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ORIGINAL PAPER

Species displacement facilitated by ascarosides between two sympatric sibling species: a native and invasive nematode

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Abstract

Competitive displacement is not only the most extreme outcome of interspecific competition, but also an important strategy for invasive species to be successful invaders. Pinewood nematode, *Bursaphelenchus xylophilus*, the causal agent for pine wilt disease and global quarantine pest, usually displaces *Bursaphelenchus mucronatus*, a native sympatric sibling species, during its invasion process. Despite this prevalent outcome, the driving forces behind this displacement remain elusive. Ascarosides, an evolutionarily conserved family of nematode pheromones, are versatile in structure and function. We hypothesize these nematode pheromones play a role in species displacement. To investigate this hypothesis, we compared the ascarosides composition of *B. xylophilus* and *B. mucronatus* by LC–MS/MS followed by bioassays to test the responses of two nematodes to both crude and synthetic ascarosides. We found that asc-C5 (ascr#9) was the most abundant component and that there were no differences in pheromone composition between the two nematode species. *B. xylophilus* had faster growth rates under competition conditions. Furthermore, low concentrations of both crude and synthetic ascarosides [asc-C5, asc-C6 (ascr#12) and their mixture] enhanced female fecundity and body length growth in *B. xylophilus* but not in *B. mucronatus*. In contrast, body length of *B. mucronatus* was suppressed by a crude extract of its own ascarosides as well as by synthetic ascarosides (asc-C5, asc-C6 and their mixture). Our results strongly suggest that ascarosides play a role in the competitive displacement between two nematode species, which could explain the phenomena observed in *B. xylophilus*-invaded forests where *B. mucronatus* widely existed prior to *B. xylophilus* invasion.

Keywords Competitive displacement · Ascarosides · *Bursaphelenchus xylophilus* · *Bursaphelenchus mucronatus*

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Key message

- The global quarantine pest, *Bursaphelenchus xylophilus*, usually displaces the native nematode *Bursaphelenchus mucronatus* during its invasion process.
- The driving forces behind this competitive displacement remain elusive.
- Here, we found that the displacement between the two nematode species was facilitated by nematode pheromones—ascarosides.
- Ascarosides can increase fecundity and female body length of *B. xylophilus* at low concentrations.
- These findings provide new information and perspectives for alleviating the negative effects of biological invasion.

Introduction

Interspecific competition is a fundamental mechanism in structuring species communities (Stewart 1996). Of the many possible outcomes resulting from interspecific competition, the most severe is competitive displacement (Reitz and Trumble 2002). The competitive exclusion principle demonstrates that two species occupying the same niche cannot coexist in the same location, resulting in one species displacing the other species (Ayala 1971). Interestingly, competitive displacement is often associated with biological invasion, and more than 80% of cases involve exotic species displacing native species or previously established exotic species (Reitz and Trumble 2002). Nevertheless, the mechanisms of competitive displacement are complex. Reitz and Trumble (2002) summarized eight mechanisms of competition causing displacement, involving in exploitation and interference competition. And these mechanisms might be mediated by many biotic and abiotic factors (Gao and Reitz 2017). Usually, competitive displacement is a result of joint action of multiple mechanisms. In Kenya, *Bactrocera invadens* displaced the indigenous species *Ceratitis cosyra* in mango agroecosystems by direct contests for resources among larvae and aggressive behaviors of the invader (Ekesi et al. 2009). Generally, displacing species are more destructive than those that are displaced, especially in biological invasions. Displacements often aggravate control measures of exotic species and might even destroy the ecological balance and reduce local biodiversity. Therefore, an in-depth study of the mechanisms of competitive displacement will be invaluable toward a better understanding of the displacement process and lead to new methods of mitigating the negative impacts of biological invasions. However, most research focuses on the winners in the competition. Usually, the winners can obtain more resources or obstruct the displaced species. So what about the losers? Do they contribute to the competitive displacement? Current research draws little attention to the underdogs in competitive displacement.

It is well known that competitive displacement is more likely to occur between species sharing host ranges or key resources. However, where there is competition over shared resources, there is information exchange, including chemical communication, possibly the most ancient and widespread form of communication among species (Haldane 1955; Günther et al. 2015; Amo and Bonadonna 2018). In nature, semiochemicals are chemicals that convey messages within or among species and mediate their behaviors (Nordlund and Lewis 1976; Dicke and Sabelis 1988; Leroy et al. 2011; Smart et al. 2014; Wyatt 2014; Evenden and Silk 2016). Because of constraints on composition and structure of semiochemicals, different species may share similar chemical signals (Berenbaum 2016), especially those species that occupy the same niche. Actually, semiochemicals are important to both inter- and intraspecific communication, so research of their impact on the specific interspecific interactions involved in "competitive displacement" is very significant.

The displacement of *Bursaphelenchus mucronatus* by *Bursaphelenchus xylophilus* is an excellent example to research the roles that semiochemicals play on competitive displacement. *B. xylophilus*, a global quarantine pest, was introduced from North America to Asia and Europe, causing severe ecological and economic losses for coniferous forests (Zhao et al. 2014; 2016). While *B. mucronatus*, a native sister species of *B. xylophilus* in China, has little pathogenicity to forests (Mamiya and Enda 1979; Yan et al. 2012; Niu et al. 2013; Pereira et al. 2013). These two species are very similar in morphological and biological characteristics (De Guiran and Bruguier 1989), and occupy the same niche (Niu et al. 2013). Interestingly, evidence indicates that *B. xylophilus* can displace *B. mucronatus* in forests (Kishi 1995). As the number of years since initial invasion increases at particular sites, the distribution frequency of *B. mucronatus* decreases, whereas that of *B. xylophilus* increases (Cheng et al. 2009). Previous studies demonstrated higher fecundity, higher growth rates, greater load onto vector beetles, stronger phenotypic plasticity of reproductive traits and unequal interspecific hybridization might help *B. xylophilus* to displace the native nematodes (Jikumaru and Togashi 2004; Vincent et al. 2008; Cheng et al. 2009; Niu et al. 2013). Nevertheless, it is still unclear whether or not semiochemicals regulate competitive displacement.

