Host-related volatile cues used by a parasitoid wasp during foraging for its woodboring host

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Abstract

Parasitoids rely mainly on infochemicals to search for their herbivore hosts to oviposit on. *Megarhyssa nortoni* (Cresson) (Hymenoptera: Ichneumonidae) is a parasitoid of siricid wasp larvae which are concealed inside tree stems of several coniferous tree species. This parasitoid is used as a biological control agent against the global pine pest *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), which engages in an obligate nutritional symbiosis with the fungus *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Russulales). We explore the chemical cues that could be used by *M. nortoni* females while foraging for hosts on the two pine species most commonly planted in commercial forests in Patagonia, Argentina, *Pinus contorta* Douglas ex Loudon var. *latifolia* Engelm. ex S Watson and *Pinus ponderosa* Douglas ex C Lawson (Pinaceae). Behavioural assays were conducted in an olfactometer and the following odour sources were tested in pairwise combinations: (1) *P. contorta*, (2) *P. ponderosa*, (3) *P. contorta* with host fungal symbiont, (4) *P. ponderosa* with host fungal symbiont, and (5) air (control). *Megarhyssa nortoni* females discriminated the odours of infested wood (host fungal symbiont-pine complex) from those of healthy trees, irrespective of the pine species. Additionally, when offered both pine species infested with the fungal symbiont, the parasitoids displayed a bias towards *P. contorta*. Even though the identity of the compounds responsible for the behavioural bias towards the symbiont was not established at this stage, our findings emphasize the relevance of chemical information derived from the fungal symbiont-pine complex during parasitoids that forage for woodboring insect hosts.

Introduction

During adult life, animals forage for resources – e.g., mates, food, and breeding sites – for survival and reproduction. Entomophagous insects, such as parasitoids and predators, often use chemical information to locate hosts or prey (Vet & Dicke, 1992; Steidle & van Loon, 2003; van Oudenhove et al., 2017). In tritrophic systems – comprising plants, herbivorous hosts, and parasitoids – foraging parasitoids commonly use infochemicals derived from their hosts, the plants on which their hosts feed, and/or from other host-associated organisms. These infochemicals are vital elements for the performance of parasitoids,

as successful host encounter translates into reproduction (Godfray, 1994; Fatouros et al., 2008; Wäschke et al., 2013). Additionally, since they can drive and modify parasitoid behaviour, knowledge on cues involved has led to studies exploring the possibility of using them to enhance the efficacy of natural enemies in reducing pest populations in multiple agricultural and forestry systems (Cook et al., 2007; Meiners & Peri, 2013).

Parasitoids attack herbivorous hosts that can display various degrees of concealment (i.e., fully exposed, leaf miners, plant galleries , stem-, wood-, or seedborers) and while searching for them, they have to contend with the reliability–detectability problem (Vet & Dicke, 1992; Godfray, 1994): host-derived stimuli are the most reliable indicators of host presence but their detectability is selected to be low; conversely, stimuli from plants on which herbivores feed may provide information which is readily detectable but not necessarily

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reliable in indicating herbivore presence. Particularly, plants release blends of volatile compounds in response to damage by herbivores (Hare, 2011), that are highly detectable by parasitoids in the long-range search process (Turlings et al., 1990; Poelman et al., 2012; Avila et al., 2016). Plant volatile blends can be diverse in their chemical composition and parasitoids cope with this chemical complexity by reacting to a limited set of volatiles released by herbivore-damaged plants. Through these, they locate specific host-plant species or varieties (Hoballah et al., 2002; Lou et al., 2006), specific hosts (De Moraes et al., 1998), and/or hosts at a susceptible developmental stage (Takabayashi et al., 1995). Nonetheless, during short-range searching, chemical information derived from the hosts themselves (oviposition marks, body odour trails), host by-products (frass, exuviae, gland secretions, etc.), and/or from host-associated microorganisms are most commonly used (Afsheen et al., 2008; Boone et al., 2008; Wäschke et al., 2013).

