Are nematodes costly to fig tree–fig wasp mutualists?

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Title page

Are nematodes associated with fig-fig wasp mutualism detrimental?

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Running title: Parasitic nematodes in fig-fig wasp mutualism

Abstract

Most mutualisms are exploited by parasites, which must strike an evolutionary balance between virulence and long term persistence. Fig associated nematodes, living inside figs and dispersed by fig wasps, are thought to be exploiters of the fig-fig wasp mutualism. The life history of nematodes is synchronized with the fig development and adapted to particular developmental characteristics of figs. We expect host breeding systems (monoecious vs. gynodioecious figs) and seasonality, to be central to this adaptation. However, the life history details of most fig nematodes are largely unknown. Here we conducted the first field surveys on the prevalence of nematodes from monoecious *Ficus microcarpa*, gynodioecious *F. hispida* and their pollinating fig wasps in two seasons and two developmental stages of figs in Xishuangbanna, China. We followed this up by quantifying the effects of nematodes on fitness related traits on fig wasps (e.g. egg loads, pollen grains, and longevity) and fig trees (seed production) in gynodioecious *F. hispida*. The magnitude of nematode infection was compared between pre- and post- dispersal pollinators to quantify the probability of nematodes being transported to new hosts. Our results showed that *Ficophagus microcarpus* was the only nematode in *Ficus microcarpa*. In *Ficus hispida*, *Martininema guangzhouensis* was the dominant nematode species, while *Ficophagus centerae* was rare. For both species of *Ficus*, rainy season and inter-floral figs had higher rates of nematode infection than the dry-hot season and receptive figs. Nematodes did not affect the number of pollen grains or egg loads of female wasps. We did not detect a correlation between seed production and nematode infection. However, carrying nematodes reduced the lifespan and dispersal ability of pollinator wasps, indicating higher rates of post-emergence mortality in infected fig wasps. Severely infected fig wasps were likely ‘filtered out’, preventing the overexploitation of figs by wasps and stabilizing the interaction over evolutionary time.

Keywords: *Ficus*, Agaonidae, mutualism, nematodes, coexistence

Introduction

Life on earth is embedded in a complex network of interactions, with mutually beneficial connections forming the backbone of most high diversity ecosystems (including but not limited to coral reefs and tropical rainforests) (Bascompte & Jordano, 2007). Such widespread and inherently stable interactions are often exploited by third-party parasites (Lee, 2015; Sachs & Simms, 2006), which pose a serious threat for mutualistic species (Sachs & Simms, 2006). While exploitative parasites may render the costs accrued by one or both mutualists to be evolutionarily unstable, a long-term exploitation strategy should result in a moderate or variable level of costs to each mutualist. Investigating the evolutionary significance of parasites is difficult, not least because most collapsed mutualisms are not possible to detect, but also because we must combine theoretical and empirical approaches in tractable systems if we are to progress.

Fig trees (*Ficus* spp.) and their pollinating wasps (Hymenoptera: Agaonidae) represent a well-known obligate mutualism (Weiblen, 2002; Wiebes, 1979). Each *Ficus* species relies on an obligate fig wasp to pollinate, and in return the trees provide nutrition and space for wasp larvae to develop inside enclosed inflorescences (‘figs’) (Weiblen, 2002). Individual figs go through several developmental stages, these include ‘A phase’ (pre-female flowers), ‘B phase’ (female flowers), ‘C phase’ (interfloral), ‘D phase’ (male flowers), and ‘E phase’ (postfloral), over a period of several weeks or months (Galil & Eisikowitch, 1968). The pollinating wasps enter receptive figs (B phase) through a specialized entrance to pollinate and oviposit in female flowers within.

Offspring of fig wasps will develop in C stage figs until they emerge (D phase), at which point alate female pollinator wasps leave the figs from channels bored by apterous male wasps to search for new receptive hosts and the life cycle anew.

In addition to obligate mutualistic pollinators, many other organisms live in the figs (enclosed inflorescences) (Borges, 2015), including various species of nematodes (Giblin-Davis et al., 1995; Kerdelhue et al., 2000; Van Goor et al., 2018). More than 20 species of nematodes associated with *Ficus* in the genus *Schistonchus* are described and are largely considered as plant parasites that lead to reduced growth of the florets and indirectly reduced seed production (Davies et al., 2015). *Schistonchus sensu stricto* was redefined to include two new genera *Ficophagus* and *Martininema* in 2015, and 18 *Ficophagus* spp., and 3 *Martininema* spp. have been described (Davies et al., 2017a; Davies et al., 2015; Davies et al., 2017b). Another genus, *Parasitodiplogaster,* is thought to consist of fig-wasp-parasitic nematodes (Giblin-Davis et al., 2006; Herre, 1993; Herre, 1995; Ramirez, 1974; Van Goor et al., 2018). Although these nematodes are specifically detected in *Ficus* and their fig wasps, the exact (facultative or obligate) association between nematodes and *Ficus* species is not usually established (Giblin-Davis et al., 1995).

The life history of nematodes is closely matched with both figs and fig wasps (Van Goor et al., 2018). In D phase, when the fig becomes mature, infective juvenile nematodes aggregate in the cavity of figs ready to infect newly emerged pollinating fig wasps (Krishnan et al., 2010; Martin et al., 1973). Once they contact the newly emerged wasps, nematodes quickly enter the body of wasps through trachea in the thoracic or abdominal cavities (Poinar & Herre, 1991). Nematodes are then carried by female fig wasps to new receptive figs, laying eggs in the host and starting their life cycle anew.

Approximately half of all *Ficus* species are monoecious and the remainder are gynodioecious (Janzen, 1979). In monoecious *Ficus* species, one fig can produce both pollens and seeds. Pollinating fig wasps and nematodes also live within the figs. When the new generation of pollinating fig wasps emerge, they carry nematodes to new figs such that the nematodes have the potential to disperse from every fig However, in gynodioecious *Ficus* species, female trees produce viable seeds (functionally female) and male trees produce pollens and support the development of fig wasps (functionally male) (Weiblen, 2002). Nematodes in female gynodioecious figs cannot disperse to a new fig as wasps are not produced. Therefore, the costs incurred by nematodes in female gynodioecious figs are considerably greater. Any costs inflicted on figs through nematode presence (e.g. consumption of fig or wasp tissue) will also differ, reducing pollen dispersal in male figs and seed production in female figs. Moreover, because fig wasp populations and fig phenology are variable across seasons (Wang et al., 2019a; Wang & Sun, 2009), we hypothesise that corresponding seasonal variation also exists for nematode prevalence, but that this is more pronounced in gynodioecious figs which have increased seasonal constraints on production. We also predict higher abundance of nematodes on gynodioecious figs because half of all vectored nematodes will languish in female figs.

Species of *Schistonchus* feed on florets and induce hypertrophy of the cells. Some *Schistonchus* nematodes feed on seed florets and aborted florets, suggesting that nematode abundance may also vary with floret development (corresponding to fig development), which may allow multiple nematodes to co-exist within one individual syconium (Center et al., 1999). Seasonal variation in *Schistonchus* nematode abundance could also be linked to seasonal dynamics in fig wasps (Davies et al., 2010). However, *Parasitodiplogaster* species have been reported to develop inside wasps and use the syconia as repositories for the dauer (dispersal stage of nematodes) juveniles (Herre, 1996; Poinar & Herre, 1991; Van Goor et al., 2018), meaning that their presence can influence pollinating wasp longevity, host location and pollination efficiency; and indirectly seed production in fig trees. The biology and impact of nematodes on the fig-fig wasp mutualism remains under studied, with most studies focusing on the taxonomy, diversity, and phylogeny of nematodes (Davies et al., 2015; Sun et al., 2017; Susoy et al., 2016; Zeng et al., 2011).

Figs, fig wasps and their parasitic nematodes provide a comparative system for assessing the influence of third parties on mutualism persistence and the cost of parasitism (Dunn et al., 2008a; Herre et al., 1999; Van Goor et al., 2018; Wang et al., 2019b). However, the effects of nematodes on the fig-fig wasp mutualism are not always clear (but see (Van Goor et al., 2018)). In this study, we conducted both field surveys and field experiments to answer the following questions: (1) What is the pattern of nematode occurrence across different seasons in Xishuangbanna, in different fig developmental stages and in different fig wasp life stages? (2) What are the effects of phoretic nematodes on pollinating wasp-fig fitness? (3) Can phoretic nematodes impact the ability of fig wasps to act as effective pollinators?

Materials and methods

Study sites

This study was carried out in the Xishuangbanna Tropical Botanical Garden in Southwest China (N 21°55′, E 101°15′). Xishuangbanna has a typical monsoon climate exhibiting a dry season between November and April and a rainy season between May and October. The dry season is characterized by a high frequency of heavy radiation fog during the night and morning, and in the rainy season, Xishuangbanna receives high rainfall from the southwest summer monsoon (Cao et al., 2006).

Study species

A locally abundant monoecious *Ficus* species (*Ficus microcarpa*) and a similarly available gynodioecious species (*Ficus hispida*) were selected to investigate the prevalence and abundance of nematodes. Both species are native to Southwest China, *Ficus microcarpa* is pollinated by the fig wasp *Eupristina verticillata* (Kong et al., 2016), and the plant parasitic nematode *Ficophagus microcarpus* has been found in the figs of this species (Davies et al., 2015; Zeng et al., 2011). *Ficus hispida* is pollinated by *Ceratosolen solmsi marchali* in Xishuangbanna (Peng et al., 2005). The plant parasitic nematodes *Ficophagus centerae* and *Martininema guangzhouensis* were found in the figs of *F. hispida* (Davies et al., 2015; Zeng et al., 2007). However, in our study, *M. guangzhouensis* is the dominant species, and *F. centerae* is rare, making up only 0.5% of the nematode records.

The figs on male trees contain galls, and male flowers, which act as nurseries for developing fig wasp larvae, while the figs on female trees contain only female flowers which produce seeds. Contrasting phenology and dispersal dynamics of monoecious and gynodioecious fig species lead to distinct expectations in terms of nematode abundance and fitness costs. For example, because female figs represent reproductive dead ends to nematodes there is likely to be stronger selection for rapid reproduction and population growth (and higher overall abundance) in colonizing nematodes in colonizing nematodes of gynodioecious figs.

Experiment 1: The prevalence of plant-parasitic nematodes in two Ficus species

We sampled C phase figs to check whether they were infested by nematodes. The figs were collected from the monoecious *F. microcarpa*, as well as male and female trees of gynodioecious *F. hispida*. Each type of sample was collected from 10 trees, and 100 fruits were collected per tree. The sampled figs were surface cleaned before each fig was cut into small pieces and put in Petri dish with distilled water. The nematodes (juveniles and adults) entered the water after 2–4 h, after which we confirmed their presence under a stereoscope (LeicaS8AP0). The figs with and without nematodes were recorded.

Experiment 2: Variation of nematode abundance in different seasons and fig developmental phases

Nematodes are transported by fig wasps to new figs. During A stage, there are no fig wasps inside the figs, therefore no nematodes were detected in the figs in the preliminary observations. In D phase, both fig wasps and nematodes are mature and ready for dispersal. Therefore, we sampled post-pollination B phase figs as well as late C phase figs as a means of investigating nematode abundance in the hot-dry season (lasting from March to April) and the rainy season (lasting from May to August). Similarly, B and C phase figs were also collected from the monoecious *F. microcarpa*, as well as male and female trees of gynodioecious *F. hispida*. Each type of sample was collected from 10 trees, and 30 fruits were collected from each tree. The Baermann funnel method was used to collect nematodes from figs (Staniland, 1954); each fruit was cut into small pieces that were placed in a funnel with a thin filter paper and distilled water. The nematodes entered the water and sank into a latex tube linked to the funnel, which was controlled by a flat jaw pinchcock. After 4–8 h, the water with nematodes was collected, and the nematodes were identified to species and counted (both juveniles and adults) under a stereoscopic microscope (Leica DM2000 and LeicaS8AP0).

Experiment 3: Nematode infection in different stages of adult pollinating fig wasps

We collected figs near D phase from male trees of *F. hispida* to observe nematode activity. Four different stages of pollinators were obtained, including unmated individuals in closed galls (unmated), mated individuals in galls (mated), and individuals both inside (before emergence) and outside figs during D phase (emerged).

Figs were cut open in a mesh bag to prevent emerged wasps from escaping. Unexited galls were dissected to obtain unmated female pollinators, while mated pollinators were obtained from galls with mating holes. All wasps inside the fig cavities were collected with tweezers. Wasps (outside the figs) flying around B phase figs were caught with a mesh net in the field. These female pollinators were dissected in distilled water in a petri dish and the nematodes were counted under a stereoscopic microscope (LeicaS8AP0). Three male trees were sampled and we collected five figs per tree, while ten wasps were randomly selected per fig for each stages (unmated, mated, before emergence, emerged) of adult pollinating fig wasps.

Experiment 4: Effects of nematodes on the amount of pollen carried by fig-pollinating wasps and their egg loads

The figs in D phase were collected from male trees of *F. hispida* and placed in mesh bags, and the fig wasps that naturally emerged from the figs and female pollinators were collected. Eleven figs were collected from four trees, and we randomly selected 10-30 female pollinators from each fig. In total, 150 female pollinators were examined. We detached the abdomen of each female pollinating wasp in a petri dish filled with distilled water using fine entomological pins. Following this we dissected the ovary, separated each ova, and then counted the number of ova and nematodes under a stereoscopic microscope (LeicaS8AP0) according to Dunn et al (2011).

The pollen grains carried inside the pollen pockets of each wasp were quantified.

We removed the pollen pockets of wasps with dissecting needles under a stereoscopic microscope (LeicaS8AP0) and then placed them into a 1.5ml centrifuge tube with 0.5ml of electrolyte solution (0.9% NaCl). The tubes were placed into an ultrasonic bath (SK1200H, Shanghai KUDOS Ultrasonic Instrument CO., LTD) for 60 seconds to detach the pollen grains. Each sample was brought to a total volume of 80ml with electrolyte solution and pollen grains were counted using a particle counter (Multisizer^{TM} 3 COULTER COUNTER®, Beckman Coulter Inc., Brea, CA). We ran each sample three times and calculated the average (Simón-Porcar et al., 2014).

Experiment 5: Comparing the lifespan of wasps with and without nematodes

The D phase figs were collected from male trees of *F. hispida* to capture female pollinators that naturally emerged from the figs. About 50 female pollinators emerging from the same fig were placed into a plastic bottle (3cm in diameter and 5cm high), and a wet filter paper was placed in the bottom to maintain moisture. The bottles were kept at room temperature (around 25℃) and approximately 75% humidity in a natural light/dark cycle. They were checked every two hours and the dead female pollinators were removed and counted (Dunn et al., 2008b). Wasps were then dissected and the number of nematodes was recorded once all were dead. In total, eight bottles of wasps (364 female pollinators, 97 with and 267 without nematodes) were monitored.

Experiment 6: Nematodes carried by fig-pollinating wasps and the effects on wasp dispersal

Nematodes were collected from 'newly emerged' wasps and those wasps that had 'arrived' in the B phase figs. For 'newly emerged' wasps, we collected ten figs in the D phase from each of two male trees of *F. hispida*. Each fig was placed into a mesh bag during wasp emergence. Ten wasps were collected from each fig, a total 200 wasps were used to count and measure nematodes. For 'arrived' wasps, we collected the female wasps flying around the B phase figs of three *F. hispida* trees. In total, 143 female wasps were collected, approximately 50 wasps per tree. Fig wasps were immediately dissected to count the nematodes in the ovary, and the body lengths of the nematodes were measured with a stereoscopic microscope (LeicaS8AP0) with a micrometer.

Experiment 7: The impact of nematode infection on seed production

The relationship between the number of nematodes and fig seeds was studied as follows. Three female trees of *F. hispida* were sampled, about ten figs in which the seeds were close to maturity were randomly selected and subsequently cut into small pieces. All the seeds were removed and the pieces were placed into a glass funnel with distilled water. The funnel was placed on a layer of mesh bags to prevent the seeds from falling into the bottom of the funnel. After two to four hours the nematodes had moved to the bottom of the funnel, after which we transferred them into a Petri dish and quantified their abundance. The seeds on the mesh bags were dried at room temperature and then counted.

Data analysis

In *F. hispida*, *M. guangzhouensis* was the dominant species and *F. centerae* was rare species in our samples, making up less than 0.5% of the nematode individuals sampled. Thereafter, the nematode abundance represented the abundance of *M. guangzhouensis* in the following analysis.

Experiment 1: The prevalence of plant-parasitic nematodes in two Ficus species

The frequency of nematodes across *F. hispida* and *F. microcarpa* was compared using Pearson's Chi-squared test with Yates' continuity correction (Kabacoff, 2010). The null hypothesis was that the proportion of nematode occurrences was equal for the two tree species. The same analysis was used to compare occurrence and frequency between female and male trees of *F. hispida*.

Experiment 2: Variation of nematode abundance in different seasons and fig developmental phases

Nematode abundance data were analyzed using a zero-inflated general linear mixed model (ZIFGLMM) to account for the high number of zeros in the data set (Brooks et al., 2017; Zuur et al., 2009). In this analysis, data were modeled as coming from two different processes: the binomial process and the counting process. Then, a binomial generalized linear mixed model (GLMM) was used to determine the probability of measuring a zero; see below for the covariates included in these models. A Poisson or negative binomial GLMM modeled the counting process, and we used a backward stepwise strategy to select the best model for these data according to the Akaike information criterion (AIC). Likelihood ratio tests were used to quantify the effects of each explanatory variable by dropping each variable in turn and comparing the results to the whole model. (Zar, 1999).

For *F. microcarpa*, nematode number was the response variable, 'season' and 'developmental phase' was set as fixed variables, and 'tree' was a random effect. For *F. hispida*, nematode number was the response variable, and 'season' (two levels: 'hot-dry' and 'rainy'), 'developmental phase' (two levels: 'B phase' and 'C phase'), 'sex of the tree' (two levels: 'male' and 'female'), and 'tree' (ten levels) were set as explanatory variables.

Experiment 3: Nematode infection in different stages of adult pollinating fig wasps

A generalized linear model fitted a Poisson distribution was used to analyze the nematode abundance in different stages (unmated, mated, before emergence, emerged) of adult pollinating fig wasps, and pair-wise multiple comparisons between different stages were performed using the Tukey contrasts.

Experiment 4: Effects of nematodes on the amount of pollen carried by fig-pollinating wasps and their egg loads

Two GLMMs were fitted to test the effects of nematodes on i) the pollen and ii) egg loads of fig-pollinating wasps, including nematode number as a fixed effect and tree as a random effect.

Experiment 5: The lifespan of wasps with and without nematodes

To compare the lifespan of wasps with and without nematodes, we categorized the number of nematodes as either nematode free (no nematodes detected) or with nematodes (more than one nematode detected). Kaplan-Meier survival curves were drawn from the survival time (hours), and the two categories were compared using the Wilcoxon test (Therneau, 2015). Additionally, a Pearson's product-moment correlation was used to analyze the relationship between the lifespan of the fig wasps and the number of nematodes they transported.

Experiment 6: Nematodes carried by fig-pollinating wasps and their effects on wasp dispersal

Welch's two-sample t-tests were used to test for a difference in body length and the number of nematodes between 'newly emerged' and 'arrived' fig wasps.

Experiment 7: The impacts of nematode infection on seed production

Pearson's product-moment correlation was used to test the relationship between the number of nematodes and seeds.

All analyses were conducted in R 3.4.3 (R Development Core Team, 2018) with R packages ‘glmmTMB’ (Brooks et al., 2017), ‘lmtest’ (Zeileis & Hothorn, 2002), and ‘survival’ (Terry, 2015).

Results

Experiment 1: The prevalence of plant-parasitic nematodes in two Ficus species

Monoecious *F. microcarpa* was colonised by the nematode species *S. microcarpus*. This nematode species occurred 12 trees sampled, and the mean prevalence was 15% (range: 3–31%) for all the detected figs. Prevalence of *S. microcarpus* different significantly among trees ($\chi^2 = 101.04$, df=11, $p < 0.001$).

Ficus hispida was mainly infested by *M. guangzhouensis*. These nematodes infested all male and female trees sampled, with an average of 78.4% of figs from male trees being infested (range: 62–93%), and 83% (range: 67–98%) of figs from female trees being infested. The prevalence of *M. guangzhouensis* did not differ significantly between male and female trees ($\chi^2 = 0.65$, df=1, $p = 0.42$, Figure 1a). However, the prevalence of *M. guangzhouensis* differed significantly among trees ($\chi^2 = 146.32$, df=19, $p < 0.001$).

The occurrence of *M. guangzhouensis* in *F. hispida* was significantly higher than the occurrence of *S. microcarpus* in *F. microcarpa* (Pearson's Chi-squared test with Yates' continuity correction: $\chi^2 = 1375.4$, df=1, $p < 0.001$, Figure 1b).

Experiment 2: Variation of nematode abundance in different seasons and fig developmental phases

For *F. microcarpa*, the ZIFGLMM failed to converge, this was because there were too many zeros under the levels of the random effect 'tree'. Thus, we excluded the random effect and used a ZIFGLM model in the count model. Significantly more

S. microcarpus were detected from figs of *F. microcarpa* in the rainy season than the dry-hot season (χ^2=200.33, df=1, p<0.001, Table 1, Figure 2a). More *S. microcarpus* were detected in C phase than B phase (χ^2=151.51, df=1, p<0.001, Figure 3a). Interactions between season and fig developmental phase were significant (χ^2=1419.7, p<0.001, Table 1). For the logistic model, both season (χ^2=115.36, df=1, p<0.001) and fig developmental phase (χ^2=13.17, df=1, p<0.001) significantly influenced *S. microcarpus* abundance.

For *F. hispida*, significantly more *M. guangzhouensis* were detected in the rainy season than the dry-hot season (count model: χ^2=5539.90, df=1, p<0.001, Table 1, Figure 2b) and more *M. guangzhouensis* were detected in C phase figs than B phase figs (count model: χ^2=11833.00, df=1, p<0.001, Table 1, Figure 3b). However, the difference in nematode abundance between sexes was not significant (count model: χ^2=6.96, df=3, p=0.07, Table 1). Interactions among season, sex, and phase were significant (χ^2=14.45, df=7, p=0.043, Table 1). For the logistic model, neither sex (χ^2=0.43, df=1, p=0.51) nor season (χ^2=1.71, df=1, p=0.19) significantly affected *M. guangzhouensis* abundance, but fig developmental phase (χ^2=23.82, df=1, p<0.001) was significantly related to *M. guangzhouensis* abundance.

The number of nematodes (*M. guangzhouensis* for *F. hispida* and *M. guangzhouensis* for *F. microcarpa*) differed significantly between *F. hispida* and *F. microcarpa* (Wilcoxon rank test, W=5*10^5, p<0.001), *F. hispida* had more nematodes per fig than *F. microcarpa* (Table 1).

Experiment 3: Nematode infection in different stages of adult pollinating fig wasps

No unmated pollinating wasps from *F. hispida* were infected by *M. guangzhouensis* nematodes (n = 50) while in their natal galls. Once the mating hole appeared in the gall, an average of 7.74±7.38 (n=50) *M. guangzhouensis* nematodes were able to enter the galls to attack the female wasps. After the wasps entered the fig cavity, an average of 43.90±36.83 (n=50) *M. guangzhouensis* infested the bodies of the wasps, finally, an average of 2.58±2.85 (n=50) *M. guangzhouensis* entered the wasp abdomen to leave the natal figs. The analysis of variation in abundance of *M. guangzhouensis* at different fig developmental stages showed a significant difference among the developmental stages of the pollinating fig wasps (χ^2=4297, df=3, p<0.001) in *F. hispida*. Multiple comparisons showed significant differences among 'mated', 'before emerged', and 'emerged fig wasps' (Figure 4). Because no nematodes were detected in unmated wasps, these also differed from all other stages.

Experiment 4: Effects of nematodes on the amount of pollen carried by fig-pollinating wasps and their egg loads

Pollinating fig wasps of *F. hispida* with *M. guangzhouensis* carried an average of 796.3±404.08 (n=70) pollen grains and 274.51±50.61 (n=70) eggs. In contrast, nematode-free wasps carried an average of 698.83±390.58 (n=80) pollen grains and 298.44±64.24 (n=80) eggs. Therefore, *M. guangzhouensis* did not affect pollinating fig wasp pollen grains (χ^2=3.20, df=1, p=0.07) or egg loads (χ^2=1.14, df=1, p=0.29).

Experiment 5: The lifespan of wasps with and without nematodes

Female pollinating fig wasps infested by *M. guangzhouensis*, had an average lifespan of 23.43±1.36 h (SD, n = 97), while female pollinators without nematodes survived 33.75±1.49 h (SD, n = 267) on average. These results suggest that nematodes significantly reduced fig wasp lifespan ($\chi^2 = 130$, df=1, p<0.001, Figure 5). Finally, there was a significant negative correlation between fig wasp life span and the number of nematodes carried (r = -0.41, p<0.001).

Experiment 6: Nematodes carried by fig-pollinating wasps and their effects on wasp dispersal

The number of *M. guangzhouensis* carried by 'newly emerged' (6.38±5.14, n=104) fig wasps and fig wasps that 'arrived' (1.47±2.40, n=100) in the B phase figs differed significantly (t=-8.80, df=147.13, p<0.001). However, the body length of *M. guangzhouensis* did not differ significantly (t=-0.28, df=76.79, p=0.78) between 'newly emerged' (0.063±0.0066mm, n=50) and ‘arrived’ (0.062±0.0062mm, n=49) pollinating fig wasps.

Experiment 7: The impacts of nematode infection on seed production

We sampled 28 female figs in mature phase from three trees of *F. hispida*; only two figs were not infested by *M. guangzhouensis*. The rest had nematodes, and no correlation was observed between seed production and the number of *M. guangzhouensis* in mature fig fruit (r=0.03, p=0.89).

Discussion

Although the nematodes (*M. guangzhouensis*) associated with *F. hispida* were frequently detected in the figs and transported by pollinating fig wasps, they had no deleterious impact on fig wasp egg loads or the number of pollen grains carried. *Martininema guangzhouensis* did not develop during the period in which they were carried inside/outside the body of the fig wasps. However, transportation of *M. guangzhouensis* reduced the lifespan of pollinator wasps, and pollinator wasps with more nematodes showed a reduced ability to reach receptive fig trees. Therefore, *M. guangzhouensis* nematodes had limited detrimental effects on either mutualist. However, based on their phylogenetic position, it is likely that these nematodes are fungi feeders (Davies et al., 2015), with the intriguing possibility that they are deleterious hitchhiker of fig wasps.

As we expected, the monoecious fig species, *F. microcarpa*, had considerably lower numbers of nematodes within its figs than the gynodioecious *F. hispida*. Increased nematode prevalence may be due to distinct biological differences and contrasting costs between these two breeding systems. Furthermore, *F. microcarpus* was the only nematode found in *F. microcarpa* and *M. guangzhouensis* was the dominant nematodes in *F. hispida*. For both *Ficus* species, higher rates of nematode infection were found in the rainy season when compared to the dry-hot season and the inter-floral phase than the receptive phase of figs.

Nematodes are thought to be specific to fig species (Vovlas et al. 1992), with fossil records indicating a long history of co-evolution between nematodes and fig wasps (Poinar 2003). However, Davies et al. (2010) and Zeng et al. (2007) showed that one *Schistonchus* nematode species might live in multiple fig tree hosts. Some nematodes are associated with more than one wasp species in Australia and Indonesia (Davies et al. 2010, Sriwati et al. 2017). These generalist *Schistonchus* nematodes are not restricted to the same wasp genus or *Ficus* subgenus/sections. Davies et al. (2011) sampled 16 morphospecies of *Schistonchus* nematodes from 13 *Ficus* species. These *Ficus* species were from subgenera *Ficus, Sycomorus,* and *Urostigma* and their pollinating fig wasps from 5 different genera, *Kradibia, Ceratosolen, Eupristina, Pleistodontes,* and *Platyscapa*. Although *Schistonchus* nematodes were mostly associated with *Ficus* species there is limited evidence to support species level specificity. Therefore, host tree identity is not a reliable ecological trait for nematode classification (Davies et al. 2015). Zeng et al. (2007) reported two nematode species living in *F. hispida* in Guangzhou, China that differ from the species in *F. hispida* in India, showing regional difference in nematode occurrence. There are about 800 *Ficus* species (Berg 1989) and approximately 67 of these species is found in Yunnan province China (Zhang and Wu 2004). Thus, nematode species diversity is likely high in Yunnan based on fig tree diversity (but see the above discussion on host specificity). Further study of the classification, phylogeny, and ecology of nematodes is required to confirm these assertions.

Nematode prevalence was higher in the rainy season than in the hot-dry season for both *F. hispida* and *F. microcarpa*, indicating high temperature and humidity is important for nematode development. In this case breeding system appears to have limited explanatory power, suggesting a similar phenology and dispersal regime between the study species. Nematode numbers were higher in the C phase figs than the B phase in both species, which indicates that nematode development continues alongside that of figs. However, no differences in nematode abundance were found between male and female figs of *F. hispida*, which implies that nematode development is not reliant on fig wasp presence because no fig wasps develop in female figs. Over ten lineages of nematodes have been reported to be associated with figs, some of them are thought derived from fungal feeders (Davies et al., 2015), others are plant parasites (Davies et al., 2009), bacterial feeders (Kanzaki et al., 2016), or entomophagous (Herre, 1995; Ramírez-Benavides & Salazar-Figueroa, 2015; Van Goor et al., 2018). *Ficophagus* and *Martininema* nematodes were included in these new genera after being split from *Schistonchus* (Davies et al., 2015). Nematodes in *Schistonchus* have long been thought to feed on plant tissue (Center et al., 1999). However, *Ficophagus* and *Martininema* are thought to be derived from aphelenchoidid fungal feeders (Davies et al., 2015). The most well-known aphelenchoidid nematode is the pinewood nematode, *Bursaphelenchus xylophilus*, this species is transported by longhorn pine beetles. The nematode feeds on pine tissues and fungi, and causes pine wilt disease (Futai, 2013; Zhao et al., 2013).

Since our focal nematodes may feed on fungi, knowledge on fungal diversity and species composition is essential for us to understand the life history and prevalence of these nematodes. However, nothing is known about the fungal community in the figs of China to date, despite the study by Martinson et al. (Martinson et al., 2012) in Panama. Are these fungi plant pathogens or are they insect necrophages? Because fungal growth is sensitive to temperature, we suppose that the fungal community within the figs will vary across seasons. Seasonal variation in temperature will substantially affect the development of figs, fig wasps, fungi and nematodes, and lead to both population and community levels oscillations.

Nematodes must enter the abdomen of fig wasps for transmission to a new host. Although, Krishnan et al. (2010) reported that *Schistonchus* nematodes could distinguish female pollinating fig wasps from wingless male fig wasps and non-pollinating fig wasps using chemical cues in *F. racemosa*. Van Goor et al (2018) provided contrasting results, in their study system both pollinating and non-pollinating fig wasps are infected by nematodes. While fig wasp assemblage size can range between three and 30 species (Compton & Hawkins, 1992) only certain wasps are likely transport nematodes from one host to another (e.g. Agaonidae, Sycoecinae and some Sycophaginae and Otitisellinae), because most non-pollinating fig wasps do not enter into the syconium to oviposit. It is not known if non-pollinating fig wasps are capable of transporting nematodes, and this would be an interesting area for future research.

The nematodes transported by *C. solmsi marchali*, the pollinator of *F. hispida* at Xishuangbanna are not parasites of the insect, because their body lengths did not increase when they entered into the abdomens of fig wasps. These results indicate that nematodes did not absorb the nutrients of wasps and only used the pollinator female for transmission. This conclusion is also supported by the fact that their close relatives are fungal feeders. Moreover, the nematodes did not hinder the capacity of fig wasps to carry pollen and eggs. It has been hypothesized that phoretic nematodes have detrimental effects on fig wasps by reducing pollen grains or egg loads (Davies et al., 2015). Our results do not support this hypothesis, possibly because pollinating fig wasps are pro-ovigenic, with egg number likely being determined during development rather than in the adult stage (Elias et al., 2018).

However, nematodes reduced the life span of pollinators and the wasps carrying more nematodes were, therefore, less likely to reach receptive trees, which may result in costs being inflicted on fig wasps by these nematodes. The number of nematodes in the abdomen in those fig wasps that 'arrived' in the B phase figs to lay eggs and pollinate was less than in newly emerged fig wasps, indicating that wasps with higher nematode loads are less likely to reach figs and might die during this period. Herre (1995) also reported that the proportion of nematode-infested fig-pollinating wasps reaching receptive figs was reduced compared to nematode free wasps. Fig wasps are small and short-lived insects, nematodes could be an encumbrance for them during the process of host location, pollination, and oviposition. Filtering out severely infected fig wasps may prevent the overexploitation by nematodes of next generation of fig-

fig wasp, and thus stabilize the evolutionary coexistence of parasitic nematodes and the fig-fig wasp mutualism.

The results of this study reflect the natural complexity of this system, suggesting isolated, artificially controlled experiments may not reveal the relationship between nematodes and fig wasps when the ecological background (e.g., the feeding mode, life cycle, and relationships between *Ficophagus* and *Martininema*, figs and fig wasps) is ignored. To better understand the effects of nematodes on the stability of the fig and fig wasp mutualism, a cross-sectional analysis is not enough, and longitudinal studies are required to give us the whole profile and the process of the influence of nematodes on fig-fig wasp mutualism. The number of aborted figs, the abundance and nematode species inside the aborted figs, and even the variation in fungal community should be studied in the future. Network thinking is required and we should treat the syconium as a whole, incorporating the interactions among figs, pollinating fig wasps, the multi-species of non-pollinating fig wasps, nematodes, fungi, and even bacteria in this enclosed ecosystem.

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Competing financial interests

The authors declare no competing financial interests.

Author Contributions

R. R. S., B. G. M., and Y. Q. P. designed and performed the study. Y. S. Z. identified nematode species, B. W. analyzed the data, and R. R. S., S. T. S., B. W., and Y. Q. P. wrote the main text of the manuscript. All the authors reviewed the manuscript.

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Figure legends

Figure 1. Mean percentage of nematodes prevalence in two *Ficus species* and between female and male *F. hispida*. *** represent p<0.001, ns represents non-significant contrasts.

Figure2. Box plot of the number of nematodes per fig fruit in *Ficus microcarpa* (a) and *F. hispida* (b) in the hot-dry and rainy seasons in Xishuangbanna. Each circle represents a data point. *** indicates p<0.001.

Figure 3. Box plot of the number of nematodes per fig fruit in *Ficus microcarpa* (a) and *F. hispida* (b) in the B phase and C phase. Each circle represents a data point. *** indicates p<0.001.

Figure 4. Box plot of the number of nematodes per fig wasp in different life stages. Each circle represents a data point. Pairwise comparisons using the Tukey contrasts: different letters represent significant differences between groups.

Figure 5. Kaplan-Meier survival curves of nematode-infested and nematode-free pollinating fig wasps.

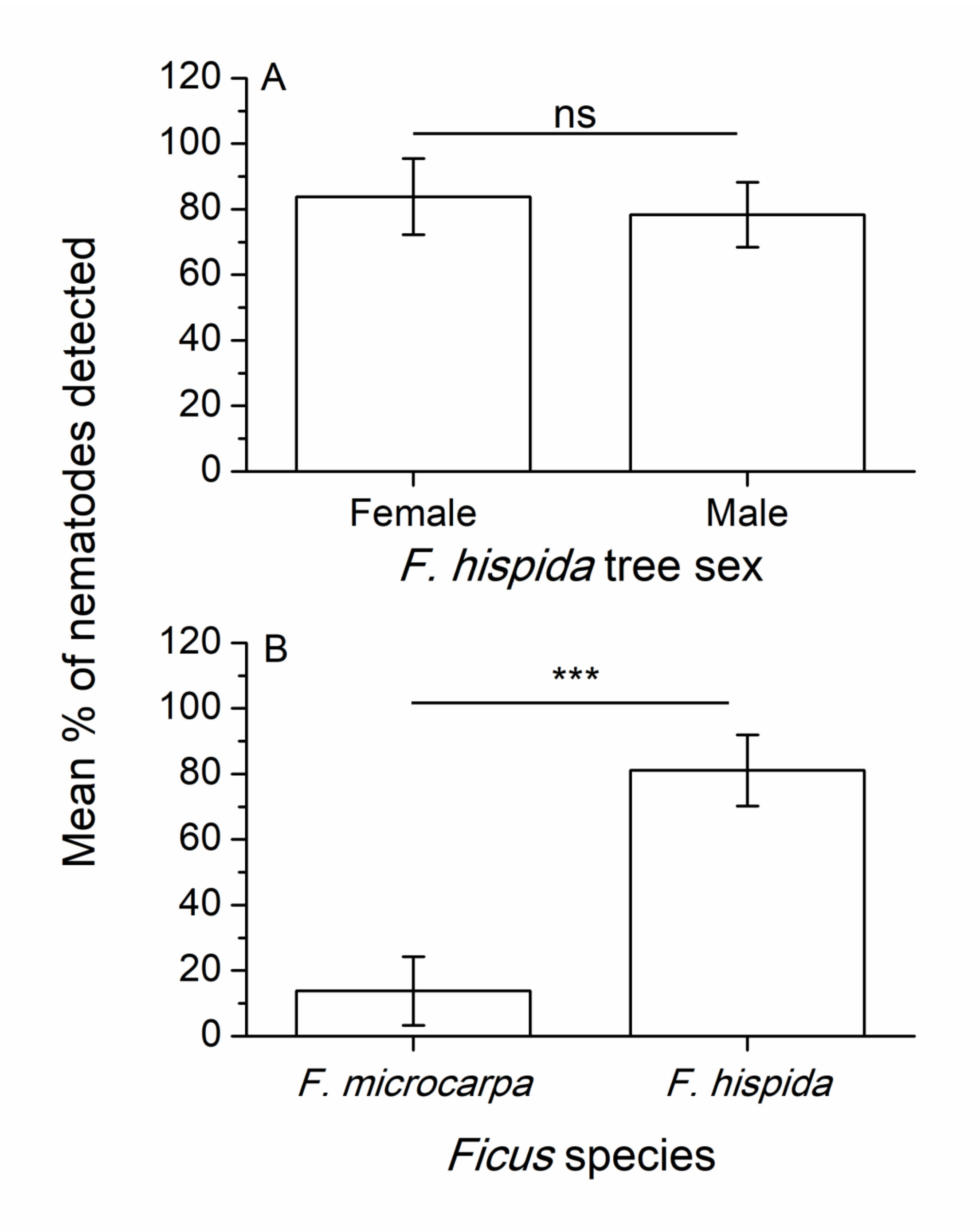

A
B
Mean % of nematodes detected
0
20
40
60
80
100
120
ns
Female
Male
F. hispida tree sex
***
F. microcarpa
F. hispida
Ficus species