Ascarosides are a group of important pheromones in nematodes (Jeong et al. 2005; Butcher et al. 2007; 2008; Srinivasan et al. 2008; Choe et al. 2012a; b; Kaplan et al. 2012; Noguez et al. 2012; Ludewig and Schroeder 2013; Manosalva et al 2015). The production of ascarosides is conserved in highly diversified nematode groups, but substantial differences exist between species (Choe et al. 2012a; b; Kaplan et al 2012; Noguez et al 2012; Manosalva et al 2015). The basic structure of ascaroside pheromones comprise the 3, 6-dideoxysugar ascarylose, connected to a fatty-acid-like side chain of varying length but might also include additional side chains like glucose as well as indole, p-hydroxybenzoyl- and (E)-2-methyl-2-butenoyl (Jeong et al. 2005; Butcher et al. 2007; 2009; Srinivasan et al. 2008; Noguez et al. 2012; Von Reuss et al. 2012). Intriguingly, analogs of dideoxysugar ascarylose with different length side chains bring functional multiplicity to ascarosides (Von Reuss et al. 2012). Several studies reported that ascarosides could mediate various nematode behaviors, including developmental diapause, sex-specific attraction, repulsion, aggregation, olfactory plasticity, foraging suppression,

adult hermaphrodites' reproduction, plant defense, population dispersal, infectivity-related behaviors and recognition of signals other nematode species (Srinivasan et al. 2008; 2012; Macosko et al. 2009; Yamada et al. 2010; Braendle 2012; Choe et al. 2012b; Izrayelit et al. 2012; Kaplan et al. 2012; Von Reuss et al. 2012; Manosalva et al 2015; Greene et al. 2016; Wharam et al. 2017; Hartley et al 2019; Shapiro-Ilan et al 2019). In *B. xylophilus*, pheromones enhanced the fecundity of invasive (China) strains while they suppressed fecundity of native (US) strains in a phenomenon termed pheromone-regulative reproductive plasticity (PRRP) (Zhao et al. unpublished). In brief, ascarosides are versatile nematode pheromones, affecting both the structure and function of nematodes.

In this study, we investigated the roles ascarosides play in the displacement of *B. mucronatus* by *B. xylophilus*. Specifically, we investigated the influence of crude and synthetic ascarosides on two nematodes in relation to egg hatch, reproduction, sex ratio, body length of female progeny, and nematode life span under starvation. Our results shed light on the fact that ascarosides are helpful to promote the displacement of both species. This study provides new clues for interpreting chemical ecology-based mechanisms of competitive displacement between two nematode species.

Materials and methods

Nematodes cultures and species identification

B. xylophilus and *B. mucronatus* were obtained from Shaanxi and Zhejiang, respectively, in China, and reared in the laboratory for several generations before experiments. Propagate stage nematodes were cultured with the fungus *Botrytis cinerea* on potato dextrose agar (PDA) plates or barley medium at 25 °C in the dark.

To differentiate the two nematode species, morphological and molecular methods were used. The females of each species are easily distinguished by their tails (Fig. S1). *B. mucronatus* has a distinct mucro (short, sharp point) at the tail terminus (Mamiya and Enda 1979; Nickle et al. 1981), whereas *B. xylophilus* has a rounded terminus (Nickle et al. 1981). However, it is difficult to differentiate males and juveniles of the two species. Thus, we adopted a molecular method, based on ITS1-PCR, according to established protocols (Zhao et al. 2005; Wang et al. 2011). By sequence analysis, the band pattern of *B. xylophilus* had sizes of 329 bp, while the pattern of *B. mucronatus* was characterized by sizes of 206 bp (Fig. S1).

In order to simplify the experiments testing competition, we used only morphological methods to identify females from both populations and used the number of females in the population to represent the dynamic change of the population.

Competitive tests in vitro

The two species were cultured as a mixture according to previously established protocol (Cheng et al. 2009). We selected 24 adults of *B. mucronatus* (female/male = 3:1) and 12 adults of *B. xylophilus* (female/male = 3:1), mixed together to imitate the initial phase of interspecific interactions, and cultured them on a PDA plate with *B. cinerea* at 25 °C for five days ($n = 5$ replicates). Five days later, we counted the number of females of each species. All nematodes were collected from five plates and suspended in 10 ml of disinfected distilled water (DDW). A 30 µl nematode suspension was transferred to a new fungus medium after full mixing (inoculated five plates) for continuous cultivation. Five days later, the above process was repeated three times in succession. Similarly, the number of females of each species was counted at each stage during the process of successive cultivation.

To compare the differences between the two species under competitive and non-competitive conditions, we cultured each species alone (24 adults of each species, female/male = 3:1) and together (12 adults of each species, female/male = 3:1) at 25 °C. Each treatment was repeated five times. Ten days later, the population of nematodes was high in media, and the number of females of each species were collected.

Ascarosides

We generated crude ascaroside extracts by cultivating the two species separately in barley media at 25 °C until propagative nematodes migrated to the wall of the bottle 1 cm away from the culture medium (high population), and extracting the whole medium (including nematodes and medium) with 30 ml high-performance liquid chromatography-grade ethanol for 30 min. Samples with only the *B. cinerea* were used as negative controls. The ethanol extracts were filtered through filter paper and dried by rotational vacuum concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Next, the dried extract residue was dissolved in 1 ml DDW, filtered through syringe filter, and stored at − 20 °C until further experimentation. (These crude ascaroside extracts were used as stock solution for the following bioassays.) One hundred fifty microliter of the sample was transferred to a target vial for liquid chromatography–mass/mass spectrometry (LC–MS/MS) analysis. Chemical analyses were performed according to established protocols (Zhao et al. 2016). These experiments were repeated at least three times.

Synthetic ascarosides were provided by Rebecca A. Butcher's Lab.

Assaying with crude ascaroside extracts

Adult nematodes were collected from synchronized cultures three days after hatching. Females and males were separated and cultured for one day (until females stopped spawning). We performed all bioassays with crude ascaroside extracts diluted with DDW to different concentrations of working solution (dilution ratio: stock solution, 10^3-, 10^6-, 10^9-, 10^{12}-fold dilution). Water served as controls. Cultures were incubated at 25 °C for all experiments.

Experiment 1. Egg hatching ratio

The juveniles (L_2) of both species were cultured on a fungus medium for three days. Next, nematodes were placed in phosphate-buffered saline with tween-20 (PBST) for 12 h to obtain eggs. Eggs (about 100 eggs per well) were transferred to a 96-well plate which contained 150 µl of pheromone solution. Each concentration was repeated five times. The number of hatched nematodes was counted after 12 h.

Experiment 2. Reproduction

Twelve individuals of each species (female/male = 3:1) were incubated in a PCR tube with 150 µl of pheromone solution. Nematodes were cultured for three days, and the number of offspring was tallied. Each treatment was repeated ten times.

Experiment 3. Life span

Three milliliter of pheromone solution or water was used for forty adult nematodes (female/male = 3:1). Each treatment was repeated three times, and the number of surviving nematodes was recorded every five days. Pheromone and water solutions were changed every 15 days. When the bodies of nematodes stayed motionless, even after blowing with a pipette, the nematodes were deemed to be dead.

Experiment 4. Sex ratio and body length

We added 150 µl of pheromone solution or water to fungus media and coated them evenly. Eighteen females and six males were transferred to different treatment plates and were incubated for five days. Then we counted the number of females and males and calculated the sex ratio. Each treatment was repeated five times. The replicates of each concentration were pooled, and about 100 female adults were randomly selected and killed by exposing them to 85 °C for 20 min. The females were photographed, and body length was measured using Olympus DP2-BSW software.

Assaying with synthetic ascarosides

To better understand how individual ascarosides may affect the competitive displacement event, we conducted reproduction assays with synthetic asc-C5 (ascr#9), asc-C6 (ascr#12), asc-ΔC6, asc-C7 (ascr#1), and asc-C9 (ascr#10). Also, we measured body length with asc-C5 and asc-C6. To better simulate natural conditions, we mixed asc-C5 and asc-C6 according to the proportion of crude ascarosides in *B. mucronatus* (asc-C5/asc-C6 = 60:1, named Bm-type ascarosides) and *B. xylophilus* (asc-C5/asc-C6 = 16:1, named Bx-type ascarosides) at an initial concentration of 3 µM, then investigated the effects of mixed ascarosides on fecundity and body length for both species. The initial concentration of synthetic ascarosides used for experiments was based on the concentrations detected in crude extracts and the concentrations of ascarosides found in natural pupal chambers of beetles (Zhao et al. 2016). The assays were repeated a second time with synthetic ascaroside blends diluted to 3 µM, 3 nM, 3 pM, 3 fM, and 3 aM, respectively.

Statistical analysis

Bonferroni-adjusted *t* test was used for population dynamics of both nematode species under different conditions. One-way ANOVA with Tukey's multiple comparison test was used for analysis of egg hatching, reproduction, sex ratio and body length data. Log-rank (Mantel-Cox) test was used for the life span data. Two-way ANOVA was used for analysis of bioassay data with the mixture of synthetic ascarosides. Independent sample *t* test was used for all other assays. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) and IBM SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) were used for statistical analyses. A value of $p < 0.05$ was considered statistically different. Standard errors (± SE) were reported for all means.

Results

Competitive displacement of *B. xylophilus* and *B. mucronatus* in laboratory

To verify the phenomenon of competitive displacement, we cultured the two nematode species together. Results showed that the numbers of *B. xylophilus* increased over time, whereas that of *B. mucronatus* decreased (Fig. 1). After 10 days of culture, there were more female *B. xylophilus* than female *B. mucronatus*; after 25 days, the mean number of female *B. mucronatus* was less than 50 while the mean number of female *B. xylophilus* was more than

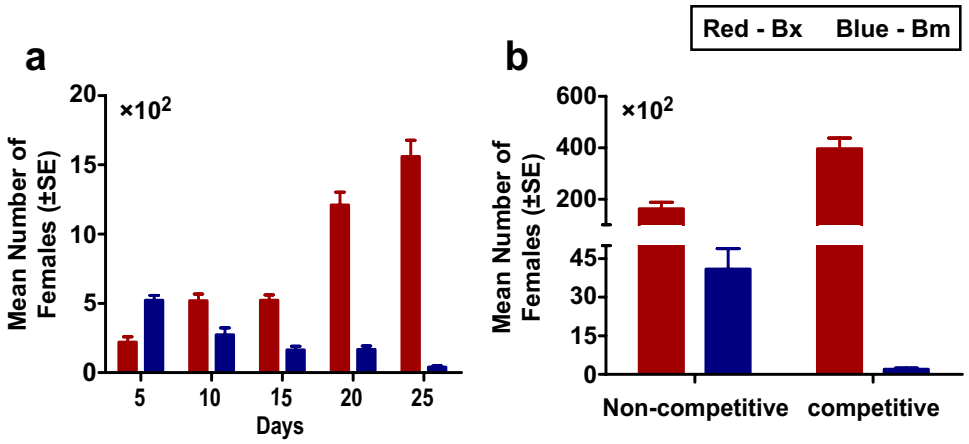

Fig. 1 Population dynamics of *B. xylophilus* (Bx) and *B. mucronatus* (Bm) in different competition conditions in laboratory culture: **a** change in mean number of females over time starting from 24 adult Bm and 12 adult Bx ($n=5$); **b** mean number of female Bx and Bm after 10 days of laboratory culture under competitive conditions (12 adults of each species in the same culture) *vs*. non-competitive conditions (24 adults of one species only per replicate). The starting sex ratio was 3:1 (F:M) in all culture. F means female, M means male; Bonferroni-adjusted *t* test, $p<0.0083$

1,500 (Fig. 1a). These results indicate that *B. mucronatus* can indeed be displaced by *B. xylophilus* under laboratory conditions.

Based on the results in the above experiment, we performed a more detailed study of the population dynamics of both species under competitive and non-competitive conditions. We found that after ten days, the number of female *B. xylophilus* was significantly higher than that of *B. mucronatus* in either conditions (non-competitive: $t=4.55$, $p<0.007$; competitive: $t=9.67$, $p<0.001$; Bonferroni correction: $\alpha=0.05/6=0.0083$). Moreover, the number of female *B. xylophilus* under competitive conditions ($39{,}633.80\pm4079.30$) was notably higher than those under non-competitive conditions ($16{,}250.40\pm2552.92$) ($t=4.86$, $p<0.002$), while that of female *B. mucronatus* under competitive conditions (202.20 ± 47.62) was lower than those under non-competitive conditions (4094.80 ± 795.82) ($t=4.88$, $p<0.002$) (Fig. 1b). The results indicated that competition was helpful to the growth of *B. xylophilus*, but adverse to *B. mucronatus*.

Bioassays of crude ascaroside extracts to *B. xylophilus* and *B. mucronatus*

To determine the effects of crude ascaroside extracts on competitive displacement of *B. xylophilus* and *B. mucronatus*, we compared fecundity, hatching success, life span under starvation in DDW, and offspring sex ratio and the body length of female progeny in fungus PDA media under crude extract treatment between the two species. The results showed that ascarosides affected the fecundity (Fig. 2a) and female body length (Fig. 2c) of both nematode species, but had no significant effects of egg hatch (Fig. 2d, Table S1), life span under starvation (Fig. 2b, Table S2) and offspring sex ratio (Fig. 2e, Table S3).

In high concentrations, crude ascarosides of either species inhibited egg production in both *B. xylophilus* (Bm ascr: $F_{5,46}=96.24$, $p<0.0001$, Bx ascr: $F_{5,50}=57.75$, $p<0.0001$) and *B. mucronatus* (Bm ascr: $F_{5,54}=9.55$, $p<0.0001$, Bx ascr: $F_{5,54}=19.33$, $p<0.0001$); however, low concentrations of ascarosides positively affected fecundity of *B. xylophilus* but had no effect on fecundity of *B. mucronatus* (Fig. 2a). Conclusively, the fecundity of *B. xylophilus* was significantly increased by ascarosides of *B. mucronatus* at both 10^{12}-fold and 10^{9}-fold dilution, whereas it was increased only by 10^{9}-fold dilution of its own ascarosides. Similarly, body length of female *B. xylophilus* was increased in 10^{12}-fold and the 10^{9}-fold dilution of *B. mucronatus* ascarosides ($F_{2,293}=28.97$, $p<0.0001$), and only in 10^{9}-fold dilution of its own ascarosides ($F_{2,297}=16.97$, $p<0.0001$) (Fig. 2c). Conversely, body length of *B. mucronatus* was suppressed by 10^{12}-fold and 10^{9}-fold dilutions of its own ascarosides ($F_{2,297}=44.25$, $p<0.0001$) and was unaffected by ascarosides of *B. xylophilus* at any dilutions ($F_{2,279}=2.11$, $p=0.12$) (Fig. 2c).

Moreover, by comparing *B. xylophilus* and *B. mucronatus*, we found that there were marked differences on hatching success, life span under starvation and offspring sex ratio between both species (Fig. 3), although the crude ascaroside extracts had no obvious effects on any of these variables (Fig. 2b, d, e). Success of egg hatch was significantly higher for *B. xylophilus* ($61.08\pm1.98\%$) than for *B. mucronatus* ($17.33\pm1.36\%$) ($t=18.01$, $p<0.0001$) (Fig. 3a). The sex ratio of offspring (females/males) was also more female-based in *B. xylophilus* (2.62 ± 0.10) than in *B. mucronatus* (1.48 ± 0.04) ($t=10.09$, $p<0.0001$)

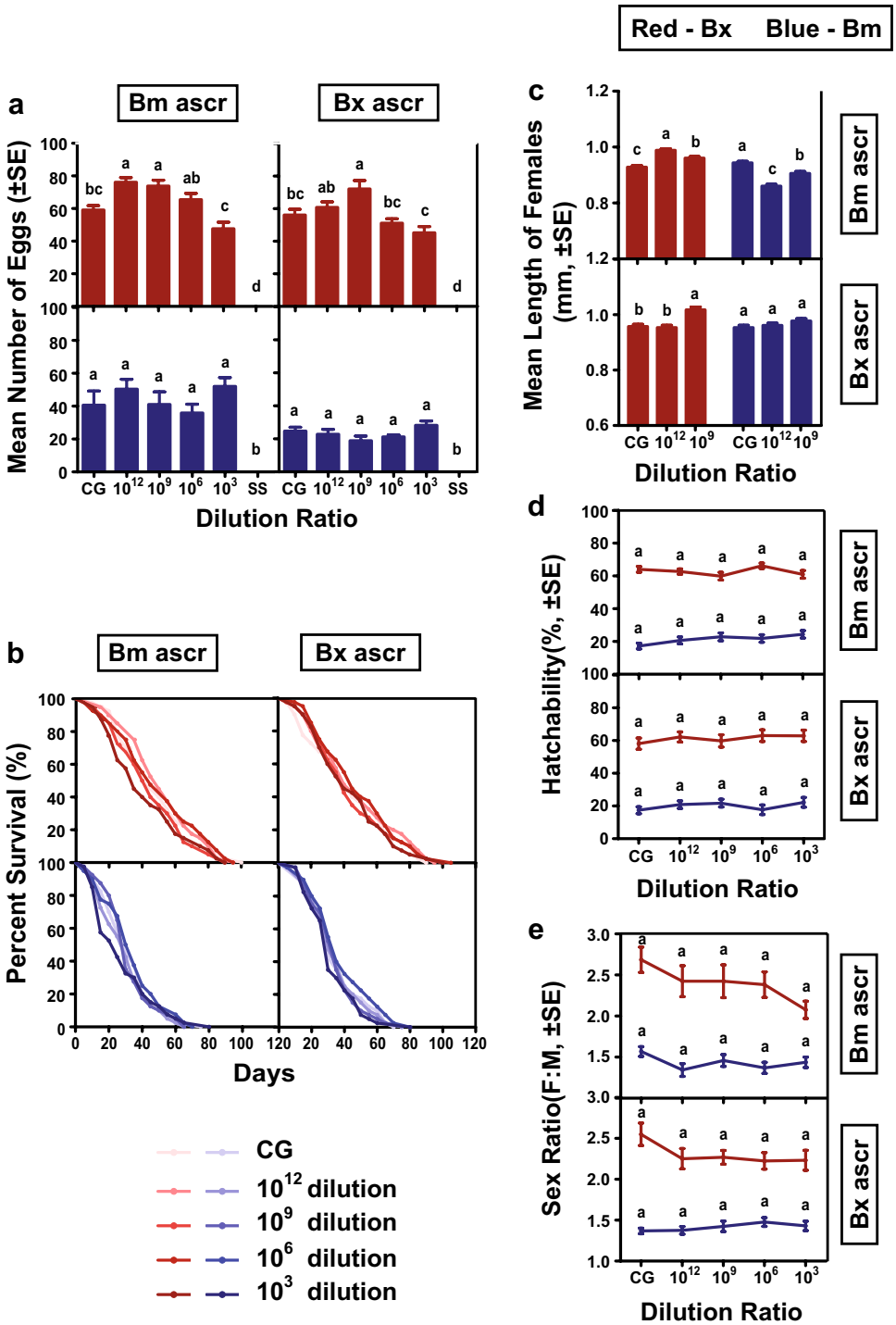

Fig. 2 Effects of dilutions of crude ascaroside extracts from Bm and Bx on fecundity (**a**), life span under starvation (**b**), female body length (**c**), hatchability (**d**) and sex ratio (**e**) of each nematode species. One-way ANOVA with Tukey's multiple comparison test for (**a**), (**c**), (**d**) and (**e**), and means with different letters were significantly different; log-rank (Mantel-Cox) test for (**b**); $p < 0.05$. F means female, M means male. Bm ascr means crude ascaroside extracts from Bm, Bx ascr means crude ascaroside extracts from Bx. CG means control group (rearing the nematodes with DDW only), and SS means stock solution of crude ascaroside extracts

(Fig. 3b). Under conditions of starvation, *B. xylophilus* (average longevity: 42.5 days) had a longer life than *B. mucronatus* (average longevity: 30 days) (Log-rank (Mantel-Cox) test: $\chi^2 = 9.72$, $p = 0.0018$) (Fig. 3c). In addition, the fecundity of both species had a distinctly different baseline. The number of eggs laid by *B. xylophilus* was much higher than *B. mucronatus* ($t = 4.12$, $p = 0.0001$) (Fig. 3d).

In brief, our evidence suggests crude ascaroside extracts may promote the displacement of *B. mucronatus* by *B. xylophilus* through regulating fecundity and female body length of both species. In addition, other characteristics of *B. xylophilus*, were higher fecundity, higher egg hatchability, longer life span under starvation and skewed sex ratios favor *B. xylophilus* females, further enhancing the displacement between the two nematode species.

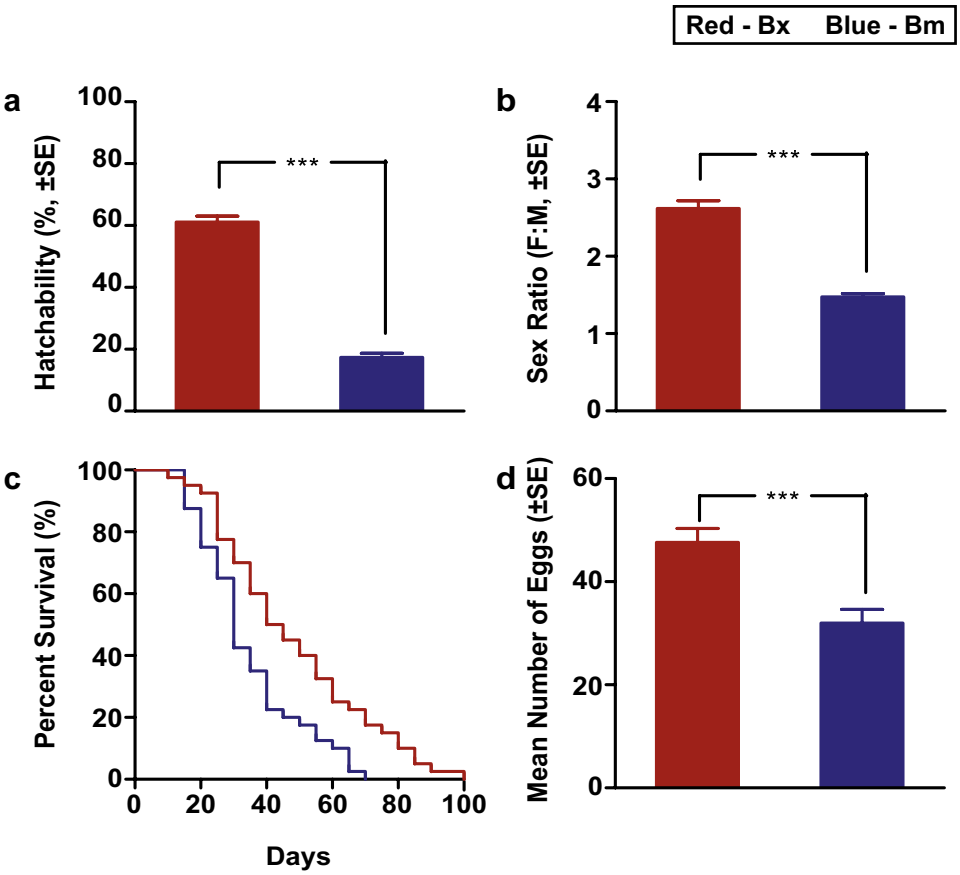

Fig. 3 Comparison of hatchability (**a**), sex ratio (**b**), life span (**c**) and fecundity (**d**) between the two nematode species. Independent sample *t* test for Fig. 3a, 3b and 3d, ***$p < 0.001$; log-rank (Mantel-Cox) test for (**c**), $p < 0.05$. F means female, M means male

Analysis of crude ascaroside extracts and bioassays of synthetic ascarosides to *B. xylophilus* and *B. mucronatus*

To ascertain exactly which ascaroside is involved in competitive displacement, we measured the amount of crude ascaroside extracts produced by both nematodes using LC–MS/MS. Five major ascarosides were produced by both species including asc-C5 ($m/z = 247.26$; mean concentrations: Bx, 3.059 µM; Bm, 4.876 µM), asc-C6 ($m/z = 261.29$; Bx, 0.192 µM; Bm, 0.081 µM), asc-ΔC6 ($m/z = 259.27$; Bx, 0.025 µM; Bm, 0.045 µM), asc-C7 ($m/z = 275.32$; Bx, 0.062 µM; Bm, 0.071 µM) and asc-C9 ($m/z = 303.37$; Bx, 0.063 µM; Bm, 0.142 µM), with asc-C5 detected in the highest concentrations (Fig. 4). No ascarosides were detected in the barley medium inoculated with only *B. cinerea*.

Of the five synthetic ascarosides tested in bioassays, fecundity of *B. xylophilus* was significantly and positively affected by low concentrations of asc-C5 ($F_{5,46} = 3.01$, $p < 0.05$) and asc-C6 ($F_{5,44} = 7.44$, $p < 0.0001$), whereas fecundity of *B. mucronatus* was not significantly affected by any concentration of asc-C5 ($F_{5,48} = 1.96$, $p = 0.10$) or asc-C6 ($F_{5,51} = 1.62$, $p = 0.17$) (Fig. 5a). None of the other synthetic ascarosides affected fecundity of either nematode species (Fig. S2, Table S4). Effects of synthetic ascarosides on body length of females differed depending on the species. Female *B. xylophilus* were significantly longer when reared on fungal media with asc-C5 ($F_{5,429} = 4.21$, $p = 0.001$) or asc-C6 ($F_{5,415} = 2.93$, $p < 0.05$), whereas those of *B. mucronatus* were significantly shorter (asc-C5: $F_{5,440} = 7.15$, $p < 0.0001$; asc-C6: $F_{5,598} = 15.49$, $p < 0.0001$).

Previous studies have shown that groups of compounds with similar structures, like ascarosides, often act synergistically (Butcher et al. 2008; Srinivasan et al. 2008; Ludewig and Schroeder 2013). In nature, ascarosides exist in a mixture; thus, we mixed asc-C5 and asc-C6 according to different ratios as determined by LC–MS/MS analyses in both nematodes. At high concentrations, the synthetic blends of Bm-type and Bx-type ascarosides suppressed fecundity of both *B. xylophilus* (Bm-type: $F_{3,28} = 16.22$, $p < 0.0001$; Bx-type: $F_{3,32} = 4.30$, $p < 0.05$) and *B. mucronatus* (Bm-type: $F_{3,31} = 13.43$, $p < 0.0001$; Bx-type: $F_{3,28} = 4.82$, $p < 0.01$); at lower concentrations, however, both Bm-type and Bx-type ascarosides increased fecundity of *B. xylophilus* and had no effect on fecundity of *B. mucronatus* (Fig. 6a). Female body length was significantly affected by concentration of ascarosides of both types in both *B. xylophilus* (Bm-type: $F_{2,282} = 9.11$, $p = 0.0001$; Bx-type: $F_{2,278} = 4.28$, $p < 0.05$) and *B. mucronatus* (Bm-type: $F_{2,278} = 19.25$, $p < 0.0001$; Bx-type:

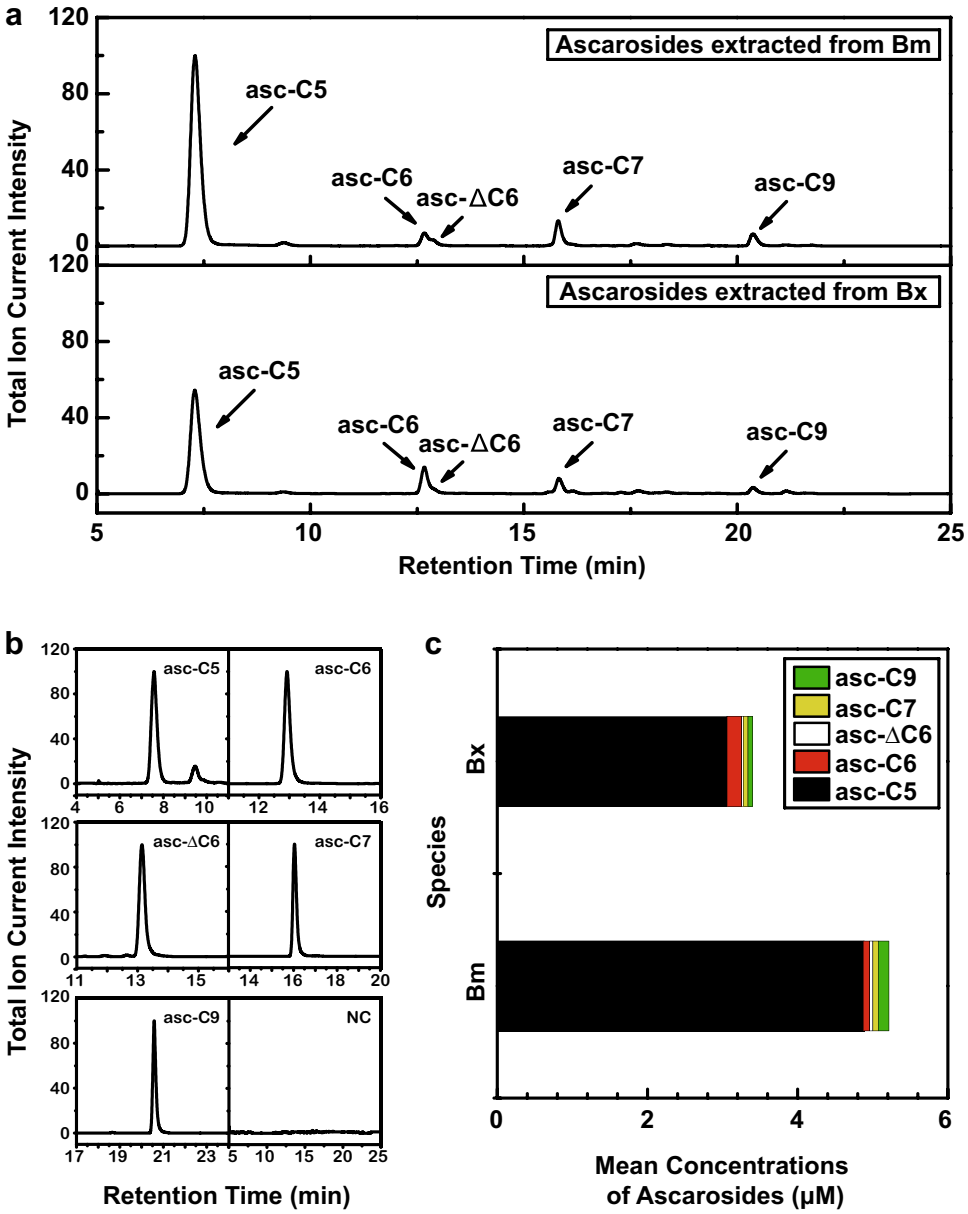

Fig. 4 Ascarosides detected using LC–MS/MS in extracts of both nematode species: **a** the elution profiles of ascarosides extracted from Bm and Bx; **b** the elution profiles of synthetic standards (asc-C5, asc-C6, asc-ΔC6, asc-C7 and asc-C9) and negative control (NC, barley medium with *B. cinerea*); **c** mean concentrations of ascarosides extracted from Bm and Bx

$F_{2,246} = 5.59$, $p < 0.01$) (Fig. 6b). However, as with fecundity, there were positive effects of low concentrations of either type of ascarosides on length of female *B. xylophilus* and negative effects of ascarosides on length of female *B. mucronatus* (Fig. 6b). These results were consistent with those of crude ascroside extracts. Meanwhile, based on the results of LC–MS/MS (Fig. 4c), the activity concentrations of crude ascaroside extracts were basically the same as that of the two synthetic ascaroside mixture (10^{12}-fold dilution corresponds to aM, 10^{9}-fold dilution corresponds to fM, stock solution corresponds to μM), indicating that asc-C5 and asc-C6 play a positive role in the process of competitive displacement between the two nematode species rather than other ascarosides (asc-ΔC6, asc-C7 or asc-C9).

To further unravel the story of competitive displacement between two nematode species driven by ascarosides, the mean number of eggs, shown in Fig. 6a, was analyzed by a two-way ANOVA (after normalization processing) for ascarosides sources (Bm-type and Bx-type) and concentrations (low concentrations: control group, aM and fM) in different species, respectively. For *B. mucronatus*, ascarosides source ($F_{1,40} = 0.03$, $p = 0.86$) and concentrations ($F_{2,40} = 2.29$, $p = 0.12$) had no significant effects on their fecundity. Interaction effects between these two factors were not obvious ($F_{2,40} = 0.26$, $p = 0.77$). However, for *B.*

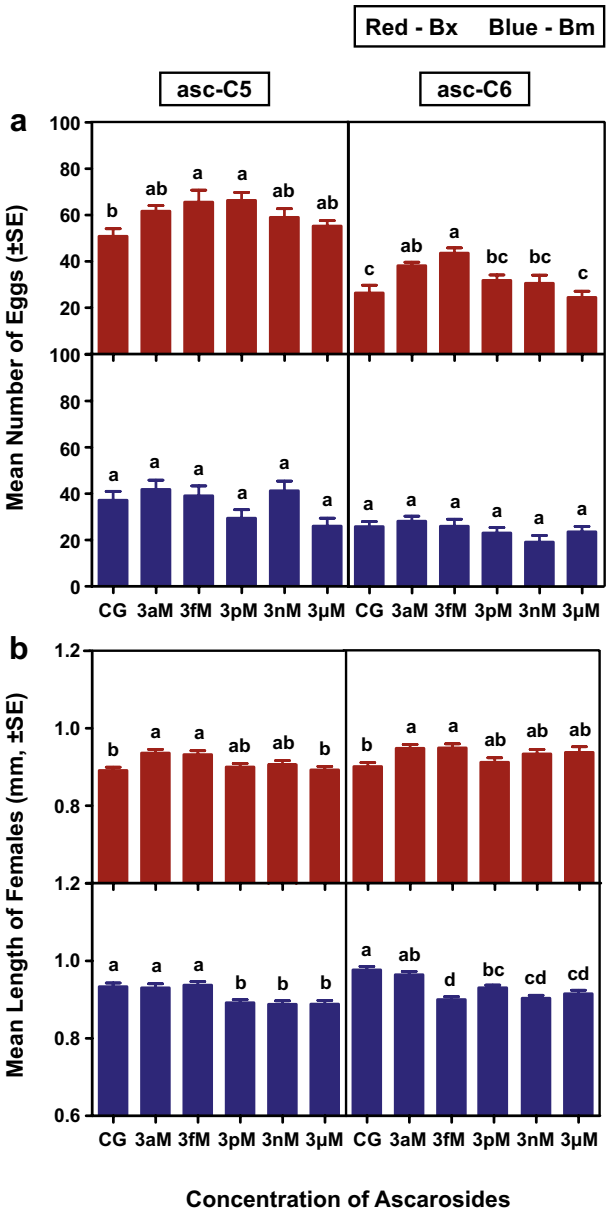

Fig. 5 Effects of asc-C5 and asc-C6 on fecundity (**a**) and female body length (**b**) of both nematode species. Means with different letters were significantly different. One-way ANOVA with Tukey's multiple comparison test, $p < 0.05$. CG means control group (rearing the nematodes with DDW only)

xylophilus, there were main effects for both factors: ascarosides source ($F_{1,39} = 6.54$, $p < 0.05$) and concentrations ($F_{2,39} = 11.96$, $p < 0.0001$), but no interactions between them ($F_{2,39} = 2.08$, $p = 0.14$). Interestingly, Bm-type ascarosides had a far greater effect on fecundity of *B. xylophilus* than Bx-type ascarosides ($p < 0.05$).

Similarly, the same analysis of female body length (data in Fig. 6b) indicated that concentrations of ascarosides (control group, aM and fM) had a significant main effect on female body length of *B. xylophilus* ($F_{2,560} = 11.94$, $p < 0.0001$), but the effect of ascaroside source was not prominent ($F_{1,560} = 1.49$, $p = 0.22$). And there were no interactions between two factors ($F_{2,560} = 1.05$, $p = 0.35$). For *B. mucronatus*, there were main effects for two factors (ascarosides sources: $F_{1,524} = 21.22$, $p < 0.0001$; concentrations: $F_{2,524} = 18.68$, $p < 0.0001$) and conspicuous interactions between them ($F_{2,524} = 5.72$, $p < 0.01$). Comparing different ascarosides sources (Bm-type or Bx-type), the inhibition effects of Bx-type ascarosides on *B. mucronatus* female body length were stronger than Bm-type ascarosides ($p < 0.0001$).

Discussion

Overall, the major finding of our study is that ascarosides may facilitate the competitive displacement between *B. xylophilus* and *B. mucronatus*. Both species can sense each other's ascarosides, yet they have starkly different responses. In this study, we extracted physiologically relevant amounts of crude ascarosides from high populations of nematodes in barley media (Fig. 4c), which were similar to ascaroside concentrations present in PDA media produced from high populations of nematodes in the competitive test (non-competitive conditions, Fig. 1b). Since both these nematode populations were high on the two different media, we can confirm the concentrations of the corresponding ascarosides present (Fig. 1b) were likely physiologically relevant. The bioassays showed that the ascarosides from *B. xylophilus* act as allomone (emitter benefits and receiver is disadvantaged or unaffected) accelerating its own fecundity and female body length but inhibiting female body length of *B. mucronatus.* At the same time, the ascarosides from *B. mucronatus* act as kairomone (emitter is disadvantaged, receiver benefits) by suppressing its own female body length but enhancing the fecundity and female body length of its competitor *B. xylophilus*. Moreover, *B. mucronatus* ascarosides make a greater contribution to the fecundity of *B. xylophilus* than those produced by *B. xylophilus*, while *B. xylophilus* ascarosides make a stronger inhibition to the female body length of *B. mucronatus* than those produced by *B. mucronatus*. In fact, across the animal kingdom, higher fecundity is usually linked with large body size. Larger females would produce more and larger offspring with superior qualities (Blueweiss et al. 1978; Parker and Begon 1986; Conover 1988; Kajita and Evans 2010; Sato and Suzuki 2010); therefore, effects of ascarosides on body length of female nematodes explain a positive feedback loop for *B. xylophilus* with negative consequences for *B. mucronatus*. When a small population of *B. xylophilus* invades a host containing *B. mucronatus*, *B. xylophilus* can sense ascarosides of both species in the environment, realizing signal amplification. In other words, the presence of *B. mucronatus* enables the total ascarosides

