RESEARCH ARTICLE

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Density-dependent fitness effects stabilize parasitic hitchhiking within a mutualism

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Abstract

1. Mutualisms are often subject to perturbations by parasitism arising from third-party interactions. How third-party perturbations are dampened is a fundamental question pertaining to mutualism stability. Phoretic organisms that turn parasitic within a mutualism may destabilize it. If the fitness cost of such phoresy is high, then density-dependent effects could be one mechanism to stabilize these interactions.

2. We experimentally examined the fitness effects of a phoretic nematode community on a brood-site pollination mutualism involving a pollinating fig wasp (the vehicle) and its associated fig species (the host for wasp and nematode development).

3. We comprehensively investigated fitness impacts of phoresy on wasp lifespan, lifetime reproductive success, dispersal ability and predation risk as well as on host brood-site volume and seed number. We employed a range of hitchhiker densities that encompassed natural and overloading levels for two nematode taxa (one plant- and one animal-parasitic type).

4. None of the plant host and vehicle fitness parameters were affected by wasps with low nematode transportation loads for either type of nematode. Furthermore, wasps arriving at their destinations carried lower densities of both animal- and plant-parasitic nematodes compared to dispersing wasps, suggesting that there is selection on hitchhiker numbers during the dispersal process and that wasps loaded with a greater density of nematodes do not successfully disperse. Overloaded wasps had shorter flight durations, suggesting limited dispersal ability; on arrival at their destination, they suffered greater predation risk. Such overloaded wasps delivered impaired pollination services and produced fewer offspring resulting in lower lifetime fitness. Therefore, the direct and indirect effects of nematodes on their vehicles are strong. These effects also translated into impacts on host plant fitness, with the overloaded pollinators promoting the development of smaller brood sites with fewer seeds, thus reducing fig tree reproductive success. The effects of the animal parasites were greater than that of the plant parasite in this study.

5. The third-party interaction is therefore self-limited and exhibits density dependence. The strong negative effects of overloading likely explain the low number of

nematodes found in nature on dispersing and arriving fig wasps. Consequently, parasitic hitchhikers are unlikely to destabilize the mutualism.

1 | INTRODUCTION

Third parties may affect mutualisms not only by increasing their complexity via direct and indirect effects, but also by changing the trajectories of costs and benefits within short timescales (Bronstein, 1994; Herre, Knowlton, Mueller, & Rehner, 1999). Third-party interactions range from being beneficial to detrimental with varied effects on mutualism stability and persistence (Bronstein, 2001; Ferriere, Bronstein, Rinaldi, Law, & Gauduchon, 2002; Maloof & Inouye, 2000; Mougi & Kondoh, 2014). How mutualisms persist in the face of destabilizing third-party interactions is a fundamental question.

Phoresy is one of the third-party interactions that is sometimes associated with mutualism (Colwell, 1986; Guerra, Romero, Costa, Lofego, & Benson, 2012; Jauharlina, Lindquist, Quinnell, Robertson, & Compton, 2012; Tschapka & Cunningham, 2004). Phoresy is defined as an interspecific relationship where one organism (phoront or traveller) uses another organism (vehicle) to disperse out of an unsuitable habitat to enable survival and/or further development of itself and its progeny (Athias-Binche & Morand, 1993; Camerik, 2010). Habitats may become unsuitable due to limitation of food, space or mates (Binns, 1982; Colwell, 1986; Farish & Axtell, 1971; Kruitbos, Heritage, & Wilson, 2009). Tight links between mutualism and phoresy may establish whether the phoront is dependent on one or both mutualistic partners; one partner might help the traveller to overcome its limited dispersal ability, while the other might provide a suitable developmental substratum (Jauharlina et al., 2012; Tschapka & Cunningham, 2004). These links might lead to synchronization of the dispersal stages of vehicle and traveller (Baldwin, Nadler, & Adams, 2004; Giblin-Davis, Davies, Morris, & Thomas, 2003). The traveller might reside inside the vehicle (endophoresy) to ensure safe dispersal, a close association that could result in paraphagy (feeding on host metabolites) which may lead to parasitism (Athias-Binche & Morand, 1993). Travellers may have positive (e.g. removal of parasites or pathogens) or negative (e.g. decreased dispersal due to increased load or paraphagy) direct or indirect impacts on the fitness of their vehicles (Hodgkin, Elgar, & Symonds, 2010; Kinn, 1971; Figure 1).

The virulence of a host–parasite relationship is believed to be dependent on transmission opportunity (Anderson & Gordon, 1982; Anderson & May, 1978; Ewald, 1983), although the virulence–transmission trade-off has received only partial empirical support (Acevedo, Dillemuth, Flick, Faldyn, & Elderd, 2019). If hitchhiking or phoresy is parasitic, how can this phenomenon be stabilized, especially within a mutualism? The regulation and

stability of such interactions, although theoretically of great interest (Anderson & May, 1978), have been scarcely empirically investigated. We therefore asked whether density-dependent fitness costs of phoresy are likely responsible for stabilizing parasitic hitchhiking in a mutualistic system where transmission/transport is directed and highly specific.

We examined the cost of phoretic interactions on an ancient, co-evolved and specialized mutualism (Figure 1). The 75+ million-year-old mutualism between figs and pollinating fig wasps has had phoretic nematodes as third-party interactants for ca 15–45 million years (Cruaud et al., 2012; Peñalver, Engel, & Grimaldi, 2006; Poinar, 2003). There are 800+ fig species, each usually associated with a specialist pollinating fig wasp and a unique nematode community (Giblin-Davis, Ye, Kanzaki, Williams, & Morris, 2006; Machado, Jousselin, Kjellberg, Compton, & Herre, 2001). Fig nematodes are obligately dependent on pollinating fig wasps for movement between fig trees (Jauharlina et al., 2012; Krishnan, Muralidharan, Sharma, & Borges, 2010). The nematode families Diplogasteridae, Parasitaphelenchidae, Rhabditidae and Aphelenchoididae are associated with figs (Giblin-Davis et al., 2006; Susoy et al., 2016). So far, only the impact of entomopathogenic fig nematodes from the genus *Parasitodiplogaster* (Diplogasteridae) on their wasp vehicles has been investigated; their relationship with pollinating wasps ranged from being necromenic, that is obtaining nutrition from insect cadavers, to having limited negative effects, to being highly virulent; the virulence of the interaction supposedly depends upon the transmission rates of these nematodes between fig trees (Herre, 1993; Ramirez-Benavides & Salazar-Figueroa, 2015; Van Goor, Piatscheck, Houston, & Nason, 2018).

We used varying degrees of natural and experimental nematode density (number of hitchhiking nematodes per wasp) to determine the fitness cost of phoretic nematodes on wasps and figs. We investigated the effect of two phoretic nematode genera, viz. *Schistonchus* (putatively plant-parasitic) and *Teratodiplogaster* (putatively animal-parasitic) on the pollinator wasp *Ceratosolen fusciceps* and its associated fig *Ficus racemosa* (Figure 1). We determined the fitness effects of phoretic nematodes on (a) the pollinator wasp (in terms of lifespan, lifetime reproductive success, flight ability and predation risk) and (b) the fig (syconium volume and seed number) to understand the overall effect of phoresy on the mutualistic association. We therefore examined density-mediated direct and indirect effects of hitchhiking per se and comprehensively explored fitness effects at various hitchhiking levels (Figure 1). Based on our findings, we speculate on how these third-party interactions could be stably maintained within this mutualism.
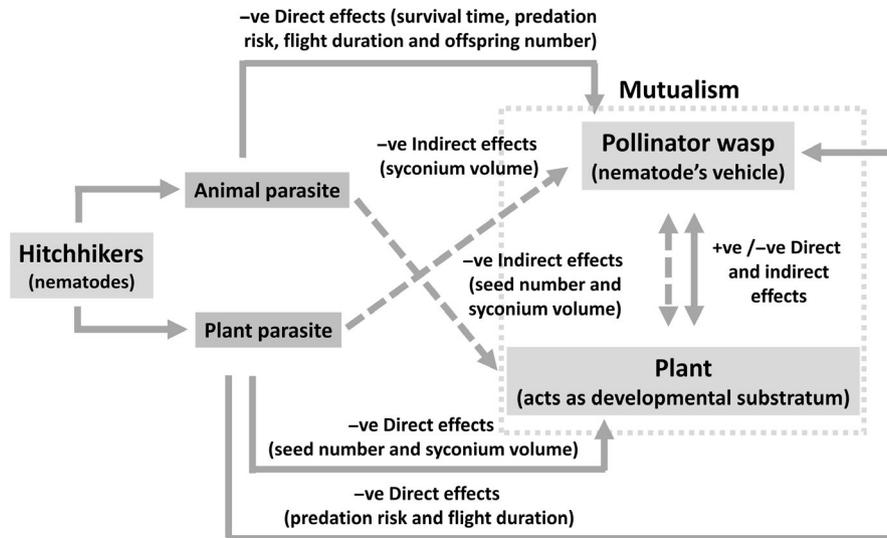
FIGURE 1 Diagramatic representation of proposed direct (shown by solid arrows) and indirect effects (shown by dotted arrows) of nematode as hichhikers in the fig–wasp–nematode tripartite system. The animal parasite can have a direct negative effect on the pollinator due to paraphagy or release of toxic metabolities, whereas it might have an indirect negative effect on the plant by reducing the pollinator's efficiency. The plant parasite can have a direct negative effect on the plant by feeding on male and female flowers, whereas reduction in the syconium volume might have an indirect negative effect on the pollinator's fitness. The plant parasite may also have a direct negative effect on the pollinator's fitness due to secretion of toxic metabolites

2 | MATERIALS AND METHODS

2.1 | Natural history of the fig–fig wasp–nematode study system

The cluster fig *Ficus racemosa* has served as an excellent model system for multitrophic interactions and wasp species dispersal (Ghara & Borges, 2010; Ranganathan, Ghara, & Borges, 2010; Venkateswaran, Shrivastava, Kumble, & Borges, 2017; Yadav & Borges, 2017). One pollinator species (*Ceratosolen fusciceps*), six non-pollinating wasp species and three nematode genera *Schistonchus* (Aphelenchoididae), *Teratodiplogaster* (Diplogasteridae) and *Pristionchus* (Diplogasteridae) develop within globular enclosed fig inflorescences or brood sites called syconia (singular = syconium) (Ghara & Borges, 2010; Susoy et al., 2016; S. Gupta, Q. Tahseen & R. M. Borges, unpublished data). Despite seven available wasp species, nematodes employ only female pollinator wasps as vehicles (Krishnan et al., 2010). The 60-day developmental cycle of the syconium commences with the entry of pollinator wasps (with endophoretic nematodes) into the syconium through an opening or ostiole at the pollen-receptive phase (or B-phase) (Figure 2). The wasps pollinate some female flowers (future seeds) and oviposit into others (forming galls that nourish wasp offspring) (Galil & Eisikowitch, 1968). After female pollinators enter the syconium, the nematodes disembark. During the interfloral or C-phase of syconium development, nematodes of all genera attain maturity, mate and lay eggs in the fig syconium (Figure 2). Nematode eggs hatch in late C-phase, mature to the dispersive stage (second juvenile phase/J2 stage) which hitchhikes on pollinator offspring in the wasp dispersal or D-phase (Figure 2; Giblin-Davis, Center, Nadel, Frank, & Ramírez,

1995; Krishnan et al., 2010; Reddy & Rao, 1986). Pulses of pollinating wasps arriving at syconia in the pollen-receptive stage and of dispersing wasps leaving syconia at the wasp-dispersal stage are prone to high predation risks from ants (Figure 2) (Ranganathan & Borges, 2009; Ranganathan et al., 2010), making rapid entry into the syconium through the ostiole essential for survival in arriving wasps.

2.2 | Study site and nematode collection for behavioural experiments

Pollinator wasps and nematodes were collected from the late C-phase of syconium development. Nematodes were introduced onto wasps while exhibiting nictation; this is characteristic of the dispersive stage; nematodes stand on their 'tails' and engage in waving their bodies to sample their chemical environment (Lee et al., 2012). Nematodes were identified using size, colour and structure of their anterior mouth parts, visible under a dissecting microscope. *Schistonchus* has a closed, blunt anterior end, is transparent whitish in colour and has a stylet and basal bulb anteriorly, whereas *Teratodiplogaster* has a sharper anterior end and a transparent silvery colour (S. Gupta, Q. Tahseen & R. M. Borges, unpublished data). All collections were made in and around the Indian Institute of Science campus in Bangalore, India (12°58′N, 77°35′E). Although *Pristionchus* is also present in this system, it is very rare, and was excluded from the study.

