# The Scent of Life: Phoretic Nematodes Use Wasp Volatiles and Carbon Dioxide to Choose Functional Vehicles for Dispersal

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## Abstract

Hitchhikers (phoretic organisms) need vehicles to disperse out of unsuitable habitats. Therefore, finding vehicles with the right functional attributes is essential for phoretic organisms. To locate these vehicles, phoretic organisms employ cues within modalities, ranging from visual to chemical senses. However, how hitchhikers discriminate between individual vehicles has rarely been investigated. Using a phoretic nematode community associated with an obligate fig–fig wasp pollination mutualism, we had earlier established that hitchhiking nematodes make decisions based on vehicle species identity and number of conspecific hitchhikers already present on the vehicle. Here we investigate if hitchhikers can differentiate between physiological states of vehicles. We asked whether phoretic nematodes choose between live or dead vehicles present in a chemically crowded environment and we investigated the basis for any discrimination. We conducted two-choice and single-choice behavioral assays using single nematodes and found that plant- and animal-parasitic nematodes preferred live over dead vehicles and used volatiles as a sensory cue to make this decision. However, in single-choice assays, animal-parasitic nematodes were also attracted towards naturally dead or freeze-killed wasps. The volatile profile of the wasps was dominated by terpenes and spiroketals. We examined the volatile blend emitted by the different wasp physiological states and determined a set of volatiles that the phoretic nematodes might use to discriminate between these states which is likely coupled with respired $CO_2$. We determined that $CO_2$ levels emitted by single wasps are sufficient to attract nematodes, demonstrating the high sensitivity of nematodes to this metabolic product.

**Keywords** Carbon dioxide · Conophthorin · Fig wasps · Nematodes · Phoresy · Wasp VOCs

## Introduction

Dispersal is crucial for organisms that reside in ephemeral microcosms. Some organisms might act as hitchhikers, needing vehicles for their movement if they are incapable of traversing the distance from a deteriorating or overcrowded habitat to a new habitat having abundant resources (Athias-Binche and Morand 1993; Bartlow and Agosta 2020; Binns 1982; Camerik 2010; Colwell 1986; Farish and Axtell 1971; Kruitbos et al. 2009). Obligate dependence of hitchhikers on vehicles necessitates host seeking and recognition of appropriate carriers for successful dispersal (Athias-Binche and Morand 1993; Farish and Axtell 1971).

Locating an appropriate dispersal agent in an ephemeral or a specialized habitat might require high discrimination ability

if there is an increased specificity for the vehicle in order to optimize the dispersal process (Fronhofer et al. 2013; Krishnan et al. 2010; Zhao et al. 2013). Increased host specificity has also been observed in phoretic organisms within tritrophic interactions (Krishnan et al. 2010; Zhao et al. 2013) where both intra- and inter-specific vehicular discrimination occurs (Gupta and Borges, unpublished; Krishnan et al. 2010). Discrimination between vehicles may occur due to differences in developmental stage, behavior, diet, immunocompetence and physiology of individual vehicles that might affect their performance (Kiffner et al. 2013; Krasnov et al. 2012; Kruitbos et al. 2009; Poulin 1996; Zuk and McKean 1996). Intra-specific discrimination in phoretic organisms has mainly evaluated factors such as developmental stage, size and sex (Binns 1982; Campbell and Luong 2016; Grossman and Smith 2008; Krishnan et al. 2010). However, a potential hitchhiker may also be required to consider the vehicle's physiological state since boarding a vehicle with poor functionality may result in failure to reach the destination affecting survival with serious fitness consequences.

The ability of the phoretic organism to discriminate and successfully locate its appropriate vehicle depends on its

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ability to perceive vehicle-specific cues in a noisy chemical environment (Krishnan et al. 2010; Vet and Dicke 1992). Phoretic organisms could use chemical, visual, tactile, and auditory cues to locate and orient towards their vehicle depending on their sensory ability and host cue availability (Binns 1982; Gibson and Torr 1999; Owen and Mullens 2004; Rea and Irwin 1994; Vet and Dicke 1992). For example, mites and phoretic insect parasitoids use semiochemicals present on the cuticle of their phoretic carriers to locate their vehicle whereas beetles produce volatiles that mimic the sex-pheromone of females of their carrier species to lure them in and hitch a ride (Huigens and Fatouros 2013; Niogret et al. 2006; Saul-Gershenz and Millar 2006).

To test whether hitchhikers can discriminate between the different physiological states of their vehicles and to determine the sensory modality involved, we selected the fig–fig wasp–nematode interaction as a model system. The fig–fig wasp interaction is a specialized brood-site pollination mutualism which harbors phoretic nematodes (Herre et al. 2008; Jauharlina et al. 2012; Krishnan et al. 2010; Shi et al. 2019). The association of figs and fig wasps is around 75 my-old and the nematode association with the wasps is dated to 15–45 mya (Cruaud et al. 2012; Peñalver et al. 2006; Poinar 2003). Fig nematodes use the pollinating fig wasps as vehicles and the fig syconium (enclosed globular inflorescence) acts as their developmental substratum (Gupta and Borges 2019; Krishnan et al. 2010); pollinator wasps are their only vehicles and fig syconia are the only habitats within which they develop and reproduce. There is therefore a requirement for high specificity in vehicle recognition in fig phoretic nematodes. There are several nematode families known to be associated with figs, viz., Diplogasteridae, Parasitaphelenchidae, Rhabditidae and Aphelenchoididae (Giblin-Davis et al. 2006; Machado et al. 2001; Susoy et al. 2016). The nematodes show commensal and/or parasitic interaction effects with both mutualistic partners, fig and fig wasps (Gupta and Borges 2019; Shi et al. 2019). The enclosed fig syconia are ephemeral in nature and have a distinct pollinator entry and exit phase (Galil and Eisikowitch 1968). During the exit phase, the nematodes not only encounter different physiological states of wasps but also enter the vehicles and reside within them (endophoresy) during dispersal (Davies et al. 2015; Dunn et al. 2008; Kanzaki et al. 2013). Further, the nematode hitchhikers are not only able to discriminate between reliable vehicular species (pollinator wasps) that live in the vicinity of other non-reliable species (non-pollinating wasps) within the fig syconium (Krishnan et al. 2010) but also discriminate between vehicles with different hitchhiker densities (Gupta and Borges unpublished).

