, with no significant difference ($\alpha \leq 0.1$), not even to the result obtained in the previous experiment (Fig. 3).

Again males occurred a day before females. Hermaphrodites took 2-3 days longer to develop than females. DJ were observed finishing development within 3-7 days after transfer of the J1 from the parent liquid culture. Of the 30 individuals which had developed into hermaphrodites in both experiments (Fig. 4), only 3 had gone through the DJ stage. All other J1s growing to hermaphrodites did not develop into DJs but had gone through a pre-dauer J2 stage, which is morphologically distinct from those J2s growing into amphimictic adults.

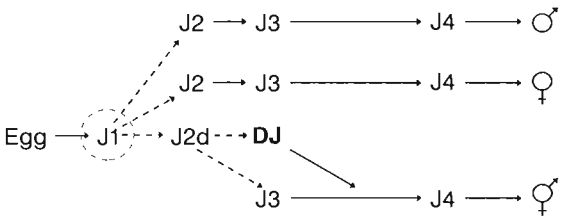

Fig. 5. *Alternative developmental pathways of* Heterorhabditis *spp. from egg to amphimictic male and female or automictic hermaphrodite. Dotted lines represent alternative pathways. Solid lines indicate obligatory developmental steps. Circle marks the sensitive first stage juvenile sensitive to environmental cues (food signal or DRIF) inducing development into either amphimictic or automictic adult (J1 - J4 = juvenile stages; J2d = pre-dauer juvenile; DJ = dauer juvenile).*

Second-stage juveniles developing to DJs had the same characters. In comparision with the J2s developing into amphimictics the pre-dauer J2 is longer, the ratio length : width is higher and the cuticle at the head region shows a corn-cob like pattern, characteristic for the J2 cuticle ensheathing the DJ. The pre-dauer J2 stages occurred simultaneously with the amphimictic preadult females (J4). The alternative developmental pathways and observed juvenile and adult stages are given in Fig. 5. Following the description of a pre-dauer J2 stage for *C. elegans* (Golden & Riddle, 1984) the pre-dauer J2 of *Heterorhabditis* is also termed J2d stage.

Discussion

The results presented clearly indicate that both amphimictic and automictic reproduction occurs in the second generation of entomopathogenic nematodes of

the genus *Heterorhabditis*. The offspring of the first generation hermaphrodite can either develop into amphimictic adults or automictic hermaphrodites, and both can occur simultaneously. Obligatory for automictic reproduction is a preceding J2d stage and this may be followed by a DJ stage which, however, is not obligatory. The alternative pathway leading to amphimictic adults is induced by favourable nutritional conditions, whereas the development into automictic hermaphrodites is induced by low concentrations of nutrients (in the present experiment created by the absence of symbiotic bacteria during the J1 stage for one day). The sensitive stage perceiving these environmental cues is the first-stage juvenile (Fig. 5).

The development of second generation automictic adults lasts at least two days longer than the development of amphimictic adults. Development into the hermaphrodite is prolonged due to the formation of a J2d and the exit from this stage. It is obvious that the development to the J2d is not reversible; however, the development of the arrested DJ stage is not obligatory and the J2d, encountering favourable nutritional conditions, can proceed to a pre-hermaphrodite J3 stage.

Automictic or amphimictic reproduction in *Heterorhabditis* is closely related to dauer formation and it was presumed that mechanisms of dauer formation in *C. elegans* are comparable with those of entomopathogenic nematodes (Fodor *et al.,* 1990). In *C. elegans* the exit from the dauer stage (recovery) is induced by favourable nutritional conditions (food signal). The developmental pathway leading to the dauer stage is induced by the Dauer Recovery Inhibiting Factor (DRIF), a pheromone secreted by all nematode stages. Antagonistic to DRIF are favourable nutritional conditions (food signal). Thus high pheromone concentrations indicating crowding together with low food signal concentrations induce dauer formation (Golden & Riddle, 1984). Culture supernatant of *S. carpocapsae* (Fodor *et al.,* 1990) and *H. megidis,* strain HSH1 (Strauch, unpubl.) also induce dauer formation, thus indicating the existence of a comparable factor produced by entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema.* In the experiments performed in cell wells (Figs 3, 4) nematode population density per ml medium was relatively low (20/200 µl and 1/40 µl) compared with the density in shaken flasks which were already inoculated with 3000 DJ/ml (Fig. 1). Thus the influence of a DRIF was of minor importance and it was mainly the food signal that influenced the development in cell wells. In cultures inoculated with about 50 eggs the medium was diluted with cell free bacterial culture supernatant. This experimental design produced relatively higher concentrations of a food signal and consequently the J2d developed to hermaphrodites (Fig. 3). In cultures inoculated with single J1 into 40 µl bacterial suspension the dilution with Ringer's solution produced relatively lower concentrations of the food signal. While in the first experiment no J2d developed to the DJ stage (Fig. 3), conditions in the latter experiment induced the development of DJs in more than 50 % of the J1s (Fig. 4A), due to relatively lower concentrations of food signal.