2.3 | Natural frequency distribution of hitchhiking nematodes in arriving versus dispersing wasps

Arriving and dispersing wasps were collected from five different trees when the syconia were in B (pollen-receptive) and D (dispersal)
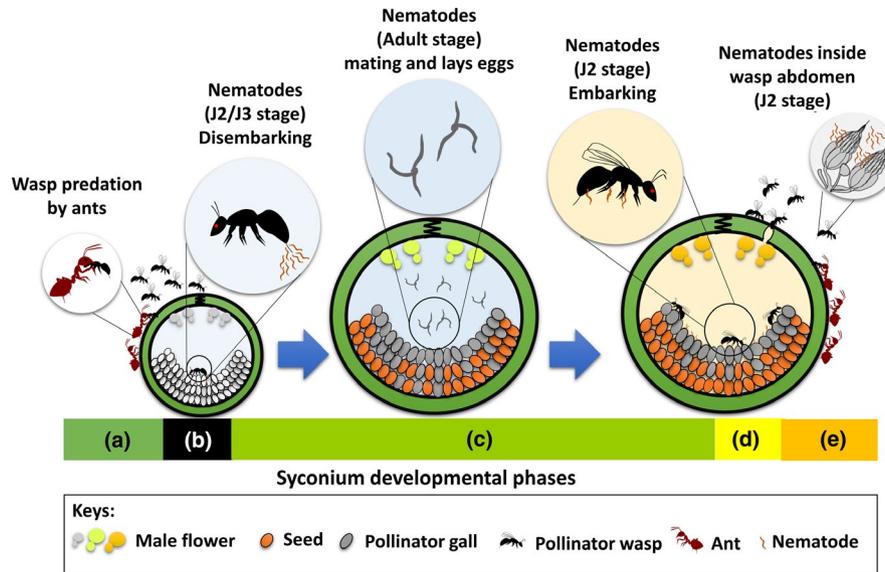
FIGURE 2 Diagramatic representation of the syconium development in relation to predatory ant presence and nematode development. In *Ficus racemosa*, the female flowers are present all throughout except at the ostiolar region where male flowers develop. The syconium development phases: A = pre-receptive phase, B = pollen-receptive phase (pollinators enter the syconium through the ostiole; nematodes disembark from pollinators at J2/J3 developmental stage), C = development phase including mating for both wasps and nematodes, D = dispersal phase of wasps (offspring of arriving nematodes embark at J2 developmental stage and enter wasp abdomens before wasps leave the syconium), and E = seed dispersal phase

phases, respectively (*n* = 100 wasps per tree). Wasps were dissected singly in distilled water; the number of animal- and plant-parasitic nematodes in each wasp was counted.

2.4 | Effect of natural nematode hitchhiking load on pollinator wasp longevity

Freshly emerging pollinator wasps were collected from D-phase syconia and placed singly at room temperature in vials with adequate aeration and moisture provided by cotton wool soaked in 1 ml water. Wasp mortality was determined at 2-hourly intervals since wasps usually live only 24 hr (Ghara & Borges, 2010), and the data were analysed using survival analyses (see Section 2.9). Dead wasps were dissected carefully on a glass slide under the microscope in distilled water. Nematodes were allowed to emerge till no more were left in the wasp and were subsequently heat-fixed by flaming the glass slide for 5 s and protected with a coverslip. The total number of nematodes, viz. *Schistonchus* and *Teratodiplogaster*, present in the wasp was counted. Wasp hind legs were also dissected and photographed to measure tibia length as surrogate for body size (Ghara & Borges, 2010) using IMAGE J software.

2.5 | Effect of hitchhiking nematodes on pollinator wasp fitness

Mated wasps devoid of hitchhiking nematodes were collected manually from late C-phase figs by opening pollinator galls. Wasps were manually loaded with 10, 20 or 30 nematodes of each genus, with each wasp receiving only one type of nematode (*n* = 110 wasps in total); these numbers were selected using the mean abundance of

nematodes of both genera together in arriving and dispersing wasps (see Section 3; Table 1); a control group of wasps was kept without loading nematodes. The process of manual loading involves picking up nematodes singly and putting them onto a wasp. Once on the wasp, the nematodes enter the wasp's abdomen. Loading was repeated till the desired numbers mentioned previously were reached. The wasps were given crushed anthers to walk over for 2 hr and were allowed to fill their thoracic pollen pockets with pollen. This was done to ensure that when these treated wasps were introduced into syconia, they carried pollen for pollination; un-pollinated syconia are aborted by fig trees. Fig bunches/clusters containing ~40 syconia were bagged with muslin cloth in their early primordial and A stages to prevent unregulated wasp entry. When these bagged syconia developed into the receptive B-phase, a single pollinator was introduced into each syconium. Syconia thus treated were allowed to develop till D-phase, and emerging wasp progeny (both male and female) were counted (*n* = 110 wasps in total). Since pollinator wasps only enter and oviposit into a single syconium where they die after pollination and/or oviposition, the offspring count obtained is the lifetime fitness of the pollinator.

2.6 | Effect of hitchhiking nematodes on predation risk faced by pollinators

Since ants are major predators of fig wasps on syconia (Ranganathan et al., 2010), the time taken by wasps to enter the syconium ostiole determines the probability of encountering these ants and is therefore an indicator of predation risk (Figure 2). Nematode loading and bagging of the syconia were performed as before. Wasps were released at around 3 mm distance from the ostiolar region, and the time taken by each individual wasp to enter the ostiole was recorded (*n* = 110 wasps).

TABLE 1 Summary statistics of nematodes present naturally in dispersing and arriving wasps

Nematode (per wasp)	Mean	Median	SD	Range	n (sample size)
Dispersing wasps					
Teratodiplogaster	16.4	16	12.1	0–46	500
Schistonchus	10.4	8	9.5	0–46	500
Nematodes of both genera per wasp	26.8	26	16.5	0–66	500
Arriving wasps					
Teratodiplogaster	6.5	2	8.9	0–42	500
Schistonchus	6.1	2	8.9	0–62	500
Nematodes of both genera per wasp	12.6	8	13.3	0–78	500

2.7 | Effect of hitchhiking nematodes on the flight ability of pollinator wasps

To determine the dispersal capacities of wasps with and without nematodes, flight recordings of tethered wasps were obtained using a custom-built optical tachometer according to standardized procedures described in Venkateswaran et al. (2017). The wasps were loaded with nematodes as in previous experiments, and total flight time of wasps in the different nematode-loaded groups was obtained by summing all flight bout durations (n = 70 wasps).

2.8 | Effect of hitchhiking nematodes on host plant fitness

Total seed count per syconium and syconium volume were used to determine the effects of nematodes on plant fitness via pollination success. Syconium variables are appropriate as measures of plant fitness since the fig plant imposes host sanctions on pollinators at the syconium level (Jandér, Herre, & Simms, 2012; Krishnan, Pramanik, Revadi, Venkateswaran, & Borges, 2014) by aborting those syconia that have received inadequate pollination. Wasp loading with nematodes and single pollinator introductions were as in previous experiments. For each syconium, change in volume (final syconium volume at D-phase – initial volume at B-phase; Krishnan et al., 2014) and the total number of seeds were recorded (n = 110 syconia).

