

Reproduction of the entomopathogenic nematode *Heterorhabditis bacteriophora* Poinar, 1976 : hermaphroditism vs amphimixis ⁽¹⁾

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Summary – The present study was aimed at elucidating the mode of fertilization (self vs cross) in 2nd generation non-male adults of the entomopathogenic nematode *Heterorhabditis bacteriophora* strain HP88. For this purpose dumpy mutants (*Hbdpy-1* and *Hbdpy-2*) were used as genetic markers. Forty hours after eggs hatching two types of juveniles were evident in the 2nd generation of either the wild-type or the mutant populations cultured *in vitro*: half of the 2nd generation individuals developed to the 4th developmental stage (J4) with discernible reproductive systems. The other individuals were, on average 1.4-2 times shorter and 1.6-3 times thinner ($p < 0.05$, *t* test) than the above described “normal” J4. They were less developed than the J4 type and had no identifiable reproductive system. Among 550 of the J4 type juveniles (either wild-type or dumpy) that had been individually transferred to culture plates, only 9 (i.e. 1.8 %) gave rise to progeny. However, when dumpy non-male adults, originating from J4 type juveniles were crossed to wild-type males, 30-71 % of them gave rise to progeny all of which were wild-type, indicating that reproduction occurred solely by cross-fertilization. These non-male adults were termed “females”. Among 105 smaller-type juveniles which had been individually transferred to culture plates, 80 % reproduced indicating a high rate of self-fertilization i.e. a high proportion of hermaphrodites. The smaller type juveniles were termed “HJ” (H for hermaphrodite). When dumpy HJ type juveniles were crossed with wild-type males, 70 % ($n = 30$) gave rise to progeny. Each successful cross yielded both dumpy (46 %-69 %) and wild-type (31 %-54 %) progeny, indicating reproduction by self as well as cross-fertilization, respectively. The importance of the co-existence of these two reproductive strategies and their implication to genetic studies are discussed.

Résumé – Reproduction du nématode entomopathogène *Heterorhabditis bacteriophora* Poinar, 1976 : hermaphroditisme ou amphimixie? – La présente étude vise à élucider le mode de fécondation (autofécondation ou fécondation croisée) des adultes non mâles de deuxième génération du nématode entomopathogène *Heterorhabditis bacteriophora* souche HP 88. Dans ce but, les mutants obèses *Hbdpy-1* et *Hbdpy-2* ont été utilisés comme marqueurs génétiques. Quarante heures après la ponte, deux types de juvéniles peuvent être distingués dans la deuxième génération de population élevées *in vitro*, qu’elles soient de type sauvage ou issues de mutants. La moitié des individus de 2^{ème} génération se développent en quatrième stade juvénile (J4) comportant un système reproducteur bien visible. Les autres individus sont, en moyenne, de 1,4 à 2 fois plus courts et 1,6 à 3 fois plus minces ($p < 0,05$, test *t*) que les individus cités plus hauts considérés comme des J4 « normaux »; ces autres individus sont moins développés que les J4 types et leur système reproducteur n’est pas visible. Sur 550 J4 (de type sauvage ou obèse) transférés individuellement sur agar, seuls neuf (ou 1,8 %) ont produit une descendance. Cependant, lorsque des adultes obèses non-mâles, provenant de J4 types, sont croisés avec des mâles sauvages, 30 à 71 % d’entre eux produisent une descendance dont tous les individus sont de type sauvage démontrant ainsi que la fécondation est uniquement de type croisé. Ces adultes non-mâles sont nommés « femelles ». Parmi 105 juvéniles de type réduit transférés individuellement sur agar, 80 % se reproduisent démontrant ainsi un taux élevé d’auto-fécondation, c’est-à-dire la présence d’une forte proportion d’individus hermaphrodites. Les juvéniles de type réduit sont nommés « HJ » (H pour hermaphrodite). Lorsque des juvéniles de type HJ obèses sont croisés avec des mâles de type sauvage, 70 % ($n = 30$) produisent une descendance. Chaque fécondation réussie donne une descendance aussi bien obèse (46-69 %) que de type sauvage (31-54 %), indiquant ainsi une reproduction par autofécondation et par fécondation croisée, respectivement. L’importance de la coexistence de ces deux types de reproduction et son implication dans les études de génétique sont discutés.

Key-words : *Heterorhabditis bacteriophora* (Strain HP 88), nematode genetics, self-fertilization, cross-fertilization, reproduction.