Figure 1

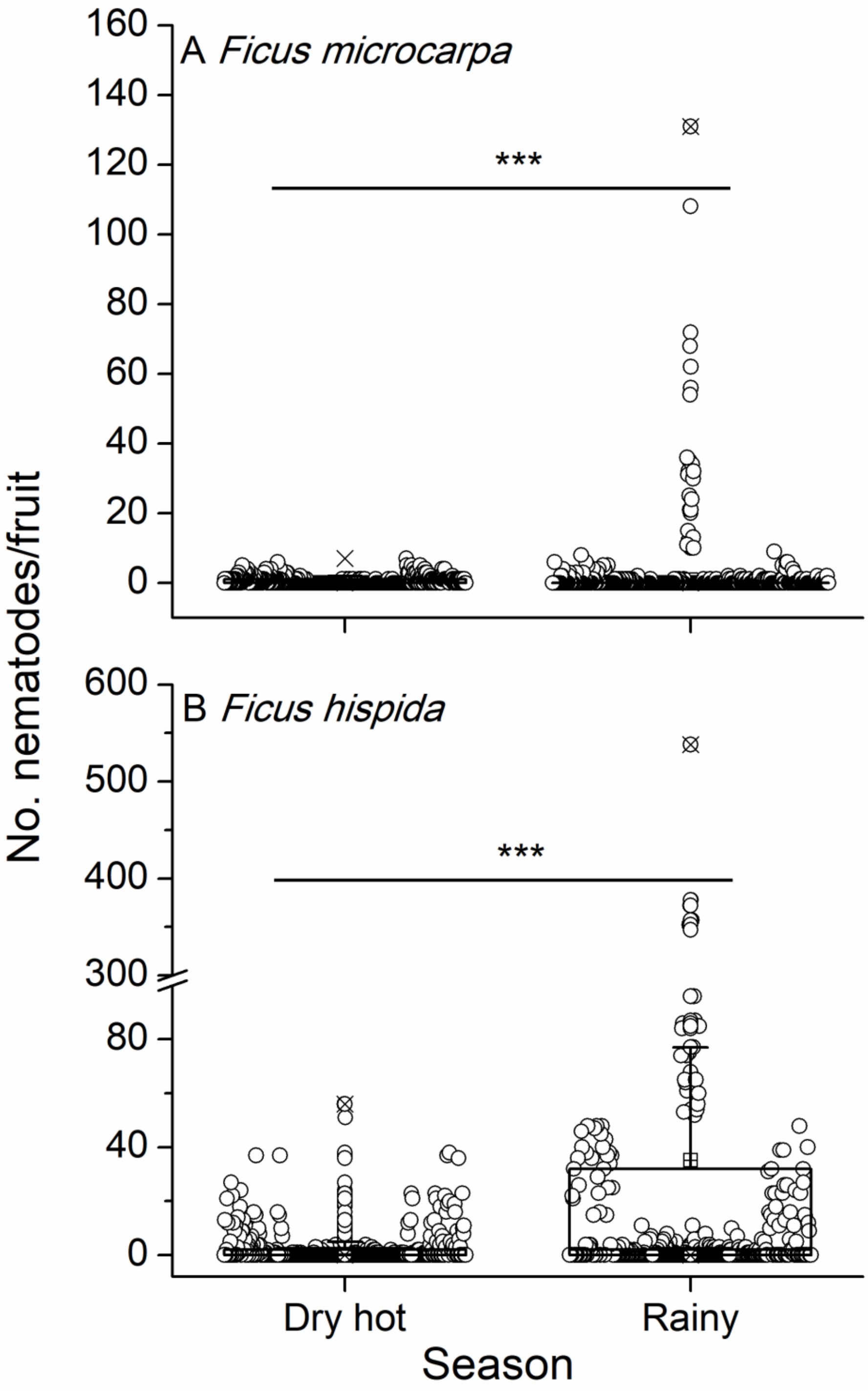
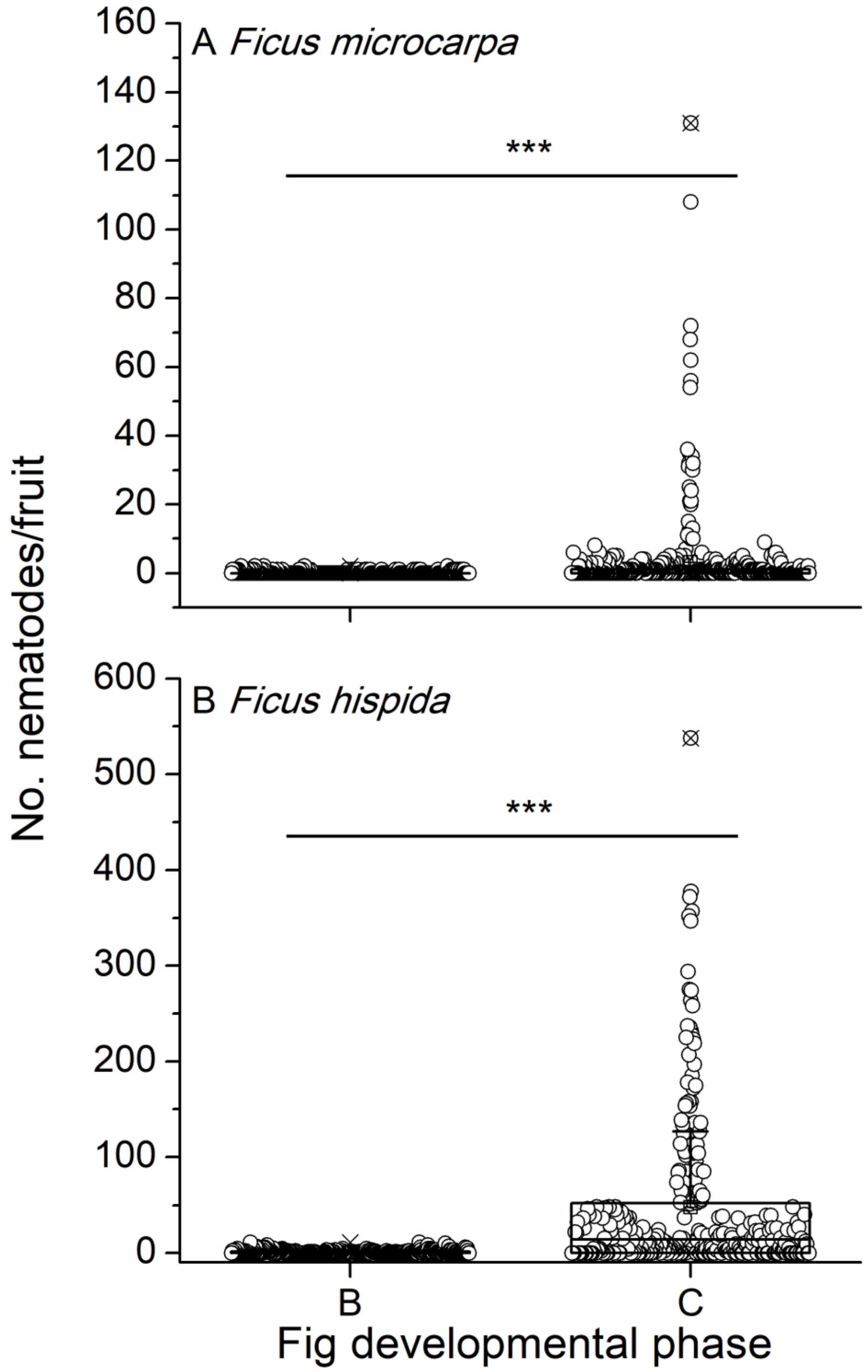
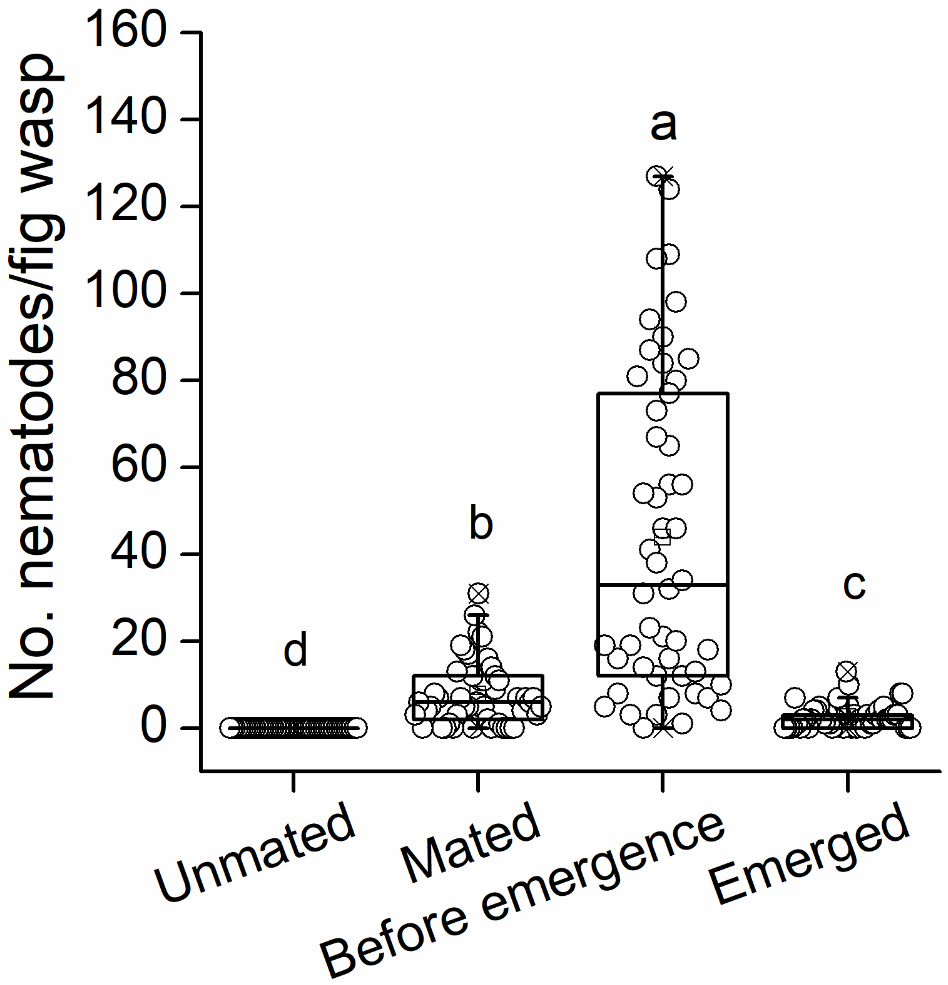