Most studies on the use of infochemicals by parasitoids during host searching have focused on given interactions such as that of aphids on brassicas (Orre et al., 2010; Najar-Rodriguez et al., 2015) or tephritids on fruits (Canale et al., 2014), but parasitoids of woodboring insect hosts have been generally understudied (but see Spradbery, 1970; Fischbein et al., 2012; Johnson et al., 2014). *Megarhyssa nortoni* (Cresson) (Hymenoptera; Ichneumonidae) is an idiobiont ectoparasitoid of siricid wasp larvae which are concealed inside tree stems of several coniferous tree species in its native habitat (Coyle & Gandhi, 2012). The parasitoid is native from North America but has been introduced into other regions (Australia, New Zealand, South America) as a biological control agent against invasive populations of *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), one of the most important pests of pine plantations worldwide (Cameron, 2012). When the females of *S. noctilio* lay their eggs into trees, they inject a phytotoxic venom and mycelium of the symbiotic fungus *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Russulales), which is essential for larval nutrition and development (Madden, 1981; Thompson et al., 2013, 2014). Developing larvae excavate galleries within the tree which in combination with fungal growth and the injected venom results in physiological stress that leads to tree death (Coutts, 1969; Ryan & Hurley, 2012).

Previous studies have determined that infochemicals derived from the fungal symbiont of *S. noctilio* can be used by the parasitoid *Ibalia leucospoides* (Hochenwarth) during host searching (Martínez et al., 2006). Fungal odours provide *I. leucospoides* females not only information on woodwasp presence and even relative densities of hosts in patches (Fischbein et al., 2012), but may also convey information about the presence of susceptible host life-stages (Jofré et al., 2016). Chemical cues are particularly important to parasitoids seeking cryptic hosts. To understand the role of non-host volatile cues used by *M. nortoni* females to locate hosts, we carried out behavioural assays in a four-arm olfactometer. We hypothesize that *M. nortoni* parasitoid females should use volatile chemical cues from the infested host plant in order to locate the hidden hosts. We expect that female parasitoids, by using blends of volatile compounds released by the fungal symbiont-pine complex, will be able to distinguish the odours of infested trees from those of healthy ones. Tests of parasitoid attraction to fungal symbiont-pine complex were performed with two tree species, *Pinus contorta* Douglas ex Loudon var. *latifolia* Engelm. ex S Watson and *Pinus ponderosa* Douglas ex C Lawson (Pinaceae). These two pine species were selected because they are the most commonly used in plantation forestry in Patagonia, Argentina. We also expect that parasitoids will be attracted to blends of volatiles released by the fungal symbiont-pine complex, irrespective of the pine tree species. The use of context information may generate adaptive advantages for parasitoids that search for concealed hosts by improving their efficiency in locating them. This may prove relevant for biological control programmes of invasive woodboring forest insects, such as *S. noctilio*.

Materials and methods

Insects

Megarhyssa nortoni adult females used in all experiments were obtained from felled trees, *P. contorta* var. *latifolia*, obtained from *S. noctilio*-attacked plantations in NW Patagonia, Argentina. Infested *P. ponderosa* are hard to come by in the study region, mainly because *S. noctilio* seldom attacks this species in pure or mixed plantations of *P. contorta* and *P. ponderosa* in Patagonia (Lantschner & Corley, 2015). Pine trees were all naturally attacked by *S. noctilio* and selected in the field by the typical oviposition symptoms (i.e., resin beads at each ovipositor insertion). After felling, trees were cut to 0.8-m-long logs and kept at natural temperature and lighting conditions in locker-type cages within the rearing facilities at IFAB (CONICET-INTA EEA Bariloche, Argentina) until wasps emerged. Adult females were collected right after emergence and kept in individual containers under controlled light and temperature (15 °C and L16:D8 photoperiod) with ad libitum water during 4 ± 2 days, until being subjected to behavioural assays. Females were assumed to be virgin and with no oviposition experience, as there were relatively short lapses between emergence and collections.