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juveniles were transferred individually to NGA plates. At this stage the nematodes are still virgin because they lack a fully developed reproductive system (Zioni *et al.*, 1992 a). To some of the plates five or six young adult wild type (6Dy) males were added and the plates were incubated 4-7 days, at 25 °C until juveniles of the next generation have developed. Third generation nematodes were obtained following the same procedure with eggs of second generation gravid hermaphrodites.

To determine nematode reproduction *in vivo* cadavers of infected *G. mellonella* were dissected 7 day post infection when the 2nd generation progeny have reached the 4th developmental stage (Zioni *et al.*, 1992 a). Juveniles were transferred individually to NGA plates and the plates were incubated 4-7 days, at 25 °C until progeny of the next generation had developed. The nematodes that had developed on each plate, at each generation, were counted and examined under a stereomicroscope Wild, M 8 (X 75), for dumpy or wild type phenotype.

MORPHOLOGICAL OBSERVATIONS

Forty hour-old juveniles that had developed from eggs on NGA plates and juveniles derived from cadavers of *G. mellonella* dissected 7 day post infection, were examined under a compound microscope (Leitz, Orthoplan) (× 300).

Results

REPRODUCTION OF THE FIRST GENERATION

Most (55 %-78 %) of the hermaphrodites, originating from IJ, which were transferred individually as J4 to NGA plates, gave rise to progeny (Table T, Nos 1-3). The offspring of wild-type or dumpy strains maintained the corresponding phenotypic characteristics of their parents (Table 1, Nos 1-3) indicating that self-fertilization had taken place.

When wild-type hermaphrodites from the first generation were placed with wild-type males, the progeny were phenotypically wild type, and could have originated from either self- or cross- fertilization (Table 1, No. 4). However, in crosses between first generation dumpy hermaphrodites and wild type males, 62-78 % of the progeny were wild type and 22-38 % were dumpy (Table 1, Nos 5, 6). Since these dumpy mutations are recessive (Koltai *et al.*, 1994), the wild type progeny of the dumpy hermaphrodites must have originated from cross-fertilization by the wild type males, whereas the dumpy progeny originated from self-fertilization.

REPRODUCTION OF THE SECOND AND THIRD GENERATIONS

Forty hours after their emergence from eggs, derived from the bodies of first generation hermaphrodites, two types of progeny were evident in either the wild-type or the dumpy strains : approximately 50 % of the progeny

Table 1. Average number and phenotype of progeny obtained from self-fertilization and crosses between wild-type (WT) as well as mutant (Hbdpy-1 & Hbdpy-2) 1st generation single hermaphrodites and wild-type males of *Heterorhabditis bacteriophora* HP88 strain.

Crosses	n ^a	Fertile	Progeny phenotype*	
			Dumpy	Wild-type
1 WT (self)	59	75	-	78 ± 31
2 Hbdpy-1 (self)	55	78	54 ± 15	-
3 Hbdpy-2 (self)	78	55	51 ± 32	-
4 (♂ ^b) WT × WT (♂)	7	71	-	47 ± 12
5 ^b (♀) Hbdpy-1 × WT (♂)	6	83	8 ± 6	28 ± 15
6 ^b (♀) Hbdpy-2 × WT (♂)	19	79	29 ± 35	48 ± 41

n^a = Number of hermaphrodites which were transferred to individual plates.

♂^b = None-male adults

* Average ± SEM.

Table 2. Measurements of two types of juveniles (J₄ and HJ) obtained from 2nd generation progeny of wild-type (WT) and mutant (Hbdpy-1 and Hbdpy-2) of *Heterorhabditis bacteriophora* HP88 strain. The nematodes were measured 40 h after egg hatching. Twenty individuals were measured for each type.

Strain	Juvenile type	Length *	Width *
WT	J ₄	0.76 ± 0.10	0.05 ± 0.01
WT	HJ	0.38 ± 0.08	0.03 ± 0.01
Hbdpy-1	J ₄	0.46 ± 0.07	0.05 ± 0.01
Hbdpy-1	HJ	0.32 ± 0.02	0.02 ± 0.01
Hbdpy-2	J ₄	0.44 ± 0.06	0.06 ± 0.01
Hbdpy-2	HJ	0.31 ± 0.04	0.02 ± 0.01

* mm (average ± SEM).

had developed to comparatively large juveniles of the 4th developmental stage (J₄) with conspicuous reproductive systems. The other progeny were significantly shorter (P < 0.05, *t* test) by 1.4-2 times and 1.6-3 times thinner (Table 2) than the "normal" J₄ described above. They were less developed than the J₄ type (Fig. 1 B) and their reproductive system was not noticeable.