The phoretic nematode community of the cluster fig *Ficus racemosa* was chosen for the study. The community consists of three nematode genera, viz., *Schistonchus* [now assigned to *Ficophagus* (Davies et al. 2015) in associations with figs] (Aphelenchoididae), *Teratodiplogaster* (Diplogasteridae) and *Pristionchus* (Diplogasteridae) (Gupta et al. unpublished; Susoy

et al. 2016). The nematodes use the mutualistic partner of the figs, i.e. the pollinator wasp (*Ceratosolen fusciceps*) as the dispersal agent or vehicle to disperse from one syconium (closed urn-shaped fig inflorescence; plural = syconia) to another (Krishnan et al. 2010). The pollinators are herbivorous gall inducers, each developing within a gall induced within a single flower. The fig syconium contains hundreds of such flowers, some developing into seeds, and others harbouring developing wasps. The entire duration of fig syconium development is ~60 days consisting of five stages: A—pre-pollen receptive phase; B—pollen receptive phase marked by entry of the pollinator along with the nematodes (endo-phoretic in nature); C—interfloral or wasp development phase (nematodes mature and lay eggs); D—wasp dispersal phase marked by exit of wasps along with the offspring of those nematodes that had entered at the B phase; and E—seed dispersal phase (Krishnan et al. 2010; Ranganathan et al. 2010; Fig. S1). At the wasp dispersal stage, live and dead pollinator wasps occur inside the syconium (Fig. 1a). Earlier experiments had indicated that nematodes are attracted to freeze-killed pollinator wasp females and prefer these over pollinator males and females of six other non-pollinator wasp species that also develop within the syconium (Krishnan et al. 2010). Individual nematodes also use access to mates and overcrowding by other nematodes as criteria while boarding vehicles (Gupta and Borges 2020).

Given that fig nematodes have an extremely narrow window of time (a few hours) to board a vehicle before all wasp vehicles disperse from the fig syconium (Fig. S1), we expected that besides all other discrimination problems, i.e. choice of vehicle species, vehicle sex and assessment of existing hitchhiker load on vehicles, the nematode hitchhikers should also be able to distinguish between vital functional states of potential vehicles.

Therefore, we asked the following questions: a) Do phoretic nematodes respond to 'live' and 'dead' wasps differently and if so, what are the cues used by these nematodes? b) Assuming that respiratory $CO_2$ is the major difference between a "live" and a freshly-dead wasp, can the amount of $CO_2$ released by a single wasp act as a cue for the phoretic nematodes to differentiate between these physiological states?

We further characterized volatiles emitted by the pollinator wasp under different physiological conditions to investigate the set of volatiles to which the hitchhiking nematodes might be demonstrating a response. We then asked the following questions: a) Is there a difference between the volatile profiles of the different physiological states? b) Which components of the wasp volatile profile can be predicted to act as vehicle recognition cues by phoretic nematodes?

## Methods and Materials

**Study Site and Species** Pollinators and nematodes were obtained from late C-phase syconia of *F. racemosa* for the behavioral experiments as described in Gupta and Borges (2019, 2020).
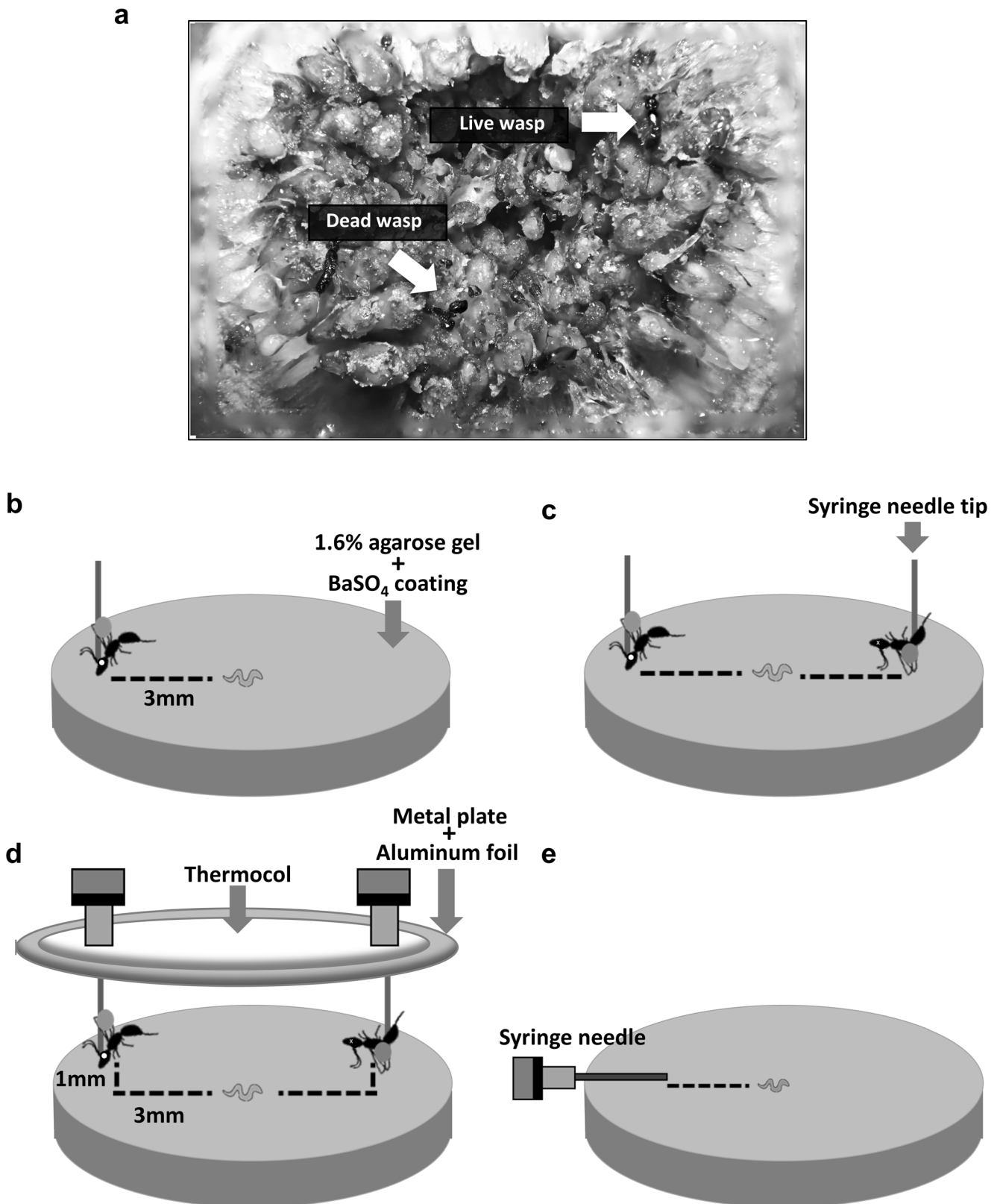
**Fig. 1** **a** Presence of live and dead wasps inside the fig syconium. **b** Diagrammatic representation of experimental set up for whole wasp attraction choice assay, **c** Two-choice assay, **d** Volatile choice assay, and **e** $CO_2$ attraction choice

Naturally eclosing pollinators were obtained from D-phase syconia for collection of wasp volatiles. Syconia were collected from fig trees in and around the Indian Institute of Science campus, Bangalore, India (12°58'N, 77°35'E). *Teratodiplogaster* and *Schistonchus* individuals were used in the experiments but not *Pristionchus* as their numbers were too low.