A Ficus microcarpa
B Ficus hispida
No. nematodes/fruit
160
140
120
100
80
60
40
20
0
600
500
400
300
***
***
Dry hot
Rainy
Season

Figure 2



No. nematodes/fruit
A *Ficus microcarpa*
160
140
120
100
80
60
40
20
0
***
B *Ficus hispida*
600
500
400
300
200
100
0
***
B
C
Fig developmental phase

Figure3



No. nematodes/fig wasp
160
140
120
100
80
60
40
20
0
a
b
c
d
Unmated
Mated
Before emergence
Emerged

Figure 4

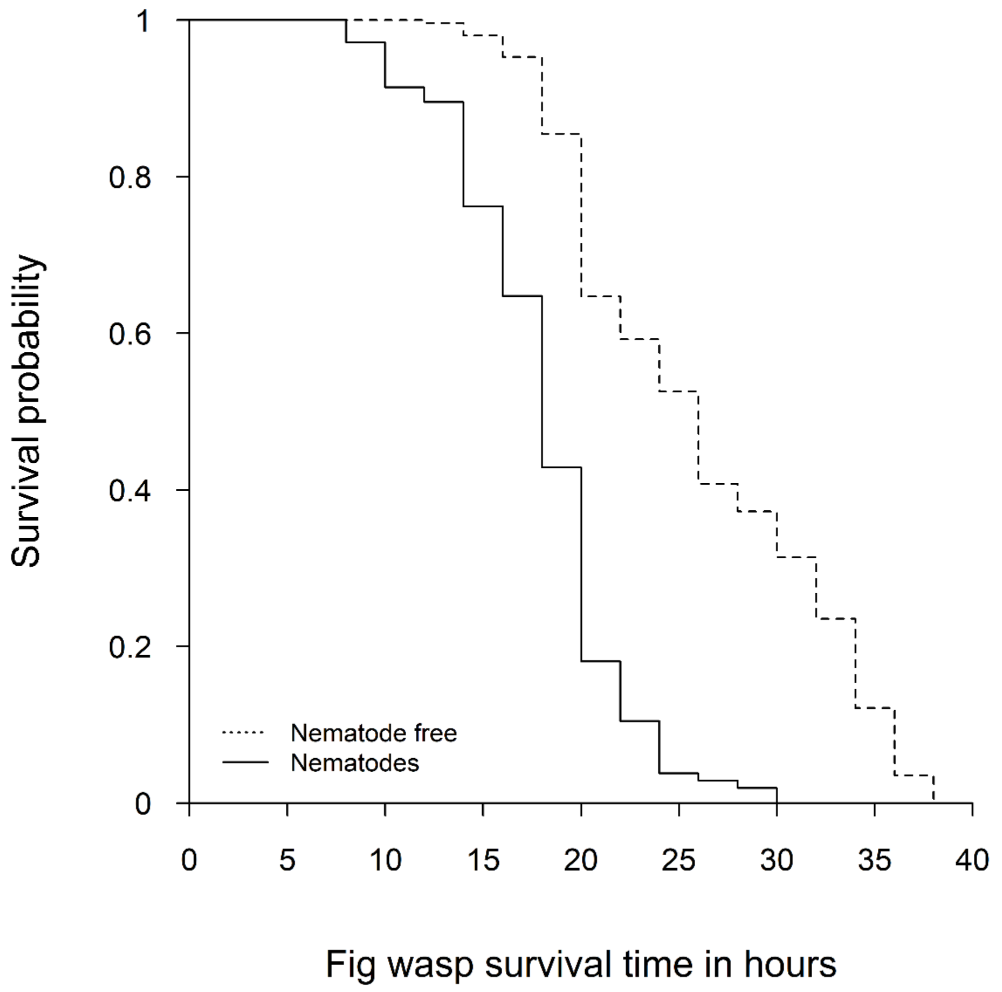

1
0.8
0.6
0.4
0.2
0
Survival probability
Nematode free
Nematodes
0
5
10
15
20
25
30
35
40
Fig wasp survival time in hours

Figure 5

Table 1. Descriptive statistics of nematodes number in *Ficus microcarpa* and *Ficus hispida* across different seasons, fig sex, and developmental phases in Xishuangbanna.

Category	**Levels**			**Mean**	**SD**	**Sample size**
F. microcarpa-season^{1}	Dry-hot			0.52	0.99	520
	Rainy			2.86	11.53	461
F. microcarpa-phase^{2}	B			0.12	0.35	500
	C			3.18	11.24	481
Interactions season×phase*	Dry-hot		B	0.065	0.26	245
			C	0.93	1.20	275
	Rainy		B	0.17	0.42	255
			C	6.18	16.68	206
Ficus***	*F. hispida*			19.44	55.69	713
	F. microcarpa			1.62	8.02	981
F. hispida-season^{1,***}	Dry-hot			2.66	7.06	346
	Rainy			35.27	73.95	367
F. hispida-sex$^{3,\ ns}$	Male			21.3	54.9	357
	Female			17.6	56.5	356
F. hispida-phase$^{2,\ ***}$	B			0.79	1.55	440
	C			49.51	81.51	273
Interactions season×sex×phase*	Dry-hot	Female	B	0.25	0.52	125
			C	10.04	12.17	52
		Male	B	0.48	0.86	125

			C	6.95	10.27	44
	Rainy	Female	B	1.13	2.13	96
			C	67.43	101.90	83
		Male	B	1.59	2.03	94
			C	75.43	86.34	94

Notes:

1. Nematodes were collected in dry-hot and rainy seasons in Xishuangbanna.
2. Figs were collected during B and C phases.
3. *Ficus hispida* is gynodioecious species with female and male individuals were collected and *Ficus microcarpa* is monoecious.

*** $p<0.001$, ns, not significant.