2.9 | Statistical analysis

Shapiro–Wilk's normality tests were conducted to determine whether the frequencies of dispersing and arriving wasps were normally distributed. To determine whether the frequency distribution of nematode infestation differed between incoming and outgoing wasps, Kolmogorov–Smirnov (K-S) two-sample tests were performed. The impact of nematodes on wasp longevity was examined using a Kaplan–Meir survival analysis; a log rank test was also performed to examine any significant difference in longevity between wasps with and without hitchhiking nematodes. Pearson's correlation coefficients

between longevity and nematode frequency (for *Schistonchus* and *Teratodiplogaster*) were calculated. A Cox proportional hazard model was constructed to examine the effect of parameters, such as wasp body size (tibia length), number of nematodes of each type (*Schistonchus* and *Teratodiplogaster*), and their interaction terms. A Kruskal–Wallis test followed by a post hoc Dunn test with Bonferroni correction was used to examine differences between the different hitchhiking groups within a nematode type for each experiment. Further, Mann–Whitney *U* tests were performed to compare the effect of nematode types with similar nematode density loading for each experiment. All statistical analyses were conducted using the software R (version 3.2.3).

3 | RESULTS

3.1 | Natural frequency distribution of hitchhiking nematodes in arriving versus dispersing wasps

The nematodes in the *F. racemosa* system are endophoretic; that is, they reside inside the wasp's abdomen during transport and disembark post-pollination (Figure 2). During the arriving and dispersing phases, the frequency of wasps carrying different numbers of hitchhiking nematodes was not normally distributed (arriving: W = 0.84, p < .001; dispersing: W = 0.97, p < .001) and was more heavily right-skewed in arriving than in dispersing wasps (Figure 3a). The mean (±*SD*) number of nematodes in arriving wasps was lower than in dispersing wasps but the ranges were similar (Table 1; D = 0.44, p < .001; Figure 3a; Table 1). Nematodes were present in lower numbers in arriving compared to dispersing wasps for *Schistonchus* (D = 0.26, p < .001; Figure 3b) and *Teratodiplogaster* (D = 0.41, p < .001; Figure 3c; Table 1). However, the number of *Schistonchus* was lower than the number of *Teratodiplogaster*, both in arriving (D = 0.086, p = .0495, Figure 3d; Table 1) and in dispersing (D = 0.27, p < .001, Figure 3e; Table 1) wasps.

3.2 | Effect of hitchhiking nematodes on pollinator longevity

Hitchhiking nematodes affected the survivorship of their vehicles significantly (χ^2 = 20.3, df = 1, p < .001); mean survival time of wasps carrying nematodes was reduced by approximately
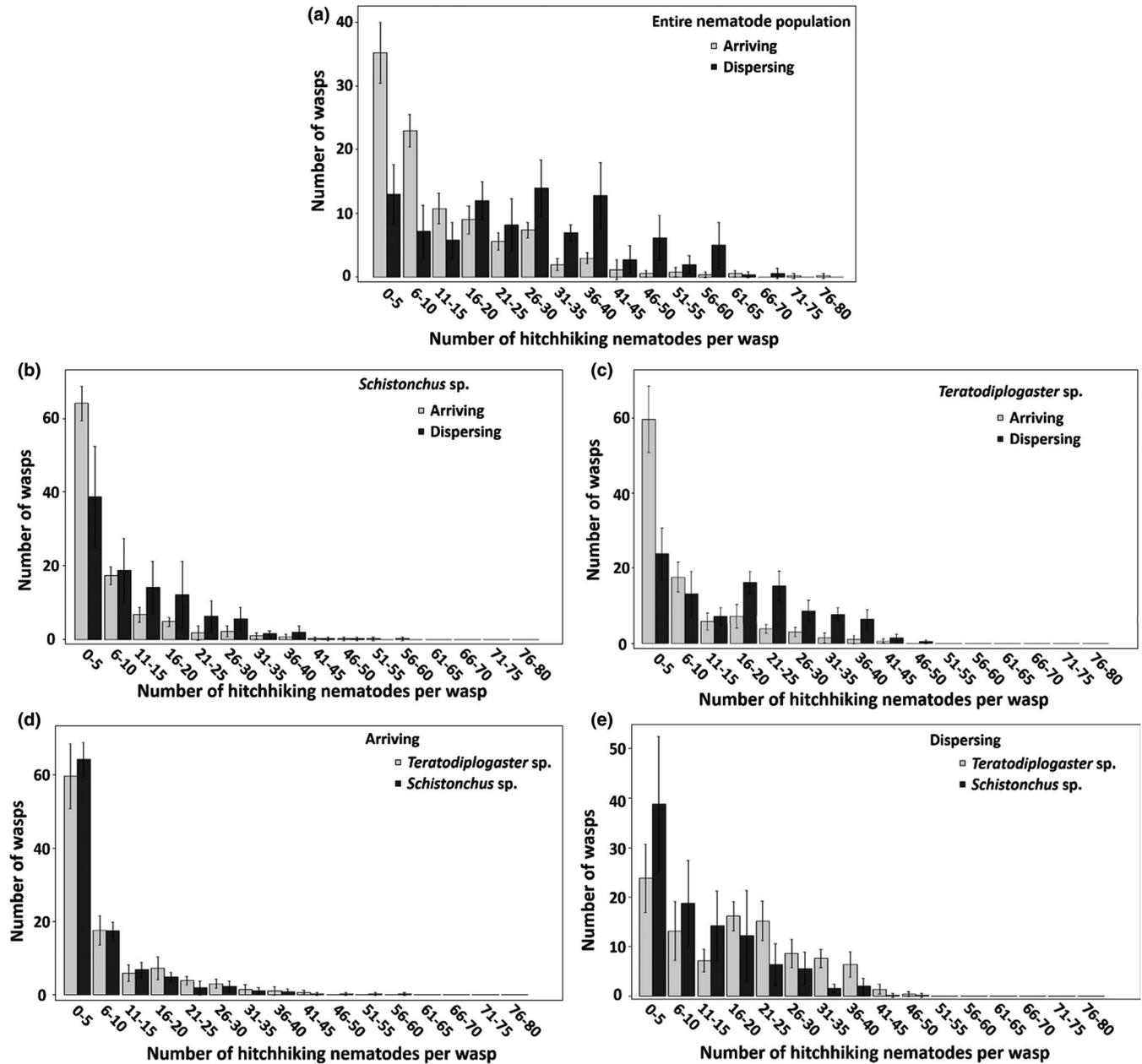
FIGURE 3 Natural frequency distribution for comparison between arriving and dispersing pollinator wasps with different nematode loads with respect to the entire nematode population (a), number of *Schistonchus* nematodes (b) and number of *Teratodiplogaster* nematodes (c). Natural frequency distribution for comparison between *Schistonchus* nematodes and *Teratodiplogaster* nematodes in arriving and dispersing pollinator wasps (d–e). The height of the bars indicates the mean and the vertical lines are the associated *SD*s (standard deviation)