**Choice Assays for Whole Vehicle (Pollinator Wasp) Attraction under Different Physiological States** To determine whether the nematodes respond to live and dead wasps differently, two kinds of choice assays were conducted. In single-choice assays, individual nematodes were placed at 3 mm distance (after Krishnan et al. 2010) from a single wasp with a defined physiological condition (live or dead) affixed to a needle using a non-toxic glue in each well of a six-well tissue culture plate (Fig. 1b). Since a rapid experimental method to obtain "dead" wasps would be to freeze-kill them (Krishnan et al. 2010), we also included a freeze-killed category besides "naturally dead" wasps. Freeze-killed wasps were obtained by keeping the wasps collected from the D- phase figs for 3 h at −20 °C. The control comprised a needle along with the glue with no attached wasp.

In two-choice assays, individual nematodes were given a choice between a pair of wasps (with different physiological states) at 3 mm distance (after Krishnan et al. 2010) from the nematode in each well of a six-well tissue culture plate (Fig. 1c). Each wasp regardless of its physiological state was affixed on a needle using a non-toxic glue, applied in equal amount on both wasps.

In both assays, 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 a 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 placed in the dark for 2 h and the final choice of the nematode as indicated by its trails was recorded. Wells containing no trails or assays in which the trails led to both wasps were designated as "no choice" (after Krishnan et al. 2010).

**Volatile Organic Compounds (VOCs) of Wasps with Different Physiological States** To determine whether the nematodes used volatiles cues to differentiate between wasps, individual nematodes were exposed to a pair of wasps with different physiological states separated from the nematode at 3 mm distance (Krishnan et al. 2010) as before. Each wasp regardless of its physiological state was fixed to a needle using a non-toxic glue and was suspended from above such that the nematode was only exposed to emitted volatiles, in each well of a six-well tissue culture plate (Fig. 1d). To eliminate the possibility of other cues such as vibration generated by live wasps, the wasps were suspended from the needle using a thermocol cube covered in aluminum foil, fixed to a steel frame (after Gupta and Borges unpublished). The experimental arena was placed in the dark for 2 h and the final choice of the nematode was recorded as before. Since $CO_2$ is also a volatile characteristic of metabolism, we attempted to remove $CO_2$ using soda lime scrubbing prior to the choice assays; however, we found that the $CO_2$ scrubbing also altered the collected volatiles (see Supplementary), and hence this was discontinued.

**Carbon Dioxide Released by a Single Wasp in Different Physiological States** To estimate the amount of $CO_2$ released by a single wasp, we used a flow-through respirometer (Lighton 2018). The measurement was carried out by a LICOR™ (Li-820) $CO_2$ gas analyzer (standardized and calibrated according to experimental procedures developed in Venkateswaran et al. 2017). For each measurement, 30 wasps of each physiological state were placed together in perforated PCR vials and kept in a large metabolic chamber (an air-tight polystyrene container). The set up was placed in the dark to mimic conditions inside the syconium. A baseline was recorded for 15 min followed by 30 min under the experimental condition. The mean $CO_2$ output was subtracted from the average baseline value and was considered the net $CO_2$ output. $CO_2$ produced was calculated as the product of the flow rate of the gas and the difference between the fractional concentrations of $CO_2$ measures obtained (Lighton 2018) and divided by wasp number to obtain $CO_2$ output per wasp per unit time. All experiments were conducted at room temperature (26–28 °C) during the day (8:00 am–12:00 pm). A total of 8 replicate tests were conducted for each physiological state.

**Attraction Assay for Different Carbon Dioxide Flow Rates** To determine if the amount of $CO_2$ released by a single wasp can act as a cue for the nematodes to differentiate between the functional state of wasps, each individual nematode was kept at 3 mm distance from the 10 μl gas-tight syringe needle tip (Hamilton®) and was subjected to different flow rates of $CO_2$ (1.5 μl/h, 2.5 μl/h and 3.5 μl/h) using a syringe pump (Model NE-1000, Multi-Phaser™) (Fig. 1e); these flow rates represent lower, actual, and higher values of estimated $CO_2$ released by a live wasp (Table 1). The experimental arena was a tissue culture petri-dish 35 mm diam, prepared in a similar way as in the previous experiment; a hole was drilled on one side to allow the syringe needle to pass through. The control

**Table 1** Amount of $CO_2$ release by a wasp (μl/h) in different physiological states

| Physiological states | Mean | Median | SD | Range | N (Sample size) |
| --- | --- | --- | --- | --- | --- |
| Live | 2.5 | 2.4 | 0.5 | 1.7–3.3 | 8 |
| Freeze-killed | 0.1 | 0.07 | 0.08 | 0.03–0.25 | 8 |
| Dead | 0.07 | 0.05 | 0.04 | 0.01–0.17 | 8 |

consisted of clean air (air bubbled through distilled water) containing ambient air $CO_2$ levels. The experimental arena was placed in the dark for 2 h and the final choice of the nematode was recorded as before.

**Volatile Organic Compounds (VOCs) of Wasps with Different Physiological States** We collected and analyzed VOCs released by wasps ($N = 400$ wasps for each sample) for three physiological states: (a) live ($N = 8$ replicates), (b) dead ($N = 10$ replicates), and (c) freeze-killed ($N = 8$ replicates). The SPME fibre was allowed to stand inside a GC-MS vial for 2 h for passive volatile collection, and to make sure that the live wasps do not come in contact with the SPME (DVB/CAR/PDMS) fibre (Supelco®), they were separated from the fibre by a steel mesh partition (Fig. S2). All samples were collected in the same manner under the same conditions in the laboratory. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) using a GC-MS instrument (Agilent-HP GC model 7890 B, MS model 5977 BMSD). The gas chromatograph was equipped with an HP 5-MS column (30 m × 250 μm × 0.25 μm). The instrumentation and temperature programs were as follows. Electronic flow control was used to maintain a constant helium carrier gas flow of 1.0 ml/min. The GC oven temperature was held at 30 °C, then increased by 2.5 °C/min to 150 °C followed by an increase by 10 °C/min to 250 °C. The GC-MS data were processed using the Agilent Chemstation software package. Chemical identification was carried out by matching the mass spectra with the Wiley 10th Edition NIST 2014 spectra library and compound retention time. For each sample run, we tentatively identified all VOCs by library and retention time matching and calculated their proportional abundance.

**Statistical Analysis** The frequency data collected from the choice assays were analyzed by a chi-square analysis and were plotted as percentages for visualization. All assays labelled "no choice" were excluded from the analyses. Kruskal–Wallis tests followed by a post hoc Dunn test with Bonferroni correction were used to examine differences between $CO_2$ released under different physiological conditions.

The volatile profiles (relative proportions of all VOCs) of wasps with different physiological states were square root transformed and standardized using a Wisconsin double standardization prior to comparison and visualisation using nonmetric multidimensional scaling (NMDS) based on the Bray-Curtis distance index (Bray and Curtis 1957). Segregation of volatile profiles between wasps with physiological states was tested for significance using permutational multivariate analysis of variance (PERMANOVA) using the function 'adonis' in the 'vegan' package with the Bray-Curtis similarity measurement and 999 permutations.

We further used the Random Forests algorithm to pick out volatile compounds that were unique to a physiological state of pollinator wasp and to classify different physiological states. This tree-based algorithm performs hierarchical clustering via multi-scale and combinatorial bootstrap resampling and is most appropriate for data where the variables (i.e. volatiles in this case) are many more than the number of samples (Grunseich et al. 2020; Ranganathan and Borges 2010, 2011; Runyon et al. 2020). We used a one versus the rest classification where "one" is the group of interest and "the rest" is the universe consisting of all other samples; we performed an all versus all classification to compare species uniqueness based on VOCs proportions. The varSelRF package for the Random Forests algorithm was used not only to find the minimum set of predictor variables but also for prediction error estimates of classification using the .632+ bootstrap method (Ranganathan and Borges 2010, 2011). All statistical analyses were performed using the software R version 3.2.3.

## Results

**Whole Wasp Single-Choice and Two-Choice Assays: Nematodes Offered Vehicles in Different Physiological States** In single-choice assays, *Teratodiplogaster* moved towards pollinator wasps regardless of their physiological states whereas *Schistonchus* moved only towards live wasps and moved away from freeze-killed or dead wasps ($x^2 = 30$ for both species; $df = 1$, $N = 30$ for all chi-square tests in the results) (Fig. 2a). Movement was random in control tests (*Teratodiplogaster*: $x^2 = 0.03$, $N = 29$; *Schistonchus*: $x^2 = 5.14$, $N = 28$) (Fig. 2a). All nematodes responded in these tests. In the two-choice assays, both species showed a significant preference for live over freeze-killed or dead wasps (*Teratodiplogaster*, live vs freeze-killed: $x^2 = 4.8$, $df = 1$, $N = 31$, live vs dead: $x^2 = 22.5$, $df = 1$, $N = 32$; *Schistonchus*, live vs freeze-killed: $x^2 = 26.1$, $df = 1$, $N = 32$, live vs dead: $x^2 = 19.2$, $df = 1$, $N = 30$) (Fig. 2b). The proportion of nonresponding wasps was low. The results of the single and two-choice assays taken together suggest that the entomopathogenic *Teratodiplogaster* might treat wasps as both food resources and vehicles while the plant-parasitic *Schistonchus* treats wasps only as vehicles.

**Wasp Volatile Two-Choice Assays: Nematodes Offered VOCs of Different Physiological States** In volatile choice assays, both nematode types exhibited a significant preference for live over freeze-killed or dead wasps (*Teratodiplogaster*, live vs freeze-killed: $x^2 = 5.8$, $df = 1$, $N = 32$, live vs dead: $x^2 = 4.8$, $df = 1$, $N = 32$; *Schistonchus*, live vs freeze-killed: $x^2 = 13.3$, $df = 1$, $N = 31$, live vs dead: $x^2 = 16.1$, $df = 1$, $N = 32$) (Fig. 2b). The proportion of non-responding wasps was low. These results suggest volatiles as a mechanism by which nematodes assess the physiological states of a vehicle.
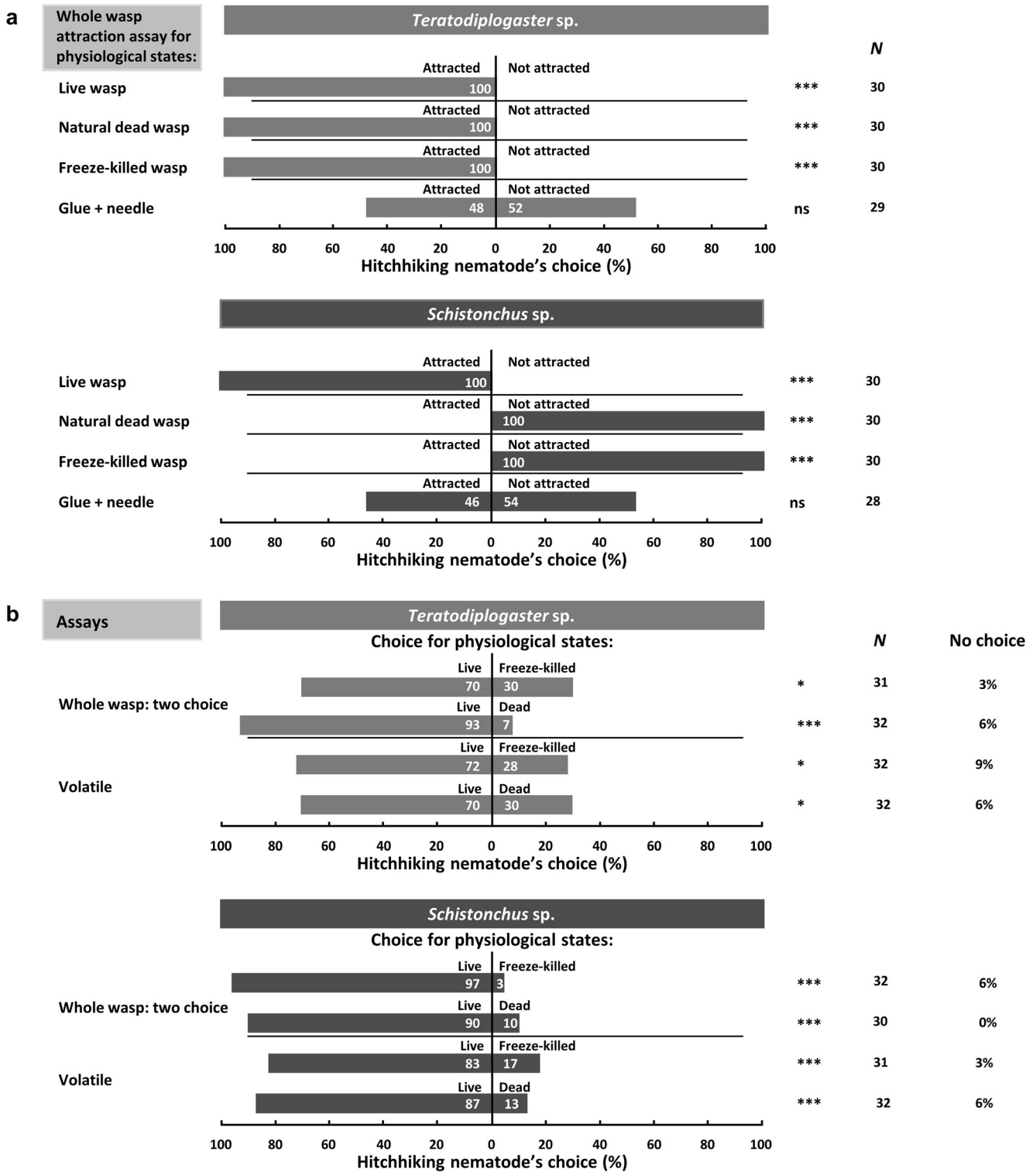
**Fig. 2** Attraction assays for both the nematode species *Teratodiplogaster* **a** and *Schistonchus* **b** for wasps having different physiological states. Choice test (%) for both the nematode species *Teratodiplogaster* and *Schistonchus* **c** for two-choice assay and volatiles emitted by different wasp physiological states. Asterisks denote significant differences obtained by chi-square tests; n.s = non-significant difference, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$
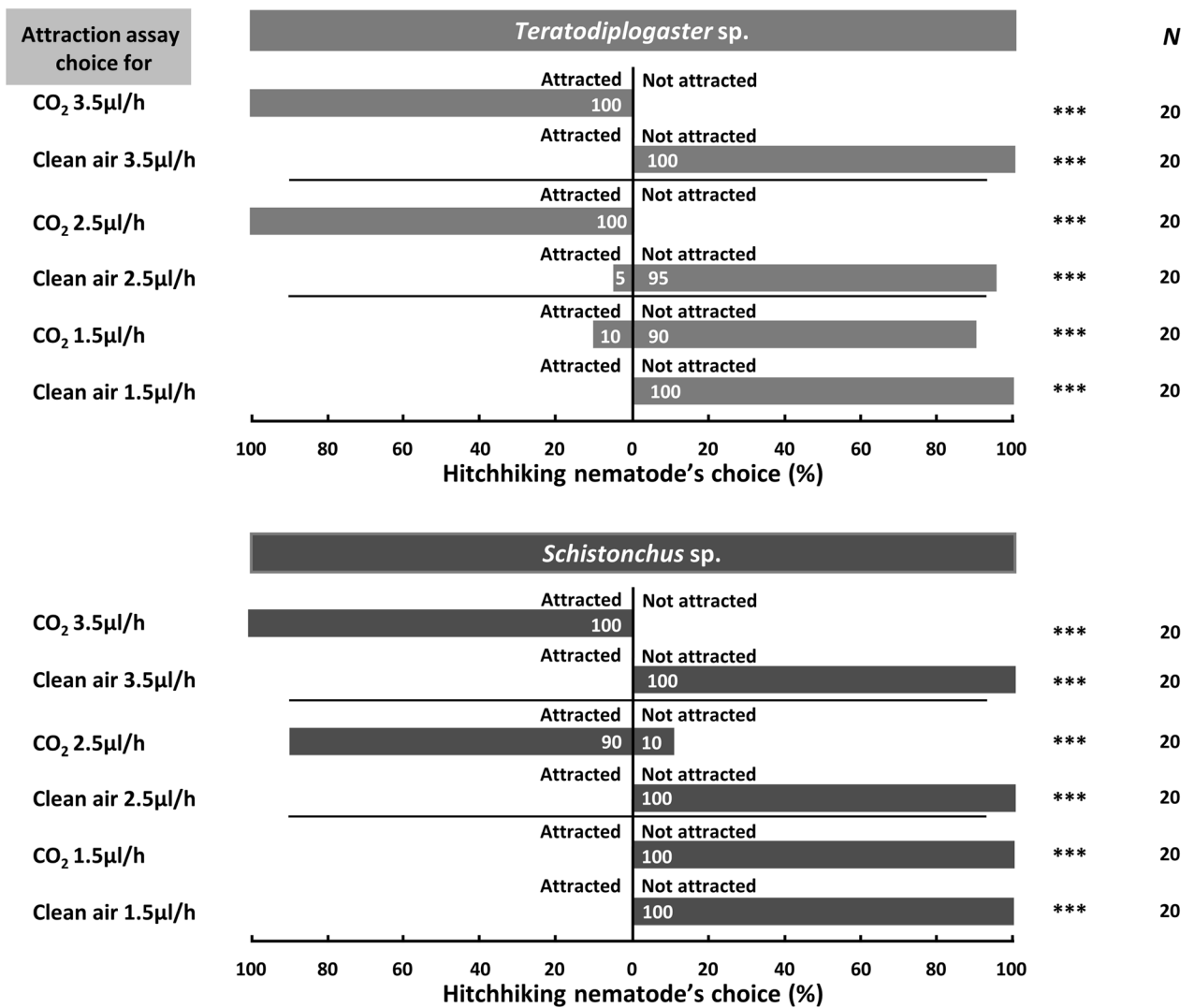
**Single-Choice Attraction Assay for Different Carbon Dioxide Flow Rates** The $CO_2$ released by wasp physiological states differed ($\chi^2 = 15.68$, $df = 2$, $P < 0.001$, $N = 24$) (Fig. 3a; Table 1; Table S1) with the mean value released by a live wasp = 2.5 μl/h. Both nematode types moved towards $CO_2$ flow rates ≥2.5 μl/h (*Teratodiplogaster*, 2.5 μl/h and 3.5 μl/h: $\chi^2 = 20$, $N = 20$;
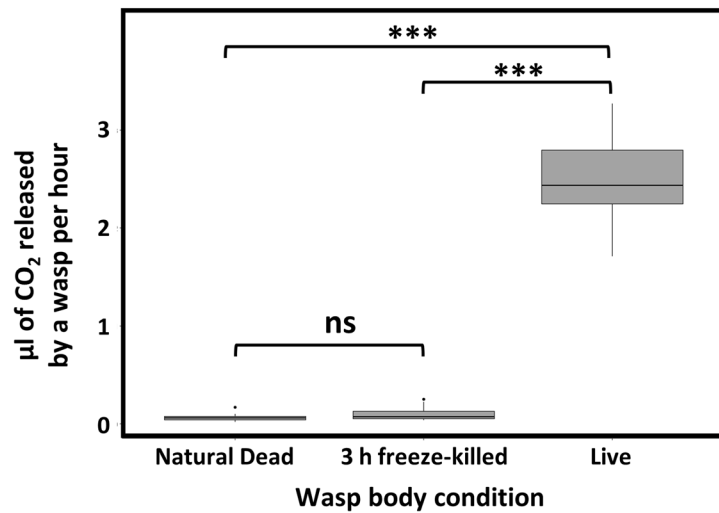
Schistonchus, 2.5 μl/h: $\chi^2 = 12.8$, 3.5 μl/h: $\chi^2 = 20$, $N = 20$) (Fig. 3b). Very few Teratodiplogaster moved towards the low range of $CO_2$ flow rates unlike Schistonchus (Teratodiplogaster, 1.5 μl/h: $\chi^2 = 12.8$, $N = 20$; Schistonchus, 1.5 μl/h: $\chi^2 = 20$, $N =$

**Fig. 3 a** Boxplot of amount of $CO_2$ released by a wasp per hour for different physiological states. Horizontal lines indicate medians, whiskers indicate the upper and lower quartiles, and solid black circles denote outliers. **b** Attraction test for both nematodes *Teratodiplogaster* and *Schistonchus* **c** for different $CO_2$ flowrates and its corresponding controls (clean air). Asterisks denote significant differences between different physiological state of wasps obtained by post hoc Dunn tests with Bonferroni corrections for pairwise comparison and significant differences between the attracted vs not attracted individuals for different $CO_2$ and control (clean air) flow rates obtained by chi-square tests; n.s = non-significant difference, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

20) (Fig. 3b). All tested nematodes did not move towards the flow rates for the control clean air sets except 5% of *Teratodiplogaster* nematodes in 2.5 µl/h (*Teratodiplogaster*, 1.5 µl/h: $\chi^2 = 20$, $N = 20$, 2.5 µl/h: $\chi^2 = 16.2$, $N = 20$, 3.5 µl/h: $\chi^2 = 20$, $N = 20$; *Schistonchus*, 1.5, 2.5 and 3.5 µl/h: $\chi^2 = 20$, $N = 20$) (Fig. 3b). The results suggest that the nematodes responded to $CO_2$ levels ≥ that released by at least one wasp.

**Volatile Organic Compounds (VOCs) of Wasps with Different Physiological States** The volatile profiles of the wasp physiological states differed significantly (PERMANOVA, $F_{2,23} =$ 6.26, *Stress* % = 17.2, $P = 0.001$; Fig. 4a; Table 2). A total of 49 compounds were found (43 identified and 2 unidentified), comprising of monoterpenes (cyclic and acyclic), sesquiterpenes, fatty acid derivatives (aliphatics and spiroketals) and shikimic acid derivatives (Table S2). Monoterpenes dominated the blend followed by spiroketals and sesquiterpenes (Table S2). The number of compounds increased as the physiological state of the wasp changed from live to dead (live = 29 compounds, freeze-killed = 43 compounds, dead = 35 compounds; Table S2). The most abundant volatile compound was the spiroketal (5S, 7S)-conophthorin in all physiological states although its proportional abundance varied (Table S2).

The results of the "one" versus "the rest" Random Forest analysis, where each physiological state was distinguished from all other samples when the identities of the other physiological states were masked, indicated a set of compounds which could be used to uniquely identify wasp physiological state; for the 'live' physiological state: α-cubebene, γ-terpinene and *trans*-linalool oxide (furan form); for the 'freeze-killed' physiological state: α-cubebene, 1,8-cineole, dimethylnonatriene, limonene, *p*-cymenene and sabinene; and for the 'naturally dead' physiological state: (*E*)-β-ocimene, *cis*-linalool oxide (furan form) and allo-neo-ocimene (Table 3). The model frequency of the 'freeze-killed' physiological state was considerably lower than the 'live' and 'dead' physiological states of the wasp (live = 96%, freeze-killed = 58%, dead = 96%; Table 3) suggesting that there may be more than one unique way to differentiate the freeze-killed group from the rest. The most abundant compound, viz. (5S, 7S)-conophthorin, was not the most unique and invariant compound in the VOC signature (Table 3).

We also performed an all versus all classification using Random Forest analysis (i.e. with the identities of each physiological state being retained) to compare physiological state uniqueness based on VOC proportions. The classification showed six compounds, viz., α-cubebene, dimethylnonatriene, (*E*)-β-ocimene, γ-terpinene, *cis*-linalool oxide (furan form) and allo-ocimene, that can be used as predictors to distinguish among all three physiological states with 54% model frequency (Fig. 4b). The proportional abundance of α-cubebene changed with the physiological state of the wasp (live > dead > freeze-killed) (Fig. 4b). Live wasps had higher percent (*E*)-β-ocimene, γ-terpinene and allo-ocimene as well but their proportional abundance was higher in freeze-killed than in dead wasps (live > freeze-killed> dead); *cis*-linalool oxide (furan form) was highest in dead wasps (dead > freeze-killed > live) whereas dimethylnonatriene was absent in dead wasps (Fig. 4b).

The use of soda lime to scrub $CO_2$ from the VOC profile wasps significantly altered the VOC profile of the naturally dead wasps in a comparison without the soda lime treatment (PERMANOVA, $F_{1,17} = 2.17$, *Stress* % = 17.6, $P = 0.021$; Fig. S3). Only 27 compounds were found with the soda lime treatment unlike 34 compounds without this treatment (Table S4) and the missing compounds were mostly alkenes. The Random Forest analysis revealed two compounds, viz. limonene and trans-linalool oxide (pyran form), that can be used as predictors to distinguish these treatments with 100% model frequency (Table S3). The proportional abundance his is fine of both compounds was higher in the treatment with soda lime (Fig. S4). Since the soda lime treatment altered the VOC profile significantly, while discontinued its use for the behavioural experiments.

## Discussion

Nematodes dispersing from fig syconia are in a rush to find suitable wasp vehicles since they have only a few hours before all fig wasps disperse and the syconia are ready to be consumed by frugivores. They therefore need reliable cues to detect functional vehicles. Consequently, when plant- or animal-parasitic fig nematodes were presented with a choice, live wasps were always preferred suggesting dispersal to be paramount at this developmental stage. Animal- and plant-parasitic nematodes used volatiles to discriminate between wasp physiological states and were also attracted to the amount of $CO_2$ respired by a single pollinator wasp suggesting that $CO_2$ alone can act as a cue to recognise a functional vehicle. Wasps with different physiological states differed in their volatile profiles and these differences along with $CO_2$ might be used by the nematodes to select functional vehicles. However, in single-choice experiments the animal-parasitic nematode was not only attracted to live wasps but also to dead and freeze-killed wasps suggesting that animal-parasitic nematodes might also be necromenic in nature as
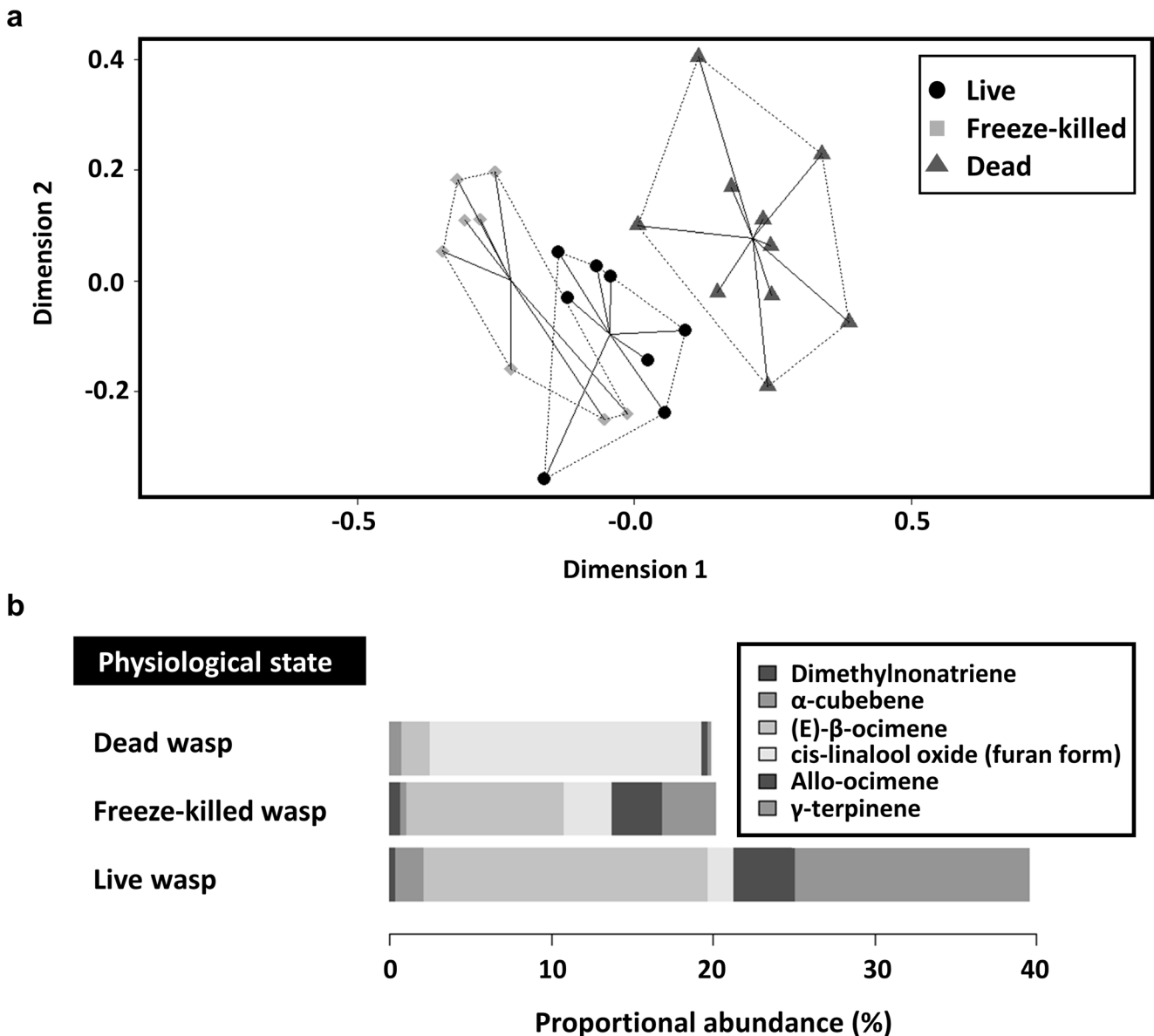
**a**



**b**



Fig. 4 **a** Non-metric multidimensional scaling (NMDS) ordination of tentatively identified volatile chemical compositions of all studied physiological states of wasps, based on Bray-Curtis distance, rotated by principal component analysis (PCA). **b** Random Forests all versus all classification using proportional abundance. For sake of clarity, only mean values are plotted for the mentioned volatile organic compounds (VOCs) for different wasp physiological states

shown for the parasitic nematode *Parasitodiplogaster* in other fig species (Ramírez-Benavides and Salazar-Figueroa 2015; Van Goor et al. 2018).

**Table 2** Permutational multivariate analysis of variance (PERMANOVA) on only volatiles of wasp physiological states

| Comparison | N | Stress (%) | Df | F | P |
|---|---|---|---|---|---|
| Global | 26 | 17.2 | 2, 23 | 6.26 | 0.001 |
| Live vs dead | 18 | 16.3 | 1,16 | 5.78 | 0.001 |
| Live vs freeze-killed | 16 | 8.5 | 1,14 | 5.14 | 0.002 |
| Dead vs freeze-killed | 18 | 13.4 | 1,16 | 7.37 | 0.001 |

*P* < 0.05 are significant values

Both nematode types were attracted to $CO_2$ levels ≥ those respired by a single wasp. Gas sensing (e.g., of carbon dioxide and oxygen) has been well documented in nematodes; gases can act as signal or cues for food, pathogens, conspecifics, predators, or hosts (Carrillo and Hallem 2015). Carbon dioxide serves as a strong attractant for entomopathogenic nematodes, and in combination with insect and plant volatiles helps them to locate their insect hosts; for soil dwelling plant-parasitic nematodes, $CO_2$ helps to locate host plant roots (Dillman et al. 2012; Farnier et al. 2012; Hallem et al. 2011; O'Halloran and Burnell 2003; Rasmann et al. 2012; Reynolds et al. 2011; Turlings et al. 2012). In a few nematodes species, the attractive response to $CO_2$ is in fact a response to low pH resulting from dissolved

**Table 3** Model frequency and predictor volatile organic compounds (VOCs) of wasp physiological states according to the Random Forests algorithm based on proportional abundance of compounds (one versus the rest)

| Wasp physiological state | Model frequency | .632+prediction error | Predictor VOCs | Proportional abundance (in %) mean±SD |
|---|---|---|---|---|
| Live | 96% | 0.1849 | α-cubebene | 1.77 ± 1.23 |
| | | | γ-terpinene | 14.5±9.94 |
| | | | *trans*-linalool oxide (furan form) | 0.27±0.48 |
| Freeze-killed | 58% | 0.1724 | α-cubebene | 0.72±0.78 |
| | | | 1,8-cineole | 0.14±0.42 |
| | | | Dimethylnonatriene | --[b] |
| | | | Limonene | 1.71±2.36 |
| | | | p-cymenene | 0.65±1.31 |
| | | | Sabinene | -b |
| Dead | 96% | 0.1019 | (*E*)-β-ocimene | 9.79±3.85 |
| | | | *cis*-linalool oxide (furan form) | 2.93±2.01 |
| | | | allo-neo-ocimene | 3.11±2.03 |

−[b] the absence of a VOC is also considered a feature unique to the group of interest

$CO_2$ rather than to $CO_2$ in gaseous form (Wang et al. 2009). In our study, $CO_2$ attraction in both nematode types might facilitate interactions with pollinator wasps capable of serving as functional vehicles. We further attempted to present the nematodes with volatiles devoid of $CO_2$ to test if volatiles alone can elicit an attraction response in these nematodes. To address this question, we used soda lime to scrub out the $CO_2$ from the volatiles (Dillman et al. 2012; Hallem et al. 2011). However, we found that soda lime altered the VOC profiles of wasps. Eight compounds were missing from the volatile profile of the 'naturally dead' physiological state scrubbed with soda lime, which therefore also altered the proportional abundance of the rest. We therefore discontinued the use of soda lime and were unable to find a suitable alternative.

Live wasp VOC profiles were preferred over both dead as well as freeze-killed profiles. Since we were unable to experimentally remove $CO_2$ from the VOC bouquets of the different physiological states without altering the bouquet itself, therefore we were unable to present nematodes with a VOC bouquet devoid of $CO_2$. Since live wasps are also a source of $CO_2$, and since we have shown that the $CO_2$ amount emitted by a single wasp is sufficient to attract nematodes, it is possible that the $CO_2$ present in live wasps was solely responsible for the attraction in our experiments. However, in two-choice experiments we have shown earlier (Krishnan et al. 2010) that nematodes in the *Ficus racemosa* system can differentiate between the freeze-killed VOCs of pollinator females versus pollinators males, and between those of single freeze-killed pollinator females and single females of six other species of fig wasps that are available but not used as vehicles. These results clearly demonstrated that nematodes use wasp species-specific VOCs in making their choices. Therefore, our results

on nematode attraction to live wasp VOCs is unlikely to be dictated by $CO_2$ alone.

In general, nematodes respond to various specific host chemicals such as cuticular hydrocarbons (CHCs), semio-chemicals and VOCs emitted by hosts (Dillman et al. 2012; Hallem et al. 2011; Krishnan et al. 2010; Rasmann et al. 2012; Rengarajan and Hallem 2016; Willett et al. 2018). For example, herbivore-induced plant volatiles (HIPVs) such as β-carophyllene and linalool produced by roots attract juveniles of entomopathogenic nematodes enable them to detect hosts and aggregate, resulting in increased host infection rates (Ali et al. 2010; Degenhardt et al. 2009; Hong and Sommer 2006; Li et al. 2015; Rasmann et al. 2005; Rasmann and Turlings 2008). Plants also produce underground volatiles (e.g., isoamyl alcohol, ethylene) which can attract or repel plant-parasitic nematodes based on their concentration (Fudali et al. 2013; Yoshida et al. 2012). We have also shown that nematodes in the *Ficus racemosa* system use volatiles to discriminate between pollinator wasp vehicles with different hitchhiker densities (Gupta and Borges 2020).

Some nematodes species use a volatile blend for dispersal and to locate hosts (Hong et al. 2008; Kaplan et al. 2012; Worthy et al. 2018). In our study, the VOC blend complexity increased as the wasp physiological state changed from live to dead in terms of number and composition of compounds. Increase in the number of compounds might mask the presence of signature compounds by reducing their proportional abundance in the blend. Also, composition changes such as increase in total percentage of aliphatic compounds and in the spiroketal (5*S*, 7*S*)-conophthorin, which is also the most abundant compound, might also serve as cues. Fatty acid derivatives such as octanal and nonanal are known to present in higher

concentration in decaying vertebrates and might contribute to a decomposition cue (Forbes and Perrault 2014). On the other hand, spiroketals are sex pheromones in insects (DeShong and Rybczynski 1991; Francke and Kitching 2001; Helms et al. 2017; Mitchell et al. 2017; Njihia et al. 2017; Zhang et al. 2002) but also have nematocidal activity (Sun et al. 2013). Thus, increase in the proportional abundance of these compounds might act as a repellant for both nematode species present in the fig system as well. Another possibility is a group of six compounds that was identified using the Random Forest all vs all analysis. The proportional abundance of all six compounds varied between wasp physiological states. α-cubebene showed a decrease in proportional abundance from live to dead physiological states. This VOC is also released by plant roots to which nematodes are attracted (Rasmann and Turlings 2007). Chemical compounds like (E)-β-ocimene and γ-terpinene are known to be a part of essential oils obtained from plants that have nematocidal property (Bai et al. 2013; Kong et al. 2007). Therefore, further investigation is required to determine if these individual compounds or their blend can serve as state-specific cues for the nematodes present in the system. How the VOC profile of the pollinators differs from that of the six other fig wasp species co-inhabiting the syconium is also unknown but is under investigation.

The relative importance of insect-specific volatiles versus $CO_2$ as attractant varies with nematode species (Dillman et al. 2012). As we failed to provide the nematodes with volatiles devoid of $CO_2$, the relative importance of $CO_2$ versus insect-specific volatiles as vehicle signatures remains unanswered for this system and awaits further investigation. However, it is highly unlikely that the nematodes would use $CO_2$ alone to make their choice of vehicle since, as mentioned earlier, there are six other species of wasps all respiring $CO_2$ within the same environment that are present in the fig syconium at the wasp dispersal stage but are unsuitable as vehicles. Using just $CO_2$ alone could lead to attraction to an inappropriate vehicle especially since the $CO_2$ production of the other wasps is within the same range (Gupta and Borges 2020; Venkateswaran et al. 2017). Therefore, the fig nematodes likely use a combination of wasp-specific VOCs to identify the correct vehicle species and $CO_2$ levels to identify the correct physiological state.

The volatile profiles of pollinator wasps were dominated by terpenes and also contained small amounts of methyl salicylate, compounds that are predominantly produced by plants. Fifteen VOCs (52%) present in the live wasp volatile profile (2 aliphatic fatty acid derivatives, 12 monoterpenes and 1 sesquiterpene) were also found in the VOC profile of the syconia of *Ficus racemosa* (Borges et al. 2013). The synthesis of terpenes such as linalool, myrcene and ocimene, as well as sequestration of methyl salicylate of plant origin have been recorded or proposed in insects (Beran et al. 2019; Morgan 2004; Opitz and Müller 2009; Zhao et al. 2018). However, very little is known about terpene synthesis in insects. For example, two monoterpene synthases in *Ips pini* (Coleoptera) and two terpene synthases in *Heliconius melpomene* (Lepidoptera) have been found (Darragh et al. 2019; Gilg et al. 2009; Martin et al. 2003; Schulz et al. 2008). Also, one novel terpene synthase gene family has been found that helps in de novo synthesis of sesquiterpenes in the striped flea beetle (Beran et al. 2016). Since the VOC profile of the pollinating fig wasp in the live condition has a large number of abundantly produced terpenes, it would be interesting to investigate if these compounds are sequestered by feeding on the plant within the galls induced by the herbivorous pollinator or if there are terpene synthase genes in pollinators as reported in other insects.

This study forms a comprehensive investigation of the chemical ecology of intra-specific discrimination of phoretic nematodes present in the mutualism between figs and fig wasps. It also illustrates that the phoretic nematodes residing in specific habitats can detect small physiological changes in their vehicles and provides new testable hypotheses in understanding the role of volatiles in species-specific interactions and their co-evolutionary processes.

## Compliance with Ethical Standards

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