Olfactory response

The olfactory response towards host-related odours was tested in a Peterson-type olfactometer (Vet et al., 1983). The olfactometer consisted of a central arena and four symmetrical arms. The length from the centre to each of the four arm ends was 17.5 cm. A square (37 × 37 cm, 4 mm thick) glass was positioned over the entire arena creating an airtight seal. The air flowed from the end of the arms towards a central exit hole at 3 l per min. Air was previously filtered in 0.5 kg of activated charcoal and then humidified through 0.5 l of distilled water. The arena was illuminated with white light-emitting diodes (LEDs) (2.5 m of LED strip set in a circumference with a diameter of 80 cm) at 150 cm over the olfactometer with 60 surface mounted diodes m^{-1} (60 W; Alic, Buenos Aires, Argentina).

Odour sources were placed inside four individual cylindrical glass chambers (7 cm long, 3 cm diameter) connected to the air stream (0.75 l per min) that entered each olfactometer arm. The olfactometer was used as a two-way, as only two odour sources were presented simultaneously to individual wasps (both odour sources in duplicate). Single females were introduced into the olfactometer arena and left for 10 min to respond. Odour sources used as stimuli were: (1) *P. contorta* var. *latifolia*, (2) *P. ponderosa*; (3) *P. contorta* var. *latifolia* with host fungal symbiont, (4) *P. ponderosa* with host fungal symbiont, and (5) air (control).

The odour sources consisted of wood plugs (25 mm long, 0.5 cm diameter) extracted with an electrical drill fitted with a plug cutter bit (1 cm diameter) 60 min before bioassays took place. Wood plugs colonized with fungal symbiont (odour sources 3 and 4) were obtained from trees naturally attacked by *S. noctilio* the previous season (i.e., attack took place between 7 and 8 months before the trees were cut down and used in experiments). Billets from these *S. noctilio*-infested trees were left under natural lighting and temperature conditions in order to obtain natural fungal growth in the wood. On the day of the experiment, the bark was removed and a *S. noctilio* oviposition site was identified visually (by the typical small black ovipositor incision left by the female wasp). A plug was extracted by drilling from the area near the ovipositor insertion. Special care was taken to avoid obtaining chemical traces that could have been left at the moment of the host oviposition by drilling adjacent to the oviposition site. Additionally, if the extracted plug contained frass (i.e., indication of a tunnel borrowed by the larva), it was discarded to ensure no chemical traces of larva were on the sample. In this way, it was possible to obtain a sample only containing traces of wood and fungi. Two olfactometer branches contained the same odour source and the other two a second contrasting odour source. The following contrasts were tested: *P. contorta* vs. air (n = 13), *P. ponderosa* vs. air (n = 14), *P. contorta* vs. *P. ponderosa* (n = 16), *P. contorta* vs. *P. ponderosa* with fungal symbiont (n = 16), *P. contorta* with fungal symbiont vs. *P. ponderosa* (n = 15), and *P. contorta* vs. *P. ponderosa*, both associated with the fungal symbiont (n = 16). When wood plugs were used as stimuli, a single plug was used per arm. When contrasting the response towards wood against clean air (control), no plugs were used in two of the olfactometer arms. After each bioassay, the olfactometer was thoroughly washed with water and alcohol. Odour sources were placed in contiguous arms (never in opposite positions), were renewed for each female tested, and odour positions relative to olfactometer arms rotated according to a pre-defined scheme every four trials to minimize orientation biases.

Female wasps were acclimatized to 22 ± 1 °C 2 h prior to the bioassay. A single wasp was introduced into the olfactometer once odour sources were in place, and its movement was registered with a web camera (Genius Facecam 1000 set at 150 cm above the olfactometer), using avi file format at 5 frames s^{-1} during the 10-min trial. The position (x and y coordinates in pixels) of the wasp in the arena was determined at each frame via analysis with ImageJ (Rasband, 2016). Once the wasp position in the arena was determined at each frame, the percentage of time spent at each odour area during the bioassay was calculated for each individual tested. It is important to note that the time spent at the central area was excluded from the analysis. For this, the central area was defined as a square with sides of 16 cm centred in the olfactometer. As data did not fit the normality assumptions, percentages of time spent in each stimulus area were compared with Wilcoxon signed rank tests in R v.3.3.2 software (R Foundation for Statistical Computing, Vienna, Austria). In order to rule out possible effects due to positional biases (i.e., position of the olfactometer), an additional Wilcoxon signed rank test was carried out considering the response of wasps in relation to each of the olfactometer arms (1–4). In this case, non-significant results are not reported.

Results

The percentages of time spent by *M. nortoni* females did not differ at each stimulus with the following choices: *P. contorta* vs. air (two-tailed Wilcoxon signed rank test: S = 13.5, d.f. = 1, P = 0.37; n = 13), *P. ponderosa* vs. air (S = −12.5, d.f. = 1, P = 0.45; n = 14), and *P. contorta* vs. *P. ponderosa* (S = 21, d.f. = 1, P = 0.30; n = 16; Figure 1A–C). As expected, females discriminated the odours
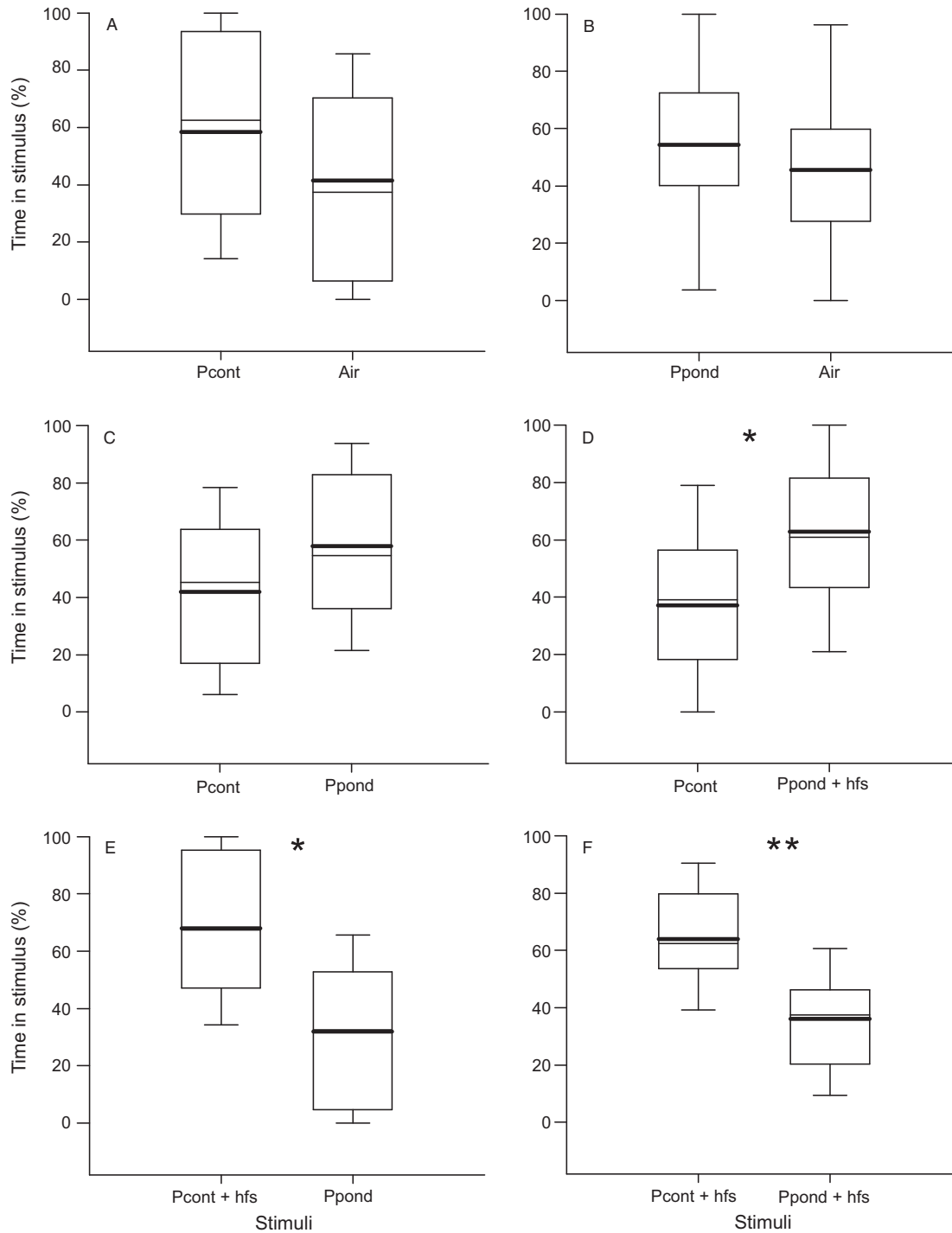
Figure 1 Boxplots of olfactory response (% time spent in stimulus section) of *Megarhyssa nortoni* females when offered two volatile cues in an olfactometer: (A) *P. contorta* (Pcont) vs. air, (B) *P. ponderosa* (Ppond) vs. air, (C) *P. contorta* vs. *P. ponderosa*, (D) *P. contorta* vs. *P. ponderosa* with fungal symbiont (Ppond + hfs), (E) *P. contorta* with fungal symbiont (Pcont + hfs) vs. *P. ponderosa*, and (F) *P. contorta* vs. *P. ponderosa* both with fungal symbiont. The top and bottom boundaries of the boxes indicate the 75th and 25th percentile, respectively. The lines within the boxes mark the median (thin) and mean (thick). Whiskers show 90th and 10th percentiles. Asterisks denote significant differences between odour sources (Wilcoxon signed rank test: *0.01<P<0.05, **P<0.01).