Among 550 of the J₄ type individuals (either wild-type or dumpy) which were transferred to separate NGA plates 500 (90 %) completed their development to fully reproductive non-male adults and 10 % developed to males. Out of the 500 non-male adults only 9 (i.e. 1.8 %) gave rise to progeny (Table 3 A, Nos 1-3, 7-9). However, when either dumpy or wild-type individuals of the non-male J₄ type were placed together with wild-

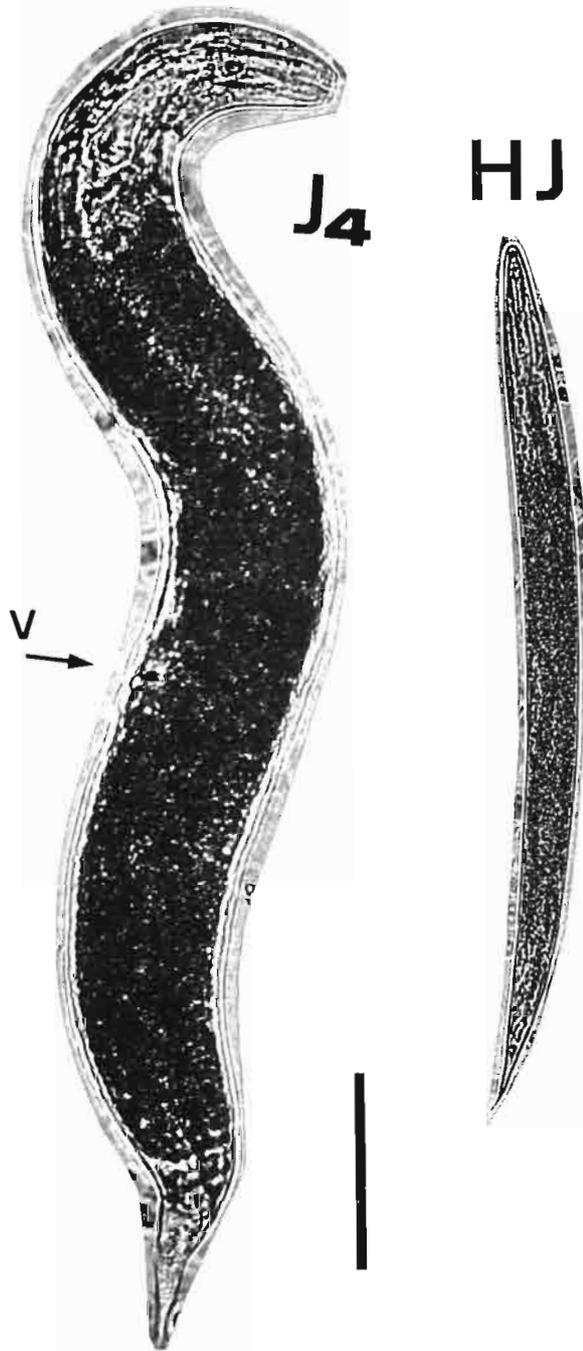


Fig. 1. Microphotograph of 40-h-old juveniles of *Heterorhabditis bacteriophora* developed from eggs on Nematode Growth Agar (NGA). The J4 will develop to amphimictic female and the HJ will develop to hermaphroditic adult (Bar = 100 μ m).

type males, reproduction increased markedly: 30%-71% of them gave rise to progeny (Table 3 A, Nos 4-6, 10-12) all of which had a wild-type phenotype. These

results indicate that where the mother was dumpy, and by extrapolation also when it was wild-type, reproduction was solely by cross-fertilization. These non-male adults were therefore termed "females".

None of the 105 smaller-type juveniles which had been individually transferred to NGA plates, developed to a male. Seventy-eight of them (74%) reproduced (Table 3 B, Nos 1-3, 5, 6), indicating a high rate of self-fertilization i.e. high proportion of hermaphrodites. Therefore these smaller type juveniles were termed "HJ" (H for hermaphrodite). When dumpy juveniles of the HJ type were placed together with wild-type males, 70% ($n = 30$) gave rise to progeny (Table 3 B, Nos 4, 7), and in all cases, dumpy (46%-69%) and wild type (31%-54%) phenotypes appeared among them indicating reproduction both by self- and cross-fertilization, respectively (Table 3 B, Nos 4, 7). Egg laying by the HJ type which reproduced by self- as well as by cross-fertilization started 3-4 days after placing them on plates.

As for the nine nematodes which reproduced by "selfing" among the J4 type it is possible that these individuals were in fact HJ type juveniles which were misidentified as J4. The egg laying by these few individuals in the J4 started, like the HJ type, 3-4 days after placing them in individual plates, whereas egg laying following cross-fertilization started 1-2 days after placing the parents on plates.

Mixed population of juveniles of J4 and HJ types were also found *in vivo* in cadavers of infected *G. mellonella* larvae dissected 7 days post infection. The number of progeny of each juvenile type varied considerably ($n =$ eight cadavers): 7 ± 35 type J4 (range 13-116) and 39 ± 28 type HJ juveniles (range 2-82) indicating roughly a 2 : 1 ratio of J4 : HJ *in vivo*. Among forty-two J4 type individuals which were transferred from the cadavers to separate NGA plates only five (12%) reproduced, indicating a low rate of self-fertilization as observed for J4 *in vitro*. On the other hand 26 out of 29 (90%) individual HJ types derived from the cadavers gave rise to progeny when transferred to individual plates, indicating a high rate of self-fertilization as observed for the HJ type *in vitro*.

Differences in fecundity were recorded between *in vitro* self-fertilizing hermaphrodites of the first and subsequent generations. While the wild-type 1st generation gave rise to an average of 78 ± 31 offspring per hermaphrodite (Table 1, No. 1), hermaphrodites developing from the HJ type produced 2.5 times more progeny (Table 3 B, No. 1).

Discussion

The results reported here indicate that there are two reproductive strategies in the second and the third generations of the population of *H. bacteriophora* HP 88, that appear in parallel: *i*) reproduction by cross-fertil-

Table 3. Number and phenotype of progeny obtained from self-fertilization and crosses between wild-type (WT) as well as mutant (*Hbdpy-1* and *Hbdpy-2*) of 2nd and 3rd generations single non-male adults, developed from : A) 4th stage juvenile (J4) or B) HJ type juveniles of *Heterorhabditis bacteriophora* HP88 strain and WT males.

Crosses			Generation	n ^a	% Fertile	Progeny phenotype *	
						Dumpy	Wild-type
A. (J4)	1	WT (self)	2nd	40	5	0 ± 0	235 ± 64
	2	<i>Hbdpy-1</i> (self)	2nd	57	7	33 ± 40	0 ± 0
	3	<i>Hbdpy-1</i> (self)	2nd	154	0	0 ± 0	0 ± 0
	4	(♀ ^b) WT × WT (♂)	2nd	47	30	0 ± 0	55 ± 31
	5	(♀) <i>Hbdpy-1</i> × WT (♂)	2nd	38	39	0 ± 0	53 ± 25
	6	(♀) <i>Hbdpy-2</i> × WT (♂)	2nd	35	40	0 ± 0	41 ± 24
	7	WT (self)	3rd	100	1	0 ± 0	24 ± 0
	8	<i>Hbdpy-1</i> (self)	3rd	100	1	50 ± 0	0 ± 0
	9	<i>Hbdpy-2</i> (self)	3rd	50	2	206 ± 0	0 ± 0
	10	(♀ ^b) WT × WT (♂)	3rd	18	56	0 ± 0	56 ± 28
	11	(♀) <i>Hbdpy-1</i> × WT (♂)	3rd	17	71	0 ± 0	29 ± 22
	12	(♀) <i>Hbdpy-2</i> × WT (♂)	3rd	26	50	0 ± 0	44 ± 24
B. (HJ)	1	WT (self)	2nd	14	79	0 ± 0	198 ± 43
	2	<i>Hbdpy-1</i> (self)	2nd	20	85	134 ± 37	0 ± 0
	3	<i>Hbdpy-2</i> (self)	2nd	29	93	147 ± 45	0 ± 0
	4	(♀) <i>Hbdpy-2</i> × WT (♂)	2nd	18	88	113 ± 61	51 ± 47
	5	WT (self)	3rd	12	67	0 ± 0	186 ± 51
	6	<i>Hbdpy-2</i> (self)	3rd	30	50	107 ± 71	0 ± 0
	7	(♀) <i>Hbdpy-2</i> × WT (♂)	3rd	12	42	54 ± 12	63 ± 20

n^a = Number of females which were transferred to individual plates.

♀^b = None-male adults; * = Average ± SEM.

ization exclusively; *ii*) reproduction by self- and cross-fertilization.

