Factors Affecting the Ratio of Female to Male of Pinewood Nematodes Bursaphelenchus xylophilus (Steiner & Buhrer) Nickle

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Abstract: The pinewood nematode Bursaphelenchus xylophilus (B. xylophilus) is responsible for a devastating disease of pine forests. Its pathogenicity is closely related to the numbers of individual B. xylophilus. The ratio of female to male (sex ratio) is key to understanding population change in this species. The sex ratio of B. xylophilus varies widely, but it is unclear how it is affected by environmental changes. The sex ratios of nematodes, isolated from different samples in the wild, varied between 0.93 and 2.20. Under laboratory conditions, maternal age and the population did not affect the sex ratio of progeny. A change from good to poor nutritional status was associated with a reduction of the sex ratio of progeny from 1.85 to 1.41, which was speculated to result from a change in the primary sex ratio. Thus, B. xylophilus effectively maintains the sex ratio with maternal age and population changes but adjusts the sex ratio of progeny on the basis of the changes in nutrition.

Keywords: pinewood nematodes; sex ratio; nutrition

1. Introduction

Sex ratio is a core concept of modern evolutionary ecology. In addition to elucidating relationships among the sexual selection, population dynamics, and evolutionary trajectories [1–4], it is also the focal point of adaptive allocation strategies in which individuals match current and expected fitness payoffs through the adjustments to their relative investment in males and females [5–7]. In both natural and experimental infections of acanthocephalan and nematode parasites, the sex ratio is often biased toward females among the adult population [8].

The pathogen of pine wilt disease—B. xylophilus is an invasive species with the potential to cause severe damage to forests worldwide. This nematode has a complex lifestyle; under suitable environmental conditions, the nematodes will reproduce and grow very rapidly from egg to adult. However, when the environment becomes unsuitable, the dispersal-stage larvae 3 (LIII) and dispersal-stage larvae 4 (LIV) appear [9,10]. Population numbers of B. xylophilus exhibit a “boom-and-bust” pattern [11]. Following infection with B. xylophilus, healthy pine trees rapidly wither. The reproduction ability of pinewood nematodes is a significant factor that influences its pathogenicity [12], and the sex ratio is an important factor determining population reproduction rate [13]. This nematode is female biased in the propagative cycle [11], but there is little information concerning the sex ratios in the dispersal stages, LIII and LIV.

Many factors are known to affect sex ratios. The sex ratio of B. xylophilus varies between 1.0 and 5.0 with temperature change [11]. When cultured in a competitive environment, the sex ratio is 4.6:1; under a noncompetitive environment, the sex ratio is 9.3:1 [14]. However, between generations were not distinguished. Therefore, it is uncertain whether the change in adult sex ratios resulted from a difference in life span between males and females or...
from changes in the sex ratio of progeny. Furthermore, to date, no research has focused on the impact of other factors on the sex ratio of *B. xylophilus*, particularly maternal age, population, and nutrition. Maternal age is known to affect the sex ratio of the progeny of other animals [15,16]. In *Caenorhabditis elegans* Maupas, the phenotypes of the progeny are affected by maternal age [17], although effects on sex ratio have not been reported. Change in nematode population contributes to density changes, thus leading to differential mortality and change in the sex ratio [18,19]. Poor nutritional conditions might change the energy allocation to reproduction to maximize the number of young produced [20]. For example, in the years of food scarcity or when the parents are in poor condition, the broods of the American kestrels Falco tend to be male biased [21].

In this study, we first investigated the distribution range of the sex ratio of *B. xylophilus* in the wild. Then, we examined the impact of maternal age, population, and nutrition on the sex ratio of the progeny under laboratory conditions. Finally, an analysis was conducted of the potential causes of these changes.

2. Materials and Methods

2.1. Sex Ratios of *B. xylophilus* in the Wild

To survey the sex ratio of nematodes in the wild, the Baermann funnel technique was used to isolate nematodes from pine trees and from the insect vector *Monochamus alternatus* Hope, as detailed in Table 1. The sex ratio of *L*$_{III}$ was calculated in accordance with Ishibashi [22], and for *L*$_{IV}$, it was calculated after the transformation into adults [10]. Male and female were identified in accordance with Ishibashi [22].

| Sampling Sites (Date) | Sampling (Separated Parts) | Nematode Morphology | Female | Male | Sex Ratio (Female/Male) |
|-----------------------|---------------------------|---------------------|--------|------|------------------------|
| Liaoning Province (July 2019) | *Pinus tabuliformis* Carr. (Lower middle trunk) | Propagative | 132 | 78 | 1.69 |
|                        |                          |                     | 90 | 63 | 1.43 |
|                        |                          |                     | 95 | 49 | 1.94 |
| Guangzhou Province (July 2019) | *Pinus massoniana* Lamb (Lower middle trunk) | | 84 | 41 | 2.05 |
|                        |                          |                     | 110 | 50 | 2.20 |
|                        |                          |                     | 105 | 55 | 1.91 |
| Liaoning Province (December 2018) | *Pinus tabuliformis* Carr. (Lower middle trunk) | *L*$_{III}$ | 357 | 382 | 0.93 |
|                        |                          |                     | 241 | 222 | 1.09 |
|                        |                          |                     | 193 | 169 | 1.14 |
|                        |                          |                     | 141 | 130 | 1.08 |
|                        |                          |                     | 344 | 315 | 1.09 |
| Zhejiang Province (September 2020) | *Monochamus alternatus* (The whole body) | *L*$_{IV}$ | 90 | 92 | 0.98 |
|                        |                          |                     | 217 | 228 | 0.95 |
|                        |                          |                     | 143 | 133 | 1.08 |
|                        |                          |                     | 234 | 240 | 0.98 |
|                        |                          |                     | 160 | 143 | 1.12 |

*L*$_{III}$ and *L*$_{IV}$ indicate dispersal-stage larvae 3 and 4 of *B. xylophilus*.

2.2. Sex Ratios and Survival Rates of *B. xylophilus* at Different Developmental Stages

*B. xylophilus* strain NXY61 (virulent) was derived from *Pinus massoniana* in Ningbo, Zhejiang Province, and was cultured on *Botrytis cinerea* Pers. on potato dextrose agar (PDA) culture medium at 25 °C in the dark.

Before investigating the factors influencing the adult sex ratio of nematodes, the sex ratios of various developmental stages and the survival rate of eggs to *L*$_2$, *L*$_3$, *L*$_4$, and adult stages, were determined. The eggs produced by adult day 1 were collected,
and before incubation at 25 °C in the dark for 24 h, almost all eggs hatched to L2. To obtain L3, L4, and adults, about 3000 L2 were inoculated onto B. cinerea for 30 h, 54 h, and 72 h, respectively. Each treatment had five replicates. About 500 worms were counted to calculate the sex ratio in each replicate. Survival rates were calculated as: (number collected of each developmental stage/number of nematodes inoculated) × 100%.

2.3. The Effects of Maternal Age and Population on the Sex Ratio

To study the effect of maternal age on the sex ratio of the progeny, the eggs produced by the adult day 1, 3, 5, and 7 were collected. To minimize potential effects of population density, the eggs, larvae, and adults were separated while the inoculation of collected adults continued on B. cinerea on day 1, 3, and 5. The collected eggs were inoculated onto the B. cinerea and cultured for 4 days to adult, and the sex ratio was calculated. Each treatment had five replicates. A total of 12 eggs were measured for each repeat, and the size of the eggs was calculated as \( V_{\text{EGG}} = \frac{4}{3}\pi ab^2 \) [23]. About 500 worms were counted to calculate the sex ratio of each replicate.

To explore the effect of the population change on the sex ratio of progeny, L4 late-stage nematodes were collected and batches of 2000, 10,000, and 50,000 nematodes were inoculated onto B. cinerea. The eggs were collected from the adult day 1. Eggs sizes and sex ratios were then calculated. Each treatment had three replicates.

2.4. The Influence of Different Nutrition

To examine the effects of nutrition, approximately 1500 L2 nematodes from eggs collected from adult day 1 were inoculated on 1*PDA (24 g PDB and 15 g agar in 1 L distilled water), 1/4*PDA (6 g PDB and 15 g agar dissolved in 1 L distilled water) or 1/16*PDA (1.5 g PDB and 15 g agar dissolved in 1 L distilled water), which had been inoculated with B. cinerea for about 7 d at 25 °C. The eggs were collected adult day 1. The survival rate of egg to adult, sex ratio, and egg size were determined as described in Sections 2.2 and 2.3. On the adult day 2, the adult nematodes were photographed with Axio Imager.A2 (Carl Zeiss GmbH, Oberkochen, Germany). Area (A), perimeter (P), length, and width were measured manually using Image J-win 64 (https://imagej.nih.gov/ij/ 1 January 2021). Each treatment had five replicates, twelve eggs, and six adult nematodes measured for each repeat, and the size of adults was expressed as \( V_{\text{Adult}} = \pi A^2/(2P) \) [23].

2.5. Statistical Analyses

SPSS v. 19.0 software (IBM Corp., Armonk, NY, USA) was applied to conduct all statistical analyses. One-way ANOVAs with Tukey’s test were carried out to detect the difference among treatments, and statistical significance was accepted at \( p < 0.05 \). The graphs were drawn using Origin 2017 (Origin Lab Corp., Northampton, MA, USA).

