Population development of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* in the larvae of *Galleria mellonella*[1]

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Summary – The population development of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* was studied in larvae of the greater wax moth, *Galleria mellonella,* after initiation with one or two infective juveniles, respectively. In both nematodes, three reproductive adult generations were observed and after 3 weeks a final population of about 150 000 infective juveniles per insect was counted. Population development of these two species differed in several aspects. Details of the population development in the insect host of these most commonly used entomopathogenic nematodes could be used to establish an optimal standard for fecundity, at least during the first generation, in *in vitro* culture.

Résumé – *Développement des populations de* **Heterorhabditis bacteriophaga** *et* **Steinernema carpocapsae** *dans les larves de* **Galleria mellonella** – Le développement de populations d'*Heterorhabditis bacteriophaga* et *Steinernema carpocapsae* dans les larves de *Galleria mellonella* a été étudié après inoculation avec un ou deux juvéniles infestants, respectivement. Pour les deux nématodes, trois générations d'adultes sont observées et, après trois semaines, une population finale de l'ordre de 150 000 juvéniles infestants par insecte est dénombrée. Cependant, le développement des populations diffère par plusieurs aspects chez ces deux espèces. Les détails du développement dans l'insecte hôte des populations de ces nématodes entomopathogènes les plus couramment utilisés pourraient servir à l'établissement d'un niveau optimal de fécondité, au moins durant la première génération en culture *in vitro*.

Key-words : *Galleria mellonella,* hermaphroditism, *Heterorhabditis bacteriophora, in vivo* culture, infective juvenile, population development, *Steinernema carpocapsae.*

Although many studies have been conducted on culturing entomopathogenic nematodes in *G. mellonella* larvae (Dutky *et al.,* 1964; Sandner & Stanuszek, 1971; Stanuszek, 1974; Kaya, 1977; Poinar, 1979, etc.), there is limited information on the population development of these nematodes. Sandner and Stanuszek (1971), working with the larvae and pupae of *G. mellonella* and *Leptinotarsa decemlineata,* made a thorough comparison of the effects of increasing the density of the first generation of female *S. carpocapsae* and of varying the size of the host on the final yields of infective juveniles, but they did not study the nematode populations as they developed, nor did they work with single female nematodes. Jaworska and Stanuszek (1986) found that populations of *Heterorhabditis* species in the apple sawfly *Hoplocampa testudinea* increased continuously over 10 days from the 5th day of infection. Zervos *et al.* (1991) showed that both temperature and inoculum size strongly influenced the development time, the duration of emergence and yields of infective juveniles of *H. heliothidis* and *S. glaseri* in *G. mellonella* larvae. However, details of nematode population development, the num-

ber of generations involved, and the contributions of each generation to the final infective juvenile population remain unknown. This present study investigates the developmental dynamics of nematode populations in an insect host beginning with one hermaphrodite *(H. bacteriophora)* or one male and one female *(S. carpocapsae).*

Materials and methods

PREPARATION OF NEMATODES AND INSECTS

Infective juveniles of the nematodes *S. carpocapsae* A24 (Leningrad strain) and *H. bacteriophora* C1 (North Carolina, USA) from monoxenic solid culture were used as inoculum. They were thoroughly washed by sedimenting and decanting with distilled water and then migrated through sieves lined with double thickness tissue paper to provide clean active infective juveniles.

The greater wax moth *G. mellonella* was reared continuously on an artificial diet consisting of Farex Blended cereal (300 g), glycerol (140 g), honey (140 g), yeast (20 g) and water (24 g), and larvae of the fifth instar weighing 0.15 ± 0.015 g were used for experiments.

INFECTION OF *G. MELLONELLA*

The infective juveniles of *S. carpocapsae* and *H. bacteriophora* were injected into be *G. mellonella* larvae under the microscope using a gas chromatography syringe with a plunger that extended to the tip of the needle so that an accurate number of nematodes was injected into each insect (Akhurst & Bedding, 1978). With *H. bacteriophora,* because of its hermaphroditic nature, to ensure that the population within a single *G. mellonella* larva was initiated from only a single reproductive, only a single infective juvenile was injected into each *G. mellonella* larva. With *S. carpocapsae,* because it is amphimictic, two infective juveniles were injected into each insect. After the injection, the insects were kept at 23 °C in Petri dishes lined with two layers of filter paper. The infected *G. mellonella* larvae that had died within 48 h of nematode injection were collected, kept at 23 °C in the Petri dishes and used for sampling and examination.

DISSECTION OF INSECTS AND EXAMINATION OF NEMATODE POPULATIONS

The cadavers were carefully dissected in Ringer's solution (NaCl 8 g, $CaCl_2$ 0.2 g, KCl 0.2 g, $NaHCO_3$ 0.2 g, distilled water 1 l) under the microscope. All developmental stages (eggs, first stage, second stage, infective stage, third and fourth stage juveniles, and adults) were identified and counted once outside the parent, using serial dilution where necessary. Dissections and nematode counts were carried out every day on at least three insect cadavers which had a successful development of nematode populations. Because of the highly viscous nature of cadavers infected with *H. bacteriophora,* dissected cadavers with this species were repeatedly irrigated with surplus Ringer's solution before counting. Ten days after injection, each remaining insect cadaver was placed individually on a White trap (White, 1927) and nematodes from both inside and outside each insect cadaver were counted.

Results

HETERORHABDITIS BACTERIOPHORA

One infective juvenile injected into a *G. mellonella* larva gave three adult generations over 14 days, but the last female generation did not oviposit. The hermaphrodite that developed from the initial infective juvenile laid eggs which produced several hundred to over one thousand offsprings that all developed into adult males and females. The hermaphrodite also retained about 500 eggs, all of which developed into infective juveniles within the body of the hermaphrodite, but contributed less than 1 % of the final population of infective that eventually emerged from each cadaver. Females derived from the original hermaphrodites also laid eggs (averaging six to ten per female) which developed into another generation of adults, but they also retained an average of about 30 eggs within the nematode body which later

became infective juveniles, contributing 5 to 10 % of the final population of infective juveniles. The 3rd (last) generation females did not oviposit and all of their eggs (averaging 40 to 50 each) hatched and developed inside the body (endotokia matricida), eventually emerging as infective juveniles. This third reproductive generation contributed over 90 % of the final infective juvenile population. From all three generations, about 150 000 infective juveniles developed in an average *G. mellonella* larva of 0.15 g in weight. Maximal populations of the infective juveniles formed after 14 days although it took about 18 days for the nematode populations to develop into their final stable stage containing only infective juveniles (Table 1, Fig. 1 A).

STEINERNEMA CARPOCAPSAE

Population dynamics of *S. carpocapsae* (Table 1) were rather different from those found in *H. bacteriophora.* Eggs and first stage juveniles were found only 72 h after nematode injection and infective juveniles formed within 1 week. Nematode populations reached their maximum levels by the 10th day after nematode injection, although only 60 % of the nematodes were infective juveniles. The final nematode population containing only infective juveniles formed after 14 days. The yields were very similar to those of *H. bacteriophora,* with about 150 000 infective juveniles per insect (Table 1).

Development of *S.carpocapsae* populations also differs from that of *H. bacteriophora.* Unlike the population development of *H. bacteriophora,* which showed a strong preponderance of infective juveniles throughout the cycle, the total population of *S. carpocapsae* remained considerably higher than the infective juvenile population for the 7 days between the 5th to 12th days after nematode injection. Compared with *H. bacteriophora,* a much larger population of second stage juveniles (mainly derived from the third generation females) was found.

Two days after nematode injection, infective juveniles developed into mature males and females; the adults mated and produced offspring after only 3 days (Fig. 1 B). The number of eggs produced by the initial female was up to 3000 to 4000 over a period of 2 to 4 days. These eggs developed into a second generation of adults which laid their eggs on the 5th and 6th days after injection. A total number of 12 000 to 14 000 third generation adults developed from the eggs laid by the second generation females, between the 6th to 9th days. Few eggs were laid by the females of the third generation and all of the eggs (average of 20 to 25 per female) hatched and developed inside the female bodies. Although most of the infective juveniles came from the last generation females, up to 20 % of the infective juveniles came from the previous second generation. Unlike *H. bacteriophora,* in which most or all infective juveniles formed inside the parent bodies, any juveniles of *S. carpocapsae* originally retained within their parent only de-

Table 1. *Population dynamics of* Heterorhabditis bacteriophora *and* Steinernema carpocapsae *in the larvae of* Galleria mellonella *after injecting one and two infective juveniles per insect, respectively.*

Days after injection	Number of nematodes of various stages per *Galleria mellonella* larva*							
	Eggs	1st stage juveniles	2nd stage juveniles	Infective juveniles	3rd or 4th stage juveniles	Males	Females	Total
HETERORHABDITIS BACTERIOPHORA								
3	0	0	0	0	0	0	1	1
4	eggs seen	0	0	0	0	0	1	1
5	285 ± 3	294 ± 4	210 ± 10	0	0	0	1	789 ± 13
6	179 ± 4	151 ± 2	441 ± 4	0	80 ± 5	0	1	852 ± 6
7	0	210 ± 6	240 ± 12	0	180 ± 4	65 ± 2	170 ± 6	864 ± 12
8	3 540 ± 42	2 180 ± 35	2 010 ± 21	0	250	280 ± 23	560 ± 31	8 820 ± 82
9	4 380 ± 23	3 250 ± 81	3 580 ± 35	2 500 ± 308	1 500 ± 69	480 ± 42	1 360 ± 120	17 050 ± 49
10	250 ± 29	2 850 ± 61	3 500 ± 236	13 750 ± 689	2 750 ± 229	1 050 ± 115	2 450 ± 126	26 600 ± 150
11	0	50 ± 22	2 500 ± 289	27 450 ± 531	3 900 ± 577	1 933 ± 73	2 500 ± 104	38 333 ± 192
12	0	0	850 ± 100	53 200 ± 702	1 550 ± 263	2 100 ± 58	2 550 ± 115	60 250 ± 250
13	0	0	0	120 500 ± 2 188	500 ± 29	1 500 ± 180	2 200 ± 76	125 150 ± 2 108
14	0	0	0	139 000 ± 2 309	0	500 ± 29	2 000 ± 125	141 500 ± 2 021
16	0	0	0	146 000 ± 2 035	0	0	1 200 ± 98	147 200 ± 2 321
18	0	0	0	149 000 ± 2 142	0	0	0	149 000 ± 2142
21	0	0	0	145 600 ± 2291	0	0	0	145 600 ± 2 291
STEINERNEMA CARPOCAPSAE								
2	0	0	0	0	0	1	1	2
3	1 686 ± 46	1 050 ± 56	0	0	0	1	1	2 738 ± 32
4	3 746 ± 81	1 800 ± 87	1 847 ± 93	0	1 200 ± 42	0	1	8 594 ± 151
5	6 967 ± 196	10 750 ± 293	3 266 ± 88	0	2 050 ± 153	600 ± 36	483 ± 19	24 116 ± 76
6	10 800 ± 321	12 200 ± 551	16 200 ± 351	15 800 ± 469	6 800 ± 208	2 200 ± 61	3 800 ± 40	67 800 ± 793
7	3 800 ± 35	4 400 ± 153	19 200 ± 436	36 000 ± 596	5 400 ± 70	5 600 ± 106	6 200 ± 98	80 600 ± 179
8	3 200 ± 40	4 200 ± 133	41 200 ± 551	38 400 ± 603	2 400 ± 44	6 600 ± 238	6 800 ± 66	102 800 ± 211
9	2 400 ± 40	3 200 ± 117	58 000 ± 2 013	64 800 ± 833	1 200 ± 64	5 600 ± 76	4 400 ± 101	139 600 ± 2 184
10	0	2 000 ± 42	56 400 ± 1 514	85 600 ± 1 286	1 600 ± 40	1 800 ± 64	2 400 ± 50	149 800 ± 1 151
12	0	1 000 ± 55	15 000 ± 306	132 000 ± 1 528	1 800 ± 95	500 ± 38	2 000 ± 32	152 300 ± 1 585
14	0	0	0	147 000 ± 3 215	1 500 ± 38	0	1 000 ± 27	149 500 ± 3 127
16	0	0	0	150 000 ± 2 080	500 ± 25	0	0	150 500 ± 2 080
21	0	0	0	149 000 ± 3 215	0	0	0	149 000 ± 3 215