4 hr (1/5th of lifespan) when compared to wasps without nematodes (Figure 4a; Table S1). Wasp longevity depended on wasp body size, number of *Teratodiplogaster* nematodes and their interaction terms (Table 2). The negative effect of *Teratodiplogaster* on survival was stronger for larger wasps when compared with smaller wasps (Figure S1). *Teratodiplogaster* reduced survival likelihood by 33% (hazard ratio = exp (coef) = 0.67; Table 2). Longevity of wasps was negatively correlated with *Teratodiplogaster* numbers per wasp ($t = -13.3$, $df = 212$, $R^2 = -0.67$, $p < .001$, $n = 214$) where there was an early steep decline (Figure 4b), whereas there was no relation

with *Schistonchus* ($t = -1.3$, $df = 212$, $p = .19$, $R^2 = 0.03$, $n = 214$; Figure 4c).

3.3 | Effect of hitchhiking nematodes on pollinator lifetime fitness

Teratodiplogaster numbers per wasp ($\chi^2 = 55.35$, $df = 3$, $p < .001$, $n = 65$) but not *Schistonchus* ($\chi^2 = 6.22$, $df = 3$, $p = .10$, $n = 65$; Figure 5a) had a significant effect on wasp offspring number compared to the control at the examined hitchhiking density levels. Moderate ($n = 20$) and high ($n = 30$) *Teratodiplogaster* density groups produced a significantly
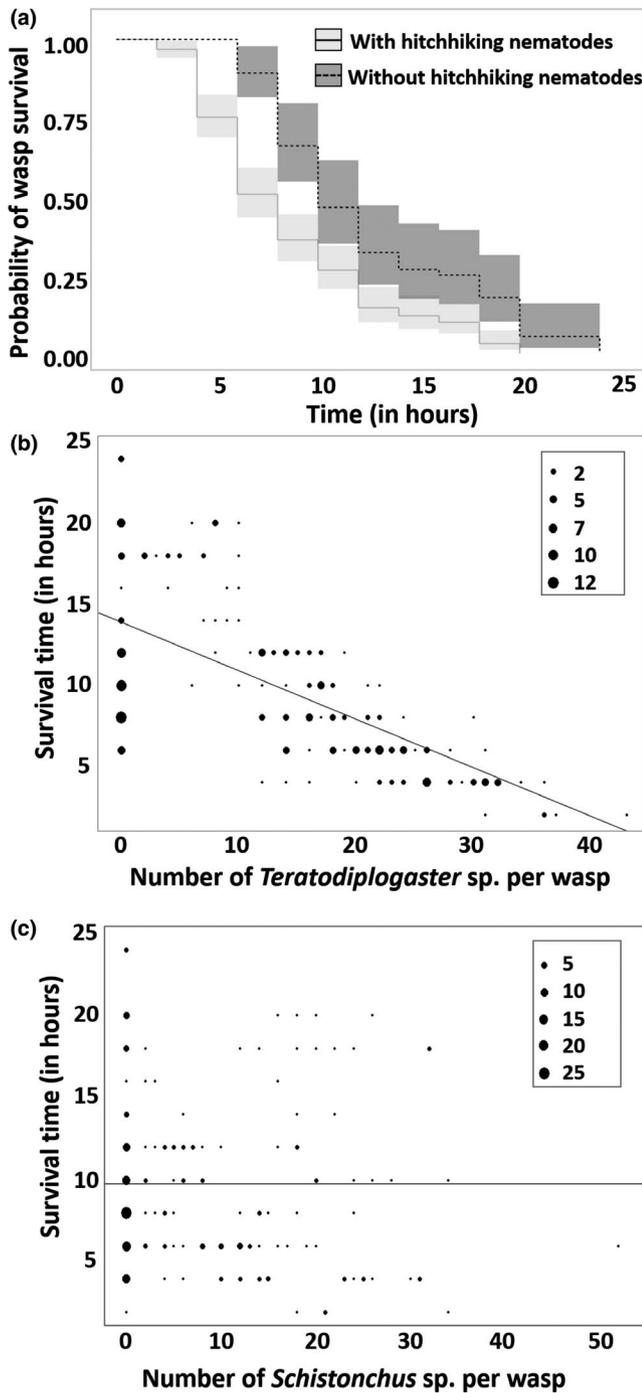
FIGURE 4 (a) Survivorship curve of pollinator wasps with and without hitchhiking nematodes, (b) relationship between wasp survival time and *Teratodiplogaster* numbers in a wasp, and (c) relationship between wasp survival time and *Schistonchus* numbers in a wasp. The differently sized black circles show the number of data points (representing number of wasps) represented by each point

lower number of offspring compared to wasps loaded with lower numbers ($n = 10$) and the control but these groups were not significantly different from each other (Figure 5a; Tables S2–S3). Fitness reduction was commensurate with numbers of *Teratodiplogaster* transported per wasp (~30% and 20% fitness reduction relative to

the control; $W = 0$, $p < .001$, $n = 30$ in both cases; Figure 5a). For lower numbers, there was no difference between the two nematode genera ($W = 64$, $p = .051$, $n = 30$), although this was close to significance.

3.4 | Effect of hitchhiking nematodes on predation risk faced by pollinator wasps

Wasps with and without *Schistonchus* ($\chi^2 = 3.94$, $df = 3$, $p < .001$, $n = 65$) and *Teratodiplogaster* differed in predation risk ($\chi^2 = 50.37$, $df = 3$, $p < .001$, $n = 65$). Wasps loaded with moderate and high *Teratodiplogaster* numbers took significantly longer to enter the ostiole (~2-fold) compared to control and lower number groups but these latter two groups were not significantly different from each other (Figure 5b, Tables S4–S6). Wasps loaded with high *Schistonchus* numbers took significantly more time to enter the ostiole with respect to all other *Schistonchus* groups; wasps with lower *Schistonchus* loading numbers and control groups were not significantly different (Figure 5b, Tables S4–S6). *Teratodiplogaster*-loaded wasps took more time to enter the ostiole: twofold higher at moderate loading levels ($W = 225$, $p < .001$, $n = 30$) and 1.5-fold higher at high loading levels ($W = 225$, $p < .001$, $n = 30$) when compared to wasps with similar loading levels of *Schistonchus* (Figure 5b, Table S4). There was no difference between the two genera for lower loading levels ($W = 141$, $p = .20$, $n = 30$).

3.5 | Effect of hitchhiking nematodes on wasp flight ability

There was a significant difference in flight duration among the tested hitchhiking groups for *Schistonchus* ($\chi^2 = 8.75$, $df = 3$, $p = .03$, $n = 40$) and *Teratodiplogaster* ($\chi^2 = 32.96$, $df = 3$, $p < .001$, $n = 40$). Wasps infested with high loads of *Schistonchus* nematodes had slightly lower flight durations than control wasps and wasps with low or moderate *Schistonchus* loads. The latter groups did not significantly differ from each other. By contrast, wasps infested with moderate and high *Teratodiplogaster* loads showed strongly reduced flight duration compared to control wasps, whereas these former groups did not differ from each other (Figure 5c, Tables S7–S9). Flight duration was reduced by ~30% at moderate and ~60% at high hitchhiking levels for wasps loaded with *Teratodiplogaster* when compared to the control. When the impact of *Teratodiplogaster* was compared to similar loading numbers with *Schistonchus*, reduction in flight duration was ~27% for moderate ($W = 0$, $p < .001$, $n = 20$) and ~54% for high loading levels ($W = 0$, $p < .001$, $n = 20$). For wasps with low numbers of nematodes, flight duration did not differ between wasps loaded with the two different nematode genera ($W = 61$, $p = .42$, $n = 20$; Figure 5c, Tables S7–S9).

3.6 | Effect of hitchhiking nematodes on plant fitness

Syconium volume was significantly smaller when syconia were entered by pollinating wasps transporting *Schistonchus* ($\chi^2 = 49.26$, $df = 3$, $p < .001$, $n = 65$) and *Teratodiplogaster* ($\chi^2 = 49.8$, $df = 3$,

TABLE 2 Survival analysis using a Cox proportional hazard model to examine the effects of number of *Teratodiplogaster* and *Schistonchus* nematodes, wasp body size and their interaction terms

Model	Coef	Z	95% CI	Pr(>|z|)
Schistonchus	−9.15E−02	−0.917	7.5e−01–1.1e + 00	0.359
Teratodiplogaster	−4.01E−01	−4.834	5.6e−01–7.8e−01	1.34e−06***
Body size of wasp	−8.95E+01	−11.742	4.3e−46–4.1e−33	<2e−16***
Schistonchus × *Teratodiplogaster*	1.49E−03	1.651	9.9e−01–1.0e + 00	0.0988
Schistonchus × body size of wasp	3.23E−01	0.615	4.9e−01–3.8e + 00	0.5388
Teratodiplogaster × body size of wasp	3.35E+00	6.596	1.0e + 01–7.7e + 01	4.22e−11***

***$p < .001$; ($n = 214$), $R^2 = 0.79$, Score (logrank) test = 295, $df = 6$, $p < .001$

$p < .001$, $n = 65$; Figure 6a, Tables S10–S12) compared to the control. The reduction in syconium volume was ~22% for moderate and high *Schistonchus* hitchhiking numbers with respect to the control, whereas the reduction was ~25% for moderate and ~35% for high *Teratodiplogaster* hitchhiking numbers with respect to the control. However, these were not significantly different from the controls. No significant difference in syconium volume was observed between syconia pollinated by wasps infested by the two nematode species for low ($W = 132$, $p = .43$, $n = 30$), moderate ($W = 95$, $p = .48$, $n = 30$) or high ($W = 76$, $p = .13$, $n = 30$) hitchhiking groups. Total seed count per syconium also differed among the *Schistonchus* ($\chi^2 = 33.8$, $df = 3$, $p < .001$, $n = 65$) and *Teratodiplogaster* hitchhiking groups ($\chi^2 = 46.1$, $df = 3$, $p < .001$, $n = 65$) (Figure 6b, Table S13–S15). Seed production per syconium was significantly lower in moderate and high *Schistonchus* hitchhiking levels compared to other groups. Similarly, seed production per syconium was significantly lower in moderate and high *Teratodiplogaster* hitchhiking groups compared to low *Teratodiplogaster* or control groups. The reduction of seed number was ~15% for moderate and ~27% for high *Schistonchus* infestation relative to the control, whereas the reduction was ~27% for moderate and ~49% for high *Teratodiplogaster* numbers relative to the control. The reduction in seed number was ~13% for moderate ($W = 48$, $p = 0.006$, $n = 30$) and ~29% for high ($W = 19$, $p < .001$, $n = 30$) *Teratodiplogaster* numbers with respect to similar numbers of *Schistonchus*. The effect of the lower hitchhiking groups was not significantly different between the two genera ($W = 86$, $p = .28$, $n = 30$).

4 | DISCUSSION

Our experimental findings indicate that none of the plant host and vehicle fitness parameters were affected in wasps with low nematode transportation load for either type of nematode. These results suggests that the third-party interaction is self-limiting and exhibits density dependence, and this might explain why low average number of hitchhiking nematodes of both species are found in nature on dispersing and arriving fig wasps. Furthermore, lower densities of both animal- and plant-parasitic nematodes were observed in arriving wasps compared to dispersing wasps, suggesting that there is selection on hitchhiker numbers during the dispersal process and

that wasps loaded with a greater density of nematodes do not successfully disperse. Overloaded wasps had shorter flight durations, suggesting that they can only reach fig trees that are within shorter flight distances; once a tree is reached, they suffered from greater predation risk, and assuming they escape predation and enter the syconium ostiole, their pollination service and oviposition resulted in fewer offspring and smaller syconia. Therefore, the direct and indirect effects of nematodes on their vehicles are strong. These effects translate into impacts on host plant fitness, with the overloaded pollinators promoting the development of smaller syconia with fewer seeds, thus impacting fig tree reproductive success. The impacts of the animal parasites were greater than those of the plant parasite in this study. Clearly, numbers of hitchhikers are regulated by strong fitness effects which help to stabilize the presence of these third parties within the mutualism by keeping their densities at levels that do not harm the mutualism.

4.1 | Effects of phoretic nematodes on wasp fitness

4.1.1 | Longevity

Teratodiplogaster infestation reduced the lifespan of their wasp vehicles indicating these nematodes to be animal-parasitic in nature. Nematodes may decrease host lifespan via endogenous or endosymbiont-derived toxic metabolites or by feeding on host tissues (Blaxter & Koutsovoulos, 2015; Bowen & ffrench-Constant 2000; Kanzaki, Giblin-Davis, Ye, Herre, & Center, 2014). Similar mechanisms may operate in *Teratodiplogaster*. These nematodes also remain around the wasp cadaver after disembarking, suggesting necromeny (S. Gupta, pers. observ.). *Schistonchus* had no effect on wasp lifespan. The negative impact of *Teratodiplogaster* on wasp survival was greater for larger than for smaller wasps. The reason for this finding is currently unknown.

4.1.2 | Lifetime reproductive success

Teratodiplogaster decreased offspring number only at higher hitchhiking densities, whereas no effects were observed for *Schistonchus*. The negative effects of *Teratodiplogaster* may be due to either reduction in egg-laying due to reduced lifespan or because hitchhiking nematodes feed on the stored lipid content present in the eggs rendering them
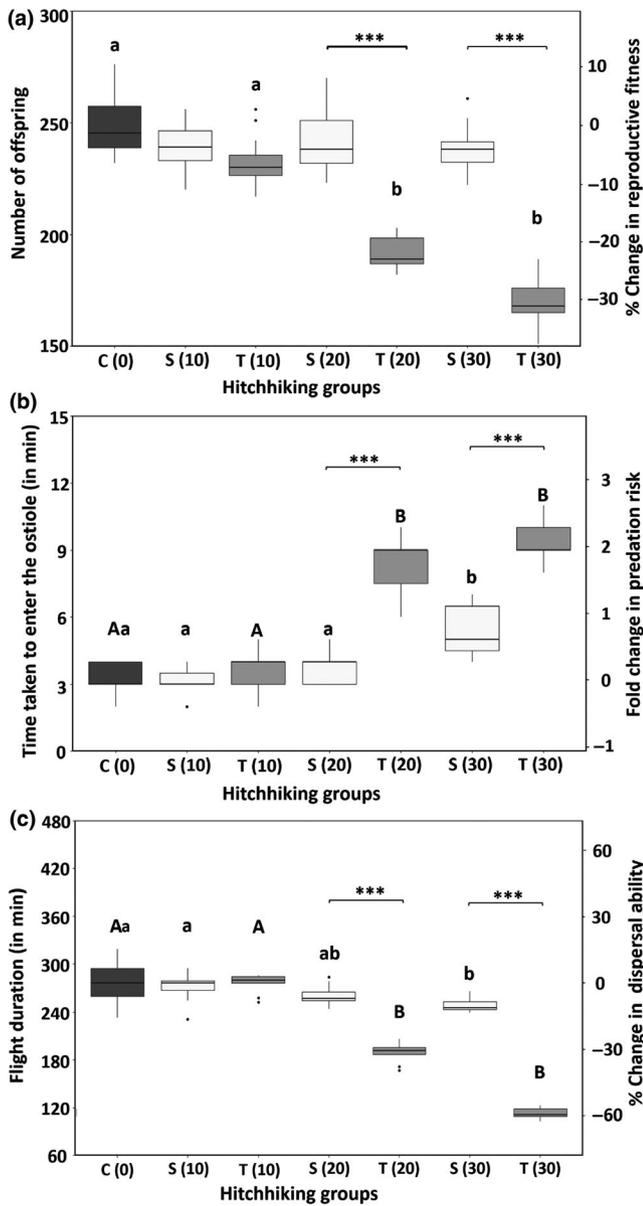
FIGURE 5 (a–c) Boxplots of the number of offspring (including % change in reproductive fitness measured using means), fold change in predatory risk (including time taken to enter the ostiole) and flight duration (including % change in dispersal ability) of a wasp at different levels of nematode load (for *Schistonchus* and *Teratodiplogaster* at increasing density per wasp). Horizontal lines indicate medians, whiskers indicate the upper and lower quartiles, and solid black circles denote outliers. Alphabets are used to denote significant differences between groups by a post hoc Dunn test with Bonferroni corrections for pairwise comparison made between the different loading groups within a nematode species (capital letters for *Schistonchus* and small letters for *Teratodiplogaster*). Asterisks denote significant differences between similar loading groups for the two genera obtained by Mann–Whitney tests; *p < .05; **p < .01; ***p < .001; Hitchhiking groups: Control denoted as 'C', *Schistonchus* denoted as 'S' and *Teratodiplogaster* denoted as 'T'; number of hitchhiking nematodes is mentioned within brackets
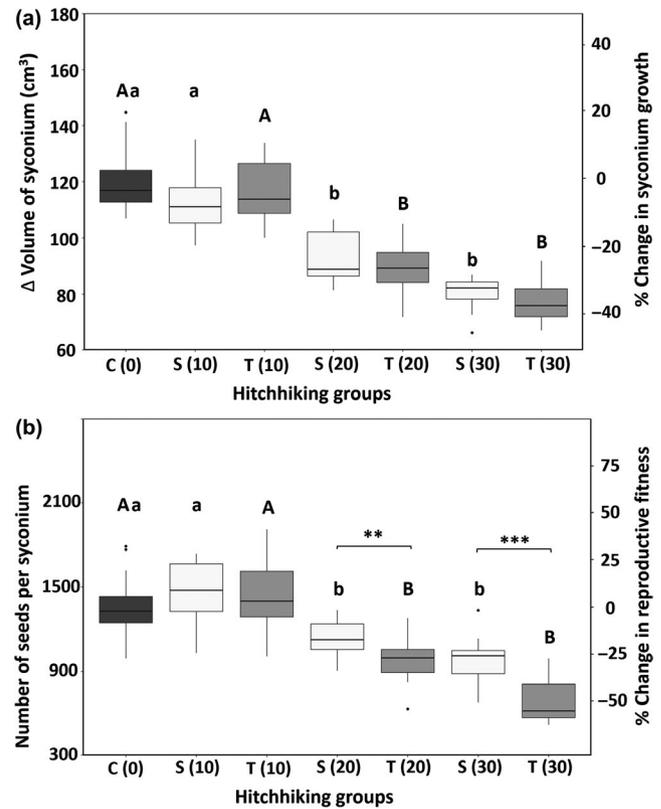
FIGURE 6 Boxplots of the change in syconium volume (including % change in syconium growth measured using means) and seed number of single syconium (including % change in reproductive fitness measured using means) at different levels of nematode infestation (for both *Schistonchus* and *Teratodiplogaster* at increasing hitchhiker density per wasp). Horizontal lines indicate medians, whiskers indicate the upper and lower quartiles, and solid black circles denote outliers. Alphabets denote significant differences between groups by a post hoc Dunn test with Bonferroni corrections for pairwise comparison made between the different loading groups within a nematode (capital letters for *Schistonchus* and small letters for *Teratodiplogaster*). Asterisks denote significant differences between similar infestation groups for the two species obtained by Mann–Whitney tests; *p < .05; **p < .01; ***p < .001; hitchhiking groups: control denoted as 'C', Schistonchus denoted as 'S', and *Teratodiplogaster* denoted as 'T'; number of hitchhiking nematodes is mentioned within brackets

non-viable. Entomopathogenic nematodes use lipids as their main energy source (Andaló, Moino, Maximiniano, Campos, & Mendonca, 2011). *Parasitodiplogaster* fig nematodes show necromeny, reduced lifespan and reduced fitness of fig wasps (Herre, 1993; Ramirez-Benavides & Salazar-Figueroa, 2015; Van Goor et al., 2018), which suggests that *Teratodiplogaster* might occupy a similar niche.