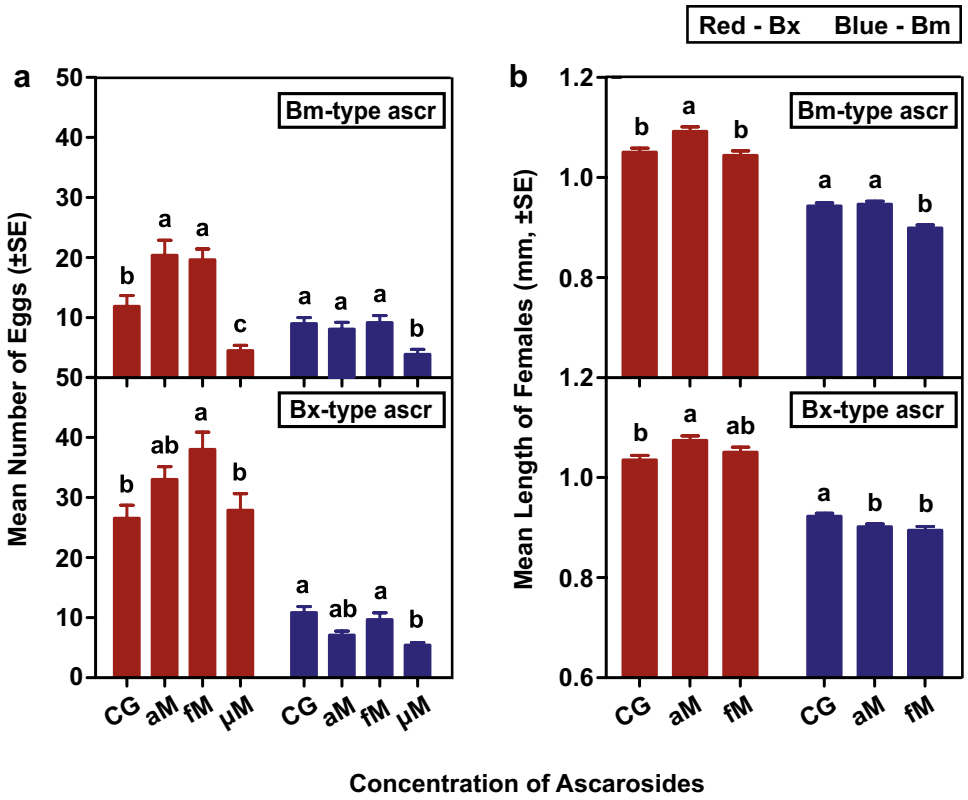

Fig. 6 Effects of different sources of ascarosides on fecundity (**a**) and female body length (**b**) of the two nematode species. Bm-type ascr means the ratio of asc-C5 and asc-C6 is 60:1, Bx-type ascr means the ratio of asc-C5 and asc-C6 is 16:1. Means with different letters were significantly different. One-way ANOVA with Tukey's multiple comparison test, $p < 0.05$. CG means control group (rearing the nematodes with DDW only)

in the environment to reach the threshold concentration that favors *B. xylophilus* reproduction and development more easily, tipping the scales in its favor, thus enabling it to build up its population faster and then achieve displacement of *B. mucronatus*, a phenomena observed in several decades of field studies (Kishi 1995; Penas et al. 2004; Cheng et al. 2009).

Competitive displacement occurs as a result of the effects from multiple mechanisms acting together (Reitz and Trumble 2002; Gao and Reitz 2017). In *B. xylophilus* and *B. mucronatus* system, our research also suggests that four mechanisms may act on displacement of *B. mucronatus* by *B. xylophilus*: higher fecundity, higher successful egg hatch, longer life span under starvation and skewed sex ratio of female progeny (Fig. 3). Collectively, ascarosides and these four traits give the *B. xylophilus* the edge in competition. These results provide partial support to previous studies which suggested that phenotypic plasticity in reproductive traits of *B. xylophilus*, based on phenotypic trade-off between egg number and egg size, shorter hatching time and more offspring, might contribute to competitive displacement between these two species (Niu et al. 2013).

Another interesting result suggests the function of ascarosides may be much wider than previously thought. Ascarosides exist extensively in Nematoda and have a variety of functions (Srinivasan et al. 2008; 2012; Macosko et al. 2009; Yamada et al. 2010; Braendle 2012; Choe et al. 2012b; Izrayelit et al. 2012; Kaplan et al. 2012; Von Reuss et al. 2012; Ludewig and Schroeder 2013; Manosalva et al 2015; Greene et al. 2016; Wharam et al. 2017; Hartley et al 2019; Shapiro-Ilan et al 2019). According to our study, ascarosides can facilitate nematode oviposition under competition for resources. Crude and synthetic ascarosides could facilitate egg laying of *B. xylophilus* under low concentrations, while the promoting effects of acarosides are not obvious or even have an inhibitory effect under high concentrations. These results suggest that ascarosides are concentration-dependent pheromones. Similarly, previous studies showed that *C. elegans*' pheromones could accelerate adult hermaphrodites' reproduction (Wharam et al. 2017). Fecundity of *C. elegans* treated with 400 nM ascr#2 (C6) and ascr#3 (C9) was slightly higher than controls (Ludewig et al. 2013). Here, our results showed that asc-C5 and asc-C6 could facilitate oviposition of *B. xylophilus* at low concentrations (Fig. 5). Notably, another function of ascarosides is regulating the body length of nematodes, which is apparent in our results. Ascarosides could increase the female body length of *B. xylophilus* but decrease that of *B. mucronatus* (Figs. 2, 5, 6). Our results also suggest that different ascaroside types and different dosages can induce different responses between species. In fact, the two nematode species had different responses to the same concentration of ascarosides. For instance, egg laying of *B. xylophilus* increased under 3 aM and 3 fM of asc-C6, but *B. mucronatus* had no obvious changes at the

same concentrations (Fig. 5). Hence, we propose a hypothesis that the underlying internal mechanism of competitive displacement between *B. xylophilus* and *B. mucronatus* may be due to differences in signal sensing and response systems of two nematodes to ascarosides. This question will be an important topic for future research.

In general, we have demonstrated that competitive displacement is not only the story of the victors, but that the displaced species may also contribute to the displacement event, albeit at their own demise. Chemical communication is an important means of information transmission within and among species. Our studies illustrate that semiochemicals play an important role in the competitive displacement between invasive and indigenous species. In the *B. xylophilus—B. mucronatus* system, ascarosides from *B. mucronatus* can kairomonally promote its own competitive displacement by triggering increased fecundity and female body length development of rival *B. xylophilus*. Furthermore, our research helps to explain the mechanism of competitive displacement from the perspective of the species that is replaced, providing potentially new information and perspectives for alleviating the negative effects of biological invasion.

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Author contribution statement JM, LZ and JS conceived and designed the research. JM and WR carried out the experiments. JM analyzed the data. JM, JDW and JS wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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