3. Results

3.1. Sex Ratios in the Wild

In the samples collected from different regions, the sex ratio of pinewood nematode varied between 0.93 and 2.20 (Table 1). The sex ratios of B. xylophilus in the propagative phase collected from the pine trees deviated significantly from 1.0 (\( N = 6, df = 5, p = 0.001 \), one-sample t-test), and were female biased. However, the sex ratios of LIII isolated from Pinus tabuliformis and the LIV isolated from M. alternatus did not deviate significantly from 1.0.

3.2. Sex Ratio at Different Developmental Stages

As it is difficult to distinguish the sex of the L2 stages, it was not included in the calculations; the sex ratios of L3, L4, and adults were not significantly different (1.90 ± 0.06, 1.90 ± 0.05, and 1.94 ± 0.03, respectively). The hatching rate of eggs to L2 approached 100%. Many nematodes died between L2 and L3; the survival rate was about 69.06%, and then the survival rate remained unchanged up to the adult stage (Figure 1).
3.1. Sex Ratios in the Wild

In the samples collected from different forest stands, the sex ratio of the progeny did not change significantly with maternal age (1.93 ± 0.08, 1.93 ± 0.06, 1.94 ± 0.06, and 1.84 ± 0.04 on adult days 1, 3, 5, and 7, respectively); the average size (volume) of eggs also did not change significantly with maternal age (Figure 2a, c). The size of parent adult nematodes was significantly influenced by differences in nutrition during their development from L2. As the nutritional status deteriorated, the length, width, and volume of nematodes decreased markedly. The percentage size decrease was larger in females than in males (1*PDA cf. 1/16*PDA, females decreased 53.9%, males

3.3. Effects of Maternal Age and Population on the Sex Ratio of Progeny

The sex ratio of the progeny did not change significantly with maternal age (1.93 ± 0.08, 1.93 ± 0.06, 1.94 ± 0.06, and 1.84 ± 0.04 on adult days 1, 3, 5, and 7, respectively); the average size (volume) of eggs also did not change significantly with maternal age (Figure 2a, c). The average size of the eggs decreased as population size increased (19,298.8 ± 207.2, 18,395.9 ± 75.6, and 16,990.8 ± 314.8 μm³ in populations of 2, 10, and 50 thousand) (Figure 2d), but the sex ratio of the progeny remained unchanged (1.94 ± 0.07, 1.85 ± 0.08, and 1.91 ± 0.10, respectively) (Figure 2b).

3.4. Influence of Different Nutrition on Nematodes

The size of parent adult nematodes was significantly influenced by differences in nutrition during their development from L2. As the nutritional status deteriorated, the length, width, and volume of nematodes decreased markedly. The percentage size decrease was larger in females than in males (1*PDA cf. 1/16*PDA, females decreased 53.9%, males
41.0%) (Table 2). However, no difference was observed in the sex ratio of parent adult under the different nutritional conditions (about 1.84).

**Table 2. The effects of different nutritional conditions on the parent adult size and sex ratio.**

| Different Nutrition | 1*PDA | 1/4*PDA | 1/16*PDA |
|---------------------|-------|---------|-----------|
| **Female**          |       |         |           |
| Length (µm)         | 1060.64 ± 18.64a | 963.84 ± 21.60b | 851.96 ± 8.00c |
| Width (µm)          | 33.06 ± 0.36a   | 29.02 ± 0.52b   | 25.18 ± 0.27c  |
| Volume (µm³)        | 657.009.80 ± 24,960.82a | 476.051.00 ± 14,296.26b | 302.878.40 ± 5530.04c |
| **Male**            |       |         |           |
| Length (µm)         | 909.22 ± 13.24a | 914.36 ± 8.05a  | 770.62 ± 7.03b |
| Width (µm)          | 27.70 ± 0.56a   | 25.56 ± 0.24b   | 22.18 ± 0.50c  |
| Volume (µm³)        | 391,535.60 ± 16,897.34a | 354,042.40 ± 6961.64a | 231,020.60 ± 19,400.00b |
| **Sex ratios**      | 1.84 ± 0.03     | 1.84 ± 0.03     | 1.82 ± 0.01    |

The data indicate average ± se. Different letter labels indicate significant difference at p < 0.05; data were analyzed by Tukey’s multiple comparison test. 1*PDA (24 g PDB and 15 g agar in 1 L distilled water), 1/4*PDA (6 g PDB and 15 g agar dissolved in 1 L distilled water) or 1/16*PDA (1.5 g PDB and 15 g agar dissolved in 1 L distilled water).

The sex ratio, egg size, and the number of progenies produced by adults that developed in 1/16*PDA were all significantly lower than these values for the progeny of adults that developed in 1*PDA; the sex ratios were 1.85 ± 0.05, 1.67 ± 0.04, and 1.41 ± 0.05 in 1*PDA, 1/4*PDA, and 1/16*PDA, respectively (Figure 3a). However, the subsequent survival rates of the egg-to-adult stage did not differ significantly (66.06%, 66.44%, and 64.09%, respectively) (Figure 3c).

**Figure 3.** The effects of different nutrition statuses on the progeny: (a) sex ratio; (b) egg size; (c) survival rate of egg to adult; (d) number of progenies produced by each female. Different letter labels indicate statistical differences among the means. Error bars represent ± se, p < 0.05. Data were analyzed by Tukey’s multiple comparison test. 1*PDA (24 g PDB and 15 g agar in 1 L distilled water), 1/4*PDA (6 g PDB and 15 g agar dissolved in 1 L distilled water) or 1/16*PDA (1.5 g PDB and 15 g agar dissolved in 1 L distilled water).
4. Discussion

The population-level sex ratio of *B. xylophilus* varied widely in the wild, suggesting the sex ratio of the progeny is adjusted depending on the environmental conditions (Table 1). In the propagation cycle, the sex ratio of *B. xylophilus* was female biased, while L_{III} and L_{IV} approached 1:1 in the dispersal phase, as revealed by field investigation and laboratory experiment.

The sex ratio of the progeny was unaffected by the maternal age or population, implying that the *B. xylophilus* is able to maintain a constant sex ratio (Figure 2a,b). In contrast, population change did affect egg size (Figure 2d). The sex ratio of the progeny declined from 1.85 to 1.41 under poor nutritional conditions (Figure 3a), while the survival rate was unchanged (Figure 3c). According to the sex-allocation hypothesis proposed by Trivers and Willard, females experiencing poor physiological conditions would lead to producing more females with lower variability [24–26]. In addition, Wiebe and Bortolotti proposed that poor nutritional conditions contribute to the production of the cheaper sex, thus maximizing the number of young produced [21]. Female *B. xylophilus* are larger than males, which means more resources are required for female progeny. As shown in Table 2, when nutrition declined, the size decrease of females was greater than that of males. Therefore, parents are potentially able to fine-tune the number and quality of progeny according to the predominant circumstances by facultative manipulating of progeny sex. As a result, parental fitness is maximized. In the wild, the nutrition becomes poorer as the trees weaken and die; the nematode larvae then enter the dispersal L_{III} state and aggregate around *M. alternatus* [27,28], L_{III} molt to L_{IV}, and L_{IV} larvae are transmitted by the beetle vector. It was suspected that the sex ratio dropped to roughly 1.0 as the nutrient supply further deteriorated. Invasion by a small number of L_{IV} founders initiates the process within a single tree [29]. When the density/number of males falls below a threshold and in the absence of such a limit on females, the population is unlikely to survive in one-dimensional models with a fixed sex ratio [30]. Moreover, the invasion is stalled by a skewed sex ratio and sex-biased dispersal [31].

The mechanisms underlying biases in the sex ratio of adult populations of nematodes and acanthocephalans are unclear. These biases in adult sex ratios might result from (1) environmental sex determination, (2) varying mortality between members of the two sexes, and/or (3) an adaptive bias in the primary sex ratio. No differences were observed in the sex ratio when L_{2} was cultured to adults at different temperatures or after different starvation time or nutritional conditions (data not shown). The haploid number of chromosomes (N) is six (2n = 12), and the sex-determination system for *B. xylophilus* might consist of XX female and XY male [32]. The sex ratios of L_{III} and L_{IV} approached 1:1. Therefore, it is unlikely that the environment determines the sex of *B. xylophilus*. Female bias is suspected to result from the differential death of males and females because a large number of nematodes died between L_{2} and L_{3} (Figure 1). However, when the sex ratio of the progeny changed (Figure 3a), no difference was observed in the survival rate (Figure 3c). Therefore, we propose that female biased and the sex ratio changed of progeny is an adaptive bias in the primary sex ratio. Biased primary sex ratios are known in other invertebrates with chromosomal sex determination and male heterogamety, including crickets [33] and nematodes [34,35].

5. Conclusions

Our observations suggest that the sex ratio of *B. xylophilus* is female-biased favorable environments. When conditions are unsuited to population reproduction, however, the numbers of females and males equalize. *B. xylophilus* effectively adjusts the sex ratio of progeny to adapt to changes in nutritional conditions.
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