4.1.3 | Susceptibility to predation

Predators can generate trophic cascades and change the trajectories of cost and benefits resulting from phoretic organisms (Buck & Ripple, 2017). Worldwide, ants are key predators of fig wasps (Bain,

Harrison, & Schatz, 2014; Ranganathan et al., 2010). Since fig syconia have a single opening, that is the ostiole, pollinating wasps have a restricted entry point which they need to locate on the fig surface, thus exposing them to patrolling predatory ants (Ranganathan et al., 2010). In our system, wasps with higher infestation of both nematode genera had slower movements (S. Gupta, pers. observ.) and took longer to enter the ostiole. This was more pronounced in wasps carrying *Teratodiplogaster* nematodes possibly resulting in higher susceptibility to predation.

4.1.4 | Flight ability

Nematodes can affect the flight performance of their vehicles; for example, pinewood nematodes reduce both flight distance and duration of their cerambycid beetle vector *Monochamus carolinensis* (Akbulut & Linit, 1999). In our study, both nematode species decreased flight duration, but *Teratodiplogaster* loading had a greater effect than *Schistonchus*. The decrease caused by these nematodes might have various reasons such as additional load, blocking the spiracles (respiratory openings in insects) thereby eliciting a respiratory deficit, and possible paraphagy (consumption of host metabolites) in *Teratodiplogaster*. Such impacts have also been observed in phoretic mites where the decreased movement or dispersal of the vehicle was attributed to increased weight, attachment to vital areas (antennae, eyes, etc.) or feeding (Kinn, 1971). These effects on flight duration are indirect effects on fitness because they reduce the probability of reaching a brood site.

4.2 | Effects of phoretic nematodes on plant fitness

In a few phoretic systems, phoronts reduce plant fitness by directly affecting the reproductive structures of the plant or indirectly by reducing rewards that the plant offers to pollinators; for example, hummingbird mites lower flower nectar and reduce pollinator visitation, thereby affecting plant fitness (Colwell, 1995; Jeppson, Keifer, & Baker, 1975). In fig systems, plant-parasitic nematodes may reside in male and female florets and cause necrosis and cavities in cortical parenchyma (Center, Giblin-Davis, Herre, & Chung-Schickler, 1999; Vovlas, Inserra, & Greco, 1992). It was proposed that fig nematodes impact plant fitness either directly by affecting reproductive structures or indirectly by decreasing pollinator fitness (Giblin-Davis et al., 1995; Van Goor et al., 2018). Syconium volume is a fairly good indicator of flower numbers; any decrease in syconium volume might lead to decrease in seed number and availability of gall flowers for the pollinator (Krishnan et al., 2014; Suleman, Quinnell, & Compton, 2013; Wang, Sun, Zheng, Shi, & Zhu, 2011). Both nematode species decreased seed count and syconium volume with increasing nematode load and hence had a negative impact on plant fitness. Syconium volume was more affected for a higher *Teratodiplogaster* than an equivalent *Schistonchus* load. For *Teratodiplogaster*, decrease in syconium volume may be attributed to lowered efficiency of the pollinators in pollination and oviposition since developing seeds and wasps act as sinks for resources that the plant diverts to individual syconia (Krishnan et al., 2014) and therefore an indirect effect on plant fitness. *Schistonchus*, on the other hand, being plant-parasitic, might directly affect plant fitness by feeding on reproductive tissues in the syconia.

4.3 | Stabilizing density-dependent effects of phoresy

Our study has shown that the animal-parasitic nematode, *Teratodiplogaster*, directly affected pollinator fitness and indirectly affected plant reproductive success, whereas plant-parasitic *Schistonchus* directly affected plant fitness and indirectly affected pollinator reproductive success. Thus, the overall impact of nematode phoresy on the fig–fig wasp mutualism was negative, although at lower hitchhiking levels, these nematodes had no detrimental effect on the mutualism. It is therefore interesting that while we used a range of hitchhiking densities in our experiments, and negative effects were only seen at higher loads, the hitchhiking rates in nature are actually commensurate with our lower experimental loading levels. Lower transportation levels might therefore be allowing the co-occurrence of these nematode species within this mutualism. We also found that ~75% of the sampled dispersing wasps contained moderate or high nematode densities unlike in the pollinator wasps associated with *Ficus petiolaris* where ~3% were overloaded with parasitic nematodes (Van Goor et al., 2018). This suggests that in our system, stronger selection occurs during the phoretic stage, rather than at the pre-dispersal stage.

Furthermore, since monoecious figs such as *F. racemosa* are less abundant and show high spatial dispersion, their pollinator wasps require great dispersal capacities, that is high active flight duration (Venkateswaran, Kumble, & Borges, 2018; Venkateswaran et al., 2017). Phoretic nematodes reduce these dispersal capacities leading to reduced fitness and curtailing their ability to reach a receptive B-phase fig after being released from their natal syconium. Hence, our observation that wasps arriving at the receptive phase of the tree had lower nematode loads than the wasps dispersing from the tree in a natural setting indicates an overall negative effect on wasp fitness and consequently also on plant fitness resulting in failure of pollen donation. Our results therefore suggest that the fig–wasp–nematode system is a self-limiting system with clear density-dependent effects where only those wasps harbouring a lower number of nematodes would be able to reach other fig trees. Since nematodes embark on their vehicles in the late juvenile phase, they also need to reach another syconium where they can disembark and attain adulthood, mate and reproduce. Therefore there are also likely to be strong fitness effects on nematodes that embark on an overloaded vehicle. It is also worth noting that we experimentally tested effects of nematode loading separately on a variety of fitness parameters but in nature nematode transportation levels would affect all these parameters in a sequential or synergistic manner. Therefore, the combined impact of phoresy is likely to be more extreme. Other than density-dependent selection effects, the mechanisms acting to achieve this limitation in the fig–wasp–nematode system need further investigation.

Our study therefore is an excellent empirical demonstration of how density-dependent fitness effects can stabilize parasitic third-party interactions within a mutualism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

S.G. and R.M.B. were involved in project conceptualization and writing of the manuscript. S.G. led the project design, data collection and analyses of data. S.G. and R.M.B. contributed critically to revisions of the manuscript and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data related to this article are available on Dryad Digital Repository http://doi.org/10.5061/dryad.c7g6573 (Gupta & Borges, 2019).

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