* Number of nematodes = mean ± standard error of the mean, n = 3.
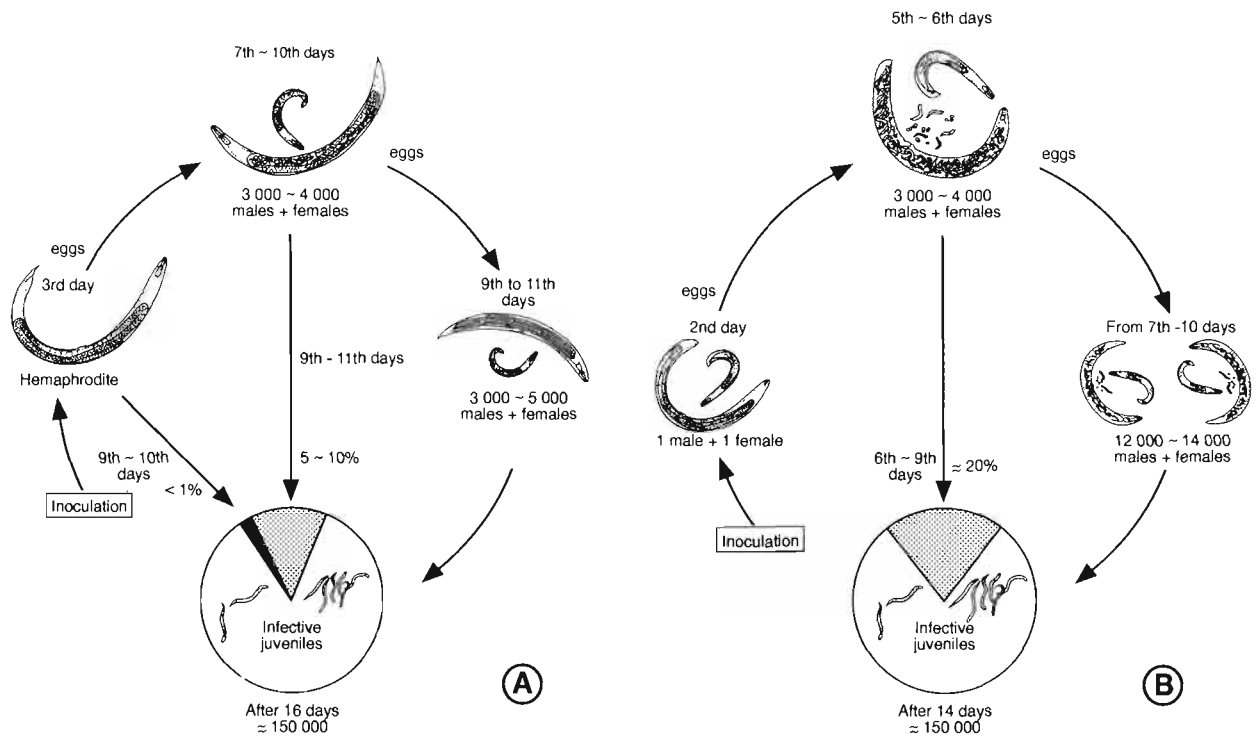
Fig. 1. Population dynamics *of* Heterorhabditis bacteriophora *(A) and* Steinernema carpocapsae *A 24 (B) in the larvae of* Galleria mellonella *after injecting one and two infective juveniles per insect, respectively.*

veloped into the ensheathed infective juveniles after leaving the body of the parent.

Discussion

The nematode populations developed through three adult generations in both nematode species. The length of time required for the development of each generation at a constant temperature of 23 °C showed that *S. carpocapsae* produced its first generation offspring 1 or 2 days earlier than *H. bacteriophora*, although both species required up to 3 weeks to develop their final populations containing only infective juveniles. Injection of infective juveniles into the insect to produce a reproductive nematode proved to be accurate and satisfactory for studying population development. Dissection of the infected larvae of *G. mellonella* at various times after infection showed that nematode development and the final yields of infective juveniles from individual insects were very similar.

In both *S. carpocapsae* and *H. bacteriophora,* populations initiated from a parent nematode resulted in about 150 000 infective juveniles per *G. mellonella* larva in 3 weeks. These yields are similar to the yields of *S. car-*

pocapsae described by Dutky *et al.* (1964) who reported an average of 1110 nematodes per mg of *G. mellonella* larvae. Sandner and Stanuszek (1971) obtained a wide variation in the yields of *S. carpocapsae* depending on the size of *G. mellonella* larvae (40 to 260 mg) and the initial number of females (from 10 to 200 per insect) in the insect host. They found that the best yield of 3091 infective juveniles per mg of *G. mellonella* larvae was obtained from an initial female number of 50, and with a body weight per insect from 140 to 180 mg. Yields with ten initial females were lower but they did not examine yields from single females.

Initiation of *S. carpocapsae* and *H. bacteriophora* populations with a reproductive nematode provides the first generation with a surplus of fresh food, which probably provides optimal conditions for maximising the number of first generation progeny per parent, while minimising any effect of feed-back mechanisms, such as pheromones, on the formation of infective juveniles during the first generation. This study indicates that the maximum number of amphimictic progeny from a *H. bacteriophora* hermaphrodite is about 800 to 1000 (plus about 500 infective juveniles) and from a *S. carpocapsae* female is

from 3000 to 4000. The infective juvenile yields per insect have probably not reached the maximum because Sandner and Stanuszeck (1971) had already shown that an inoculum of 50 females produced higher final yields than with 10. Our general observations on *S. carpocapsae* (Wang & Bedding, unpubl.) indicate that the larger the inoculum the smaller the number of second generation parents produced; with optimal numbers inoculated, there may even be no or negligible second generation reproduction, and certainly no third generation. The same effect results from a greatly decreased host size. With only one generation, most reproduction will occur in the most suitable environment including high bacterial populations, limited degradation of food, limited excretory products and perhaps limited pheromone (Fodor *et al.,* 1990); this is probably why the final yield of infective juveniles (up to 350 000 per cadaver) found by Sandner and Stanuszek (1971) is much higher than yields in the present study (150 000).

The situation with *H. bacteriophora* is similar, but with the important difference that when there are large initial numbers of hermaphrodites, or when the host is very small, most of the infective juveniles produced in the cadaver may come from the original self fertilizing hermaphrodite and are therefore genetically less diverse. Population regulation as a result of infective juvenile formation is also achieved in a different manner from *S. carpocapsae.* Infective juveniles are only formed within parent nematodes in *H. bacteriophora* and all eggs retained within parents form infective juveniles; this never occurs in *S. carpocapsae.* Thus whereas infective juvenile formation by *S. carpocapsae* must be a direct result of a feedback mechanism (probably a combination of reduction of quantity and/or quality of food, perhaps interacting with a pheromone), in *H. bacteriophora* feed back mechanisms, if they occur, must influence the ratio of eggs laid to eggs retained within the parent. This in turn will be influenced by the size of the parent and it would seem more likely that it will be affected by food quantity and quality rather than by any possible pheromone. *H. bacteriophora* has the mechanism for safely sequestering infective juveniles in each hermaphrodite or adult female soon after these parents have matured, while at the same time " risking " some of its progeny in the extra-parental environment within the cadaver for the potential of further immediate reproduction.

In most populations competing for food in a density dependent situation, some mortality would be expected at high densities. However negligible mortality was observed in either *S. carpocapsae* or *H. bacteriophora* in these *in vivo* experiments. This is because of the phenomenon of infective juvenile formation, which is a major mechanism for population control in these species (and many other Rhabditida). As food is depleted, and probably as feed-back mechanisms such as pheromones come into play, a higher and higher proportion of the progeny become infective juveniles which no longer

compete for food and probably compete less for oxygen than other stages. The end result is that as much of the insect as possible is converted into another generation of infective juveniles able to enter the environment and survive there without feeding for long periods ready to infect new hosts as they become available.

Acknowledgments

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