These strategies correspond to two types of juveniles that emerged from eggs : *i*) comparatively large, J4 which developed into adults that reproduced amphimictically, and will be referred to as females and males. The females did not reproduce by self-fertilization and when males were available cross-fertilization occurred; *ii*) smaller, HJ juveniles that reproduced by self as well as by cross-fertilization, and will be referred to as hermaphrodites. These hermaphrodites, even when fertilized by males and producing progeny as a result of this originated from cross-fertilization, maintained in parallel the self-fertilization mode of reproduction.

In this study we used two recessive mutants isolated previously in our laboratory (Zioni *et al.*, 1992 *b*; Koltai *et al.*, 1994) as markers which enabled us to determine the mode of reproduction (self *vs* cross-fertilization) of *H. bacteriophora* HP 88 in different generations, regardless of culture conditions and other external effects.

The larger (J4) and smaller (HJ) types of juveniles were observed in the wild-type as well as in the mutant strains (Fig. 1). While their different modes of reproduction could be ascertained, using the mutants as phenotypic markers, only for the mutant strains, it is likely

that they occur in the wild-type strain as well. These two types of juveniles and their different modes of reproduction were observed also *in vivo*, indicating that these phenomena are not an artifact of the *in vitro* culture conditions.

Dix *et al.* (1992) were not able to propagate *H. bacteriophora* following injection of early second generation immature females into *G. mellonella* larvae without the presence of males. They concluded that the initial progeny of *H. bacteriophora* hermaphrodites reproduce exclusively by amphimixis. These findings are in agreement with those of the present study regarding the 2nd generation J4 type juveniles. However, since in the study of Dix *et al.* (1992) only those individuals from the 2nd generation which could be reliably identified as immature females were individually picked and used for crosses, it is likely that the small and less developed HJ type individuals, which we describe in the present study, were not tested by these authors.

Despite the distinct morphological differences between the J4 type and the HJ type reported here, we found some rare incidents of reproduction by self-fertilization among the former type *in vitro* as well as *in vivo*. Dix *et al.* (1992) also reported one case in which progeny were detected in a cadaver that had been injected

only with females. They speculated that this resulted from misidentification of a male nematode that had been accidentally injected along with the females. It is possible that in both present and Dix *et al.* (1992) studies the apparent self-fertilization of J4 type resulted from misidentification of HJ type which developed earlier and were indistinguishable from the J4 type. Noteworthy in our previous studies (Glazer *et al.*, 1991; Zioni *et al.*, 1992 *b*; Koltai *et al.*, 1994) the level of successful self-fertilization was relatively low (36%; unpubl.) when 2nd generation juveniles were arbitrarily transferred to separate plates without differentiating between those that were of the HJ and J4 types.

The two reproductive strategies may have different contributions for survival of the species: first, reproduction by cross-fertilization, characteristic of females derived from the J4 type of juveniles, is essential for preservation of genetic variability. Second, reproduction mostly by self-fertilization, typical of HJ-derived hermaphrodites, is important for mass proliferation of the population, avoiding the need of mating with the rare males. To our best knowledge simultaneous appearance of hermaphrodites and females in the same generation has not been reported in other nematodes, including *Caenorhabditis elegans*.

In this study, the first generation gave rise to fewer progeny than either of the subsequent generations, or the first generations as reported by Zioni *et al.* (1992 *a*). This could be the result of our culture conditions, unknown for now. It should be noted however that in cases reported in the present study where an individual hermaphrodite reproduced by self as well as by cross-fertilization, we could not find any statistically significant difference between the fecundity of progeny originating from self-fertilization and those originating from cross-fertilization (*t* test; Tables 1, 4). Fecundity may depend on the time during the hermaphrodites life at which cross-fertilization actually occurs.

The delayed egg laying phenomenon of 2nd generation hermaphrodites observed here is attributed to the general slow development noted for the HJ type progeny. Different rates of development of the two types of juvenile described here might have an important role in the timing of cross *vs* self-fertilization in the population *H. bacteriophora* population. However, the factors influencing the course of juvenile development to J4 or HJ and subsequently to the two reproductive types of adults, remain to be determined. Nor have the stages of development of the newly identified HJ been described. Nevertheless, our results allow the establishment of procedures for genetic studies of *H. bacteriophora*. Thus, inbred lines, e.g. homozygous for a desired mutation/trait, are created by self-fertilization of individuals developing from HJ juveniles. On the other hand nematodes developing from J4 juveniles can be used for cross-fertilization, e.g. in order to combine two desired traits in a single line.

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