The question arises as to why, first of all, conditions induce the development to the J2d and later the development into the DJ is not completed. Instead a hermaphrodite is formed. An explanation would be that nutritional conditions inducing development of the J1 to the J2d stage are not the same as those causing the J2d to complete dauer formation. The concentration ratio of DRIF : food signal inducing the development of the J2d may be lower than the ratio necessary to terminate the formation to the DJ. This would represent a well adapted strategy to changing culture conditions. The J1 enters the development to the J2d in order to secure survival in the dauer stage, but, if nutritional conditions do not deteriorate the J2d can terminate the development to the DJ in order to take up the reproductive life cycle again. To experimentally prove this hypothesis a purification of food signal(s) and DRIF is urgently needed.

For *C. elegans* it was supposed that starvation during the J1 stage does not induce dauer formation (Golden & Riddle, 1984). Fodor *et al.* (1990) could also not induce dauer formation by starvation in steinernematids. However, the present results show that starvation induces DJ formation and automictic reproduction in *Heterorhabditis* spp. as the majority (94 %) of the single J1 kept for one day in Ringer's solution without any bacteria (no food signal) developed to DJs or hermaphrodites. A single J1 probably does not produce enough DRIF to induce its own development to a DJ.

The " y " or " λ " type copulation behaviour of heterorhabditid nematodes excludes fertilization under liquid culture conditions due to the lack of any solid surface and/or surface tension needed to attach to the female body. Curling behaviour of males, as observed for *S. anomali,* is certainly necessary for successful insemination in liquid culture. With this knowledge the occurrence of unfertilized female phenotype adults in liquid cultures can now be explained and it is evident that these adults, unable to self-fertilize, represent the amphimictic part of the F1 population. Thus for creation of inbred lines of *Heterorhabditis* spp. propagation in liquid culture is the ideal method as cross-fertilization can be excluded.

Poinar (1990) doubted that autotokous reproduction of second generation " amphimictic females " occurs and considered that a continuous line of hermaphrodites is only possible when an infective juvenile (DJ) is formed. Our observations give evidence that only a preceding J2d stage, and not a complete development of a DJ, is necessary for autotokous reproduction of the F1 hermaphrodite. The hermaphroditic juvenile (HJ) described by Koltai *et al.* (1995) is obviously the J2d stage and in accordance with Koltai *et al.* (1994) we observed the simultaneous occurrence of the preadult amphim-

ictic J4 and the J2d when cultures were started with synchronized J1.

The results of the present studies can now explain conflicting results previously published. Poinar (1975) described the *in vivo* life cycle of *H. bacteriophora* grown in *G. mellonella*. Favourable nutritional conditions resulted in anphimictic adults in the F1 generation. The same is true for the experiments of Dix *et al.* (1992). No offspring were observed when five F1 generation females isolated from *G. mellonella* corpses 6-7 days post-infection were injected into *G. mellonella* and the authors suggest that F1 adults are not autotokous. An early isolation of F1 offspring favours development into amphimictic adults as early juveniles still encounter favourable nutritional conditions. However, in five cases reproduction was observed. Dix *et al.* (1992) suggested that either a misidentification of a male nematode (strain K122) or a selection of inseminated females (strain HP88) were the reason for nematode reproduction. Although these explanations may still be correct we can now also suggest that either a J2d was isolated (strain K122) or the inseminated females were hermaphrodites (strain HP88).

In contrast, Glazer *et al.* (1991) and Zioni *et al.* (1992 *a, b*) stated that F1 and successive generations exclusively reproduce automictically in the absence of males. They were able to produce homozygous inbred lines by transfer of single preadult female phenotype nematodes to fresh culture media over several generations. Their *in vitro* culture conditions (Dogfood Agar) may have favoured the development of hermaphrodites, but amphimictic females were certainly present. Koltai *et al.* (1995) cultured HP88 under the same conditions and report the simultaneous development of amphimictic and automictic adults. Although Glazer *et al.* (1991) and Zioni *et al.* (1992 *b*) did not record how many of their cultures failed to reproduce it must be concluded that those plates inoculated with single amphimictic females were unable to produce offspring. This was certainly attributed to regularly occurring mortality and not interpreted as impossible reproduction of amphimictic females in the absence of males.

It is impossible to morphologically differentiate between amphimictic females and hermaphrodites. The same is true for early J4 stages. The only opportunity to identify a hermaphrodite is during sperm production in the late preadult J4 stage. However, this would make necessary an observation with high magnification (400×) and it is nearly impossible to continue culturing these individuals monoxenically after observation under the microscope. Although a J2 can easily be distinguished from a J2d this identification would also require a microscopic observation at high magnification.

The present results have a significant impact not only on selection of non-self or self-fertilizing nematodes for cross-breeding experiments but also on mass production of *Heterorhabditis* spp. in liquid culture. A comprehensive knowledge of the environmental cues which promote the development of DJ is needed to manage nematode population dynamics in order to avoid or minimize the development of non-reproductive amphimictic stages in liquid culture production systems.

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