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**BACTERIA MODULATE THE DEGREE OF AMPHIMIX OF THEIR SYMBIOTIC
ENTOMOPATHOGENIC NEMATODES (*Heterohabditis* spp) IN RESPONSE TO
NUTRITIONAL STRESS**

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Abstract: Facultatively sexual entomopathogenic nematodes are a promising model for the experimental study of the adaptive values of sex. Our experiments in the laboratory showed that Entomopathogenic nematodes display at least two different strategies in regulating the degree of amphimix as a response to nutritional stress. One strategy promotes the production of males, amphimix and the genetic variability of the offspring, improving the chances for a successful new adaptation. Another strategy increases the production of hermaphrodites at the expense of males, increasing the total number of reproductive individuals and thus the total number of offspring produced. Surprisingly, the strategy used depends upon the strain of symbiotic bacteria the nematodes are growing. The relevance of the results, in helping to discriminating between rival theories for the evolutionary maintenance of sex, is discussed.

INTRODUCTION: Although sex is ubiquitous, there is no generally accepted explanation for its existence (Hurst & Peck, 1996). Do sex helps organisms to adapt to new or to changing environments by increasing genetic variance? Or, do organisms fare better under environmental stress when they are able to boost their reproductive output by switching to asexual reproduction? A recent theoretical model suggests that sex may be advantageous in solving evolutionary problems in highly competitive situations, if adaptation can proceed at a moderate pace and if the environmental changes force adaptive changes in several different genes simultaneously (Jaffe 1996, 2000). The model predicts that under environmental stress, a reversion from sex to no sex (or from bisexual to monosexual reproduction) is advantageous if the adaptation requires the involvement of a few (<4) genes. The reverse is true if the adaptation to the new environmental demands require the simultaneous change in various genes or loci.

The nematodes in the family Heterorhabditidae are insect parasites that cultivate and feed on symbiotic bacteria of the genus *Photorhabdus*, which in turn feed on dead insects (Kaya & Gaugler 1993). The life cycle of these entomopathogenic nematodes consists of eggs, four juvenile stages (instars) and adults. Third instars may become infective agents when, covered with the cuticle of the second instars, they stop feeding and leave the dead insect taking some bacteria with them in the intestinal tract to infect new insects. When infecting a live insect, the nematode enters the hemolymph, releases its symbiotic bacteria and substances that kill the insect in about 24-72 hours. Once inside an insect the nematodes reproduce either amphimictic (producing males and females) or by producing hermaphrodites which reproduce either automictic (auto fecundation), amphimictic (copulating with males) or through matricidal endotoky (Strauch et al. 1995). In this endotoky, eggs are retained in the body of the mother and the juveniles grow inside their mother killing her and then forming infective juveniles. When nematodes are starved and/or nematode population densities are high, more infective juveniles are formed (Wang & Bedding 1996). This phenomenon may be regulated by pheromones (Golden & Riddle, 1984). The relationship between the nematode and the symbiotic bacteria seems to be species and strain specific (Wilkinson & Hay, 1997). These nematodes are known to produce variable proportions of males under different circumstances (Strauch et al. 1995). Here we explore the strategies used by these nematodes in regulating amphimix as a response to nutritional stress, as a test case for the proposed theoretical model.

MATERIALS AND METHODS: Nematodes of the species *Heterorhabditis bacteriophora* (Hb) and *H. indica* (Hi) were used. Samples of Hb were Hb1: FRG01 (Basse Terre, Guadeloupe, French Antilles), Hb2: HP88adams and Hb3: HP88 (both from Ohio, USA); and samples of Hi were Hi1: FRG20 (Guadeloupe) and Hi2: FRG22 (St. Barthelemy), all provided by the Laboratoire des Nématodes Parasites des Insectes, INRA, Guadeloupe. Nematodes were reared on *Galleria mellonella* as described

by Woodring & Kaya (1988). The bacteria were isolated using the "hanging drop" method described by Poinar & Thomas (1966) and reared on Petri dishes at 26–28°C over McConkey agar. Bacteria were named using the code for the nematode strain they originated from, preceded by the letter "B". Tests for presence of the correct bacteria included assessing the presence of catalase using hydrogen peroxide and assessing the presence of antibiotics by testing production of toxins against *Micrococcus luteus*.

The bacteria were grown on Petri dishes for 48 h and then either 50, 100 or 200 nematodes producing initial densities of 5, 10 and 20 infective juveniles per cm² (Zioni et al., 1992; Koltai et al., 1995) were placed in the dish. The dish contained for agar1 and agar2 respectively 16 and 8 g/l of meat extract nutrient broth, 11 and 0 ml/l wheat oil and 15 g/l agar-agar. After 6 days (when second generation adults matured), nematodes were killed and fixed in TAF (2 % triethanol-amine + 3 % Formaline) and individuals were counted (juveniles and adults: males, females and hermaphrodites). At least five replicate experiments were performed for each combination of variables and at least 3 different samples were taken from each dish for counting the nematodes.

