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Hopping on: Conspecific traveller density within a vehicle regulates parasitic hitchhiking between ephemeral microcosms

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Abstract

1. Hitchhikers (phoretic organisms) identify their vehicles using species-specific visual, chemical and vibrational cues. However, what factors influence their choice between vehicles of the same species has rarely been investigated.
2. Hitchhikers must not only avoid overcrowded vehicles but may also need to travel with conspecifics to ensure mates at their destination. Hence, a trade-off between overcrowding and presence of conspecifics likely determines the choice of a vehicle especially when destination sites are distant, ephemeral and unique.
3. Here, we investigate whether a trade-off between the presence of conspecifics versus overcrowding by conspecifics or heterospecifics on a vehicle affects hitchhiker choice. We also investigate the sensory modality responsible for this choice. We experimentally examine these questions using a phoretic nematode community (containing plant- and animal-parasitic taxa) obligately associated with a brood-site pollination mutualism. In this model system, nematodes co-travel with conspecifics and heterospecifics on pollinators as vehicles, between ephemeral plant brood sites to complete their developmental life cycle. In this system, hitchhiker overcrowding has proven negative impacts on vehicle and plant fitness. We expected nematodes to respond to conspecifics and heterospecific density on offered vehicles when making their choice.
4. We found that animal-parasitic nematodes preferred vehicles containing some conspecifics within a certain density range. However, plant-parasitic nematodes preferentially boarded vehicles that were devoid of conspecifics or had few conspecifics. Plant parasites that preferred empty vehicles likely hitchhiked in pairs. Both nematode types employed volatile cues to discriminate between vehicles with different conspecific nematode densities. Our results suggest that vehicle overcrowding by conspecifics, most likely, guaranteed access to mates at the destination determined hitchhiker choice. Surprisingly, and contrary to our expectations, plant- and animal-parasitic nematodes did not respond to heterospecific crowding on vehicles and did not discriminate between vehicles with different heterospecific nematode densities. The reason for this lack of response to heterospecific presence is unknown.
5. This study not only shows that phoretic organisms use different strategies while choosing a vehicle but also confirms that density-dependent effects can ensure

the stability and persistence of phoretic interactions in a mutualism by balancing overcrowding against reproductive assurance.

KEYWORDS

density-dependent effects, fig wasps, hitchhiking nematodes, overcrowding, parasitism, phoresy, reproductive assurance, volatiles

1 | INTRODUCTION

Dispersal is essential but difficult for organisms with low mobility that reside in specialized ephemeral habitats. The use of other organisms with higher mobility as vehicles can overcome the dispersal predicament. This association of organisms, where one species acts as a vehicle while the other is a phoront (hitchhiker) is termed phoresy (Athias-Binche & Morand, 1993; Camerik, 2010; Krishnan et al., 2010). Hitchhikers are often dependent on their vehicles, a feature which necessitates recognition and location of appropriate vehicles for successful dispersal (Athias-Binche & Morand, 1993; Farish & Axtell, 1971). Hitchhikers use a combination of chemical, visual, tactile and/or auditory cues to orient themselves towards their carriers (Gibson & Torr, 1999; Owen & Mullens, 2004; Rea & Irwin, 1994).

Several classic examples of vehicle–hitchhiker relationships occur across highly diverse taxa, for example, hummingbirds/bumblebees–flower mites, beetles/wasps–nematodes, flies–pseudoscorpions, tree frogs–ostracods and crabs–annelids (Houck, 2009; White et al., 2017). When such relationships occur in systems involving parasitism or mutualism, an increase in specificity and specialization can be expected (Krishnan et al., 2010; Lopez et al., 2005; Zhao et al., 2013). Increased specialization might result in high discrimination ability for their vehicles in hitchhikers resulting in optimization

of the dispersal process (Fronhofer et al., 2013). This is because differences between vehicles in behaviour, diet, immunocompetence and physiology affect their suitability as phoretic hosts. If reliable vehicles live in close syntopy or proximity with other non-reliable species (Krishnan et al., 2010) or if there is a great deal of within-species variation among vehicle populations, the ability to sift through potential vehicles and locate those that are suitable becomes crucial for survival and reproduction (Kiffner et al., 2013; Krasnov et al., 2012; Poulin, 1996; Zuk & McKean, 1996).

Most studies on intraspecific vehicle discrimination have examined size- and sex-based factors (Campbell & Luong, 2016; Grossman & Smith, 2008; Krishnan et al., 2010). However, a potential hitchhiker may also need to consider the numbers of travellers already on a vehicle, and the type of traveller, especially when destination sites are ephemeral and the availability of mates at the destination are uncertain. Therefore, overcrowding of a vehicle and the presence of conspecific travellers on a vehicle are likely major selection forces in determining vehicular choice.

In a few vehicle–hitchhiker relationships, high hitchhiker density severely compromised the behaviour and physiology of the vehicle, affecting its dispersal (Gupta & Borges, 2019; Kinn, 1970). Therefore, under an overcrowding scenario (Figure 1a), whether caused by heterospecific or conspecific travellers, the probability of successful dispersal of the hitchhiker may decrease with

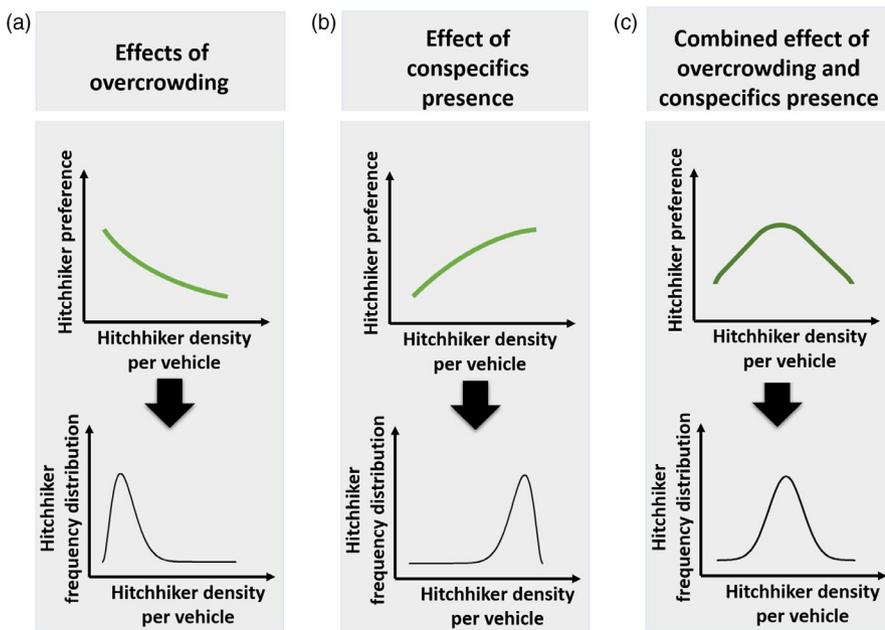


FIGURE 1 Diagrammatic representation showing expected graphs for hitchhiker preference and its frequency distribution in relation to hitchhiker density under the different vehicular selection criteria. (a) effect of overcrowding, (b) effect of conspecific presence, and (c) combined effect of overcrowding and conspecific presence

co-traveller numbers. Subsequently, hitchhikers may choose vehicles with lower hitchhiker density resulting in a nematode's frequency distribution to be left-skewed for hitchhiker density per vehicle (Figure 1a). In another scenario the hitchhiker may prefer vehicles with a greater number of conspecific travellers if, for example, the availability of mates at the destination site is uncertain; this may lead to a nematode's frequency distribution to be right-skewed for hitchhiker density per vehicle (Figure 1b), under the assumption that overcrowding does not affect the dispersal ability of the vehicle. These two opposing selection forces, that is, overcrowding, and the presence of conspecifics might result in an optimum intermediate frequency distribution of hitchhiker density per vehicle (Figure 1c).

Overcrowding may be caused by heterospecific and conspecific travellers. An overcrowded vehicle may therefore contain few conspecifics and thereby few potential mates. Phoretic mites and nematodes avoid resource patches containing heterospecifics if they are competitors, whereas they aggregate if conspecifics are present (Grewal et al., 1997; Koenraadt & Dicke, 2010; Shapiro-Ilan et al., 2014). Therefore, we asked the question whether hitchhikers can use conspecific and heterospecific density on a vehicle to discriminate between vehicles while hitchhiking and whether these choices are driven by overcrowding per se or by the presence and density of conspecifics.

To test if hitchhikers consider the trade-off between overcrowding per se and the presence of conspecifics while selecting a vehicle, we used the fig–fig wasp–nematode interaction as a model system and designed experiments to tease these opposing factors apart. The fig–fig wasp mutualism is a 75+ my old, specialized brood-site mutualism where nematodes have an obligate ancient vehicle–hitchhiker relationship with pollinating wasps (15–45 mya; Cruaud et al., 2012; Jauharlina et al., 2012; Krishnan et al., 2010; Peñalver et al., 2006; Poinar Jr., 2003). There are 800+ fig species, each usually hosting a specialist pollinator and a unique nematode community belonging to nematode families Diplogasteridae, Parasitaphelenchidae, Rhabditidae and Aphelenchoididae (Giblin-Davis et al., 2006; Machado et al., 2001; Susoy et al., 2016). The hitchhikers are mostly juveniles and reside inside the vehicle (endophoresy) during the dispersal duration to assure safe dispersal (Davies et al., 2015; Kanzaki et al., 2013). The hitchhikers not only show commensal (*Parasitodiplogaster* sp.) or parasitic (*Teratodiplogaster* sp. and *Schistonchus* sp.) interaction effects with the mutualistic partners but also show variation in hitchhiker density per vehicle within the dispersing wasp population (Gupta & Borges, 2019; Jauharlina et al., 2012; Van Goor et al., 2018).

Since we had established that nematode hitchhikers have density-dependent negative fitness effects on the pollinating wasp vehicles and on the fig plants (Gupta & Borges, 2019), we predicted that the hitchhikers would discriminate between vehicles based on co-traveller density. We used varying nematode densities of both heterospecifics and conspecifics to determine the selection criteria that hitchhiking nematodes consider while boarding a vehicle. In addition, we investigated the sensory cues that the nematodes might

use to discriminate between conspecific and heterospecific nematode densities within a wasp vehicle.

2 | MATERIALS AND METHODS

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

Ficus racemosa L. (Moraceae), a widespread and a common tropical keystone fig species, was selected as the study system. It harbours a hitchhiker nematode community comprising of three nematode genera *Schistonchus* [now assigned to *Ficophagus* (Davies et al., 2015) in associations with figs] (Aphelenchoididae), *Teratodiplogaster* (Diplogasteridae) and *Pristionchus* (Diplogasteridae) that utilizes the pollinator wasp (*Ceratosolen fusciceps*), as the vehicle for dispersing from one syconium (closed urn-shaped fig inflorescence; plural = syconia) to another (Gupta & Borges, 2019; S. Gupta, Q. Tahseen & R.M. Borges, unpubl. data). Nematodes choose female pollinator wasps as vehicles despite the presence of six other species of wasp co-developing within the syconium (Krishnan et al., 2010). The fig forms a mutualistic association with the pollinator wasp where the syconia act as developmental microcosms, providing habitats for the development of both wasps and nematodes (Ghara & Borges, 2010; Gupta & Borges, 2019). The syconium undergoes a ~60-day developmental cycle where single or multiple pollinators carrying the hitchhiker nematodes enter it at the pollen-receptive phase (B phase; Figure S1). Nematodes disembark from the pollinator while she is pollinating and laying eggs in the female flowers; pollinators die within the syconium after this process. The nematodes attain maturity, mate and lay eggs in the inter-floral or C phase of syconium. These eggs hatch at the late C phase and reach the dispersal stage which hitchhikes on pollinator offspring in the wasp dispersal or D phase when the fig syconia ripen and become unfit to support further generations (Figure S1; Giblin-Davis et al., 1995; Krishnan et al., 2010; Reddy & Rao, 1986). Syconia are thus ephemeral resources and nematodes must enter new syconia, using female pollinators to reach their maturation sites. Hitchhiking nematodes demonstrated negative density-dependent effects on the dispersal and reproductive success of their pollinator vehicles and the fig (Gupta & Borges, 2019). Consequently, wasps arriving at their destinations within their brief 24-hr adult life span carried lower densities of both *Teratodiplogaster* (animal)- and *Schistonchus* (plant)-parasitic nematodes compared to dispersing wasps suggesting strong selection pressures on wasps with high hitchhiker numbers during the dispersal process (Gupta & Borges, 2019).

2.2 | Study site and nematode collection for behavioural experiments

Pollinator wasps were collected by dissecting open their galls at the late C phase of syconium development. Nematodes were

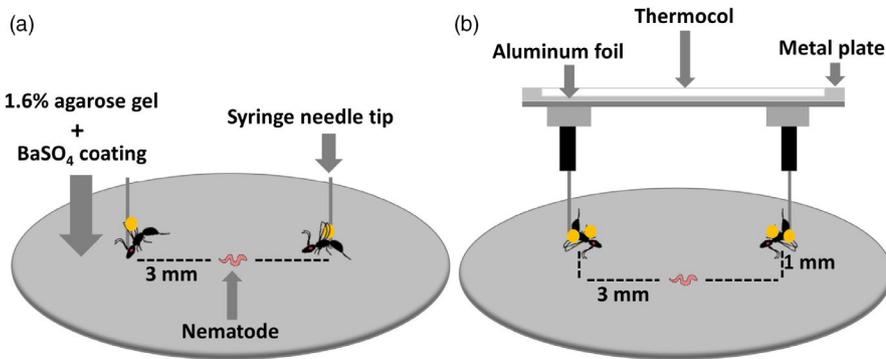


FIGURE 2 Diagrammatic representation of experimental set up for whole wasp choice assays (a) and volatile choice assay (b)

collected at the stage when they exhibit nictation (a behaviour in which nematodes stand on their 'tails' and engage in waving their bodies to sample their chemical environment) or host-seeking behaviour (Supporting Information: Video S1; Gupta & Borges, 2019). Nematodes were identified to genus under a dissecting microscope prior to the behavioural experiments (Gupta & Borges, 2019). All collections were made in and around the Indian Institute of Science campus, Bangalore, India (12°58'N, 77°35'E). Experiments were conducted only with *Teratodiplogaster* and *Schistonchus* since the numbers of *Pristionchus* were too low for experimentation. Individual *Teratodiplogaster* and *Schistonchus* in *F. racemosa* syconia are unisexual (male or female); no hermaphrodites have been found (S. Gupta, Q. Tahseen & R.M. Borges, unpubl. data).

2.3 | Whole vehicle (pollinator wasp) choice assay for different conspecific and heterospecific hitchhiker densities

Three nematode density levels (numbers per wasp) were chosen for these experiments using the mean (*Teratodiplogaster* = 16.4, *Schistonchus* sp. = 10.4) and median (*Teratodiplogaster* = 16, *Schistonchus* = 8) densities found in naturally dispersing wasps (Gupta & Borges, 2019). Wasps were manually loaded (as described in Gupta & Borges, 2019) with low (*Teratodiplogaster* = 10, *Schistonchus* = 6), medium (*Teratodiplogaster* = 20, *Schistonchus* = 12) and high (*Teratodiplogaster* = 30, *Schistonchus* = 18) nematode density levels per wasp. As the experiments were technically challenging (sporadic availability of fig syconia at the wasp dispersal stage and the narrow window of time of a few hours before the wasps leave the syconia with their nematode load), we chose three density pairs, that is, zero–low, low–medium and medium–high, to obtain a comprehensive evaluation of nematode choice for different conspecific and heterospecific hitchhiker densities. Further, we also added a fourth density comparison, that is, zero–high, to ascertain the choice of both nematodes for vehicles loaded with conspecifics.

To determine the choice of nematodes for these different conspecific or heterospecific nematode density levels on wasp vehicles, individual nematodes placed at a central point were given a choice between a pair of live wasps (with different nematode density levels) at 3 mm distance from the test nematode (according

to standardized experimental procedures developed in Krishnan et al., 2010) in each well of a six-well plate (Figure 2a). Each wasp was affixed on a needle (using a non-toxic glue, applied in equal amount on both wasps). Each well was half-filled with 1.6% agarose in a buffer containing 1 mM CaCl_2 , 1 mM MgSO_4 and 50 mM potassium phosphate (pH 6.0; after Brenner, 1974) and coated with 2% BaSO_4 layer to visualize the trail left by nematodes on the surface while making their choice (after Krishnan et al., 2010). In all cases, the experimental arena was kept in the dark for 2 hr and the final choice of the nematode as indicated by its trails was recorded. Wells which contained no trails or in which the trails led to both wasps were labelled as 'no choice' (after Krishnan et al., 2010) and excluded from further analysis.

Since *Teratodiplogaster* showed a graded response to different conspecific densities (see Section 3), we further tested its response to four additional hitchhiker densities (5, 10, 15 and 20 nematodes) to determine a precise tipping point in its decision to ride on or avoid a vehicle.

Similarly, we also conducted choice tests with different heterospecific nematode density levels on wasp vehicles, using the low, medium and high relative densities mentioned earlier. We also gave nematodes a choice between wasps loaded with heterospecifics or conspecifics, using the appropriate relative density levels.

2.4 | Volatile choice assay for vehicles (pollinator wasps) with different hitchhiker densities

To determine if the nematodes used volatiles as a cue to differentiate between wasps loaded with conspecifics, we subjected the test nematode to low versus high nematode densities. Individual nematodes were exposed to a pair of live nematode-loaded wasps separated from the nematode at 3 mm distance as before. Each wasp was fixed to a needle suspended from the top such that the nematodes were only exposed to nematode-loaded wasp volatiles, in each well of a six-well plate (Figure 2b). A thermocol cube covered in aluminium foil, fixed to a steel frame, was used to suspend the needles such that no other cues such as vibrations generated by live wasps were responsible for hitchhiker choice of vehicle. The experimental arena was placed in the dark for 2 hr and the final choice of the nematode was recorded as before.

2.5 | Statistical analysis

A chi-square analysis was performed on the frequency data obtained from the above choice assays using the software R (version 3.2.3) and the data were plotted as percentages for visualization. All assays labelled 'no choice' were excluded from the analyses. Therefore, the null or expected chi-square distribution is equal distribution between Wasp A and Wasp B, where Wasp A and Wasp B are the pairwise choices presented in each experiment to the test nematode.

3 | RESULTS

3.1 | Whole wasp choice: Nematodes offered vehicles loaded with different conspecific densities

Teratodiplogaster nematodes showed a significant preference for wasps with either low or high conspecific nematode density than for wasps devoid of nematodes (zero vs. low: $\chi^2 = 10.8, n = 33, df = 1, p < 0.01$; zero vs. high: $\chi^2 = 4.8, n = 33, df = 1, p < 0.05$; Figure 3a). However, *Teratodiplogaster* consistently preferred wasps with lower nematode density levels (low vs. medium: $\chi^2 = 13.3, n = 32, df = 1, p < 0.001$; medium vs. high: $\chi^2 = 8.53, n = 31, df = 1, p < 0.01$; Figure 3a) when both vehicles had conspecific co-travellers. A

significantly larger number of *Teratodiplogaster* (80%) tested preferred wasps loaded with five nematodes than those devoid of nematodes ($\chi^2 = 10.8, n = 31, df = 1, p < 0.01$) but when given a choice between 5 versus 10 nematodes ($\chi^2 = 0.31, n = 30, df = 1, p = 0.57$) and 10 versus 15 nematodes ($\chi^2 = 0.13, n = 32, df = 1, p = 0.71$), no preference was observed. Further, when given a choice between 15 versus 20 nematodes, wasps loaded with 15 nematodes were preferred significantly ($\chi^2 = 6.53, df = 1, n = 31, p < 0.05$; Figure 3b). This suggests that *Teratodiplogaster* avoids boarding vehicles with <5 and greater than 15 conspecifics.

Schistonchus consistently preferred vehicles with low or zero nematode density levels over those loaded with high nematode densities (zero vs. low: $\chi^2 = 8.53, n = 31, df = 1, p < 0.01$; low vs. medium: $\chi^2 = 13.3, n = 33, df = 1, p < 0.01$; and medium vs. high: $\chi^2 = 16.13, n = 34, df = 1, p < 0.001$; zero vs. high: $\chi^2 = 9.14, n = 30, df = 1, p < 0.01$; Figure 3c). *Schistonchus* therefore preferred to board empty vehicles unlike *Teratodiplogaster*.

3.2 | Whole wasp choice: Nematodes offered vehicles loaded with different heterospecific densities

Teratodiplogaster did not discriminate between wasps loaded with a range of heterospecific (*Schistonchus*) density levels (zero vs. low:

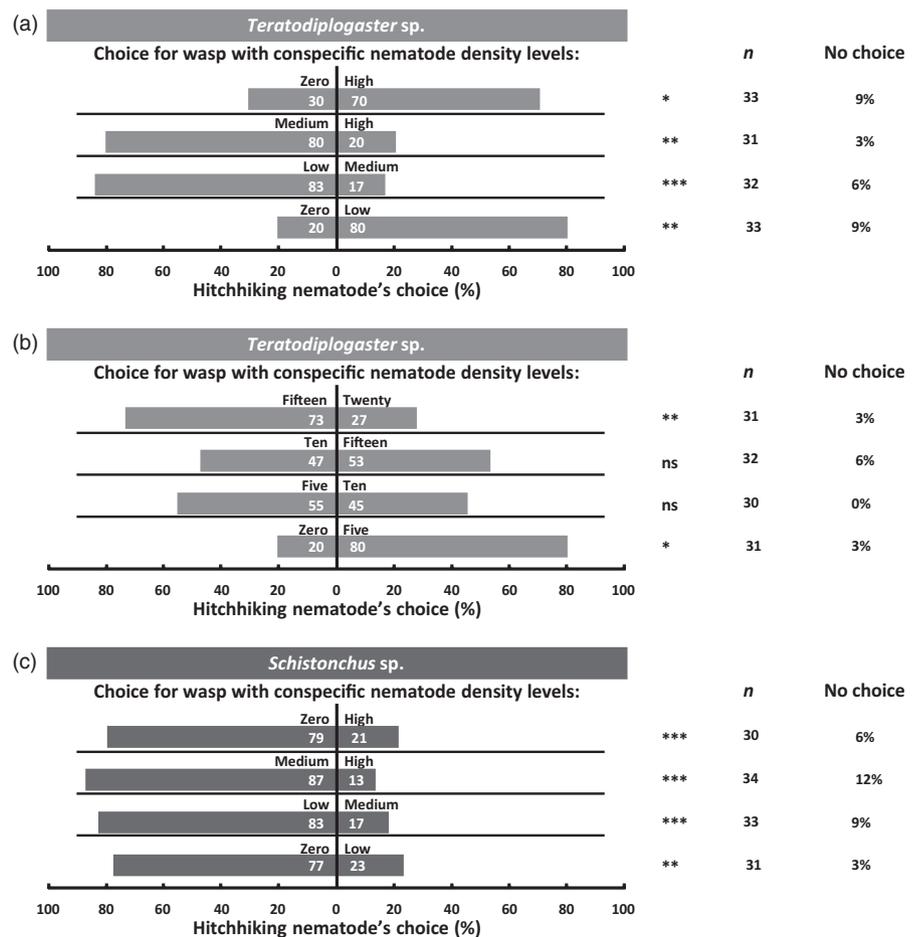


FIGURE 3 Choice tests (%) for both the nematode species *Teratodiplogaster* (a, b) and *Schistonchus* (c) for wasps loaded with different nematode density of conspecifics; viz. zero, low, medium or high as described in the Methods. Asterisks denote significant differences between the different density infestation groups for the two genera obtained by chi-squared tests; n.s = non-significant difference, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

$\chi^2 = 0.03$, $n = 30$, $df = 1$, $p = 0.84$; low vs. medium: $\chi^2 = 0$, $n = 30$, $df = 1$, $p = 1$; medium vs. high: $\chi^2 = 0.13$, $n = 33$, $df = 1$, $p = 0.71$; Figure 4a). Similar results were found for *Schistonchus* offered choices between wasps loaded with a range of heterospecific (*Teratodiplogaster*) density levels (zero vs. low: $\chi^2 = 0.03$, $n = 31$, $df = 1$, $p = 0.85$; low vs. medium: $\chi^2 = 0.31$, $n = 29$, $df = 1$, $p = 0.57$; and medium vs. high density: $\chi^2 = 0.03$, $n = 28$, $df = 1$, $p = 0.84$; Figure 4b). These results suggest that both nematode types were unresponsive to the presence of heterospecifics on potential vehicles.

3.3 | Whole wasp choice: Nematode offered vehicles with same relative density level of conspecifics and heterospecifics

Teratodiplogaster nematodes preferred wasps loaded with conspecific wasps over heterospecifics (*Schistonchus*) at both low ($\chi^2 = 4.8$, $n = 30$, $df = 1$, $p < 0.05$) and high ($\chi^2 = 7$, $n = 29$, $df = 1$, $p < 0.01$) relative density levels (Figure 5a). However, *Schistonchus* nematodes showed a preference for wasps loaded with heterospecifics

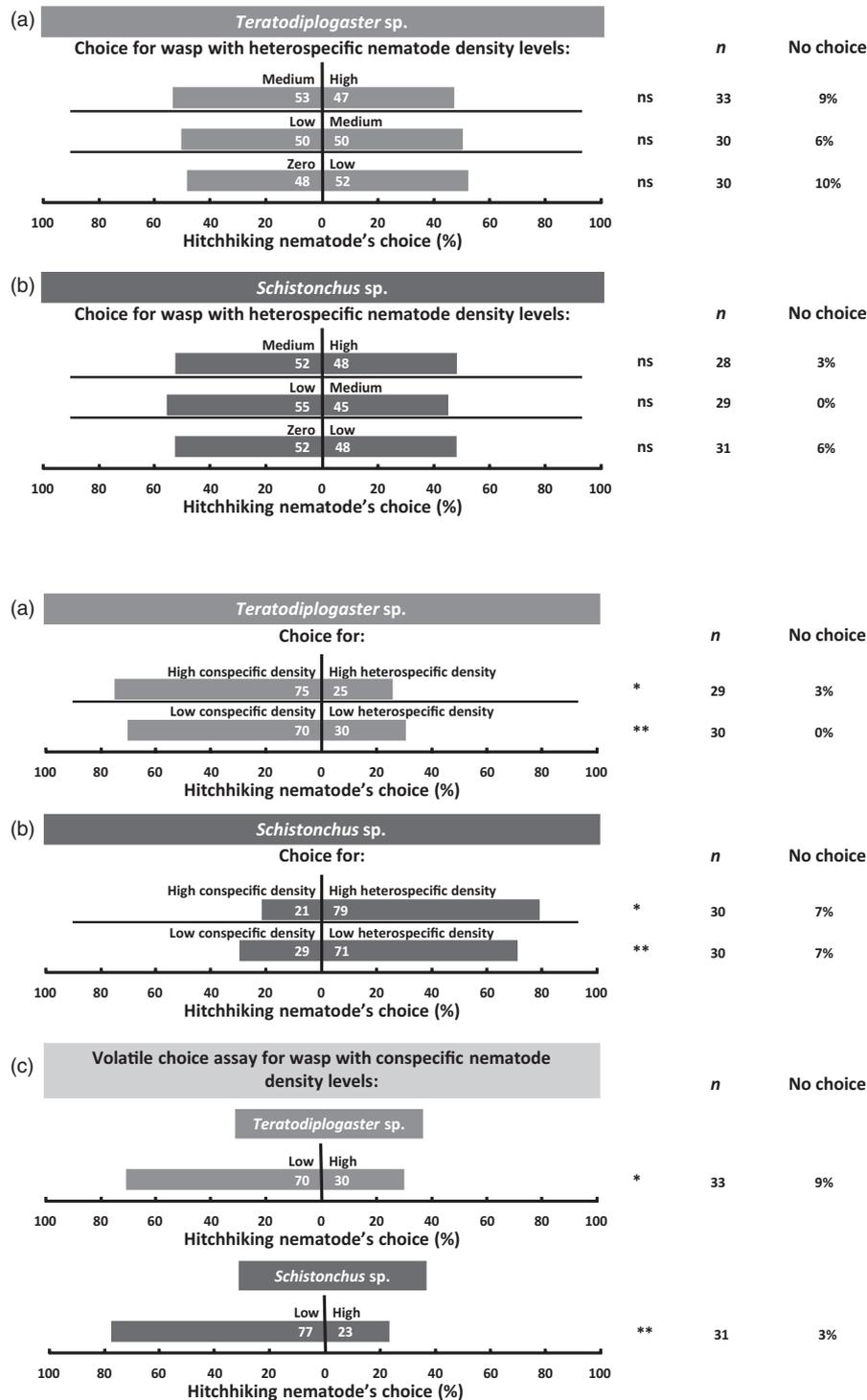


FIGURE 4 Choice tests (%) for both the nematode species *Teratodiplogaster* sp. (a) and *Schistonchus* sp. (b) for wasps loaded with different heterospecific densities viz., zero, low, medium or high as described in the Methods. Asterisks denote significant differences between the different density infestation groups for the two genera obtained by chi-squared tests; n.s = non-significant difference, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

FIGURE 5 Choice tests (%) for wasps loaded with conspecifics and heterospecifics at the same relative density levels. (a) Choice of *Teratodiplogaster* nematodes for wasp loaded with conspecifics versus heterospecifics at low- and high-density levels. (b) Choice of *Schistonchus* nematodes for wasps loaded with conspecifics versus heterospecifics at low level and high-density levels. (c) Choice tests (%) for the nematode species *Teratodiplogaster* sp. and *Schistonchus* sp. for volatiles emitted by the wasps loaded with different conspecific densities (low and high). Asterisks denote significant differences between the different density infestation groups for the two genera obtained by chi-squared tests; n.s = non-significant difference, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

(*Teratodiplogaster*) over conspecifics (*Schistonchus*) at both low ($\chi^2 = 5.1$, $n = 30$, $df = 1$, $p < 0.05$) and high ($\chi^2 = 9.1$, $n = 30$, $df = 1$, $p < 0.01$) relative density levels (Figure 5b). These results are consistent with those of the previous experiments which suggested that *Teratodiplogaster* preferred boarding vehicles with conspecifics (albeit within a limited density range) while *Schistonchus* preferred vehicles that do not have conspecific hitchhikers when given a choice. They further corroborated the results that nematodes are non-responsive to heterospecific densities.

3.4 | Wasp volatile choice assay with different hitchhiking density levels

In volatile choice assays, both nematode types exhibited a preference for wasps loaded with low rather than high conspecific densities (*Teratodiplogaster*: $\chi^2 = 4.8$, $n = 33$, $df = 1$; *Schistonchus*: $\chi^2 = 8.53$, $n = 31$, $df = 1$, $p < 0.01$; Figure 5c). These results suggest that volatiles could be one mechanism by which nematodes assess the density of existing hitchhikers on a vehicle.

4 | DISCUSSION

Our experimental findings indicate that the two nematode types (i.e. animal- and plant-parasitic taxa) associated with their pollinator wasp vehicles present in the *Ficus racemosa* system can discriminate between wasps loaded with different densities of conspecific nematodes. The animal- and plant- parasites use different strategies to hitchhike on a vehicle. The animal-parasitic nematodes preferred wasps which already had low densities of conspecific nematodes but avoided empty vehicles as well as vehicles overcrowded with conspecific nematodes. In contrast, plant-parasitic nematodes not only preferred empty vehicles but consistently preferred vehicles with lower conspecific numbers. The primary choice factor for animal-parasitic nematodes appeared to be the presence of conspecifics, whereas overcrowding by conspecifics was the main factor for plant-parasitic nematodes. Both nematode types used volatiles as cues to discriminate between wasps with different levels of conspecific loading. However, these nematodes did not show any discrimination between wasps with different levels of heterospecific loading. Overcrowding by conspecifics clearly determined the choice of vehicles. Further, boarding such crowded vehicles would be likely linked to reproductive assurance as well.

For successful completion of the life cycle, dispersal to another site where these specialized fig nematodes will mature, mate and reproduce is vital. Since these nematodes hitchhike at the juvenile stage, choosing an empty vehicle devoid of conspecific travellers can be a problem. This is because even in the event of successful dispersal to a new site (pollen-receptive phase fig syconium, Figure S1), nematodes might not be able to successfully reproduce due to lack of mating partners if no other conspecifics have co-travelled with them on the same vehicle. In *Ficus racemosa*, the number

of pollinators entering the fig syconium was 5.35 ± 0.70 (mean \pm SE) and one pollinating wasp per syconium was the most common number (Jauharlina, 2014). This lack of mating partners may become especially acute if no other pollinator wasps carrying nematodes have entered the same syconium; in figs, syconia with single wasp foundress females often occur (Zavodna et al., 2007). Furthermore, in this model system, high numbers of both nematode types reduce the fitness of their wasp vehicles in terms of reduction in flight duration and increase in predation risk (Gupta & Borges, 2019); hence, overcrowding will not only have a detrimental effect on the vehicle but also on the travellers who may fail to reach their destination.

Since we were unable to determine the sex ratio of the nematodes at the time of phoresy or the extent of monogamy among the species (given that the nematodes hitchhike at their juvenile stage, wherein they lack any phenotypic feature that would allow us to determine sex, and that they develop and mate inside of figs during their mature stage), there may be other possibilities as to why the nematodes were attracted to wasps with conspecific loading. Both evolutionary theory and some empirical data suggest that sex ratios tend to be female biased in systems where the prevalence of infection of hosts and/or the intensity of infection of hosts are low (to avoid in-breeding) and tend to be approximately equal otherwise (Li et al., 2017; Poulin, 1997). Our system may fall into the former category. Vehicle overcrowding among nematode parasites of fig wasps has been linked previously to other (possibly related) factors besides reproductive assurance, including, for example, increased opportunities for transmission (and subsequent promotion of virulence; Herre, 1993). Attraction to conspecifics in phoretic nematodes might also arise to avoid the impact of Allee effects (i.e. decreased fitness due to low population density at the destination) as proposed in other species (Clotuche et al., 2013; Courchamp et al., 1999; Fronhofer et al., 2013; Kramer et al., 2018).

The animal-parasitic nematode *Teratodiplogaster* showed the expected hitchhiking strategy of avoiding empty vehicles and vehicles overcrowded with conspecifics; they preferred a density range of between 5 and 15 conspecific co-travellers which lies between the naturally occurring low and medium densities of conspecific nematodes present in a vehicle dispersing from a fig syconium (Gupta & Borges, 2019). Although the plant-parasitic nematode *Schistonchus* avoided vehicles overcrowded with conspecifics, it entered empty vehicles. Plant-parasitic nematodes are often observed in pairs at the time of hitchhiking (Supporting Information: Video 1), unlike the animal-parasitic nematodes, and this strategy may explain their choice of empty vehicles if such pairing solves the problem of lack of mates at the destination site. In other fig species, only mated *Schistonchus* females hitchhike on the pollinating wasps (Giblin-Davis et al., 1995; Ye et al., 2007; Zeng et al., 2010). In such fig species, *Schistonchus* may also fail to avoid empty vehicles, but this has not yet been investigated. Since hermaphrodites do not occur in *Teratodiplogaster* and *Schistonchus* in our system (S. Gupta, Q. Tahseen & R.M. Borges, unpubl. data), it appears that the two nematode types have adopted different strategies to guarantee mating partners at the destination site; the former avoids vehicles without conspecifics and the

latter likely boards empty vehicles in pairs. In nematodes, transitions to self-fertilization often occur when access to mates is limited (Theologidis et al., 2014), and nematodes in fig phoretic systems may have similarly evolved a variety of strategies to circumvent the problem of mate access.

Our study also shows that the nematodes used volatiles as cues to discriminate between wasps with different nematode density levels. Nematodes respond to various types of chemical cues emitted by their hosts such as cuticular hydrocarbons (CHCs), pheromones, host volatiles and even carbon dioxide (Dillman et al., 2012; Hong & Sommer, 2006; Krishnan et al., 2010; Linit & Stamps, 2001; Okumura & Yoshiga, 2014; Willett et al., 2018). Juveniles of plant-parasitic nematodes are attracted to below-ground herbivore-induced plant volatiles (HIPVs), for example, β -caryophyllene, enabling them to detect hosts and aggregate, resulting in increased host infection (Ali et al., 2011; Degenhardt et al., 2009; Rasmann & Turlings, 2008). We have earlier shown in this system that nematodes use wasp volatiles and CHCs not only to distinguish pollinator wasps from other non-pollinator wasps but also to discriminate between male and female pollinators (Krishnan et al., 2010). Nematodes can also cause physiological changes in their hosts which might influence the amount of CO₂ released (Ramos-Rodríguez et al., 2007). The fig nematodes might also sense differences in CO₂ levels released by wasps at different hitchhiker density levels and utilize this for discrimination. Nematodes also produce non-pheromonal volatile compounds (e.g. prenol) that act as repellants and that in turn can also alter the volatile chemistry of the host (Kin et al., 2019). Volatile differences between wasps with different hitchhiker loadings may also arise due to tissue damage that nematodes might inflict on the wasps while entering them and/or to nematode odour; however, these factors have not been investigated.

In contrast to our expectations, neither nematode types discriminated between wasps with different heterospecific densities and did not use overcrowding by heterospecifics as a selection criterion for hitchhiking. This might be due to failure to recognize heterospecific presence (Janssen et al., 1999), with heterospecifics being treated as 'empty' vehicles. Some nematodes, however, can respond to heterospecific cues such as pheromones (Kaplan et al., 2020). This lack of response to heterospecifics may also be expected if the nematodes occupy different niches in the syconium such that inter-species competition between nematodes is low in their destination and maturation sites within the fig syconium. Consequently, during phoresy, nematodes are more responsive to future conspecific competitors and/or mates rather than non-competitive heterospecifics. Since the animal parasites reside in and around the pollinator wasps whereas the plant parasites reside in the inner layers of the syconium wall (Gupta & Borges, 2019; Jauharlina et al., 2012), it is possible that interspecific competition is indeed low. However, since both nematode types and the animal-parasite in particular are detrimental to the performance of their vehicles especially at high densities (Gupta & Borges, 2019), both species were expected to be responsive to the other's presence on the vehicle. There was also either no relationship or a weak positive relationship between the natural densities of

Schistonchus and *Teratodiplogaster* co-travelling on individual wasps during the hitchhiking period (Table S1; fig tree-wise data from Gupta & Borges, 2019). This also suggests that the nematode types are not negatively affected by each other's presence on their vehicles. This finding requires further investigation.

Our study, therefore, clearly demonstrates that hitchhikers use strategies based on the density of conspecific co-hitchhikers to select an appropriate vehicle for its dispersal. This intraspecific discrimination likely contributes to restrictions observed in the hitchhiker's natural distribution on their vehicles during hitchhiking which might help in the persistence of such vehicle-hitchhiker relationships within mutualisms.

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AUTHORS' CONTRIBUTIONS

S.G. led the project design, data collection, analyses of data and initial draft of the manuscript. Both the authors were involved in project conceptualization, contributed critically to revisions of the manuscript and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.vt4b8gtr0> (Gupta & Borges, 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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