of infested pine (fungal symbiont-pine complex) from those of non-attacked pine, irrespective of the pine species. That is, differences were found in the trials carried out between *P. contorta* vs. *P. ponderosa* with the fungal symbiont (one-tailed Wilcoxon signed rank test: S = 37, d.f. = 1, P = 0.028; n = 16), with female response biased towards the *P. ponderosa* with fungal symbiont (Figure 1D). In the same way, *P. contorta* with fungus vs. *P. ponderosa* trials resulted in individuals biased towards *P. contorta* with the fungus (S = 39, d.f. = 1, P = 0.012; n = 15; Figure 1E). Finally, wasps subjected to *P. contorta* with fungus vs. *P. ponderosa* also with fungus displayed a biased response towards the *P. contorta* with fungus (two-tailed Wilcoxon signed rank test: S = −52, d.f. = 1, P = 0.005; n = 16; Figure 1F).

Discussion

Female *M. nortoni* parasitoid wasps were attracted to odours of various *S. noctilio*-infested pine tree species (fungal symbiont-pine complex), such as *P. contorta* and *P. ponderosa*, and did not display any attraction towards clean air or healthy trees. Additionally, *M. nortoni* was more attracted to *P. contorta* infected with fungus than to *P. ponderosa* plus fungus. These results suggest that volatiles released by *A. areolatum*, the fungal symbiont growing inside the wood, might be of relevance to *M. nortoni* during foraging. Attraction to the host symbiont may be a way to bypass the reliability-detectability problem faced by parasitoids that attack *S. noctilio* eggs and larvae. The host odours restricted to microhabitats along the tree are unequivocal information of host location but maybe hard to detect; however, the fungus colonizing a tree could become a detectable and reliable odour signal of accessible hosts for foraging *M. nortoni*. Responses to *A. areolatum* volatiles have also been reported for *I. leucospoides* and even for *S. noctilio* females (Fernández Ajó et al., 2015; Jofré et al., 2016). Additionally, other parasitoid species also respond to cues produced by host-associated microorganisms; for example, it has been demonstrated that some bark beetle parasitoids respond to olfactory cues produced by blue-stain fungi associated to their hosts (Sullivan & Berisford, 2004; Boone et al., 2008). Similarly, fungal volatiles associated with *S. noctilio* likely are part of a set of other chemical cues that indicate host habitat for *M. nortoni*. Alternatively, the parasitoids may not respond exclusively to fungal volatiles but to a combination of fungal and plant volatiles. Fungus-infected trees may generate blends of volatiles in the affected tissues that differ quantitatively and qualitatively from those associated with healthy trees.

Parasitoids can respond to both specific and generic volatile cues, but specialist parasitoids more frequently use specific cues whereas generalists commonly use generic cues (Steidle & van Loon, 2003; van Oudenhove et al., 2017). However, the degree of host specialization acquired by parasitoids results from physiological and behavioural interactions between them and their hosts in given environments and, thus, may be an attribute of a population (established through local adaptation) rather than of a species throughout its geographic range (Fox & Morrow, 1981). When characterizing the degree of specialisation of species, scale, and context matter (Loxdale et al., 2011), a flexible response to a wide array of volatile blends by parasitoids is likely of relevance in nature, given that different generations of the host and parasitoids probably develop on different host plants. *Megarhyssa nortoni* is reported to have the potential to attack various species of siricids (of two subfamilies) associated with two species of symbiotic fungi of the genus *Amylostereum* (Schiff et al., 2012). As an oligophagous parasitoid in its native range, *M. nortoni* should use host-related information of intermediate specificity in order to forage successfully, and the fungal symbiont-pine complex may meet these characteristics, even in the absence of the host larva itself.

Interestingly, when wasps were subjected to infested *P. contorta* vs. infested *P. ponderosa*, females responded towards the *P. contorta* plugs bearing the fungal symbiont. That is, even after 4 days in absence of both hosts and host-related cues, the wasps were selective towards the pine species they developed in and with which they had had first contact as early adults. This result suggests that females could gain experience with the volatile complex of the natal host plant during either immature development or immediately upon adult emergence and such experiences may increase the responsiveness towards a particular volatile blend. Indeed, it is known that pre-emergence and early-adult exposure to a given chemical complex of a host plant can lead to adult olfactory preferences (Storeck et al., 2000; Gandolfi et al., 2003; Dukas, 2008; Takemoto et al., 2012; Giunti et al., 2015). In any case, interpretation of these results must be done with care. Reciprocal tests (i.e., tests of *M. nortoni* emerging from *P. ponderosa*) were not carried out because infested *P. ponderosa* are difficult to obtain in pine stands in the study region (Lantschner & Corley, 2015). Alternatively, *P. contorta* could be preferred irrespective of the natal host plant. That is, differences in chemical profiles between infested pine species, if any, may result in a differential attraction of parasitoids simply because one chemotype is more attractive than another.

Knowledge of infochemicals used by parasitoid insects to locate hosts is relevant in the context of biological control because it provides a better understanding of the steps

leading to successful parasitism. In addition, in classical biological control programs in which non-native natural enemies are introduced, ensuring that only the target species is attacked is crucial. Establishing whether the candidate natural enemy innately uses infochemicals that are specific to the host-plant complex of the pest of interest, is one way to determine its specificity. This is also relevant when new pest-host plant associations develop in the invaded range, such as that of European *S. noctilio* with North American pines. The ecological significance of infochemical use warrants further investigation in parasitoids of woodboring insect hosts, as we showed that the first trophic level may moderate host location efficiency by *M. nortoni*.

Acknowledgements

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