RESULTS: Nematode populations cultured on bacteria growing on the poorer agar2 were always smaller than those cultured on richer agar1 ($p < 0.0001$, $t = 21.1$, $df = 174$, t -test). The percentage of males among the adult population changed between agar2 and agar1, depending on the bacteria present in the culture (Figure 1A). A breakdown Anova respecting grouping variables gave values of $F = 5.9$, $df = 23 \times 71$, $p < 0.0001$ and $F = 6.3$, $df = 23 \times 69$, $p < 0.0001$ for data on graphs a and b of Figure 1 respectively. That is, nematodes Hb2 and Hi2 cultured on their symbiotic bacteria (BHb2 and BHi2 respectively) produced a lower percentage of males in poor agar compared to richer agar, whereas nematodes Hb1 cultured on their symbiotic bacteria produced higher percentages of males in the poorer agar. Nematodes Hb3 cultured on bacteria BHb3 produced only juveniles and hermaphrodites while the same nematodes cultured on BHb2 and BHb1 produced also males (not shown). Nematodes Hi1 and Hi2 cultured on BHi1 did not grow under the experimental conditions used (not shown). When we exchanged the bacteria between the nematode strains, a pattern, practically identical to that shown in Fig 1A was observed (Fig. 1B). Thus, the pattern of male production among nematodes depended on the bacteria on which they were feeding rather than on the nematode strain. The exceptions were BHb2/Hb1-seed 100 and BHb1/Hb2-seed 200, where the nematode strain seemed to be a better predictor for the relative pattern of male production on the two types of agars. Extreme examples were Bacteria BHb3, which always made the nematode strain growing on them produce only hermaphrodites. A multiple regression analyzing the effect of bacteria strain, nematode strain, initial number of nematodes and agar ($F(4,171) = 2.6$, $p = 0.038$) showed that the bacteria strain predicted variance at $p = 0.008$, nematode strain at $p = 0.25$, initial number of nematodes

at $p = 0.31$ and agar at $p = 0.56$.

DISCUSSION: Results showed that at least two different strategies for male production under dietary stress are used by these nematodes. One strategy increases the proportion of males produced, whereas the other increases the proportion of hermaphrodites produced when nematodes suffer dietary stress. The type of strategy chosen is in great part dependent on the symbiotic bacteria. Both strategies have adaptive advantages. An increased production of males increases the chances for amphimictic reproduction, eventually increasing genetic variability of the offspring, improving the chances for successful adaptation to dietary stress. An increased production of hermaphrodites (and thus lower production of males), increases the total number of reproductive individuals and thus the total number of offspring produced, increasing the chance that a few may survive to infest new insects. Similar work with trematodes (Trouvé et al., 1999) showed that the proportion of males increased with the number of adult individuals. Here we found that the proportion of males related to the amount of adults negatively (Correlation coefficient $r = -0.33$, $p = 0.006$, $n = 68$) when bacteria Hb1 were present and positively ($r = 0.46$, $p < 0.0004$, $n = 54$) with bacteria Hi2, suggesting that the strategy of the trematodes coincides with that of BHi2 which reduces the proportion of males produced as a response to nutritional stress. It is intriguing that the strain of symbiotic bacteria affects the strategy the nematode is to use, the more so as the specific adaptive advantage for the bacteria or for the nematode of each strategy are unknown. It is though tempting to speculate, based on the results of the theoretical model, that the mechanisms uncovered here optimize the speed of adaptation. That is, the bacteria induce the switch to sexual reproduction in nematodes whenever they are overwhelmed by environmental change, whereas asexual (or monosexual) reproduction is favored when bacteria can cope with the environmental change, as they themselves reproduce mainly monosexually.

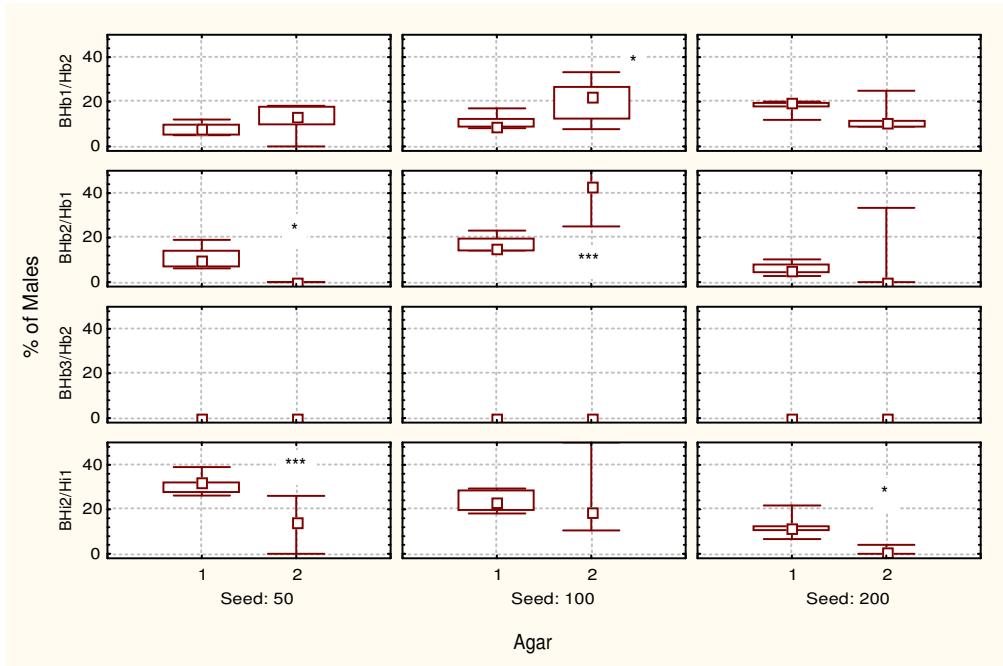
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Figure 1: Min-max, 25%-75% range and Median value for the percentage of males among the adult population in experiments with different bacteria/nematode combinations growing on two different agars (Agar1 has about twice as much nutrients as agar2). A: Experiments growing nematodes on their own bacteria. B: Experiments growing nematodes on bacterial cultures from other nematode strains. Seed indicates the number of nematodes initially placed in the dish. Data with * and *** differ from those of agar1 at $p < 0.05$ and $p < 0.001$ respectively using LSD on transformed data using formula $y = \arcsin(\sqrt{x/100})$.

A:



B: